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Myelin Gene Expression: Implications for Alcohol Abuse and Dependence

A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Pharmacology and Toxicology at Virginia Commonwealth University.

By

Sean Patrick Farris

Bachelor of Science in Forensic Science from Eastern Kentucky University 2005

Directed by: Michael F. Miles, M.D., Ph.D.,
Professor of Pharmacology and Toxicology

Virginia Commonwealth University

Richmond, Virginia

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ABSTRACT

Myelin Gene Expression: Implications for Alcohol Abuse and Dependence

By: Sean P. Farris

A Dissertation submitted in partial fulfillment of the requirements for the degree of
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Major Director: Michael F. Miles, M.D., Ph.D.

Professor, Departments of Pharmacology and Toxicology, and Neurology

Acute behavioral responses to ethanol have predictive value for determining an individual's risk of long-term drinking behavior. Although the neurobiology of alcohol abuse is complex, prior studies from our laboratory demonstrated differential myelin-associated gene expression (MAGE) in medial prefrontal cortex (PFC) as one potential mechanism influencing acute ethanol behaviors between C57BL/6J (B6) and DBA/2J (D2) mice. Our laboratory and others have also shown MAGE is reduced in PFC of alcoholics. Herein, I have extended these findings through expression profiling of PFC

into chronic models of ethanol self-administration from non-human primates and mice. Together, these results suggest that regulation of MAGE may be relevant to behavioral phenotypes witnessed in alcoholism. The pathogenesis of alcoholism progresses through multiple stages of drug exposure and withdrawal, however, genetic predisposition is also a major contributing factor for this disease. Therefore, I tested the hypothesis that not only does ethanol have direct effects on MAGE, but also variation in basal MAGE within the PFC is a molecular endophenotype underlying ethanol behavioral sensitivity. Bioinformatics of basal MAGE across the BXD recombinant inbred panel (n=29), derived from B6 and D2 mice, revealed a densely correlated myelin gene network associated with several ethanol behavioral phenotypes. Literature association tools identified *Fyn* kinase as potential regulator of MAGE. *Fyn* knockout mice are known to be more sensitive to the sedative-hypnotic properties of ethanol in the loss of righting reflex (LORR) paradigm. Microarray analysis of *Fyn* knockout mice revealed a significant decrease in MAGE, suggesting MAGE may be an underlying factor for LORR. In support of this premise, microarray analysis of genetic variance in LORR across Inbred Long Sleep and Inbred Short Sleep mice, as well as congenics for the *Lore5* quantitative trait locus, also demonstrated an inverse relationship between MAGE and duration of LORR. The hypothesis was further investigated using cuprizone to model demyelination in B6 mice and test them in a battery of acute ethanol behaviors. Cuprizone-treated mice had a significantly greater duration in LORR ($p < 0.01$), demonstrating that myelin is an important contributor to the genetic variance in LORR. Thus, through genetic, genomic, and pharmacological tools I have 'molecularly triangulated' a myelin gene network as a contributing factor influencing acute ethanol

behavioral sensitivity. The ability of myelin to alter acute ethanol sensitivity may warrant a prospective study of myelin in humans as a predictive molecular phenotype for an individual's risk of developing alcohol dependence. Additionally, further genomic dissection of MAGE architecture and associated networks may aid in developing novel pharmacotherapies for an alcohol use disorder. *Supported by NIAAA grants F31 AA018615 to SPF.*

CHAPTER 1: INTRODUCTION

Alcohol abuse and alcohol dependence, collectively referred to as an alcohol use disorder, is a serious debilitating disorder with long-standing implications on society and the global disease burden. An epidemiological survey of the United States determined approximately 9% of the population, 17.6 million adult Americans, suffer from an alcohol use disorder (Grant et al., 2004). Perhaps even more staggering, a report by the World Health Organization estimates more than 140 million individuals around the world suffer from alcoholism; however, this number may be woefully underestimated due to the variation in screening, diagnostic criterion, and absence of adequate data from developing countries. Alcohol abuse is defined by the 4th edition of the Diagnostic and Statistical Manual of Mental Disorders as a maladaptive pattern of alcohol usage in spite of reoccurring adverse consequences such as failure to fulfill major obligations, physically hazardous situations, alcohol-related legal issues, and recurrent social or interpersonal problems. In addition to the symptoms of alcohol abuse, the manifestation of alcohol dependence is more extensively characterized by the development of tolerance, withdrawal, loss of control, preoccupation of use, and increased habitual drinking patterns leading to the presence of physical dependence (2000).

The socioeconomic impact of alcohol abuse and dependence in the United States is estimated to account for roughly 220 billion dollars per year (University, 2006); with an estimated economic cost worldwide ranging between one and six percent of a country's gross domestic product (Organization, 2004). The dollar amount associated

with alcohol or substance abuse; however, pales in comparison to the tremendous personal costs arising from the physiological and psychological consequences in afflicted individuals, as well as their surrounding friends and family. The average lifespan of an individual enduring an alcohol use disorder is cut short 10 to 15 years, with the lifetime risk of occurrence among developed countries being greater than 20% for men and 8 to 10% for women (Schuckit, 2009a). In spite of these dramatic personal, societal, and financial expenditures there is no medicinal cure for an alcohol use disorder, and few effective pharmacotherapies exist to date for treatment regimens. Medication is often paired with cognitive-behavioral studies, but often show minimal success rates for preventing alcoholic recidivism (Mariani and Levin, 2004).

Similar to other medical conditions such as heart disease and obesity, there is no singular cause of alcoholism. The underlying molecular biology regulating the risk for developing this disorder has remained elusive despite numerous years of scientific innovations and inquiry. Family and twin-based adoption studies suggest that 40-60% of the risk of developing an alcohol use disorder is inherited through an individuals' genetic background with the remainder associated with gene-environment interactions (Prescott and Kendler, 1999, Schuckit et al., 2001). Genetic variation (i.e. polymorphisms) in the metabolizing enzymes guiding the breakdown of alcohol into acetaldehyde has been identified in Asian populations as being protective against dependence, but the entire complement of genetic variants guarding against or predisposing the risk of dependence remains undefined. Further complicating the identification of causal variants is interpreting how such DNA polymorphisms contribute to the range of

endophenotypes, which subsequently interact with the environment, and lead to alcoholism.

Additional risk factors for alcohol dependence such as age of onset (Chou and Pickering, 1992, Grant and Dawson, 1997) and impulsivity (Dick et al., 2010), among many others, have been repeatedly. As many as 80% of men and 60% of women experiment with alcohol consumption at some point throughout their lifetime (Teesson et al., 2006). In the United States nearly 50% of all individuals surveyed report having consumed an alcoholic beverage within the past 30 days (Administration 2005). However, only 20% of the population consumes over 80% of all the alcohol sold (Dawson, 2000), and not everyone exposed to alcohol or genetically predisposed for the condition will go on to develop an alcohol use disorder. Furthermore, risk factors are not predictive of treatment outcomes (Vaillant, 2003). Thus, a complex interaction of abuse liability, acute experiences, and habitual drinking behavior contributes to physiological and psychological dependence on alcohol.

Preclinical *in vitro* and *in vivo* models have accelerated our insights into the molecular targets involved with the varying stages of addiction (Koob, 2006). Such preclinical models are not without limitation. The low molecular weight, low potency, relatively poor reactivity, and distribution into total-body water complicate characterizing the precise pharmacological profile of ethanol. Changes in the expression or function of cell signaling mechanisms typically are of small effect size with changes of only 30% not uncommon (Lovinger and Crabbe, 2005). Overcoming these hurdles has been met with significant rigor by the scientific community through the creation of numerous molecular assays, behavioral models, and genetically engineered mouse models. Although animal

models do not perfectly mimic the human condition as a whole, lacking the operative psychopathology and diagnostic criteria, they do model varying specific aspects of the disease (Bennett et al., 2006).

Studies directly addressing the consilience between human and animal models for understanding the molecular basis of endophenotypes in alcohol use disorders has been sparse (Crabbe et al., 2010a) but are not beyond our grasp. For example, quantitative trait loci (QTL) mapping studies have identified syntenic regions within the mouse and human genome for alcohol-related phenotypes suggesting the existence of evolutionary conserved genetic factors influencing the behavioral response to alcohol (Ehlers et al., 2010). Combining QTL mapping with expression profiling studies, now commonly referred to as genetical-genomics, has been suggested to further facilitate the identification of genes underlying complex traits (Jansen and Nap, 2001). Compared to other scientific fields of inquiry the neuroscience community has been slow to adopt the use of functional genomics, certain genetic methodologies, and large-scale databases or web-based resources (Geschwind and Konopka, 2009); however, these methods have shown promise in the discovery of novel quantitative trait genes (QTGs) shaping the phenotypic variance of disease (Schadt et al., 2003, Schadt et al., 2005).

Single gene approaches utilizing genetic strategies within the central nervous system have received considerable attention in regards to the effect of these genes on ethanol behavioral phenotypes (Crabbe et al., 2006b, Crabbe et al., 2010a). None of these studies have produced the proverbial silver bullet in alcohol abuse or dependence, with many of these studies showing only modest effect. An alternative and more inclusive approach are to examine the phenotypic variance of several

endophenotypes alongside the accompanying genotypic variance. By using genetic linkage to anchor both gene expression networks and behavioral sub-phenotypes, the actual goal of defining an “alcoholism gene” or quantitative trait gene becomes less important, shifting the focus from a single gene to a series of genes or gene networks. The underlying premise for this approach is that gene expression *per se* can be treated as a quantitative trait just as with behavioral phenotypes, and that genetically driven alterations in gene expression might underlie much of the genetic contribution to complex traits (Farris et al., 2010, Farris and Miles, 2011). Changes in gene expression following acute and repeated drug administration are proposed as critical molecular adaptations in promoting substance dependence (Nestler and Aghajanian, 1997). Additional variation in the baseline abundance of gene networks may also functionally contribute to predisposition for abuse and long-term maintenance of drinking behavior. Given the complex role of pleiotropic and polygenic effects of genes on the expression of behavior, gene networks may offer improved mechanistic insight into the underlying neuropathology and risks for developing an alcohol use disorder.

Herein, this dissertation will discuss and show evidence to test the hypothesis for the role of a myelin-associated gene expression network in acute ethanol sensitivity and long-term drinking behavior across mouse, monkey, and human model systems. Studies within the medial prefrontal cortex utilizing molecular triangulation through the combination of genetic, genomic, and pharmacological approaches has produced support for the role of a myelin-associated network underlying acute ethanol sensitivity as measured by the loss of righting reflex behavior and in phenotypic variance of ethanol drinking behavior. In part, these studies provide confirmation of a network

approach to unraveling specific endophenotypes associated with ethanol. Furthermore, this body of work may warrant a prospective study of CNS myelin within human populations as an inherent risk factor for the development of alcoholism. Lastly, future genomic dissection of myelin-associated gene expression and associated networks may aid in the development of novel pharmacotherapies for treatment and prevention of alcoholism.

CHAPTER 2: BACKGROUND AND SIGNIFICANCE

The overall framework leading to the onset of alcohol dependence involves the progression from initial acute exposure toward compulsive drug use with frequent intermixed reoccurring bouts of tolerance and withdrawal (Koob and Volkow, 2010). The disease involves reward seeking, formation of compulsive and habitual behavior, aversive stimuli (e.g. withdrawal) and multiple other behavioral facets. There is likely no single causative factor in alcoholism, and thus each facet of this disorder may provide an important area of scientific inquiry. For example, interpreting the gene network structure of an organism undergoing withdrawal may impart novel mechanistic information contributing to the neuroadaptations driving relapse behavior. The overall phenotype of alcoholism could thus be considered a “behavioral vector” that is made up of multiple component vectors sub serving endophenotypes as mentioned above (Figure 1). Vectors of interacting neuronal/glia networks across multiple brain regions, in turn, likely control each of these endophenotype vectors. Drilling down yet further, these neural networks are ultimately controlled by the regulation and function of multiple gene networks expressed within individual neurons or glial cells. As depicted in [Figure 1](#), this hierarchy of nested response vectors, extending from the molecular to the behavioral, likely explains the tremendous difficulty encountered in studying mechanisms of diverse complex traits such as alcoholism. This degree of complexity also explains why efforts to correlate function/expression of single genes to a complex disease are exceedingly difficult. When considered in reference to this framework, it becomes apparent that progress in studying the mechanisms of complex disease

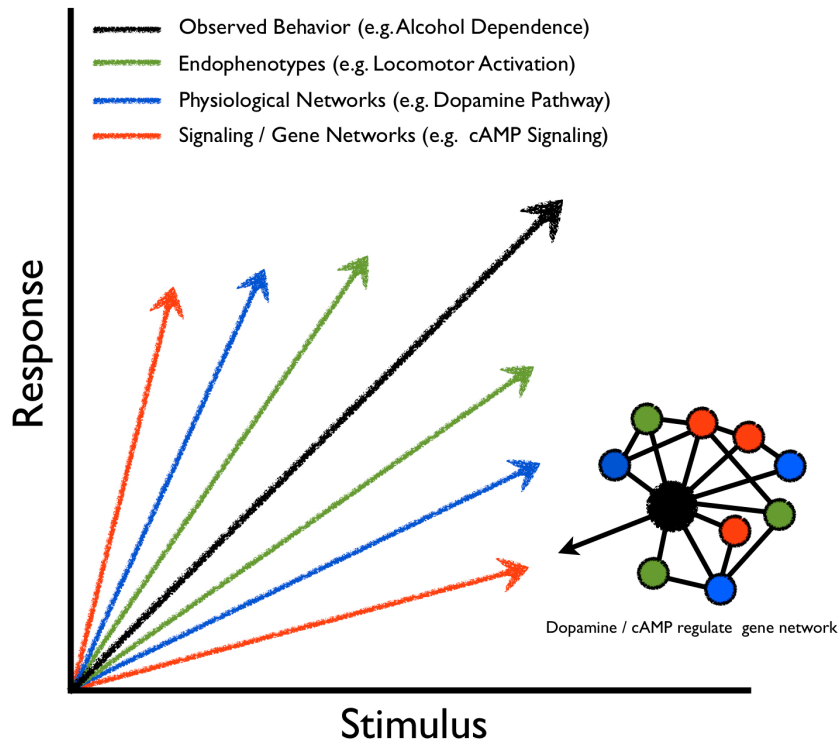


Figure 1. Diagram of multiple potential subcomponent vectors contributing to the observed behavioral phenomenon of alcohol dependence. The figure illustrates the premise that observed behavioral phenotypes studied in humans or animal models are actually made up of multiple behavioral subcomponents (e.g. endophenotypes) that are in turn controlled by neural networks. The gene networks derived by genomic studies are a syncytium of gene regulation events occurring within multiple cells comprising a given neural network.

requires a combined distillation of traits into endophenotypes and the amalgamation of brain regional gene function/expression into networks relevant to the trait vectors. Once the network structure of these varying endophenotypes is successfully mapped, we may be able to identify major genetic hubs for developing more rational pharmacotherapies in the treatment of alcoholism.

Genetic Contribution to Alcoholism and Ethanol-Related Behaviors in Humans

Alcohol abuse and dependence has been documented throughout history, dating back to Biblical time-periods and early Egyptian culture. The term 'alcoholism' is thought to be initially coined by Dr. Magnus Huss describing a chronic relapsing physiological condition attributed to the repeated ingestion of alcohol (Marcet, 1860). However, formal recognition of alcoholism as a disease is often attributed to Dr. Elvin Morton Jellinek in his published work *The Disease Concept of Alcoholism* (Jellinek, 1960). Recognition of alcoholism as a disease, rather than dismissed as problematic behavior or moral weakness, contributed to a need for the medicinal community to determine the underlying cause, and prompt pharmacological and psychological intervention.

The underlying factors causing alcoholism are unknown, but human research suggests 40-60% of the risks for the development of this condition are genetic in origin with the remaining factors due to environmental influences (Schuckit and Smith, 2006, Timberlake et al., 2007). Familial inheritance is a well-recognized factor in the risk of alcoholism (Cotton, 1979, Lieb et al., 2002), with early evidence suggestive of an 82% chance of disease co-occurrence within family members (Lucero et al., 1971). Distinguishing whether the trait was biologically inherited or a consequence of being

exposed to a shared hazardous environment is problematic when interpreting familial risk studies. Complementary studies in monozygotic and dizygotic twins; however, have also established a genetic component for alcoholism. Identical twins have higher rates of alcohol abuse and dependence compared with fraternal twins (Pickens et al., 1991). This is not due entirely to a shared environment as cross fostering studies of adopted identical siblings from an alcoholic parent into an the household of non-biological relative without substance abuse remain at a significantly increased risk of developing alcoholism (Cloninger et al., 1981). Collectively family, twin, and adoption-based studies suggest a sizable genetic component contributes to predisposition for the disease.

Discovering which genes are responsible or causal for an alcohol use disorder has been an area of intensive inquiry. One complication in the elucidation of risk alleles is the presence of comorbid mental health diseases; 37% of individuals with alcohol related issues also suffer from mental disorders such as schizophrenia and antisocial personality disorder (Regier et al., 1990). The common disease-common variant hypothesis has proposed the genetic risk of common diseases is due to the relatively increased frequency of common disease causing alleles (Lander, 1996). Heritability of alcoholism and related psychiatric conditions may thus be due in part to a collection of individual genetic variants of small effect size that have an additive effect in driving susceptibility of disease. For example, variants in the corticotropin-releasing factor system (*CRHR1* and *CRHBP*) are predictive of comorbid alcohol use disorder in schizophrenic patients (Ribbe et al., 2011). It is possible that no single or set of common characteristics may be necessary or sufficient in complex diseases across all

populations, and concerns have been raised regarding the 'common disease-common variant' model (Becker, 2004, Dickson et al., 2010). Although no single study can prove or refute common-disease-common variant disease model, large- and small-scale initiatives do jointly add to our knowledge base for unraveling the genetic architecture of multifaceted diseases including alcoholism.

The Collaborative Study on the Genetics of Alcoholism (COGA) was one of the first large-scale initiatives from treatment centers across the United States aimed at the identification of genes contributing to alcohol dependence. Linkage and genome-wide association studies from this collaborative and others have made progress in detecting common genetic variants conferring vulnerability or protective effects for an alcohol use disorder. These recognized variants are involved in multiple physiological systems: alcohol metabolism (i.e. alcohol dehydrogenase and aldehyde dehydrogenase enzymes), dopamine system, GABAergic system, glutamatergic system, opioid system, cholinergic system, serotonin system, and Neuropeptide Y signaling processes (Michael D. Kohnke 2008). Genetic loci related to alcoholism are spread out across the genome with evidence on chromosomes 1, 2, 6, 7, 10, 12, 14, 16 and 17 (Hill et al., 2004). Although several candidates have been identified some subsequent studies have failed replication. For example, Blum et al. initially reported allelic variation associated with alcoholism for the dopamine receptor *DRD2*, a key component of the mesocorticolimbic 'reward' pathway (Blum et al., 1990). But, further investigation into the *DRD2* gene has been controversial with regard to mixed results and phenotypic outcome measures (Kidd, 1993, Noble, 1998). Additionally, the *DRD2* is in strong linkage disequilibrium with *ANKK1* (ankyrin repeat and kinase domain containing 1), which is involved in numerous

signal transduction pathways. The close physical association of *DRD2* and *ANKK1* may thus make it difficult to discern which gene is responsible for driving the observable phenotype (Neville et al., 2004). Discovery and disentanglement of all the genetic-factors, including the relationship between *DRD2* and *ANKK1*, involved in an alcohol use disorder may further hinge on the investigation of genotype-environment interactions(Young-Wolff et al., 2011).

In spite of significant advancement in the areas of genetics and genomics it is a sobering reality that there is a clear lack in effective long-term pharmacotherapies for addiction (Goldman et al., 2005). Arguably expensive genomic inquiries concerning drug and alcohol abuse may be of low priority relative to other inherited diseases due to an individual's choice for continued consumption and heterogeneous etiology (Merikangas and Risch, 2003). Improvement in the identification of candidate genes and defining the causal mechanisms of action underlying disease progression may come from the integration across scientific disciplines. Animal studies offer a suitable extension for scientific inquiry into the underlying biology of substance abuse, offering a more homogenous genetic background, better-controlled environmental variables, and allow studies that are not necessarily ethical or amenable to human research.

Model Organisms for the Genetic and Genomic Analysis of Complex Traits

Model organisms offer an extraordinary window into the physiological and pathological processes governing complex traits. Animal models may not necessarily reflect the exact disposition shaping disease as whole, but certainly can model distinctive characteristics (or endophenotypes) underlying disease. Alcohol abuse and

dependence are human delineations of a psychiatric diagnosis. Model organisms lack the functional criteria for psychiatric diagnosis, but are able to mimic human conditions with two overall goals: (i) testing of mechanistic hypothesis and (ii) test and validate drug treatments (Bennett et al., 2006, Crawley, 2007).

The laboratory mouse, or *Mus musculus*, is one of the most important model organisms and an indispensable tool in medicinal research (Boguski, 2002). Completion of the human genome project (Lander et al., 2001, Venter et al., 2001) was swiftly followed with an initial draft of the mouse genome, based on the C57BL/6J strain that was developed in the 1920s, in order to connect clinical and basic research scientists through comparative genomics (Waterston et al., 2002). This initial sequencing has been complimented through the determination of subspecific origin, haplotype diversity, and identical by descent maps for 198 samples (including 100 classical strains) across three different subspecies of *Mus musculus* (Yang et al., 2011). Additionally, at the time of this dissertation, there are now at least seventeen different inbred strains of mice that have been fully sequenced, which are representative of the most commonly used laboratory strains (Keane et al., 2011). Such a tremendous amount of genetic diversity can help dissect phenotypic variation of complex traits within multiple homogeneous reference populations and controlled environmental contexts.

Mus musculus are an excellent genetic tool for the analysis of complex traits in medicinal research sharing nearly 99% of their genes with humans, which account for approximately 30,000 protein-coding genes (Waterston et al., 2002). Additionally, mice are a mammalian organism that have a comparable physiological profile to humans and share common inherited diseases such as diabetes and several neurological disorders

(Peters et al., 2007). Spontaneous mutations, chemical mutagenesis, or targeted deletion through homologous recombination, for the loss of function of specific genes within the mouse genome has provided significant insight into the function of individual genes and some of the underlying biological processes governing disease states (Paigen, 2003b, a). For example, single gene mutations in mice for the hormone leptin has uncovered some of the pathophysiological aspects managing body weight that are related to obesity (Zhang et al., 1994, Friedman and Halaas, 1998). Such experimental evidence continues to provide credence to the fact that there are genetic pathways perturbed by various diseases, which are biological in nature and beyond the naïve or misguided perspective of a lack in willpower or moral control.

Although genetically engineered mice have certainly provided discernible clues into the underlying biology of complex traits, approximately only 10% of all the genes within the mouse genome have been utilized in generating knockout mice (Austin et al., 2004). The lack of null alleles for a large portion of the mouse genome serves as an untapped resource that has led to the founding of 'The Knockout Mouse Project,' an ongoing high-throughput international effort to systematically construct knockout alleles for all mouse genes and place them into the public domain with accompanying phenotypic information (Austin et al., 2004). Certain null mutations can however result in embryonic-lethality or have deleterious effects on development and metabolism, but molecular techniques are continually being refined to incorporate temporal and spatial control over gene targeting strategies allowing for unprecedented precision in characterizing a variety of phenotypes, including mammalian brain function (Lewandoski, 2001). Deciphering the down-stream effects of null and conditional-allele

approaches, not to mention the amount of time and financial contribution required for such a task are well beyond the resources of any single collective or research group, requiring collaborative efforts on global scale (Grimm, 2006). The International Knockout Mouse Consortium was established to meet the growing needs of the research community in the post-genome era for genetic analysis of all protein-coding genes within the mouse genome and following phenotyping efforts (Skarnes et al., 2011). The availability of knockout mouse resources continues to expand through international collaboration and is poised to uncover the phenotypic impact of every gene within the mammalian genome (Guan et al., 2010). Ascertaining the shared relationship between the genome and phenome may very well promise to be next the hurdle to overcome (Houle et al., 2010).

Interpretation of the phenotypes, especially those related to behavioral research, which emerge due to single genetic null mutations should be examined with caution as phenotypic outcome measures may vary due to the background genotype (Sibilia and Wagner, 1995, Gerlai, 1996, 2001), compensatory mechanisms (Gerlai, 2001), and laboratory environment (Crabbe et al., 1999, Wahlsten et al., 2003). Careful evaluation of relevant individual endophenotypes in animal models concerning neuropsychiatric diseases including alcoholism are particularly challenging given the unknown etiology of psychiatric diseases and potential impact of multiple genes and environmental factors (Takao et al., 2007). Genetic variance among differing inbred strains is however markedly robust for several behavioral phenotypes (Crabbe et al., 1999) and can establish a baseline level of performance prior to any mutagenesis assays (Tarantino et al., 2000). Single gene mediated responses may be of small effect size and possibly be

obscured by background genotype if there is strong intra-strain variability for a particular response. Therefore, it is important to carefully consider which endophenotype and mouse strain is most appropriate for testing any hypothesis. Certainly no result can stand entirely on its own and consensus among different laboratories for behavioral results among mice carrying a specific null mutation is essential before concluding the impact of any single gene on a distinct behavioral domain (Crabbe et al., 1999, Tarantino and Bucan, 2000).

Complex traits such as neuropsychiatric disorders are multifaceted ailments of complex origin involving non-Mendelian modes of inheritance. The mouse has emerged as the preeminent model system for the dissection of complex traits (Rosenthal and Brown, 2007). Significant advances in the manipulation of the mouse genome and derivation of accompanying phenotypes have spurred large-scale collaborations for a systemic assault of every gene-phenotype relationship throughout the mammalian genome. The Mouse Phenome Database and EuroPhenome are just two examples of collaborative open-source repositories for helping capture some of the genetic-genomic complexities underlying complex traits using mouse models (Grubb et al., 2009, Morgan et al., 2010). Integrating findings from mouse models with human data as well as additional model organisms will assist in defining causal mechanisms of gene action. Model organisms allow investigators to circumvent some of the challenges faced in human studies such as uncontrolled environmental variables or the influence of genetic heterogeneity, and put disease-associated genes within a biological context (Aitman et al., 2011). Model organisms also offer the ability to discover novel intrinsic factors regulating genes and the potential manifestation of disease. For example,

microRNAs are short ribonucleic acids initially discovered in *Caenorhabditis elegans* that modulate gene regulation through complementary binding of a target genes messenger RNA (Lee et al., 1993). Following this discovery it is now known that hundreds of conserved and non-conserved microRNAs exist across species (Bentwich et al., 2005) and are involved in behavioral processes relevant to complex traits such as substance abuse (Kocerha et al., 2009, Hollander et al., 2010).

The genetic underpinnings of complex traits are amenable to functional evaluation and novel discovery through the use of model organisms. Environmental pressures and epistatic interactions may initially complicate the interpretation of genetic associations with varying endophenotypes; however, they are also fertile areas for basic science and translational research. It may not only be essential to consider the impact of individual gene effects on complex traits, but also the entire complement of genes throughout the genome across multiple different genetic backgrounds and experimental conditions (Chadman et al., 2009, Doetschman, 2009). Human and model systems are complementary approaches needed for effectively defining causal genetic associations with disease. Even in the post-genomic era uncovering and prioritizing candidate genes in the analysis of complex traits will require a plethora of genetically homogeneous and heterogeneous stocks, available mutants for suspected variants, and precise definition of reproducible quantitative traits (i.e. endophenotypes), which accurately model differing aspects of human disease (Bucan and Abel, 2002).

Mapping Quantitative Traits in Model Organisms for Disease

Physically mapping the human and mouse genomes has provided the necessary platform in comparative cross-species functional and genetics studies for understanding complex traits (Gregory et al., 2002, Keane et al., 2011). Although the identification of sequence variants driving quantitative traits has shown limited success, genetic mapping studies have demonstrated favorable agreement in the genetic architecture of complex traits between humans and model organisms (Flint and Mackay, 2009). A quantitative trait is a measurable difference in a continuous phenotypic distribution within a population that stems from variation in genetic and/or environmental influences (Abiola et al., 2003). Genetic variation may arise from several sources, including spontaneous mutations, chemical or genetic mutagenesis, and selective breeding strategies of inbred mice (Peters et al., 2007). Advantages and disadvantages exist for the separate approaches, but working in concert with one another provide exquisite insight into complex traits associated with human diseases (Belknap et al., 2001).

The chromosomal regions harboring genetic variants responsible for phenotypic variation are referred to as quantitative trait loci (QTL) (Geldermann, 1975, Lander and Botstein, 1989). Mapping quantitative trait loci begins with selectively mating two parental strains (usually demonstrating contrasting phenotypes of interest) to produce F₁ offspring, followed by F₂ progeny, and subsequent intercrosses of brother-sister matings for 20+ generations to establish a mosaic of recombinant inbred strains with a distinctive complement of the parental genomes (Figure 2) (Peters et al., 2007, Wu et al., 2010). Leveraging the genetic variation derived from selective breeding for a panel of recombinant inbred mice against resulting phenotypic variation can discern a chromosomal region containing the genetic variant associated with the defined trait

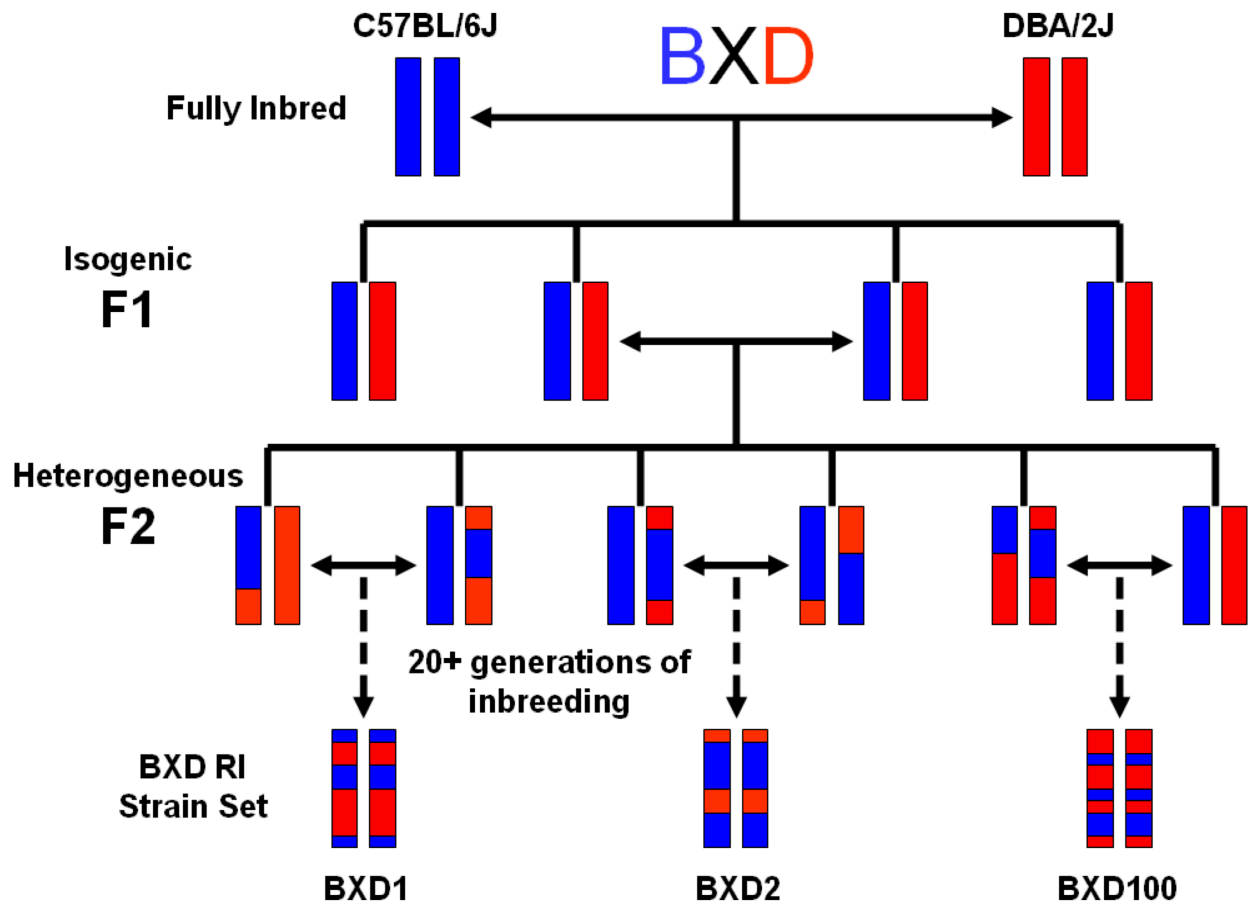


Figure 2. Schematic of BXD recombinant inbred mice. Illustration of the breeding strategy used to create BXD RI strains of mice. Two genetically distinct strains of mice (i.e. C57BL/6J and DBA/2J) are mated to create an isogenic F1 generation, consisting of 50% of each progenitor. F1 mice are then used to generate a heterogeneous F2 population, then selectively inbred for 20+ generations to create a mosaic of individual BXD lines carrying a unique genomic combination of the two progenitor strains.

(Darvasi, 1998). Distinguishing a significant QTL amid competing genetic and environmental factors relies on the statistical association of sufficiently divergent trait data and genetic marker information (Tanksley, 1993, Belknap and Atkins, 2001, Doerge, 2002, Henderson et al., 2004). Scale of the experiment and the density of DNA markers determine the size of the genomic region beneath a defined QTL interval, which can span hundreds of genes depending on the number of recombination events previously occurring in a defined region (Mackay, 2001). Multiple interacting genetic loci may be involved in complex traits, complicating the associating between the polymorphic alleles and phenotypic variation (Glazier et al., 2002, Ehrenreich et al., 2010). Behavioral traits in particular, may be influenced by multiple genetic variants of small effect size, and not always attributed to an individual quantitative trait locus (Plomin, 1990). However, QTLs can be replicated across independent studies for behavioral traits (Belknap and Atkins, 2001, Henderson et al., 2004). In the absence of potential epistatic interaction, improved phenotypic resolution for a single QTL may be obtained through the use of reciprocal interval-specific congenics, which are genetically identical to their respective progenitor strain except at the suspected QTL interval (Flaherty, 1981, Bennett, 2000). Narrowing the implicated QTL region to a quantitative trait gene (QTG) poses a significant challenge for further characterizing phenotypic differences at the molecular level (Flint et al., 2005). Integrating conventional QTL strategies with differential gene expression and directed sequencing may assist in the identification of QTGs (Wayne and McIntyre, 2002, Hitzemann et al., 2003). Following candidate gene identification molecular signatures (i.e. QTGs) of phenotypic variance

can be further dissected using null mutations or transgenic animal models, as well as pharmacological interventions (Hoffman and Tabakoff, 2005).

Candidate genes or candidate loci derived from model organisms furthers the identification of genetic variants in human diseases. Human and mouse genomes have genes ordered in a syntenic fashion, occurring in the same order in the differing species (Pennacchio, 2003, Peters et al., 2007). Less than 1% of the genes within the mouse and human genome lack a clear homologue between the two species (Okazaki et al., 2002, Waterston et al., 2002). The BXD recombinant inbred mouse lines, derived from C57BL/6 and DBA/2 mice, and the LXS recombinant inbred mouse lines, derived from Inbred Long Sleep (ILS) and Inbred Short Sleep (ISS) mice, have been widely used as reference populations to identify QTLs associated with alcohol behavioral phenotypes (Johnson et al., 1992). Additional QTL evidence for alcohol-related behavioral traits has been garnered through a multiple cross-mapping strategy from a panel of inbred strains (Hitzemann et al., 2002, Malmanger et al., 2006). Mouse QTL information can be viewed alongside human linkage data for alcohol related endophenotypes to interrogate consilience across species and further the identification of putative QTGs (Uhl et al., 2008, Ehlers et al., 2010). Syntenic mapping of mouse and human data for alcohol associated traits has identified two examples of concordance and suggested the inwardly-rectifying potassium channel subunit *Kcnj9* as a high-priority candidate gene for alcohol-related responses (Ehlers et al., 2010). A null mouse mutant for *Kcnj9* exhibits less withdrawal signs from sedative-hypnotic agents, including alcohol, suggesting it may be a QTG for alcohol withdrawal syndrome (Kozell et al., 2009).

The multiple PDZ domain protein (*Mpdz*) has been formally identified as a quantitative trait gene for alcohol withdrawal severity (Shirley et al., 2004). C57BL/6 (B6) and DBA/2 (D2) mice display contrasting acute ethanol withdrawal behaviors (Crabbe et al., 1983). Genetic mapping studies using B6 and D2 mice as progenitors identified four chromosomal loci influencing acute alcohol withdrawal severity, including on mouse chromosome 4 (Buck et al., 1997). Acute alcohol withdrawal scores within this genetic population were significantly correlated to a subsequent study on pentobarbital withdrawal, suggesting common genetic factors underlie these drug-induced behavioral phenotypes (Buck et al., 1999). Additional evidence identified a QTL on chromosome 4 using an F₂ intercross between B6 and D2 mice (B6D2F₂) for chronic alcohol withdrawal severity (Buck et al., 2002). Congenic animals fine-mapped the QTL for withdrawal severity to a < 1-centimorgan interval on chromosome 4, with *Mpdz* being recognized as virtually the only gene within this region showing variation in sequence and/or gene expression (Fehr et al., 2002). Additionally, *Mpdz* co-segregated with acute alcohol withdrawal severity in mice selectively bred for this phenotypic difference (Fehr et al., 2004). The QTL on chromosome 4 was further fine-mapped using six additional interval-specific congenic strain, leaving *Mpdz* as the only candidate displaying genotype-dependent differences in coding sequence and/or expression, also witnessed at the protein level; confirming *Mpdz* as a QTG for alcohol withdrawal severity in mice (Shirley et al., 2004). Although the function of *Mpdz* in alcohol withdrawal remains to be elucidated, the phenotype is thought to manifest through the interaction of *Mpdz* with serotonin 5-HT_{2c} and gamma-amino butyric acid receptor B (GABA_B) (Shirley et al., 2004, Milner and Buck, 2010).

Genotyping of two human populations combined with a genetical-genomics analysis of HXB/BXH recombinant inbred rat strains identified variation GABAergic pathways for alcohol consumption (Tabakoff et al., 2009). Polymorphisms in *MPDZ* were also significantly associated with alcohol intake, which may suggest a signaling pathway involving MPDZ and GABAergic signaling mechanisms influence alcohol-drinking behavior (Tabakoff et al., 2009). Sequence variants in *MPDZ* were also associated with alcohol dependence in European Americans, but not alcohol withdrawal symptoms (Karpyak et al., 2009). Convergent evidence from *Mus musculus*, *Rattus norvegicus*, and *Homo sapiens* suggests genes involved in alcohol associated endophenotypes within animal models, such as *Mpdz*, are relevant risk factors for alcoholism in human populations. Continued insights into the genetic underpinnings of human diseases will emerge through the integration of existing datasets and genetical genomics approaches combined with novel genetically diverse reference populations such as the Collaborative Cross (Churchill et al., 2004). The pleiotropic effects of single genetic variants also necessitates the need for an extensive collection of carefully defined phenotypes (Houle et al., 2010).

Neuropharmacology of Acute and Chronic Alcohol Exposure

Ethanol readily distributes into total-body water, exhibiting it's pharmacological properties within multiple biological compartments and organ systems including most notably the central nervous system. A number of molecular and neurobiological pathways have been implicated in acute and chronic responses to ethanol. In comparison with other drugs of abuse, such as cannabinoids and opioids, ethanol does

not have a clearly defined receptor or binding profile. The biophysical properties of ethanol result in poor reactivity and low potency preventing many pharmacological techniques from assessing molecular interactions as putative drug targets for ethanol (Lovinger and Crabbe, 2005). *In vitro* and *in vivo* evidence from model systems has helped delineate some of the major direct and indirect sites of ethanol action including, but not limited to, the GABAergic system, glutamatergic system, opioid system, and the mesocorticolimbic dopaminergic system, as well as a host of intracellular signaling pathways. Changes within these target systems facilitate the acute reinforcing effects of ethanol and the molecular adaptations causing tolerance and dependence following repeated exposure.

The neurobiology of substance abuse has long suspected vulnerability within the mesocorticolimbic dopaminergic pathway in the development and maintenance of addiction (Koob and Nestler, 1997, Koob and Volkow, 2010). Exposure of the dopaminergic system to ethanol and other drugs of abuse reduces the threshold for electrical stimulation associated with reinforcement (Olds and Milner, 1954, Wise, 1996). The ventral tegmental area (VTA), nucleus accumbens (NAC), and prefrontal cortex (PFC) constitute the three major brain structures within this reinforcing dopaminergic neural pathway (Nestler, 2001). In support of premise that ethanol exerts its effects on the mesocorticolimbic system, it has been shown that acute low-dose ethanol administration to unanesthetized rats increases the firing rate of dopaminergic neurons and the release of dopamine within the mesolimbic pathway, whose release is often associated with rewarding stimuli (Gessa et al., 1985, Di Chiara and Imperato, 1988). Additionally, ethanol is readily self-administered into the ventral tegmental area

by rats selectively bred for alcohol preference (Waller et al., 1984, Gatto et al., 1994). Self-administration of ethanol, but not water or total fluid intake, is decreased in rats following site-specific delivery of the dopaminergic antagonist fluphenazine into the nucleus accumbens (Rassnick et al., 1992). Similarly, the oral ingestion of ethanol (1 ml/kg) in humans leads to reduced binding of the dopamine receptor ligand [¹¹C] raclopride within the ventral striatum (Boileau et al., 2003), which is suggested to correlate with the euphoric effects of dopamine release (Drevets et al., 2001). The availability of dopamine receptors within ventral striatum of humans and mammalian models, and other brain structures such as prefrontal cortex, may therefore regulate the consumption and subjective effects of ethanol (Hodge et al., 1997, Yoder et al., 2005). Although the midbrain dopamine system may serve as a point of convergence for multiple drugs of abuse, activation of this reward circuitry can occur via disparate molecular mechanisms (Nestler, 2005). Acute ethanol acts as a positive allosteric modulator of GABA_A receptors (Ticku et al., 1986, Allan and Harris, 1987), which may facilitate the loss of inhibitory control over dopaminergic tone (Mereu and Gessa, 1985, Diana et al., 1993). GABA_A receptors are ligand-gated ion channels composed of multiple subunits classes: $\alpha(1-6)$, $\beta(1-3)$, $\gamma(1-3)$, δ , ϵ , θ , π , and $\rho(1-3)$ (Olsen and Sieghart, 2008, 2009). The heteromeric subunit composition of GABA_A receptors substantially effects ethanol sensitivity over a wide range of concentrations, but in general ethanol is capable of enhancing GABAergic neurotransmission at low to moderate intoxicating concentrations (3-30 mM) (Lobo and Harris, 2008). Chronic exposure to ethanol differentially alters GABA_A receptor subunit gene expression (Mhatre and Ticku, 1992) and can impair ion channel function at blood ethanol concentrations associated with withdrawal syndrome

(Morrow et al., 1988). Alterations in the function and distribution of GABA_A receptor subunits likely underlies the manifestation of tolerance and dependence; however, assessing the contribution of individual subunits on ethanol-associated behaviors is best achieved through genetically modified mouse models.

Variation in a cluster on chromosome 4 of GABA_A receptor subunits (GABRG1, GABRA2, GABRA4, and GABRB1) are associated with alcohol dependence and endophenotypes related to neural excitation; the most prominent and reproducible association was detected for the GABRA2 subunit (Edenberg et al., 2004). A null mutation for *Gabra2* in mice cause a decrease in the sedative-hypnotic properties of ethanol, decrease in acute ethanol withdrawal, and decreased ethanol preference (Boehm et al., 2004c). Knock-in of *Gabra2* that retains normal function, but are insensitive to ethanol-induced potentiation, have altered ethanol-intake and increased sensitivity to ethanol-induced sedation without affecting ethanol-induced motor incoordination (Blednov et al., 2011). Ethanol insensitive mutants for *Gabra1*, the most abundant alpha subunit (Kralic et al., 2002), do not show any alterations in ethanol drinking behavior, sedation, locomotor stimulation or cognitive impairment (Werner et al., 2006). The coordinate action of ethanol on specific behavioral processes is thus related to different GABA_A receptor subunits, as well as non-GABergic targets that may indirectly influence GABA-mediated responses (Kumar et al., 2009). Although GABA_A receptors are influential targets of ethanol action, the molecular binding site(s) of ethanol for the different subunits and the specific behavioral responses mediated by GABA_A receptor subunits and non-GABergic targets warrant further investigation (Crabbe et al., 2006b, Addolorato et al., 2011).

The neurotransmitter γ -aminobutyric acid (GABA) is synthesized by glutamate decarboxylase from the precursor glutamate (Walker, 1983). There are both ionotropic and metabotropic glutamatergic receptors present within the mammalian central nervous system with more than 20 glutamate receptors having been identified (2011, Harvey and Shahid, 2011). *N*-methyl D-aspartate (NMDA) is one of the main ionotropic glutamate receptors comprised of a heteromeric assembly of genes encoding NR1, NR2A-NR2D, and NR3A-NR3B subunits (Stephenson et al., 2008). NMDA receptor function is attenuated in a concentration-dependent manner by physiological relevant amounts of ethanol (Lovinger et al., 1989). Site-directed mutagenesis assays suggest ethanol is a non-competitive partial antagonist that may interact within the third transmembrane domain of the NR1 subunit (Ronald et al., 2001); however, ethanol most potently inhibits NR2A and NR2B containing receptors (Woodward, 2000, Smothers et al., 2001). Prolonged exposure to ethanol in cultured neurons and animal models alters the expression of NR2A-B receptor subunits and ion-channel function, suggesting neuroadaptive changes in this system that may compensate for the attenuating effects of ethanol (Snell et al., 1996, Kalluri et al., 1998, Chandler et al., 1999).

Animals will readily substitute NMDA receptor antagonists for ethanol in discriminative stimulus paradigms (Shelton and Balster, 1994), which occurs due to the involvement of NMDA receptors within the nucleus accumbens, and interactions with other brain structures (Hodge and Cox, 1998). The neural circuitry underlying substance abuse suggests the dorsal striatum is an important brain region that is associated with the nucleus accumbens and additional brain regions such as the ventral tegmental area

(Volkow et al., 2011). Dorsal striatum is involved in goal-directed actions and formation of habitual behavior, aiding in the progression of addiction (Everitt and Robbins, 2005, Yin and Knowlton, 2006). Repeated exposure to ethanol leads to a long-lasting increase in the activity of NR2B containing receptors within dorsomedial striatum, which if inhibited using the NR2B selective antagonist ifenprodil, causes a significant reduction in ethanol self-administration and seeking behavior (Wang et al., 2010). Ifenprodil can also lessen the severity of ethanol withdrawal and the acute sedative-hypnotic effects of ethanol (Malinowska et al., 1999), suggesting NR2B-containing receptors as prominent sites of ethanol action. These pharmacological studies are partially supported by a conditional knockout for NR2B in mouse forebrain, where mice carrying the null mutation are more sensitive to the sedative-hypnotic properties of ethanol without differing in their pharmacokinetic profile (Badanich et al., 2011). The acute intoxicating effects of ethanol relative to NMDA receptor inhibition are however subunit specific, as a null mutation for NR2A does not significantly alter ethanol-induced sleep time (Boyce-Rustay and Holmes, 2005). The ensuing downstream effects elicited by ethanol administration may thus act via a complex, yet selective system of ionotropic receptors normally managed by selective neurotransmitters such as glutamate and γ -aminobutyric acid under physiological conditions.

In addition to modulating the biochemical and behavioral properties of neurotransmitter systems, acute and chronic ethanol augments the function of endogenous neuropeptides, endorphins, and enkephalins. Endogenous opioids, acting on their corresponding receptors, are one of the mitigating factors associated with ethanol-induced reward and continuous self-administration (Vengeliene et al., 2008).

The opioid receptor antagonist naltrexone slows the acquisition of ethanol drinking behavior (Phillips et al., 1997), reduces ethanol self-administration in rodents (Altshuler et al., 1980, Middaugh et al., 2000), and is a prescribed pharmacotherapy that decreases habitual drinking among high-level consuming alcoholics (McCaul et al., 2000). A genetic variant in the μ -opioid receptor is significantly associated with naltrexone response among alcohol dependent subjects (Oslin et al., 2003). Interestingly, μ -opioid receptor mice knockout mice do not readily self-administer ethanol under multiple different paradigms (Roberts et al., 2000), and the human equivalent of the μ -opioid receptor variant leads to enhanced ethanol drinking behavior in non-human primates (Barr et al., 2007).

The κ -opioid and δ -opioid receptors are two additional internal substrates for endogenous opioids, whose cellular signaling can be affected by acute and chronic ethanol exposure. Dynorphin is an endogenous opioid peptide that primarily mediates its psychoactive effects through κ -opioid receptors (Chavkin et al., 1982). Genetic deletion or pharmacological inhibition of dynorphin show an increased response for ethanol-induced conditioned place preference, suggesting κ -opioid receptors may exhibit a negatively regulatory tone for ethanol reward like behavior (Nguyen et al., 2012). Direct activation of the kappa opioid receptor system using a kappa agonist, such as endolin or bremazocine, leads to decreased ethanol self-administration in rodents and rhesus monkeys (Holter et al., 2000, Cosgrove and Carroll, 2002). Ethanol-drinking behavior, however, is increased δ -opioid receptors knockout mice (Roberts et al., 2001). Selective agonism of δ -opioid receptor subtypes can also augment the

rewarding properties of ethanol (van Rijn et al., 2011), and attenuate anxiety-like behavior (van Rijn et al., 2010). Collectively, the opioid receptor system encompassing μ -, κ -, and δ -opioid receptors plays an extensive role in mediating several ethanol behavioral phenotypes; especially those phenotypes interconnected with ethanol drinking behavior (Camarini et al., 2010). Pharmacological intervention targeting the opioid receptor system may thus be of crucial importance in the armamentarium against an alcohol use disorder (Hillemacher et al., 2011), with consideration for the opposing roles of each opioid receptor subtype and any potential interacting druggable targets (Kroeze and Roth, 2006).

The acute behavioral responses and enduring neuroadaptations occurring in response to a substance of abuse is initiated by multiple extracellular systems such as the dopaminergic and opioid system, but is ultimately achieved through a series of intracellular signaling cascades and transcriptional events (Koob and Nestler, 1997, Robison and Nestler, 2011). There is substantial heterogeneity in the molecular transducers required in order to facilitate the psychoactive effects of ethanol and other drugs of abuse; however, a few key targets have emerged as the principle focus in the short- and long-term neurobiology of ethanol (Nestler, 2005, Ron and Jurd, 2005, Heilig et al., 2011). The cyclic adenosine monophosphate response element binding protein (CREB) is a stimulus-inducible transcription factor that regulates the expression for a large abundance of genes within the nervous systems (Lonze and Ginty, 2002, Conkright et al., 2003). Acutely ethanol can lead to increased expression and phosphorylation of CREB (Yang et al., 1996, Asyyed et al., 2006). Conversely, chronic ethanol can cause the opposite effects on CREB phosphorylation and downstream

factors (Yang et al., 1998a, Yang et al., 1998b, Pandey et al., 2001). The opposing roles of acute and chronic ethanol on CREB activation may suggest the transcription factor is acting as a molecular switch in the predisposition and development of alcohol dependence. For example, CREB haplo-insufficient mice display increased anxiety-like behavior and increased ethanol preference (Pandey et al., 2004). Haplo-insufficiency for CREB also results in corresponding decreases in the expression of neuropeptide Y (NPY) and brain-derived neurotrophic factor (BDNF) (Pandey et al., 2004). Reduced expression of either NPY or BDNF causes increased ethanol consumption (Thiele et al., 1998, McGough et al., 2004), suggesting CREB activation and subsequent transcription of select downstream targets may be a pathophysiological mechanism governing ethanol intake patterns.

Upstream of CREB are multiple protein kinases important in ethanol behavioral and molecular processes, that promote CREB-mediated transcriptional events (Moonat et al., 2010). Among several others, cAMP-dependent protein kinase (also known as PKA) is a prominent regulator of CREB activation through phosphorylation of serine-133; blockade of which abolishes transcriptional activity (Gonzalez and Montminy, 1989). In order to initiate PKA signaling events ethanol induces production of the second messenger cyclic adenosine 3' 5'-monophosphate (cAMP) through adenylyl cyclases coupled to stimulatory guanine nucleotide binding proteins (Sutherland et al., 1962, Coles, 1994, Diamond and Gordon, 1997). Nine different isoforms of adenylyl cyclase have been identified in mammalian species (Hanoune and Defer, 2001). Comparatively, type VII adenylyl cyclase is 2-3 times more sensitive for cAMP production in response to intoxicating amounts of ethanol (Yoshimura and Tabakoff,

1995, 1999). Increases in cAMP causes the release of PKA regulatory subunits, exposing the catalytic subunits that phosphorylate neighboring proteins (Mochly-Rosen, 1995). Activation of PKA also causes translocation of the catalytic subunit into the nucleus, triggering CREB and nuclear dependent gene expression (Dohrman et al., 1996). Repression of the cAMP dependent kinase pathway via pharmacological or genetic inhibition of PKA significantly alters acute ethanol sensitivity and drinking behavior (Moore et al., 1998, Wand et al., 2001).

The multiple points of convergence between ethanol and the cAMP-PKA-CREB pathway provides strong evidence it is a major signaling cascade involved in the molecular and behavioral processes associated with alcoholism (Ron and Jurd, 2005). However, substantial cross-talk exists among intracellular signaling mechanisms perturbed by ethanol exposure, which makes it exceedingly difficult to elucidate the precise machinery and develop targeted pharmacotherapies for substance abuse (Lee and Messing, 2008). Pharmacotherapies aimed at disrupting protein kinases is active area of CNS drug development, but lags behind other disease oriented fields such as cancer (Chico et al., 2009). Therapeutic potential exists for both single- and multi-targeted kinases inhibitor, which one day could exploit the entire complement of kinases throughout the genome (Sebolt-Leopold and English, 2006). Additionally, kinase inhibitors already approved by the Food and Drug Administration for oncology and other disorders may provide important information in developing therapies for an alcohol use disorder. Through inhibition of a serine/threonine kinase known as mTORC1 (mammalian target of rapamycin complex 1) the immunosuppressant rapamycin, also known as sirolimus, decreases ethanol seeking behavior, conditioned place preference,

and consumption in a preclinical animal model (Neasta et al., 2010). Inhibition of this particular pathway is not associated with altered motivation states (Neasta et al., 2010, Neasta et al., 2011), and is well tolerated in humans without any cognitive deficits (Lang et al., 2009).

A horde of extracellular and intracellular targets are responsible for the physiological and behavioral effects of ethanol (Harris et al., 2008). However, the pathophysiology underlying alcohol abuse and alcoholism is still largely unknown and much of the published research has presented a spherical cow of a very complex psychiatric condition. Understanding how the multitude of receptors and signaling pathways interact in the neuropharmacology of acute and long-term ethanol exposure may rest upon a systems biology approach (Spanagel, 2009, Farris et al., 2010). Systems biology utilizes advancements in molecular biology, mathematical modeling, and technology in order to simultaneously examine all the working elements within a functioning biological system (Ideker et al., 2001). Deciphering the working environment of cellular systems, including the neurobiological factors perturbed by ethanol, is possible through the use of high-throughput genomic and proteomic strategies paired with computational tools and large-scale, systematic scientific efforts (Lockhart and Winzeler, 2000, Tyers and Mann, 2003). The blending of computational approaches with modern techniques for both spatial and temporal control of cellular biology will ultimately shape our comprehension of synaptic plasticity, and neural networks governing behavior (Kotaleski and Blackwell, 2010). Such blending will also integrate information from a diverse set of mammalian brain cells (Okaty et al., 2011) and structural abnormalities intrinsic to individual differences in human behavior (Kanai and Rees,

2011). Guarded with the existing knowledge across multiple biological systems translational research will help advance the development of personalized treatments for alcoholism and other psychiatric disorders (Goldman et al., 2005, Heilig et al., 2011).

Neurobiology of Myelin and Animal Models of Demyelination

The central nervous system is fundamentally wired through the extensive interconnections of neurons and neuronal synapses; however, glial cells, which occupy more than half of the volume of a mammalian brain, are essential elements in brain development, structure, function, and disease (Barres, 2008, Eroglu and Barres, 2010). Astrocytes, oligodendrocytes, and microglia are the three major glial cell-types within the central nervous system, each serving specialized responsibilities (Haydon, 2001). Oligodendrocytes are the myelin-forming glial cells of the CNS that follow a distinct lineage classified by differences in proliferation, migration capability, morphology, and the presence of cellular markers (Rowitch and Kriegstein, 2010). A single mature oligodendrocyte can ensheath as many as 50 separate neuronal axons in a lipid-rich membrane (Peters and Proskauer, 1969). Lipid-rich membrane ensheathments form the 'white matter' of the brain, capable of axo-glial communication and CNS plasticity (Karadottir et al., 2005, Micu et al., 2006, Fields, 2010). The majority of white matter (~70-75% of its dry weight) are lipids such as cholesterol, galactosylceramide, and sulfatide (Gielen et al., 2006); however, hundreds of proteins have been characterized within myelin fractions of mice and humans that may be important in the pathology of myelin-related diseases (Ishii et al., 2009).

Myelin is the most abundant membrane structure in the CNS, which has evolved in concert with vertebrate physiology (Baumann and Pham-Dinh, 2001). Axon-dependent signals promote proliferation and survival of mature oligodendrocytes to match the surface area required for myelination (Barres and Raff, 1999). Following maturation oligodendrocytes extend processes that tightly spiral around neuronal axons in 150-200 μm segments (Bunge, 1968), extruding cytoplasm to form compacted multi-lamellar myelin layers (Bauer et al., 2009). Myelin forms near concentric layers, characterized by the presence of major and minor dense (also known as intraperiod) lines, increasing transverse resistance and decreasing capacitance that allows for the efficient communication of action potentials between neurons along nodes of Ranvier (Figure 3) (Poliak and Peles, 2003).

Facilitated by myelin the formation of nodes of Ranvier is under strict developmental control, causing an abundant clustering of Na^+/K^+ ATPases, $\text{Na}^+/\text{Ca}^{2+}$ exchangers, and Na^+ voltage-gated channels along short regularly spaced axonal intervals (Waxman and Ritchie, 1993). Although not fully elucidated this axonal-glia interface is possible through the interaction of several cell-surface adhesion molecules and the specialized function of multiple individual myelin proteins (Sherman and Brophy, 2005). Myelin-associated glycoprotein (*Mag*) is a member of the immunoglobulin family that mediates neuronal-oligo interaction, promoting the formation and maintenance of myelin in the CNS (Schachner and Bartsch, 2000). Once myelin biogenesis is initiated precise sorting and trafficking of distinct protein-lipid interactions occur within the intracellular space of oligodendrocytes (Maier et al., 2008). Myelin basic protein (Mbp) and proteolipid protein (Plp1) constitute the two most predominant myelin proteins

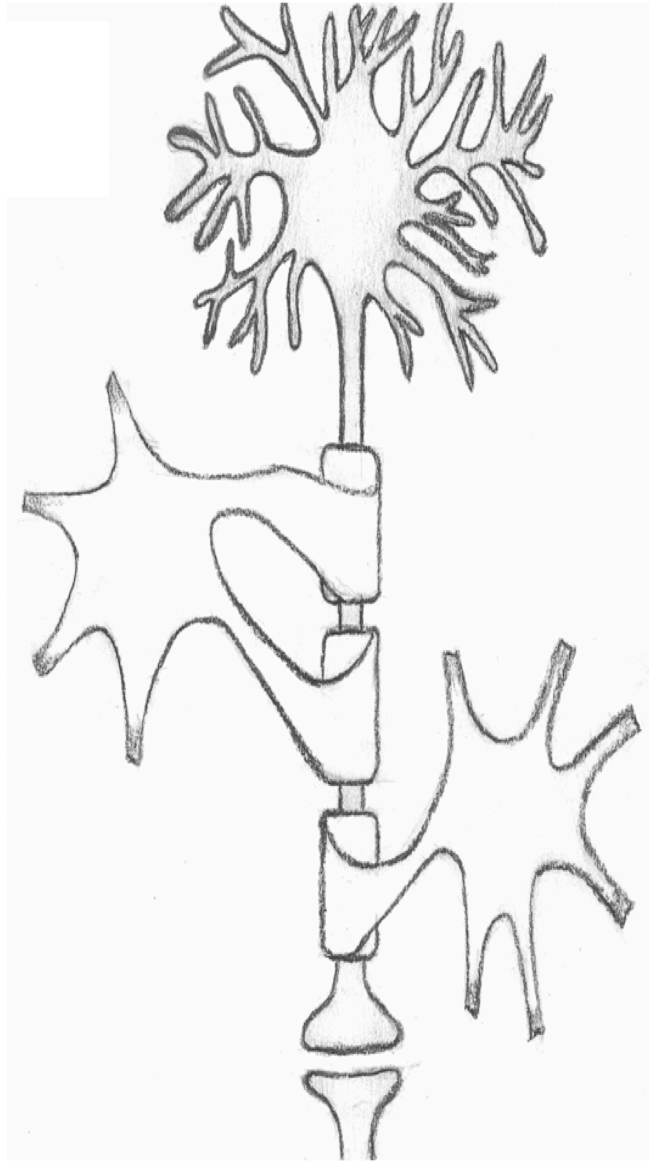


Figure 3. Pictorial of myelinated axon. Oligodendrocytes ensheathing a neuronal axon, permitting the efficient transmission of action potentials along Nodes of Ranvier.

within the CNS (Jahn et al., 2009), which are spatially and temporally regulated through different intracellular mechanisms for the proper assembly of myelin sheaths (Pfeiffer et al., 1993). Mbp and Plp1 are ubiquitously expressed throughout the mammalian brain to facilitate the formation of compact myelin sheaths (Figure 4) (Lein et al., 2007).

Proteolipid protein is the most abundant myelin protein in the CNS, representing approximately 50% of total CNS myelin protein (Nave et al., 1987). Interspersed intracellular and extracellular loops for proteolipid protein (Plp1) are involved in the formation of the intraperiod line through the proper apposition of myelin membrane extracellular surfaces (Popot et al., 1991). Proteolipid protein is synthesized by processes associated with the secretory pathway, which coordinates many of the intracellular events during myelin biogenesis (Lin and Popko, 2009). Pathophysiology studies have demonstrated mutant forms of proteolipid protein cause retention within the endoplasmic reticulum, activating the unfolded protein response, and eventually leading to apoptosis (Dhaunchak and Nave, 2007). Proteolipid protein associates with galactosylceramide and cholesterol to form proteolipid rafts within the Golgi, which may be important for intracellular trafficking and formation of the myelin sheath (Simons et al., 2000). Palmitoylation of the N-terminal region of proteolipid protein may also be important for stabilizing protein-lipid interactions, and is necessary for transporting proteolipid protein to the myelin membrane (Schneider et al., 2005).

Myelin basic protein is the second most abundant CNS protein, accounting for 30% of total CNS myelin protein (Kamholz et al., 1986). Although multiple isoforms exist, the two major MBP isoforms are 18.5 kDa and 14 kDa in size, for humans and

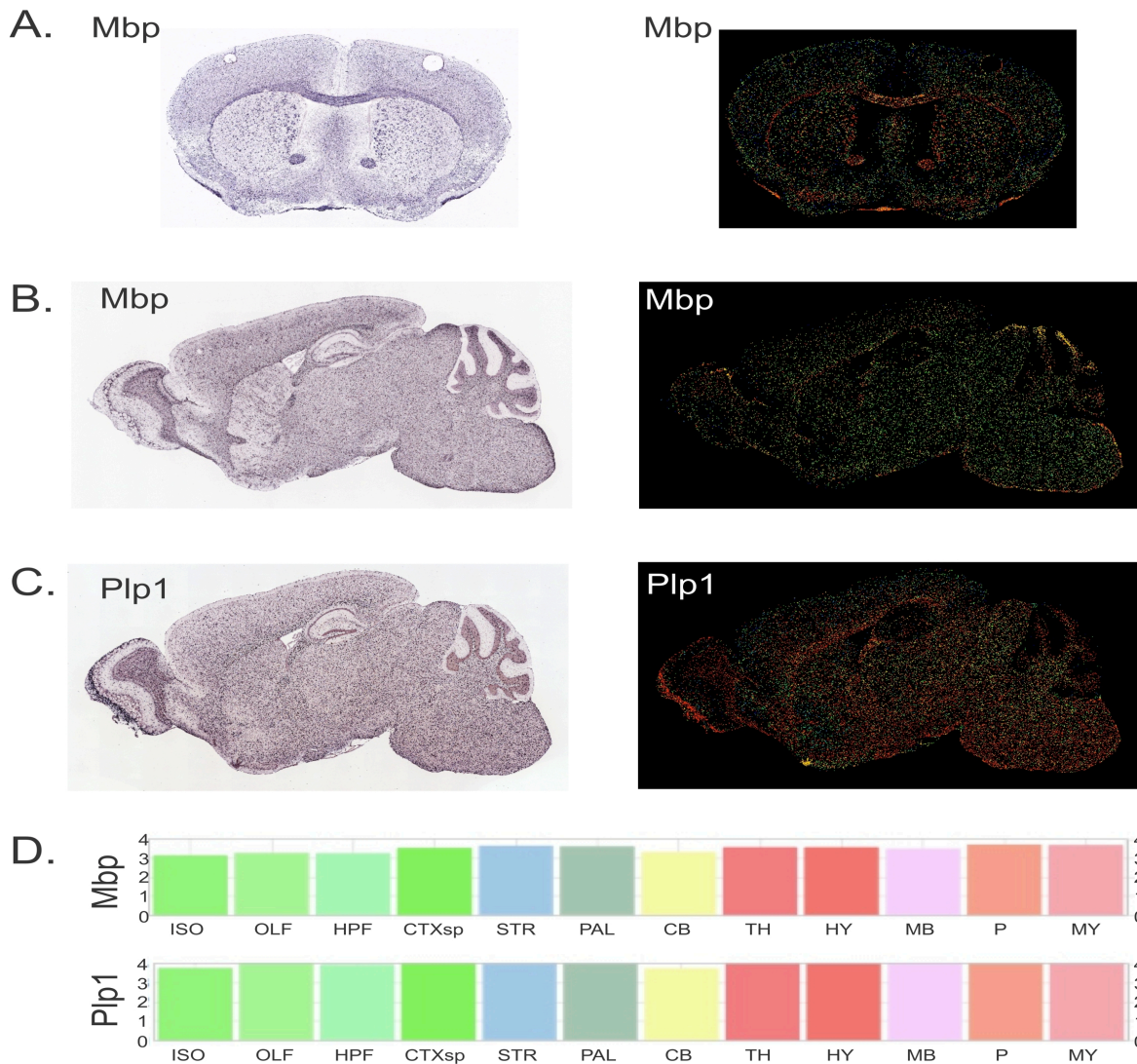


Figure 4. Myelin Basic Protein (*Mbp*) and Proteolipid Protein (*Plp1*) mRNA expression in mouse brain. (A) In situ hybridization of *Mbp* in a coronal section of mouse brain. (B) In situ hybridization of *Mbp* in a sagittal session of mouse brain. (C) In situ hybridization of *Plp1* in a sagittal session of mouse brain. (D) Relative expression of *Mbp* and *Plp1* in different brain regions, showing abundant and near uniform myelin expression throughout the brain (ISO = isocortex, OLF = olfactory areas, HPF = hippocampal formation, CTXsp = cortical subplate, STR = striatum, PAL = pallidum, CB = cerebellum, TH = thalamus, HPY = hypothalamus, MB = midbrain, P = pons, MY = medulla). Data available through <http://www.brain-map.org/>.

rodents respectively (Boggs, 2006). Myelin basic protein mRNA, as well as closely related myelin genes such as myelin oligodendrocyte basic protein (*Mobp*), are trafficked to distal cellular processes by heterogeneous ribonucleoproteins that bind to the three-prime untranslated region (3'-UTR) of the myelin mRNA (Raju et al., 2008); allowing for *Mbp* to be locally translated into protein at the cell surface. Guiding *Mbp* mRNA to the membrane for protein synthesis prevents unfavorable adhesion to non-myelin intracellular membranes and increases interactions with the negatively charged polar head groups of lipids at sites of active myelin biogenesis (Barbarese et al., 1999). Binding of myelin basic protein to lipid-rich moieties enables the compaction of opposing cytoplasmic membrane surfaces, forming the major dense line within the myelin sheath (Readhead et al., 1987). Presence of the major dense line is essential for compact myelin and thus MBP has been referred to as the executive molecule of CNS myelin (Moscarello, 1997).

Shiverer (*shi /shi*) mice carry an autosomal recessive mutation for the MBP gene, that is caused by genomic recombination on chromosome 18 leading to deletion of a 20-kilobase region within the MBP gene (Molineaux et al., 1986). Behaviorally shiverer mice are characterized by generalized tremors, abnormal gait, and seizure activity (Chernoff, 1981). Depending on the genetic background, shiverer mice have a shortened lifespan between 50 and 100 days (Readhead and Hood, 1990). Mice bearing this mutation fail to assemble compact myelin and exhibit a CNS hypomyelination (Rosenbluth, 1980). Similarly, Long Evans shaker rats harbor a mutation in the MBP causing a dysmyelinating disorder (Carre et al., 2002). Abnormal molecular and behavioral phenotypes are reversed in transgenic mice expressing a

single wild-type MBP gene (Readhead et al., 1987, Kimura et al., 1989). Additionally, the shiverer phenotype can be introduced using an anti-sense MBP transgene (Katsuki et al., 1988), suggesting a central role for MBP in compact myelin. Deficits in myelin basic protein do not result in oligodendrocyte degeneration or axonal loss; however, deficits can lead to aberrant axonal cytoskeletons and impaired conduction velocities (Brady et al., 1999, Nave, 2010).

Mutations in proteolipid protein can also cause CNS hypomyelination and adversely affect the physiology of humans and other vertebrates (Duncan, 2005). Myelin-deficient rats do not express proteolipid protein reduced white matter, generalized tremors, and eventually premature death (Csiza and de Lahunta, 1979, Zeller et al., 1989). Mouse models of proteolipid protein abnormalities depict a similar picture as myelin-deficient rats. The mutant mouse jimpy is hypomyelinated in the CNS due to alternative splicing and a 74 nucleotide deletion, which alters the the carboxyl terminus of proteolipid protein (Nave et al., 1986). Conservative amino acid substitutions in PLP, replacing valine for alanine, can cause a similar phenotype (Gencic and Hudson, 1990). The rump-shaker mouse mutant for PLP, substituting isoleucine for threonine, also results in CNS hypomyelination, but unlike jimpy mice have normal longevity and morphologically normal oligodendrocytes (Schneider et al., 1992).

In addition to genetic mutations, homeostatic control over the expression of myelin genes is important for CNS myelin formation. Transgenic mice over-expressing proteolipid protein halts oligodendrocyte maturation and survival, bringing about dysmyelination (Kagawa et al., 1994). Aberrant myelination arising from transmitting

extra copies of proteolipid protein may be due to a breakdown in the endoplasmic reticulum-Golgi apparatus system (Kagawa et al., 1994). The transport of abnormal PLP is restricted within the endoplasmic reticulum preventing PLP from participating in myelin formation at the oligodendrocyte processes (Roussel et al., 1987, Gow et al., 1994). During transport through the secretory pathway proteolipid protein may associate with lipid rafts containing cholesterol and galactosylceramide (Simons et al., 2000). However, the lipid composition of CNS myelin in jimpy and shiverer mice is unaltered (Ganser et al., 1988a, b). This may suggest an organized series of pathways participate in myelin architecture that depend on specific protein-lipid interactions for compact myelin ensheathments (Baron and Hoekstra, 2010).

The sphingolipid galactocerebroside and its sulfated derivative sulfatide constitute the two major lipids expressed within the myelin sheath (Coetzee et al., 1998). Galactocerebroside, which acts as a marker for oligodendrocytes, is synthesized by the enzyme uridine diphosphate-galactose:ceramide galactosyltransferase (CGT or UGT) that transfers galactose from uridine diphosphate-galactose to ceramide (Morell and Radin, 1969, Raff et al., 1978). Mice deficient in CGT assemble myelin with normal ultrastructure, but exhibit tremors, mild ataxia, and eventually develop hindlimb paralysis (Coetzee et al., 1996). CGT-deficient mice also display reduced conduction velocities, comparable to unmyelinated axons (Bosio et al., 1996). Phenotypic abnormalities of CGT-deficient mice are rescued by over-expression of a CGT transgene under the control of an oligodendrocyte promoter (Zoller et al., 2005). Due to the expression of galactosylceramide in neurons and astrocytes, oligodendrocyte specific reintroduction of CGT indicates a central role of myelinating glial cells in the phenotypes accompanying

CGT deficiency (Zoller et al., 2005). Additionally, the presence of galactolipids produced by CGT are important in the formation of nodes and paranodes, demonstrating a fundamental role for lipids in mediating interactions between the myelin sheath and neuronal axons (Dupree et al., 1998).

Regardless of the numerous potential proteins and lipids shaping the myelin sheath, it is evident that white matter ensheathment is necessary for the development and maintenance of axonal integrity (Poliak and Peles, 2003). Myelin enables the needs of a complex nervous system existing within a confined space, forcing the transmission of action potentials along nodes of Ranvier and reducing the metabolic requirements for neuronal communication (Boron and Boulpaep, 2009). Dysmyelinating and demyelinating mutations within the CNS cause aberrant axonal conduction and behavioral phenotypes (Werner et al., 1998). Although myelin in the central and peripheral nervous system (PNS) serve similar purposes, the CNS and PNS are morphologically and biochemically distinct (Morell et al., 1999, Quarles et al., 2006). For example, shiverer mice, lacking MBP and compact CNS myelin, have normal myelin morphology and periodicity in the PNS (Kirschner and Ganser, 1980). Structural and mechanistic differences existing between the CNS and PNS may be expected as each system serves distinct functions (McKinley and O'Loughlin, 2006). White matter anomalies affecting the CNS are heterogenous in nature, observed in a wide range of human psychiatric disorders including alcoholism and drug abuse (Fields, 2008). The context of the relationship between psychiatric illness and CNS myelin is unknown, however, it most likely affects both white matter and neuronal integrity (Pfefferbaum and Sullivan, 2005). In essence, dysfunction of white matter creates a pathogenic loop

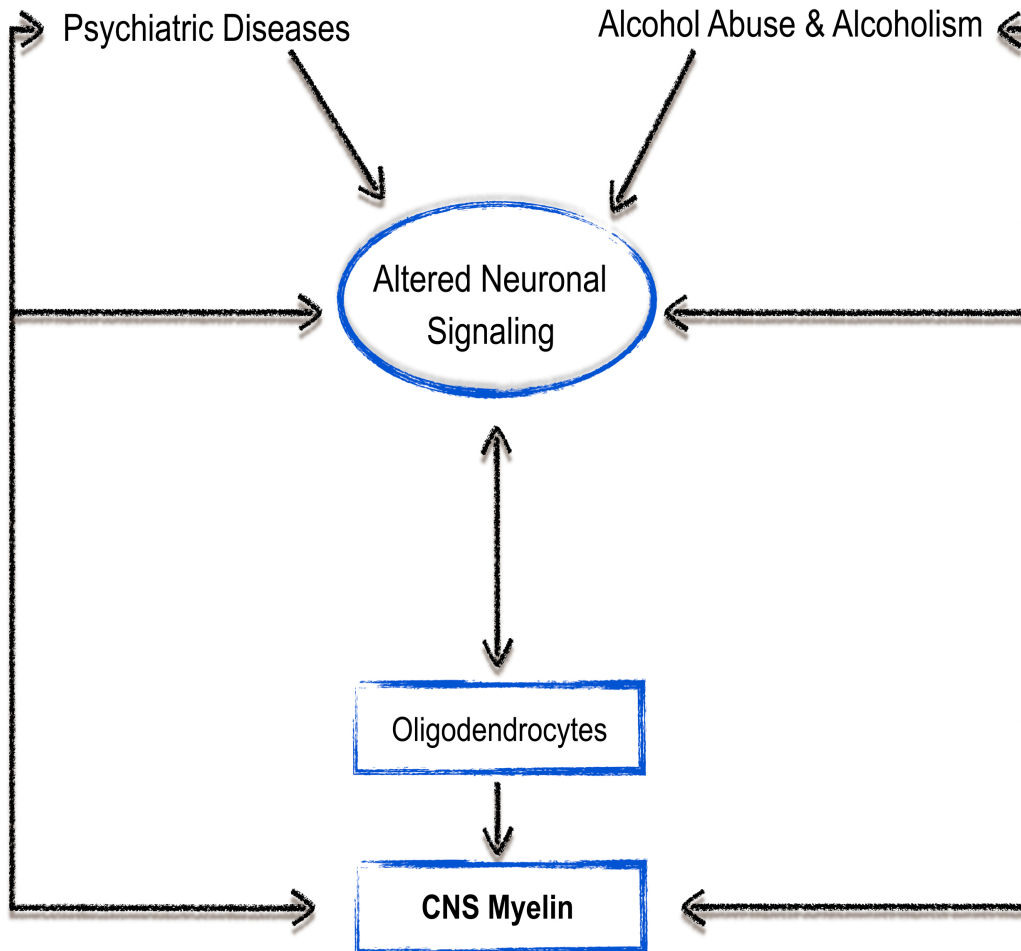


Figure 5. Myelin-associated pathogenic loop. Diagram of potential convergence for psychiatric diseases with alcohol abuse and alcoholism through dysfunction of CNS myelin. Abnormal CNS myelin feeds back to alter neuronal signaling and influence cross-talk between neurons and oligodendrocytes leading to vulnerability to disease; adapted from (Feng, 2008).

affecting neuronal signaling and psychiatric disorders, including alcohol abuse and dependence (Figure 5).

Brain atrophy, also commonly referred to as brain shrinkage due to the loss of both white and grey matter within the CNS, has often been attributed to the excessive life-long consumption of alcohol (Kril and Halliday, 1999). The total brain weight of alcoholics is significantly less than non-alcoholics (Harper and Blumbergs, 1982), which may be associated with the rate and total lifetime consumption of alcohol (Harding et al., 1996). Moderate alcohol drinking behavior, defined as those individuals having consumed 15-20 drinks per week on average and no greater than a lifetime consumption of 170-240 kg, does not adversely affect the size of male or female brains (de Bruin et al., 2005). Heavy (418.1 g/week), but not moderate (181.2 g/week), social drinking among non-alcoholics is associated with brain atrophy (Kubota et al., 2001), suggesting a neurotoxic effect of excessive alcohol drinking behavior. Although precise definitions of moderate and heavy drinking behavior is not well established and varies among scientific reports, conclusions generally support the role of recurring patterns of excessive alcohol intake leading to brain atrophy and cognitive impairment. The neurobiological alterations found with brain atrophy due to alcoholism, however, may be multifactorial in origin and target differing areas of the human brain (Neiman, 1998).

Individuals suffering from an alcohol use disorder are at risk for liver cirrhosis and severe nutritional deficiencies, which may also affect brain structure (Guerrini et al., 2009, Rehm et al., 2010). Liver dysfunction, in the absence of nutritional deficits, is significant correlated to a decrease grey matter, white matter, and total brain volume in

alcohol dependent patients (Chen et al., 2011). Reduction in total brain volume may be due to alcohol induced dysfunction of the liver-brain axis, causing oxidative stress and the production of toxic lipids capable of crossing the blood brain barrier (de la Monte et al., 2009). A preexisting metabolic condition or dietary problems leading to progressive inflammation may further exacerbate alcohol-related neurodegeneration (de la Monte et al., 2009). However, morphological analysis of uncomplicated alcoholics, those without confounding liver or nutritional issues, has shown brain abnormalities and cognitive deficits (Pfefferbaum et al., 1997).

Wernicke-Korsakoff syndrome, characterized by the presence of Wernicke encephalopathy and Korsakoff psychosis, is a frequently antemortem under diagnosed condition associated with alcoholism and malnutrition (Zubaran et al., 1997). Resulting from thiamine (vitamin B₁) deficiency due at least in part to an alcohol use disorder, Wernicke-Korsakoff syndrome leads to brain atrophy and structural changes in multiple brain regions that markedly impairs cognitive function (Sullivan and Pfefferbaum, 2009). Alcoholism induced brain atrophy, and brain structure, is largely due to the loss of CNS myelin (Harper and Kril, 1985, de la Monte, 1988). The most profound changes being detected in the prefrontal cortex white matter of alcoholics with Wernicke-Korsakoff syndrome (Kril et al., 1997); a brain region implicated in higher-order executive functions, motor control, and substance abuse. Additionally, alcoholism is associated with a decrease in the volume of the corpus callosum, the major white matter commissure of the brain that permits the exchange of information between cerebral hemispheres (Arnone et al., 2006). A rat model of alcohol consumption using pyriethiamine, a thiamine inhibitor, revealed a compounding effect of alcohol and

thiamine deficiency on the corpus callosum (He et al., 2007). Importantly, ethanol induced neurotoxicity can be readily distinguished from nutritional deficiencies associated with Wernicke-Korsakoff syndrome and other disorders affecting CNS white matter, such as Marchiafava-Bignami syndrome (Charness, 1993).

Alcoholics with and without Wernicke encephalopathy differentially affect the extent and location of corpus callosum damage, suggesting fundamental differences in neurotoxicity and malnutrition (Lee et al., 2005b). The loss of brain weight in alcohol dependence has been shown to occur independently from the presence of Wernicke encephalopathy (Harper and Blumbergs, 1982). Non-malnourished canines chronically exposed to alcohol experience a similar pathology, with regional loss of white matter in the neocortex and corpus callosum, resulting in enlargement of the lateral ventricles (Hansen et al., 1991). Structural magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) have shown degradation of white matter microstructure within the corpus callosum of alcoholics (Pfefferbaum et al., 2006). Neuroimaging has also identified multiple brain regions susceptible to the neurotoxic effects of ethanol in uncomplicated alcoholics (Gallucci et al., 1989, Sullivan and Pfefferbaum, 2009). Selective *in vivo* monitoring of patients through imaging technologies may help diagnose and differentiate Wernicke encephalopathy, Korsakoff amnesic state, and alcohol dependence (Victor, 1990). Although some of the neurotoxic effects of ethanol may be permanent, continued abstinence from alcohol reverses the loss of white matter in uncomplicated alcoholics (Shear et al., 1994, Pfefferbaum et al., 1995). The recovery of white matter deficits from chronic alcohol perniciousness is paralleled by improved metabolic function and neuropsychological performance (Bartsch et al., 2007).

Neurodevelopment proceeds through multiple stages in the maturation to adulthood. The prefrontal cortex in particular undergoes active development during early childhood and adolescence (Giedd et al., 1999, Thompson et al., 2000). White matter within the frontal cortex progressively increases throughout childhood and adolescence reaching maximum between the second and third decade of life (Pfefferbaum et al., 1994, Sowell et al., 1999). Similar to adults, adolescents (13-17 years of age) and young adults (18-21 years of age) with an alcohol use disorder have diminished white matter volume within the prefrontal cortex, which inversely correlates with alcohol consumption (De Bellis et al., 2005). Deficits in white matter are not due to comorbid psychiatric or neurological disorders (Medina et al., 2008). Binge-drinking adolescents without a history of drug or alcohol abuse, exhibit diminished white matter integrity, suggesting heavy episodic drinking adversely affects neurodevelopment processes related to CNS myelin (McQueeney et al., 2009).

Alcohol consumption during pregnancy can grievously affect a developing fetus, leading to the development of fetal alcohol spectrum disorder; the most serious form of which is diagnosed as fetal alcohol syndrome (Medina, 2011). Prenatal alcohol exposure adversely impacts multiple biological processes, including the central nervous system (Sulik, 2005, Davis et al., 2011). Post-mortem studies have shown widespread brain atrophy and structural anomalies occur in infants prenatally exposed to alcohol (Jones and Smith, 1973, Clarren et al., 1978). Alterations in brain morphology contribute to the continuum of neurobehavioral abnormalities associated with prenatal alcohol exposure (Mattson and Riley, 1998). Overall reductions in brain volume for fetal alcohol spectrum disorder is due to a greater reduction in white matter than grey matter,

affecting numerous major white matter fiber tracts throughout the brain (Archibald et al., 2001, Lebel et al., 2008). The degree of brain damage occurring is influenced by the amount and pattern of alcohol exposure, as well as developmental timing (Maier et al., 1996). Although ethanol-induced teratogenesis may transpire throughout the entire brain, *in vivo* studies have revealed some brain regions may be more sensitive to the neurotoxic effects of ethanol than others (Riley et al., 2004).

Corpus callosum, the major white matter fiber tract of the brain, undergoes significant alterations in cases of fetal alcohol syndrome (Riley and McGee, 2005). Children with fetal alcohol syndrome show significant thinning of the corpus callosum, and in some severe cases complete agenesis (Mattson et al., 1992, Clark et al., 2000). The rate of corpus callosum agenesis is considerably higher than the general population, 6.8% versus 0.3% (Roebuck et al., 1998). Additionally, the abnormalities within the frontal cortex are among the most consistent findings from case reports of prenatal ethanol exposure (Norman et al., 2009). Decreases in white matter and structural irregularities within the frontal lobe persist long after prenatal alcohol exposure on the developing brain (Sowell et al., 2002). Persistent defects within the forebrain, due to prenatal alcohol insult, may underlie concomitant behavioral problems associated with a lack of inhibition and impaired executive function (Olson et al., 1998, Connor et al., 2000). Unlike alcoholic adults, the detrimental changes in white matter and structural integrity for individuals afflicted with fetal alcohol syndrome or spectrum are irreversible (Kodituwakku et al., 2001, Alfonso-Loeches and Guerri, 2011).

Animal models have accelerated our understanding of the teratogenic properties

of ethanol on development (Cudd, 2005). Craniofacial and forebrain abnormalities in mice prenatally exposed to alcohol at specific developmental stages, correspond to phenotypic abnormalities in children suffering from fetal alcohol syndrome (Webster et al., 1980, Sulik et al., 1981). The extent of malformation depends on the severity of alcohol exposure and coincidence with critical developmental windows (Riley and McGee, 2005). Interestingly, defects in facial development correlate with the severity of brain deformities and associated behavioral problems (Riley et al., 2011). Alcohol exposure during periods of rapid CNS growth significantly reduces weight of rat forebrain (Maier et al., 1997). Decreases in brain weight are due in part to the reduction in myelin thickness and number of myelinated fibers following alcohol exposure during rat development (Phillips et al., 1991). Akin to their human counterparts, animals prenatally exposed to alcohol exhibit thinning and partial agenesis of the corpus callosum (O'Leary-Moore et al., 2011). Alcohol exposure during critical developmental periods reduces the number of oligodendrocytes, delays oligodendrocyte maturation, and reduces the expression of myelin basic protein, without affecting neuronal expression of neural cell adhesion molecule (Phillips and Krueger, 1992, Ozer et al., 2000).

Alcohol affects the myelin architecture at all of the stages of human development, spanning from *in utero* exposure to adulthood. Although the mechanisms of ethanol-induced neurotoxicity remain to be fully elucidated, the adverse consequences of excessive alcohol consumption on neurobehavioral abnormalities are well substantiated in both humans and animal model. The etiology and pathophysiology of psychiatric diseases and alcoholism may be due in part to imperfections arising from

oligodendroglia and myelin (Sokolov, 2007). Impairments in myelin expression and formation likely disrupts axonal integrity, putting individuals at risk for continued or future substance abuse (Bava et al., 2009, Harper, 2009). Dysfunction of white matter in discrete brain regions confers control over specific cognitive and motor impairments (Pfefferbaum et al., 2010). Continued scientific inquiry into the fundamental aspects governing white matter, brain structure, and behavior will help delineate the genetic risk factors and neurobiology of alcohol abuse and dependence (Zahr et al., 2011).

Application of DNA Microarrays on Ethanol and Ethanol-related Phenotypes

Through an unbiased, parallel examination of mRNA expression from virtually the entire genome, DNA microarrays can identify genes or gene expression patterns associated with a given phenotype (drug response, disease, developmental stage, etc.) (Lockhart and Winzeler, 2000, Lockhart and Barlow, 2001). Gene expression provided by such studies can provide novel information regarding the mechanisms underlying disease. Microarrays provide a complementary approach to genetic mapping for the non-biased identification of disease-related genes. Since the inception of microarrays a growing number of studies have applied expression profiling towards the neurobiology of alcohol abuse and dependence. Although differences exist among the various platforms, microarrays depend on similar underlying molecular fundamentals. Fluorescent-labeled probes are generated from RNA isolated from a desired tissue or cell type. Target molecules may be either RNA or DNA based, depending on the particular microarray platform. A target gene of interest, through the basic principle of

complementary base pairing of nucleotides, adheres to small probes attached to a solid-phase support such as a glass slide, bead, or nylon membrane. The probes are generally DNA oligonucleotides 25–70 nucleotides in length. Following hybridization and washing, fluorescent signals are detected and these correlate directly to specific mRNA abundance to monitor changes in gene expression (Chee et al., 1996). Monitoring simultaneous changes in gene expression can identify the function of uncharacterized genes and the pharmacological mechanisms of action for drugs of unknown function (Hughes et al., 2000).

Alcoholism is a complex disease influenced by a multitude of potential factors, both genetic and environmental in origin (Spanagel, 2009). As described previously, the genetic predisposition for the risk of developing alcoholism has been well documented through a host of publications on the familial origin of substance abuse (Merikangas et al., 1998, Hill et al., 2008). Genetic inquiries of human and animal models have to date only identified a few well-documented genes contributing to the risk for alcoholism or genetic variation in behavioral responses to ethanol. This difficulty is likely due to the occurrence of one or more rare polymorphisms of small effect size in a large number of genes being causal elements in complex traits such as alcoholism. Additionally, the alteration in mRNA transcript abundance evoked by ethanol or substance abuse are proposed as mechanisms underlying enduring neuroadaptations leading to abuse and addiction (Nestler and Aghajanian, 1997). Disrupted homeostatic control of gene networks is also a possible mechanism underlying CNS toxicity from compulsive ethanol or drug use. Furthermore, genetic differences in gene expression responses to ethanol are also thought to be an important mechanism underlying a predisposition to

alcoholism or other complex traits (Schadt et al., 2003, Chesler et al., 2005).

Differences in ethanol-induced signaling events are a dynamic aspect influencing the maturation of alcohol dependent behaviors. Divergent sensitivity to an initial acute exposure to ethanol is correlated with the potential for long-term drinking behavior (Schuckit, 1994), suggesting divergence in acute ethanol-induced signaling events may be an important risk factor for developing alcoholism. Alterations in gene expression can occur at biologically relevant concentrations of ethanol within 4 hours after initial exposure and persist for extended periods of time (Miles et al., 1991). Expression profiling of neuroblastoma cells identified some of the major gene targets of ethanol (Thibault et al., 2000). Genomic profiling pointed to catecholamine metabolism (dopamine B-hydroxylase), cellular survival and oxidative stress, and cyclic-AMP (cAMP) signaling mechanisms. The cAMP system is a known factor underlying acute and chronic ethanol action; however, profiling of neuroblastoma cells also exemplified that ethanol has unique signaling mechanisms owing to the fact that not all of the same genes were regulated solely by cAMP (Thibault et al., 2000, Hassan et al., 2003, Thibault et al., 2005). Characterizing these differences in gene expression both validated the use of microarrays by uncovering genes known to contribute to ethanol responses (i.e. cAMP signaling genes), and demonstrated the utility of genomic approaches to identify novel genes unique to an ethanol-induced response.

Microarray studies on brain tissue for complex traits related to alcohol abuse and dependence can be subdivided into human versus animal model, acute versus chronic, basal versus treated, and whole brain versus brain regional. In general, for the animal model studies, two different experimental approaches have been used. The first entails

profiling of 'basal gene expression' across two or more lines of animals that are known to have important differences in ethanol drinking or other behavioral responses to ethanol. The second approach looks at responses to acute or chronic ethanol exposure or ethanol withdrawal. The two approaches have been merged in a limited number of studies where ethanol responses across different lines of animals are also studied. As a general conclusion, all of these approaches have produced statistically significant results, generating prodigious gene lists under continued evaluation and validation.

Genomic studies on ethanol-naive inbred strains of mice differing in ethanol-sensitive behavioral phenotypes have been important for determining genes and molecular pathways potentially involved in ethanol-related physiological pathways. For example, whole-brain expression profiling of inbred long sleep (ILS) and inbred short sleep mice (ISS), specifically bred for their initial sensitivity to a sedative-hypnotic dose of ethanol, show expression differences for approximately 41 genes (Xu et al., 2001). Expression differences between ILS and ISS mice suggested the involvement of several assorted gene ontology categories. One prospective category included glycolysis, implicated through differential expression of pyruvate dehydrogenase E1-a subunit, a key enzyme in the conversion of pyruvate to acetyl coenzyme A (acetyl-CoA). Additional follow-up analysis of ILS and ISS samples suggested potential splice variants for unknown cDNA clones contributes to variation in the sedative-hypnotic effects of acute ethanol (Xu et al., 2001).

A meta-analysis of differential gene expression across a panel of alcohol naive inbred mice strains that are known to differ in voluntary ethanol drinking behavior pointed to a diverse collection of genes and molecular pathways in the predisposition for

ethanol consumption (Mulligan et al., 2006). An additional meta-analysis of selectively bred ethanol-naïve mice, recombinant inbred mice, and a large heterogeneous panel of inbred mice identified greater than 8000 transcripts related to ethanol preference (Tabakoff et al., 2008). Whole-brain expression profiling differences revealed by the two meta-analysis strongly suggests the involvement of thousands of genes spanning several functional pathways are involved in alcohol drinking behavior. Brain-region specific differences further complicate defining the gene-sets governing alcohol consumption. Microarray analysis of individual brain regions has demonstrated within region gene expression differences that correlate with alcohol intake (Mulligan et al., 2011, Wolstenholme et al., 2011), suggesting numerous genomic integrative processes across multiple brain regions influence drinking behavior.

A number of laboratories are interested in brain region specific differences contributing to ethanol behavioral phenotypes. Genotype specific differences have been demonstrated within individual brain sections using microarray analysis across five distinct brain regions for eight different strains of inbred mice from priority group A of the Mouse Phenome Project (Bogue and Grubb, 2004, Letwin et al., 2006). Correlation of expression profiling results in drug-naïve mice identified a subset of genes solely within the ventral striatum related to the N-methyl-d-aspartate (NMDA)/glutamate signaling pathway for total distance traveled following 1.5 g/kg ethanol, suggesting a brain region specific expression pattern associated with locomotor activity. Correlation of basal gene expression with an ethanol behavioral phenotype across multiple inbred mouse lines suggested the potential power of combining genomic profiling with genetic analysis of ethanol behaviors (Chesler et al., 2005, Letwin et al., 2006).

C57BL/6J (B6) and DBA/2J (D2) mice are two inbred strains, commercially available from the Jackson Laboratory (<http://www.jax.org/>), that differ in a range of ethanol behavioral phenotypes (McClearn and Rodgers, 1959). For example, D2 mice demonstrate greater acute ethanol-induced locomotor activation, withdrawal-induced seizures, and reduced ethanol preference/consumption compared to B6 mice (Goldstein, 1973, Belknap et al., 1993, Phillips et al., 1994, Metten et al., 1998a). An initial global whole-brain expression profiling experiment of B6 and D2 mice acutely treated with 6 g/kg ethanol revealed a small number of genes responding to ethanol related to cell signaling, gene regulation, and homeostasis/stress response (Treadwell and Singh, 2004). Several of the ethanol-responsive genes differing between B6 and D2 mice resided within known ethanol behavioral QTLs; however, only small number of genes (16 total) were differentially regulated by acute ethanol. The low extent of differential expression between the two strains was attributed to heterogeneity of the brain tissue from performing genomic comparisons on whole brain homogenates.

Basal and acute ethanol-responsive neurogenomics differences within the ventral tegmental area (VTA), nucleus accumbens (NAC), and prefrontal cortex (PFC) between D2 and B6 mice have also been recently characterized (Kerns et al., 2005). Extensive multivariate analysis of VTA, NAC, and PFC uncovered basal and ethanol-evoked brain region specific gene expression profile differences. Acute ethanol induced coordinated changes in gene expression patterns that suggested an overall functional involvement with neuroplasticity. Region specific functional relationships were determined for retinoic acid signaling (VTA), Bdnf signaling and neuropeptide expression (NAC), and glucocorticoid signaling and myelination (PFC). A subset of differentially expressed

genes mapped to QTLs that have been previously implicated in acute ethanol behavioral phenotypes. The overlap of ethanol-responsive genes and genes contained within support intervals identified for behavioral QTLs provided a glimpse of the power that microarrays might have for prioritizing candidate genes for quantitative traits. Overall, this study strongly suggested that the activity of divergent brain region-specific gene expression patterns, and their cognate signaling mechanisms, might play an important role in the differing ethanol behaviors witnessed between B6 and D2 mice.

The studies from Kerns et al. (2005) also defined coordinate regulation of myelin associated genes within the prefrontal cortex. Myelin-associated genes showed divergent basal and ethanol-responsive effects between the B6 and D2 mice, which might be determinants in acute ethanol sensitivity and long-term drinking behavior (Figure 6). Ethanol regulation of myelin gene expression is further supported by genomic analysis of postmortem human brain tissue, which demonstrates a coordinate decrease of myelin gene expression within the frontal cortex (Figure 7) (Lewohl et al., 2000, Mayfield et al., 2002, Iwamoto et al., 2004). Co-ordinate down-regulation of myelin expression was also confirmed at the level of protein (Lewohl et al., 2005) as well as neuropathology and neuroimaging studies (Krill and Harper, 1989, Rosenbloom et al., 2003). Microarray evidence of myelin gene dysregulation has also been identified in studies on schizophrenia and cocaine addiction, suggesting that PFC myelin gene expression may be sensitive to dopaminergic signaling (Sokolov, 2007).

A number of other functional categories, such as glutamate signaling, protein trafficking, and cyclic adenosine monophosphate (cAMP) signaling, has also been revealed through genomic studies of postmortem alcoholic brain tissue (Mayfield et al.,

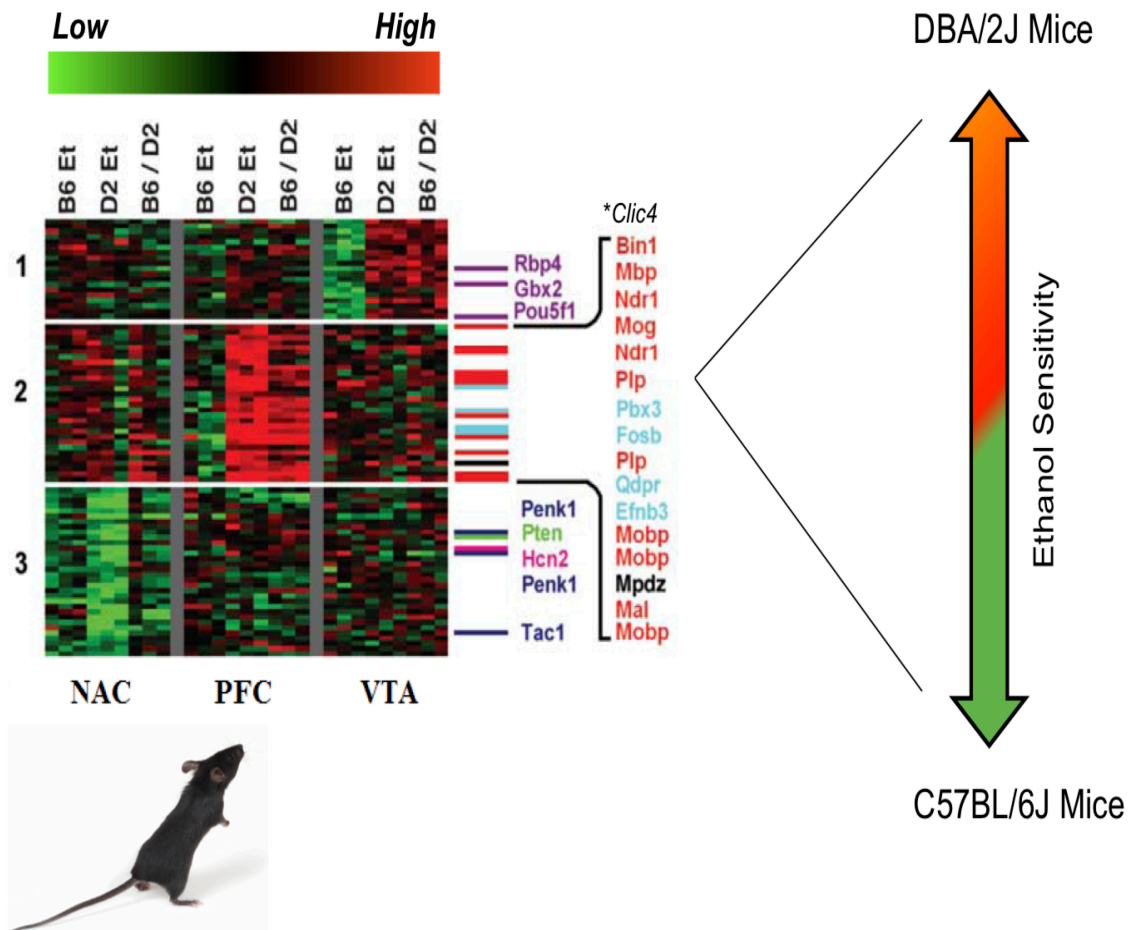


Figure 6. Microarray analysis of acute ethanol-responsive gene expression in B6 and D2 mice. Cluster shows differential basal and acute ethanol-responsive gene expression across B6 and D2 mice PFC. Triplicate experiments were conducted on saline- or ethanol-injected (2 g/kg) B6 AND D2 mice. Brain sections were micro-dissected after 4 hours and RNA prepared for microarray analysis using Affymetrix Mg_U74A2 oligonucleotide arrays. Red= increased expression. Green= decreased expression. Black = no change in expression. Figure amended from Kerns et al. 2005.

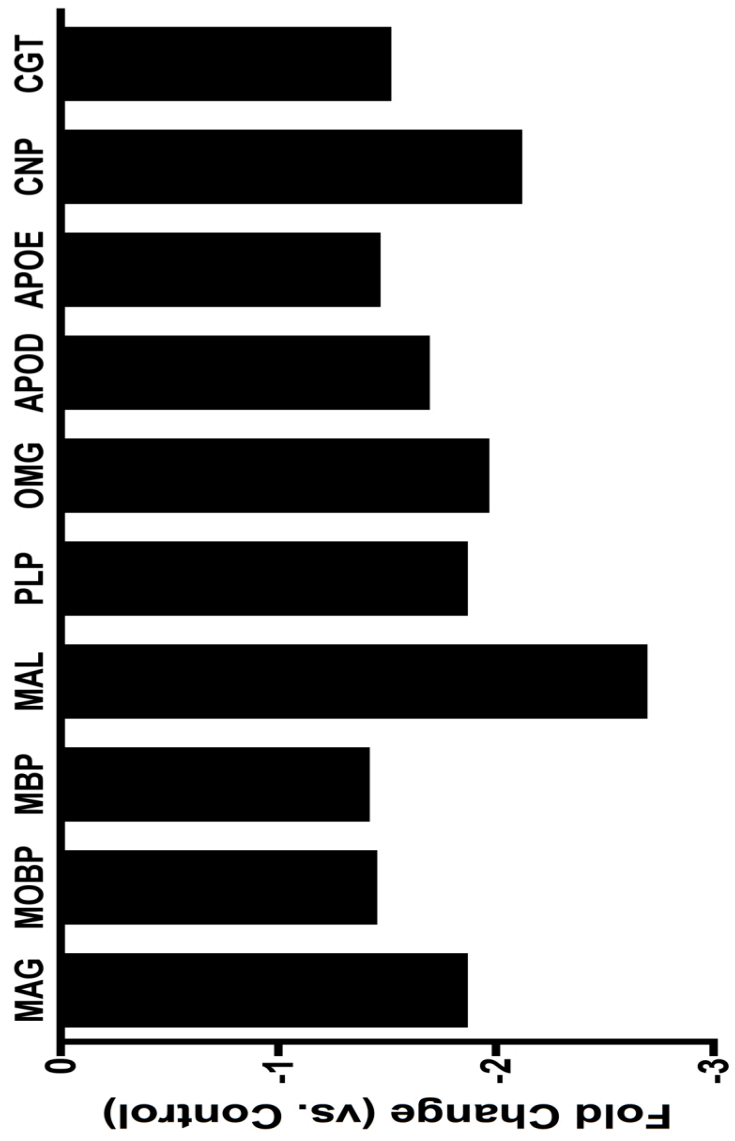


Figure 7. Decrease myelin-associated gene expression from human PFC. Bar graph showing coordinate decrease fold change in myelin-associated gene expression in frontal cortex from post-mortem alcoholic brain tissue; derived from data presented in (Lewohl et al., 2000).

2002, Sokolov, 2007). These changes reflect a large cohort of cell signaling mechanisms cooperating in an orchestrated series of CNS plasticity occurring with prolonged ethanol exposure. The cAMP-signaling pathway, for example, has been implicated in a large number of experimental models relevant to alcohol abuse (Diamond and Gordon, 1997). A potential weakness of microarray studies on postmortem brain tissue is they only represent the final end point of the disorder, and not necessarily the neuroadaptations occurring over time that lead to the development of dependence. The possibility of other confounding environmental or population stratification factors affecting the microarray results also cannot be discounted.

Although challenging in terms of experimental design, chronic ethanol exposure in animal models is a significant etiological factor for understanding the neurobiology of alcoholism (McBride and Li, 1998, Knapp and Breese, 2012). Animal models lack many of the functional criteria for clinical diagnoses of alcoholism, but are able to mimic different phenotypic components of the disorder. Brain region-specific differences in gene expression have been detected in the NAC shell and central nucleus of the amygdala of alcohol-preferring rats following an 8-week model of alcohol binge-like behavior (McBride et al., 2010). Little overlap in gene expression results was found between this study and a previous analysis from the same research group of the NAC within the same strain following ethanol operant self-administration, suggesting that differences in behavioral procedures and the neural circuits they activate may contribute to deviations in expression-profiling results (Rodd et al., 2008, McBride et al., 2010).

Chronic intermittent rather than continuous ethanol exposure causes increased ethanol self-administration (O'Dell et al., 2004, Lopez and Becker, 2005). Intermittent

episodes of ethanol vapor exposure permits control over dose and duration of exposure, maintaining stable blood-ethanol concentrations, producing dependency and withdrawal in mice and rats (Becker, 2000). Expression profiling on cingulate cortex and amygdala in rats exposed to the chronic intermittent ethanol exposure found striking alterations in expression for genes involved with glutamate neurotransmission, synaptic plasticity, and mitogen-activated protein kinase signaling (Rimondini et al., 2002). Expression changes were detected three weeks after withdrawal from the last ethanol vapor exposure, suggesting important molecular adaptations underlying escalating ethanol intake. However, these studies used only a single time point and only two microarrays per treatment/ brain region group, and thus may be susceptible type I and type II errors.

Altered gene expression profiles have been demonstrated for acute and chronic ethanol withdrawal within hippocampi for B6 and D2 mice, revealing strain and treatment specific changes for ethanol-induced withdrawal behavior (Daniels and Buck, 2002). Contrasting gene expression patterns for ethanol-induced withdrawal phenotypes for B6 and D2 mice is associated with the neural circuitry of physical dependence and tolerance (Chen et al., 2009a). Combining genomics with genetic null mutations that target specific behavioral traits can help refine the potential molecular pathways and candidate genes for ethanol-responsive endophenotypes. Protein kinase C gamma (γ -PKC), confined to the CNS (Yoshida et al., 1988), is an influential substrate for tolerance to ethanol (Bowers et al., 1999). Microarray analysis of cerebellar tissue from γ -PKC knockout mice, which do not develop tolerance to ethanol, identified a novel potassium channel (*Twik-1*) dependent on the presence of γ -PKC for the development of tolerance (Bowers et al., 2006). However, the association of γ -PKC and tolerance, as

well as shared differential gene expression, is dependent on genetic background (Bowers et al., 1999). Additionally, basal differences in gene expression arise in γ -PKC mutants that may be important for interpreting the behavioral response to ethanol (Smith et al., 2006). Although it is difficult to establish a causative association between variation in gene expression and complex traits, convergent evidence from multiple resources can help to determine novel relationships beyond our existing knowledge (Ponomarev et al., 2004, Baker et al., 2012). Cross-species analysis incorporating human genetic variation and gene expression can also prioritize candidate genes for a comprehensive translational approach for novel genes and gene networks underlying complex traits (Bertsch et al., 2005, Rodd et al., 2007).

Neuropsychiatric conditions including alcoholism are multifaceted diseases of complex origin. Extensive efforts across multiple fields of scientific research have actively sought to explain the origins of alcoholism; however, no solitary molecular mechanism has yet been established. Evidence from genetic epidemiology, genetic variation, and differential gene expression studies has demonstrated multiple genes may account for the manifestation of psychiatric disease. Variation in gene expression may act as an intermediate phenotype between genotypic differences and neurobehavioral responses (Abiola et al., 2003, Cheung et al., 2003, Nica and Dermitzakis, 2008). In order to understand the functional importance of genetic variation and differential gene expression, visualizing the shared interconnection among gene products using network-based approaches may become essential for the analysis of complex traits.

Bioinformatics and Network Analysis of Gene Expression

Once genes differentially expressed across different conditions or animal strains relative to the disease model have been identified or robust expression data has been collected across a panel of phenotypes and genotypes, a relational network needs to be established across individual genes. A gene network could be considered either a set of genes that interact functionally (e.g. metabolite processing, protein–protein interaction, or protein modification) with one another to accommodate the needs of the cellular environment or a set of genes that share a common regulatory mechanism and show highly correlated expression patterns. In many cases these two factors may overlap within a given gene network. However, associating biological function with a gene network for a behavioral phenotype might lead to new hypotheses regarding the underlying molecular mechanisms and avenues for intervention. Such an approach is certainly the hope of using genetical genomics and bioinformatics for studies on ethanol-related phenotypes. The types of analyses required are however contingent upon the source of gene expression data and the hypothesis in question (Slonim, 2002).

Organizing gene expression data entails determining whether or not a given gene set has some coherent biological function and if the results are greater than expected by chance. Performing over-representation analysis requires proficient databases of known gene ontologies covering individual categories nested within three broad domains: cellular component, biological process, and molecular function (Ashburner et al., 2000). These interconnected domains are derived from the existing biomedical literature, and defined by their shared functional involvement such as protein–protein interactions, gene–gene interactions, or cellular signaling systems. Although gene

ontologies have drawbacks and limitations, including incomplete categories and pre-existing biases, the collaborative effort is continually evolving to incorporate new biological discoveries and meet the needs of the scientific community (Harris et al., 2004, Khatri and Draghici, 2005). A number of resources and bioinformatic tools are readily available to determine if a particular data set is enriched for a functional category (<http://www.geneontology.org/>). The Database for Annotation, Visualization and Integrated Discovery (DAVID) for example provides a comprehensive package for determining biological meaning behind extensive gene sets (Dennis et al., 2003, Huang et al., 2009). DAVID also links to additional shared resources such as the Kyoto Encyclopedia of Genes and Genomes (KEGG), which is a database of manually curated molecular pathways (Kanehisa, 2000). Pathway analysis of gene expression data, or other large-scale methodologies, may help determine common genes and molecular pathways underlying drug addiction (Li et al., 2008).

ToppGene Suite is another multifaceted resource that can mine gene expression outcomes for enrichment of ontologies, as well as human and mouse phenotypes (Chen et al., 2009c). Additionally, ToppGene prioritizes candidate genes for targeted validation of genes in downstream molecular and behavioral processes. The ranking or prioritizing of candidate genes is assigned based upon functional annotation and phenotypic data (Chen et al., 2007) or protein–protein interaction networks (Chen et al., 2009b). Conducting over-representation analysis of gene expression results can thus help organize an abundance of results into biologically meaningful information current with existing knowledge. Additionally, gene ontologies can be used to help construct gene networks, generate novel predictions regarding gene function, and identify genes

potentially missed in up-stream analyses (Mostafavi et al., 2008, Warde-Farley et al., 2010).

Literature association analysis is a complementary bioinformatics approach to gene ontology analysis that is often used for querying the functional and chemical relationships existing within large-scale gene sets. Biological databases solely derived from manual input cannot keep pace with the exponential growth of peer-reviewed literature (Hunter and Cohen, 2006, Krallinger et al., 2008), and thus have integrated text mining approaches for probing the underlying biology gene expression data, or other omic technologies (Krallinger et al., 2010). Ingenuity Pathway Analysis (<http://www.ingenuity.com/>) is an example of bioinformatics program for exploring and visualizing the network structure of data sets based on computer-generated and manually curated literature associations, biological function, and cell signaling mechanisms. Other commercial programs such as GeneGo (<http://www.genego.com/>) and free programs such as Chilibot (Chen and Sharp, 2004) are available for literature mining and composing network diagrams among gene sets. The network structure of gene sets can reveal innovative associations between genes outside of predetermined functional categories, leading to novel hypotheses and follow-up experimental biology.

The Search Tool for the Retrieval of Interacting Genes/ Proteins (STRING; <http://string-db.org/>) is an in-depth meta-resource tool for illustrating functional network associations of genes and proteins across 630 organisms (Snel et al., 2000, Jensen et al., 2009). Networks are based upon a composite of known functional associations and protein–protein interactions existing within the biomedical literature, as well as predicted relationships in order to potentially provide a more comprehensive systems biology

outlook (Szkarczyk et al., 2011). The predicted functional relationship among given gene sets are determined through an array of experimentally derived conditions from phylogenetic and co-expression profiles (von Mering et al., 2007). Additionally, predicted associations are incorporated into STRING using natural language processing of the peer-reviewed literature (Saric et al., 2006) and conserved organismic transfer of protein interactions (von Mering et al., 2005). Resulting networks can be filtered for confidence scores, and used as a prominent exploratory tool for investigating all of the possible biological relationships within a potentially more limited user-defined gene set. Caution must be exercised in over-interpreting the results of such network associations and verifying the implied literature interrelationships (Auffray et al., 2009). Deriving the same or similar network structure from different bioinformatics resources or through combinations of literature association, functional group over-representation, and expression correlation is a reassuring indication of some meaningful functional relationship amongst gene expression data.

Empirical assessments may also be determined *de novo* for gene network analysis or gene–gene relations, based upon expression/expression, expression/genotype or expression/phenotype correlations. Partnerships between expression, genotype, and phenotype depend upon the inherent correlation structure generated by datasets, such as microarray expression studies. Clustering of expression profiles due to genes with highly correlated mRNA abundances across a large number of different experimental conditions or genotypes are hypothesized to share biologically relevant relationships. Cluster analysis of the inherent patterns produced by correlated gene expression can provide insights into the function of uncharacterized genes or the biological mechanisms

underlying a given drug action or phenotypic trait (Eisen et al., 1998, Hughes et al., 2000). However, without sufficient biological variance in the expression of individual genes, correlation based network approaches can produce statistically parsimonious relationships between genes that might have little biological relevance (Baginsky et al., 2010). In view of the fact environmental or technical factors can produce artifactual correlations, experimental biases should also be considered in the statistical and biological principles of pattern recognition (Kerr and Churchill, 2001).

Despite the potential problems and limitations facing the identification of coordinately regulated gene expression patterns, the concept of gene networks is successfully and increasingly being used in systems biology of complex traits (Chen et al., 2008, Horvath and Dong, 2008). A large number of methodologies exist and are continually emerging to classify co-expression networks (Butte et al., 2000; Baldwin et al., 2005; Chesler et al., 2005; Zhang and Horvath, 2005). Not surprisingly, correlation matrices can clarify the role of genes with unknown function (van Noort et al., 2003); however, gene co-expression networks can also identify components specific to a singular model organism or conserved across an evolutionary diverse set of species (Stuart et al., 2003). Although many genes remain stationary over time, dynamic relevance networks may also exist in response to differing stimuli and adapt throughout an organisms' lifetime (Reis et al., 2001, Yeung et al., 2011). Combined with genetic mapping strategies (i.e. QTL analysis) and detailed phenotyping, heritable variation in gene expression networks can help chart complex traits associated with human disease (Rockman and Kruglyak, 2006, Sieberts and Schadt, 2007, Cookson et al., 2009).

Although human interactome data is receiving increased attention for network-

based approaches to human disease (Barabasi et al., 2011), model organisms and *in silico* analysis provide an experimental platforms for fundamental questions in network biology (Joyce and Palsson, 2006). Yeast models have successfully demonstrated that integrating gene expression with transcription factor binding information and protein-protein interaction data can reconstruct gene networks capable of predicting complex system behavior (Zhu et al., 2008). Characteristic patterns in gene expression emerge from elaborate global networks, often referred to as sub-networks or modules, which function as a group and may be essential in physiological or pathophysiological processes (Milo et al., 2002, Yeager-Lotem et al., 2004). Genes forming tightly co-regulated gene expression patterns comprising sub-networks are more likely to be interacting protein complexes cooperating in a shared molecular function (Laub et al., 2000, Ge et al., 2003). Additionally, distinct functional modules existing within a larger framework are more likely to be evolutionary conserved across species (Wuchty et al., 2003). The global organization and interaction of biological networks is controlled through intra-modular genes that may be prime targets for therapeutic intervention (Han et al., 2004). Pharmaceutical agents can however functionally interact, as well as potentially impact interlinked genomic and proteomic networks (Yeh et al., 2006, Yildirim et al., 2007, Nichols et al., 2011). The majority of disease-oriented genes do not necessarily encode for essential hub genes in humans (Goh et al., 2007), further suggesting the focus for understanding disease mechanisms should part ways with reductionism and be directed towards perturbed gene expression networks rather than single alleles (Nelson, 2008, Schadt et al., 2009).

Treating gene expression as a quantitative trait has helped identify some of the

key drivers of physiological and behavioral phenotypes within living systems (Brem et al., 2002, Schadt et al., 2003); however gene expression networks have also illuminated the substantial complexity underlying disease mechanisms (Schadt, 2009). Using a conditional correlation model for gene expression data, genotypic variation, and phenotypic measures, approximately one hundred gene expression traits have been predicted for the susceptibility to obesity across a segregating mouse population derived from C57BL/6J and DBA/2J mice (Schadt et al., 2005). Knockout or transgenic mouse models in a subsequent analysis validated eight out of nine top candidate genes for abdominal obesity, originally identified by the conditional correlation results (Yang et al., 2009). Importantly, expression profiling of the mouse models for abdominal obesity determined they influenced common pathways and gene networks, suggesting gene networks and not a singular gene is responsible for common human diseases (Yang et al., 2009). In addition to obesity, gene co-expression networks have also been widely implicated in a variety of other complex traits associated with human health, including neural development disorders and psychiatric disease (Chen et al., 2008, Derry et al., 2010, Miller et al., 2010, Potkin et al., 2010, Penagarikano et al., 2011).

Concluding Remarks

Higher-order organizational frameworks composed of biological relationships, physical interactions, expression correlation structures, and predicted associations encompass some of the current network approaches designed for understanding the molecular basis of disease. Multiple sources of information and theoretical frameworks can be readily utilized to obtain gene networks. How to build the most informative

networks is open to interpretation, but undoubtedly will rely on some type of an organizing framework such as correlation structures or functional associations. The infrastructure of gene networks shares similar properties with other complex systems, namely scale-free topology (Price, 1965, Barabasi and Albert, 1999, Barabasi and Oltvai, 2004, van Noort et al., 2004). In some instances gene co-expression networks may be evolutionary conserved with coherent biological function (Milo et al., 2002, Stuart et al., 2003). Genetic and genomic studies have identified a large number of candidate genes that may contribute to a variety of phenotypic traits related to alcoholism; however, the adoption of network analyses for understanding alcoholism is still in a state of infancy. Transcriptional variation and insertional mutagenesis within *Drosophila melanogaster* has indicated gene networks, rather than an individual gene, are important for sensitivity to alcohol (Morozova et al., 2011).

Heterogeneity and complexity of the mammalian brain has hindered the progress of functional genomics for elucidating the mechanisms of neurological and psychiatric disease (Mirnics et al., 2000, Geschwind and Konopka, 2009). Expression profiling of brain tissue from humans and animals, in combination with existing research, is opening the door to the discovery of fundamental networks inherent in alcohol use disorders (Spanagel, 2009). Through systems biology brain region-specific networks can be constructed for endophenotypes associated with alcohol abuse and dependence (Figure 8). Although each individual layer of detail may hold novel insights, coordinately regulated gene expression networks can serve as an informative intermediate between the genotype-phenotype relationship (Nica and Dermitzakis, 2008, Cheung and Spielman, 2009). Identifying gene networks with a conserved role in both biological and

phenotypic variation may be essential for understanding the neurobiology of drug actions and susceptibility to disease (Berger and Iyengar, 2009, Pujol et al., 2010).

Alcohol Abuse and Dependence Endophenotypes

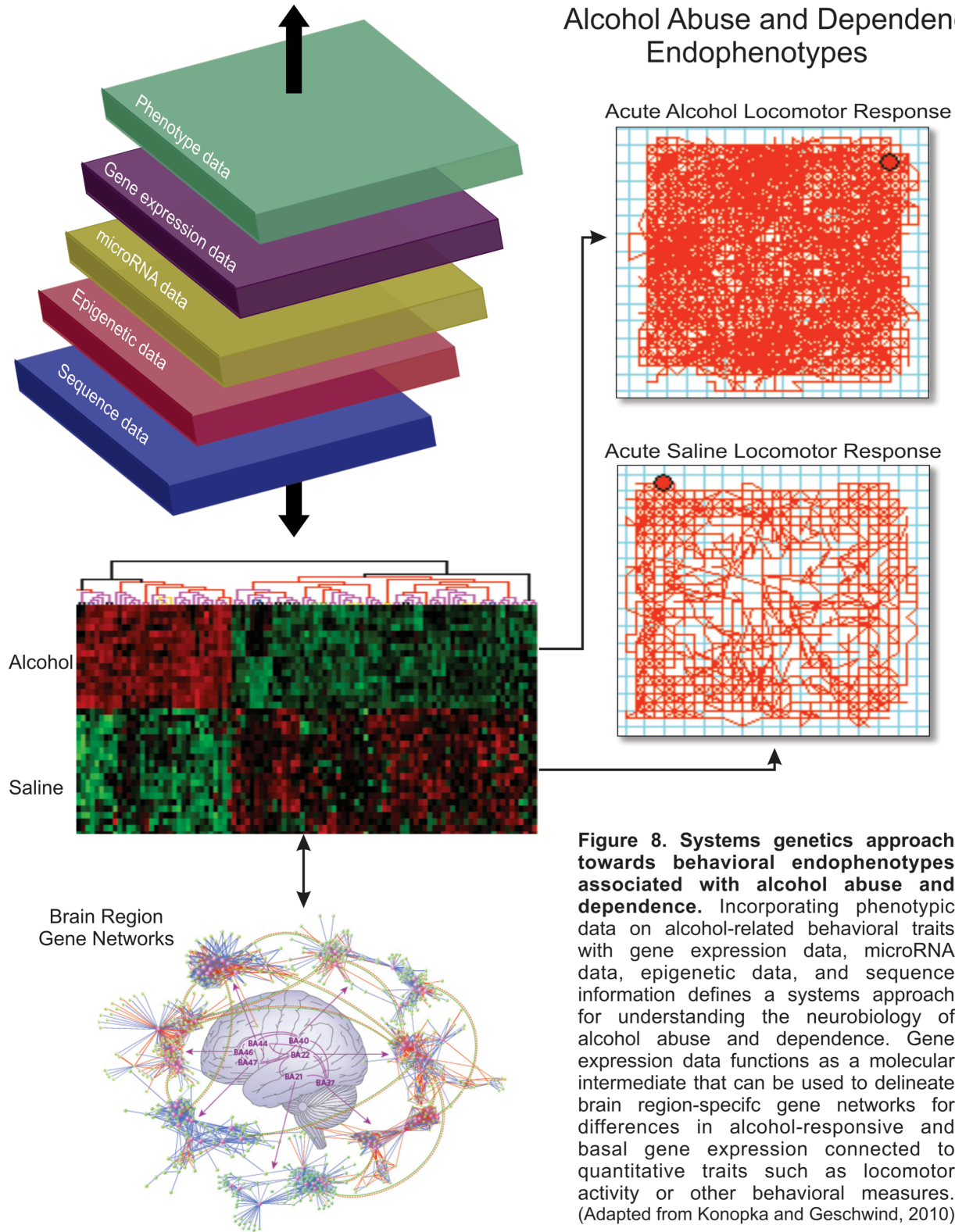


Figure 8. Systems genetics approach towards behavioral endophenotypes associated with alcohol abuse and dependence. Incorporating phenotypic data on alcohol-related behavioral traits with gene expression data, microRNA data, epigenetic data, and sequence information defines a systems approach for understanding the neurobiology of alcohol abuse and dependence. Gene expression data functions as a molecular intermediate that can be used to delineate brain region-specific gene networks for differences in alcohol-responsive and basal gene expression connected to quantitative traits such as locomotor activity or other behavioral measures. (Adapted from Konopka and Geschwind, 2010)

CHAPTER 3:

GENE NETWORKS OF CHRONIC ALCOHOL ADMINISTRATION ACROSS SPECIES

Introduction

Alcoholism is a chronic relapsing condition resulting from excessive alcohol consumption associated with altered cognition and behavior. Individual differences as well as disrupted homeostatic control over brain mechanisms involving positive and negative reinforcement underlie the neurobiology of maladaptive alcohol drinking behavior (Koob, 2003, Spanagel et al., 2010). Although no animal model completely encompasses every aspect of alcoholism (McClearn, 1979, Crabbe, 2008), preclinical models have been valuable tools for investigating the genetic and environmental factors influencing alcohol consumption (Becker, 2000, Pautassi et al., 2010). Non-human primate models in particular are powerful experimental models due to their genetic, neuroanatomical, physiological, behavioral, and social similarities to humans (Barr et al., 2003, Weerts et al., 2007). Alcohol drinking behavior in non-human primates parallels several factors witnessed in some rodent models and human alcoholics, potentially demonstrating one or more components related to alcohol consumption: voluntary self-administration, intoxication, dependence, withdrawal, and significant variation in individual alcohol intake (Grant and Bennett, 2003). The biological and behavioral similarities between non-human primates and humans, as well as certain facets of other animal models, present a unique opportunity for translational studies on alcohol abuse and dependence (Barr and Goldman, 2006, Zahr and Sullivan, 2008).

Non-human primates have played an important role in understanding the neurobiology of disease (Capitanio and Emborg, 2008). Comparative transcriptomics reveals an elaborate array of gene expression differences between non-human primates and human brain (Caceres et al., 2003, Hsieh et al., 2003). Expression differences, largely resulting from increased gene expression in human brain, may be a consequence of accelerated brain evolution (Enard et al., 2002, Gu and Gu, 2003), which is reflected in the increased volume and complexity of the human brain (Gazzaniga, 2009). Differing regions of the mammalian brain are remarkably heterogeneous, potentially capable of accommodating hundreds of contrasting cell types (Levitt et al., 1997, Geschwind, 2000). Despite the inherent and evolving complexity, coordinately regulated gene expression networks are conserved among discrete brain regions (Oldham et al., 2006). Additionally, there is a robust organization to the mammalian brain transcriptome (Oldham et al., 2008). Deciphering the overall framework of gene co-expression networks among brain regions provides a systematic approach to understanding the neurobiology of disease mechanisms (Miller et al., 2010, Torkamani et al., 2010, Voineagu et al., 2011). The extent to which co-expression networks are associated with the underlying neurobiology of addiction remains to be elucidated; however, integrative cross-species analyses provides important details concerning the molecular biology of phenotypes involved in neuropsychiatric diseases (Konopka and Geschwind, 2010).

Genetic factors explain approximately 50% of the vulnerabilities leading to excessive alcohol intake (Schuckit, 2009b). Cross-species investigations have focused on individual genes associated with specific endophenotypes related to alcohol abuse

and dependence (Kapfhamer et al., 2008, Corl et al., 2009, Lasek et al., 2011, Schumann et al., 2011), suggesting there are evolutionary conserved factors in the pathogenesis of alcoholism. Whole-brain microarray meta-analysis of alcohol naïve mice known to differ markedly in alcohol drinking behavior demonstrates variation in gene expression contributes to the predisposition for abuse (Mulligan et al., 2006, Tabakoff et al., 2008). Basal and drug-responsive differences in expression patterns are dependent upon both genotype and brain-regions, conferring distinct neurobehavioral phenotypes (Sandberg et al., 2000, Kerns et al., 2005, Letwin et al., 2006). Gene expression differences within discrete brain-regions are correlated to individual differences in alcohol drinking behavior (Mulligan et al., 2011, Wolstenholme et al., 2011). The gene expression abundance for GABA and NMDA receptor subunits is altered in cynomolgus monkeys (Hemby et al., 2006, Acosta et al., 2010). Changes in the expression of receptor subunits alter receptor and behavioral sensitivity to alcohol (Haughey et al., 2008, Hurley et al., 2009), suggesting variation in gene expression is important for the acute and long-term effects of alcohol. Inter- and intra-species variation in gene co-expression networks are significantly associated with phenotypic differences in alcohol intake. Defining conserved gene networks across species permits a translational approach for understanding the molecular mechanisms governing alcohol-drinking behavior, and a foundation for further scientific hypotheses.

As part of the Integrative Neuroscience Initiative on Alcoholism (INIA) consortium, a comparison of microarray profiles from brain tissue are discussed herein for differing evaluations of alcohol drinking behavior in non-human primates and mice, as well as a previous microarray analysis of human alcoholic brain tissue. Female cynomolgus

macaques were analyzed across the prefrontal cortex (PFC), nucleus accumbens (NAC), hippocampus (HIP), and amygdala (AMY) in comparison to control subjects. Gene expression profiles within prefrontal cortex were also analyzed for male cynomolgus macaques, a separate cohort of male rhesus macaques, and a panel of heterogeneous mice. The prefrontal cortex is part of the mesocorticolimbic dopaminergic 'reward' pathway involved in numerous behavioral functions and the neurobiology of addiction (Ross and Peselow, 2009, Goldstein and Volkow, 2011). Previous research from our laboratory and others has shown chronic alcohol abuse causes decreased expression of myelin-associated genes within the frontal cortex of postmortem brain tissue (Lewohl et al., 2000, Mayfield et al., 2002). Coordinate down-regulation for the expression of a myelin-associated gene network may be an underlying component in CNS plastic events of alcohol dependence. Rather than emphasizing individual genes, gene expression networks are constructed to illustrate some of the global properties presiding over alcohol drinking behavior in mammalian species (Zhao et al., 2010b). We hypothesize that variation in an evolutionary conserved gene networks, including a network related to myelin-associated gene expression, are important determinants of alcohol behavioral phenotypes. Networks are not directly intended to infer causality, but are meant to convey an integrative viewpoint of molecular signatures underlying the neurobiology of endophenotypes associated with alcohol dependence, as well as set the stage for additional scientific inquiries. Our approach imparts a unique systems hypothesis of an evolutionary conserved network(s) involved in alcohol drinking behavior across mice, monkeys, and humans.

Materials and Methods

Non-human primate subjects and Self-Administration of Alcohol: Studies of non-human primates were part of a collaborative study through the Integrative Neuroscience Initiative on Alcoholism (INIA). Primate procedures were approved by the Oregon National Primate Research Center Animal Care and Use Committee and carried out according to the National Institutes of Health Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research. Overall three different groups were analyzed for gene expression differences: female cynomolgus macaque (*Macaca fascicularis*; INIA cohort #3, n = 12 alcohol drinkers and n = 4 controls), male cynomolgus macaque (*Macaca fascicularis*; INIA cohort #2, n = 11 alcohol drinkers and n = 4 controls & n = 7 housing keeping controls) and male rhesus macaque (*Macaca mulatta*, INIA cohort #4&5, n = 19 alcohol drinkers and n = 5 controls). As part of the INIA collaborative effort by our group, nucleus accumbens, amygdala, hippocampus, and prefrontal cortex (areas 24, 25, and 32 pooled together) were sampled from subjects for microarray analysis of INIA cohort #3. Prefrontal cortex (areas 24, 25, and 32 pooled together) was sampled from subjects for microarray analysis of INIA cohort #2, as well as INIA cohorts #4 and #5. Cynomolgus macaques were purchased from a commercial vendor (World Wide Primates, Miami FL); rhesus macaques were obtained from the Oregon National Primate Research Center with verified birth dates and pedigree information.

The schedule-induced polydipsia (SIP) method for inducing monkeys to drink alcohol was utilized to establish alcohol self-administration (Vivian et al., 2001). Monkeys were trained to operate a drinking panel in their cage and to place a finger

inside an opening that broke a light beam to deliver a banana pellet. This training was used to document food intake during scheduled meals (Grant et al., 2008). Monkeys then were induced to consume water (in the volume of a 1.5 g/kg dose of 4% (w/v) ethanol in water) in daily 22-h sessions by delivering banana pellets at a fixed time interval of 300 s in which no response was required.

After 1 month of induction, ethanol (4% w/v) was the only fluid present for the initial SIP component of each daily 22-h session. The dose of ethanol induced by SIP in the first component of the session was increased in a stepwise fashion over 30-session epochs. Monkeys were induced to drink ethanol, 0.5 g/kg/day (two or three drinks), for 30 days, then 1 g/kg/day (four or five drinks) for 30 days, and finally 1.5 g/kg/day (six or seven drinks) for 30 days. For these purposes a drink is operationally defined as the equivalent of 0.25 g/kg ethanol (17 grams of alcohol in a 70 kg human) (Kalant and Poikolainen, 1999). After the induced dose of ethanol was consumed, only water was available for a period of 3 hours. Following that period, water and the remaining food were available for the remaining session time. Voluntarily alcohol self-administration was started in the fifth month, wherein monkeys were allowed free access to 4% ethanol and water for 22 hours/day (11:00 AM to 9:00 AM). The quantity and pattern of self-administered ethanol was measured accurately by mass displacement from a fluid reservoir and highly correlated with measurements of blood ethanol concentration (Figure 9).

After one year of open access to ethanol, monkeys in the chronic intoxication (CI) group were killed and necropsied (INIA Cohorts #3, 4, and 5). INIA cohort #2 was subjected to repeat sustained abstinence or repeated bouts of withdrawal. INIA cohort

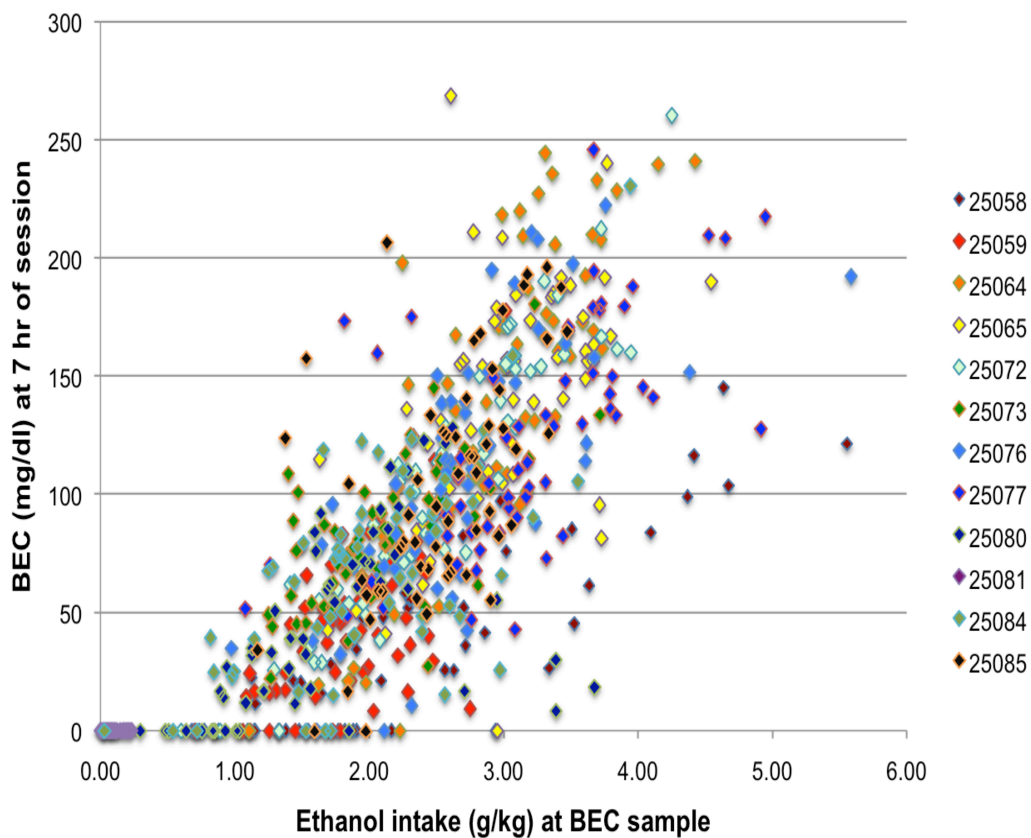


Figure 9. Correlation of blood ethanol concentration (BEC) with ethanol self-administration in cynomolgus macaques. Blood ethanol concentrations at 7th hour of self-administration session and corresponding ethanol intake at the time of sample, taken every 5th day for 12 months of ethanol drinking. Courtesy of Dr. Kathleen Grant and colleagues, Oregon Health and Science University.

#2 group self-administered ethanol according to the same protocol as the chronic intoxication monkey groups, but were given an additional 4 months of open access followed by 28 days of abstinence; this sequence was repeated two more times, and then were necropsied at the end of the third abstinence period.

The necropsy procedures were performed by trained staff members at Oregon Health and Science University according to published protocols (Daunais et al., 2010). Monkeys were sedated with ketamine (15 mg/kg, i.m.), and hair was removed from the head, neck, and calves. A catheter was inserted into the saphenous vein and secured to the skin with tape. A surgical plane of anesthesia was established by slowly injecting 20–35 mg/kg sodium pentobarbital through the i.v. catheter and flushing with an equal volume of saline. The adequacy of surgical anesthesia was verified by the absence of the corneal, palpebral, and hindlimb withdrawal reflexes. Oregon Health and Science University affiliated veterinarian monitored cardiac and respiratory function and blood pressure closely throughout the procedure. Incisions were made in the scalp along the sagittal suture from bregma to the third cervical vertebra and perpendicularly along the coronal suture and occipital ridge. Then the skull was exposed by reflecting the temporal, frontal, and occipital muscles and by reflecting the muscles along the sagittal suture and the occipital ridge. The craniotomy was carried out using bone rongeurs after drilling a hole 1 cm in diameter in the right parietal bone. Once the bone was removed, the calvaria was removed from the dura mater, and the occipital ridge was removed. The exposed brain was covered gently with saline-soaked gauze. The animal was turned onto its back, and sequential incisions were made through the skin from the clavicle to the pelvis and then through the abdominal and pectoral muscles and

diaphragm. The ribcage was cut, and the pericardium was opened. The right atrium was cut to drop blood pressure, and a 16-gauge cannula was inserted into the ascending aorta and clamped into place. The descending aorta was clamped in the lower thorax. The animal was perfused over 2 min with 1.5 L of oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) NaCl 124, KCl 5, NaH₂PO₄ 3, MgSO₄ 2, D-glucose 10, NaHCO₃ 26, and CaCl₂ 2 (290–300 mOsm, pH 7.3–7.4, adjusted by 95% O₂/5% CO₂). After perfusion, the animal was turned over immediately, and the dura mater was cut at the posterior extent of the occipital lobes and along the mid-sagittal line and was reflected. The falx cerebri was removed, the occipital lobes were elevated with a scalpel handle, and then the tentorium cerebelli was removed. The cervical cord was cut at C3 using a scalpel, and the cranial nerves were dissected bluntly from the brain using the scalpel's handle. The brain was removed from the skull in a caudal-to-rostral fashion. The brainstem was separated using a razor blade by sectioning just rostral to the pontomedullary junction and by sectioning the cerebellar peduncles. The tissue block was submerged in a small beaker of oxygenated, low sodium, ice-cold ACSF (in mM) composed of sucrose 220, KCl 2, NaH₂PO₄ 1.5, MgSO₄ 1.2, D-glucose 10, NaHCO₃ 26, and CaCl₂ 0.2 and was transported to the electrophysiology laboratory. Once the brainstem was isolated, the remaining brain and tissues of the body were prepared for additional studies by other investigators. Individual brain sections for the purposes of the studies performed here at Virginia Commonwealth University were flash frozen using liquid nitrogen and stored at -80°C until over-night shipment on dry ice.

Administration of Chronic Ethanol and Micro-dissection in Mice: A total of 121 mice (85 males and 36 females representing 43 BXD RI strains along with progenitor

strains, F2 generation, and positive controls) were initially included in the study BXD recombinant inbred mice (Figure 2) were obtained from University of Tennessee Health Science Center (Memphis, Tennessee); C57BL/6J mice (positive controls) obtained from Jackson Laboratories (Bar Harbor, ME). This study was conducted as the first part of an overall design, with the second part to be completed using complementary male and female mice of the corresponding genotypes used in the present study. The general study design involved typically one mouse per experimental cell (n= 1/genotype/sex/group). A positive control condition (C57BL/6J male mice) was included in the study (n = 6-8/group).

An established mouse model for dependence and repeated bouts of alcohol drinking behavior was utilized for investigating the molecular signatures underlying alcohol consumption (Becker and Lopez, 2004, Lopez and Becker, 2005). Alcohol intake was measured using a two-bottle choice (15% v/v ethanol vs. water) limited access (2 hr/day) drinking model; baseline intake was determined for each mouse genotype over a period of six weeks of access. After establishing baseline intake, mice from each genotype received 4 weekly cycles of chronic intermittent ethanol (CIE) vapor exposure (EtOH group) or air exposure (CTL group) in inhalation chambers (16 hr/day x 4 days + 72 hr forced abstinence) alternated with weekly test cycles in which ethanol intake was measured during five consecutive limited access daily drinking sessions. Mice were not food or water deprived throughout the duration of the study. Ethanol concentrations in the inhalation chambers were uniformly set for all genotypes to yield blood ethanol levels in the range of 200-300 mg/dl, using the aldehyde dehydrogenase inhibitor pyrazole (Griffin et al., 2009). Blood samples were collected from all mice on the 2nd and

4th day of each exposure cycle (mean BEC values for each exposure cycle are presented). Body weights were recorded weekly for each subject to determine the amount of alcohol intake per kilogram of body weight. All mice received a fifth-cycle of CIE exposure cycle and EtOH and CTL groups were sacrificed by decapitation at 72 hr post removal from inhalation chambers. As part of the INIA collaboration blood and tissue samples were collected from brain and various organ systems during the same necropsy sessions. Per our part of the genomics investigation, brain tissue from prefrontal cortex, nucleus accumbens, and ventral tegmental area was acquired via a micro-punch dissection and stored as previously described (Kerns et al., 2005). The analysis herein will focus on data derived from prefrontal cortex; results from additional brain regions collected shall be presented in future investigations.

Human Alcoholic Brain Tissue: Raw gene expression data was retrieved from the Gene Expression Omnibus (GEO; GSE29555) for alcoholic and control brain tissue from frontal cortex, central nucleus of amygdala, and basolateral nucleus of amygdala (Ponomarev et al., 2012). Expression profiling was processed using the Illumina HumanHT-12 V3.0 Expression beadchip. Autopsy brain samples were obtained from the New South Wales Tissue Resource Centre at the University of Sydney. The Centre is funded in part by the National Institute on Alcohol Abuse and Alcoholism to provide brain tissue for alcoholism research. Fresh-frozen sections of tissue from the central (CNA) and basolateral nucleus (BLA) of amygdala, as well as the superior frontal cortex (CTX), were obtained from 32 cases (17 alcoholics and 15 matched controls; 30 males and 2 females). These regions are important substrates in the reward circuitry that is involved

in the development of alcohol dependence and alcoholism (Koob and Volkow, 2010). Cases were matched as closely as possible by age, gender, postmortem interval (PMI), and brain pH. Diagnoses were confirmed by physician interviews, review of hospital medical records, questionnaires to next-of-kin, and pathology, radiology, and neuropsychology reports. Cases were also chosen on the basis that agonal hypoxia did not appear to have differed significantly from the study group. Moreover, none of the brains showed evidence of hypoxic encephalopathy; further suggesting that agonal hypoxia was minimal. We did not accept cases that suffered prolonged agonal states. Cases with a history of polydrug abuse were excluded. Cases were matched for smoking history. In addition, cases with concomitant diseases, such as cirrhosis of the liver, Korsakoff psychosis, or Wernicke or hepatic encephalopathies, were excluded. The concentration and quality of all RNA samples was determined, and degraded (RNA integrity number, RIN <4) and/or contaminated RNA samples were excluded from the analysis. The Diagnostic and Statistical Manual of Mental Disorders-IV diagnosis assigned to each case was based on a detailed and standardized clinical assessment summary created by specially trained staff with a background in psychiatry and/or psychology (Ponomarev et al., 2012).

Sample Processing, Microarray Hybridization and Scanning: Brain tissue for each sample from individual mouse and monkey subjects was homogenized in STAT-60 reagent (Tel-Test, Friendswood, TX) using a Tekmar homogenizer. Total RNA was isolated according to the manufacturer's protocol (<http://www.tel-test.com/prod02.htm>). RNA concentration was determined by absorbance at 260 nm, and RNA quality was

analyzed by agarose gel electrophoresis and 260:280 nm absorbance ratios. Total RNA derived from each sample was reverse transcribed into double-stranded cDNA using the One-cycle Targeting and Control Reagent kit (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA was synthesized from cDNA, purified, and fragmented according to Affymetrix instructions. Individual samples per experiment, treatment, and brain-region were hybridized to an individual microarray for each of all of the brain-regions studied. Labeled cRNA non-human primate samples were analyzed on Affymetrix oligonucleotide microarrays (Rhesus Macaque Genome Array; Affymetrix, Santa Clara, CA) that contain over 52,000 probe sets to interrogate the known genes in the monkey genome. Labeled cRNA mouse samples were analyzed on Affymetrix oligonucleotide microarrays (Mouse Genome Array 430 2.0; Affymetrix, Santa Clara, CA) that contain over 45,000 probe sets targeting over 34,000 well substantiate mouse genes. Hybridization and scanning were performed separately for each experiment, and exactly according to the protocol of the manufacturer and as described previously (Thibault et al., 2000, Kerns et al., 2005). Arrays were the washed, stained with streptavidin-phycoerythrin, and scanned according to standard protocols supplied Affymetrix.

Microarray Data Acquisition and Analysis: Microarray data were initially processed GeneChip Operating Software (GCOS; Affymetrix). Arrays were normalized to median total hybridization intensity, target average intensity, equal to 190). Array quality was assessed by accepting only arrays with a scaling factor of < 3.0 and a 3'-5'-actin ratio of <2 and by examining chip validity and linearity of intensity values. Arrays passing quality control measures were subjected to microarray suite version 5.0 (MAS 5.0) to eliminate

probesets consistently called 'absent' across all samples (McClintick and Edenberg, 2006), using the R environment for statistical computing (Team, 2010). Probe-level data from MAS5.0 filtered datasets were further summarized using robust multi-array average (RMA) algorithm (Irizarry et al., 2003) and the significance scores algorithm (S-score) (Zhang et al., 2002). The S-score algorithm compares hybridization signals from two differing treatment samples (e.g. Control Sample #1 versus Alcohol Sample #1); for monkey samples controls were averaged to a single factor for calculating the S-score of each individual probeset. S-scores for mouse samples were calculated using the within genotype air control sample, but an average of C57BL/6J air controls was used for calculation of the positive controls group. The relatively low number of replicates (e.g. only 4 control samples for female cynomolgus macaques) may have limited detection of more subtle changes in gene expression, particularly of low-abundance genes; however, the S-score analysis methodology is useful for having limited number of Affymetrix arrays because the method uses the statistical power of all oligonucleotide pairs for a given probeset (Zhang et al., 2002). Additionally, the S-score algorithm provides summary statistic which is normally distributed with a mean = 0 and standard deviation = 1, with signals correlated to fold-change in gene expression between alcohol treatment and controls. An S-score of 2.0 corresponds to a p-value = 0.0455, not corrected for biological variability or multiple comparisons. INIA cohorts #3 and #2 (female and male cynomolgus macaques) were tested separately using the rank-based permutation method statistical analysis of microarrays (SAM) (Tusher et al., 2001) to evaluate genes differentially expressed between brain-regions (for female cynomolgus macaques) and treatment groups. SAM significant genes were further filtered for an

average S-score of ≥ 1.5 or ≤ -1.5 representing a composite significance of p-value < 0.01 , uncorrected for multiple comparisons. All SAM analyses used a false discovery rate of $\leq 5\%$ to avoid eliminating genes that may be biologically important and could assist in interpretation of expression patterns in multivariate studies. Multivariate and bioinformatic analyses were conducted on gene lists determined by SAM using The Institute for Genome Research Multi-experiment Viewer (TMeV) (Saeed et al., 2003, Saeed et al., 2006). Mouse Affymetrix probesets were annotated using the information available through the data sharing zone of GeneNetwork (<http://www.genenetwork.org/>), current as of November 11, 2011. Monkey Affymetrix probesets were annotated using information available through Ingenuity Pathway Analysis (<http://www.ingenuity.com/>), current as of November 15, 2011.

Bioinformatics Analysis of Microarray Data: Hierarchical (Eisen et al., 1998) or k-means clustering (Soukas et al., 2000) of gene expression data using TMeV or Cluster 3.0 and TreeView (Page, 1996, de Hoon et al., 2004). Toppgene Suite (Chen et al., 2009c) and was used for data exploration of functional classification among gene expression profiles using gene ontology categories, mouse phenotype data, and public pathway databases. ToppFun (functional enrichment analysis within the Toppgene Suite) calculations were set to a 5% false discovery rate, with gene limits of $n \geq 3$ and ≤ 300 to identify representative *a priori* ontological categories. Primary categories investigated were those defined by the Gene Ontology Consortium: biological processes, molecular function, and cellular component (Ashburner et al., 2000). Literature association analysis of statistically significant genes was investigated using Ingenuity Pathway

Analysis (<http://www.ingenuity.com/>), a curated bioinformatic resource for the discovery of biological interaction among differing genes based on literature association, biological function, and cell-signaling mechanisms. GeneGo was additionally used to identify shared literature and pathway relationships among gene products.

Results

Differential Gene Expression Among Brain regions in Female Cynomolgus Macaques

Basal differences in the expression of genes among disparate brain regions may be a contributing factor to alcohol drinking behavior in human populations. Non-human primates provide a sensible substitute as an experimental model for studying the underlying neurobiology of substance abuse. In order to illustrate some of the potential factors that may influence the predisposition for alcohol drinking behavior, and highlight individual differences among brain regions within the reference samples, we analyzed the RMA expression measures for differences among the prefrontal cortex (PFC), nucleus accumbens (NAC), hippocampus (HPC), and amygdala (AMY) in the female cynomolgus macaques non-drinking control samples (Figure 10A and 10B). Gene expression differences were unevenly distributed among the four brain regions with PFC >> AMY > HPC > NAC (Figure 10C). Relative to the average intensity across the four brain structures, the PFC exhibited the largest number of differentially expressed genes with more genes down regulated than up regulated. (Figure 10D) By and large, functional over-representation analysis suggests differential expression of genes

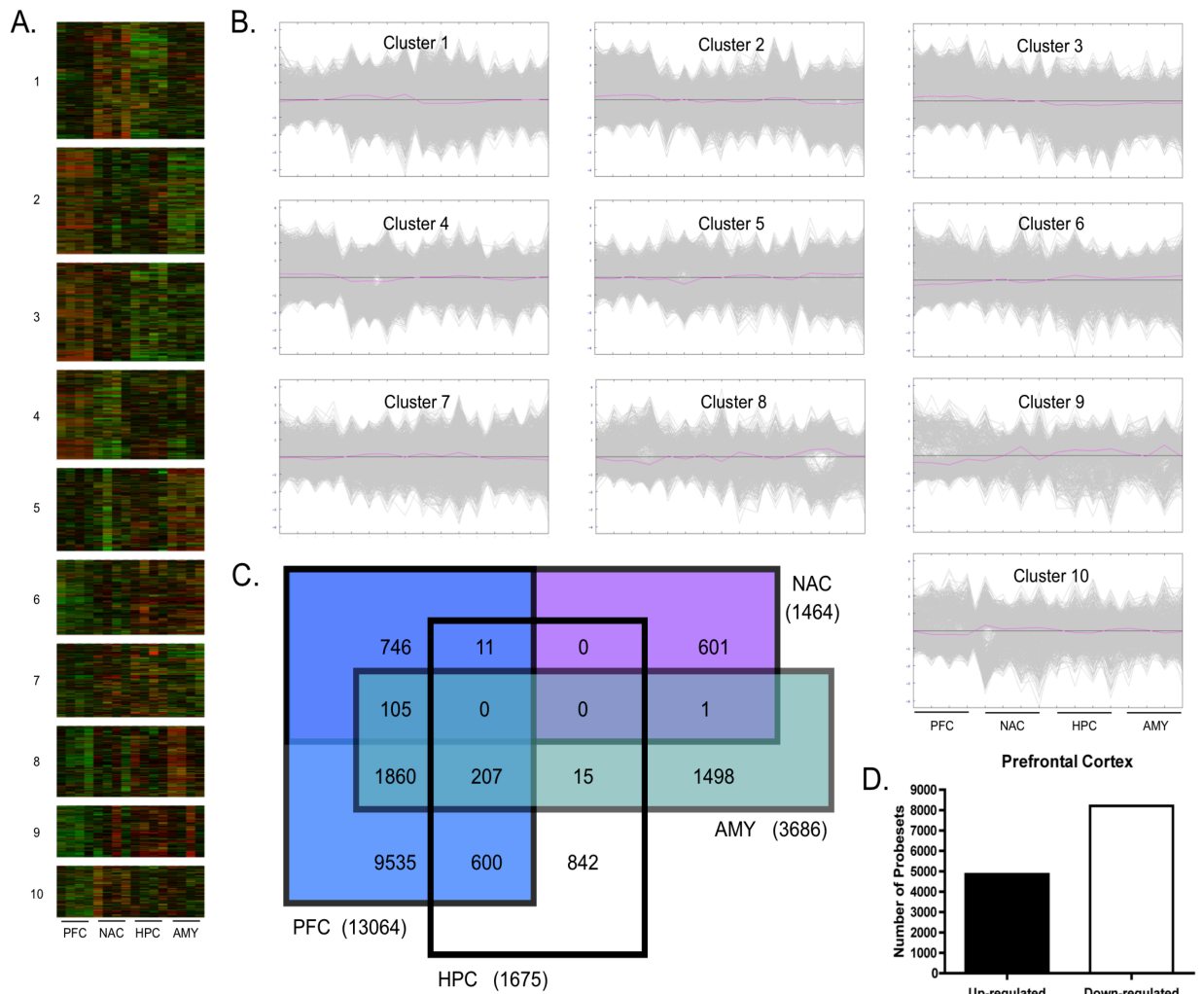


Figure 10. Basal gene expression differences among different brain regions in female cynomolgus macaques. (A) K-means clustergram relative gene expression showing differential gene expression among prefrontal cortex (PFC), nucleus accumbens (NAC), hippocampus (HPC), and amygdala (AMY); Red= increased expression. Green= decreased expression. Black = no change in expression. Figure amended from Kerns et al. 2005. (B) Expression graphs for clusters presented in 10A, grey lines show expression for individual genes, pink line indicates mean expression across all genes for each sample, y-axis corresponds to S-score values and x-axis corresponds to samples for each brain region. (C) 4-way venn diagram showing overlap of basal gene expression among the PFC, NAC, HPC, and AMY. (D). Bar graph showing the number of total probesets up-regulated and down-regulated in their expression pattern.

involved in nerve growth factor signaling (PFC), GTPase activity and synaptic vesicles (NAC), cAMP metabolic process (HPC), and kinase activity (AMY) (Table 1). The biological processes operating within each brain region may underlie variation in alcohol drinking behavior. For example, nerve growth factor plasma concentrations are abnormal in alcohol-dependent patients (Yoon et al., 2006), and has been suggested as a biomarker for the differing stages of alcohol abuse and dependence (Jockers-Scherubl et al., 2007). Nerve growth factor activity also interacts with chronic alcohol exposure in rodents (Aloe et al., 1993, Gericke et al., 2006), suggesting variation in nerve growth factor signaling may influence alcohol-drinking behavior.

Gene Expression in Chronic Alcohol Drinking Female Cynomolgus Macaques

Persistent alterations in the transcriptome as a result of chronic alcohol consumption may be a key-determining factor in compulsive behavior and alcoholism (Saito et al., 2004, Worst et al., 2005). SAM analysis, using an FDR correction less than or equal to 5%, identified shared and discrete gene expression differences between the PFC, NAC, HPC, and AMY (Figure 11 A&B). All four brain regions demonstrated more genes were up-regulated than down-regulated, with unequal overall differences showing the PFC > HPC > NAC > AMY (Figure 11C). A total of 169 probesets (representing ~113 unique genes), differentially regulated by alcohol drinking female cynomolgus macaques, are in common among the PFC, NAC, HPC, and AMY (Figure 11 A&B). The overlapping genes are over-represented for kinase signaling, sphingomyelin synthesis (SGMS2 and SPTLC1), ribosomal components, and mitochondrial function (Table 2). Abnormal expression of mitochondrial genes and mitochondrial dysfunction actively

Table 1. Functional over-representation for brain-region differences in female cynomolgus macaques

| Category | ID | Name | P-value | % Targeted in Category |
|------------------------|------------------------|------------|-------------|------------------------|
| PFC | GO: Biological Process | GO:0048011 | 4.00E-06 | 60.71428571 |
| | GO: Biological Process | GO:0046039 | 1.28E-03 | 55.51470588 |
| | GO: Cellular Component | GO:0030427 | 2.27E-03 | 63.26530612 |
| | GO: Cellular Component | GO:0016585 | 4.01E-03 | 61.61616162 |
| | GO: Molecular Function | GO:0004842 | 2.00E-06 | 60.18099548 |
| | GO: Molecular Function | GO:0003713 | 2.87E-04 | 56.41025641 |
| | GO: Molecular Function | GO:0019900 | 1.20E-03 | 53.53535354 |
| | Human Phenotype | HP:0002311 | 5.65E-03 | 45.52845528 |
| | Human Phenotype | HP:0001251 | 5.65E-03 | 44.32624113 |
| | Human Phenotype | HP:0001268 | 5.65E-03 | 47.8021978 |
| | GO: Molecular Function | GO:0003924 | 4.20E-05 | 15.48672566 |
| | GO: Biological Process | GO:0045664 | 7.14E-03 | 12.83018868 |
| | GO: Biological Process | GO:0016358 | 9.30E-03 | 19.1011236 |
| | GO: Biological Process | GO:0048489 | 9.30E-03 | 25.53191489 |
| MAC | GO: Cellular Component | GO:0030424 | 1.00E-06 | 15.4676259 |
| | GO: Cellular Component | GO:0030426 | 4.50E-05 | 21.27659574 |
| | GO: Cellular Component | GO:0008021 | 2.88E-04 | 18.34862385 |
| | Mouse Phenotype | MP:0002207 | 2.90E-05 | 19.8757764 |
| | Mouse Phenotype | MP:0002204 | 5.08E-03 | 25.3968254 |
| | Mouse Phenotype | MP:0002945 | 5.36E-03 | 25.86206897 |
| | GO: Molecular Function | GO:0051020 | 4.18E-04 | 11.88811189 |
| | GO: Molecular Function | GO:0015185 | 9.74E-04 | 60 |
| | GO: Molecular Function | GO:0042923 | 1.08E-03 | 17.77777778 |
| | GO: Molecular Function | GO:0031402 | 3.18E-03 | 42.85714286 |
| | GO: Biological Process | GO:0007212 | 1.00E-06 | 43.47826087 |
| | GO: Biological Process | GO:0046058 | 2.00E-05 | 13.60544218 |
| | GO: Biological Process | GO:0042136 | 2.40E-05 | 42.85714286 |
| | GO: Cellular Component | GO:0005834 | 3.27E-04 | 21.05263158 |
| HPC | GO: Cellular Component | GO:0030665 | 1.67E-03 | 12.63157895 |
| | GO: Cellular Component | GO:0043197 | 2.11E-03 | 12.94117647 |
| | GO: Molecular Function | GO:0003735 | 3.00E-06 | 26.70807453 |
| | GO: Molecular Function | GO:0019900 | 1.10E-05 | 22.22222222 |
| | GO: Molecular Function | GO:0015108 | 1.28E-04 | 57.14285714 |
| | GO: Molecular Function | GO:0003714 | 1.54E-04 | 23.52941176 |
| | GO: Biological Process | GO:0006414 | 1.00E-06 | 33.06451613 |
| | GO: Biological Process | GO:0006415 | 1.00E-06 | 36.17021277 |
| | GO: Biological Process | GO:0043624 | 1.00E-06 | 30.51948052 |
| | GO: Cellular Component | GO:0022626 | 1.00E-06 | 37.5 |
| | GO: Cellular Component | GO:0043197 | 5.20E-05 | 29.41176471 |
| | GO: Cellular Component | GO:0044309 | 5.20E-05 | 29.41176471 |
| | GO: Cellular Component | GO:0005891 | 5.30E-05 | 47.82608696 |
| | Mouse Phenotype | MP:0004753 | 5.00E-06 | 43.75 |
| Mouse Phenotype | MP:0001417 | 2.10E-05 | 36.61971831 | |
| AMY | GO: Biological Process | GO:0048011 | 4.00E-06 | 60.71428571 |
| | GO: Biological Process | GO:0046039 | 1.28E-03 | 55.51470588 |
| | GO: Cellular Component | GO:0030427 | 2.27E-03 | 63.26530612 |
| | GO: Cellular Component | GO:0016585 | 4.01E-03 | 61.61616162 |
| | GO: Molecular Function | GO:0004842 | 2.00E-06 | 60.18099548 |
| | GO: Molecular Function | GO:0003713 | 2.87E-04 | 56.41025641 |
| | GO: Molecular Function | GO:0019900 | 1.20E-03 | 53.53535354 |
| | Human Phenotype | HP:0002311 | 5.65E-03 | 45.52845528 |
| | Human Phenotype | HP:0001251 | 5.65E-03 | 44.32624113 |
| | Human Phenotype | HP:0001268 | 5.65E-03 | 47.8021978 |
| | GO: Molecular Function | GO:0003924 | 4.20E-05 | 15.48672566 |
| | GO: Biological Process | GO:0045664 | 7.14E-03 | 12.83018868 |
| | GO: Biological Process | GO:0016358 | 9.30E-03 | 19.1011236 |
| | GO: Biological Process | GO:0048489 | 9.30E-03 | 25.53191489 |
| GO: Cellular Component | GO:0030424 | 1.00E-06 | 15.4676259 | |
| GO: Cellular Component | GO:0030426 | 4.50E-05 | 21.27659574 | |
| GO: Cellular Component | GO:0008021 | 2.88E-04 | 18.34862385 | |
| Mouse Phenotype | MP:0002207 | 2.90E-05 | 19.8757764 | |
| Mouse Phenotype | MP:0002204 | 5.08E-03 | 25.3968254 | |
| Mouse Phenotype | MP:0002945 | 5.36E-03 | 25.86206897 | |
| GO: Molecular Function | GO:0051020 | 4.18E-04 | 11.88811189 | |
| GO: Molecular Function | GO:0015185 | 9.74E-04 | 60 | |
| GO: Molecular Function | GO:0042923 | 1.08E-03 | 17.77777778 | |
| GO: Molecular Function | GO:0031402 | 3.18E-03 | 42.85714286 | |
| GO: Biological Process | GO:0007212 | 1.00E-06 | 43.47826087 | |
| GO: Biological Process | GO:0046058 | 2.00E-05 | 13.60544218 | |
| GO: Biological Process | GO:0042136 | 2.40E-05 | 42.85714286 | |
| GO: Cellular Component | GO:0005834 | 3.27E-04 | 21.05263158 | |
| GO: Cellular Component | GO:0030665 | 1.67E-03 | 12.63157895 | |
| GO: Cellular Component | GO:0043197 | 2.11E-03 | 12.94117647 | |
| GO: Molecular Function | GO:0003735 | 3.00E-06 | 26.70807453 | |
| GO: Molecular Function | GO:0019900 | 1.10E-05 | 22.22222222 | |
| GO: Molecular Function | GO:0015108 | 1.28E-04 | 57.14285714 | |
| GO: Molecular Function | GO:0003714 | 1.54E-04 | 23.52941176 | |
| GO: Biological Process | GO:0006414 | 1.00E-06 | 33.06451613 | |
| GO: Biological Process | GO:0006415 | 1.00E-06 | 36.17021277 | |
| GO: Biological Process | GO:0043624 | 1.00E-06 | 30.51948052 | |
| GO: Cellular Component | GO:0022626 | 1.00E-06 | 37.5 | |
| GO: Cellular Component | GO:0043197 | 5.20E-05 | 29.41176471 | |
| GO: Cellular Component | GO:0044309 | 5.20E-05 | 29.41176471 | |
| GO: Cellular Component | GO:0005891 | 5.30E-05 | 47.82608696 | |
| Mouse Phenotype | MP:0004753 | 5.00E-06 | 43.75 | |
| Mouse Phenotype | MP:0001417 | 2.10E-05 | 36.61971831 | |

Table 2. Functional over-representation for overlapping expression profiles between PFC, HPC, NAC, and AMY in female cynomolgus macaques

| Category | ID | Name | P-value | % Targeted in Category |
|------------------------|------------|--|----------|------------------------|
| GO: Molecular Function | GO:0019887 | protein kinase regulator activity | 2.24E-03 | 4.04040404 |
| GO: Molecular Function | GO:0019207 | kinase regulator activity | 4.34E-03 | 3.361344538 |
| GO: Molecular Function | GO:0016455 | RNA polymerase II transcription mediator activity | 1.12E-02 | 6.896551724 |
| GO: Molecular Function | GO:0003735 | structural constituent of ribosome | 1.24E-02 | 2.48447205 |
| GO: Molecular Function | GO:0030551 | cyclic nucleotide binding | 1.27E-02 | 6.451612903 |
| GO: Biological Process | GO:0006686 | sphingomyelin biosynthetic process | 2.96E-04 | 40 |
| GO: Biological Process | GO:0006684 | sphingomyelin metabolic process | 2.25E-03 | 15.38461538 |
| GO: Biological Process | GO:0042775 | mitochondrial ATP synthesis coupled electron transport | 4.62E-03 | 4.918032787 |
| GO: Biological Process | GO:0042773 | ATP synthesis coupled electron transport | 4.62E-03 | 4.918032787 |
| GO: Biological Process | GO:0046513 | ceramide biosynthetic process | 5.88E-03 | 9.523809524 |
| GO: Biological Process | GO:0006119 | oxidative phosphorylation | 6.25E-03 | 4.411764706 |
| GO: Cellular Component | GO:0005840 | ribosome | 5.23E-03 | 2.380952381 |
| GO: Cellular Component | GO:0016592 | mediator complex | 1.17E-02 | 6.451612903 |
| GO: Cellular Component | GO:0030426 | growth cone | 1.37E-02 | 3.191489362 |
| GO: Cellular Component | GO:0030427 | site of polarized growth | 1.53E-02 | 3.06122449 |
| GO: Cellular Component | GO:0030175 | filopodium | 2.00E-02 | 4.87804878 |
| GO: Cellular Component | GO:0032983 | kainate selective glutamate receptor complex | 2.10E-02 | 25 |
| Human Phenotype | HP:0000712 | Emotional lability | 8.63E-04 | 6.52173913 |
| Human Phenotype | HP:0001296 | Mood alterations | 1.62E-03 | 5.263157895 |
| Human Phenotype | HP:0100852 | Abnormal fear/anxiety-related behavior | 4.00E-03 | 3.846153846 |
| Human Phenotype | HP:0003380 | Decreased number of myelinated fibers | 7.54E-03 | 6.451612903 |
| Human Phenotype | HP:0010993 | Abnormality of the cerebral subcortex | 8.52E-03 | 6.060606061 |
| Human Phenotype | HP:0003546 | Exercise intolerance | 1.06E-02 | 5.405405405 |
| Human Phenotype | HP:0002520 | Abnormal myelination | 1.13E-02 | 2.654867257 |
| Human Phenotype | HP:0000231 | Thin vermilion border of upper lip | 1.30E-02 | 33.33333333 |

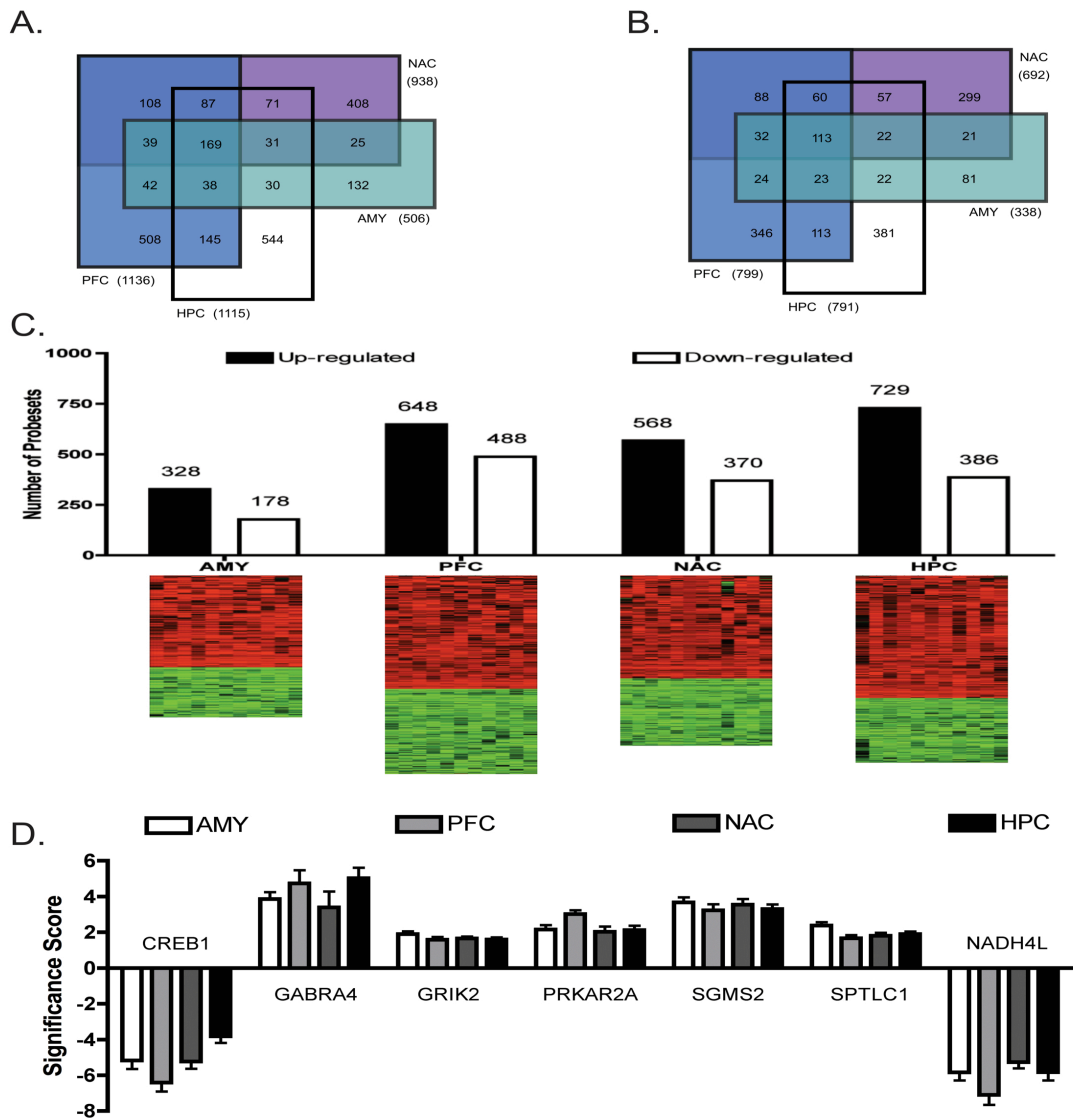


Figure 11. Microarray analysis of alcohol-responsive gene expression for female cynomolgus macaques. (A) 4-way venn diagram of alcohol-responsive probesets among differing brain regions. (B) 4-way venn diagram of alcohol-responsive genes, based on unique annotated gene symbols, among differing brain regions. (C) Bar graph for number of probesets up-regulated (black) and down-regulated (white) for each brain region, clustergrams for each brain region are shown beneath the x-axis; Red= increased expression. Green= decreased expression. (D) Selected alcohol-responsive genes overlapping within all four brain regions; prefrontal cortex (PFC), nucleus accumbens (NAC), hippocampus (HPC), and amygdala (AMY).

participates in alcohol drinking behavior and neurotoxicity (Li et al., 2002a, Sapag et al., 2009). The overlapping expression profiles between PFC, HPC, NAC, and AMY may thus represent system-wide brain targets of chronic alcohol exposure that contribute to continued drinking behavior. In addition to mitochondrial genes, several potential key alcohol-responsive targets were differentially expressed in all four-brain structures (Figure 11D).

The transcription factor *CREB1* is *significantly* down regulated in all four regions, which is consistent with previous reports of decreases in *CREB* leading to escalated alcohol intake (Misra and Pandey, 2003, Pandey et al., 2004). Signaling components upstream of *CREB*, such as cAMP-dependent protein kinase (which may include regulatory subunits such as *PRKAR2A*), modulates alcohol consumption (Wand et al., 2001); however, differing PKA subunits show different outcomes on alcohol intake (Thiele et al., 2000). Decreased expression of *CREB1* alongside increased *PRKAR2A* expression in drinking non-human primates may suggest compensatory effects within a signaling pathway. Additional intersecting targets among the differing brain structures suggest GABAergic (*GABRA4*) and glutamatergic (*GRIK2*) processes are important for heavy alcohol consumption. Variation in the expression *GABRA4* will mediate alcohol intake and preference (Rewal et al., 2009). *GRIK2* encodes for an ionotropic glutamate receptor subunit belonging to the kainate family. The expression of *GRIK2* is also altered in PKC γ knockout mice consuming a chronic alcohol diet (Bowers et al., 2006), suggesting *GRIK2* is an important downstream element of chronic alcohol exposure. Although these 169 probesets are differentially expressed within all four-brain structures examined, they also work in concert with brain-region specific differences in gene

expression. Brain region specific differences are important for substance abuse and alcohol-related behavioral phenotypes (Kerns et al., 2005, Melendez et al., 2011).

Amygdala Chronic Alcohol Gene Expression

The amygdala is a critical brain structure in stress, anxiety, and alcohol dependence (Heilig and Koob, 2007, Tye et al., 2011). As shown in Figure 11A and 11B ~338 genes were differentially regulated by chronic alcohol administration in amygdala. These genes primarily participate in axonogenesis, construction and organization of cellular components, and abnormal long-term potentiation (Table 3). Literature association analysis established connections among gene products surrounding histones and histone deacetylase (Figure 12A). Histones and histone deacetylases contribute to chromatin remodeling underlying enduring epigenetic modifications (Hondele and Ladurner, 2011). Acute and chronic alcohol induces amygdaloid chromatin plasticity through histone modification (Pandey et al., 2008). Systemic treatment with a histone deacetylase inhibitor augments the anxiolytic effects of alcohol and alcohol drinking behavior in rodents (Wolstenholme et al., 2011, Sakharkar et al., 2012). Epigenetic modifications in mammalian brain underlie transcriptional alterations in human drug abusers and alcoholics (Zhou et al., 2011). The non-human primate amygdala network (Figure 12A) indicates a series of gene expression differences with shared biological function bordering epigenetic targets of chronic alcohol exposure, suggesting these expression changes are influential in alcohol drinking behavior and associated behavioral traits such as anxiety.

Table 3. Functional over-representation for alcohol-responsive genes in different brain regions of female cynomolgus macaques

| Category | ID | Name | P-value | % Targeted in Category | |
|------------------------|------------------------|---|---|------------------------|--------------|
| AMY | GO: Molecular Function | kinase regulator activity | 3.30E-05 | 8.403361345 | |
| | GO: Molecular Function | protein kinase regulator activity | 2.62E-04 | 8.080808081 | |
| | GO: Molecular Function | pyruvate dehydrogenase (acetyl-transferring) kinase activity | 1.65E-03 | 50 | |
| | GO: Biological Process | cellular protein complex disassembly | 4.88E-03 | 5.194805195 | |
| | GO: Biological Process | positive regulation of axonogenesis | 5.05E-03 | 9.756097561 | |
| | GO: Biological Process | regulation of long-term neuronal synaptic plasticity | 1.54E-02 | 9.677419355 | |
| | GO: Cellular Component | histone deacetylase complex | 7.32E-03 | 8.510638298 | |
| | GO: Cellular Component | postsynaptic membrane | 2.32E-02 | 4.093567251 | |
| | Human Phenotype | Abnormality of the glia cells | 4.95E-04 | 6.818181818 | |
| | Human Phenotype | Abnormal fear/anxiety-related behavior | 2.04E-03 | 6.41025641 | |
| | PFC | GO: Molecular Function | GABA receptor activity | 1.76E-04 | 27.27272727 |
| | | GO: Molecular Function | protein N-terminus binding | 1.60E-03 | 11.458333333 |
| | | GO: Molecular Function | GTPase binding | 1.86E-03 | 9.79020979 |
| | | GO: Biological Process | dendrite development | 1.30E-05 | 15.73033708 |
| | | GO: Biological Process | synaptic vesicle transport | 9.50E-05 | 19.14893617 |
| | | GO: Biological Process | regulation of long-term neuronal synaptic plasticity | 1.95E-04 | 22.58064516 |
| | | GO: Cellular Component | synaptosome | 1.30E-05 | 12.76595745 |
| GO: Cellular Component | | axon part | 7.50E-05 | 12.12121212 | |
| Human Phenotype | | HP:0001251 Ataxia | 1.87E-03 | 6.737588652 | |
| Human Phenotype | | HP:0002311 Incoordination | 6.34E-03 | 6.504065041 | |
| NAC | | GO: Molecular Function | nucleocytoplasmic transporter activity | 5.50E-05 | 38.46153846 |
| | | GO: Molecular Function | protein tyrosine phosphatase activity | 8.40E-05 | 12.38095238 |
| | | GO: Molecular Function | dopamine receptor binding | 4.38E-03 | 30 |
| | | GO: Molecular Function | potassium channel regulator activity | 4.96E-03 | 15.625 |
| | | GO: Biological Process | negative regulation of synaptic transmission, glutamatergic | 9.90E-05 | 50 |
| | | GO: Biological Process | mRNA transport | 6.95E-04 | 10.61946903 |
| | | GO: Biological Process | synapse assembly | 1.10E-03 | 13.55932203 |
| | GO: Biological Process | response to ethanol | 2.73E-03 | 10.84337349 | |
| | GO: Cellular Component | postsynaptic density | 4.28E-04 | 11.00917431 | |
| | GO: Cellular Component | presynaptic membrane | 6.90E-04 | 14.28571429 | |
| | GO: Molecular Function | protein N-terminus binding | 4.33E-04 | 12.5 | |
| | GO: Molecular Function | syntaxin-1 binding | 2.42E-03 | 26.66666667 | |
| | GO: Biological Process | cognition | 4.00E-06 | 12.90322581 | |
| | GO: Biological Process | synaptic vesicle transport | 1.40E-05 | 21.27659574 | |
| | GO: Biological Process | neurotransmitter secretion | 1.80E-05 | 14.42307692 | |
| | GO: Biological Process | regulation of long-term neuronal synaptic plasticity | 1.90E-04 | 22.58064516 | |
| | HPC | GO: Cellular Component | clathrin coated vesicle membrane | 0.00E+00 | 17.89473684 |
| GO: Cellular Component | | synaptic vesicle | 1.00E-06 | 15.59633028 | |
| GO: Cellular Component | | coated vesicle | 2.00E-06 | 11.46788991 | |
| GO: Cellular Component | | synaptosome | 3.00E-06 | 13.4751773 | |
| Mouse Phenotype | | MP:0002064 seizures | 7.00E-06 | 10.75268817 | |
| Mouse Phenotype | | MP:0002910 abnormal excitatory postsynaptic currents | 4.50E-05 | 16.4556962 | |
| Mouse Phenotype | | MP:0000950 abnormal seizure response to pharmacological agent | 4.66E-04 | 13.13131313 | |

Prefrontal Cortex Chronic Alcohol Gene Expression

Multivariate analysis of PFC microarrays revealed this brain region had the greatest overall differences in gene expression due to chronic alcohol administration (Figure 11A&B). This may suggest relative to the other brain areas examined the PFC is the most malleable CNS structure subsequent to habitual alcohol intake. At least five GABA receptor subunits were all up-regulated following chronic alcohol exposure (Figure 12B). The PFC is one of the most complex brain areas of the mammalian brain (Abernathy et al., 2010), shows complex regional variation in GABA receptor expression for alcohol dependence (Grobin et al., 2000). *GABRA5* and *GABRB3*, next to the previously discussed *GABRA4*, had the highest changes in expression. *GABRA5* and *GABRB3* are part of the chromosome 15 cluster associated with alcoholism during paternal transmission (Song et al., 2003). However, the network suggests these two receptor subunits may cooperate with a group of genes altered by chronic alcohol. Deletion of *GABRD* subunits does not alter the discriminative stimulus properties of alcohol (Shannon et al., 2004), but subjected to certain assemblies can be highly sensitive to low concentrations of alcohol (Glykys et al., 2007). Consistent with other investigations (Kerns et al., 2005) a number of genes regulated by alcohol within the PFC are neuronal development or plasticity and synaptic vesicles (Table 3). Some of these synapse related elements might be involved in alcohol behavioral phenotypes. For example, alpha-synuclein (*SCNA*) is differentially expressed and maps to a QTL for alcohol preference in alcohol-preferring and non-preferring rats (Carr et al., 1998, Liang et al., 2003). Additionally, genetic variation in alpha-synuclein is associated with craving in human populations (Foroud et al., 2007). In contrast to microarray studies on

postmortem alcoholic frontal cortex (Figure 7) (Lewohl et al., 2000) no myelin-associated genes were found to be decreased or regulated in alcoholic female cynomolgus macaques.

Nucleus Accumbens Chronic Alcohol Gene Expression

The nucleus accumbens is a major component of the mesocorticolimbic reward pathway (Volkow et al., 2011). Chronic alcohol self-administration regulated the expression of an extended dopaminergic network with the NAC (Figure 12C). The dopamine receptor (*DRD2*) was up regulated and connected to multiple factors within the alcohol-drinking network. Alcohol and drug addiction phenotypes are significantly associated with dopaminergic receptors, including *DRD2* (Le Foll et al., 2009). The *DRD2* receptor physically interacts with the adenosine receptor (*ADORA2A*) (Kamiya et al., 2003), which is associated with functional antagonism (Tozzi et al., 2011). Neurons within the NAC containing adenosine A2 receptors and dopamine D2 receptors are hypersensitive to alcohol, synergistically activating downstream signaling cascades (Mailliard and Diamond, 2004). Inhibition of adenosine A2 receptors with 3,7-dimethylpropargylxanthine decreases alcohol consumption (Thorsell et al., 2007), suggesting increased expression of *ADORA2A* is directly correlated to increased alcohol drinking behavior. Expression of the mu (*OPRM1*) and kappa (*OPRK1*) –opioid receptor are increased in alcohol drinkers. Similar to the adenosine A2 receptor, pharmacological blockade of the mu- and kappa-opioid receptors reduces alcohol intake (Anton et al., 2008, Walker et al., 2011). Regulator of G-protein signaling 9 (*RGS9*) is largely localized to ventral striatum where it indirectly inhibits the mu-opioid receptor

(Rahman et al., 1999), as well as modulates dopaminergic signaling and behavior (Rahman et al., 2003). To the best of our knowledge no studies have directly addressed the role of RGS9 in alcohol drinking behavior; however, increased expression of *RGS9* in alcohol consuming cynomolgus macaques suggests it may be a viable target mediating alcohol-drinking behavior. Genetic deletion of *Trpv1* (transient receptor potential cation channel subfamily V, member 1) in mice, which is decreased in the NAC of alcohol consuming macaques, increases alcohol consumption and preference (Blednov and Harris, 2009). The neuropeptide Y receptor (*NPY2R*) is increased in expression in response to chronic alcohol administration. Alcohol dependence and alcohol withdrawal symptoms are significantly associated with sequence variation in *NPY2R* (Wetherill et al., 2008). Under voluntary access conditions mice carrying a null-mutation for *Npy2r* consume less alcohol compared to controls (Thiele et al., 2004), which might argue increased expression of this NPY receptor may lead to increased alcohol drinking behavior, as evident in the network analysis of NAC. Collectively, literature association analysis within the NAC identified a core group of genes with prominent roles in alcohol drinking behavior that are consistent with directional changes observed in cynomolgus macaques. This provides experimental evidence of a gene expression network overseeing alcohol-drinking behavior within a discrete brain region.

Hippocampus Chronic Alcohol Gene Expression

Alcoholism causes long-term changes in hippocampal structure (Sullivan et al., 1995, Laakso et al., 2000). Deficits in hippocampus function induced by alcohol can lead to behavioral complications, such as impaired learning and memory (Ryabinin,

1998). Chronic alcohol self-administration resulted in the increased gene expression for a host of glutamate receptors (Figure 12D). The largest increased expression was observed for the obligatory NMDA receptor subunit *GRIN1*. Chronic alcohol exposure in rodents also triggers an up-regulation of glutamate receptors in hippocampus (Trevisan et al., 1994, Snell et al., 1996). Increased abundance of excitatory glutamate receptors may act as a compensatory mechanism for persistent inhibition of glutamate. However, the rise of excitatory glutamate receptors within the hippocampus causes a biological kindling process. This process results in CNS hyper-excitability and seizures during alcohol withdrawal episodes (Becker, 1998). Over-representation analysis identified seizures and abnormal post-synaptic currents as consequences of chronic alcohol regulation of genes confined to the hippocampus (Table 3). Avoidance of negative withdrawal states related to CNS dysfunction has been suggested to drive the compulsive use of alcohol and other drugs of abuse (Koob, 2009).

In addition to an up-regulation of glutamate receptors, chronic alcohol self-administration led to an increase in *NRXN1*, *NCAM1*, and *NTRK2* expression connected to the glutamate sub-network (Figure 12D). Neurexin (*NRXN1*) is involved in the recruitment of NMDA receptors and other neuronal synapse components in CNS plasticity (Sudhof, 2008). Sequence variants in *NRXN1* and *NCAM1* are associated with alcohol dependence (Yang et al., 2005, Yang et al., 2008). The neurotrophic tyrosine kinase receptor *NTRK2*, the cognate receptor for brain-derived neurotrophic factor (BDNF), is involved in development and maintenance of the nervous system (Lipsky and Marini, 2007). BDNF plays a prominent role in hippocampal synaptic plasticity (An et al., 2008). Rodent models of self-administration have shown BDNF mediates alcohol-

drinking behavior (Hensler et al., 2003, Jeanblanc et al., 2009). Additionally, a polymorphism in BDNF is associated with treatment outcomes in alcohol dependence (Wojnar et al., 2009). Elevated BDNF/NTRK2 signaling promotes the interaction of NTRK2 and NMDA receptors (Wang et al., 2011). NMDA receptor stimulation activates transcription of BDNF within hippocampal neurons (Tian et al., 2009), suggesting the interaction of BDNF signaling and a glutamatergic gene network may be important for alcohol drinking behavior.

Gene Expression in Chronic Alcohol Drinking Male & Female Cynomolgus Macaques

Male cynomolgus macaques were subjected to schedule-induced polydipsia followed with voluntary self-administration of alcohol with planned bouts of repeated withdrawal. Microarray analysis of PFC from these non-human primates revealed a total of 1717 probesets (~1209 unique gene symbols) differentially expressed due to chronic ethanol exposure (Figure 13A). In contrast to the female cynomolgus macaques there were more probesets/genes down regulated than up-regulated (Figure 14), with 919 probesets down- and 798 probesets up-regulated in their expression in the males (Figure 13A). Similar to females, males demonstrated altered GABA receptor subunit expression; however males and females appear to have a different complement of GABA receptor subunits (Figure 12B and Figure 13B). Both males and females have increased *GABRA4* and *GABRA5* expression, but males have a decreased expression of *GABRA2*. Polymorphisms in *GABRA2* are associated with alcohol dependence and endophenotypes related to neural excitability in humans (Edenberg et al., 2004, Lydall et al., 2011). This particular subunit accounts for approximate 26% of GABA-A receptors

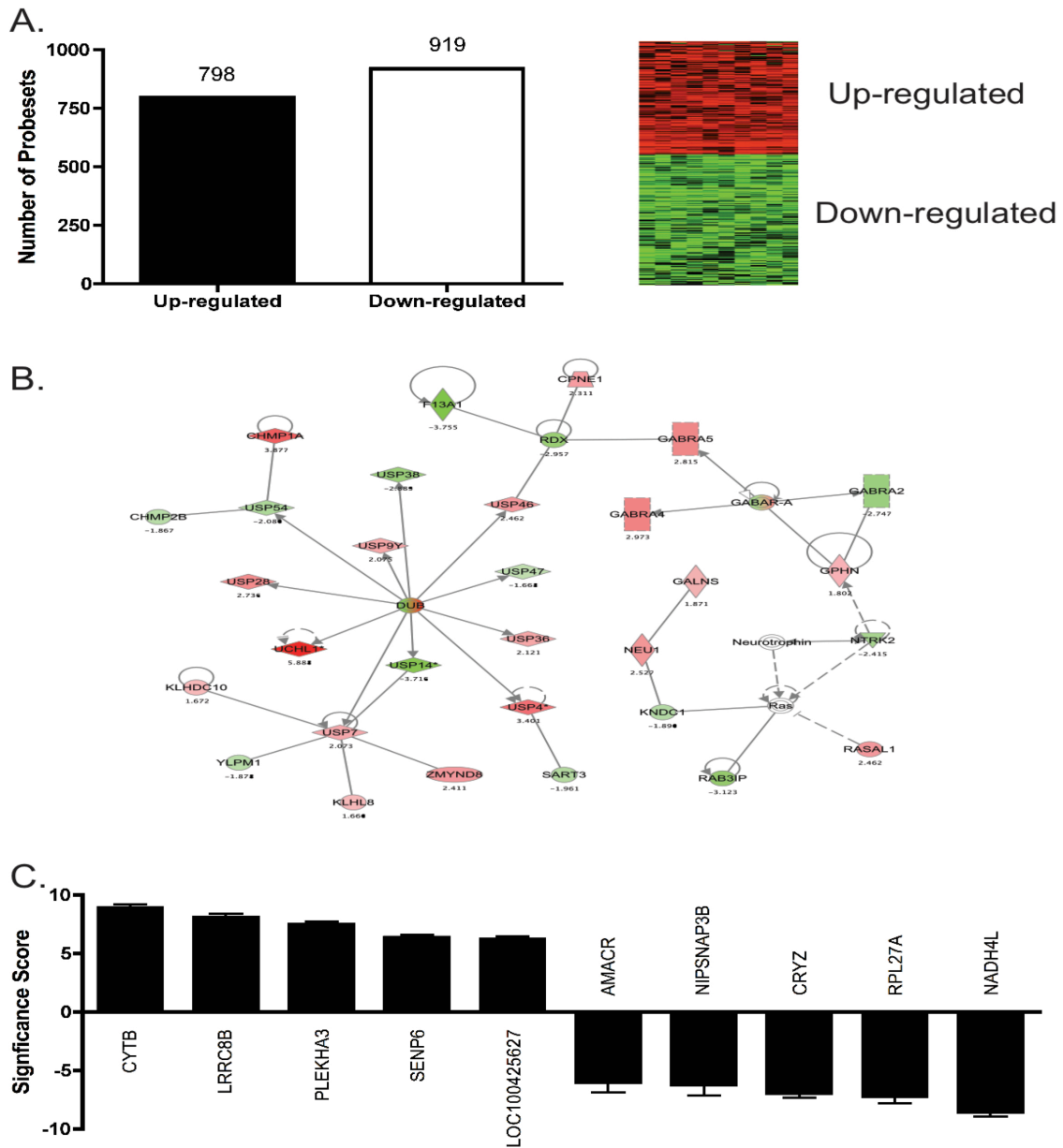


Figure 13. Alcohol-responsive gene expression in male cynomolgus macaque prefrontal cortex. (A) Bar graph showing number of alcohol-responsive probesets up-regulated (black) and down-regulated (white), and clustergram for alcohol responsive gene expression; Red= increased expression. Green= decreased expression. Black = no change in expression. (B) Ingenuity pathway analysis network showing altered GABA receptor expression. Red= increased expression. Green= decreased expression. (C) Bar graph showing the top five alcohol-responsive genes up-regulated and down-regulated in male cynomolgus macaque PFC.

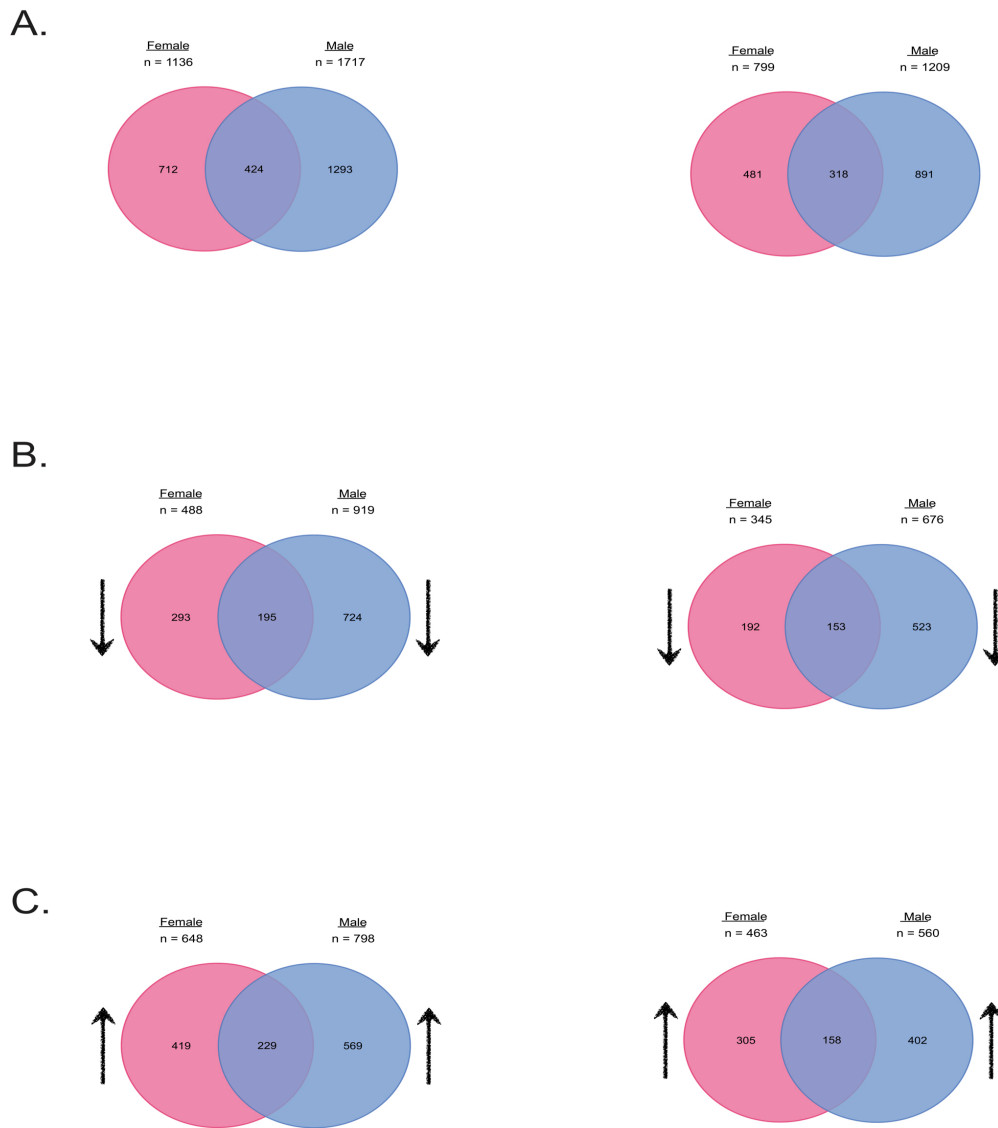


Figure 14. Venn diagram for alcohol-responsive gene expression in female and male cynomolgus macaques PFC. Left column indicates number of probesets altered by alcohol consumption in PFC, right column indicates number of unique gene symbols. Pink = Female PFC, Blue = Male PFC. (A) total number of overlapping and non-overlapping probesets and genes altered by chronic alcohol (B) number of overlapping and non-overlapping probesets and genes down-regulated by chronic alcohol (C) number of overlapping and non-overlapping probesets and genes up-regulated by chronic alcohol.

in the mammalian brain (McKernan and Whiting, 1996). A genetic knock-in for an insensitive form of *Gabra2* altered alcohol behavioral phenotypes (Blednov et al., 2011), suggesting decreased expression of *GABRA2* in male cynomolgus macaques is important for increased alcohol drinking behavior.

The BDNF receptor NTRK2 was decreased in females, but increased in response to chronic alcohol in males. NTRK2 may interact with GABA receptor signaling through the neuronal assembly protein gephyrin (*GPHN*) (Figure 13B). Gephyrin controls the mobility and clustering of GABA receptors to the cell surface of neurons (Jacob et al., 2005). Increased expression of *GPHN* may facilitate altered trafficking of GABA receptors following withdrawal from chronic alcohol (Diaz et al., 2011). Literature association analysis of gene expression results from male monkeys chronically consuming alcohol also suggests the cytoskeletal structure is influenced through the expression of genes involved in ubiquitination (Figure 13B), which is involved in intracellular trafficking and recycling. Altered expression of ubiquitin genes, such as decreased expression of USP14 in neuronal tissue, causes motor impairment and ataxia (Crimmins et al., 2006). Dysregulation of ubiquitin processes may interfere with neuronal inhibition through altered trafficking of synaptic GABA-A receptors (Arancibia-Carcamo et al., 2009). Thus, interfering with homeostatic expression of a gene network involving ubiquitin and GABA receptors may influence neuronal signaling and influence alcohol-drinking behavior.

Previous research conducted in our laboratory has investigated alterations in the mouse transcriptome due to individual differences in alcohol drinking behavior within a single inbred strain of mice (Wolstenholme et al., 2011). Gene expression results from

female and male PFC cynomolgus macaques were overlapped with genes correlated to alcohol consumption in mice to identify down-stream targets of chronic alcohol administration (Figure 15). Six genes (CLIC4, STAM2, SLC30A7, SORBS1, PPP2R5C, and DENR) were identified across species that may serve as critical points of convergence for alcohol intake. Network analysis suggests these eight genes interact through common signaling pathways such as PKC, PKA, and Fyn kinase (Figure 15A), which are involved in various behavioral and molecular aspects of alcohol and other drugs of abuse (Lee and Messing, 2008).

Cluster analysis illustrated differences and similarities of chronic alcohol induced expression patterns between male and female cynomolgus macaques (Figure 16A). Cluster 2 showed increased expression patterns only in females involved in the regulation of synaptic plasticity (Figure 16A&B). A portion of the genes in cluster 2 are involved in the regulation of glutamatergic signaling. For example, *DLG4* (also known as post-synaptic density protein 95, PSD95) is a scaffolding protein for NMDA receptors. Females also showed a unique decreased gene expression pattern in cluster 3, over-represented for DNA binding elements. Interestingly, *CREB1* and the glucocorticoid receptor *NR3C1* were decreased in females PFC, but not in male PFC. Decreased glucocorticoid receptors are consistent with increased brain glucocorticoid concentrations that are important in the development of alcohol dependence and neurotoxicity (Rose et al., 2010). There was an inverse relationship between male and females for not only clusters 2 and 3, but also clusters 4, 5, 6, and 7 suggesting a diverse set of gene expression patterns driving alcohol consumption in the differing sexes. However, clusters 8, 9, and 10 showed some commonalities in expression

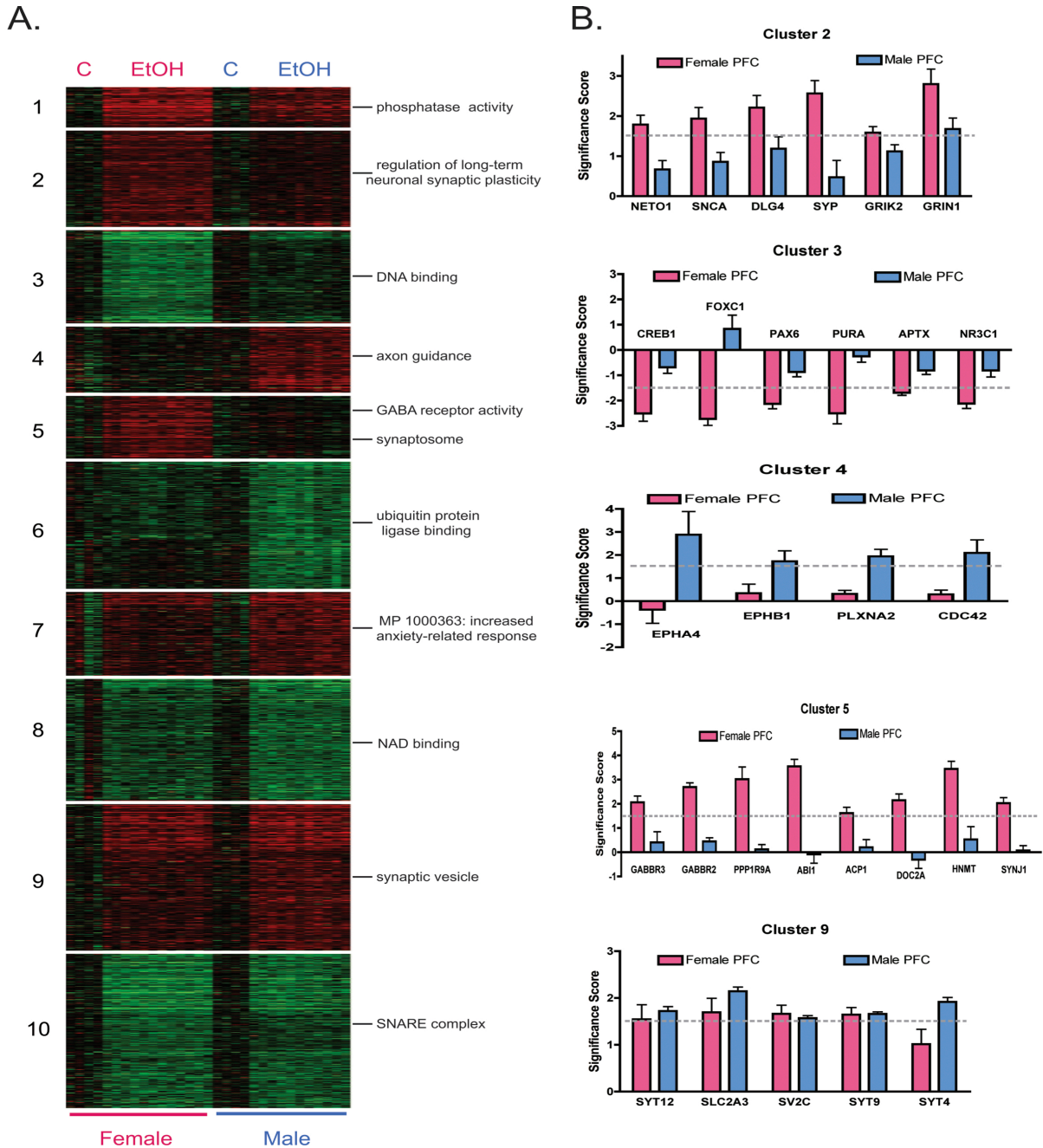


Figure 16. Male and female cynomolgus macaque PFC differential expression and gene ontology analysis. (A) Heatmap of control and alcohol-responsive expression for S-scores in male (blue) and female (pink) PFC; Red= increased expression. Green= decreased expression. Black = no change in expression. Significant gene ontology of interest are listed to the right of individual clusters (B) Bar graphs for alcohol-responsive gene expression for individual genes within gene ontology categories for selected k-means clusters in 16A; pink = female, blue = male, grey dashed line = level of significant S-score.

profiles between males and females. Cluster 9 show-increased expressions of genes involving ion channels and synaptic vesicles (Figure 16 A&B). Contrary to our initial hypothesis neither males nor females exhibited decreased myelin-associated gene expression in the PFC in response to chronic alcohol consumption. However, Cluster 8 demonstrates decreased expression of genes involving mitochondrial function and nicotinamide adenine dinucleotide (NAD) binding, which may be major factors underlying alcohol drinking behavior and alcohol-induced neurotoxicity (Quintanilla et al., 2006, Manzo-Avalos and Saavedra-Molina, 2010).

NADH dehydrogenase subunit 4L (*NADH4L*, Probeset: MMU.6048.1.S1_s_at) demonstrated the largest relative decrease in gene expression for both female and male PFC (Figure 11D and Figure 13C). Additionally, *NADH4L* was also markedly decreased in female AMY, NAC, and HPC (Figure 11D), making it a viable candidate for follow-up verification. Two replicated quantitative real-time polymerase chain reaction (Q-rtPCR) of *NADH4L* however showed no differences in gene expression for female cynomolgus macaques PFC (Figure 17A). The Q-rtPCR melt curve product showed two separate peaks for alcohol drinkers and controls (Figure 17B), suggesting a different product between the two groups. Principal component analysis (PCA) of the sequencing results showed a clear separation of alcohol drinkers and controls (Figure 17C). Preliminary analysis of the sequence differences suggested the *NADH4L* gene codes for different protein products between the two experimental groups (Figure 17D). Thus, the decreased expression results are most likely due to polymorphic differences between the two groups relative to the design of the microarray probeset for detecting *NADH4L*. Combined with the over-representation of mitochondrial genes between alcohol drinkers

and controls suggested expression differences might be due to the effect of ancestral or germline transmission and not solely the result of chronic alcohol exposure. Subsequent sequencing analysis by our collaborators confirmed our suspicion of differences in the ancestral origin of controls and alcohol drinkers (Figure 18). Whole genome microarrays can also be utilized to detect potential problems due to genetic variation within a single species (Gresham et al., 2008). Pairwise comparison of microarrays for the male *Macaca fascicularis* further indicated batch differences, which are likely due to differences in ancestral origin (Figure 19A); however, pairwise comparison of microarrays for the male *Macaca mulatta* revealed no distinguishing differences among the two groups (Figure 19B). Lack of discernible differences in the male *Macaca mulatta* subjects suggests this experimental group may be more suitable for the detection of differences in chronic alcohol-induced drinking expression. A further advantage of the rhesus macaques is that they are selected from the pedigreed population at the Oregon National Primate Research Center. The rhesus macaques also demonstrate a greater distribution of alcohol consumption compared to the cynomolgus groups (Figure 20), presenting the opportunity to extend our analysis beyond a two-group analysis and leverage the variance in gene expression against phenotypic variation for subjects selected without common grandparents. Using a platform designed against the rhesus macaque genome, microarrays can accurately assess variation in gene expression between individuals (Oleksiak et al., 2002).

| AnimalID | Cohort | Ancestry | CCR4:420 | CD40:25 | CD4:192 | CYP11A1:150 | IL2RA:559 | INHBB:131 | MAOA:116 | NR3C1:458 | SASH1:527 | NPY:485 | SASH1:578 | TNF:621 | CCR7:397 | CD44:471 |
|-----------|----------|--------------|----------|---------|---------|-------------|-----------|-----------|----------|-----------|-----------|---------|-----------|---------|----------|----------|
| AG051519 | standard | Viet/Camb | C/A | C/C | C/C | G/G | G/G | C/T | C/C | A/A | G/G | C/C | A/A | T/T | G/T | T/T |
| AG23454 | standard | Viet/Camb | A/A | C/C | C/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | T/T | T/T |
| AG23742 | standard | Viet/Camb | C/A | T/C | C/C | G/G | G/G | C/T | C/C | A/A | G/G | C/C | A/A | C/T | T/T | T/T |
| AG23761 | standard | Viet/Camb | A/A | C/C | C/C | G/G | G/G | C/C | C/C | A/A | G/G | C/C | C/A | T/T | G/T | T/T |
| 4009 | standard | Indon/Mau/Ph | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | T/T | T/T |
| 4012 | standard | Indon/Mau/Ph | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | C/A | T/T | T/T | C/T |
| 27906 | standard | Indon/Mau/Ph | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | C/C | T/T | T/T | T/T |
| 28658 | standard | Indon/Mau/Ph | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | T/C | C/A | T/T | T/T | C/C |
| 25081 con | 3 | Indones | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | C/C | T/T | G/G | C/T |
| 25089 con | 3 | Indones | A/A | C/C | T/T | G/A | G/G | C/C | C/C | A/A | G/G | T/T | C/C | T/T | G/G | T/T |
| 25088 con | 3 | Indones | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | T/T | A/A | T/T | T/T | T/T |
| 25089 con | 3 | Indones | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | T/C | C/C | T/T | T/T | T/T |
| 25081 | 3 | Viet/Cam | A/A | C/C | C/C | G/G | G/G | C/C | C/C | T/A | A/A | C/C | A/A | T/T | T/T | C/T |
| 8996 | 1 | Indones | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | C/A | C/T | G/T | T/T |
| 8992 | 1 | Indones | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | G/T | C/T |
| 25080 | 3 | Viet/Cam | A/A | C/C | C/C | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | C/T | G/T | T/T |
| 25098 | 2 | Viet/Cam | A/A | C/C | C/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | G/T | T/T |
| 25059 | 3 | Viet/Cam | C/A | C/C | C/C | G/G | G/G | C/C | C/C | A/A | A/G | C/C | A/A | T/T | G/T | T/T |
| 8996 | 1 | Indones | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | G/G | C/T |
| 25099 | 2 | Viet/Cam | A/A | C/C | C/C | G/G | G/G | C/T | C/C | A/A | A/A | C/C | A/A | T/T | G/G | T/T |
| 25094 | 2 | Viet/Cam | A/A | C/C | C/C | G/G | G/G | C/C | C/C | A/A | A/G | C/C | A/A | T/T | T/T | C/T |
| 8994 | 1 | Indones | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | T/C | C/A | T/T | G/T | T/T |
| 25096 | 2 | Viet/Cam | C/A | C/C | C/T | G/G | G/G | C/C | C/C | A/A | A/G | C/C | A/A | T/T | G/T | C/T |
| 25097 | 2 | Viet/Cam | A/A | T/C | C/C | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | G/T | C/T |
| 25096 | 2 | Viet/Cam | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | A/G | C/C | C/A | T/T | G/G | T/T |
| 25095 | 2 | Viet/Cam | C/C | C/C | C/C | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | G/T | T/T |
| 25091 | 2 | Viet/Cam | A/A | C/C | C/C | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | T/T | T/T |
| 25073 | 3 | Viet/Cam | C/A | C/C | C/C | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | T/T | C/T |
| 25090 | 2 | Viet/Cam | A/A | C/C | C/T | G/G | G/G | C/T | C/C | A/A | A/G | C/C | A/A | T/T | G/T | T/T |
| 25076 | 3 | Viet/Cam | C/A | C/C | C/C | G/G | G/G | C/C | G/C | A/A | G/G | C/C | A/A | T/T | T/T | C/T |
| 25072 | 3 | Viet/Cam | x | C/C | C/C | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | T/T | T/T |
| 25087 | 2 | Viet/Cam | A/A | T/C | C/T | G/G | G/G | C/C | G/G | A/A | A/G | C/C | A/A | T/T | G/T | C/T |
| 8999 | 1 | Indones | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | T/C | C/A | T/T | G/T | T/T |
| 25085 | 3 | Viet/Cam | A/A | T/C | C/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | G/T | C/T |
| 25084 | 3 | Viet/Cam | C/A | C/C | C/C | G/G | G/G | C/T | C/C | A/A | G/G | C/C | A/A | T/T | T/T | T/T |
| 8993 | 1 | Indones | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | C/A | T/T | G/G | T/T |
| 8987 | 1 | Indones | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | T/C | C/A | T/T | T/T | C/T |
| 25058 | 3 | Viet/Cam | A/A | C/C | C/C | G/G | G/G | C/T | G/G | A/A | G/G | C/C | A/A | T/T | G/G | T/T |
| 25085 | 3 | Viet/Cam | C/A | C/C | C/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | T/T | T/T |
| 25084 | 3 | Viet/Cam | C/A | C/C | C/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | T/T | C/T |
| 25077 | 3 | Viet/Cam | C/A | C/C | C/C | G/G | A/G | C/T | C/C | A/A | G/G | C/C | A/A | T/T | G/T | T/T |
| 8988 | 1 | Indones | A/A | C/C | T/T | G/G | G/G | C/C | C/C | A/A | G/G | C/C | C/C | T/T | G/G | C/T |
| 25093 | 2 | Viet/Cam | A/A | C/C | C/C | G/G | A/G | C/C | C/C | A/A | G/G | C/C | A/A | T/T | T/T | C/T |

Figure 18. Sequencing confirmation of genetic differences between controls and alcohol-drinking cynomolgus macaques. Single nucleotide polymorphism analysis (SNP) showing differences in ancestry for controls and alcohol drinking cynomolgus macaques; pink indicates control samples (water drinkers). Courtesy of Dr. Kathleen Grant and colleagues, OregonHealth and Science University.

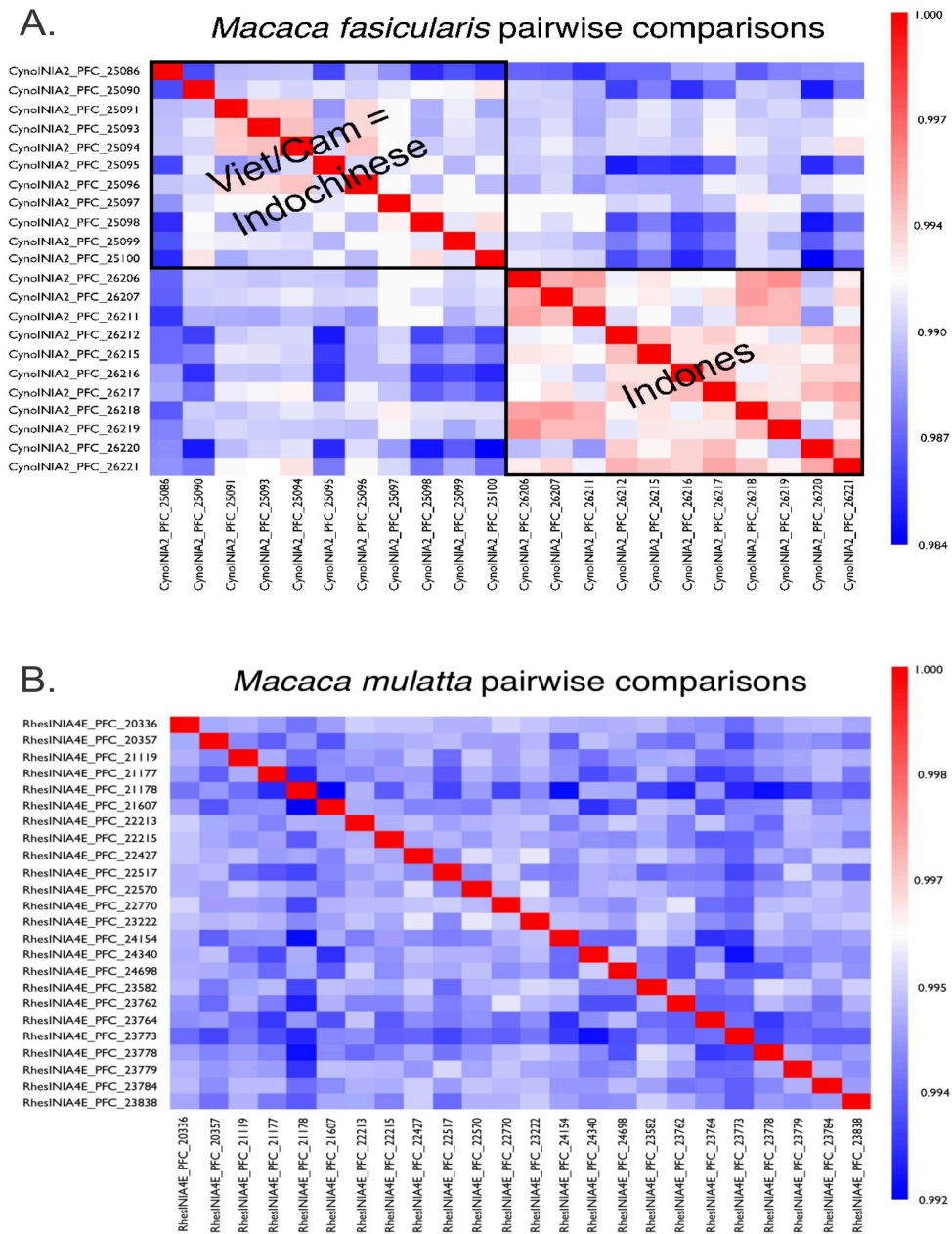


Figure 19. Pairwise comparison of male cynomolgus macaque and rhesus macaque PFC microarrays. (A) Quality control from pairwise microarray comparisons for male PFC cynomolgus macaques (*Macaca fascicularis*) showing batch differences based on genotype. (B) Quality control from pairwise microarray comparisons for male PFC rhesus macaques (*Macaca mulatta*) showing lack of genotype effect ; red = 100 percent correlation.

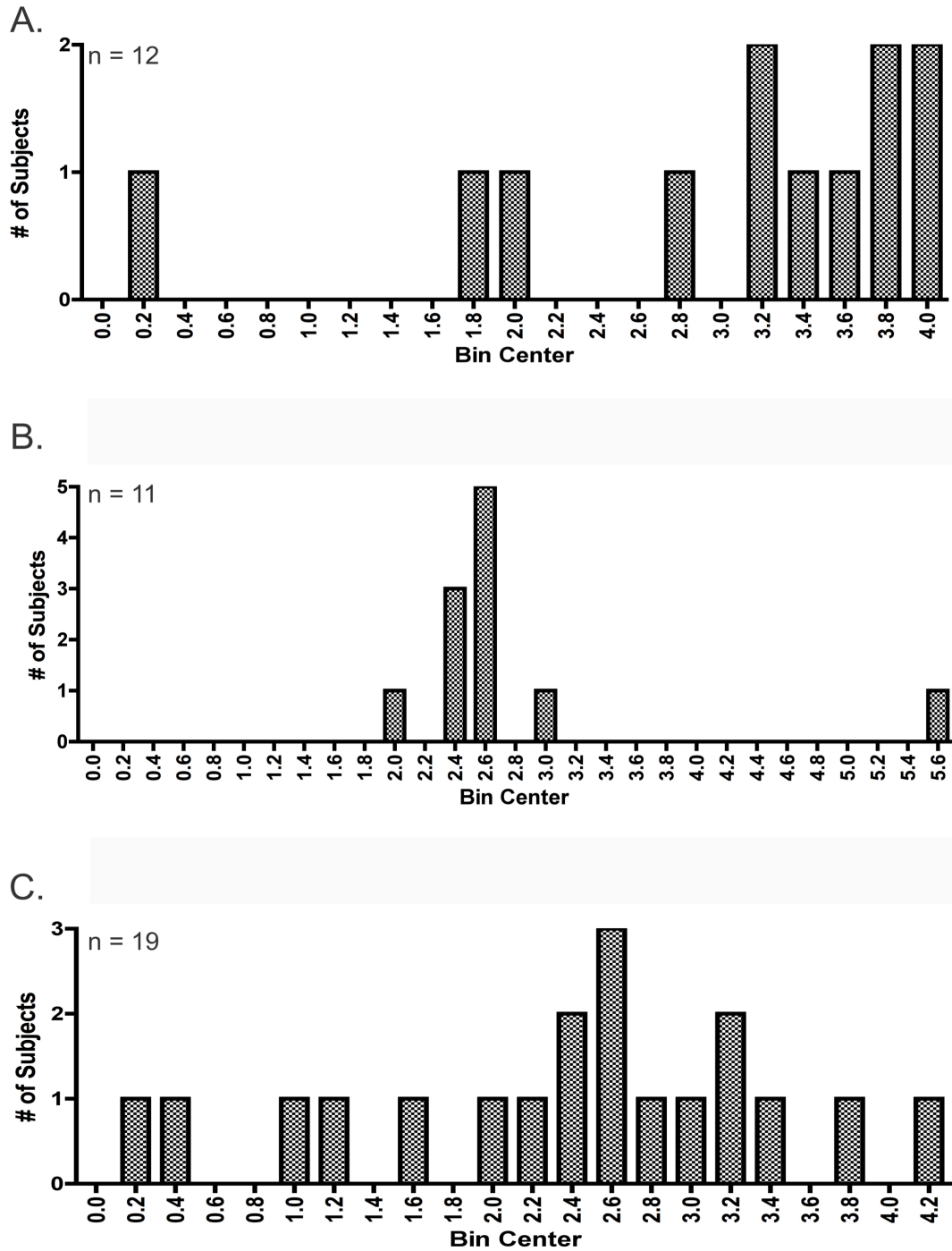


Figure 20. Drinking histogram frequencies for each of the three monkey cohorts. Binned drinking behavior for average g/kg/day intake in (A) female cynomolgus macaque ($n = 12$), (B) male cynomolgus macaque ($n = 11$), and (C) male rhesus macaque ($n = 19$).

Network Analysis of Gene Expression in Rhesus Macaques

Male rhesus macaques ranging from ~5.5 to 10 years of age (the equivalent of ~19-42 years of age in humans) consumed between an average of 0.27 – 4.12 g/kg/day over a 12 month period. Weighted gene co-expression network analysis (WGCNA) was conducted for gene expression data from PFC to determine association between the amount of alcohol consumed and modules of interconnected genes (Langfelder and Horvath, 2008). Focusing on gene networks, rather than individual genes differentially regulated due to alcohol, alleviates the multiple testing problems inherent in microarray data (Zhao et al., 2010b). WGCNA of S-scores derived from rhesus macaques identified 174 modules (gene networks) spanning 30 (lavender) to 1835 (turquoise) probesets in size (Figure 21). The first principal component of each module, also referred to as the module eigengene, is a representation of the gene expression profile within a module. Among the 174 co-expression modules, the module eigengene of 10 differing networks was significantly correlated (Pearson's product-moment correlation coefficient, p -value ≤ 0.05) to the average daily intake covering twelve months (Figure 22 & 24). Significantly correlated networks range in size from 31 to 481 probesets (Figure 23). Directionality of correlations to alcohol intake was evenly split with five networks showing a positive relationship and five networks showing a decreased relationship. Each of the modules are involved in differing biological processes (Table 4). The 'Green' module, possessing the greatest number of probesets of any network associated with alcohol drinking, was over-represented for myelin-associated gene expression. In contrast to the observation of decreased myelin within the brain tissue of human alcoholics, the myelin-related gene network was positively correlated to alcohol drinking behavior. Paired with our previous

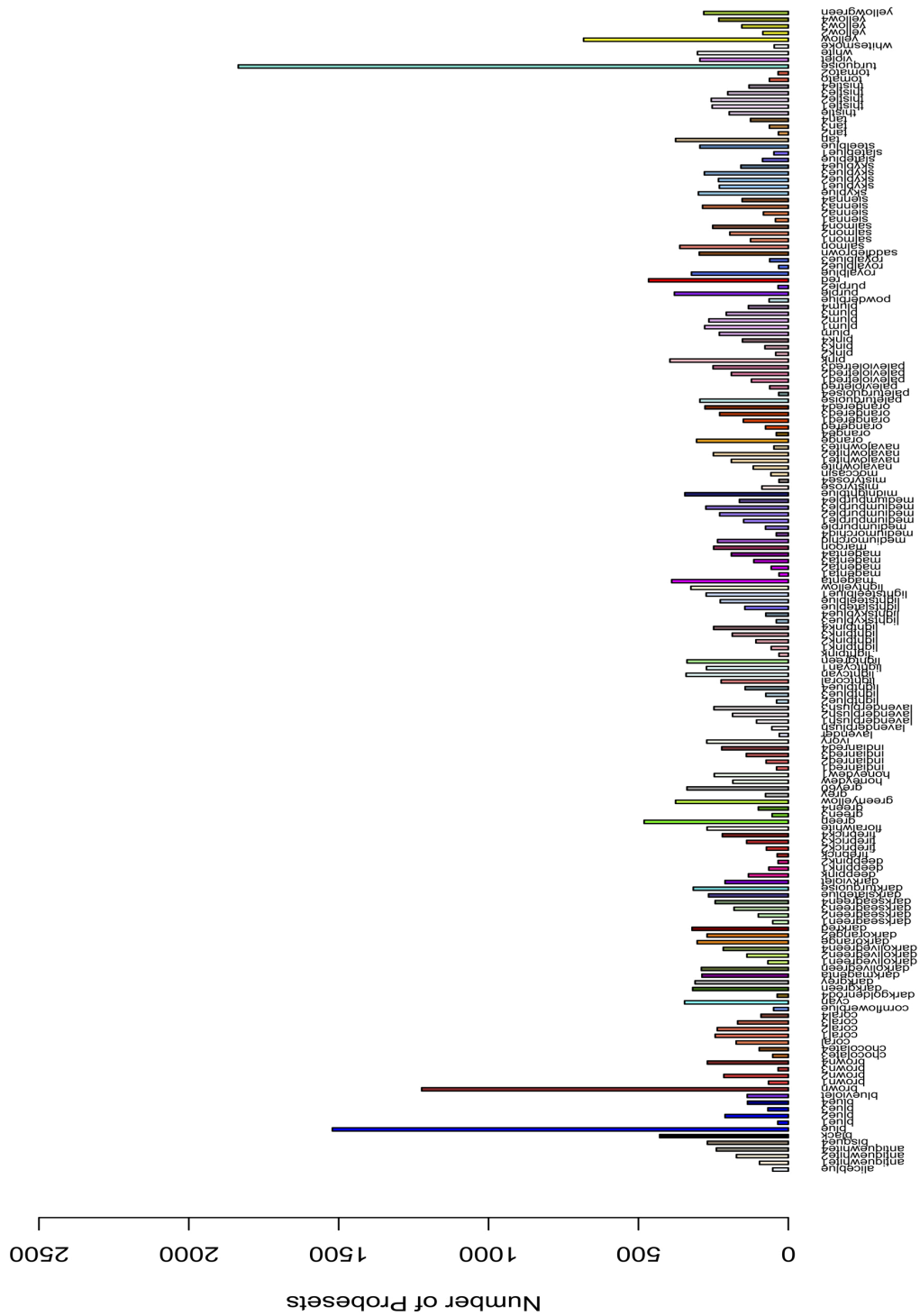


Figure 21. Weighted gene co-expression network analysis (WGCNA) for rhesus macaque PFC. Distribution for the size of 174 modules identified by WGCNA based on the number of probesets (y-axis), colors correspond to different modules listed on the x-axis; smallest module = 30 probesets (lavender), largest module = 1835 probesets (turquoise).

Module-trait relationship

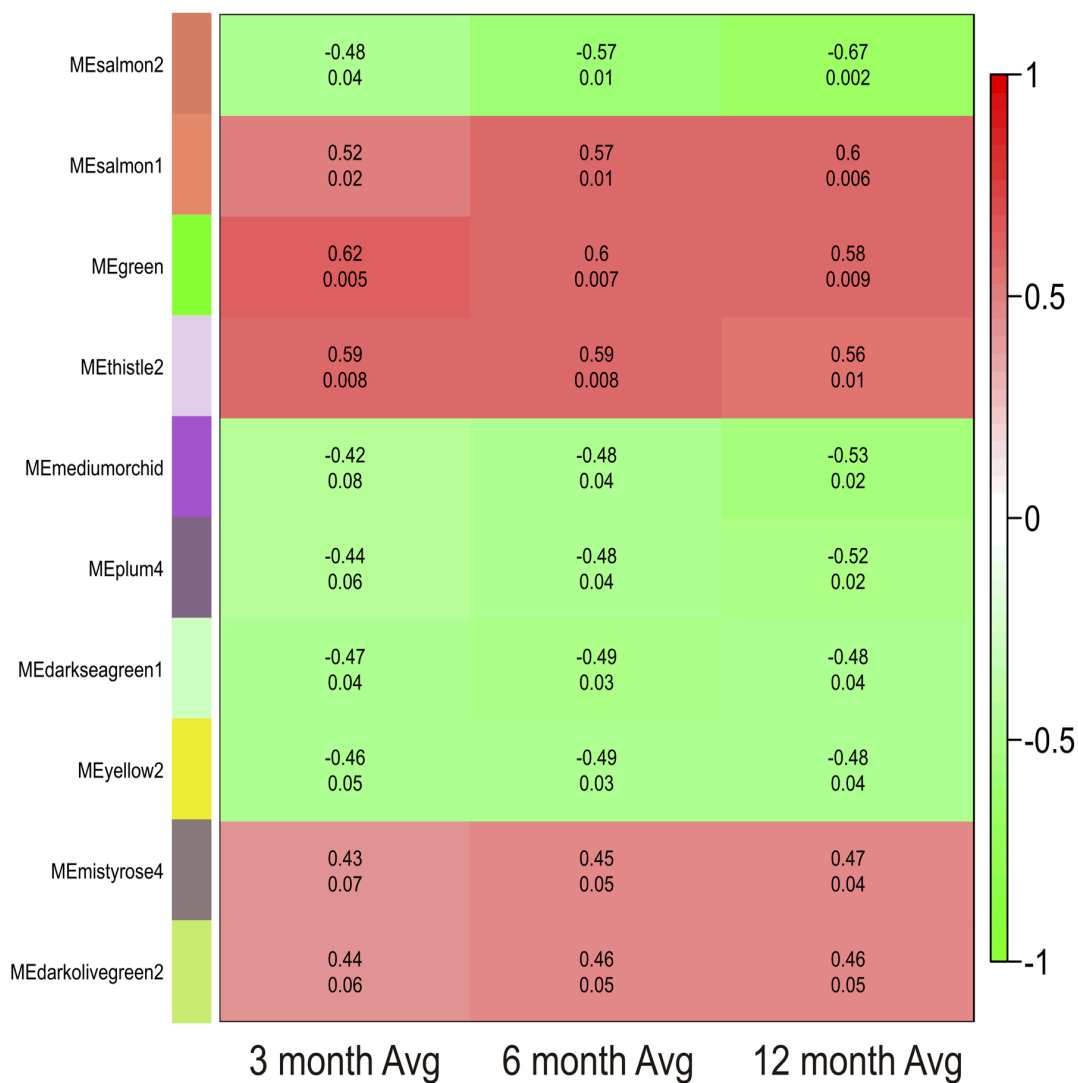


Figure 22. S-scored modules significantly correlated to alcohol drinking behavior in male rhesus macaque PFC. Heatmap of correlation for the module eigengene for the 10 modules significantly correlated to average alcohol intake (g/kg/day) for 365 days of consumption. Red = positive correlation, Green = negative correlation, x-axis = duration of alcohol drinking exposure, y-axis WGCNA modules from S-score data.

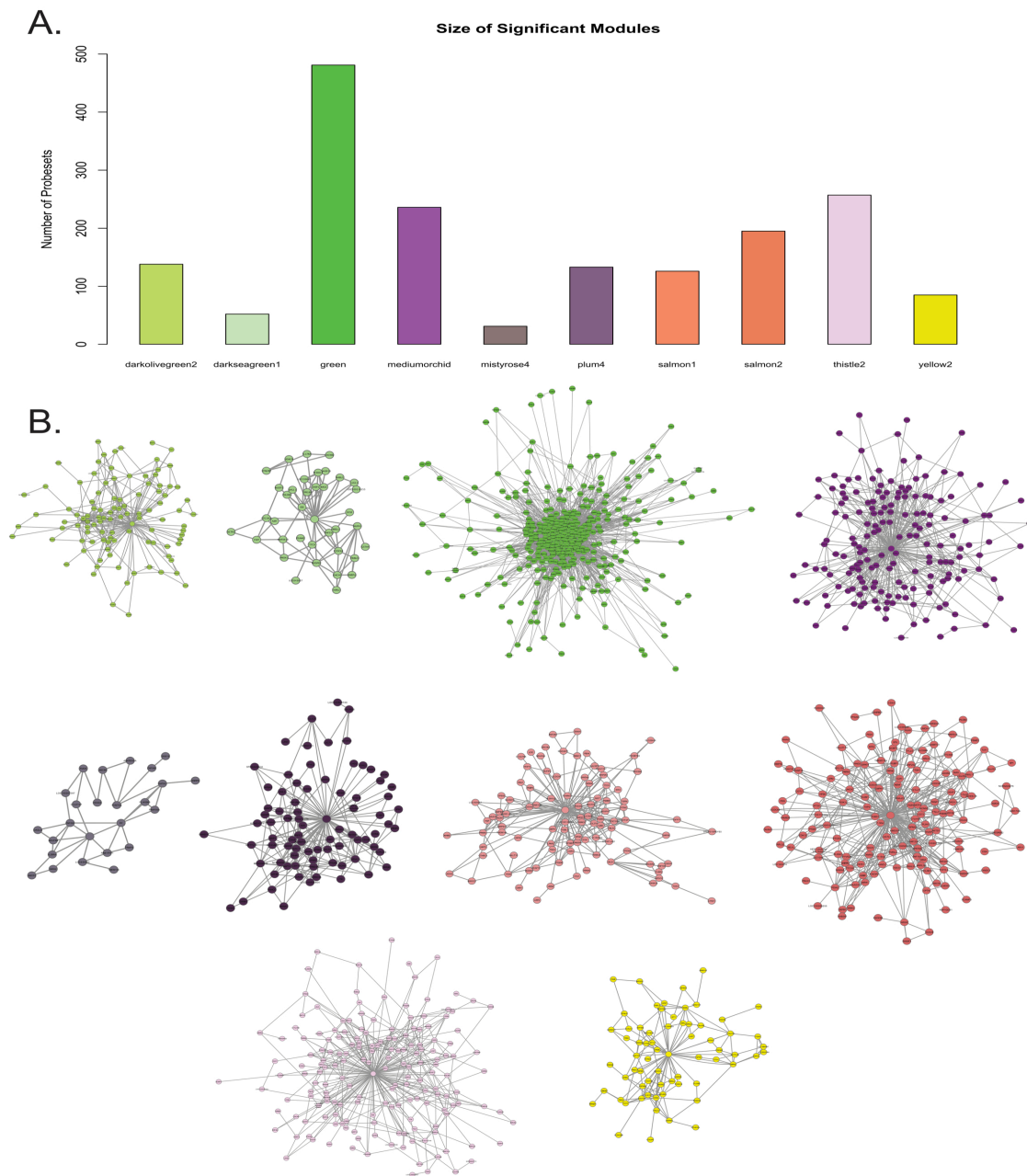


Figure 23. S-scored modules significantly correlated to alcohol drinking behavior in male rhesus macaque PFC. (A) Distribution in the number of probesets contained within PFC modules significantly correlated to alcohol drinking behavior of male rhesus macaques (B) Representative gene networks for significantly correlated modules, illustrated networks are based on Pearson correlation coefficients between genes $\geq |0.70|$.

Table 4. Functional over-representation for rhesus macaque PFC modules associated with alcohol drinking behavior

| Category | ID | Name | P-value | % Targeted in Category |
|-----------------|-------------------------------|---|-----------------|------------------------|
| Salmon2 | GO: Molecular Function | tubulin-tyrosine ligase activity | 4.87E-03 | 13.33333333 |
| | GO: Biological Process | regulation of antigen processing and presentation | 1.50E-04 | 66.66666667 |
| | GO: Biological Process | pyrimidine nucleotide biosynthetic process | 5.62E-04 | 13.04347826 |
| | GO: Biological Process | mRNA cleavage | 3.73E-03 | 15.38461538 |
| | GO: Biological Process | regulation of cell killing | 2.67E-03 | 7.692307692 |
| Salmon1 | GO: Molecular Function | prostaglandin receptor activity | 1.02E-03 | 20 |
| | GO: Molecular Function | NADH dehydrogenase activity | 2.01E-02 | 4.444444444 |
| | GO: Biological Process | regulation of fatty acid beta-oxidation | 1.49E-03 | 16.66666667 |
| | GO: Biological Process | regulation of synaptic transmission, GABAergic | 4.61E-03 | 9.523809524 |
| | GO: Biological Process | peptide hormone processing | 5.51E-03 | 8.695652174 |
| | Mouse Phenotype | abnormal circulating corticosterone level | 2.38E-04 | 6.557377049 |
| | GO: Molecular Function | structural constituent of myelin sheath | 4.64E-07 | 80 |
| | GO: Molecular Function | cAMP-dependent protein kinase activity | 6.11E-03 | 28.57142857 |
| | GO: Molecular Function | motor activity | 3.98E-02 | 4.225352113 |
| | GO: Biological Process | myelination | 1.75E-07 | 15.625 |
| Thistle2 | GO: Molecular Function | oligodendrocyte differentiation | 1.35E-04 | 13.33333333 |
| | GO: Molecular Function | protein tyrosine kinase activity | 9.51E-03 | 3.472222222 |
| | GO: Biological Process | regulation of chromosome segregation | 2.47E-04 | 21.42857143 |
| | GO: Biological Process | positive regulation of MAPK cascade | 8.96E-04 | 4.87804878 |
| | GO: Molecular Function | protein tyrosine/threonine phosphatase activity | 8.26E-04 | 40 |
| Mediumorchid | GO: Molecular Function | calcium channel regulator activity | 9.01E-04 | 14.28571429 |
| | GO: Molecular Function | transmembrane receptor protein tyrosine kinase | 3.56E-03 | 5.882352941 |
| | GO: Biological Process | mRNA catabolic process | 2.14E-04 | 6.451612903 |
| | Human Phenotype | Immunodeficiency | 1.57E-02 | 6.666666667 |
| | GO: Molecular Function | ATP-dependent DNA helicase activity | 6.92E-03 | 6.451612903 |
| Plum4 | GO: Biological Process | aromatic compound biosynthetic process | 7.04E-03 | 6.666666667 |
| | GO: Biological Process | cellular component maintenance | 7.04E-03 | 6.666666667 |
| | Mouse Phenotype | increased motor neuron number | 2.39E-03 | 12.5 |
| | GO: Molecular Function | ligand-dependent nuclear receptor binding | 3.77E-04 | 13.33333333 |
| | GO: Biological Process | cellular response to nutrient levels | 7.34E-04 | 3.191489362 |
| DarkSeaGreen1 | GO: Biological Process | macroautophagy | 9.30E-04 | 8.333333333 |
| | GO: Biological Process | negative regulation of proteolysis | 3.11E-03 | 4.545454545 |
| | Mouse Phenotype | abnormal double-strand DNA break repair | 8.38E-04 | 13.33333333 |
| | GO: Biological Process | negative regulation of canonical Wnt receptor signaling pathway | 1.16E-02 | 3.773584906 |
| | GO: Biological Process | gene silencing | 2.52E-02 | 2.5 |
| Mistyrose4 | GO: Cellular Component | intrinsic to endoplasmic reticulum membrane | 3.45E-03 | 2.631578947 |
| | GO: Molecular Function | ATP catabolic process | 1.77E-02 | 1.098901099 |
| | GO: Biological Process | methylesterase activity | 2.67E-02 | 0.884955752 |
| | Mouse Phenotype | abnormal neuron differentiation | 3.62E-02 | 0.793650794 |
| | GO: Molecular Function | cytochrome-c oxidase activity | 9.40E-03 | 6.896551724 |
| Darkolivegreen2 | GO: Molecular Function | specific RNA polymerase II transcription factor activity | 1.28E-02 | 3.125 |
| | GO: Biological Process | regulation of glycogen biosynthetic process | 1.81E-04 | 13.63636364 |
| | GO: Cellular Component | postsynaptic density | 1.82E-02 | 2.727272727 |

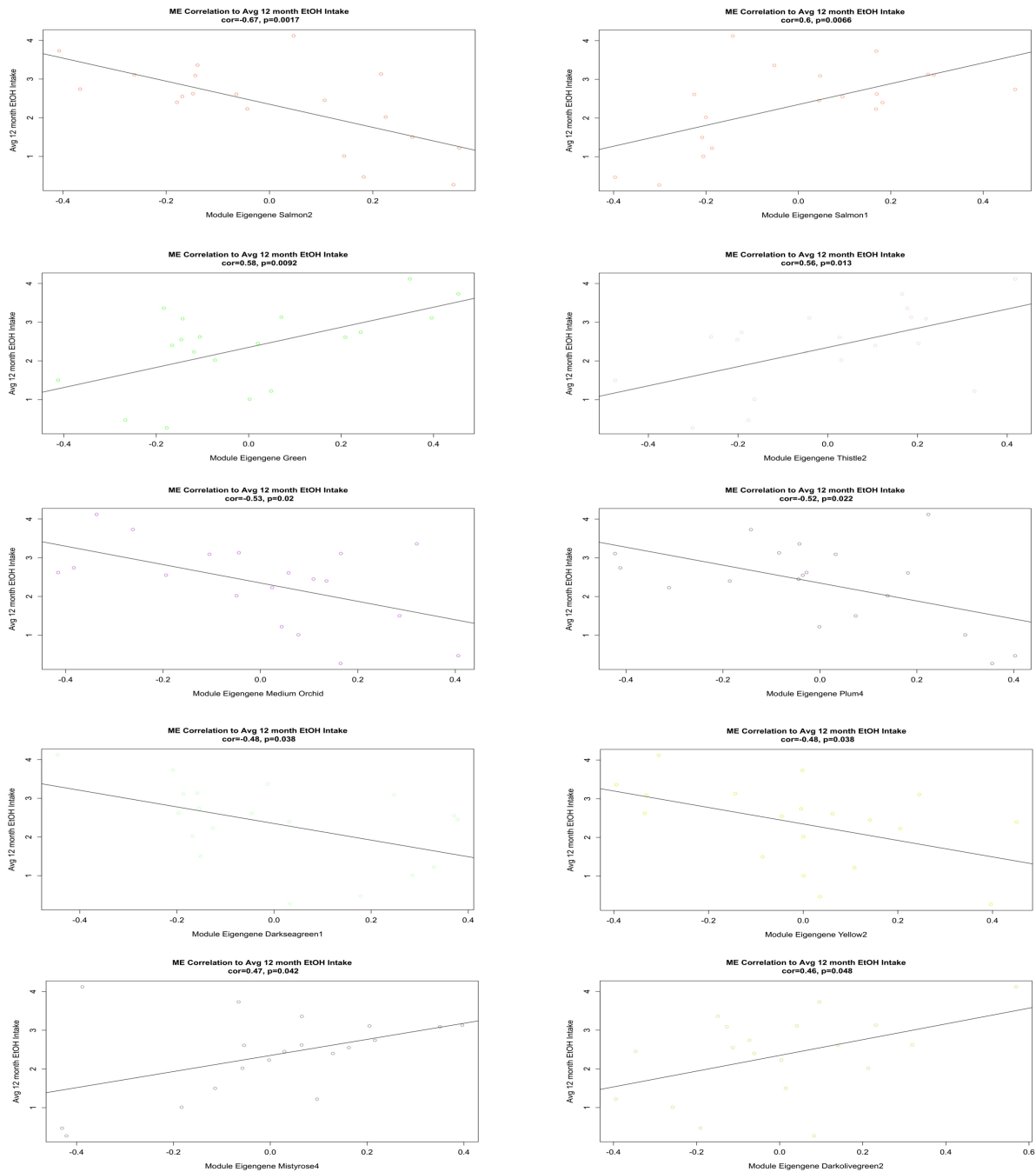


Figure 24. Male rhesus macaque PFC module eigengene correlations to alcohol intake. Scatter plots for the module eigengene expression (first principal component for an individual module) based on S-scores from PFC against average daily intake (g/kg/day) over 12-month period; y-axis = EtOH intake, x-axis = module eigengene based expression for each separate module listed. Bolded are the associated correlation coefficients and corresponding p-value.

expression profiling of mice with differential sensitivity to alcohol (Kerns et al., 2005), may suggest during acute or short-term alcohol exposure myelin gene expression influences alcohol behavioral phenotypes.

Substance abuse, as well as other psychiatric diseases, is influenced through the coordinate regulation of gene expression via microRNAs (Li and van der Vaart, 2011). MicroRNAs are short ribonucleic acid molecules acting as post-transcriptional elements, which bind complementary sequences of messenger RNA and influence gene regulatory networks (Bartel, 2009, Su et al., 2011). Combining the 10 alcohol drinking associated modules into a single network identified over-represented microRNAs (Table 5) The most abundant microRNA in brain mir-124 (Lagos-Quintana et al., 2002), targets at least 25 genes in this combined alcohol drinking network (p -value = 0.0001). Potentially serving as core elements within this network, individual microRNAs target multiple genes within differing modules (Figure 25). MicroRNA-26B (33 genes, p -value = 0.000042) is an intrinsic factor underlying neural differentiation and neurogenesis (Dill et al., 2012), which may be important in addiction related plasticity. Susceptibility to cocaine addiction is regulated through mir-212 (Hollander et al., 2010). The regulation of cocaine intake involving mir-212 is mediated by dynamic interactions with BDNF and the transcriptional repressor methyl CpG binding protein 2 (MeCP2) (Im et al., 2010). MeCP2 is regulated by mir-132 (33 genes, p -value = 0.000042), controlling chromatin remodeling (Alvarez-Saavedra et al., 2011). Expression of mir-132 is also a necessary factor for differentiation of dopamine neurons (Yang et al., 2012).

Within the alcohol-drinking combined network (Figure 25) mir-9 and mir-219 were the top two over-represented microRNAs, p -value < 0.00001 and p -value = 0.000014

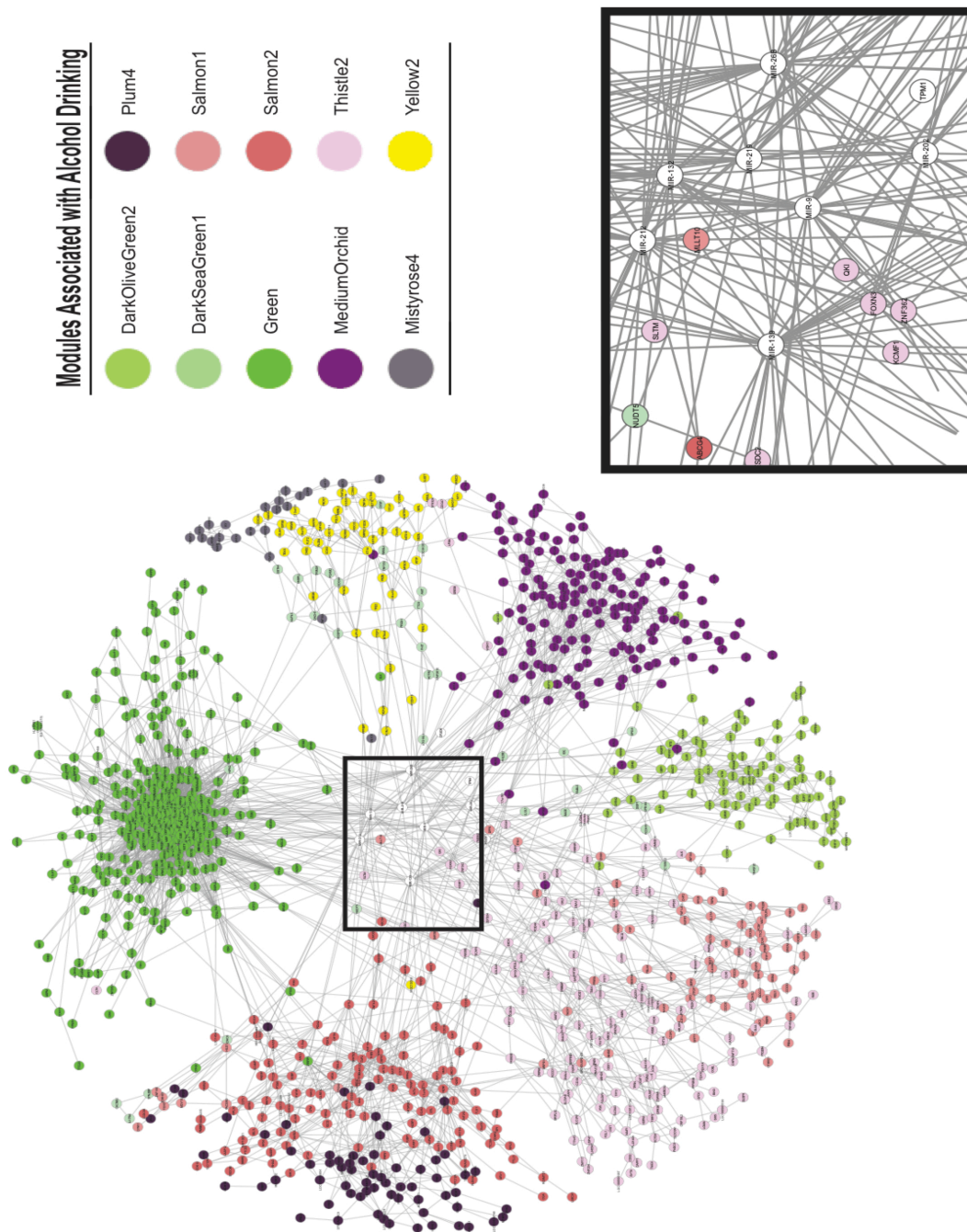


Figure 25. MicroRNA network for rhesus macaque PFC associated alcohol-drinking modules. Cytoscape network for top over-represented microRNAs for the ten PFC rhesus macaque gene expression networks correlated to alcohol intake. Connections among microRNAs is based on binding potential, connections among colored nodes (i.e. genes) are based on Pearson correlation coefficients between genes $\geq |0.70|$.

Table 5. Functional over-representation of microRNAs associated with alcohol drinking networks

| Category | ID | Name | P-value | % Targeted in Category |
|------------------------|--|---|-----------------|------------------------|
| GO: Molecular Function | GO:0011911 | structural constituent of myelin sheath | 0.00E+00 | 100 |
| GO: Molecular Function | GO:0004714 | transmembrane receptor protein tyrosine kinase activity | 3.53E-04 | 17.64705882 |
| GO: Molecular Function | GO:0015197 | peptide transporter activity | 4.80E-04 | 38.46153846 |
| GO: Molecular Function | GO:0004312 | fatty acid synthase activity | 1.56E-03 | 40 |
| GO: Molecular Function | GO:0017147 | Wnt-protein binding | 1.71E-03 | 25 |
| GO: Molecular Function | GO:0022832 | voltage-gated channel activity | 3.94E-03 | 10.55276382 |
| GO: Biological Process | GO:0007272 | ensheathment of neurons | 8.30E-05 | 19.40298507 |
| GO: Biological Process | GO:0008366 | axon ensheathment | 8.30E-05 | 19.40298507 |
| GO: Biological Process | GO:0042552 | myelination | 2.16E-04 | 18.75 |
| GO: Biological Process | GO:0001508 | regulation of action potential | 6.68E-04 | 14.5631068 |
| Mouse Phenotype | MP:0000953 | abnormal oligodendrocyte morphology | 2.00E-05 | 31.25 |
| Mouse Phenotype | MP:0003871 | abnormal myelin sheath morphology | 8.06E-04 | 22.5 |
| MicroRNA | TAGCTTT,MIR-9,TAGCTTT,MIR-9:MSigDB | | 0.00E+00 | 14.66666667 |
| MicroRNA | GACAATC,MIR-;GACAATC,MIR-219:MSigDB | | 1.40E-05 | 15.67164179 |
| MicroRNA | hsa-miR-139-5p; hsa-miR-139-5p:TargetScan | | 1.90E-05 | 12.5 |
| MicroRNA | hsa-miR-212;Tair;hsa-miR-212:TargetScan | | 4.20E-05 | 11.66077739 |
| MicroRNA | hsa-miR-132;Tair;hsa-miR-132:TargetScan | | 4.20E-05 | 11.66077739 |
| MicroRNA | TACTTGA,MIR-26;TACTTGA,MIR-26B:MSigDB | | 4.20E-05 | 11.66077739 |
| MicroRNA | TACTTGA,MIR-26;TACTTGA,MIR-26A:MSigDB | | 4.20E-05 | 11.66077739 |
| MicroRNA | ATACCTC,MIR-20;ATACCTC,MIR-202:MSigDB | | 6.40E-05 | 13.52941176 |

respectively. Alcohol induces the expression of mir-9, playing a key role in the behavioral and molecular processes underlying tolerance to alcohol (Pietrzykowski et al., 2008). Adaptations in the glutamatergic system, such as NMDA receptors, contributes to alcohol tolerance and the development of alcohol dependence (Krystal et al., 2003). Inhibition of mir-219 in mouse PFC disrupts NMDA receptor transmission, disturbing downstream behavioral responses associated with glutamatergic signaling (Kocerha et al., 2009). Additionally, mir-219 is critical in oligodendrocyte differentiation and myelination (Dugas et al., 2010), corresponding to the green module, the largest module within the alcohol-drinking network. Dual regulation of NMDA receptor function and myelination by mir-219 suggests these two systems may interact in regulating behavioral response to alcohol and other drugs of abuse. Enrichment for multiple microRNAs pertinent to neurogenesis, myelin gene expression, and behavioral responses to substances of abuse suggests the gene expression networks determined through WGCNA are important intermediate molecular phenotypes underlying alcohol-drinking behavior. The myelin-related network (i.e. the green module) may be particularly interesting since it demonstrates the most striking correlation between module membership and alcohol intake for each individual gene (Figure 26).

Conservation of Alcohol-Related Gene Expression Networks Across Species

The known association of decreased myelin in the PFC of alcoholics and correlation to alcohol intake in rhesus macaques led to the hypothesis that variation in the expression of a myelin-related gene network may be a substantial factor presiding over alcohol consumption across species. Issues with inadequate controls for the

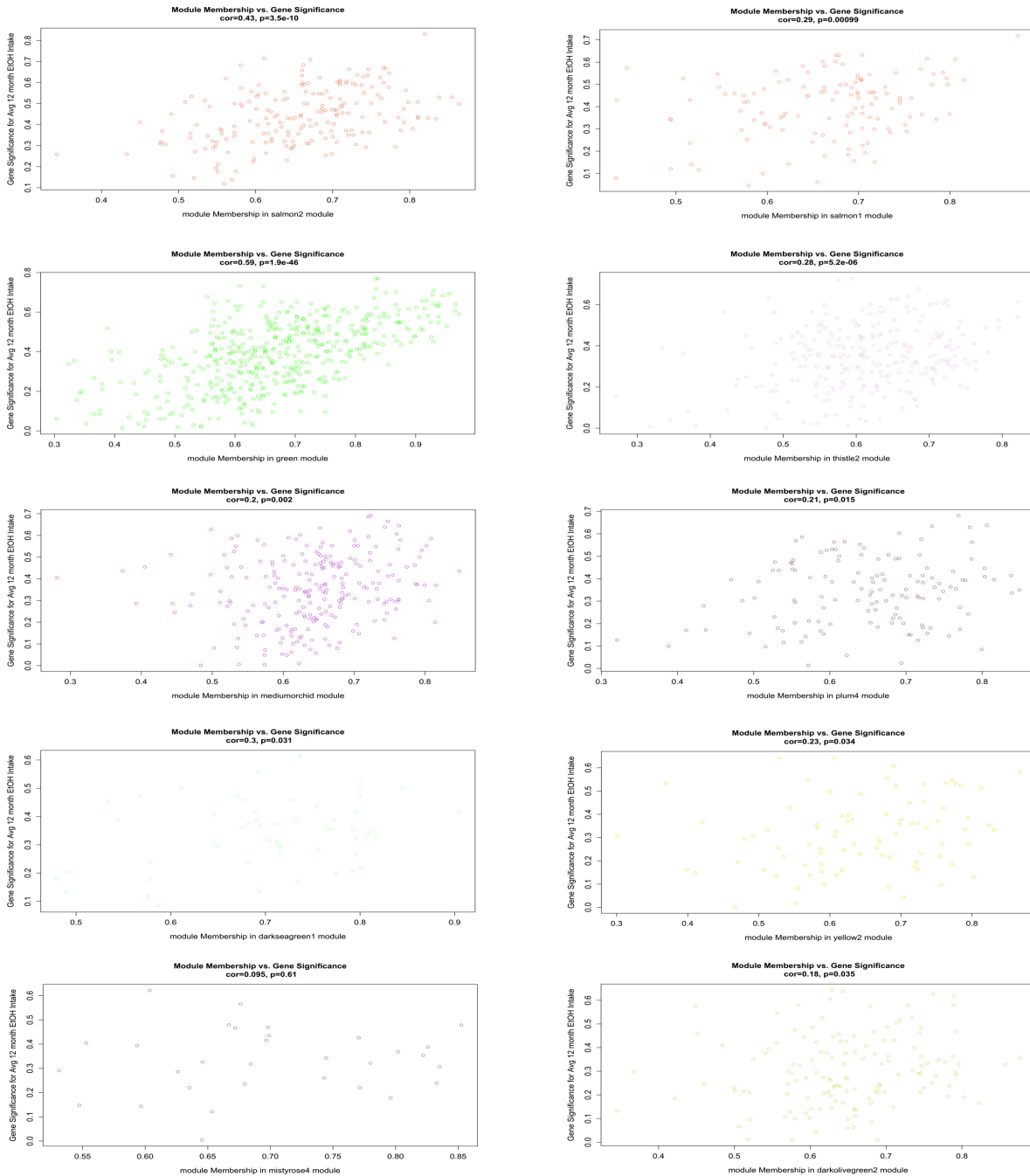


Figure 26. Module membership correlation for rhesus macaque PFC associated alcohol-drinking modules. Scatter plots for the module membership of individual genes based on S-scores from PFC against alcohol drinking correlation for individual genes (gene significance) in each of the ten alcohol drinking modules. y-axis = Pearson correlation of individual genes to alcohol intake , x-axis = module membership based on Pearson correlation of individual genes to the module eigengene . Bolded are the associated correlation coefficients and corresponding p-value.

cynomolgus macaque cohorts and non-Affymetrix gene expression profiling of human alcoholic brain tissue available from the Gene Expression Omnibus (GEO) however necessitated using a probe set expression summarization technique other than the S-score algorithm. WGCNA of gene expression data using the robust multi-array average (RMA) algorithm identified eleven networks significantly correlated to alcohol drinking behavior (Figure 27). Modules ranged between 38 and 582 probesets in size. The red module was the largest among the eleven identified groups, and demonstrated the highest correlation to the average daily alcohol intake over 12 months of exposure. Similar to the largest S-score module, the red RMA module was strongly over-represented for multiple myelin-related gene ontology categories. A total of 220 probesets were significantly shared between the red RMA module and the green S-score module (Fisher's exact test, p -value = 1.42×10^{-272}), suggesting the myelin-associated network is a highly robust module using two different microarray algorithms for evaluating the correlation structure of genes associated with alcohol drinking behavior. Additionally, this suggests RMA based expression values are appropriate for assessing the conservation of myelin-related gene expression network across species.

In order to compare gene expression networks across species, as well as closely related monkeys, multiple probesets measuring the expression of the same gene needs to be collapsed into a single representative measurement within each microarray study (Miller et al., 2011). Ranked gene expression and ranked connectivity for the alcohol drinking rhesus macaque and BXD recombinant inbred mouse PFC datasets were compared using four separate collapsing strategies: maximum mean expression ('Max'), maximum expression variance ('Var'), maximum mean expression + maximum

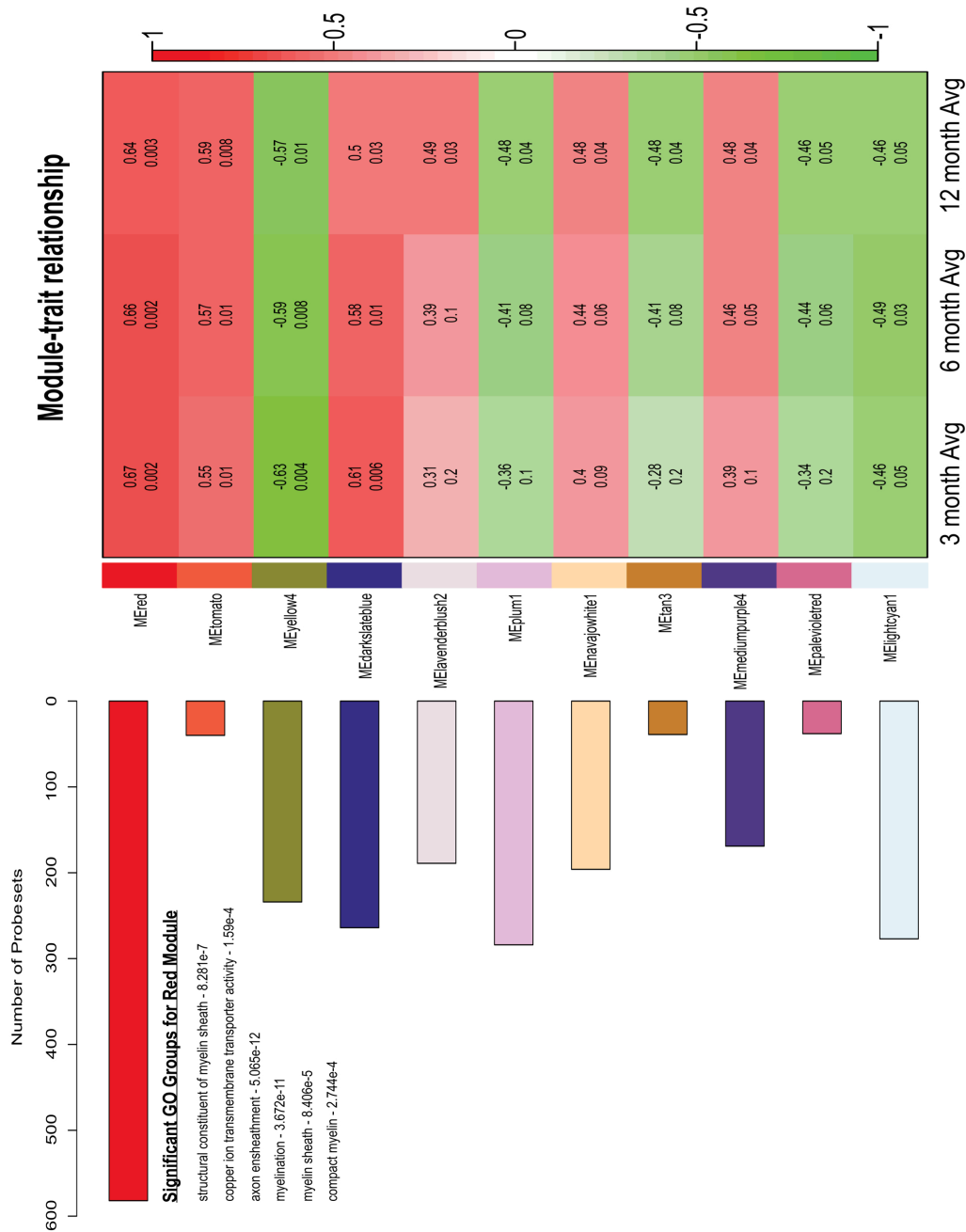


Figure 27. RMA modules significantly correlated to alcohol drinking behavior in male rhesus macaque PFC. Right panel shows heatmap of correlation for the module eigengenes significantly correlated to average alcohol intake (g/kg/day) for 365 days of consumption. Red = positive correlation, Green = negative correlation, x-axis = duration of alcohol drinking exposure, y-axis WGCNA modules from RMA data. Left panel shows the distribution of modules significantly associated with alcohol drinking behavior, based on the number of probesets within each module. The ‘red’ module was enriched for gene ontology categories related to myelin-associated gene expression in PFC.

connectivity ('kMax'), and maximum expression variance + maximum connectivity ('kVar') (Figure 28). Aggregating the data using the maximum mean expression method demonstrated the most significant relationship between rhesus macaque PFC and mouse PFC. The same result was observed when comparing rhesus versus humans, rhesus versus female cynomolgus, and rhesus versus male cynomolgus, suggesting the 'Max' function was the most appropriate method for collapsing the differing microarray experiments. Ranked expression for each of the independent datasets revealed the highest correlation coefficient for each of the profiled comparisons (Figure 29). Relative strength of the overall correlations was dependent upon the microarray platform, evolutionary distance between species or genus, and sex of the subjects. Human ranked expression data was also significantly correlated to ranked results from mice (Pearson's r -value = 0.5, p -value < 1 e-200).

WGCNA was applied separately within each of the five datasets to identify the correlation structure of gene expression for well-annotated genes spanning (~6000) mice, monkeys, and humans (Figure 30). A total of 10 to 21 modules were determined within each of the five separate studies. Representative modules varied in expression patterns across individual samples (Figure 31). Comparing mouse, human, male and female cynomolgus macaques to rhesus macaques determined statistically significant over-lapping genes among modules (Figure 32). For example, the greenyellow module in rhesus macaque PFC and the black BXD mouse PFC singled-out 45 common genes within the two modules, which was the most statistically conserved group between any of the modules (Fisher's exact test, p -value = 4.34 e-25). *MBP* and *PLP1* were two of the characteristic myelin genes shared among the identified 45 common genes. The

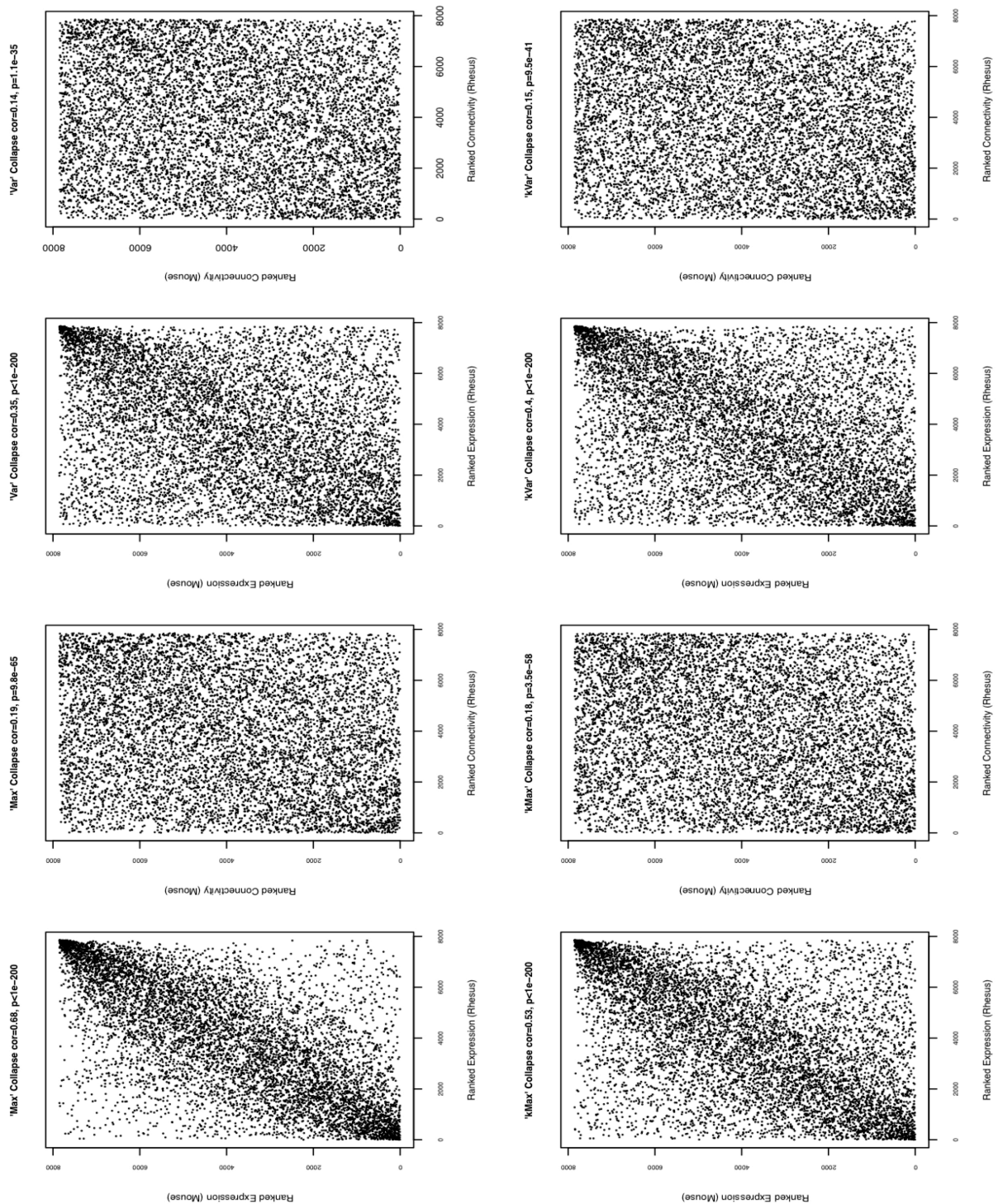


Figure 28. Comparison of methods for collapsing probes to genes across mice and rhesus macaques. Scatter plots for ranked expression and ranked connectivity of the PFC RMA expression for BXD RI mice and rhesus macaques using maximum mean expression ('Max'), maximum expression variance ('Var'), maximum mean expression + maximum connectivity ('kMax'), and maximum expression variance + maximum connectivity ('kVar').

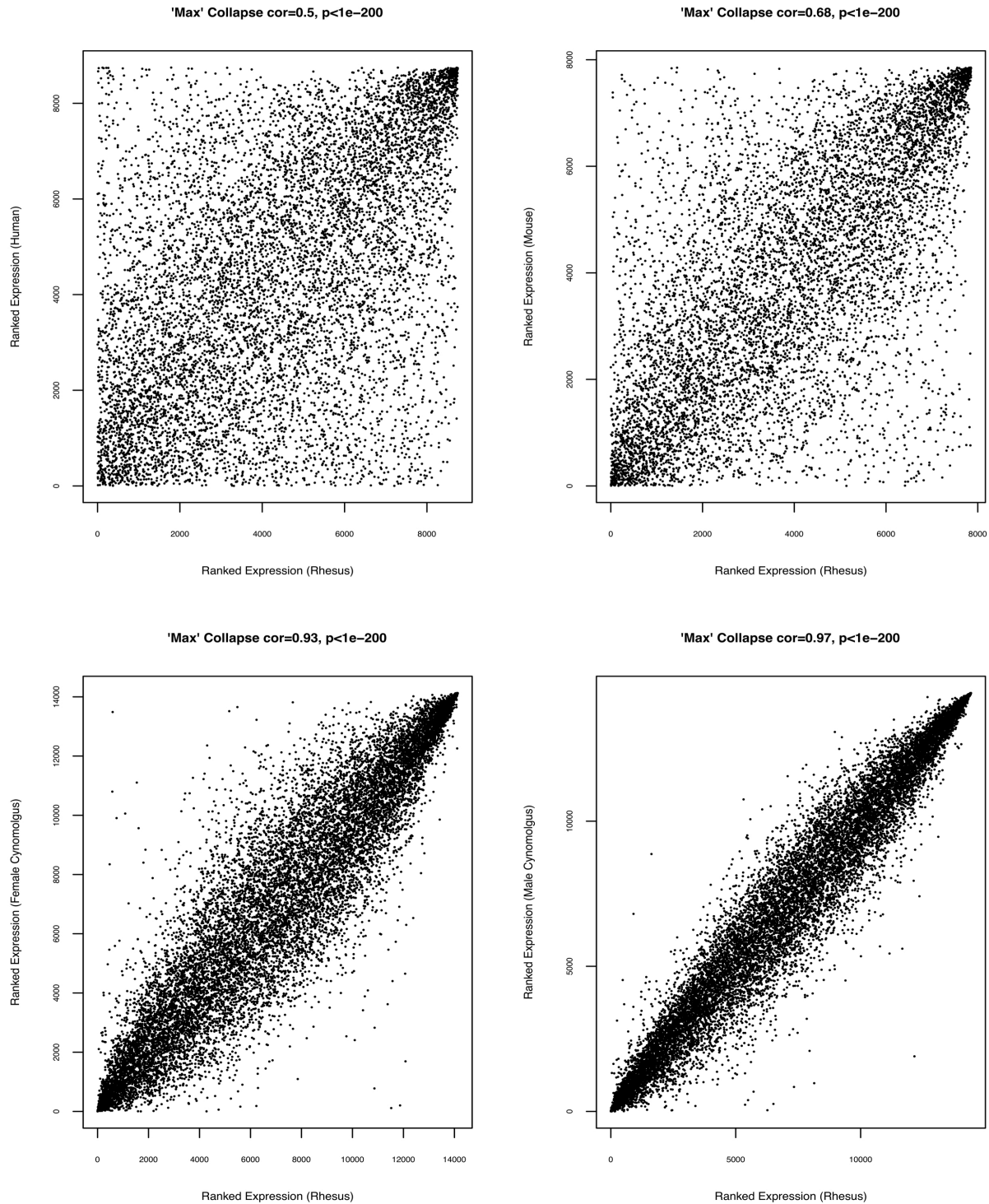


Figure 29. Ranked gene expression for maximum mean expression. Scatter plots for ranked expression using maximum mean expression ('Max'), comparing rhesus macaque PFC RMA gene expression (x-axis) to humans (top left panel), mice (top right panel), female cynomolgus macaque (bottom left panel), and male cynomolgus macaque (bottom right panel).

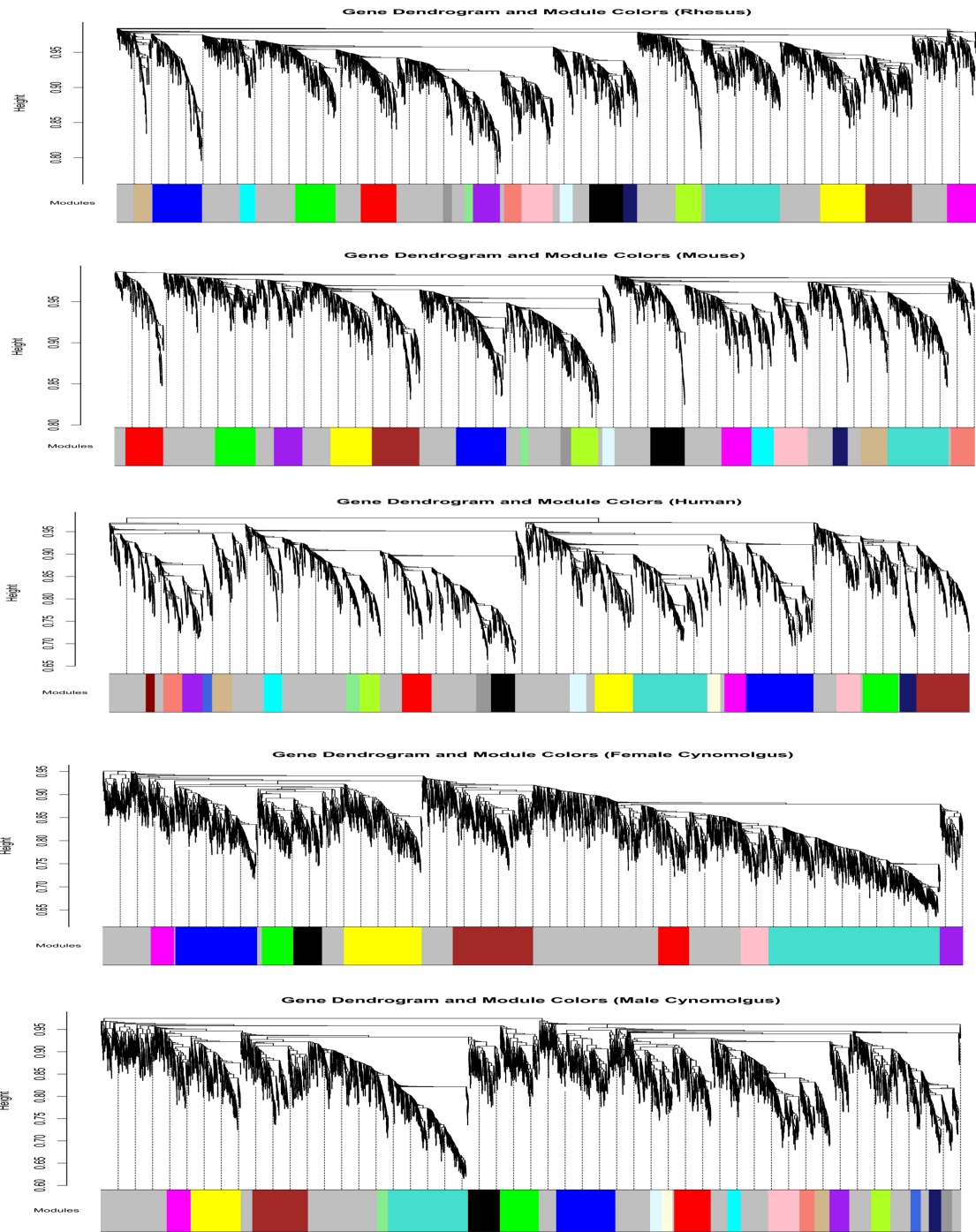


Figure 30. Cross-species weighted gene co-expression PFC dendrograms. Average linkage hierarchical clustering of gene expression for rhesus macaques, BXD RI mice, humans, female cynomolgus macaques, and male cynomolgus macaques (top to bottom), beneath each dendrogram are the identified colored modules based on correlated expression profiles; width of bars correspond to the number of genes within the module, grey regions indicated unassigned genes within each species' dataset.

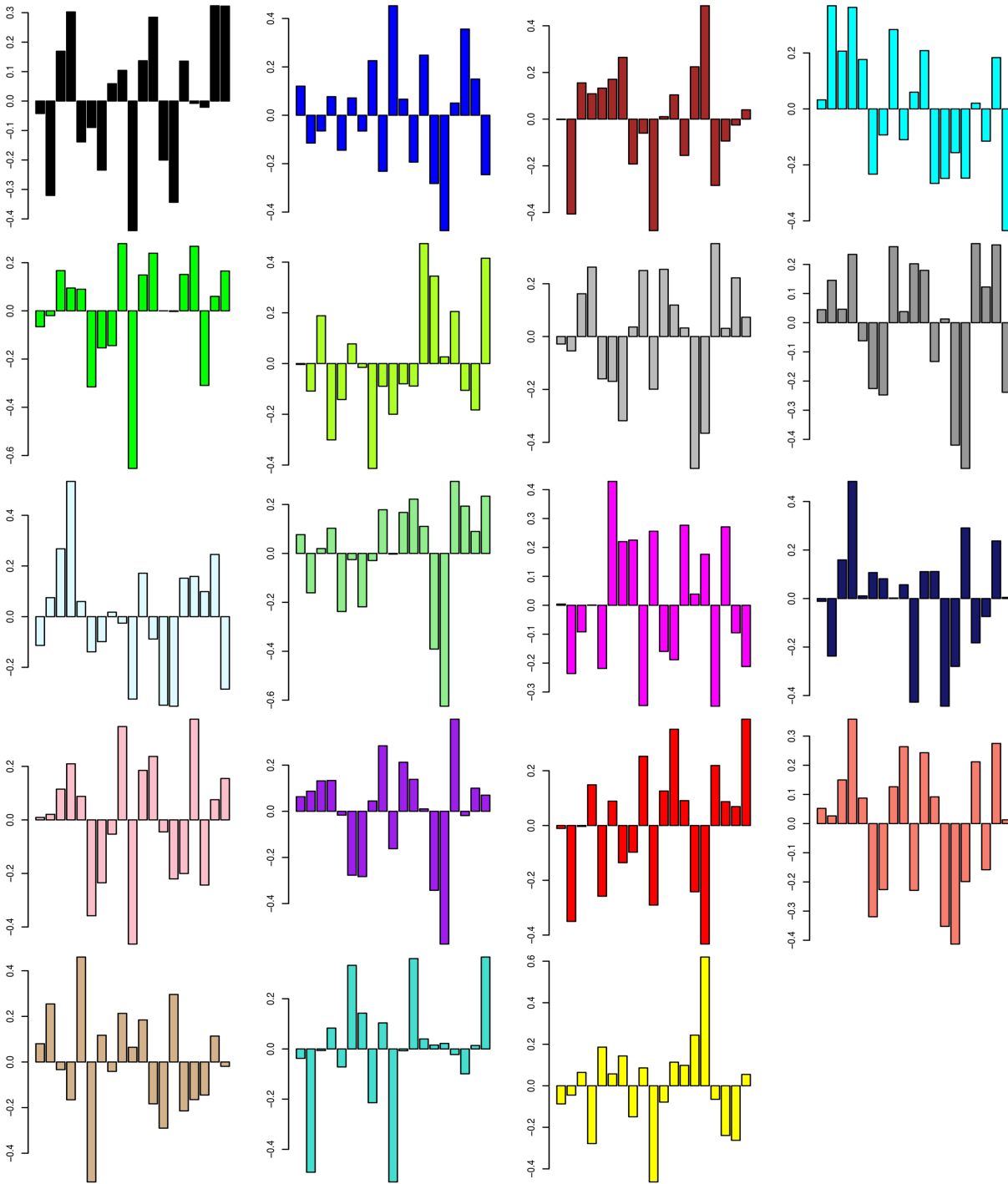


Figure 31. Module eigengene expression for collapsed rhesus macaque PFC dataset. Representative module eigengene (ME) expression for the nineteen modules identified through weighted gene co-expression network analysis of the collapsed RMA rhesus macaque PFC dataset; showing variation in relative expression (y-axis) between individual samples (x-axis / single bars); ME expression is based on the first principal component of each colored module.

myelin network was the most robustly conserved across groups: rhesus-human (Fisher's exact test, p-value = 2.23×10^{-40}), mouse-human (Fisher's exact test, p-value = 4.58×10^{-16}), rhesus – female cynomolgus (Fisher's exact test, p-value = 4.20×10^{-34}), and rhesus – male cynomolgus (Fisher's exact test, p-value = 1.34×10^{-44}). Similar to the gross overall comparison of every gene present within each experiment (Figure 29), robustness of the myelin module conservation was generally dependent upon evolutionary distance between species or genus, and sex of subjects. In addition to conserved correlation structure, myelin-related genes are highly homologous in protein coding sequence across mice, monkeys, and humans (Figure 33). The major myelin-related gene PLP1 shows 100% sequence conservation across all three species.

Overlapping all WGCNA results for the myelin-related modules identified 21 genes shared in all five data sets, and 47 genes shared in four out of five data sets. MBP was present in four of five myelin modules. Network analysis of the non-reduced datasets showed MBP present within the myelin module, suggesting the MBP gene should be included for a cross-species network. Over 90% of this conserved gene set is enriched in oligodendrocytes, the myelin forming cells of the CNS (Cahoy et al., 2008). All of the genes within the myelin-related modules were positively correlated to one another, suggesting they may form protein-protein interactions (Ge et al., 2003). Most importantly, relative expression of individual myelin genes, such as NDRG1, as well as expression for the myelin-associated gene network significantly correlated to alcohol drinking behavior in rhesus macaque and BXD RI mice PFC (Figure 34, p-value < 0.01). Genes within the conserved network were ranked by their average module membership across all five datasets and correlation to alcohol drinking behavior to highlight some of

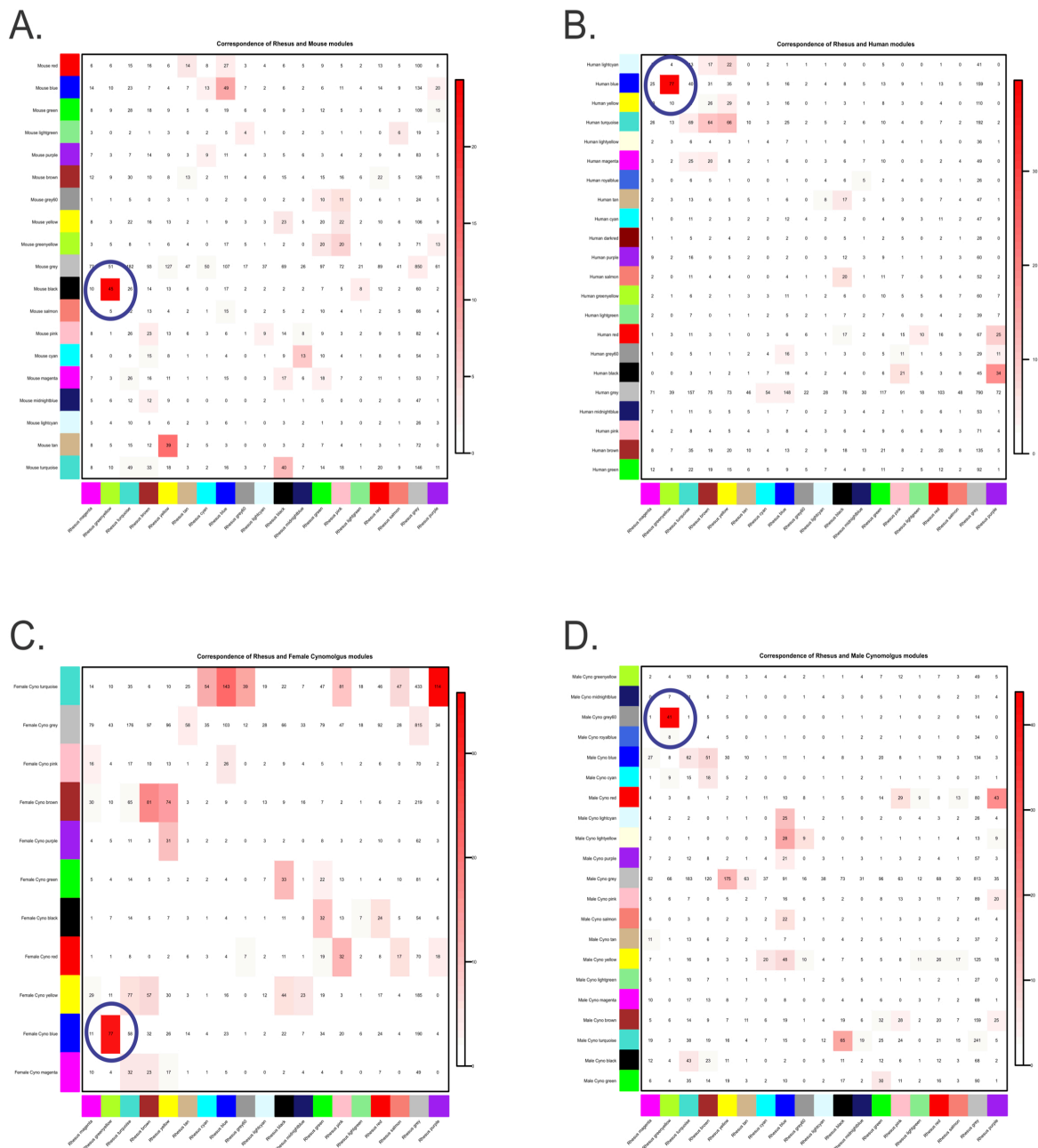


Figure 32. Overlap of weighted gene co-expression modules across species. Heatmap of fisher's exact test for significant overlap in the number of genes between the rhesus macaque WGCNA modules (x-axis) and mouse WGCNA modules (A), human WGCNA modules (B), female cynomolgus macaque WGCNA modules (C), and male cynomolgus macaque WGCNA modules (D); numbers correspond to the number of genes shared between modules, red indicates the $-\log p$ -value for fisher's exact test, blue circles indicate the significant overlap of myelin-associated gene expression.

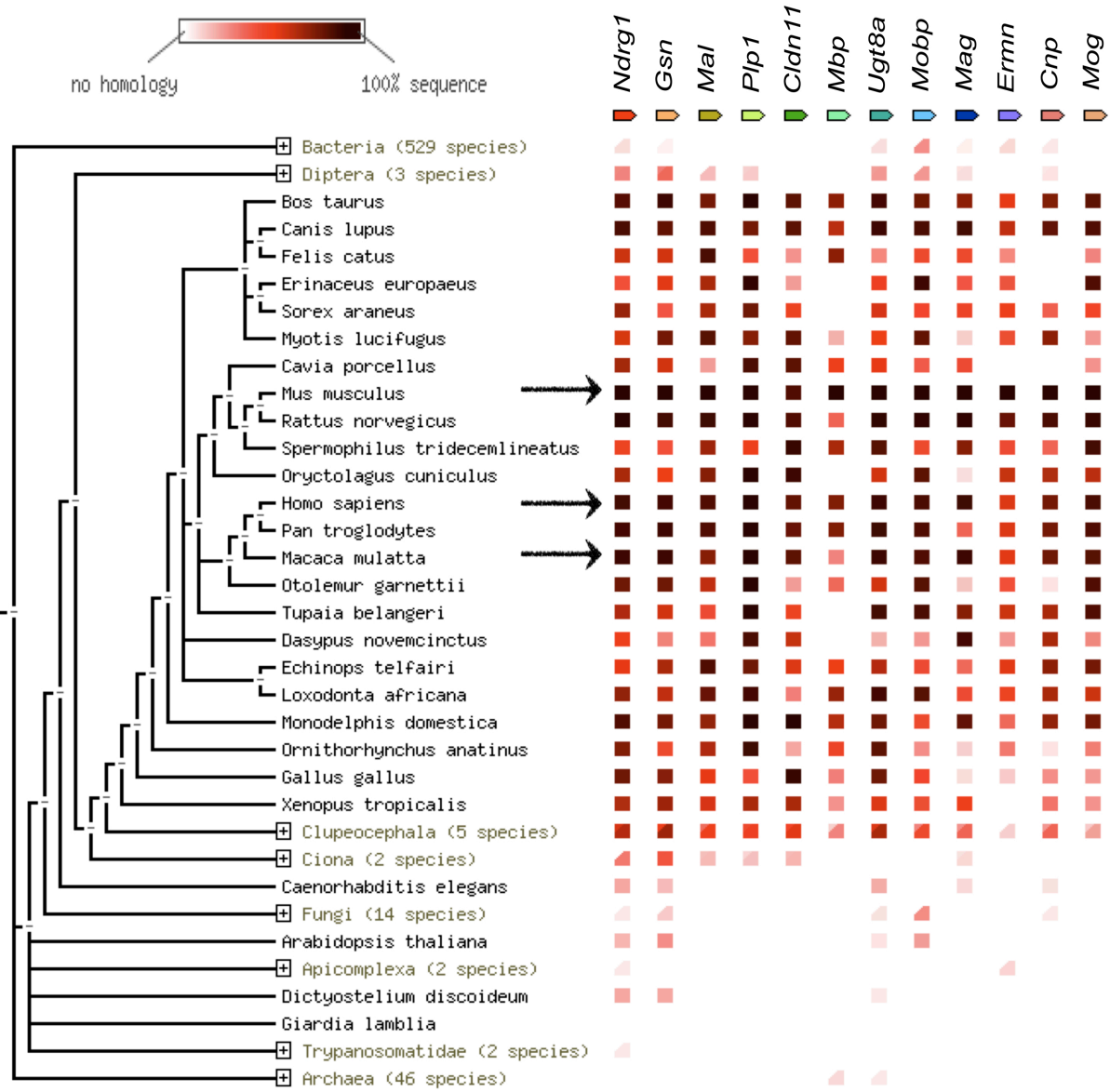


Figure 33. Phylogenetic display of myelin conservation between species. Phylogenetic profile of myelin protein coding sequence conservation compared to mouse for twelve representative myelin genes; degree of colored blocks show relative sequence homology with black indicating 100% homology, and white (or no box) indicating no homology; arrows point out mice (*Mus musculus*), rhesus (*Macaca mulatta*), and humans (*Homo sapiens*). Myelin is highly conserved across mice, monkeys, and humans with PLP1 having 100% sequence homology.

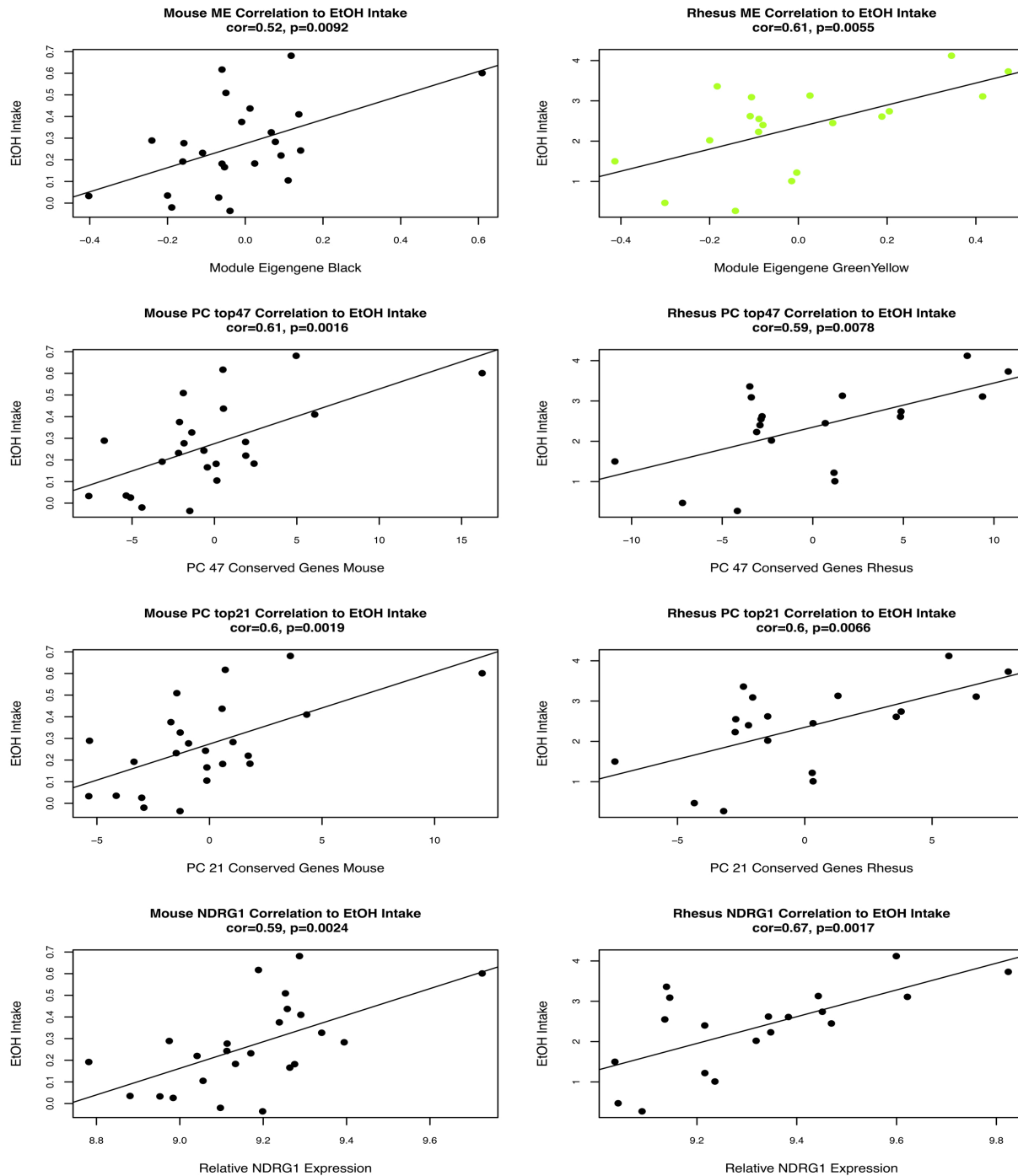


Figure 34. Association of myelin gene expression with alcohol drinking behavior. Correlation of alcohol intake and myelin-related gene expression within PFC for BXD RI mice (left side) and rhesus macaque (right side); significant scatter plots are shown for the within species module (1st row), top 47 myelin-related genes across 4 out of 5 data sets (2nd row), top 21 myelin-related genes across all 5 datasets (3rd row), and the individual myelin-related gene *NdrG1* (4th row).

the most 'important' genes. MBP and NDRG1 each ranked within the top-five prioritized genes. CNTN2, which is a key component of myelin integrity and organization (Savvaki et al., 2010), also ranked within the top-five priority genes (Figure 35). However, the expression of all of these genes exists within the confines of a molecular network (Figure 35). Existing as a molecular network these myelin-associated genes participate in a shared biological function and are significantly associated to alcohol drinking behavior across species.

Discussion

Myelin is a vital component of the central nervous system, which facilitates the efficient transmission of action potentials for neuronal communication. Alcohol abuse causes a significant decrease in myelin abundance within the frontal cortex of adults (Mayfield et al., 2002), and successively binge-drinking adolescents (McQueeney et al., 2009). Abstinence enables the recovery of myelinated membranes from the neurotoxic influence of chronic alcohol exposure (Ende et al., 2005). Transition to uncontrolled alcohol drinking behavior is inversely associated with acute behavioral sensitivity to alcohol (Schuckit, 1994). Expression profiling of mice, known to differ in acute alcohol sensitivity and long-term alcohol drinking behavior, demonstrated differences in the coordinate regulation for basal abundance and acute alcohol responsiveness of myelin-associated gene expression (Kerns et al., 2005). Dynamic alterations in the amount of myelin due to chronic alcohol exposure, abstinence, and acute alcohol responsiveness, as well as basal variation in myelin gene expression, suggests myelin is a major constituent underlying CNS plasticity associated with alcoholism. Through a network

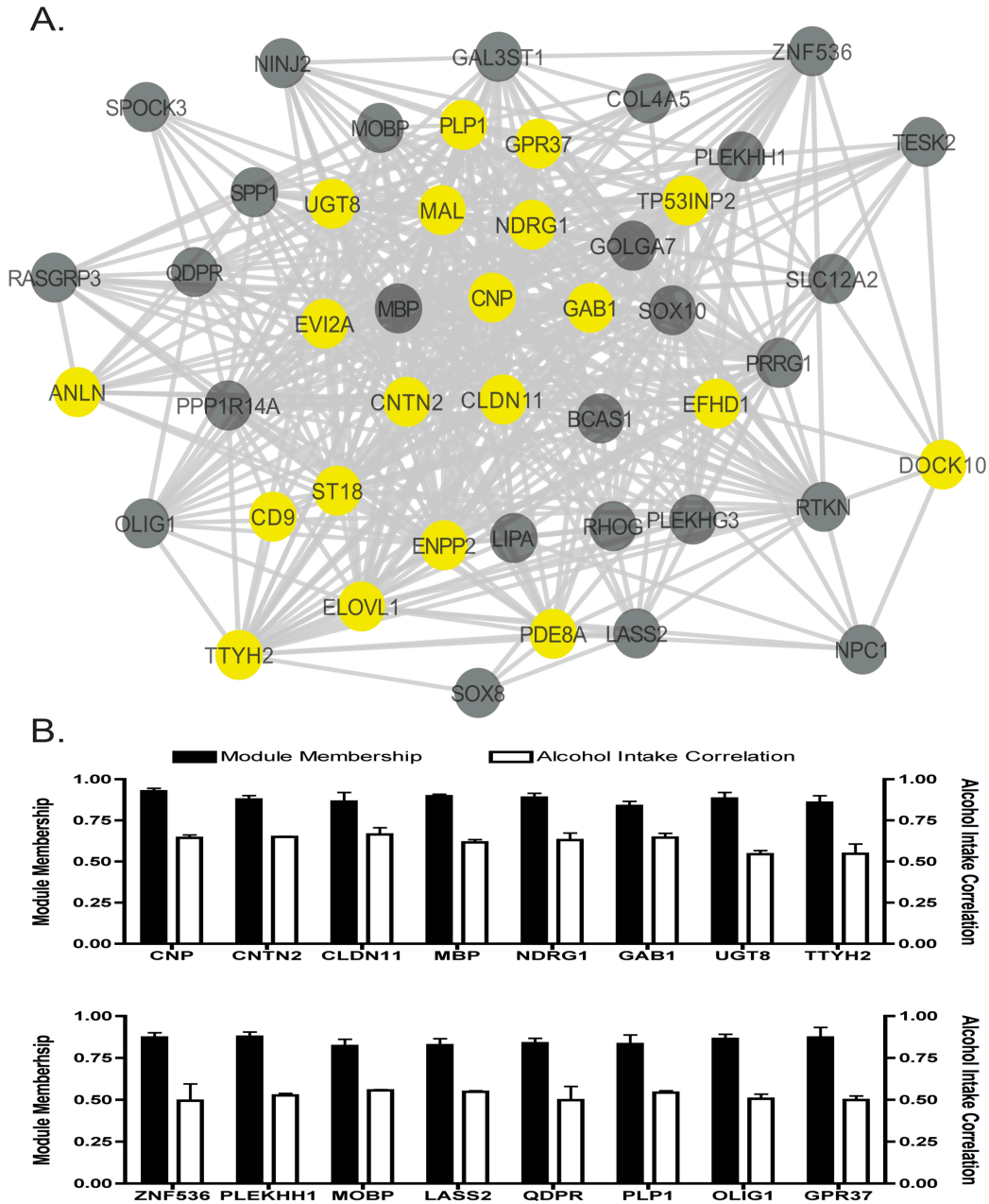


Figure 35. Cross-species myelin gene expression network. (A) Cytoscape network of myelin-related genes for the frontal cortex with connections among nodes (i.e. genes) derived from Pearson correlation coefficients between genes $\geq |0.70|$; yellow indicates presence in all five datasets (B) bar plot of top 16 ranked genes within the myelin network, black indicates the average module membership across all five datasets, white bars indicated the average correlation coefficient to alcohol intake in BXD RI panel and rhesus macaques. Data are presented as Mean \pm SE.

approach that uses gene expression as a quantitative trait, our results indicate myelin-associated gene expression is a conserved biological network significantly associated with variation in alcohol drinking behavior across species.

Inclusion of only four to five non-drinking control subjects for the non-human primate studies likely limits the detection of gene expression patterns relevant to individual differences, which may substantially vary between individuals within a single species (Enard et al., 2002). Sex-differences may significantly influence alcohol drinking behavior and complications accompanying substance abuse (Ceylan-Isik et al., 2010). Therefore, considering only brain-region specific differences in female subjects may also provide an incomplete assessment of gene expression patterns relevant to alcohol abuse and dependence. As part of the INIA-stress consortium a limited number of control subjects were utilized to maximize the allocation of resources, as well as ethical limitations on the number of non-human primates appropriate in biomedical research. Although important, identifying region-specific expression patterns on alcohol-naïve subjects that may predispose individuals for an alcohol use disorder was not the immediate focus of this particular inquiry. The principal objective for characterizing profiling variation in gene expression among chronically exposed non-human primates, and additional mammalian models, was to test the hypothesis that myelin-associated gene expression is intertwined with alcohol intake, and direct future unbiased hypotheses for understanding the neurobiology of alcohol drinking behavior.

Applying a systems based approach provides a broader perspective of genetic architecture of neurological diseases (Courtney et al., 2010, Ponomarev et al., 2012). Gene expression networks serve as molecular signatures driving the manifestation and

maintenance of complex traits associated with disease (Baudot et al., 2009). Rhesus macaques are a close phylogenetic relative to humans (Magness et al., 2005), widely used as a model organism in biomedical and neuroscience research (Carlsson et al., 2004). Genetic and physiological similarities of non-human primates to humans imparts a unique animal model for deciphering the origin of multifactorial diseases such as alcoholism (VandeBerg and Williams-Blangero, 1996). A total of ten gene expression networks were significantly correlated to the average daily alcohol intake during one year of voluntary self-administration (Figure 22). These ten gene networks were over-represented as targets for a common subset of microRNAs important in CNS plasticity and neurobehavioral dysfunction (Figure 25). Variation in microRNAs, and subsequently their downstream targets, are suspected factors in the pathogenesis of neuropsychiatric disorders (Xu et al., 2010) and alcohol dependence (Lewohl et al., 2011). Differences in the expression of microRNAs were not directly examined; however, our analysis highlights potential microRNAs associated with alcohol drinking behavior. Additionally, our analysis provides information regarding the co-expression of specific biological networks that may serve as key intermediate phenotypes presiding over alcohol drinking behavior. The largest identified network was enriched for genes localized within oligodendrocytes and genes involving myelination.

WGCNA defined coherent patterns of gene expression for five separate studies, comprised of alcohol drinking cohorts from BXD RI mice, rhesus macaques, male and female cynomolgus macaques, and human alcoholics. Consistent with the high degree of evolutionary conservation in mammals (Nave, 1994), myelin-related gene expression was by far the most robust network across each of the independent analyses. In

addition to alcohol dependence, altered homeostatic expression of myelin-related genes has been implicated in schizophrenia (Hakak et al., 2001, Katsel et al., 2005), major depression (Aston et al., 2005), and cocaine abuse (Lehrmann et al., 2003, Bannon et al., 2005). Dysregulation of myelin-associated gene expression from multiple neuropsychiatric disorders and substances of abuse may denote a common molecular substrate in the neurobiology of disease (Thomas, 2006). The heterogeneous nature of psychiatric illness and addiction nonetheless necessitates defining which behavioral endophenotypes are associated with coordinate regulation of myelin gene expression.

Variation in myelin-associated gene expression within PFC was significantly correlated to alcohol drinking behavior in both mice and rhesus macaques. The correlation does not necessarily infer a causal association with the development of alcohol dependence; however, it establishes a connection between a conserved mammalian network of myelin-associated genes and alcohol consumption. In contrast to human alcoholic brain tissue that shows decreased myelin gene expression (Lewohl et al., 2000), a two-group analysis (alcohol drinkers versus non-drinkers) of model organisms failed to identify chronic alcohol-induced changes in myelin gene expression. Systematic decreases of myelin in alcoholic brain tissue compared to controls may be related to a longer duration of alcohol exposure than the time-course applied to mice or monkeys (Sullivan et al., 1995). Alterations in myelin-associated gene expression may be secondary to the direct effects of alcohol, or may function as a predisposing factor in the susceptibility of persistent alcohol drinking behavior. To our surprise, rather than inversely correlated to average alcohol intake as might be advocated by decreased myelin in the PFC of alcoholics (Lewohl et al., 2000), myelin was positively correlated to

alcohol drinking behavior in both mice and nonhuman primates. Given the shorter duration of alcohol exposure than witnessed in humans, a positive correlation may suggest a higher relative abundance of myelin gene expression is associated with increased alcohol intake during initial drinking behavior.

Overall, our results support the hypothesis that a myelin-associated gene network acts as an underlying factor in alcohol behavioral phenotypes. The conserved expression of myelin-related genes within the frontal cortex and correlation to alcohol consumption behavior across species provides experimental evidence that mice are an appropriate animal model for testing future hypotheses regarding the role of myelin gene expression in acute alcohol sensitivity and long-term drinking behavior.

CHAPTER 4:
ALCOHOL-RELATED BEHAVIORAL PHENOTYPES
AND VARIATION OF MYELIN GENE EXPRESSION IN PREFRONTAL CORTEX

Introduction

Myelination is a dynamic process accomplished by oligodendrocytes within the central nervous system (Zuchero and Barres, 2011). Proper formation of the myelin sheath is essential for the cytoskeleton structure of neuronal axons (Brady et al., 1999), and downstream behaviors. Improved function of prefrontal cortex (PFC) neurocircuitry during development occurs with ongoing myelination (Clark et al., 2008). Abnormalities within the PFC lead to the dysregulation of behaviors associated with addiction (Goldstein and Volkow, 2011). Variation in gene expression is a contributing factor the vulnerability of neurobehavioral diseases such as alcoholism (Geschwind, 2003). Characterizing myelin-associated gene expression in PFC may thus provide novel insights into the neurobiology of the neurobiology of alcohol-related behaviors and the susceptibility for an alcohol use disorder.

Marked decreases in protein trafficking and myelin-associated genes are witnessed in the frontal and motor cortices of alcoholics (Lewohl et al., 2000, Mayfield et al., 2002). Alterations in gene expression detected within postmortem human brain samples are not due to confounding aspects involving death (Franz et al., 2005). Observed decreases in myelin mRNA expression translate to the down regulation of myelin structural proteins in alcoholic brain tissue (Lewohl et al., 2005). Although myelin genes are consistently reduced in the frontal cortices of alcoholics, individual variability

exists within their gene expression profiles (Liu et al., 2004b, Liu et al., 2006). The risk of developing a substance abuse disorder is influenced by individual differences of tasks directed by the PFC (Perry et al., 2011). Differences between C57BL/6J and DBA/2j mice within the PFC for basal and ethanol-responsive increases in myelin-associated gene expression are suggested to be important determinants of acute alcohol sensitivity and compulsive drinking behavior (Kerns et al., 2005). Although limited to only two genetically divergent strains of mice, this latter study provides preliminary evidence that individual differences in sensitivity to alcohol or baseline abundance of myelin may contribute to the long-term effects of alcohol.

Gene expression can serve as a quantitative trait linked with variability to molecular, anatomical, and behavioral phenotypes (Williams, 2006). Animal models mimic differing behavioral aspects of human delineations meeting the criterion for a DSM-IV-R diagnosis of alcohol dependence (Crabbe et al., 2011). Genetic factors influence the level of behavioral responses to alcohol in humans and animal models (Foroud and Li, 1999). The initial level of response to alcohol is inversely associated with the risk of maladaptive alcohol drinking behavior (Schuckit and Smith, 1996, Schuckit et al., 2006). Large-scale gene expression studies from mouse whole brain have identified a complex web of genes involved in alcohol drinking behavior (Mulligan et al., 2006, Tabakoff et al., 2008). Variation gene expressions among discrete brain regions affect behavioral and molecular differences between two or more strains of mice (Pavlidis and Noble, 2001). Merging functional genomics data from whole brain homogenates and separate brain regions with quantitative trait loci (QTL) for behavioral phenotypes facilitates the identification of candidate genes responsible for complex

traits (Hitzemann et al., 2004). Selection of the proper brain region for investigation is challenging given limited resources and inadequate knowledge regarding which brain region(s) are accountable for alcohol or drug-related behavioral traits. Altered alcohol-induced expression of myelin-associated basic protein (*Mobp*) within mouse whole brain resides within a known QTL region on chromosome 9 for alcohol preference, suggesting *Mobp* is a candidate gene for alcohol consumption (Weng et al., 2009). Our previous analysis of mice, monkeys, and humans chronically exposed to alcohol indicated the expression of *Mobp* varies along with a network of myelin-associated genes in PFC that is correlated to alcohol drinking behavior.

Myelin asserts protective effects on axonal integrity and function (Nguyen et al., 2009). Changes in myelin gene expression leads to blunted neuronal conduction velocities that cause altered anxiety-like behaviors, reduced prepulse inhibition, deficits in spatial learning, and working memory impairments (Tanaka et al., 2009). Majority of drug-induced adaptations in gene expression are short-lived, returning to baseline between 4-12 hours post drug exposure (Rhodes and Crabbe, 2005). However, drugs of abuse can lead to sustained aberrations in gene expression which linger long after drug exposure ceases (Nestler, 2008). The duration of alcohol exposure required for persistent reductions of myelin-associated gene expression within the PFC of alcoholics is unknown. Down-regulation of myelin-related gene expression in postmortem brain tissue contrasts with acute alcohol-induced up-regulation of myelin gene expression in the PFC of mice. Differential effects of acute and chronic alcohol, as well as basal differences, on myelin gene expression may be important in alcohol-related behaviors. We hypothesize that coordinate baseline variation of myelin-associated gene

expression within the PFC of animals are important determinants in the short and long-term behavioral effects of alcohol. Weighted gene co-expression network analysis (WGCNA) of two alcohol naïve recombinant inbred reference panels (LXS and BXD) demonstrates significant correspondence for the expression of a myelin-associated gene network in PFC across 72 strains of mice. Expression of this myelin network is largely dissimilar than observed in the NAC and VTA, suggesting a unique myelin gene expression profile in PFC. Correlation of the PFC myelin gene expression pattern against a public repository for phenotypes derived from BXD mice revealed that basal variation of myelin-associated gene expression is associated with multiple behavioral measures of acute alcohol sensitivity and alcohol drinking behavior. These results independently reaffirm and extend our previous observations regarding baseline differences in myelin gene expression between C57BL/6J and DBA/2J mice (Kerns et al., 2005). Examining single nucleotide polymorphisms (SNPs) of myelin genes in humans determined no association for myelin genes in alcohol dependence; however, SNPs within a set of myelin genes demonstrate a clustering pattern for intermediate phenotypes in alcohol use disorders. Overall, our analysis suggests individual differences for a myelin-associated gene network is a relevant biological factor underlying endophenotypes associated with alcohol abuse and dependence.

Materials and Methods

BXD Recombinant Inbred (RI) Mice from a previous study conducted in the laboratory of Dr. Michael Miles were used as a reference population for prefrontal cortex (PFC), nucleus accumbens (NAC), and ventral tegmental area (VTA) mRNA expression in

response to a saline injection across 27-35 RI mice, as well as C57BL/6J and DBA/2J progenitors. All samples from a total of 468 adult male animals were obtained from Jackson Laboratory and housed in a standard laboratory environment. An average of 8 males per strain were used to measure anxiety-like behavior in response to restraint and saline treatment in the light-dark transition model of anxiety. Four hours after treatment, animals were rapidly sacrificed by cervical dislocation, brains were removed, chilled in saline for one minute, and micro dissected on ice as previously described by our laboratory (Kerns et al., 2005). All RNA isolation and subsequent probe generation and hybridization to microarrays were completed using a supervised randomization procedure to minimize potential technical batch effects. Affymetrix M430 type 2.0 microarrays were used for hybridization according to the manufacturer's protocol. Expression analysis was conducted by estimating the relative abundance of over 45,000 transcripts in the PFC, NAC, and VTA in response to a saline injection using Robust Multi-array Average (RMA) (Irizarry et al., 2003).

Male mice were obtained at 8-9 weeks of age from the Jackson Laboratory (Bar Harbor, ME). Animals were treated, behaviorally tested for anxiety-like behavior, and dissected as described above by member of Dr. Michael Miles' laboratory. Following an hour acclimation period to the behavioral room, animals were restrained for 15 minutes, immediately given an intraperitoneal injection (I.P.) of physiological saline or 1.8 g/kg of ethanol, and 5 minutes later placed in the light-dark box for a 10-minute session. All behavioral testing occurred between 10:00 AM and 1:00 PM during the light phase over a 12-month period beginning August 2005. Four hours after treatment, animals were rapidly sacrificed by cervical dislocation; brains were removed, cooled and micro-

dissected as previously described. Prefrontal cortex tissue was isolated by micro-dissection using a wedge-shaped slice taken from a 4-mm thick brain slice extending rostrally from the optic chiasm. The wedge was centered on the inter-hemispheric fissure and extending 2-mm laterally on each side and ventrally to just above the corpus callosum. This tissue and all other brain regions (including the NAC and VTA) were dissected in less than 5 minutes per mouse and were immediately frozen in liquid nitrogen followed by storage at -80 °C prior to RNA isolation. Individual brain regions were pooled from 3 individual mice of the same strain to generate RNA samples for hybridizing to an individual microarray. Samples were randomized across all strains and treatment groups for each stage of data collection.

LXS Recombinant Inbred (RI) Mice from a previous collaborative study with Drs. Beth Bennett, Chris Downing, and Thomas E. Johnson at the Institute of Behavioral Genetics, University of Colorado Boulder, were used as a second reference population of mice. The LXS genetic reference panel of recombinant inbred strains has been inbred for more than 23 generations (F23). The LXS data set provides gene expression estimates for basal expression of mRNA from the prefrontal cortex (PFC) of 43 inbred strains of mice generated by crossing Inbred Long Sleep (ILS) and Inbred Short Sleep (ISS) mice. All samples are from a total of 376 adult male animals raised in a standard laboratory vivarium. An average of 8 males per strain were habituated to intraperitoneal injection (I.P.) of physiological saline for a period of two days. Following habituation, four animals of each strain were then given I.P. of saline or ethanol (4.1 g/kg). Abiding by previous protocols in our laboratory animals were rapidly sacrificed by cervical

dislocation and brains removed, cooled and micro-dissected as described previously (Kerns et al., 2005). All animal treatments and dissections were conducted by Dr. Chris Downing and colleagues at the Institute for Behavioral Genetics, between 9:30 AM and 11:30 AM. The PFC was isolated as described above. Individual PFC samples from 3-4 mice, balanced for strain and treatment group, were pooled for a single biological replicate. All RNA isolation and subsequent cDNA generation and hybridization to microarrays were done using a supervised randomization procedure to minimize potential batch effects due to technical artifacts. Unlike the BXD samples, LXS samples were hybridized to Affymetrix M430A 2.0 arrays according to the manufacturer's protocols. Affymetrix 430A 2.0 arrays capture expression of approximately 14,000 well-characterized mouse genes. Basal expression measurements were collected using Robust Multi-array Average (RMA) (Irizarry et al., 2003).

Gene Expression of Chronic Ethanol Inhalation. Global transcriptome expression data for chronic alcohol treatment across six brain regions, including frontal cortex, was collected Courtesy of Dr. Susan E. Bergeson at Texas Tech University Health Sciences Center. Data is available through the Alcohol Research Integrator Data Base, an interactive repository for microarray data (<http://aridb.ttuhscc.edu/cgi-bin/genedb.pl>). Adult male DBA/2J were given a 1.5 g/kg loading dose of ethanol (i.p.) and chronically exposed to 72 hours of ethanol vapor. Dissected tissue from DBA/2J mice was immediately frozen in liquid nitrogen and stored at -80 °C. RNA (2µg) was extracted according to the manufacturer's protocol using RNA STAT-60™ (Tel-Test, Inc. Friendswood, TX, USA). The Genisphere Array 350 kit (Hatfield, PA, USA) was used for

creation of labeled cDNA, hybridized to custom in-house complementary DNA (cDNA) microarrays printed at the University of Texas (Mulligan et al., 2006, Mulligan et al., 2008). A total of 13801 probes are present on the array, using SOURCE as a reference database (<http://source.stanford.edu/cgi-bin/source/sourceSearch>).

Alcohol-Related Behavioral Differences for C57BL/6J AND DBA/2J Mice. Male C57Bl/6J (B6) and DBA/2J (D2) mice were obtained from Jackson Laboratory (Bar Harbor, ME) from 8-9 weeks of age. Animals were treated according to protocols for animal care established by Virginia Commonwealth University and the National Institute of Health. Mice were singly housed with *ad libitum* access to water and standard rodent chow (catalog #7912; Harlan Teklad, Madison, WI) for a one-week habituation period prior to testing. Following habituation, B6 and D2 mice were studied for alcohol drinking behavior using a two-bottle choice protocol similar to those described elsewhere (Blednov et al., 2003). Mice were given a period of 3 days of two-bottle access to water tubes in order to acclimate to behavioral testing, establish baseline intake, and prevent possible neophobia. After 3-days of water baseline, mice were allowed to choose between water and ethanol. Ethanol containing tubes increased in concentration every 5 days for 3%, 6%, 10%, and 15% (w/v) ethanol. Water and ethanol tubes were switched every other day to mitigate any potential side biases. Animals were weighed weekly during cage cleaning to monitor general health and calculate the amount of ethanol consumed per kilogram of bodyweight.

A separate cohort of mice (house 4 per cages) was assessed for locomotor activity behavior using an open-field activity chamber (30 cm x 30 cm x 30 cm). Animals

were placed into the behavioral room and allowed to habituate for a period of one hour prior to testing. All animals were tested between 8:00 AM and 12:00 PM eastern standard time. Following habituation animals were given an intraperitoneal injection of saline or 2 g/kg of ethanol and immediately placed inside the activity chamber. Boxes are enclosed in a sound attenuating chamber equipped with overhead lighting and ventilation system, interfaced with Med Associates software (Med Associates Inc., St. Albans, VT, USA). The software enables automatic measurement of activity using a set of 16 infrared beam sensors along the X-Y plane. All animals were tested for a total of 10 minutes in the chambers and then returned to their home cage environment. Between sessions all chambers were cleaned with ammonia and paper towels, then given 2 to 5 minutes to air dry. At completion of the experiment all animals were returned to the animal vivarium and monitored for a period of 24 hours.

Statistical Analysis. All data are reported as mean \pm standard error of the mean and analyzed separately using an unpaired t-test, factorial analysis of variance paired with a Tukey-Kramer post-hoc analysis, or Pearson product-moment correlations. Statistical analyses were conducted using either the R project for statistical computing (Ihaka and Gentleman, 1996) or GraphPad Prism (Motulsky, 1999), with a p-value \leq 0.05 considered statistically significant.

Results

Myelin-associated genes are highly conserved across differing classes of mammalian species in terms of protein coding sequence and gene co-expression

networks. Alcohol drinking behavior is significantly correlated to variation in a network of myelin-associated genes within the PFC of mice and rhesus macaques (Figure 34). Transitioning from gene expression networks to a phenotypic outcome measure may essentially be explained by three experimental models: (i) causal association, (ii) independent association, (iii) and interaction of basal variation with environmental or drug exposure (Figure 36). First, alcohol-related behavioral traits such as drinking behavior may be caused by the initial action of alcohol on primary and secondary targets. These initial targets of alcohol in turn lead to alterations in myelin gene expression that drives phenotypic behaviors (i.e. alcohol consumption). Secondly, the variance in myelin-related gene expression and behavioral phenotypes might be independently acted upon by alcohol. Or lastly, the phenotypic outcome(s) of alcohol action may be influenced by baseline variation in myelin gene expression, which with repeated exposure to the neurotoxic effects of alcohol changes in expression over time.

Chronic Alcohol Effects on Myelin Gene Expression.

Adult male DBA/2J mice, which are more acutely sensitive to the effects of alcohol on myelin gene expression and behavioral traits, were chronically exposed to alcohol vapor for a period of 72 hours following a 1.5 g/kg (i.p.) loading dose of alcohol. Gene expression was sampled from frontal cortices for alcohol-exposed animals immediately post vapor exposure, six-hours post alcohol, twenty-four hours post alcohol, and air exposed controls. Similar to our previous findings in DBA/2J mice (Kerns et al., 2005) the relative abundance of myelin gene expression was increased six hours following alcohol exposure. However, 24 hours post alcohol exposure the

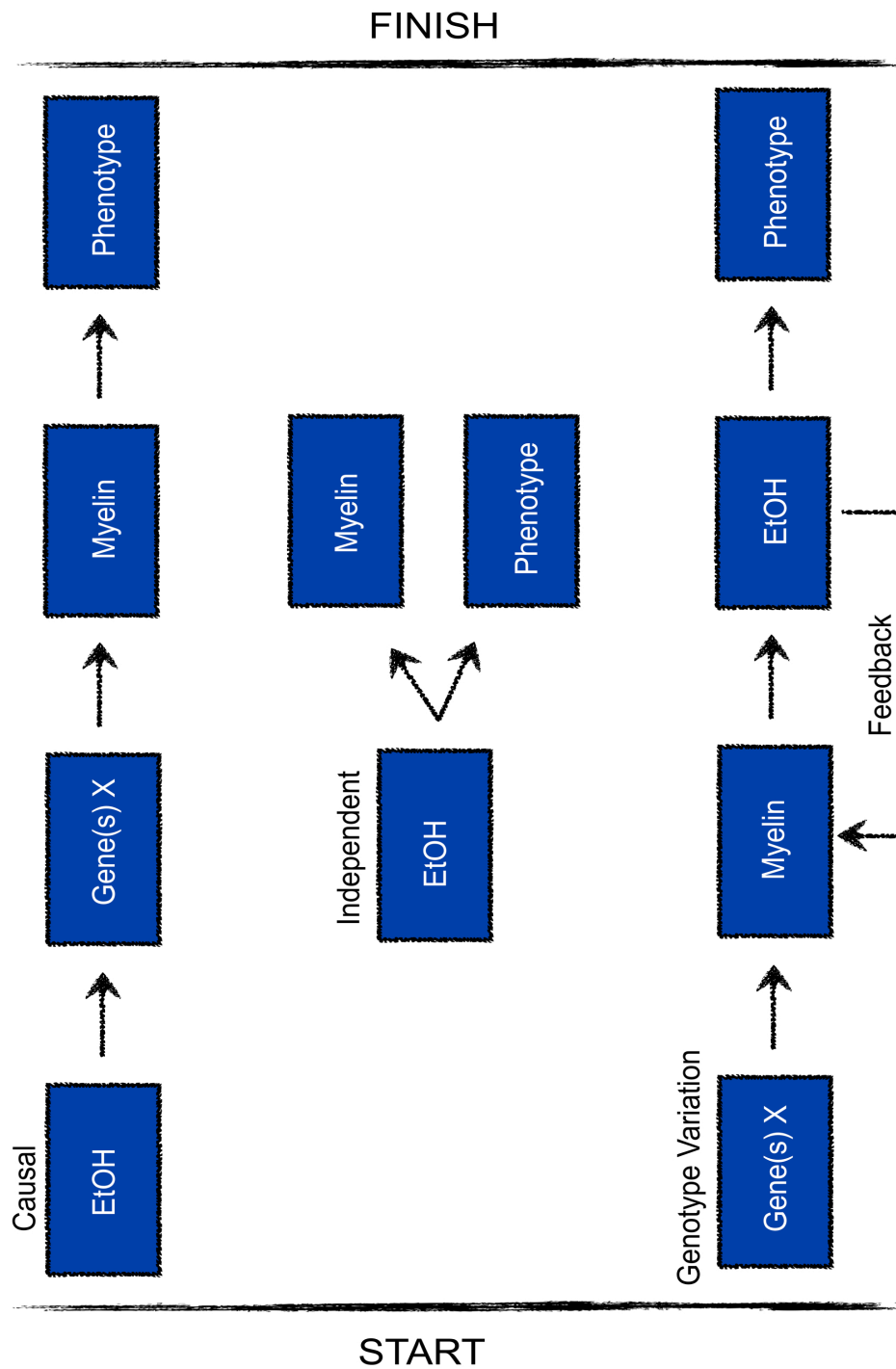


Figure 36. Model for genotype to phenotype relationship. Transitioning from gene expression networks to a phenotypic outcome measure may be explained by three experimental models: (i) causal association, (ii) independent association, (iii) and interaction of basal variation with environmental or drug exposure causing feedback on the gene expression network over time.

expression of myelin-associated genes returns to baseline levels (Figure 37). Transient increases in myelin-related gene expression may reflect that the expression of myelin genes is reactive to alcohol exposure and not an enduring CNS adaptation underlying maladaptive alcohol-drinking behavior.

Basal Variation in Myelin Gene Expression.

C57BL/6J (B6) and DBA/2J (D2) mice are distinct inbred strains of mice that serve as progenitors for the BXD recombinant inbred panel (Figure 2). B6 mice voluntarily consume more alcohol ([F (3, 56) = 3.310, p-value = 0.0265]) and are less sensitive to acute locomotor activating effects of alcohol in comparison to D2 mice ([F (1,20) = 5.86, p-value = 0.0253]) (Figure 38). These results are consistent with several previous alcohol behavioral studies in B6 and D2 mice (Phillips et al., 1994, Metten et al., 1998b); suggesting the phenotypic variance for these two differing inbred strains of mice is reproducible across different environmental settings. The baseline abundance of major myelin-associated genes, including Mbp and Plp, is higher in B6 mice compared to D2 mice within the PFC (Figure 39). Derived from B6 and D2 mice, the BXD recombinant inbred panels represent a mosaic of background genotypes, each strain with a differing pattern of gene expression. The PFC of alcohol naïve BXD recombinant inbred mice also exhibits baseline variation in myelin gene expression, with some strains having higher and other strains having lower myelin expression than their progenitors (Figure 39). We hypothesize those differences in baseline levels of myelin-associated genes within the PFC are a contributing factor to the phenotypic variance inherent in acute alcohol sensitivity and long-term drinking behavior.

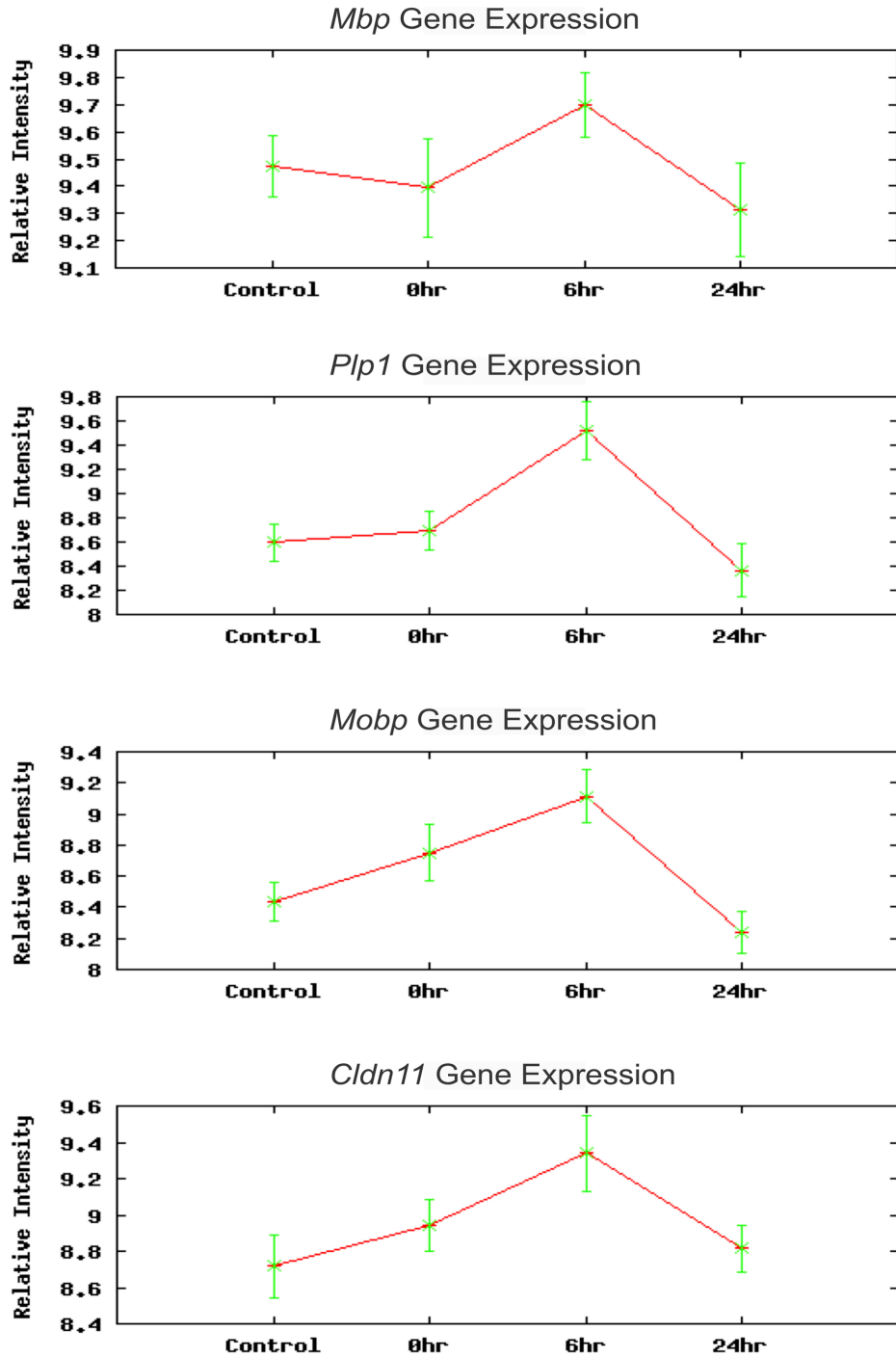


Figure 37. Time-course of myelin-associated gene expression from DBA/2J mice. Relative expression of available myelin gene expression for *Mbp*, *Plp1*, *Mobp*, and *Cldn11*, following removal from alcohol vapor exposure at 0-hr, 6-hr, and 24-hrs compared to air exposed (control) group. Data available courtesy of work performed by Dr. Susan Bergeson through the Alcohol Research Integrator Data Base (<http://aridb.tuhsc.edu/cgi-bin/genedb.pl>).

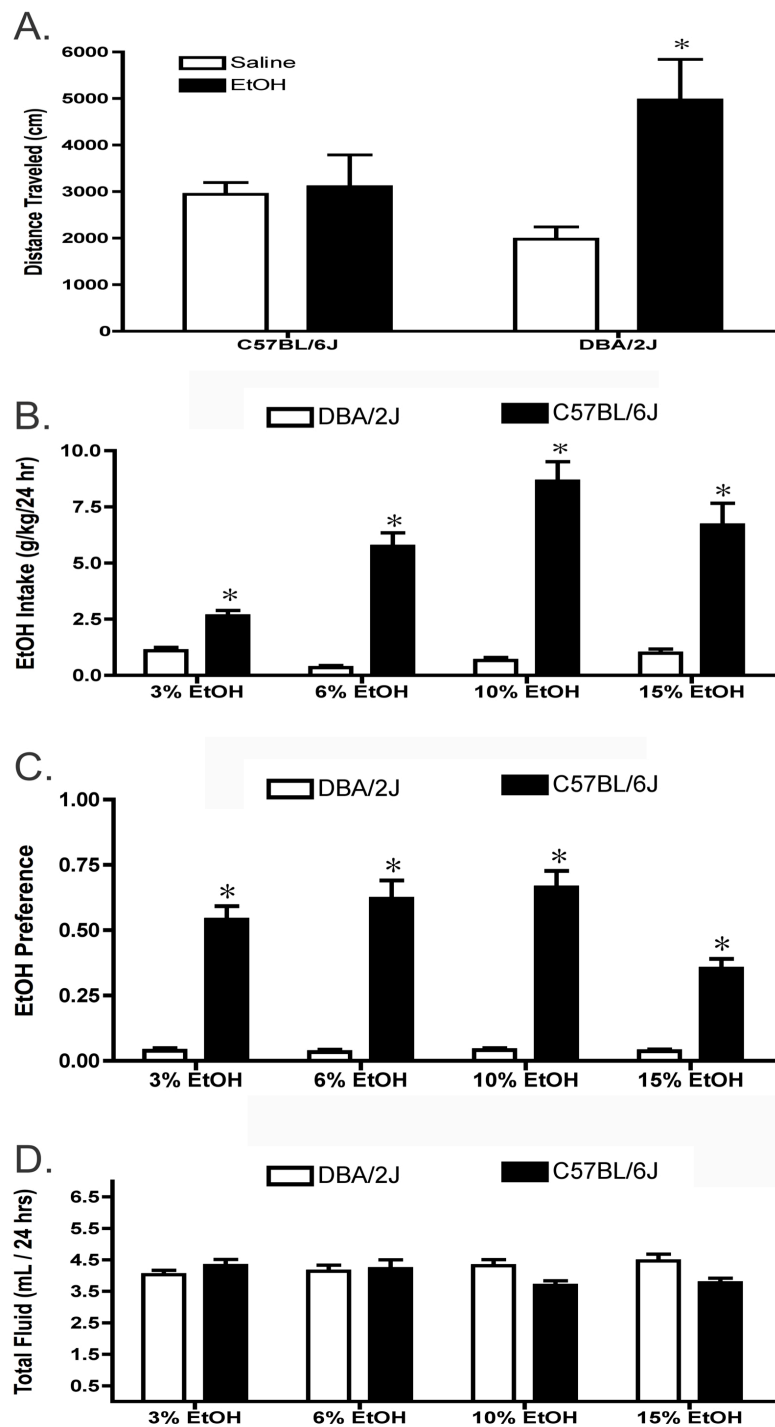


Figure 38. Subset of alcohol behavioral phenotypes for C57BL/6J and DBA/2J mice. (A) Distance traveled in locomotor chamber following 2 g/kg (i.p) alcohol administrations [F (1,20) = 5.86, p-value = 0.0253], (B) Two-bottle choice alcohol intake [F (3, 56) = 3.310, p-value = 0.0265], (C) Two-bottle choice alcohol preference [F (3, 56) = 5.626, p-value = 0.0019], (D) Total fluid intake; * p-value < 0.05.

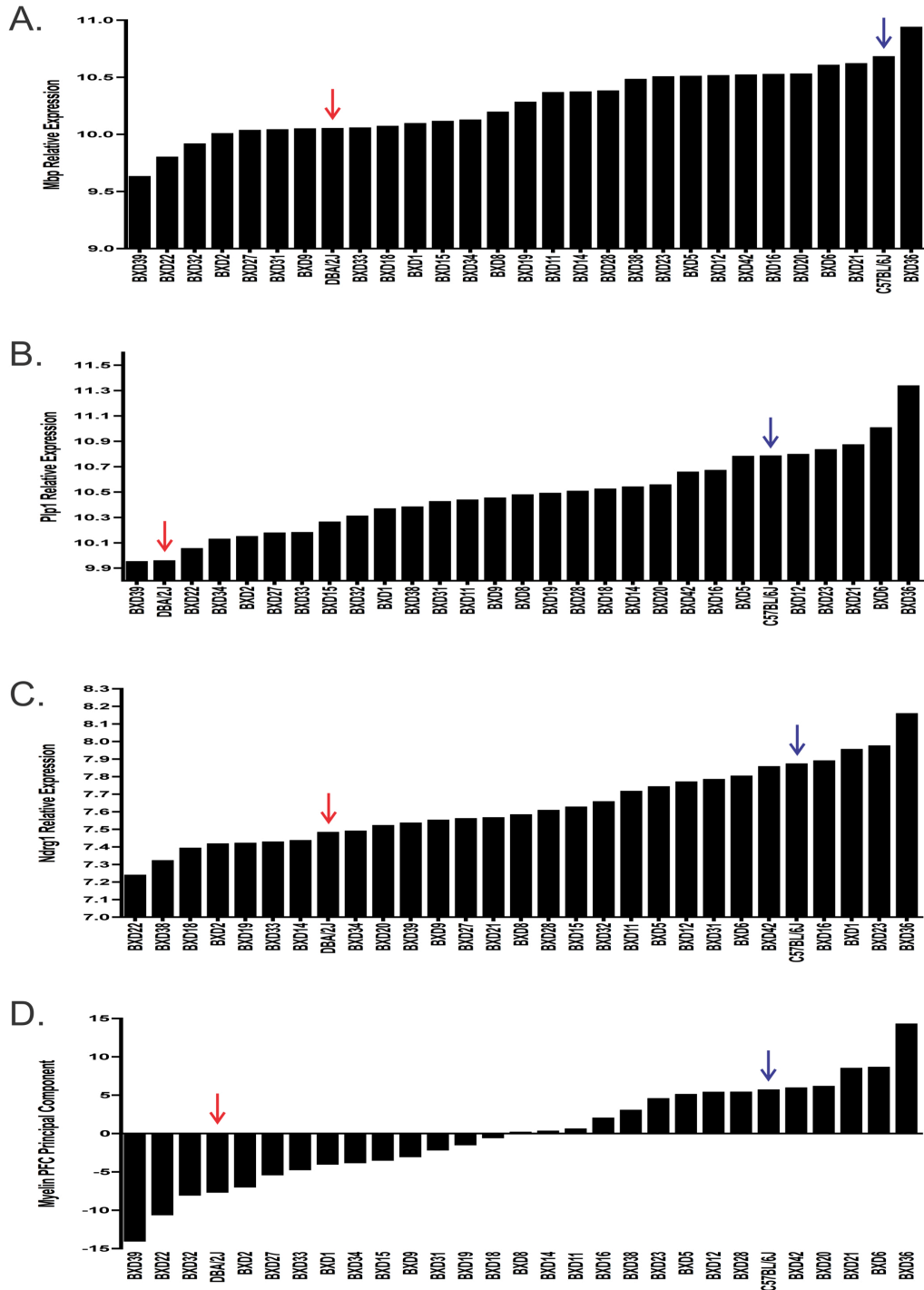


Figure 39. Myelin gene expression variation between C57BL/6J and DBA/2J, and BXD RI mice. Control (saline) relative mRNA expression for the PFC of (A) Myelin basic protein (B) Proteolipid protein (C) N-myc downstream-regulated gene 1, and (D) principal component of myelin-associated network; red arrow denotes location of DBA/2J, blue denotes location of C57BL/6J.

Network Analysis of Basal Variation in the PFC of Mice.

Weighted gene co-expression network analysis (WGCNA) was applied to mouse PFC datasets to identify a basal network of coordinately expressed myelin-associated genes. In addition to the PFC of saline treated BXD mice and progenitors (n = 29), WGCNA was also used on the PFC of alcohol naïve LXS mice (n = 43). The LXS RI panel is a completely separate mouse population from BXD mice, derived from inbred long sleep (ILS) and inbred short sleep (ISS) mice. ILS mice and ISS mice were selectively bred for maximum differences to the sedative-hypnotic effects of high-dose alcohol (McClearn and Kakihana, 1981). LXS RI strains of mice demonstrate marked differences for an assortment of acute alcohol related behaviors influenced by both high and low-doses of alcohol (DeFries et al., 1989, Erwin et al., 1990). The BXD and LXS gene expression measurements were collapsed to include only the probesets common to both datasets (n = 22,626 probesets). WGCNA identified 40 modules for the LXS basal PFC dataset, and 62 modules for the BXD basal PFC dataset (Figure 40). The LXS darkorange module (170 probesets) and BXD orangered4 module (104 probesets) significantly overlapped with one another (Fisher's exact test, p-value = 2.71 e -21). Using the biological network gene ontology tool (BiNGO) (Maere et al., 2005), each of these modules were over-represented for gene ontology categories related to myelin and ensheathment of neurons (Figure 41). Functional enrichment for genes involved in myelination firmly shows coordinate basal expression of myelin-associated genes within the PFC across 72 genetically distinct strains of mice.

Initial expression profiling between B6 and D2 mice indicated differences in basal abundance for myelin-associated genes were uniquely observed in the PFC (Kerns et

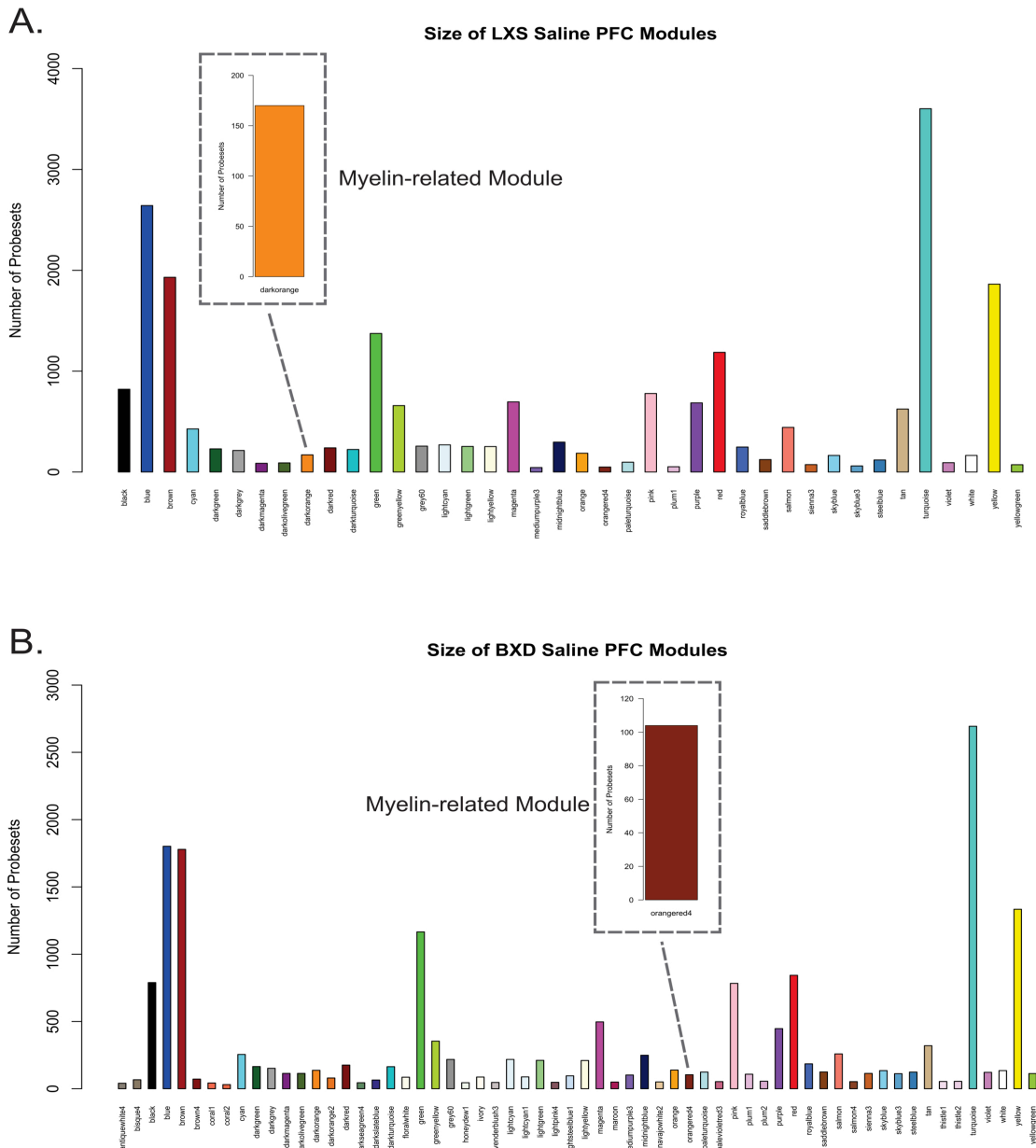


Figure 40. Weighted gene co-expression network analysis of LXS and BXD PFC. Network analysis of common probesets between LXS and BXD datasets, showing distribution in the size of modules for (A) LXS and (B) BXD saline PFC. The darkorange module in LXS and the orangered4 module in BXD is enriched for myelin gene expression and significantly overlap between the two PFC basal datasets.

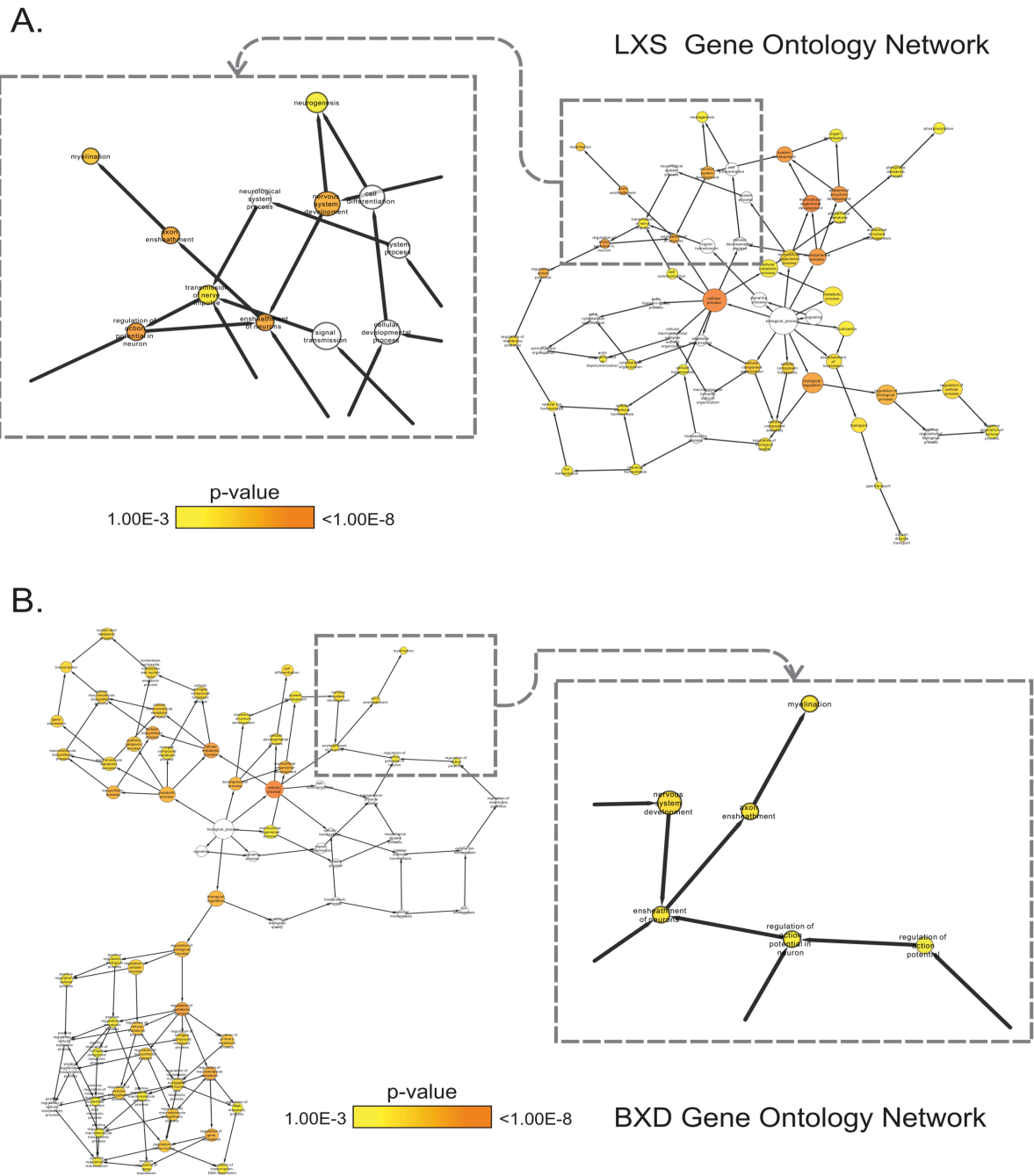


Figure 41. Functional over-representation analysis of LXS and BXD PFC modules. Network visualization of the myelin-associated modules using the biological network gene ontology tool (BiNGO) (Maere et al., 2005), showing enrichment for genes related to multiple myelin-related categories. Categories are colored based on p-values with darker orange indicating 'high' enrichment and white indicating no association.

al., 2005), and not in NAC or VTA. A subsequent investigation using WGCNA extended this myelin observation across the BXD RI panel. Principal component analysis of the myelin module identified by WGCNA in the PFC across B6, D2, and BXD mice showed a wide distribution for myelin-related gene expression (Figure 39D). Replicating our previous results, baseline expression for the PFC myelin gene network had higher relative expression in B6 mice than detected in D2 mice. The 104 probesets from the PFC myelin module were collectively compared to gene expression for NAC and VTA across the BXD RI panel ($n = 37$). At this time gene expression data for the NAC and VTA is not available across the LXS RI panel. Principal component analysis of NAC and VTA for basal expression of the same 104 probesets constituting the myelin gene network in PFC did not identify any significant association between PFC and NAC or VTA (Figure 42A). The PFC demonstrated higher average module membership than the other two brain regions (Figure 42B, $[F(2,100) = 360.5, p\text{-value} < 0.001]$). However, the average basal expression for these 104 probesets was significantly lower in the PFC than compared to either the NAC or VTA (Figure 42C, $[F(2,741) = 95.63, p\text{-value} < 0.001]$). Thus, the basal expression of a closely regulated myelin-associated gene network within PFC is differently expressed and not associated with the same group of genes in the NAC or VTA across 29 strains of mice.

Myelin Gene Expression Network and Behavioral Phenotypes.

An integrated public repository, known as WebQTL or GeneNetwork, maintains a large collection of alcohol-related behavioral traits for the BXD recombinant inbred panel (Chesler et al., 2005) (<http://genenetwork.org/webqtl/main.py>). The open web resource,

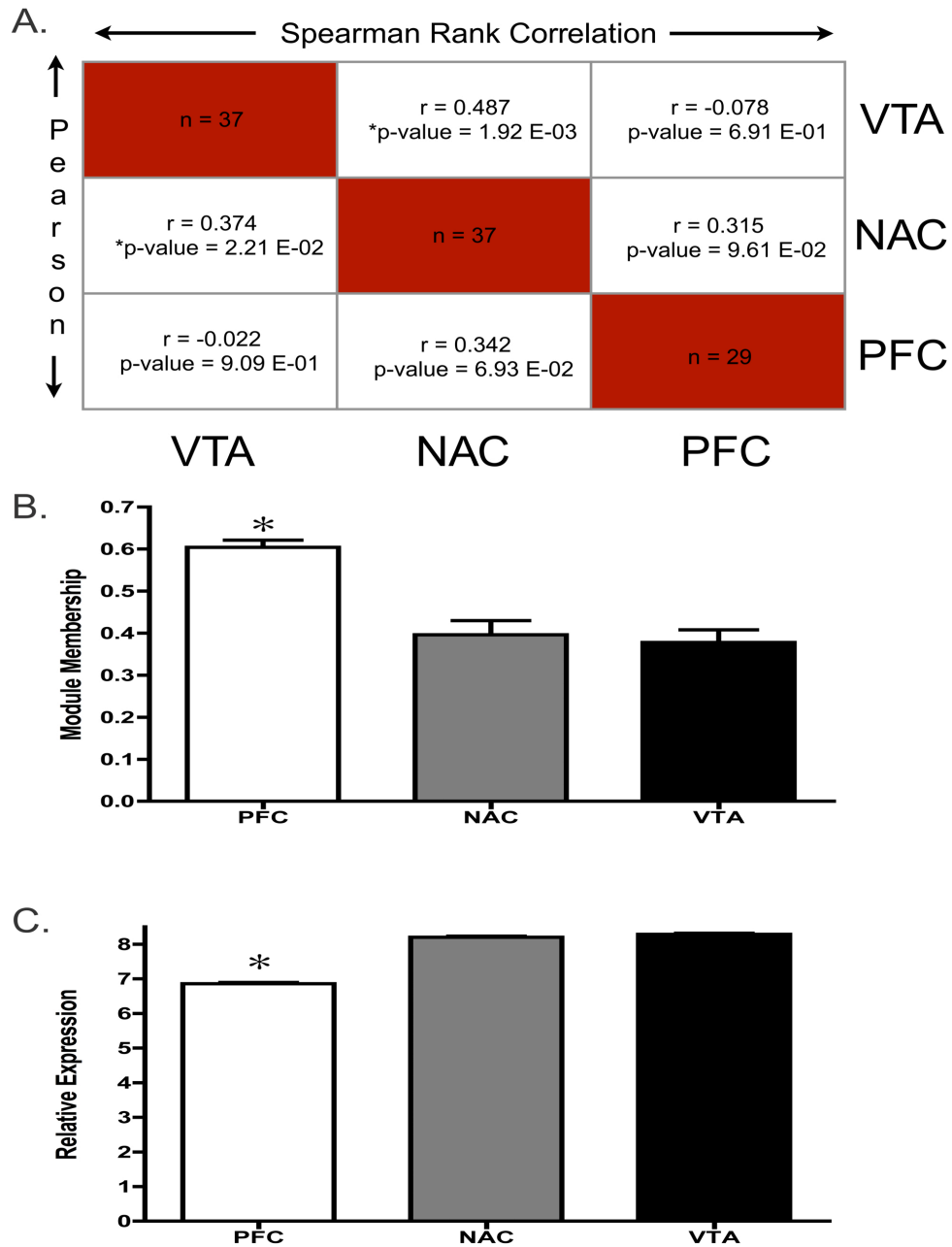


Figure 42. Brain-region specific differences in the myelin-associated gene network. (A) Correlation matrix for the BXD myelin module comparing PFC, NAC, and VTA; Spearman rank correlation are across the top, with Pearson's correlation across the bottom, (B) Mean module membership for genes with the PFC myelin module compared to the NAC and VTA data sets * [F (2, 309) = 45.36, p-value < 0.0001] (C) Mean relative saline expression for the PFC myelin module compared to the NAC and VTA datasets * [F (2, 309) = 544.4, p-value < 0.0001]. PFC = prefrontal cortex, NAC = nucleus accumbens, VTA = ventral tegmental area.

GeneNetwork, also contains all of the raw gene expression data generated by the arrays described herein. Correlating alcohol naïve expression data from the PFC of BXD mice against variance in phenotypic traits will determine if basal variation of a myelin-related gene network is associated with alcohol behavioral phenotypes. The basal PFC myelin expression network was pared down to 13 high-priority genes (MBP, PLP1, CNP, OLIG1, QDPR, MOBP, MAL, NDRG1, UGT8A, RHOG, PDE8A, PPP1R14A, and ANLN) using the network of myelin-associated genes conserved across species chronically consuming alcohol. All 13 of these genes are localized to oligodendrocytes, with several of these genes, such as MBP, PLP1, and NDRG1, coding for myelin structural proteins. The first principal component of these 13 genes significantly correlated to 126 phenotypic records within GeneNetwork (Pearson product-moment correlation, p -value < 0.05). Strikingly, basal variation in gene expression for the myelin network showed a significant positive correlation to several studies on alcohol consumption and a negative correlation to studies related to acute alcohol sensitivity and acute functional tolerance (Table 6). The inverse associations relative to myelin-related gene expression suggests that basal variation of myelin within the PFC is a contributing factor in acute alcohol sensitivity and long-term drinking behavior. Similar results are obtained from basal expression of the 47 myelin-related genes from the cross-species investigation of PFC (Figure 35) and the 104 probesets determined by WGCNA of basal expression data across the PFC of BXD, B6, and D2 mice. A total of 12 strains are in common between the BXD mice chronically exposed to alcohol and acute saline treated animals. Although limited in number, basal variation in

Table 6. Alcohol behavioral phenotypes associated with myelin gene expression in PFC

| ALCOHOL BEHAVIORAL PHENOTYPE | PUBMED ID | SAMPLE | R | P-VALUE |
|---|-----------|--------|----|----------|
| Ethanol response (10% in water, po), preference during free choice period for females using two bottles, choice [mis 10.0% ethanol/mis total fluid] | 8651451 | 0.86 | 9 | 1.35E-03 |
| Ethanol response (10% in water, po), preference during free choice period for females using two bottles, choice [mis 10.0% ethanol/mis total fluid] | 8651451 | 0.86 | 9 | 1.35E-03 |
| Ethanol response (3 day exposure to 8-11 g/kg ethanol in vapor chamber with pyrazole 1 mM/kg/day), handling-induced convulsion (HIC) withdrawal severity score | 6683363 | -0.83 | 9 | 4.03E-03 |
| Ethanol response (10% in water, po), voluntary consumption ratio using a two bottle choice test [gms ethanol/kg body weight] | 8651451 | 0.83 | 9 | 4.05E-03 |
| Ethanol response (10% in water, po), consumption during free choice period for females using two-bottle choice [gms ethanol/kg body weight/day] | 8651451 | 0.81 | 9 | 5.99E-03 |
| Ethanol response (10% in water, po), preference during 3-week free choice period using two bottle choice test for males [mis 10% ethanol/mis total fluid] | 8651451 | 0.77 | 9 | 1.15E-02 |
| Ethanol response (2-bottle choice consumption), 2 hour access after cycle 2 of ethanol exposure in vapor chamber, average of 5 days ethanol intake [g/kg/2h] | 6683363 | 0.64 | 10 | 4.34E-02 |
| Ethanol response (3 day exposure to 8-11 g/kg ethanol in vapor chamber with pyrazole 1 mM/kg/day), handling-induced convulsion (HIC) withdrawal severity score | 8627537 | -0.64 | 22 | 1.19E-02 |
| Ethanol response (dose route), tolerance measured as the fold-increase to ethanol-induced ataxia [ratio of maximal threshold divided by onset threshold] | | -0.62 | 12 | 1.36E-03 |
| Ethanol response (2-bottle choice 2 h access consumption), week 4 [log transformed g/kg/2h] | 8748968 | -0.62 | 21 | 2.39E-02 |
| Ethanol response (2-bottle choice consumption), 2h access after cycle 1 of Air exposure in vapor chambers. Average of 5 days ethanol intake [g/kg/2h]. Air_T1_Etqkqave. | | 0.60 | 11 | 4.81E-02 |
| Ethanol response (dose route), tolerance, difference between ethanol-induced ataxia (maximal threshold) minus onset threshold | 865537 | 0.59 | 16 | 2.87E-03 |
| Ethanol response (2-bottle choice 2 h access consumption), week 1 [log transformed g/kg/2h] | 7095038 | 0.59 | 16 | 4.57E-03 |
| Ethanol response (2-bottle choice 2 h access consumption), week 2 [log transformed g/kg/2h] | | 0.57 | 17 | 4.99E-02 |
| Ethanol response (3% g/kg in 0.2% saccharin and tap water), consumption using a two-bottle choice test vs. tap water, mean of day 2 and day 4 of a 4-day 24- | 7978106 | 0.57 | 17 | 1.65E-02 |
| Ethanol response (2 mg/kg ip), locomotor tolerance or sensitization in males [difference between activity after fourth ethanol trial and first trial] | 7480533 | -0.54 | 18 | 1.90E-02 |
| Ethanol response (10% in water, po), acceptance and consumption of 10% ethanol in 24 hours after voluntary ethanol consumption | 7978106 | 0.54 | 17 | 2.49E-02 |
| Ethanol response (2.25 g/kg ip), motor coordination effects, tolerance assay, difference in time on rotarod between saline and ethanol for males [sec] | 7625571 | 0.53 | 19 | 1.70E-02 |
| Ethanol response (2.25 g/kg ip), time on rotarod after saline minus time on rotarod after ethanol for males and females [sec] | 19958391 | 0.51 | 22 | 1.41E-02 |
| Ethanol response (10%, g/kg in 0.2% saccharin and tap water), consumption using a two-bottle choice test offered vs. tap water, mean of day 2 and day 4 of a 4 | 19958391 | 0.49 | 23 | 1.68E-02 |
| Ethanol response (dose ip), ethanol-induced ataxia following first of five injections (initial sensitivity), onset threshold [mg/kg] | 7978106 | 0.48 | 17 | 4.70E-02 |
| Ethanol response (2 mg/kg ip), locomotor tolerance or sensitization in males, difference between experimental group (two prior exposures to ethanol) and saline | 8627537 | 0.48 | 22 | 2.31E-02 |
| Ethanol response (2 mg/kg ip), locomotor tolerance or sensitization in males, difference between experimental group (two prior exposures to ethanol) and saline | 7480533 | -0.48 | 18 | 4.38E-02 |
| Ethanol response (2 mg/kg ip), locomotor tolerance or sensitization in males, difference between experimental group (two prior exposures to ethanol) and saline | 8627537 | -0.47 | 23 | 2.77E-02 |
| Ethanol response (2.25 g/kg ip), motor coordination effects, difference in time on rotarod between training session and ethanol for males and females [sec] | 19958391 | -0.43 | 22 | 4.28E-02 |

myelin-associated gene expression is significantly correlated to alcohol intake within the mouse cohort chronically exposed to alcohol (Pearson's $r = 0.62$, $p\text{-value} = 2.99 \times 10^{-2}$).

Myelin Single Nucleotide Polymorphisms and Alcoholism.

Approximately 40% to 60% of the risk for developing an alcohol use disorder is explained by genetic factors (Schuckit, 2000). In recognition of a genetic basis for alcoholism the Collaborative Studies on the Genetics of Alcoholism (COGA) was established to identify specific genes conferring the risk of developing alcohol dependence (Almasy and Borecki, 1999). In collaboration with Dr. Danielle Dick of Virginia Commonwealth University, a member of COGA, the basal myelin-related gene network was assessed for polymorphisms associated with a diagnosis for alcohol dependence and a subset of alcoholism-affiliated endophenotypes. Similar to previous genome wide association studies (GWAS) no polymorphisms in myelin genes reached genome-wide significance (Figure 43). However, using the nominal p -values for the differing myelin genes as descriptive measure may suggest that genotypic variation in myelin-associated genes underlie more immediate endophenotypes of alcoholism. Clustering the descriptive p -values inside a heatmap shows single nucleotide polymorphisms (SNPs) for a set of myelin genes are grouped according to self-reports for the maximum number of drinks consumed within a 24-hour period, alcohol craving, and subjective measures of alcohol withdrawal (Figure 43). Combined with the WGCNA of basal and chronic alcohol exposure of myelin-associated gene expression may imply that myelin variation within the PFC is an underlying risk factor for alcohol related behavioral traits.

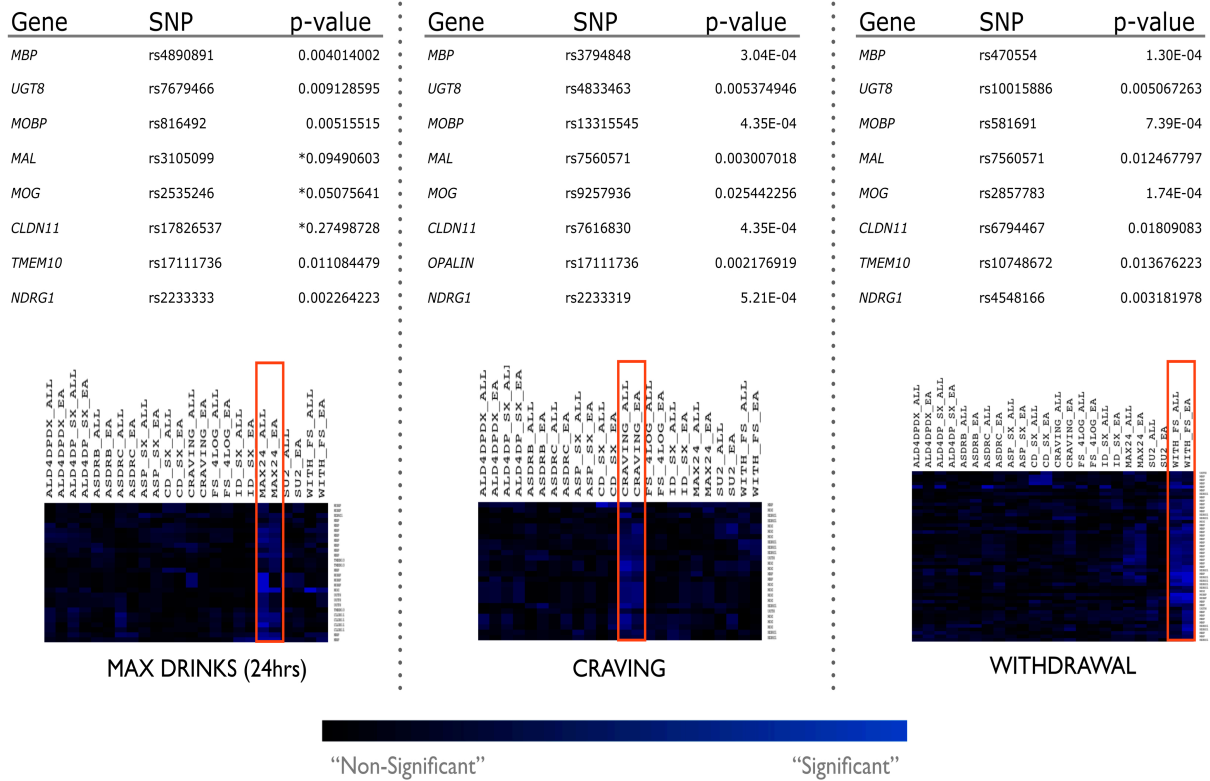


Figure 43. Pilot study on genome wide-association of myelin genes for endophenotypes of alcohol dependence. K-means clustergrams for nominal p-values of individual single nucleotide polymorphisms (SNPs) associated with ‘Max Drinks in 24 hours,’ ‘Craving,’ and ‘Withdrawal.’ Listed left to right (ALD4DPDX = DSM-IV Alcohol Dependence, ALD4DP_SX = DSM-IV Alcohol symptom counts, ASDRB = Conduct disorder, ASDRC = Anti-Social Personality, ASP_SX = Anti-Social Personality symptom counts, CD_SX = Conduct disorder symptom counts, CRAVING = Craving, FS_4LOG = -log factor score of alcohol related variables, ID_SX = Illicit drug symptom counts, MAX24 = Maximum number of drinks in 24 hours, SU2 = Suicide, WITH_FS = withdrawal items factor score).

Discussion

A large degree of biological and environmental heterogeneity exists among individuals afflicted with a substance abuse disorder (Sturgess et al., 2011). Structural variation within fronto-striatal brain systems are involved in neurocognitive impairments associated with addiction to psychoactive stimulants (Ersche et al., 2012). The PFC in particular, is a major brain area underlying vulnerability for the development of addiction (Perry et al., 2011). Degradation of white matter in frontal and limbic fibers is detected consistently within the brains of alcohol dependent subjects (Buhler and Mann, 2011). A mouse model of chronic alcohol exposures causes transient changes in myelin gene expression; however, our analysis suggests basal variation of a myelin-associated gene network is associated with acute behavioral sensitivity and the long-term risk of drinking behavior in mice.

Sequence variation of myelin genes may be pertinent risk factors entailing endophenotypes associated with alcohol dependence. Withdrawal and craving are two endophenotypes that contribute to compulsive drinking behavior and alcoholic recidivism (Koob, 2003). The quantity of myelin changes during chronic alcohol abuse, withdrawal, and abstinence (Agartz et al., 2003). In contrast to decreased myelin gene expression in frontal and motor cortices of alcoholics (Liu et al., 2004b), adult DBA/2J mice showed an increased relative abundance for myelin gene expression following a continuous 72-hr exposure to alcohol. Repeated episodes of withdrawal from chronic alcohol exposure lead to reduced brain volume and neurotoxicity, which may be related the expression and sensitivity of NDMA receptors (Self et al., 2005, Raeder et al., 2008). NMDA receptors are expressed in the myelinating processes of oligodendrocytes

(Karadottir et al., 2005), and may cause damage and eventual loss of the myelin sheath during disease states (Micu et al., 2006, Burzomato et al., 2010). Pharmacological antagonists for NMDA receptors facilitate neurocognitive improvements in alcohol-dependent patients (Cheon et al., 2008). Symptomatic improvements are also seen with NMDA receptor antagonists in Wernicke-Korsakoff syndrome (Rustembegovic et al., 2003), a myelin-related disorder often associated with alcoholism (Harper, 2009). The lack of reduced myelin gene expression in mice continuously exposed to alcohol may therefore be related to the absence of intermittent withdrawal and accompanying neuroadaptations. Increases in myelin gene expression do support our previous findings on acute ethanol regulation of myelin genes in DBA/2J mouse PFC (Kerns et al., 2005). The regulation of myelin-associated gene expression may also depend upon genetic differences in initial sensitivity to alcohol or the complexities of alcohol drinking behavior in preclinical models (Crabbe et al., 2010b).

Susceptibility for psychiatric disorders is conferred by the complex interaction of environment influences and multiple genetic variants (Collins et al., 2003). Single nucleotide polymorphisms in myelin genes did not achieve statistical significance for a genome-wide association approach. Identifying the genetic variants responsible for complex traits has shown limited success with few genes achieving genome-wide significance due to a lack of adequate phenotypes and methodological issues (i.e. poor sample size) (Bondy, 2011). Complex traits manifest through the additive actions from multiple genes of small effect size (Plomin et al., 2009). Studying function and structure distinct brain regions is beginning to elucidate the role of individual genes and groups of genes in complex traits (Thompson et al., 2010). Dysfunction in PFC dopaminergic

transmission occurs in drug addiction and schizophrenia (Meyer-Lindenberg et al., 2002, Luscher and Malenka, 2011). Specific genetic variants for the dopamine metabolizing enzyme catechol-O-methyltransferase (COMT) are associated abnormal neuronal transmission in PFC (Meyer-Lindenberg et al., 2006). The volume of gray matter within discrete brain regions is altered due to the presence of a SNP in the promoter region of COMT (Honea et al., 2009). COMT is also in epistasis with sequence variants for other candidate genes implicated in disease processes and regulate prefrontal cortical GABA function (Nicodemus et al., 2007, Marengo et al., 2010). Genetic variation of myelin-related genes can lead to CNS demyelination. Polymorphisms within specific myelin genes are associated with vulnerability to schizophrenia (Wan et al., 2005, Georgieva et al., 2006, Peirce et al., 2006). The presence of polymorphisms in myelin genes predicts decreased expression within the PFC of schizophrenics (Mitkus et al., 2008). Little attention has been directed at SNPs for myelin-associated genes in the risk of developing an alcohol use disorder. Our analysis shows variation in expression and sequence of myelin genes may be important in endophenotypes relevant to alcohol abuse and alcoholism.

Previous examination of gene expression for mice, monkeys, and humans chronically consuming alcohol revealed a conserved network of myelin-associated genes across species. Expression profiling across the BXD RI panel of mice chronically exposed to alcohol vapor with periodic two-bottle choice drinking determined a correlation between the expression for a network of myelin-associated genes and alcohol intake. Analyzing a separate cohort of alcohol naïve BXD RI mice indicated the association between alcohol consumption and myelin is due to genetic variation in the

basal abundance of myelin gene expression. Genetic differences between mice contribute to acute alcohol sensitivity (Chesler et al., 2012) and control over alcohol drinking behavior (Ozburn et al., 2010). Acting as a molecular intermediate, variation within the expression for a particular gene network may underlie the susceptibility for disease (Schadt et al., 2005, Schadt, 2009). Genetic factors can influence the overall abundance and integrity of myelin in different brain regions, which impacts the neurodevelopment and cognitive function of healthy individuals (Jahanshad et al., 2012). The etiology of alcoholism and other complex diseases is attributed to the combination of genetic variants and biological networks (Li et al., 2011). Co-variation in a conserved myelin gene network alongside alcohol-related behavior implies myelin gene expression may be a risk factor in the development of alcohol dependence.

Further investigation is needed to ascertain the extent of basal differences in the myelin gene expression in alcohol behavioral phenotypes, as well as the molecular components that regulate myelin gene expression. Myelin is tightly regulated by a number of differing signaling mechanisms and demonstrates substantial plasticity throughout the lifetime of a vertebrate nervous system (Emery, 2010). Reciprocal communication between axonal impulse activity and myelin form an intricate CNS framework controlling cognition and behavior (Lee and Fields, 2009). In spite of mounting evidence for the role of myelin in psychiatric illness, little information exists regarding which intermediate phenotypes are directly affected by variation in myelin gene expression and myelin dysfunction (Takahashi et al., 2011). Probing the molecular mechanisms governing myelination and the myelin-associated gene network itself will

further our understanding for the neurobiology of acute and lasting behavioral phenotypes underlying alcohol abuse and dependence.

CHAPTER 5:

FYN-DEPENDENT GENE NETWORKS IN ACUTE ALCOHOL SENSITIVITY

Introduction

Acute sensitivity to alcohol is a predictive indicator of the long-term risk of abusive drinking behavior in humans and animal models (Schuckit, 1994, Metten et al., 1998b). Fyn kinase, a non-receptor protein tyrosine kinase widely expressed in the central nervous system, modulates the acute sedative-hypnotic properties of alcohol (Miyakawa et al., 1997, Yaka et al., 2003b) and in some studies has been shown to reduce two-bottle choice consumption in rodents (Boehm et al., 2003, Cowen et al., 2003). A large number of studies suggest that Fyn modulation of NMDA and GABA receptor function (Kitazawa et al., 1998), particularly in regard to the NR2B subunit of NMDA receptors, at least partially underlies Fyn modulation of alcohol behavioral traits. Genetic variation in Fyn kinase is associated with alcohol dependence and alcohol related phenotypes such as withdrawal and craving behavior in humans (Schumann et al., 2003), supporting the premise that Fyn modulation of acute alcohol behaviors contributes to the risk for the development of alcohol dependence.

In addition to acute sensitivity to alcohol, Fyn kinase has also been shown to be important in complex aspects of neurodevelopment (Liu et al., 2004a, Chen and Charness, 2008), myelination (Sperber et al., 2001), and learning and memory (Kojima et al., 1997). Although gene-targeting strategies, such as those mentioned above for *Fyn* have been widely used to study the neurobiology of alcohol and drug abuse (Crabbe et al., 1994a, Crabbe et al., 2006b), the interpretation of such results is difficult

given the multiple system-wide effects of genes such as Fyn and the multivariate nature of complex diseases such as alcohol use disorders (AUDs). Even ignoring complications such as developmental compensation in gene-targeted animals, the deletion of a single gene such as a kinase of widespread action like Fyn, could augment alcohol behavior by triggering network-wide alterations in the function or expression of genes downstream of Fyn, in addition to mechanisms related to direct targets of Fyn phosphorylation.

The mesolimbic dopaminergic reward pathway, encompassing the prefrontal cortex (PFC), nucleus accumbens (NAC), and ventral tegmental area (VTA), is activated by acute alcohol and other drugs of abuse (Koob, 1992). Baseline differences or drug-induced alterations in gene expression within the mesolimbocortical dopamine pathway may play an important role in the transition from initial drug exposure to the development of dependence (Nestler and Aghajanian, 1997, Farris et al., 2010). Previous research from our laboratory has shown divergent basal and acute alcohol-evoked patterns of gene expression within the dopamine reward pathway may contribute to acute alcohol behavioral sensitivity (Kerns et al., 2005). It is our hypothesis that altered expression or function of Fyn kinase may produce network level changes in gene expression within the mesolimbic dopamine pathway. Thus, providing a mechanism modifying subsequent behavioral responses to alcohol.

Using expression profiling we sought to define Fyn-dependent gene networks underlying alcohol behavioral traits; with emphasis on alcohol-induced loss of righting reflex (LORR) due to the reproducible association of Fyn kinase genotype with this particular behavioral phenotype (Miyakawa et al., 1997, Boehm et al., 2003, Yaka et al.,

2003b, Boehm et al., 2004b). Expression profiling has been previously used to determine downstream signaling mechanisms altered by single gene knockout animals exposed to acute (Repunte-Canonigo et al., 2010) and chronic alcohol exposure (Bowers et al., 2006). However, a detailed characterization of alcohol-responsive gene expression patterns in mice carrying a null mutation for *Fyn* has yet to be reported. Given the fundamental role of Fyn kinase in neurodevelopment, receptor function, behaviors, and mediating numerous signaling cascades, identifying gene expression patterns in Fyn kinase knockout animals is important for understanding the neurobiology of Fyn kinase and regulation of acute alcohol sensitivity.

Our expression profiling and bioinformatics results suggest multiple Fyn-related mechanisms, especially those affecting the expression for a myelin-associated gene network within the medial PFC, as contributing factors to the sedative-hypnotic properties of alcohol. Variation in the expression of these Fyn-dependent gene networks may be critical molecular endophenotypes affecting the initial level of response to acute alcohol and lasting phenotypes associated with the development of an alcohol use disorder.

Materials and Methods

Animal micro-dissection and acute alcohol administration. Animals were treated according to protocols for animal care established by Virginia Commonwealth University and the National Institute for Health. Adult (requested 8-9 weeks of age at time of their shipment) male B6129SF2/J and B6;129S7-Fyn^{tm1Sor}/J mice from the Jackson Laboratory were housed 4-5 animals per cage with *ad libitum* access to water and

standard rodent chow (Harlan Teklad #7912, Madison, WI, USA). Animals were observed daily for general health and wellness with bedding (Harlan Sani-chips, #7090A, Harlan Teklad) changed weekly. All animals were allowed to habituate to the animal vivarium on a 12-hour light/dark cycle for at least two weeks prior to start of the experiment.

Mice were administered intraperitoneal (i.p.) injections of saline for 3 days to habituate animals to the injection process; on day 4 mice received either an injection of saline or 3 g/kg (20% v/v) of alcohol, a sedative-hypnotic dose (Ponomarev and Crabbe, 2004). Animals were sacrificed by cervical dislocation at a 4-hour time-point. Previous work in our laboratory has shown the 4-hour time-point captures a spectrum of early, intermediate and late gene expression responses to alcohol. Mouse brains were then extracted and chilled for one minute in phosphate buffer saline solution on ice before micro-dissection. Micro-dissection of individual brain regions was conducted as previously described (Kerns et al., 2005). Individual brain sections were immediately frozen using liquid nitrogen, and subsequently stored at -80°C until isolation of total RNA.

Brain tissue pooled from three mice was homogenized in PureZol Reagent (Bio-Rad Laboratories, Hercules, CA) using a Tekmar homogenizer. Total RNA was isolated according to standard protocols following the Aurum Total RNA Fatty and Fibrous Tissue Kit. RNA concentrations were determined by absorbance at 260 nm and RNA quality was assessed by 260:280 nm absorbance ratios and electrophoretic analysis (Experion; Bio-Rad Laboratories, Hercules, CA) with RQI values ≥ 8.0 . Double-stranded cDNA and

biotin-labeled cRNA was synthesized according to instructions provided by Affymetrix protocols.

Microarray hybridization and scanning. Biological replicates (n = 3) from pooled samples within each treatment group and genotype were hybridized to an individual microarray for prefrontal cortex (PFC), nucleus accumbens (NAC), and ventral midbrain areas (VMB) (n = 36 total microarrays). Arrays for a single brain region were processed together in a single day, using a supervised randomization method to order samples to minimize potential batch effects. Labeled cRNA samples were analyzed on oligonucleotide arrays (Affymetrix Mouse Genome 430 2.0 arrays; Affymetrix, Santa Clara, CA) that contain ~36,000 genes and expressed sequence tags. Hybridization, washing, staining, and scanning were performed according to the manufacturer's protocols (<http://www.affymetrix.com/estore/index.jsp>).

Microarray data analysis. Microarray data were initially processed using the Gene Chip Operating Software (GCOS), formerly referred to as Microarray Suite version 5.0. Arrays were normalized to common median total hybridization intensity values (target average intensity = 190). Arrays passing quality control were computationally processed for any potential batch effects (Johnson et al., 2007). Processed microarrays were first filtered for MAS 5.0 absent and present calls (McClintick and Edenberg, 2006) to eliminate probesets consistently called "absent" across all samples, and then subjected to Significance score (S-score) analysis (Zhang et al., 2002). The S-score algorithm, developed in Dr. Miles' laboratory for analyzing Affymetrix arrays was applied to compare hybridization signals between two arrays from differing treatment samples.

Resulting S-scores are independent of initial analysis from the MAS 5.0 algorithm, and has a normal distribution with a mean of 0 and standard deviation of 1, which are correlated to the fold-change in gene expression. An S-score equaling 2 corresponds to a p-value = 0.0455, uncorrected for biological variability or multiple comparisons. S-scores were generated using all of the pairwise comparisons between alcohol treated controls and Fyn kinase knockout mice, as well as basal differences between genotypes. The average S-score from all individual pairwise comparisons between subjects was used to represent a single biological replicate and downstream bioinformatic analyses. Within each brain structure and treatment, S-scores were divided by the greater of 1 or the group standard deviations from same/same comparisons to reduce the contribution of biological or technical noise. Our laboratory, as well as others, has found this method to reduce the contribution of technical variance across experimental replicates, although at the cost of generating a more conservative estimate of gene expression (Hughes et al., 2000).

A one-class statistical analysis of microarrays (SAM) was used within each treatment group and brain region to determine those genes with S-scores significantly different from zero (Tusher et al., 2001). Differences in alcohol-induced regulation of gene expression were determined using a two-class SAM within each brain region (i.e. Fyn KO versus Control). Within each independent SAM analysis S-scores were filtered for an average ≥ 1.5 or ≤ -1.5 (composite significance, p-value < 0.01) to focus on more biologically relevant measurements. All SAM analyses were corrected for a median false-discovery rate (FDR) $\leq 5\%$. An FDR of 5% was chosen to avoid eliminating genes that may be biologically important and could assist in the interpretation of functional

expression profiles for multivariate studies. All significant genes/probesets were subsequently combined and subjected to k-means clustering procedures (Eisen et al., 1998) to determine coordinately regulated gene expression patterns, and demonstrate brain-region, treatment, and genotype specific differences in gene expression. The number of groups for k-means clustering was estimated using principal component analysis that accounted for $\geq 90\%$ of the total experimental variance.

The inclusion of only three biological replicates likely limited detection of more subtle expression changes, particularly of low-abundance genes that may be important for complex psychiatric conditions. The S-score methodology, however, is particularly useful for studies having limited numbers of Affymetrix microarrays because the method uses the statistical power of all of the oligonucleotide pairs for a given gene (Zhang et al., 2002). Additionally, incorporating a method for decreasing the contribution of technical or biological noise increases the yield of statistical filtering measures such as SAM, by decreasing the overall variance within the data set. Such measures are unlikely to contribute misleading results because the replicated bioinformatic studies should only detect genes with coherence in their biological function.

Bioinformatics analysis of microarray data. Toppgene Suite (Chen et al., 2009c) and ErmineJ (Lee et al., 2005a) were used for data exploration of functional classification among gene expression profiles operating within the limits of gene ontology categories, mouse phenotype data, signaling pathways, and public empirical databases. Function enrichment analysis within the Toppgene Suite, referred to as ToppFun, was performed using a 5% FDR, with gene limits set between 3 and 300 to identify representative a

priori ontological groups. ErmineJ gene set analysis was implemented for over-representation analysis (ORA) using the best scoring gene replicate and a gene score threshold of 1.5; ErmineJ reported p-values are corrected for the ErmineJ default of a 10% FDR. Literature association analysis of statistically significant genes were also investigated using Ingenuity Pathway Analysis (<http://www.ingenuity.com/>), a curated bioinformatics resource for the discovery of biological interaction among differing genes based on literature association, biological function, and cell-signaling mechanisms.

Fyn-centric correlation networks were constructed using the intersection of Fyn kinase knockout data and gene expression correlates for Fyn kinase mRNA abundance (Pearson's product moment correlation, $p\text{-value} \leq 0.01$) across the BXD and LXS recombinant inbred mouse lines (available at <http://genenetwork.org/webqtl/main.py>). Due to the redundant design of Affymetrix microarrays with multiple probesets representing a single gene, all probesets representing a single gene were considered for analysis with multiple probesets represented as a single node, but with the removal of duplicated edges between two genes and self-loops among the same gene. Visualization of gene correlation networks was rendered in the platform for complex network analysis and visualization: Cytoscape (<http://genenetwork.org/webqtl/main.py>) (Shannon et al., 2003). Resulting Fyn-centric correlation networks were submitted to GeneMANIA (Mostafavi et al., 2008) to separately assess potential protein and genetic interactions, cell-signaling pathways, co-expression, co-localization, and protein domain similarity as well as identify candidate genes not directly identified through our microarray analysis.

Results

Literature association, using Ingenuity Pathway Analysis of our previous microarray data contrasting gene expression profiles for C57BL6/J and DBA/2J mice pointed to a few selected genes, which may confer basal and alcohol-evoked differences in the molecular and behavioral responses between strains (Figure 44A). A subsequent investigation using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (Szklarczyk et al., 2011) largely corroborated the Ingenuity findings (Figure 44B). Fyn kinase directly targets the NMDA receptor subunit NR2B (*Grin2b*) through NMDA receptor scaffolding proteins (Yaka et al., 2003a). Fyn kinase is suggested to mediate acute functional tolerance through phosphorylation of the NR2B subunit (Miyakawa et al., 1997). NMDA receptor signaling events also activate the transcription factor cAMP response element-element binding protein (*Creb1*) (Almeida et al., 2009), regulating downstream effects on gene expression. The transcription factor CREB1 promotes expression of the major myelin-associated gene myelin basic protein (*Mbp*) (Afshari et al., 2001) and other myelin genes such as claudin-11 (*Cldn11*) (Lui et al., 2007). Participating within the confines of a molecular network, Fyn kinase may thus be a key factor controlling myelin-associated gene expression and the short- and long-term consequences of alcohol exposure.

Gene Expression Pattern Changes in Fyn knockout mice

Genome-wide expression profiling across the mesolimbocortical dopamine pathway of saline or ethanol-treated Fyn null mice was conducted in order to characterize the molecular mechanisms underlying altered behavioral responses to

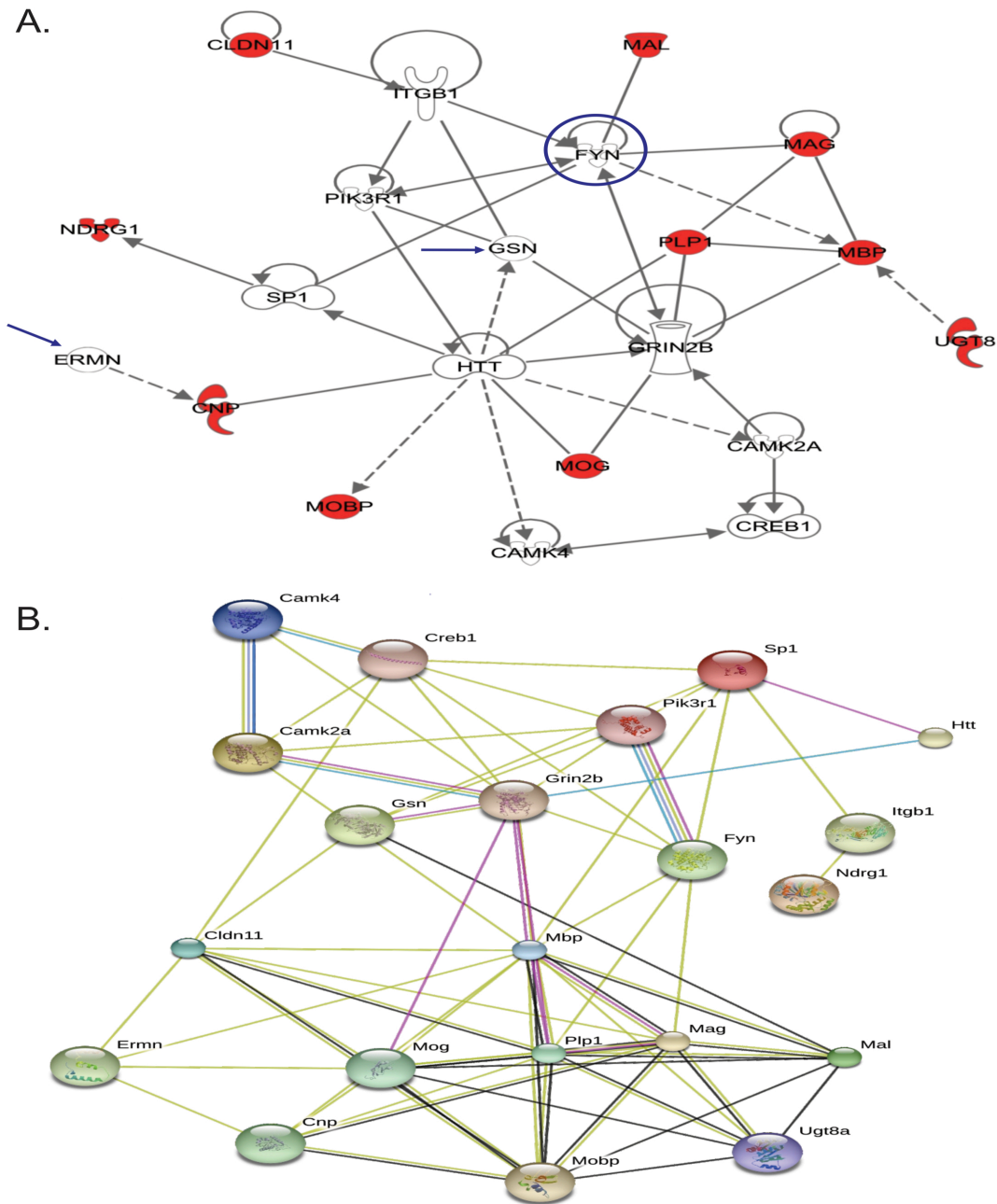


Figure 44. Literature association analysis of myelin gene expression. (A) Ingenuity Pathway analysis of myelin-associated genes from Kerns et al., 2005 (Cluster 2) identifying Fyn kinase; blue circle denotes the location of Fyn kinase, blue arrows denote location of other myelin-related genes not identified by Kerns et al., 2005 (B) Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) network of myelin-associated genes and Fyn kinase.

ethanol in this mutant mouse line. Direct or indirect changes in gene expression resulting from elimination of *Fyn* were determined through one-class and two-class SAM analysis of S-scores for either basal or ethanol-responsive gene expression. SAM results were additionally filtered for absolute expression values greater than or equal to 1.5 to further reduce the potential contribution of technical or biological noise. Multivariate analysis using k-means clustering identified region specific patterns of gene expression (Figure 45A); basal and ethanol-treated gene expression across each of the three brain regions was combined to increase the statistical power of k-means clustering. As expected from the overall expression distributions (Figure 45B & C), most clusters showed basal (KO/CTL) differences that were unique to a single or two brain regions. Several clusters showed possible trends toward differences in ethanol responses, as well as basal expression changes with the *Fyn* KO, particularly in the VMB (see clusters 2, 3, 4, 6, 9, 11 and 12 for VMB). By and large, differences in basal and ethanol-responsive gene expression were distributed unevenly among the three brain regions with basal alterations ranging NAC > VMB > PFC (Figure 45B) and ethanol- response varying in the order of VMB > NAC > PFC (Figure 45C).

Bioinformatics analysis of basal expression changes in Fyn Knockout Mice

In order to assess the potential biological significance of basal expression differences due to a null mutation for *Fyn*, we performed a functional over-representation analysis for each individual brain-region using the ToppGene Suite web-portal (FDR \leq 0.05, Table 7 Basal Gene Ontology Categories;). Evaluation of functional changes may be important for determining the role of *Fyn* within different brain-regions

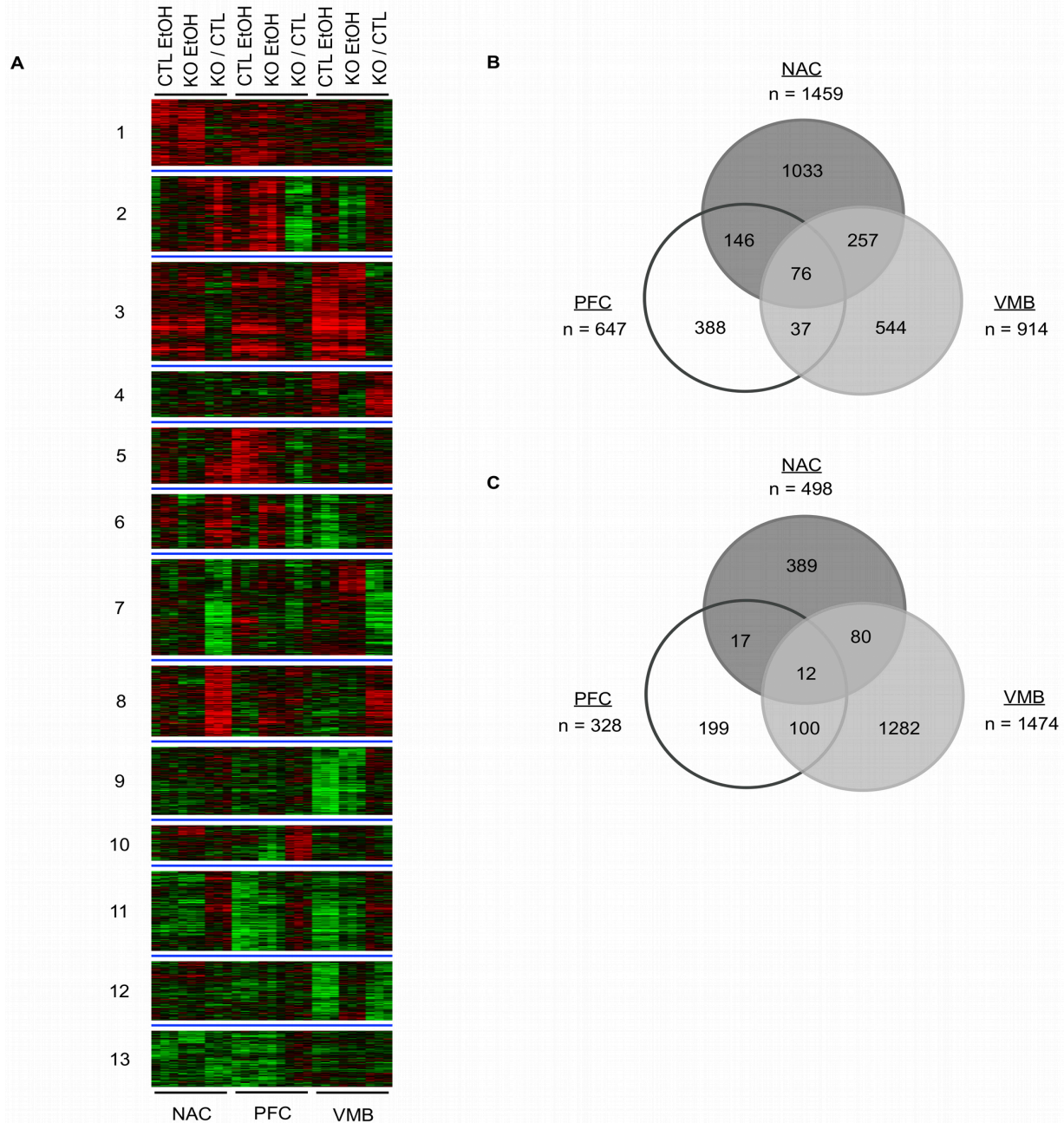


Figure 45. Basal and alcohol-responsive gene expression differences between *Fyn* knockout mice and controls. (A) K-means clustering analysis of differential gene expression for S-scores; green = decreased relative expression, red = increased relative expression, black = no change in expression (Control EtOH = CTL EtOH, KO EtOH = *Fyn* Knockout EtOH, KO / CTL = *Fyn* Knockout Saline / Control Saline). (B) Venn diagram of overlapping and non-overlapping genes differentially expressed between saline treated *Fyn* knockout mice and controls. (C) Venn diagram of overlapping and non-overlapping genes differentially expressed between acute ethanol (3 g/kg) treated *Fyn* knockout mice and controls. (NAC = nucleus accumbens, PFC = prefrontal cortex, and VMB = ventral midbrain).

Table 7. Functional over-representation for Fyn knockout mice basal gene expression

| Category | ID | Name | P-value | % Targeted in Category | | |
|------------------------|------------------------|---|---|---|-------------|-------------|
| MAC | GO: Molecular Function | GO:0008022 | protein C-terminus binding | 1.90E-05 | 18.27956989 | |
| | GO: Molecular Function | GO:0019911 | structural constituent of myelin sheath | 1.90E-05 | 100 | |
| | GO: Molecular Function | GO:0005158 | insulin receptor binding | 1.90E-05 | 33.33333333 | |
| | GO: Molecular Function | GO:0022843 | voltage-gated cation channel activity | 4.60E-02 | 18 | |
| | GO: Biological Process | GO:0007612 | learning | 7.79E-03 | 26.92307692 | |
| | GO: Biological Process | GO:0050890 | cognition | 2.57E-02 | 19.35483871 | |
| | GO: Biological Process | GO:001508 | regulation of action potential | 2.57E-02 | 22.33009709 | |
| | GO: Biological Process | GO:0007611 | learning or memory | 2.63E-02 | 19.44444444 | |
| | GO: Biological Process | GO:0019228 | regulation of action potential in neuron | 2.63E-02 | 23.52941176 | |
| | GO: Biological Process | GO:0001505 | regulation of neurotransmitter levels | 2.63E-02 | 19.56521739 | |
| | GO: Biological Process | GO:0071375 | cellular response to peptide hormone stimulus | 4.65E-02 | 15.85366584 | |
| | PFC | GO: Molecular Function | GO:0035254 | glutamate receptor binding | 1.25E-04 | 31.03448276 |
| | | GO: Molecular Function | GO:0019900 | kinase binding | 1.25E-04 | 9.427609428 |
| | | GO: Molecular Function | GO:0019901 | protein kinase binding | 1.25E-04 | 9.881422925 |
| | | GO: Molecular Function | GO:0008022 | protein C-terminus binding | 1.25E-04 | 10.75268817 |
| | | GO: Molecular Function | GO:0019911 | structural constituent of myelin sheath | 1.25E-04 | 80 |
| | | GO: Molecular Function | GO:0005250 | A-type (transient outward) potassium channel activity | 1.25E-04 | 100 |
| | | GO: Molecular Function | GO:0022843 | voltage-gated cation channel activity | 1.25E-04 | 10.66666667 |
| | | GO: Molecular Function | GO:0005516 | calmodulin binding | 1.25E-04 | 10.66666667 |
| GO: Molecular Function | | GO:0017124 | SH3 domain binding | 1.25E-04 | 11.47540984 | |
| GO: Molecular Function | | GO:0003924 | GTPase activity | 1.25E-04 | 8.849557522 | |
| GO: Molecular Function | | GO:0017075 | syntaxin-1 binding | 1.25E-04 | 33.33333333 | |
| GO: Molecular Function | | GO:0005057 | receptor signaling protein activity | 1.25E-04 | 11.40350877 | |
| GO: Molecular Function | | GO:0005249 | voltage-gated potassium channel activity | 1.26E-04 | 12.12121212 | |
| GO: Molecular Function | | GO:0015271 | outward rectifier potassium channel activity | 1.42E-04 | 44.44444444 | |
| GO: Molecular Function | | GO:0015276 | ligand-gated ion channel activity | 1.52E-04 | 10.60606061 | |
| GO: Molecular Function | | GO:0022834 | ligand-gated channel activity | 1.52E-04 | 10.60606061 | |
| GO: Molecular Function | | GO:0015631 | tubulin binding | 4.97E-02 | 10.60606061 | |
| GO: Biological Process | | GO:0048167 | regulation of synaptic plasticity | 7.42E-04 | 18.60465116 | |
| GO: Biological Process | | GO:0050804 | regulation of synaptic transmission | 4.29E-03 | 11.95652174 | |
| GO: Biological Process | GO:0051969 | regulation of transmission of nerve impulse | 7.44E-03 | 11.16751269 | | |
| GO: Biological Process | GO:0031644 | regulation of neurological system process | 7.44E-03 | 10.69767442 | | |
| GO: Biological Process | GO:0006813 | potassium ion transport | 7.44E-03 | 11.35135135 | | |
| GO: Biological Process | GO:0048169 | regulation of long-term neuronal synaptic plasticity | 2.87E-02 | 25.80645161 | | |
| GO: Biological Process | GO:0007215 | glutamate signaling pathway | 3.85E-02 | 24.24242424 | | |
| GO: Biological Process | GO:0018107 | peptidyl/threonine phosphorylation | 3.85E-02 | 23.52941176 | | |
| GO: Biological Process | GO:0032886 | regulation of microtubule-based process | 3.85E-02 | 16.17647059 | | |
| GO: Biological Process | GO:0031111 | negative regulation of microtubule polymerization or depolymerization | 3.85E-02 | 26.92307692 | | |
| GO: Biological Process | GO:0001505 | regulation of neurotransmitter levels | 3.85E-02 | 11.5942029 | | |
| GO: Biological Process | GO:0031644 | regulation of neurological system process | 6.77E-03 | 13.48837209 | | |
| GO: Biological Process | GO:0071845 | cellular component disassembly at cellular level | 1.61E-02 | 12.24489796 | | |
| GO: Biological Process | GO:0022411 | cellular component disassembly | 1.61E-02 | 12.04819277 | | |
| GO: Biological Process | GO:0051969 | regulation of transmission of nerve impulse | 3.50E-02 | 12.69035533 | | |
| VTA | | | | | | |

and subsequent phenotypic impact of a *Fyn* null mutation. Although some categories are certainly redundant, the overall number of differentially expressed genes for each brain-region was disproportionate to the number of significant functional groups. PFC had the smallest change in total number of genes among the three brain structures, but this region also showed the largest number of significant functional categories.

The medial PFC has important modulatory effects on the dopaminergic reward system through glutamatergic feedback to the nucleus accumbens and ventral midbrain (Sesack and Pickel, 1992). Functional over-representation analysis implicated *glutamate receptor binding* (GO:0035254; p-value = 0.000125) and *glutamate signaling pathway* (GO:0007215 p-value = 0.038472), with a decrease in the NMDA receptor obligatory subunit *Grin1* and increase in *Grin2b* expression. Other genes in these functional groups that showed basal expression differences in the *Fyn* KO, included *Dlg4* (Psd-95) and *Homer1*, which both showed decreased expression in the null mice. Basal variation in this system within PFC is important due to prior evidence of ethanol-mediated long-term facilitation of glutamate receptors containing the NR2B (*Grin2b*) subunit, which is phosphorylated by *Fyn* kinase in a brain region specific manner (Wang et al., 2007). In addition to *Fyn* kinase, H-ras and *Src* can also regulated glutamatergic receptors function in response to alcohol (Suvarna et al., 2005). *Fyn* null mice exhibited increases in basal transcript abundance of *Hras* and *Src*, suggesting a compensatory response to loss of *Fyn* activity. *Hras* and *Src* were contained in multiple over-represented ontological categories including *regulation of synaptic plasticity* (GO:0048167, p-value = 0.000742) and *regulation of synaptic transmission* (GO:0050804, p-value = 0.004285). NMDA subunit receptor composition is altered following exposure to

ethanol with a relative increase in the NR2B subunit within the membrane. This functional change in receptor subunit composition is due to H-Ras activation and inhibition of Src kinase (Suvarna et al., 2005).

Clusters 7 and 8 (Figure 45A) showed strong inverse relationships in basal gene expression within NAC. Although the NAC is important in the neurobiology of addiction, it also has a recognized role in learning and memory (Setlow, 1997). Gene ontology analysis of NAC showed a broad range of categories centered on the abnormal expression of genes in Fyn knockout mice involving neuronal transmission and biological processes related to learned behavior (Table 7). In support of these findings, Fyn knockout mice have been previously reported to exhibit abnormal spatial learning (Grant et al., 1992) and hyper-responsiveness to fear inducing stimuli (Miyakawa et al., 1994). Our expression results suggest that Fyn-regulated gene expression in the NAC may contribute to observed learning and memory differences exhibited by Fyn kinase knockout animals.

Tissue from the ventral midbrain (VMB), encompassing the ventral tegmental area, had several clusters differentially expressed between knockouts and controls (see Clusters 4, 7, 8, and 12). The overall functional impact of these expression patterns as a whole for VMB was less obvious compared to PFC and NAC with ontologies related to altered nervous system function (Table 7. GO: 0031644 & GO:0051969) and the breakdown of cellular components (Table 7. GO: 0071845, GO:0022411). Expression profile of genes altered in the knockout mouse within VMB is possibly consistent with the known role of Fyn kinase in neurodevelopment (Chen and Charness, 2008).

Myelin-Associated Gene Expression

In agreement with previously published research (Umemori et al., 1994, Goto et al., 2004), our analysis detected a significant decrease of myelin-associated gene expression in Fyn kinase knockout mice. The 74 genes with altered basal expression across all three brain regions (Figure 45B) were over-represented for members of 'structural constituent of the myelin sheath (GO: 0019911; p-value = 0.000105 uncorrected for multiple comparisons). Unfortunately, this ontological category is limited by the inclusion of only 5 reference genes (*Mal*, *Mbp*, *Plp1*, *Tspan2*, and *Mobp*), only 2 of which (*Mbp* and *Plp1*) were determined to have decreased expression in the knockout mice. To perform a more comprehensive gene ontology analysis for genes involved in myelination we used ErmineJ (Lee et al., 2005a) to test functional over-representation for a dozen myelin-associated genes based on their absolute expression within each individual brain region (Figure 46 A&C, FDR \leq 10%). As shown in Figure 46C, the NAC and PFC but not the VMB was significantly over-represented for a decrease in myelin-related gene expression. Literature-association analysis (Figure 46B) further showed the functional relationship between Fyn kinase and myelin, as well as other interesting genes such as prodynorphin (*Pdyn*) and myelin basic protein expression factor 2 (*Myef2*). Both *Pdyn* and *Myef2* both reside within the *Lore2* quantitative trait loci (QTL) for alcohol-induced loss of righting reflex (Ehringer et al., 2001). Decreased myelin gene expression results suggest that a hypomyelination phenotype for Fyn knockout animals may be important for the interpretation of alcohol behavioral phenotypes. As previously mentioned, our previous transcriptome study on B6 and D2 mice also identified divergent basal levels of myelin-associated gene

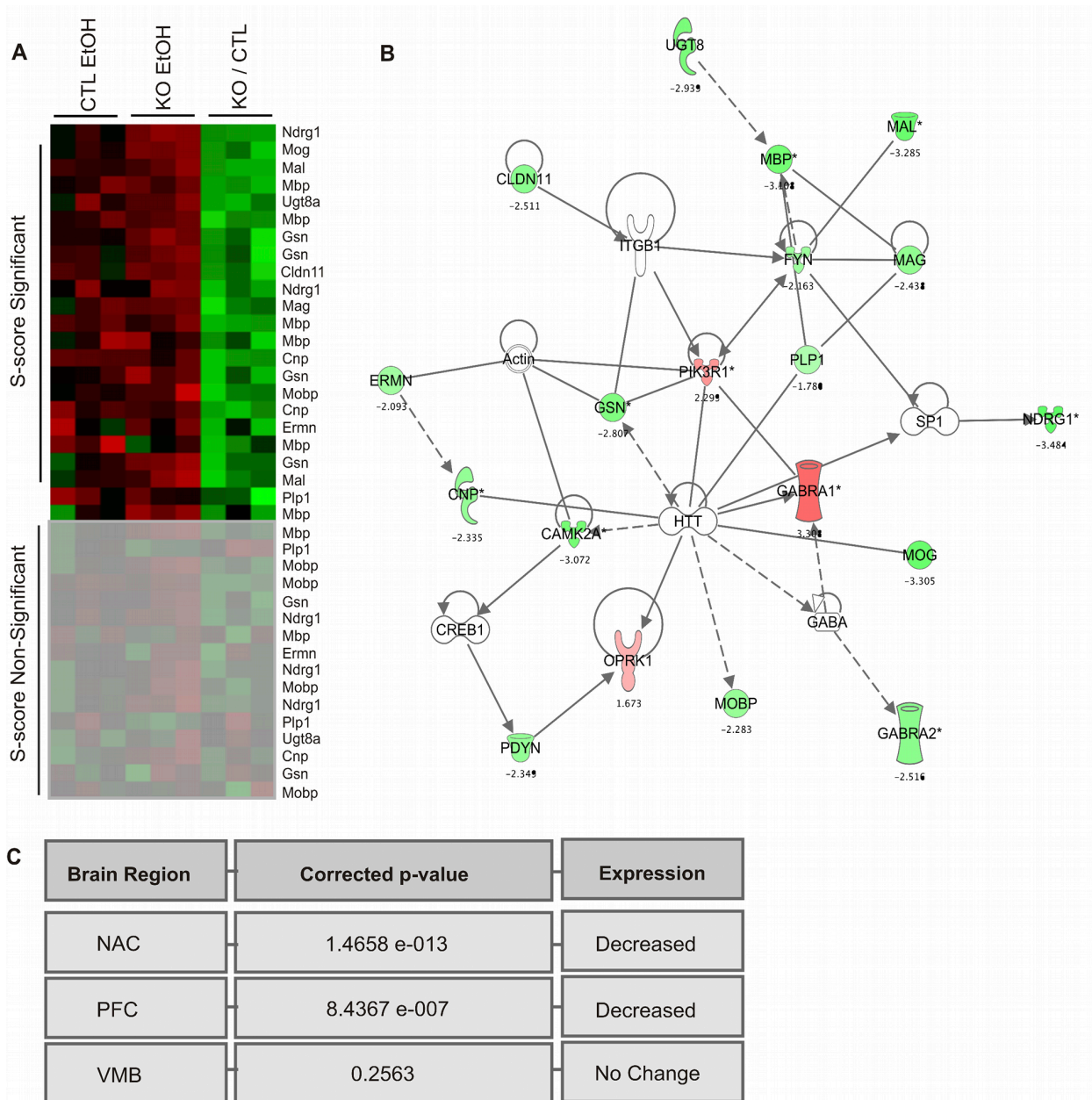


Figure 46. Over Representation Analysis (ORA) of Myelin-Associated Gene Expression. (A) ErmineJ heatmap of NAC gene expression showing a coherent decrease for myelin-related basal gene expression in *Fyn* knockouts compared to control. (B) Ingenuity Pathway Analysis (IPA) of myelin-related genes and literature associations; numbers shown are mean S-score of three biological replicates. (C) Table of ErmineJ corrected p-values (10% FDR) for myelin gene expression in NAC, PFC, and VMB.

expression in PFC as an important determinant for alcohol behavioral phenotypes (Kerns et al., 2005).

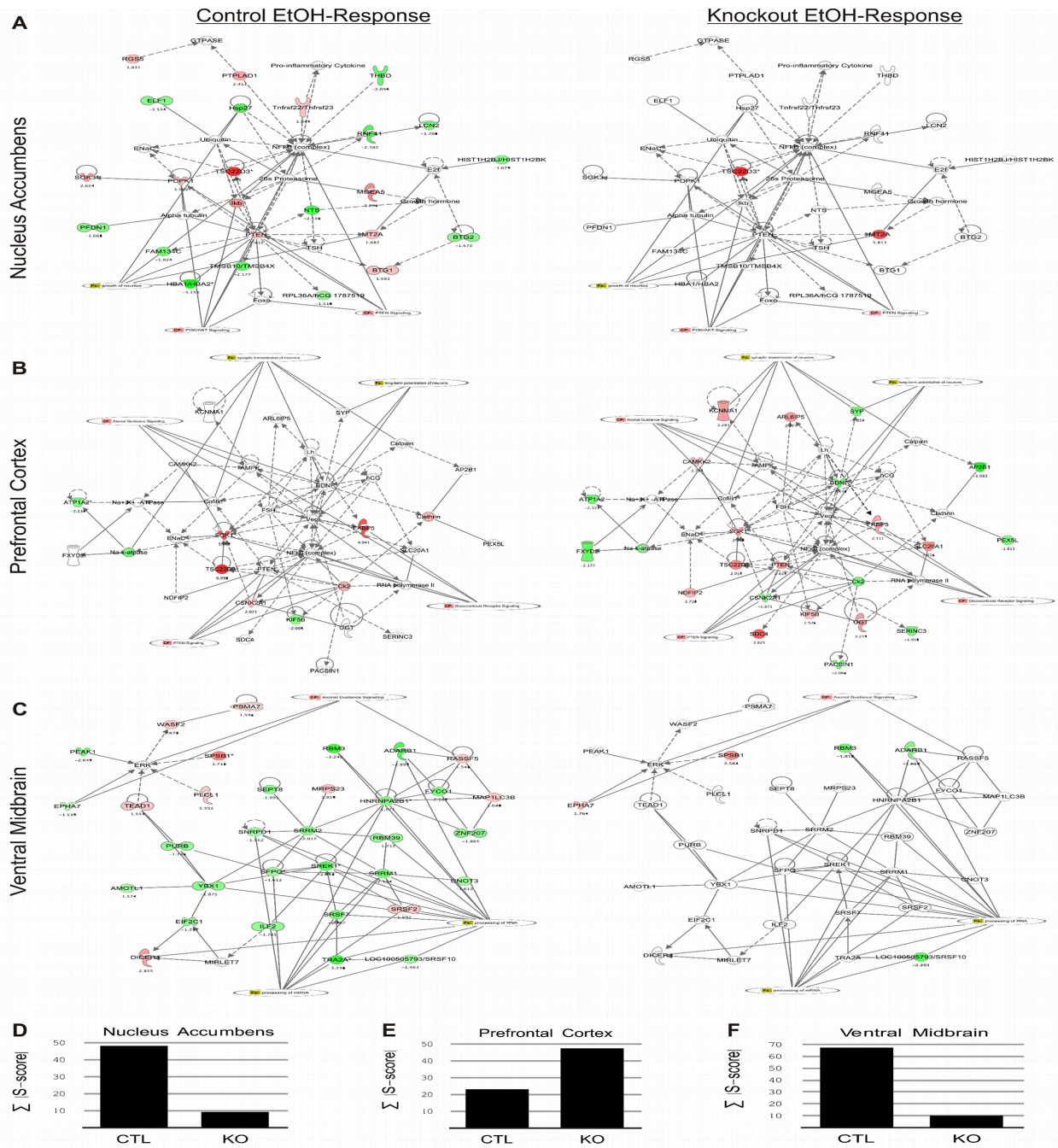
Alcohol-Responsive Gene Expression

In addition to alterations in the baseline abundance of gene expression, differences in alcohol-induced signaling events may contribute to differences the short- and long-term consequences of alcohol. Using gene expression as a surrogate measure of signaling mechanisms evoked by acute alcohol, we conducted whole-genome microarray analysis of NAC, PFC, and VMB from knockout and control animals 4-hours post an acute alcohol injection (Figure 45A). Preliminary studies from our laboratory showed a 4-hour time-point captures a range of early, intermediate, and late gene expression responses to alcohol. A 3 g/kg (i.p.) dose of acute alcohol was chosen for our studies on Fyn kinase knockout mice, attempting to minimize potential oxidative-stress reactions that may occur during high-dose alcohol exposure (Figure 45A, Cluster 3). A 3 g/kg (i.p.) dose of alcohol has been previously used in regards to behavioral genetics for alcohol-induced sedative-hypnotic effects (Ponomarev and Crabbe, 2004),

Global changes in gene expression due to acute alcohol administration were captured for each individual brain structure using a one-class SAM analysis and included alongside basal differences for k-means clustering (Figure 45A). Differences in alcohol-responsive gene expression were determined using a two-class SAM analysis within each brain region (Figure 45 A&C). Unlike our previous studies comparing B6 and D2 mice, region specific alcohol patterns were difficult to discern visually from k-means clustering. The lack in obvious alcohol-induced gene expression profiles may be due to

the large number genes of altered by acute 3 g/kg of alcohol on this particular genetic background and predominant differences in overall basal abundance (Figure 45B). Subsequently, we further analyzed these resulting acute ethanol expression differences for separate brain areas using a multivariate bioinformatics approach described in Materials and Methods. Surprisingly, unlike our analysis for ORA of basal differences (Table 7) gene ontology analysis at a 5% FDR yielded few significant groups related to gene function. This may suggest that basal variation of system-wide changes in expression (i.e. glutamate receptor function and myelin-associated gene expression) is a stronger predictor of acute ethanol sensitivity in the *Fyn* kinase knockout mouse. Alternatively, it may suggest that our use of a 5 % FDR is overly conservative in determining ontological groups related to gene function following a transient event such as with a single ethanol exposure on this specific genetic background. Literature association analysis using curated resources from Ingenuity Pathway Analysis and GeneGo were examined for more a detailed account of potential signaling mechanisms represented by differences in ethanol-responsive gene expression between knockout and control animals.

Examination of alcohol-responsive networks in the NAC depicts a set of functionally related genes regulated by acute alcohol, which are essentially blocked in their mRNA expression for mice carrying a *Fyn* null mutation. Members of this NAC network are involved in the phosphoinositide 3-kinase (PI3K)/AKT and phosphatase and tensin homolog (PTEN) signaling pathway. Acute alcohol causes a coordinate activation of the AKT signaling within the NAC. Inhibition of AKT signaling events within the ventral striatum attenuates operant self-administration and binge drinking of alcohol (Neasta et



al., 2011). These published observations and our microarray results suggest that this Akt/Pi3k/Pten network may both function in mediating basal alcohol seeking behavior and Fyn-dependent neuroadaptations in response to an initial alcohol exposure for the nucleus accumbens.

Molecular alterations within alcohol sensitive strains of mice, such as Fyn-dependent mice, may provide mechanistic information regarding acute alcohol sensitive behaviors. Although these changes may only occur within the context of a null or loss of function mutation for Fyn kinase, identifying these neuroadaptations are important for understanding the molecular pathways involving Fyn kinase and alcohol behavioral phenotypes. Several genes related to neurogenesis, long-term potentiation, and synaptic transmission were uniquely regulated by acute alcohol within the PFC of Fyn kinase knockout mice, but were non-responsive in the control group (Figure 47B). For example, expression of brain derived neurotrophic factor (*Bdnf*) and synaptophysin (*Syp*) were decreased by acute alcohol in the PFC of Fyn knockout animals but were unresponsive to alcohol in control animals (Figure 47B).

Fyn is important in the induction of long-term potentiation in adult murine forebrain neurons (Kojima et al., 1997), but the facilitation of long-term potentiation may also occur through other effectors. Increased PFC expression of BDNF following cocaine withdrawal facilitates activity induced long-term potentiation, sensitizing excitatory synapses within PFC (Lu et al., 2010). *Bdnf* gene expression is decreased in response to alcohol in response to alcohol in Fyn kinase knockout animals, which may suggest these knockout animals are at an enhanced risk for blunted long-term potentiation in the presence of acute alcohol exposure. Acute alcohol under normal

conditions can lead to increased neuronal levels of *Bdnf* and altered voluntary intake (Logrip et al., 2009).

Glucocorticoid-related gene expression is increased in the PFC following acute alcohol exposure (Kerns et al., 2005). Alcohol-responsive increases in glucocorticoid gene expression were blunted in the PFC of Fyn kinase knockout animals in comparison to alcohol treated controls (Figure 47B). Glucocorticoids dose-dependently decrease the sedative-hypnotics properties of acute alcohol exposure (Sze, 1993). Thus, decreased responsiveness of glucocorticoid-related gene expression in Fyn null mutants may contribute to increased susceptibility for alcohol-induced loss of righting reflex behavior in mice.

Top ranking literature association networks for alcohol-responsive differences within VMB were broadly related to RNA processing, cell signaling, and neurogenesis. Acute alcohol caused down-regulation of the Eph receptor 7a (*Epha7*) in controls, while leading to increased expression in Fyn knockout mice (Figure 47C). Ephrin receptors are one of the largest families of tyrosine kinase with an important role in synaptic function and neurodevelopment (Klein, 2009). Genetic deletion of *Epha7* causes a significant decrease in neural progenitor cell death and expansion of the ventral forebrain (Depaepe et al., 2005). Additionally, EphA7 activation leads to phosphorylated ERK (Nakanishi et al., 2007), which can then regulate the expression of downstream effectors involved in multiple signaling processes involving neuronal development and plasticity (Thomas and Huganir, 2004). As shown in Figure 47C, acute alcohol alters the expression of multiple genes targeting ERK within the VMB that are not altered in Fyn knockout animals.

Acute alcohol exposure caused a significant decrease for large category of genes within VMB related to RNA processing, unaltered in the *Fyn* kinase knockout mouse (Figure 47C). Conversely, the relative mRNA abundance of *Dicer1* was increased in control animals, but not regulated in the knockouts. DICER is a critical element in mouse development (Bernstein et al., 2003), processing of microRNAs that in turn then regulate mRNA expression (He and Hannon, 2004), and has a functional role in midbrain dopaminergic neurons and associated dopaminergic behaviors (Kim et al., 2007). Our analysis suggests that *Fyn* kinase may regulate acute alcohol sensitivity, at least in part, through the regulation of a gene network involving RNA processing within the VMB. An RNA processing network has also been implicated in a systems genetic analysis of alcohol sensitivity for *Drosophila melanogaster* (Morozova et al., 2011), further suggesting a network of RNA processing genes in behavioral sensitivity to alcohol.

Fyn-related Gene Network Analysis of LORR

A null mutation in *Fyn* increases duration in the loss of righting reflex behavior following acute alcohol exposures (Miyakawa et al., 1997, Boehm et al., 2003, Yaka et al., 2003b). However, our microarray results insinuate differences in alcohol behavioral responses may also be due to developmental compensation and altered signaling events in *Fyn* kinase knockout animals. In order to extend these previous studies and determine a *Fyn*-dependent behavioral gene network, we compared our microarray analysis of *Fyn* null mice with gene expression correlates of LORR or *Fyn* expression across the BXD (n = 29) and LXS (n = 42) panels of recombinant inbred mice (Figure

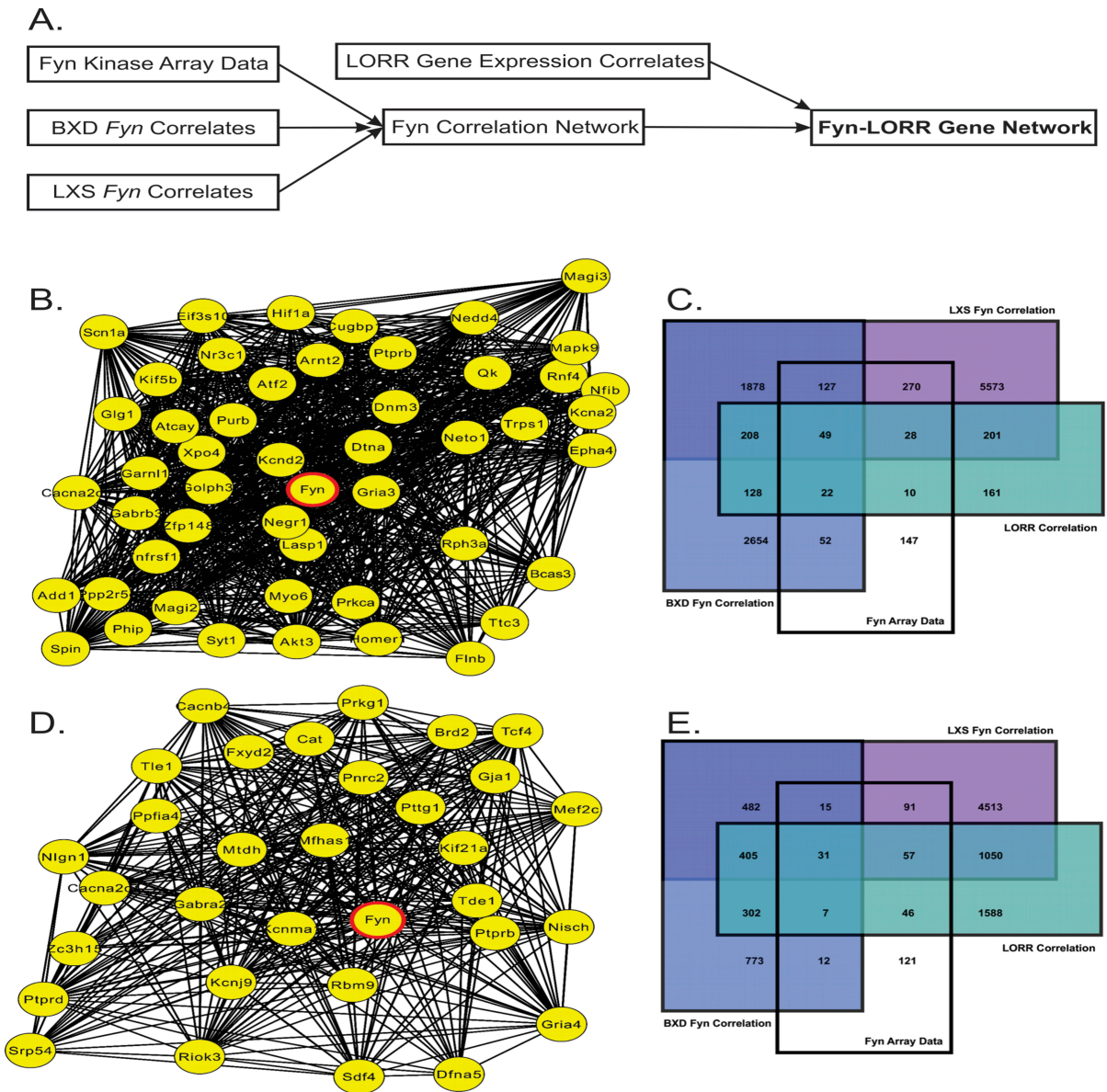


Figure 48. Fyn-LORR Correlation Network within PFC. Network analysis of concurrent variation of gene expression amongst PFC *Fyn* knockout array data, *Fyn* expression correlates across the BXD RI PFC, *Fyn* expression correlates across the LXS RI PFC, and LORR – BXD gene expression correlates in PFC. (A) Diagram of general approach used to define a Fyn-dependent gene network for the loss of righting reflex behavior. (B) Basal Fyn-dependent gene network showing correlations among BXD RI RMA saline dataset (p -value ≤ 0.01). (D) Alcohol-responsive Fyn-dependent gene network showing correlations among BXD RI S-score dataset (p -value ≤ 0.01). Red circle indicates the position of *Fyn*. (C) 4-way Venn diagram of basal datasets from Figure 48B; (E) 4-way Venn diagram of Alcohol-Responsive datasets from Figure 48D. Numbers shown represent unique gene symbols excluding *Fyn* itself. White = Fyn kinase array data, Blue = BXD *Fyn* Correlates, Purple = LXS *Fyn* Correlates, and Green = LORR gene expression correlates.

48A). Additionally, our analysis included gene expression from the PFC of BXD mice correlated with a previously published LORR study across a subset of BXD RI mice (Figure 49 GeneNetwork ID: 10589 (Rodriguez et al., 1995)). Combining data from these four gene expression datasets, allowed us to define a Fyn-LORR gene network (Figure 48). The intersection of multiple datasets increased the chance of a type II error; the over-lapping dataset defined a distinct set of 'highly' correlated genes to a specific behavioral trait. Although these genes are strongly connected with Fyn kinase, the gene network correlation is qualitatively greater than *Fyn* expression alone (Figure 49).

Analysis of basal gene expression from the Fyn null mice by the scheme mentioned above identified a network of 50 highly inter-correlated genes (Figure 48B&C); the first principal component of these 50 genes significantly correlated to LORR ($r = -0.83$, $p\text{-value} = 5.05 \times 10^{-6}$). Functional over-representation analysis of this gene network highlighted ion channel activity, function/localization to the post-synaptic density, and regulation of action potentials (Table 8). *Neto1*, *Kcnd2*, *Dnm3*, *Gria3*, and *Homer1* are all localized to the post-synaptic density and also may play a role in modulating synaptic N-methyl-D-aspartic (NMDA) acid receptor function. *Neto1* knockout mice have abnormal long-term potentiation, learning, and memory due to altered NMDAR subtype abundance (Ng et al., 2009). *Kcnd2*, also known as *Kv4.2*, is a voltage-gated potassium channel that regulates spontaneous NMDAR activation and downstream calcium signaling mechanisms (Jung et al., 2008). In the postsynaptic membrane *Dnm3* is a binding partner of *Homer1* (Gray et al., 2003), a scaffolding protein associated with type1 metabotropic glutamate receptors and the NMDAR

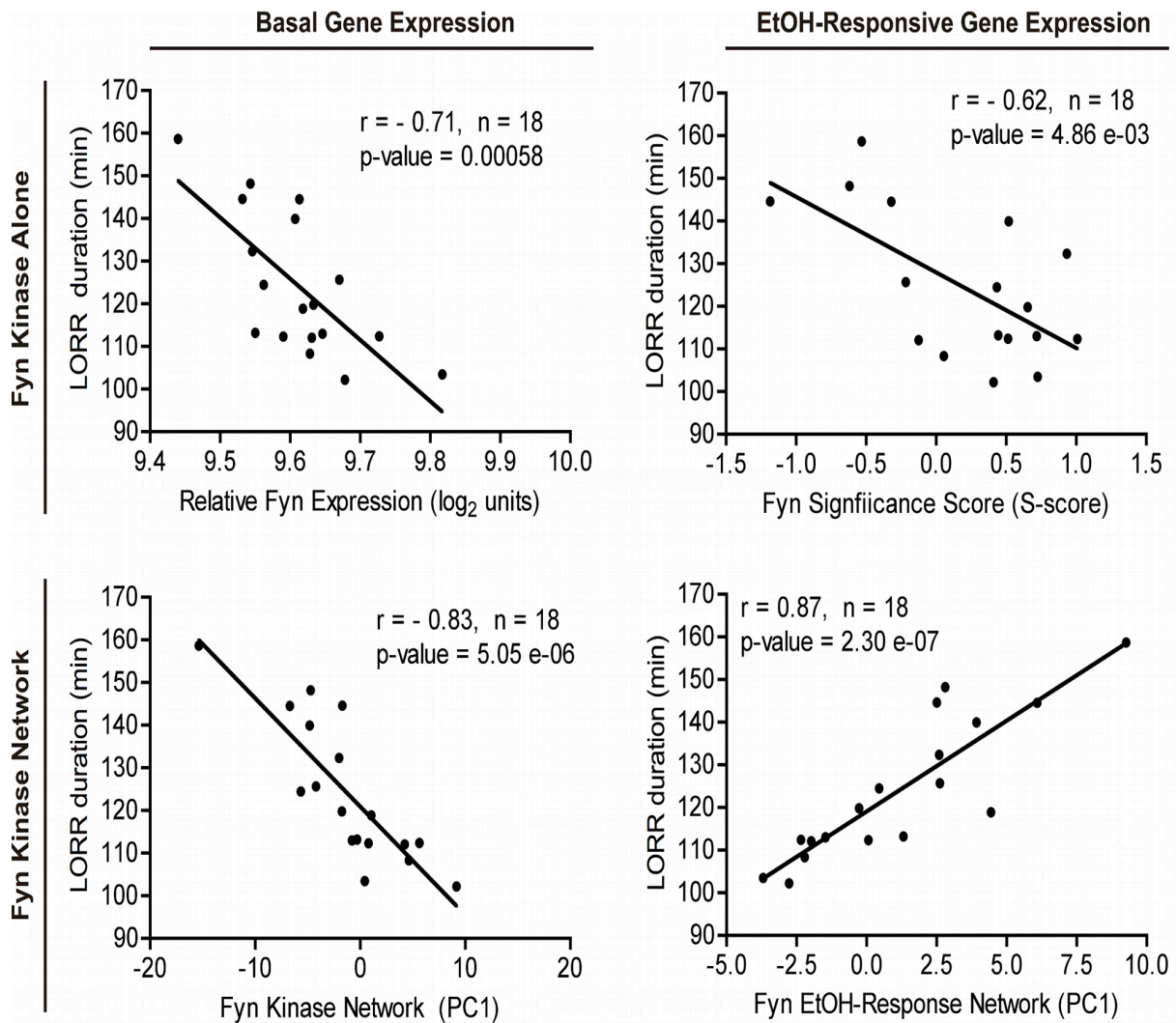


Figure 49. *In silico* correlation analysis of Fyn and LORR behavior. Pearson product moment correlations analysis of saline (RMA) and ethanol-responsive (S-score) alongside the LORR behavior (Rodriguez et al., 1995; WebQTL Record ID: 10589) for *Fyn* and *Fyn*-dependent networks from Figure 48B & 48D.

Table 8. Functional over-representation for Fyn knockout mice basal dependent gene network

| Category | ID | Name | P-value | % Targeted in Category |
|------------------------|------------|--|----------|------------------------|
| GO: Molecular Function | GO:0042608 | T cell receptor binding | 6.80E-05 | 40 |
| GO: Molecular Function | GO:0003690 | double-stranded DNA binding | 7.40E-05 | 3.012048193 |
| GO: Molecular Function | GO:0000900 | translation repressor activity, nucleic acid binding | 1.01E-04 | 33.33333333 |
| GO: Molecular Function | GO:0015271 | outward rectifier potassium channel activity | 2.42E-04 | 22.22222222 |
| GO: Molecular Function | GO:0043566 | structure-specific DNA binding | 3.20E-04 | 2.202643172 |
| GO: Molecular Function | GO:0030371 | translation repressor activity | 3.69E-04 | 18.18181818 |
| GO: Molecular Function | GO:0090079 | translation regulator activity, nucleic acid binding | 5.21E-04 | 15.38461538 |
| GO: Molecular Function | GO:0051015 | actin filament binding | 5.84E-04 | 4.838709677 |
| GO: Molecular Function | GO:0022843 | voltage-gated cation channel activity | 6.51E-04 | 2.666666667 |
| GO: Molecular Function | GO:0005244 | voltage-gated ion channel activity | 1.85E-03 | 2.010050251 |
| GO: Molecular Function | GO:0022832 | voltage-gated channel activity | 1.85E-03 | 2.010050251 |
| GO: Molecular Function | GO:0045182 | translation regulator activity | 3.83E-03 | 5.714285714 |
| GO: Molecular Function | GO:0032182 | small conjugating protein binding | 4.98E-03 | 5 |
| GO: Molecular Function | GO:0005267 | potassium channel activity | 5.63E-03 | 2.189781022 |
| GO: Molecular Function | GO:0005261 | cation channel activity | 6.19E-03 | 1.433691756 |
| GO: Molecular Function | GO:0003774 | motor activity | 6.22E-03 | 2.112676056 |
| GO: Molecular Function | GO:0005516 | calmodulin binding | 7.37E-03 | 1.986754967 |
| GO: Molecular Function | GO:0005230 | extracellular ligand-gated ion channel activity | 1.63E-02 | 2.702702703 |
| GO: Molecular Function | GO:0030165 | PDZ domain binding | 2.02E-02 | 2.409638554 |
| GO: Biological Process | GO:0018107 | peptidyl/hreonine phosphorylation | 9.20E-05 | 8.823529412 |
| GO: Biological Process | GO:0043618 | regulation of transcription from RNA polymerase II promoter in response to | 9.80E-05 | 33.33333333 |
| GO: Biological Process | GO:0010035 | response to inorganic substance | 1.16E-04 | 2.006668963 |
| GO: Biological Process | GO:0018210 | peptidyl/hreonine modification | 1.19E-04 | 8.108108108 |
| GO: Biological Process | GO:0032909 | regulation of transforming growth factor beta2 production | 1.37E-04 | 28.57142857 |
| GO: Biological Process | GO:0032906 | transforming growth factor beta2 production | 1.37E-04 | 28.57142857 |
| GO: Biological Process | GO:0001508 | regulation of action potential | 1.45E-04 | 3.883495146 |
| GO: Biological Process | GO:0043620 | regulation of DNA-dependent transcription in response to stress | 1.82E-04 | 25 |
| GO: Biological Process | GO:0042921 | glucocorticoid receptor signaling pathway | 1.82E-04 | 25 |
| GO: Biological Process | GO:0034765 | regulation of ion transmembrane transport | 2.23E-04 | 2.336448598 |
| GO: Biological Process | GO:0031958 | corticosteroid receptor signaling pathway | 2.91E-04 | 20 |
| GO: Biological Process | GO:0007416 | synapse assembly | 4.78E-04 | 5.084745763 |
| GO: Biological Process | GO:0034644 | cellular response to UV | 5.02E-04 | 15.38461538 |
| GO: Biological Process | GO:0046885 | regulation of hormone biosynthetic process | 5.02E-04 | 15.38461538 |
| GO: Cellular Component | GO:0014069 | postsynaptic density | 9.00E-06 | 4.545454545 |
| GO: Cellular Component | GO:0019717 | synapsome | 3.10E-05 | 3.521126761 |
| GO: Cellular Component | GO:0044309 | neuron spine | 6.70E-05 | 4.705882353 |
| GO: Cellular Component | GO:0043197 | dendritic spine | 6.70E-05 | 4.705882353 |
| GO: Cellular Component | GO:0034702 | ion channel complex | 2.28E-04 | 2.314814815 |
| GO: Cellular Component | GO:0060076 | excitatory synapse | 2.88E-04 | 20 |
| Mouse Phenotype | MP:0002064 | seizures | 3.50E-05 | 3.169014085 |
| Mouse Phenotype | MP:0009745 | abnormal behavioral response to xenobiotic | 3.90E-05 | 3.636363636 |
| Mouse Phenotype | MP:0008026 | abnormal brain white matter morphology | 1.60E-04 | 4.316546763 |
| Mouse Phenotype | MP:0004753 | abnormal miniature excitatory postsynaptic currents | 2.30E-04 | 7.843197255 |

complex. The ionotropic glutamate receptor *Gria3* is a member of the AMPA-receptor family, which facilitates fast excitatory synaptic transmission.

In addition to *Kcnd2* and *Gria3*, the ion channels *Kcna2*, *Gabrb3*, *Cacna2d1*, *Scn1a* were present in Figure 47B, suggesting Fyn kinase may augment the function of multiple ion channels in addition to the NMDA receptor. The beta3 subunit of GABA-A receptor has been previously reported to be functionally altered in Fyn kinase knockout mice contributing to reduced sensitivity to etomidate, a beta-2/beta-3 selective compound, in the LORR behavior for Fyn knockout mice (Boehm et al., 2004a). *Scn1a* is expressed in the nodes of Ranvier of motor neurons regulating the propagation of action potentials (Duflocq et al., 2008) and resides on chromosome 2 within a known QTL for alcohol preference (Bice et al., 2006). The regulation and localization of sodium channels is altered in mouse models of adult-onset demyelination (Rasband et al., 2003). The Fyn-dependent basal gene network is significantly over-represented for 'abnormal white matter morphology' (p-value = 0.000003; MP:0008026), and as shown in Figure 45C white matter related genes are significantly decreased in the PFC of Fyn knockout mice. *Fyn* is also correlated to quaking (*Qk*) (Figure 48B). Quaking is an RNA binding protein that regulates the transport of *Mbp* mRNA outside of the nucleus (Larocque et al., 2002). Defects in quaking function results in a severe hypomyelination phenotype (Larocque et al., 2002, Zhao et al., 2006). Fyn specifically targets the activity of quaking through phosphorylation of tyrosine residues with the C-terminal domain of quaking (Lu et al., 2005). Basal variation of gene expression for the *Fyn*-associated network may thus be a major determinant of myelin gene expression as well as acute alcohol-induced behavioral sensitivity to the LORR.

Establishing both basal and alcohol-responsive gene expression networks may be crucial for a more holistic understanding of the neurobiology of alcohol behavioral traits. The S-score provides a reliable measurement of alcohol-response (i.e. EtOH / Saline). Correlation analysis of alcohol-responsive gene expression identified a set of 32 distinct genes across the four different datasets. The first principal component of the alcohol-responsive network (Figure 49C) was significantly correlated to the LORR ($r = 0.87$, p -value = 2.30×10^{-7}), greater in magnitude for Fyn kinase ethanol-response alone. Coordinate regulation in the alcohol-response of this network may be an additional underlying component involved in the LORR behavior mediated by alcohol.

Of the 32 genes identified only 2 genes (*Cacna2d1* and *Ptprb*) were in common with the basal network other than Fyn kinase itself, suggesting the alcohol-responsive network is largely distinct from basal differences in expression. This Fyn centric alcohol-responsive network similar to the basal network was over-represented for functional categories related to ion channels (Table 9); notably including *Kcnj9*, *Cacnb4*, *Kcnma1*, and *Gabra2*. Several of these genes have been characterized for their association with alcohol-related phenotypes in mice and humans; however, their association with Fyn kinase has yet to be investigated. *Kcnj9* has been identified as a potential quantitative trait gene (QTG) for sedative-hypnotic withdrawal from alcohol (Kozell et al., 2009). *Kcnj9* resides with a portion of mouse chromosome 1 which is syntenic to region of human chromosome 1 implicated in the vulnerability to alcohol dependence and related phenotypes (Dick et al., 2002, Hill et al., 2004, Ehlers et al., 2010). Single nucleotide polymorphisms in *Gabra2* are associated with alcohol-elicited cues in the medial frontal cortical area (Kareken et al., 2010), alcohol dependence (Edenberg et al., 2004), and

Table 9. Functional over-representation for Fyn knockout mice alcohol dependent gene network

| Category | ID | Name | P-value | % Targeted in Category |
|------------------------|------------|---|----------|------------------------|
| GO: Molecular Function | GO:0022843 | voltage-gated cation channel activity | 1.23E-04 | 2.67 |
| GO: Molecular Function | GO:0019198 | transmembrane receptor protein phosphatase activity | 4.32E-04 | 11.11 |
| GO: Molecular Function | GO:0005261 | cation channel activity | 1.28E-03 | 1.43 |
| GO: Molecular Function | GO:0015276 | ligand-gated ion channel activity | 1.49E-03 | 2.27 |
| GO: Molecular Function | GO:0003714 | transcription corepressor activity | 3.07E-03 | 1.76 |
| GO: Molecular Function | GO:0043021 | ribonucleoprotein complex binding | 4.32E-03 | 3.51 |
| GO: Molecular Function | GO:0005230 | extracellular ligand-gated ion channel activity | 7.18E-03 | 2.70 |
| GO: Molecular Function | GO:0005262 | calcium channel activity | 9.38E-03 | 2.35 |
| GO: Molecular Function | GO:0005249 | voltage-gated potassium channel activity | 1.26E-02 | 2.02 |
| GO: Molecular Function | GO:0017124 | SH3 domain binding | 1.86E-02 | 1.64 |
| GO: Biological Process | GO:0050804 | regulation of synaptic transmission | 3.06E-04 | 2.173913043 |
| GO: Biological Process | GO:0051969 | regulation of transmission of nerve impulse | 3.97E-04 | 2.030458853 |
| GO: Biological Process | GO:0001764 | neuron migration | 3.98E-04 | 3.703703704 |
| GO: Biological Process | GO:0034765 | regulation of ion transmembrane transport | 5.43E-04 | 1.869158879 |
| GO: Biological Process | GO:0031644 | regulation of neurological system process | 5.52E-04 | 1.860465116 |
| GO: Biological Process | GO:0006816 | calcium ion transport | 7.11E-04 | 1.739130435 |
| GO: Biological Process | GO:0007214 | gamma-aminobutyric acid signaling pathway | 8.27E-04 | 8.333333333 |
| GO: Biological Process | GO:0051932 | synaptic transmission, GABAergic | 8.98E-04 | 8 |
| GO: Biological Process | GO:0051924 | regulation of calcium ion transport | 1.13E-03 | 2.586206897 |
| GO: Biological Process | GO:0070838 | divalent metal ion transport | 1.15E-03 | 1.526717557 |
| GO: Biological Process | GO:0007628 | adult walking behavior | 1.38E-03 | 6.451612903 |
| GO: Cellular Component | GO:0045211 | postsynaptic membrane | 1.86E-04 | 2.339181287 |
| GO: Cellular Component | GO:0034702 | ion channel complex | 4.51E-04 | 1.851851852 |
| GO: Cellular Component | GO:0005891 | voltage-gated calcium channel complex | 6.77E-04 | 8.695652174 |
| GO: Cellular Component | GO:0034704 | calcium channel complex | 1.23E-03 | 6.451612903 |
| GO: Cellular Component | GO:0034703 | cation channel complex | 1.68E-03 | 2.127659574 |
| GO: Cellular Component | GO:0043195 | terminal button | 2.59E-03 | 4.444444444 |
| GO: Cellular Component | GO:0043679 | axon terminus | 6.88E-03 | 2.702702703 |
| Mouse Phenotype | MP:0009745 | neuron projection terminus | 7.78E-03 | 2.53164557 |
| Mouse Phenotype | MP:0002578 | abnormal behavioral response to xenobiotic | 5.68E-04 | 2.272727273 |
| Mouse Phenotype | MP:0005620 | impaired ability to fire action potentials | 7.66E-04 | 15.38461538 |
| Mouse Phenotype | MP:0009747 | abnormal muscle contractility | 1.27E-03 | 1.901140684 |
| Mouse Phenotype | MP:0002972 | impaired behavioral response to xenobiotic | 1.29E-03 | 4.411764706 |
| Mouse Phenotype | MP:0000738 | abnormal cardiac muscle contractility | 2.59E-03 | 2.173913043 |
| Mouse Phenotype | MP:0002189 | impaired muscle contractility | 3.83E-03 | 1.851219512 |
| Mouse Phenotype | MP:0004036 | abnormal myocardial trabeculae morphology | 6.63E-03 | 2.479338843 |
| Mouse Phenotype | MP:0000010 | abnormal muscle relaxation | 9.52E-03 | 4.347826087 |
| Mouse Phenotype | MP:0001522 | abnormal abdominal fat pad morphology | 1.03E-02 | 2.112676056 |
| Mouse Phenotype | MP:0002912 | impaired swimming | 1.79E-02 | 3.125 |
| Mouse Phenotype | MP:0005334 | abnormal excitatory postsynaptic potential | 1.79E-02 | 3.125 |
| Mouse Phenotype | MP:0006319 | abnormal fat pad morphology | 1.86E-02 | 1.694915254 |
| Mouse Phenotype | MP:0006319 | abnormal epididymal fat pad morphology | 1.90E-02 | 3.03030303 |

the acute effects of alcohol in humans (Haughey et al., 2008). Genetic knock-in of an ethanol insensitive mutant for *Gabra2* in mice show increased acute alcohol-induced hypnosis, loss of motor stimulation, and altered alcohol-drinking behavior (Blednov et al., 2011). Acute alcohol modulates the voltage and Ca^{++} sensitivity of BK potassium channels such as *Kcnma1* (Dopico et al., 1998). Activation of *Kcnma1* by alcohol causes acute intoxication in *Caenorhabditis elegans* (Davies et al., 2003) and is associated with the level of response to alcohol in humans (Schuckit et al., 2005). Convergent correlation between *Fyn* and the aforementioned set of genes following acute ethanol (Figure 48D&E) may thus suggest modulation of a Fyn kinase dependent gene network(s) are important for the interpretation of alcohol-behavioral responses.

Resulting Fyn-related basal and alcohol-responsive gene networks were submitted to GeneMANIA to assess our network across multiple independent datasets related to gene co-expression, genetic interaction, and protein interaction, and predict any possible genes that may have been missed by our analysis. The networks from GeneMANIA are qualitatively similar to our networks within PFC, suggesting these genes are associated with one another across independent datasets. Strikingly, approximately 65% of all links between two genes within the basal network is due to protein-protein interactions (i.e. physical interactions) (Figure 50A). Although not directly tested, this network provides *in silico* evidence for coherence in gene and protein associations surrounding Fyn kinase and the LORR behavior. The alcohol-responsive network (Figure 50B) is slightly different with approximately 81% of all connections derived from co-expression of genes; however, a subset of genes showed physical interactions with Fyn kinase. GeneMANIA datasets do not necessarily address the

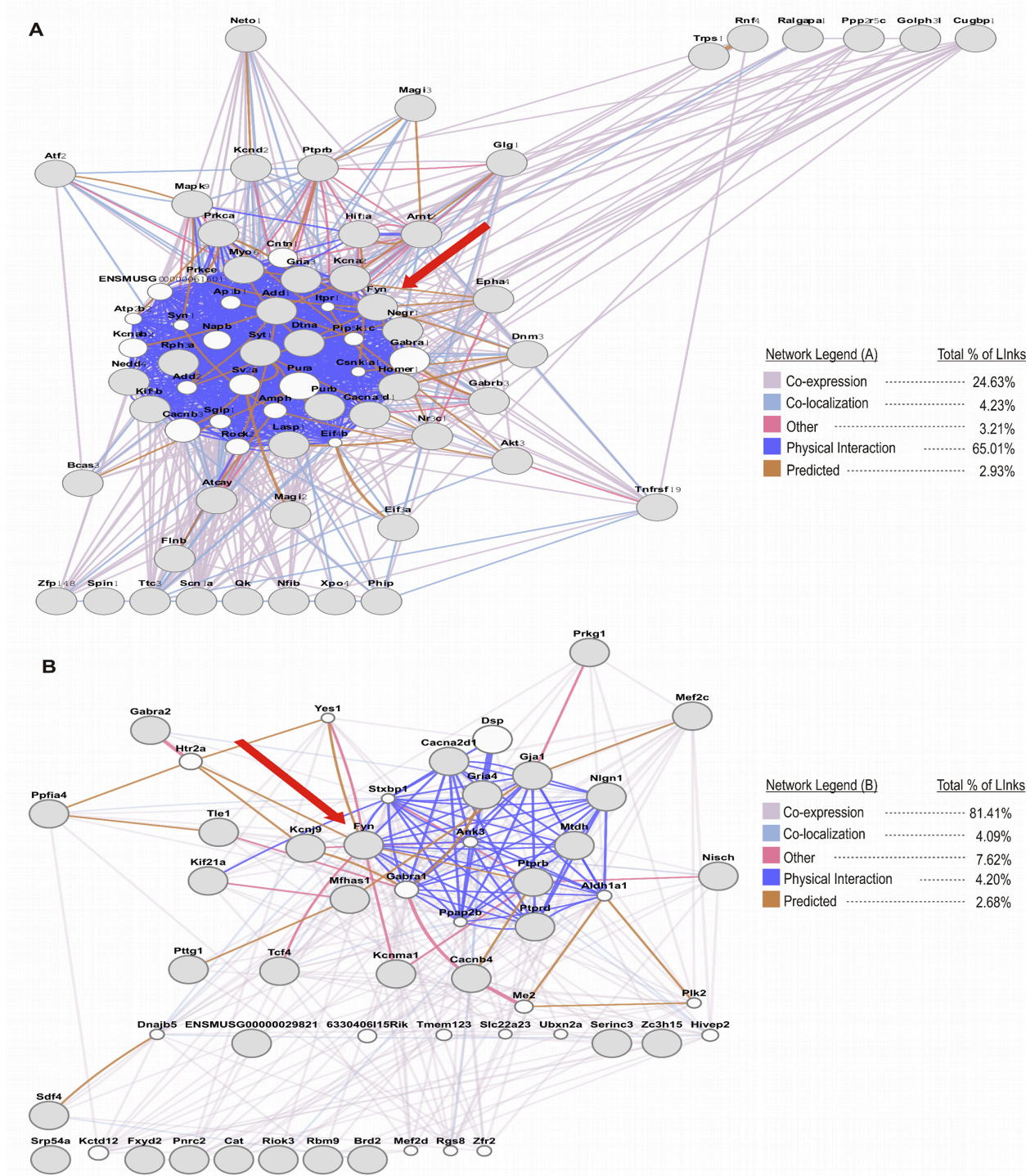


Figure 50. Independent assessment of Fyn kinase correlation networks.

GeneMANIA web-tool (<http://genemania.org/>) analysis using public resources for *Fyn*-related gene expression networks: (A) Reconstruction of basal network from Figure 48B. (C) Reconstruction of ethanol-responsive network from Figure 48D. Grey nodes are query genes and white nodes are genes predicted from the GeneMANIA algorithm. Red arrow indicates the location of Fyn kinase.

gene-gene interactions following the effects of alcohol exposure and may underestimate the degree of physical interactions under such particular experimental conditions.

GeneMANIA predicted the membership of multiple other candidate genes to both the basal and alcohol-responsive networks (labeled in white). Notably, the GeneMANIA algorithm predicted the neurotrophic receptor *Ntrk2* and the phosphatase *Pten* for the basal network, a subunit for the serotonin (*Htr2a*) and GABA-A (*Gabra1*) receptor within the alcohol-response network. All of which have been previously reported to have associations with the molecular and behavioral responses to alcohol. Therefore, our analysis has defined a set of Fyn-related correlation networks from the measurement of steady state mRNA following either an acute dose of saline (basal) or alcohol.

Intersecting these results with behavioral correlates of the LORR across the BXD RI lines has lead to the identification of a Fyn-dependent network associated with acute alcohol behavioral phenotypes. Although this approach may have inflated the correlation coefficient above that of Fyn kinase alone due to our bias LORR correlates, it highlights coherent networks related to a specific ethanol behavioral trait functionally altered within Fyn kinase knockout mice.

Discussion

A variety of mouse models, including null mutants, have been widely used to study the underlying neurobiology of alcohol and associated behavioral phenotypes (Crabbe et al., 2006b). Fyn kinase has been reproducibly reported to modify the sedative-hypnotic effects of alcohol as determined by duration of the LORR behavior (Miyakawa et al., 1997, Boehm et al., 2003, Yaka et al., 2003b). Herein, we have

conducted a genomic analysis of the mesocorticolimbic system from *Fyn* knockout mice in the presence and absence of alcohol (3 g/kg) to determine gene expression patterns associated with the LORR that may have underlying associations beyond a single null mutation. The explicit brain-regions involved in acute ethanol sensitivity and preference is unknown (Wand et al., 2001). Our *in silico* analysis using the WebQTL database (Chesler et al., 2005) showed that variation in the expression of *Fyn* within PFC is significantly correlated to a published report of the LORR across the BXD recombinant inbred strains of mice. Additionally, *Fyn* and the LORR are correlated to a common set of genes altered in the *Fyn* knockout mouse, suggesting a *Fyn*-related network of genes influencing this behavioral phenotype. In agreement with previous research our analysis suggested a deregulation of glutamatergic and GABAergic function; however, our analysis also suggests multiple other systems are perturbed in their expression, especially those related to abnormal myelin-associated gene expression.

Basal abundance of myelin-associated gene expression was significantly disrupted in forebrain of *Fyn* knockout mice. Myelin gene and protein expression is impaired in the frontal cortex of human alcoholics (Lewohl et al., 2000, Mayfield et al., 2002). Myelin is also suggested to be a dynamic aspect of substance abuse and comorbid disorders such as schizophrenia and manic depression (Ogden et al., 2004, Sokolov, 2007). Differential expression of basal and acute ethanol-responsive myelin gene expression within medial PFC has also been previously suggested as an important aspect of lasting alcohol behavioral phenotypes between B6 and D2 mice (Kerns et al., 2005). Alcohol drinking behavior is correlated to the expression of a dense network of myelin-associated genes within the PFC across species. Extending these observations across

the PFC a panel of alcohol naïve mice determined basal variation in myelin-related gene network is associated with alcohol consumption and acute behavioral sensitivity to alcohol. Acute functional tolerance was one of the behavioral measures significantly connected to variation in myelin-associated gene expression. The LORR is a behavioral measure of initial sensitivity and acute functional tolerance (Ponomarev and Crabbe, 2002, 2004, Radcliffe et al., 2006). Thus, we hypothesize variation of a myelin-associated gene expression is a contributing factor in the sedative-hypnotic effects related to acute functional tolerance and contributes, at least in part, to the previously documented changes in alcohol-induced LORR for *Fyn* null mice.

Acute functional tolerance due to various genetic factors, signaling processes, and neuroadaptations is important for interpretation of the LORR. *Fyn* kinase phosphorylation of the NMDA receptor subunit NR2B may account for one mechanism involved in mediating the duration for LORR behavior. Our analysis has focused exclusively on coordinate expression of stable steady-state messenger RNA and may not necessarily reflect phosphorylation events, post-translation modifications, or changes in protein expression. Concordance between gene expression and protein levels is not always a simple linear relationship and may depend on the individual genes, local environment, preexisting conditions, the model organism in question, or protein half-life (Greenbaum et al., 2003). Although protein abundance and function are important for cellular machinery transcript abundance may be more predictive of the overt phenotype (Ghazalpour et al., 2011).

In conclusion we have characterized a set of gene expression patterns or gene networks are important in the interpretation of acute ethanol sensitivity within a *Fyn*

kinase knockout mouse. Our results are consistent with previous research related to Fyn kinase and behavioral phenotypes previously reported in the literature; however, they suggest a novel gene network perspective within individual brain regions contributing to acute alcohol sensitivity. Convergent results from three different microarray studies as well as genes correlating with the LORR identified a significant network within PFC related to alcohol-induced behavioral sensitivity. In particular, basal variation in gene expression identified a set of genes related through protein-protein interaction, including Fyn kinase. Thus these results may suggest a network of genes, and down-stream elements such as myelin gene expression, and not just a single allele such as Fyn are important in the sedative-hypnotic properties of ethanol. Although not a perfect correlation in all studies, the inverse relationship between acute ethanol sensitivity and risk of long-term drinking behavior may suggest these networks are important in underlying processes governing ethanol consumption. Furthermore, continued inquiry into basal and drug-induced variation in gene networks across genetically diverse strains of mice, knockout mice, and demyelinated animal models may assist in our understanding of the neurobiology and pathogenesis of alcoholism.

CHAPTER 6:

MYELIN GENE EXPRESSION AND THE LOSS OF RIGHTING REFLEX BEHAVIOR

Introduction

Complex behavioral traits are influenced by a collection of genetic factors arising from two or more segregating populations. Mapping quantitative trait loci (QTLs) in several model organisms, including mice and rats, has identified multiple regions of the genome underlying behavioral responses to alcohol and other drugs of abuse (Spence et al., 2009, Drews et al., 2010). Inbred Long-Sleep (ILS) and Inbred Short-Sleep (ISS) mice are two inbred strains that were selectively bred for divergent behavioral responses to the acute sedative-hypnotic effects of alcohol (McClearn and Rodgers, 1959, Sanders et al., 1978, McClearn and Kakihana, 1981). Multiple QTLs have been identified in mediating the loss of righting reflex (LORR) due to ethanol (LORE) using recombinant inbred lines (RIs) and an F2 population derived from ILS and ISS mice (Markel et al., 1996, Bennett et al., 2002). Reciprocal interval specific congenic mice have been created in order to further genetically dissect QTL intervals inherent to the LORE, residing beneath four of the largest effect size QTLs: *Lore1*, *Lore2*, *Lore4*, and Genetic markers, flanking a defined phenotypic region, are used to generate congenic animals by inserting a QTL interval from a donor strain onto a genetically different recipient strain (Rogner and Avner, 2003, Armstrong et al., 2006). Polymorphisms residing within the donor QTL region are suspected to influence phenotypic variability. The use of reciprocal congenic mice allows for further genetic dissection QTL regions impacting quantitative traits related to behavioral outcomes for alcohol and other drugs

of abuse (Ferraro et al., 2005, Palmer et al., 2006, Ferraro et al., 2007, Crabbe et al., 2010b).

Discerning the regulatory variation for quantitative trait gene(s) underlying QTL(s) in complex traits has been considerably time-consuming, and will take nearly 1,500 years to track down all the potential QTGs beneath known QTL at the present pace (Finn et al., 2003, Flint et al., 2005). Isolation of the *Lore* QTL regions has identified molecular variants within coding regions for several candidate genes; however, no causal factor has yet been established (Ehringer et al., 2001, Ehringer et al., 2002). Genetic disequilibrium may be a prominent factor complicating the identification of QTG, with polymorphic differences existing among numerous genes beneath a given phenotypic QTL. Functional evaluation of candidate genes may be facilitated by *a priori* knowledge of a biological system, but prioritizing genes based solely on existing evidence potentially limits novel discovery. Additionally, too many polymorphic genes may sometimes exist within a QTL for adequate selection and downstream validation.

DNA microarray technologies allow essentially whole-genome expression analysis (Hughes et al., 2000); enabling non-biased, parallel evaluation of gene expression linked to suspected QTL regions (Chesler et al., 2005, Schadt et al., 2005). Regulatory variation of cis- and trans-acting QTL intervals is associated with divergent gene expression and susceptibility to disease (Farber and Luskis, 2008). Therefore, a complementary approach for narrowing prospective candidate genes is to restrict the analysis of differential expression to targets within a defined QTL acting on complex traits (Wayne and McIntyre, 2002, Hitzemann et al., 2003, Farber and Luskis, 2008). Not surprisingly, pairing expression profiling with genetic models, including recombinant

inbred and congenic mice for defined QTLs, has refined a subset of candidate genes for an array of phenotypic traits (Schadt et al., 2003, Drake et al., 2005, Mehrabian et al., 2005). Anchoring the suspected causal gene to a reference QTL, genomic profiling further permits a comprehensive exploration of coordinately expressed gene networks related to behavioral responses, improving mechanistic insight into more global complex traits (Chesler et al., 2005, Geisert et al., 2009).

Initially characterized in an F2 cross between ILS and ISS, the *Lore5* QTL on mouse chromosome 15 possessed a maximum logarithm of the odds (LOD) score of 4.0 at approximately 46 cM and accounted for 8.4% of the genetic variance (Markel et al., 1997). The *Lore5* QTL was subsequently introgressed onto both ILS (ILS.ISS-*Lore5*) and ISS (ISS.ILS-*Lore5*) backgrounds, using markers flanking the peak LOD score. Using oligonucleotide microarray profiling of prefrontal cortex (PFC) from ISS, ILS, and *Lore5* congenic mice we sought to determine important regulatory genes and gene networks underlying the LORR behavioral response specific to *Lore5*. Previous expression profiling of saline and alcohol-responsive gene expression in the PFC of C57BL/6J and DBA/2J mice (Kerns et al., 2005), as well as the PFC of Fyn kinase knockout mice, revealed a coordinate regulation of myelin-associated gene expression. Fyn kinase is key regulatory factor of myelin gene expression (Goto et al., 2004) and alcohol-induced LORR (Miyakawa et al., 1997). Based on our previous microarray investigations, we hypothesized that genetic variation in the expression pattern for a myelin-associated gene network within PFC of ILS, ISS, and reciprocal congenics is a decisive aspect functioning in the LORR behavior.

Studying both basal and alcohol-responsive gene expression in a selectively bred

model for the LORR behavior lead to the identification of N-myc downstream-regulated gene 1 (*Ndr1*) as a strong candidate gene underlying the *Lore5* QTL. *Ndr1* is a myelin-related gene expressed within oligodendrocytes (Okuda et al., 2008, King et al., 2011), suggesting *Ndr1* and myelin oriented gene expression is a selective mediator of LORR for acute alcohol. Using the cuprizone model of adult onset demyelination (Kipp et al., 2009) we tested the role of myelin gene expression in a subset of behavioral phenotypes. Consistent with our previous observations decreased myelin-associated gene expression in PFC was inversely correlated to acute behavioral sensitivity to alcohol as demonstrated by the LORR behavior. Our results point towards a novel genetical-genomics derived network of myelin genes contributing to divergent acute alcohol behavioral sensitivity.

Materials and Methods

Administration of acute alcohol, animal micro-dissection and RNA Isolation (LORE study). All animal procedures were approved by the University of Colorado Institutional Animal Care and Use Committee, in accordance with National Institute of Health guidelines. Male ILS, ISS, ILS.ISS-*Lore5* (hereafter referred to as L.5S) and ISS.ILS-*Lore5* (hereafter referred to as S.5L) mice (10 mice per strain, 55-85 days of age) were injected intraperitoneal (i.p) with saline for 6 consecutive days. On day 7, mice were injected with either saline (5 mice per strain) or 4.1 g/kg (w/v) ethanol (5 mice per strain). Four hours later, mice were sacrificed by cervical dislocation. We chose a 4-hour time point (post ethanol or saline injection) and prefrontal cortex (PFC) for these initial

studies based upon our prior work showing a robust collection of ethanol-responsive genes using this brain region and time point (Kerns et al., 2005). Brains were excised and chilled for 1 minute in phosphate buffer on ice prior to dissection. Dissections were completed 5-7 minutes from time of sacrifice. Prefrontal cortex (PFC) was excised, placed into individual tubes, frozen immediately in liquid nitrogen and subsequently stored at -80°C until shipment to the Miles laboratory at Virginia Commonwealth University. Other brain regions were dissected and stored for future analysis. This experimental protocol was performed in triplicate, for a total of 120 mice.

Tissue pooled from 4-5 animals was homogenized in STAT-60™ reagent (Tel-Test, Inc., Friendswood, TX) using a Tekmar homogenizer and total RNA was isolated according to the STAT-60™ protocol. RNA concentration was determined by absorbance at 260 nm, and RNA quality was analyzed by agarose gel electrophoresis and 260/280 absorbance ratios. Total RNA (7 µg) derived from each pool was reverse transcribed into double-stranded cDNA using Invitrogen Superscript II System (Invitrogen, Carlsbad, CA). Biotin-labeled cRNA was synthesized from cDNA using BioArray High Yield RNA Transcript Labeling Kit (ENZO Diagnostics, Farmingdale, NY) according to manufacturer's instructions, purified using the RNeasy Mini Kit (Qiagen, Mountain View, CA), and quantified by absorbance at 260 nm.

Microarray Hybridization, Scanning, and Data Analysis. Each treatment group (saline or ethanol), strain (ILS, ISS, L.5S or S.5L) or replicate (n=3) was hybridized to an individual microarray (n=24 total microarrays). Labeled cRNA samples were analyzed on oligonucleotide microarrays (Murine GeneChip™ U74Av2, U74Bv2, U74Cv2,

Affymetrix, Santa Clara, CA) that contain over 30,000 named genes and expressed sequence tags (ESTs). Array hybridization, staining and scanning were performed according to the manufacturer's protocol and exactly as described previously (Thibault et al., 2000, Kerns et al., 2005) using 10 µg of fragmented cRNA (in 200 µl master mix) for hybridization to arrays.

Microarray data was initially processed using Microarray Suite Software version 5.0 (MAS, Affymetrix). Arrays were normalized to a median total hybridization intensity (TGT = 190). All arrays had scaling factors <2.3, 3'-5'-actin ratio below 2, and linear intensity values. Arrays were further analyzed in three steps to identify genes with altered expression patterns. First, genes with consistently low expression values were filtered to eliminate genes with MAS 5 intensity values below 50 in all samples. Secondly, the S-Score algorithm, developed in this laboratory for the analysis of Affymetrix oligonucleotide arrays (Zhang et al., 2002, Kerns et al., 2005, Kennedy et al., 2006a, Kennedy et al., 2006b), was applied to compare hybridization signals between two randomly selected arrays from different treatment samples. S-score results have a normal distribution with mean 0 and SD of 1, and are correlated with the fold change. An S-score of $|2|$ corresponds to a $p=0.0455$, uncorrected for biological variability or multiple comparisons. S-scores were calculated within each experiment replicate for basal *Lore5*-specific changes across strains (S.5L-saline vs. ISS-saline; L.5S-saline vs. ILS-saline; and ISS-saline vs. ILS-saline) or within-strain responses to ethanol (S.5L-ethanol vs. S.5L-saline; L.5S-ethanol vs. L.5S-saline; ISS-ethanol vs. ISS-saline; and ILS-ethanol vs. ILS-saline). These comparisons generated a total of 21 different S-scores, 7 for each experimental replicate. Finally, S-scores from the intensity trimmed

gene list were loaded into The Institute for Genomics Research MultiExperiment Viewer (TMEV) (Saeed et al., 2003, Saeed et al., 2006) for further statistical and multivariate analyses. Statistical Analysis of Microarrays (SAM), a rank-based permutation method, was used to identify genes with S-scores significantly different from zero (Tusher et al., 2001) using a multi-class analysis. A false discovery rate of 10% or lower was used to avoid eliminating genes that may be biologically important and could assist in interpretation of expression patterns in multivariate studies. This gene list was used for subsequent multivariate or bioinformatic analyses.

Statistically filtered genes were further analyzed for correlated gene expression patterns by application of k-means clustering after estimating the number of nodes by principle component analysis as described previously (Kerns et al., 2005). We also used post-hoc template matching (PTM) (Pavlidis and Noble, 2001) to detect genes with basal expression or ethanol regulation correlating with the *Lore5* region. A cutoff of *p-value* < 0.001 was used to detect genes with statistically similar basal or ethanol responses for strains having either a ISS *Lore5* interval (L.5S and ISS) or a ILS *Lore5* interval (S.5L and ILS).

Bioinformatic Analysis of Microarray Data. Expression Analysis Systematic Explorer (EASE v 1.21) (Hosack et al., 2003) was used to identify biological themes among gene expression profiles and group genes into functional classifications developed by several public databases. The following annotation groupings were analyzed for over-representation in gene lists: chromosome, SwissProt keyword, GenMAPP pathway, Gene Ontology Consortium Biological Process, Molecular Function, and Cellular

Component. EASE results were filtered to remove categories with EASE score > 0.05. Gene Ontology Consortium (2001) categories with over 250 members on the U74Av2 array were considered too broad and removed. Redundant categories with the same gene members were removed to yield a single representative category. Additional bioinformatics analysis of genes showing altered basal expression or regulation by ethanol was done by identifying the chromosomal location of all such genes and superimposing this on the *Lore* support intervals. Chromosome locations for genes probed by the Affymetrix U74Av2, U74Bv2, and U74Cv2 arrays were obtained through the UCSC Mouse Genome database (<http://genome.ucsc.edu/>). Genes were also probed for linkage of basal expression to published ethanol behavioral data and to defined chromosome locations through the WebQTL database (<http://genenetwork.org/webqtl/main.py>). The WebQTL database contains expression data on whole brain or brain regions from recombinant inbred lines and can be used to correlate gene expression, genetic marker information and behavioral QTL data (Chesler et al., 2005).

Northern Blot Analysis. Total RNA (5 µg/sample) was analyzed by denaturing gel electrophoresis and transferred to nylon membranes for Northern blot analysis as described previously (Thibault et al., 2000). Probes for *Ndr1* and *C430014K11Rik* were prepared by reverse transcriptase PCR (rtPCR) amplification from total RNA isolated from prefrontal cortex of ILS mice. Primer pairs were as follows: *Ndr1* -- forward primer: 5'- CCAAAGGCAAGAAGTTCAG, reverse primer: 5'- GGAAACAGGAAGTAGGCAGGTTG; *C430014K11Rik* (AW049397) -- forward primer: 5'-CACTCCCACGGAAACCATTG, reverse primer: 5'-

GCTTACAAAGTCTGCCCCCTTCTC; GAPDH -- forward primer: 5'-
ACCACAGTCCATGCCATCAC, backward primer: 5'-TCCACCACCCTGTTGCTGTA.
Probe sequences were generated using PCR, a T7 promoter was ligated onto the 3'
end using the LigN'Scribe kit (Ambion). Labeled antisense probe sequences were
generated by in vitro transcription with 32P-CTP using the StripEz Kit (Ambion).

Genetical Genomics Analysis of Ndr1 in LXS recombinant inbred lines. Male mice (6-8
per strain) from 42 recombinant inbred (RI) lines of the LXS RI battery (Williams et al.,
2004) were injected with saline IP and brain regions harvested by microdissection four
hours later. RNA isolation and microarray analysis were as described above except that
all strains were randomized at each step of the procedure so as to minimize potential
batch effects. Microarray analysis utilized Affymetrix 430A type 2 arrays. Genetic
analysis of *Ndr1* expression across the LXS lines was done using RMA intensity
scores (Irizarry et al., 2003) and the batch analysis feature of GeneNetwork to generate
an interval map analysis of *Ndr1* with significance levels determined by permutation
analysis.

*Administration of acute alcohol, animal micro-dissection and RNA Isolation (Cuprizone
Study).* All animals were treated according to protocols for animal care established by
Virginia Commonwealth University and the National Institute of Health. Adult male
C57BL/6J mice (6 to 7 week-old, The Jackson Laboratory, Bar Harbor, ME) were singly
housed with *ad libitum* access to water and standard rodent chow (catalog #7912;
Harlan Teklad, Madison, WI) or 0.2% cuprizone (w/w) rodent chow and a 12 hour light/

dark cycle. Cuprizone chow was made by grinding standard rodent chow weighing 100 grams with 0.2% cuprizone (Bis(cyclohexanone)oxaldihydrazone; Sigma-Aldrich #14690). All mice were fed cuprizone for a period of five weeks to induce demyelination. Animals were weighed once weekly during cage changes to assess any potential differences and body mass between controls and cuprizone fed mice. The Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University approved all experimental procedures (#AM10332 to Dr. Michael F. Miles).

Mice were given an intraperitoneal saline injection once daily for three days in order to habituate to the injection and animal handling processes. On day four mice received either an injection of saline or 20% ethanol in saline for the appropriate testing procedure described the below. After four hours animals were killed by cervical dislocation. Brain tissue was harvested as tree recently described in Kerns et al., 2005. Brain regions were placed in individual tubes, frozen immediately with liquid nitrogen, and stored at -80°C until isolation of total RNA was performed on individual brain sections. Brain tissue from medial prefrontal cortex was homogenized with STAT-60 reagent (Tel-Test, Firendswood, TX) using a Tekmar homogenizer. Total RNA was isolated per Qiagen protocols using RNeasy Midi Kit (#75144). RNA quantifican and quality was determined by agarose gel electrophoresis using Bio-Rad Experion RNA analysis kits (#700-7104).

Quantitative real-time reverse transcription – PCR. Total RNA (1 µg) passing quality control was reverse transcribed into double-stranded cDNA using iScript cDNA synthesis kit (Bio-Rad # 170-8891) according to the protocol from the manufacturer.

Briefly, total RNA was incubated with oligo-dT, random hexamer primers, and reverse transcriptase at the 55°C for 5 min., 42°C for 30 min., and 85°C for 5 min. Following synthesis cDNA was immediately used or stored at -20°C. Quantitative real-time PCR performed using the iCycler iQ system (Bio-Rad) according to the instructions of the manufacturer for iQ SYBER Green Supermix (Bio-Rad #170-8880). Three technical replicates for performed for each cDNA preparation and average prior to statistical analysis. All primers were designed to minimize secondary structure formation and cross intron-exon boundaries to reduce amplification of possible genomic DNA contamination. Relative abundance of target transcripts was normalized to the housekeeping gene GAPDH.

Locomotor and Anxiety -Like Behavior. Animals were placed into the behavioral room and allowed to habituate for a period of one hour prior to testing. All locomotor / anxiety-like experiments were conducted between 8 AM and 12 PM eastern standard time. Following habituation animals were given an intraperitoneal injection of saline or 2 g/kg ethanol and placed immediately in the light-dark box chamber facing the dark compartment to start on entry. Two equally sized compartments (30 cm x 30 cm x 15 cm) or separated by a black plastic partition with an opening in the middle to allow for light – dark transitions (Med Associates Inc., St. Albans VT). The boxes were enclosed in a sound attenuating chamber equipped with overhead lighting in and ventilation system was interfaced with Men Associates software enabling automatic measurement of activity using a set of 16 infrared beam sensors along the X – why plane. All animals were tested for a total of 10 minutes in the chamber using 5 minute bins and then

returned to their home cage. Between sessions all chambers were cleaned with ammonia and paper towels given 2 to 5 minutes to air dry. At completion of the experiment animals are returned to their home cage monitored for period 24 hours.

Loss of righting reflex (LORR) due to ethanol. Animals were placed into the behavior room and allowed to habituate for a period of one hour prior to testing. All LORR experiments were conducted between 8 AM and 12 PM eastern standard time.

Following habituation animals were given an intraperitoneal injection of saline or 4 g/kg ethanol and placed in a clean cage with bedding labeled with appropriate information.

Each animal was timed for the onset of the LORR from the moment of injection until acquiring the LORR using a laboratory timer. Any animals not acquiring LORR within 5 minutes were deemed an outlier and removed from the study. Animals were deemed to have acquired the LORR when placed on their back V-shaped cage-top and remained in that position for no less than 20 seconds. Following acquiring the LORR animals were scored for the duration of the LORR, defined as the amount of time it takes an animal to right itself onto all four paws 2 to 3 times within 60 seconds. At completion of the experiment animals are returned to their home cage monitored for period 24 hours.

Gait analysis of mouse locomotor behavior. Animals were placed into the behavior room and allowed to habituate for a period of one hour prior to testing. All gait analysis experiments were conducted between 8 AM and 12 PM Eastern Standard Time using two groups of animals of equal group sizes on alternating days. Following habituation animal's paws were painted with Crayola non-toxic paint. Front paws were painted with

blue and rear paws were painted in red. On day one each animal was immediately placed the gait analysis box and allowed to run to the end of the chamber. Following this initial run animals were each given a second opportunity to run through the chamber, returned to their home cage, then given a intraperitoneal saline injection and run the third time representing time zero. Each animal was run again at 5 minutes, 15 minutes, 30 minutes, and 60 minutes under saline conditions to complete day one. On day two for each group each animal was given an intraperitoneal 2 g/kg ethanol injection and immediately placed in the chamber for time zero then run again at 5 minutes, 15 minutes, 30 minutes, and 60 minutes. On day three for each group each animal was given an intraperitoneal 2.75 g/kg ethanol injection and immediately placed in the chamber for time zero then run again at 5 minutes, 15 minutes, 30 minutes, and 60 minutes. Animal groups were rotated to give each group at least one day off prior to repeated testing. Paper strips for each animal were hung to dry and measured using a standard ruler for stride length, stance length, and sway length. Measurements were sampled using the average of six repetitive steps from the midsection of the paper.

Acute Alcohol Handling-Induced Convulsion Behavior. Adult male C57BL/6J mice pre-exposed to +/- cuprizone treatment were tested for acute alcohol (4 g/kg) withdrawal seizures using handling-induced convulsions (HIC) (Metten and Crabbe, 2005). HIC is a powerful behavioral model for assessing shared genetic/genomic influences across different pharmacological agents, including alcohol (Belknap et al., 1988, Crabbe et al., 2002). Individual-animals were tested immediately pre- and post-injection of alcohol, then every hour between 2-8 hours. Video was recorded for individual subjects were

lifted by their tail, gently spun at a 180-360° arc, and scored by a blinded observer on a rating scale from 0 to 7. Scoring was determined as 0 (no convulsions), 1 (no convulsions when lifted by tail, only presence of a facial grimace after spinning), 2 (no convulsions when lifted by tail, but tonic convulsion elicited by spinning), 3 (no convulsion when lifted by tail, but tonic-clonic convulsion after spinning), 4 (tonic convulsion when lifted by tail), 5 (tonic-clonic convulsion when lifted by the tail, often with onset delayed by 1-2 seconds), 6 (severe, tonic-clonic convulsion when lifted by the tail, with quick onset and long duration, often continuing for several seconds after the mice are released, and 7 (severe, tonic-clonic convulsion, with quick onset and long duration: spontaneous, or elicited by mild environmental stimulus, such as lifting of the cage-top). Overall withdrawal scores were computed as the area under the curve (AUC) between 2 and 8 hours after alcohol administration. The average baseline HIC Score for each animal was then subtracted from its AUC score to calculate a total alcohol withdrawal score, corrected for individual differences in baseline HIC.

Statistical Analysis. All data are reported as mean \pm standard error of the mean and analyzed using the appropriate statistical tests for each independent behavioral assessment as discussed in the text. All the behavioral measures were collected independently of one another, with no repeated behavioral testing of any subject to prevent any possible carryover effects. Statistical analyses were performed in SPSS and Prism, data was considered significant at the p-value less or equal to 0.05 confidence level. Graphs were rendered using Prism (GraphPad).

Results

LORR behavioral differences between ISS, S.5L, ILS, and L.5S mice. The LORR reflex behavior following acute alcohol administration was verified for Inbred Short-Sleep (ISS) and Inbred Long-Sleep mice (ILS). Reciprocal introgression of the *Lore5* QTL region onto the ILS background, ILS.ISS-*Lore5* (L.5S), and the ISS background, ISS.ILS-*Lore5* (S.5L), led to a statistically significant reversal in duration of LORR (Figure 51A). Behavioral differences were relatively small in magnitude, but only one truncated chromosomal region (*Lore5*) was under investigation. Pairing differences in gene expression profiles for saline and acute alcohol administration (Figure 51B with mean behavioral differences in the LORR between strains we sought to define gene networks associated with acute behavioral sensitivity to alcohol. As described above and in previous chapters, we hypothesized from our earlier microarray analyses that basal variation myelin-associated gene expression within PFC is an underlying factor of acute behavioral sensitivity (i.e. LORR). Leveraging variation in gene expression within a defined QTL for LORR further prioritized candidate genes, which helped explain the genetic architecture for this specific alcohol behavioral response.

Refining the Lore5 QTL interval by single nucleotide polymorphism (SNP) and dinucleotide marker analysis. Advances in large-scale sequencing methodologies and SNP genotyping for hundreds of mouse strains, in addition to interrogating micro-satellite markers within the L.5S and S.5L congenics, allows for a more in-depth analysis of sequence diversity within the *Lore5* QTL interval. According to previous analysis by Johnson and colleagues, the *Lore5* QTL region extends from *D15Mit278*

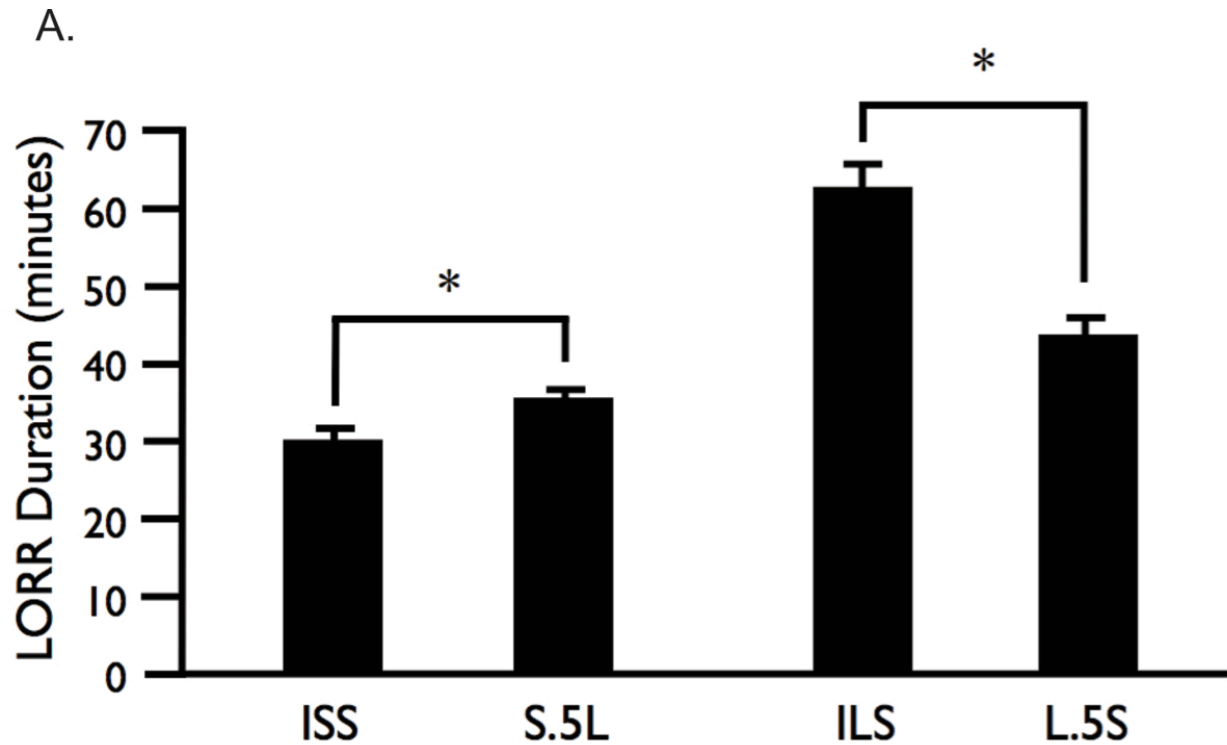
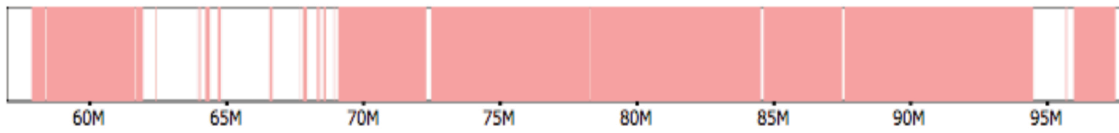


Figure 51. Behavioral genomics of ILS, ISS, L.5S, S.5L. (A) duration in the loss of righting reflex behavior for ILS, ISS, L.5S, and S.5L mice following 4.1 g/kg (i.p.) alcohol administration, * p-value < 0.01; (B) Hierarchical clustering of basal and alcohol-responsive gene expression within PFC for ILS, ISS, L.5S, and S.5L mice 4-hours post i.p. injection; Red= increased expression. Green= decreased expression. Black = no change in expression.

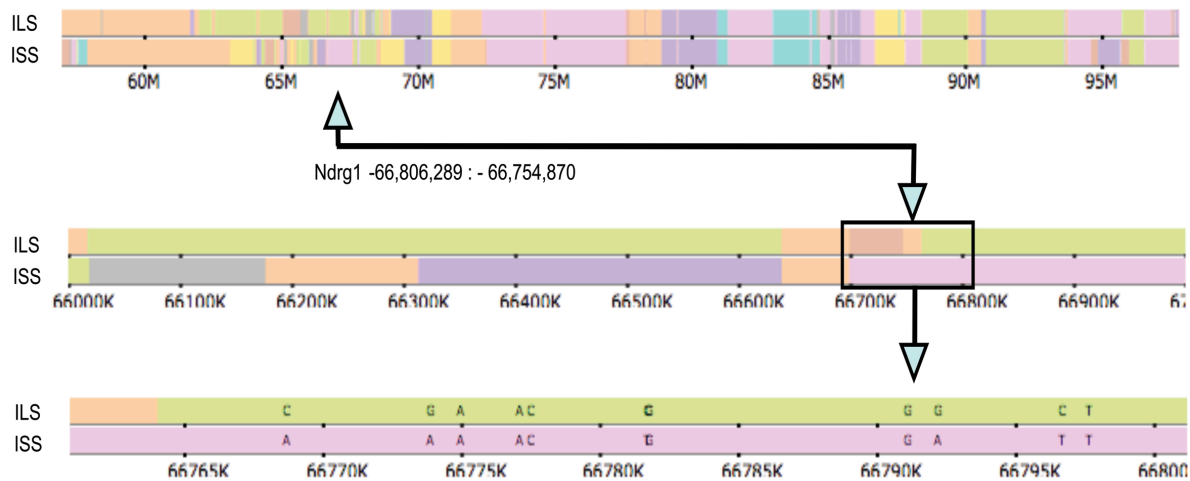
(56.99 Mb) to *D15Mit43* (97.82 Mb). Using publicly available data from a genome-wide mapping study, analyzing SNPs data across 36 wild caught mice, 62 wild-derived laboratory strains, and 100 classical mouse strains (including ILS and ISS mice), identified a large portion of the *Lore5* QTL candidate region (72.64-94.71 Mb) is identical by descent (IBD) between ILS and ISS strains (Figure 52A). This suggests the remaining non-IBD regions likely harbors the causal variant predisposing ILS and ISS (as well as reciprocal congenics) for phenotypic differences to the LORR. Within the 22 Mb IBD region (from marker *D15Mit169* to SNP *rs13482721*), there are a total of 121 SNPs and 44 di-nucleotide markers exhibiting no polymorphic differences between ILS and ISS mice. However, within the non-IBD region *D15Mit107* (84.21 Mb) and *D15Mit171* (89.88 Mb) are two polymorphic elements between ILS and ISS. Variation in *D15Mit107* and *D15Mit171* may be a result of the relatively high mutation rate existing among micro-satellite markers (Li et al., 2002b). Pseudo-colored images for haplotype blocks between ILS and ISS mice (Figure 52B) illustrated allelic differences within a small region of the *Lore5* QTL interval. The IBD region haplotype block structure was nearly identical across ILS, ISS, and C57BL6/J, further illustrating a lack of sequence divergence for a large portion of the *Lore5* QTL region. Overall, the *Lore5* interval contains 407 genes or predicted genes but after eliminating the IBD region, 78 genes (*Lore5*-NonIBD) remained as probable candidates for the alcohol behavioral phenotype LORR.

Differential gene expression between Lore5 congenics, ILS and ISS. Affymetrix genechip arrays (U74Av2, Bv2 and Cv2) were used to investigate both basal and

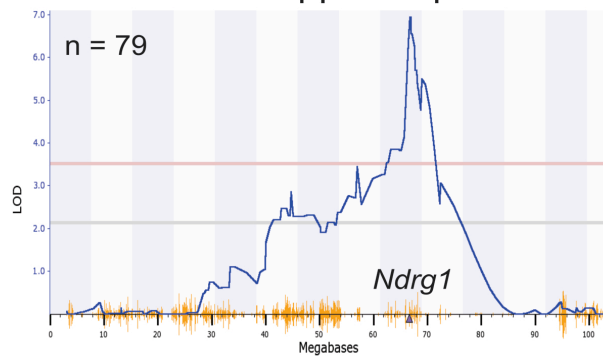
A. Identical By Descent (IBD) - colored in pink



B. Haplotype Blocks



C. LXS Hippocampus



D. LXS Prefrontal Cortex

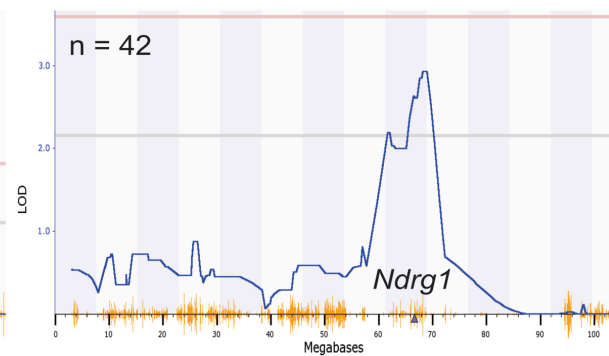


Figure 52. Genetic analysis of *Lore5* genotype. (A) identification of regions identical by descent (IBD) between ILS and ISS mice for the *Lore5* interval; pink indicates regions IBD, (B) Haplotype block analysis of ILS and ISS for the *Lore5* interval using pseudocolored blocks, (C) cis-eQTL for *Ndr1* basal gene expression in hippocampus of the LXS RI panel (n = 79), (D) cis-eQTL for *Ndr1* basal gene expression in prefrontal cortex of the LXS RI panel (n = 42). (<http://msub.csbio.unc.edu/> & <http://www.genenetwork.org/webqtl/main.py>)

alcohol-responsive gene expression within the medial prefrontal cortex (PFC) of ILS, ISS, L.5S, and S.5L mice. The PFC was selected based off our previous expression profiling studies concerning a suspected role for myelin-associated gene expression, and associated gene networks, influencing differences in acute and long-term alcohol behavioral phenotypes. Additionally, despite disputes of evolutionary differences in the neuroanatomical profile of the PFC, rodent PFC has a diverse role in a number of behavioral processes, including motor execution and reward-associated behavior (Dalley et al., 2004). Therefore, a functional genomics evaluation of this brain region in mice may be important for pinpointing genetical-genomic factors relevant to the LORR and the *Lore5* behavioral QTL.

Similar to some of our previous studies, gene expression analysis was conducted using the S-score algorithm (Zhang et al., 2002, Kennedy et al., 2006) for baseline differences or differences following 4.1 g/kg of acute (i.p.) alcohol at a 4-hour time point. Previous studies from our laboratory have utilized this time point to evaluate brain-region specific variation in alcohol elicited gene expression alongside baseline dissimilarities (Kerns et al., 2005). Following a statistical filtering with permutation analysis at a 10% FDR (Tusher et al., 2001) in the current analysis of ILS, ISS, L.5S and S.5L animals, probesets were further filtered for an average S-score ≥ 1.5 or ≤ -1.5 across three biological replicates within each treatment group. Selecting gene expression differences represented by an average S-score ≥ 1.5 or ≤ -1.5 eliminates subtle variations in gene expression influenced by biological or technical noise. Changes in gene expression due to acute alcohol exposure were inferred as surrogate events for signaling mechanisms contributing to the acute behavioral response.

Evaluating coordinate differences in baseline and alcohol-evoked gene expression led to an overall assessment of the system(s) between mice specifically bred for divergence in the LORR and a specific chromosomal interval (i.e. *Lore5*).

A total of 573 differentially expressed genes were determined due to background strain or alcohol treatment. Congruent with behavioral observations ILS mice were more sensitive to the sedative-hypnotic dose of alcohol than ISS mice. Congenic animals followed a similar patterns as their respective progenitors with L.5S having more alcohol-induced differences in gene expression compared to S.5L mice. Genotype based differences in expression depended upon background strain, which is expected because ILS and ISS mice were selectively mated for phenotypic differences related to the LORR and congenic mice only differed from progenitors at a limited chromosomal region. Thus, L.5S congenics were more similar to ILS mice and S.5L congenics were more similar to ISS mice in terms of their gene expression profile within PFC. All SAM positive S-scores filtered for values ≥ 1.5 or ≤ -1.5 were subsequently combined to visualize global patterns of gene expression using multi-variant hierarchical clustering (Figure 51B). Overt differences among the interaction of background genotype and alcohol exposure, as also previously evident from Fyn kinase knockout mice, were difficult to discern because most genes showing basal heterogeneity were also robustly regulated by an acute dose of alcohol. In order to narrow potential differences of the *Lore5* QTL interval our analysis was then restricted to comparing ILS > ISS or ILS < ISS, L.5S > ILS or L.5S < ILS, and S.5L > ISS or S.5L < ISS. Thus, only genes consistently altered either by alcohol or genetic background, falling within the *Lore5* locus, remained as candidate regulators for the behavior.

The *Lore5* interval, including both the IBD and non-IBD region, contained approximately 407 genes. The amalgamation of microarrays accounted for ~391 representative genes within the *Lore5* region; however, the arrays accounted for all of the genes within the non-IBD region. Variation in the entire *Lore5* genotype coincided with 13 differentially expressed probesets, representing 11 distinct gene products. Of the 11 genes consistently altered with the *Lore5* genotype, only N-myc downstream-regulated gene 1 (*Ndr1*) and solute carrier family 38 member 2 (*Slc38a2*) were located in the non-IBD region. The two suspected factors are located at two disparate sites of the QTL interval with *Ndr1* being located at ~ 66.76 Mb and *Slc38a2* located at ~ 96.52 Mb of chromosome 15. Comparing genotypic differences in gene expression using other analytical techniques for microarray data, such as robust multi-array analysis (RMA), further demonstrated co-variation in *Lore5* genotype and expression of *Ndr1*, but not *Slc38a2*. Consistency in expression of basal expression for *Ndr1* and the *Lore5* genotype strongly suggests *Ndr1* as a prominent factor related to the sedative-hypnotic behavioral effect of acute alcohol. Correspondent changes in *Lore5* genotype and *Ndr1* were only witnessed for basal differences, whereas alcohol-responsiveness of *Ndr1* expression appeared to be controlled by the background genotype outside of the *Lore5* interval.

Ndr1 expression as a quantitative trait for the LORR behavior. Northern blot analysis confirmed the expression patterns seen on microarrays for *Ndr1* (Figure 53A). Differential expression of *Ndr1* within a defined QTL for the LORR suggests *Ndr1* may be a quantitative trait gene (QTG) for the LORR. Adult male C57BL/6J, DBA/2J,

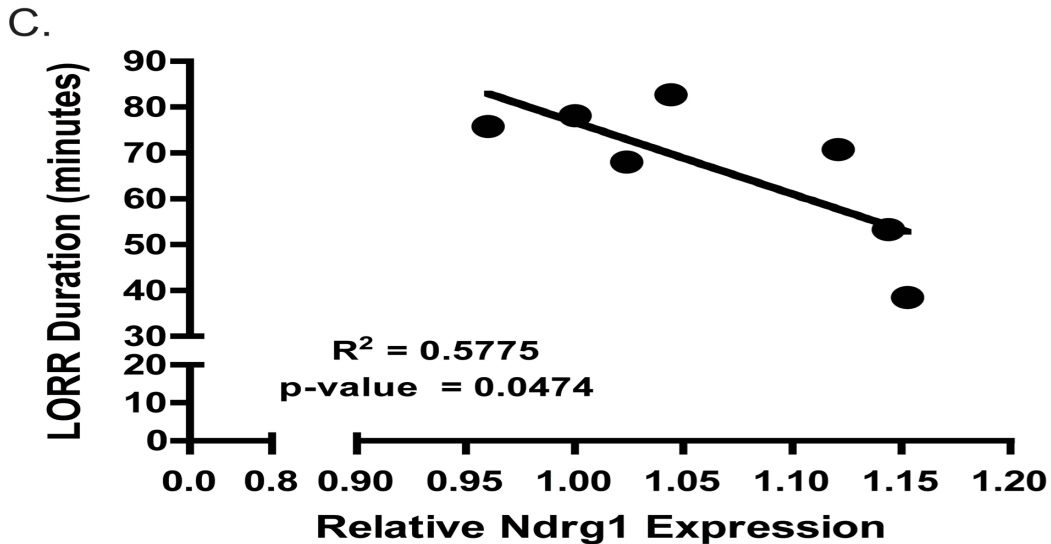
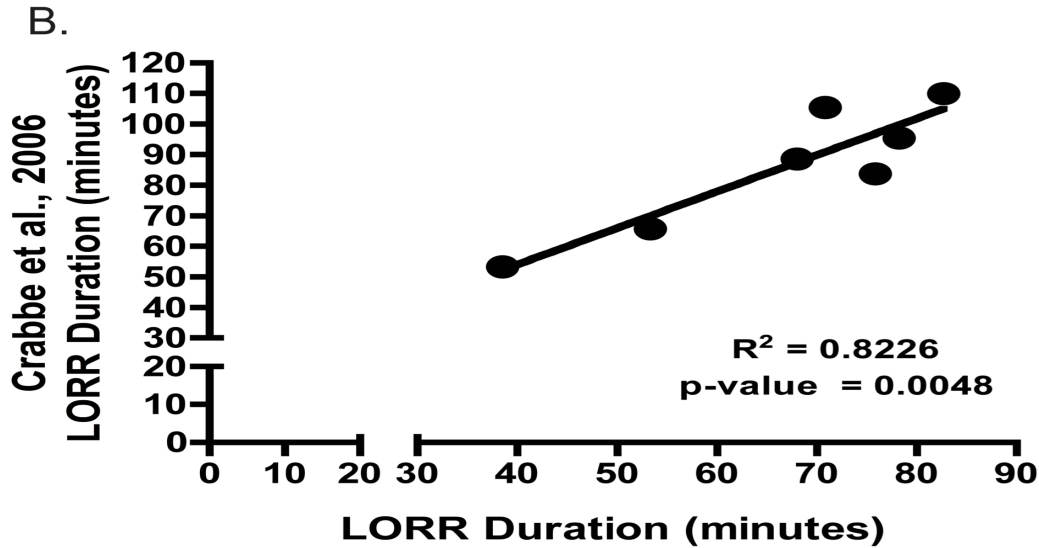
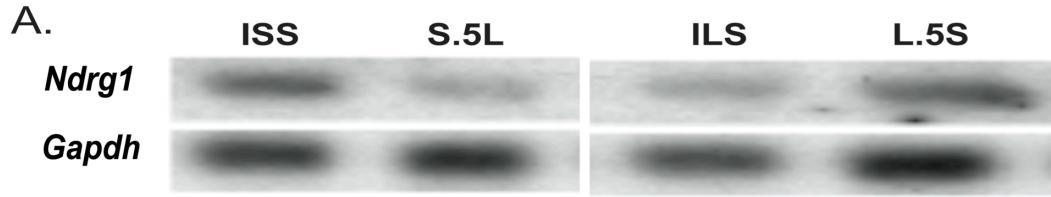


Figure 53. PFC basal *NdrG1* expression and LORR behavior. (A) Northern blot analysis of *NdrG1* mRNA within ISS, S.5L, ILS, and L.5S PFC, showing inverse relationship between *NdrG1* expression and duration in LORR, (B) confirmation of LORR phenotype in differing laboratories for C57BL/6J, DBA/2J, C3H/HeJ, BALB/cByJ, 129S1/SvImJ, A/J, and FVB/NJ mice using data from Crabbe et al., 2006, (C) significant correlation of basal PFC expression of *NdrG1* to the LORR duration across C57BL/6J, DBA/2J, C3H/HeJ, BALB/cByJ, 129S1/SvImJ, A/J, and FVB/NJ mice.

C3H/HeJ, BALB/cByJ, 129S1/SvImJ, A/J, and FVB/NJ mice were assessed for co-variation of LORR and *Ndr1* gene expression within the PFC to test the relevance of *Ndr1* as a QTG for the LORR behavior. Environmental variables may change the outcomes of alcohol-related behavioral traits conducted in different laboratories (Crabbe et al., 1999, Wahlsten et al., 2003). Therefore, durations in the LORR across the seven strains mentioned above were correlated to previously reported outcome measures for the LORR from a different laboratory, within the same strains of mice (Crabbe et al., 2006a). Duration in the LORR from the seven genetically divergent strains of mice was significantly correlated across laboratory environments (Figure 53B; $R^2 = 0.8226$, p -value = 0.0048), suggesting the behavioral phenotype is reproducible across laboratories. Basal expression of *Ndr1* significantly correlated to duration in the LORR (Figure 53C; $R^2 = 0.5775$, p -value = 0.0474). The significant association of basal abundance for *Ndr1* within PFC and alcohol-induced LORR behavior across a heterogeneous stock of mice further suggests variation in the expression of *Ndr1* is prominent factor for this specific behavioral measure.

Linkage of Ndr1 gene region to expression divergence in ILS/ISS-Lore5 genotype. Variation in the expression of the candidate gene *Ndr1* reproducibly associates with susceptibility to the duration of the LORR. Haplotype analysis of ILS and ISS animals further suggested polymorphic differences in *Ndr1* between these two strains. As part of our ongoing collaboration, Johnson and colleagues genotyped micro-satellite markers spanning the location of *Ndr1*, confirming the locus was non-IBD and each respective congenic (L.5S and S.5L) possessed an identical form of *Ndr1* from

the appropriate parental strain (S.5L = ILS, L.5 = ISS). Six genetic markers were detected within *Ndr1*, four of the markers verified genotypic differences aligned with the *Lore5* interval corresponding to sequence divergence of *Ndr1*.

Using WebQTL online resource (www.genenetwork.org) basal variation in gene expression from two available datasets (including prefrontal cortex) was analyzed alongside selective recombination events in 42 LXS recombinant inbred strains of mice, derived from ILS and ISS progenitor strains. Individually probing for *Ndr1* identified the presence of an expression quantitative trait loci occurring exactly at the location of *Ndr1* within the hippocampus (Figure 52C) and the prefrontal cortex (Figure 52D). Calculating genetic linkage for the expression of *Ndr1* at its own genomic location firmly demonstrates a cis-eQTL, suggesting differences in the assembly of nucleotides between ILS and ISS influences expression of *Ndr1* under baseline conditions. The presence of a cis-eQTL for *Ndr1* at the location of the phenotypic *Lore5* QTL further suggests that *Ndr1* is a candidate gene regulating the sedative-like behavioral response to acute alcohol exposure.

Ndr1 expression correlates with a cluster of myelin genes across microarray studies in ISS, ILS, C57BL/6J, and DBA/2J mouse strains. Although our genetical genomic analysis singled out variation of *Ndr1* as a causal factor for acute alcohol sensitivity, we hypothesized that *Ndr1* participated within the framework of a gene network to drive downstream behavioral responses to alcohol. Data from previous chapters herein, as well as previous data from our laboratory, has demonstrated a reoccurring association between *Ndr1* and large cohort of myelin-associated genes in terms of coordinate gene expression. Thus, *Ndr1* may function within the context of a

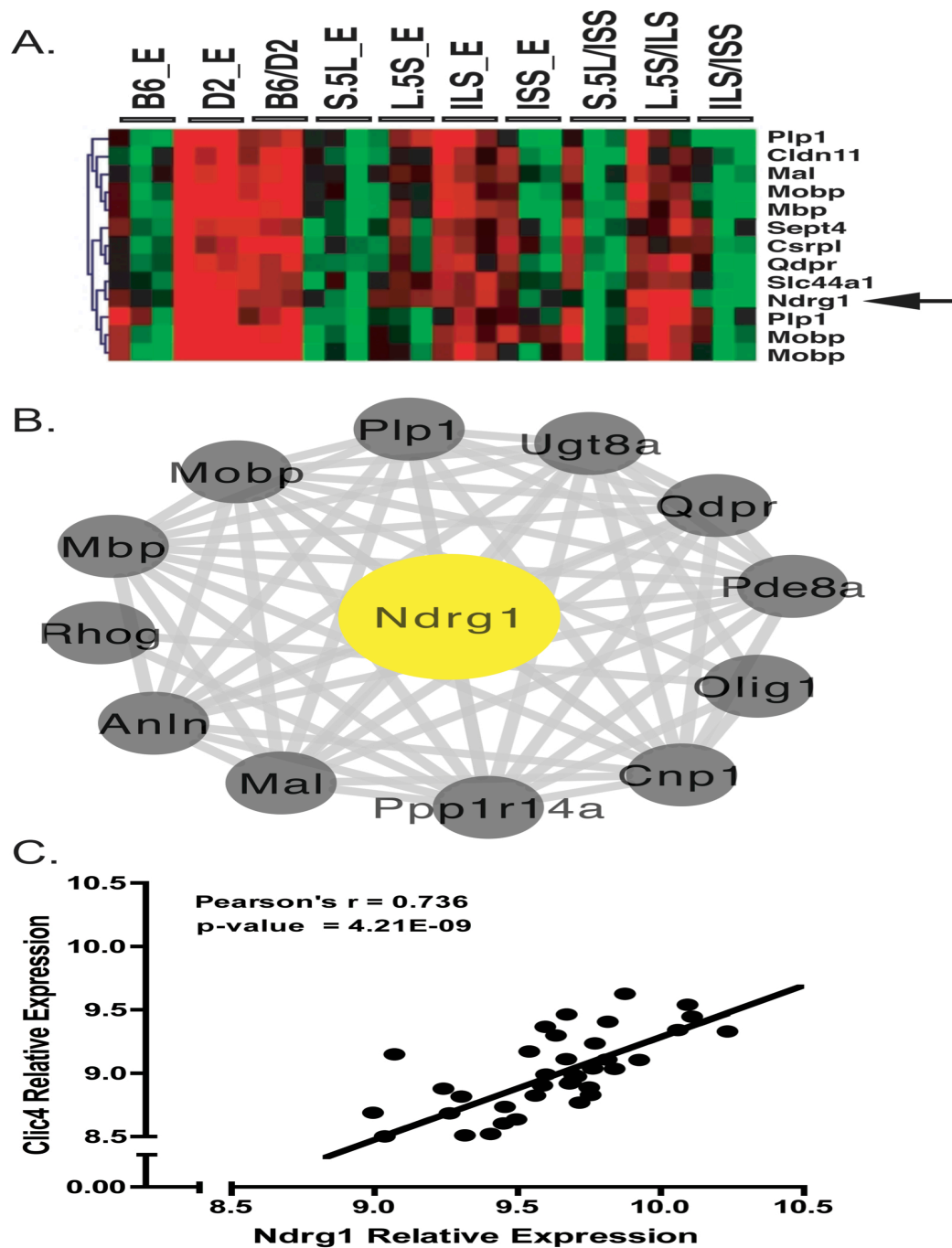


Figure 54. *Ndrg1* and associated gene expression. (A) Hierarchical clustering of gene expression for basal and alcohol-responsive gene expression six strains showing *Ndrg1* association with myelin genes and inverse relationship to acute alcohol sensitivity, (B) Cytoscape network of 13 strongly basally correlated myelin genes, including *Ndrg1*, across the LXS RI PFC, (C) Correlation of *Ndrg1* expression with *Clic4* expression across the LXS RI PFC, another gene known to influence the LORR behavior in mice.

myelin-related network across species to gauge an initial level of response and susceptibility to long-term drinking behavior. Interpreting the association between a *Ndr1* / myelin gene network and alcohol behavioral response is important because together with Dr. Adron Harris and colleagues, our previous microarray studies have shown myelin is significantly down regulated in the PFC of human alcoholics (Lewohl et al., 2000); implying a myelin network containing *Ndr1* has immediate clinical significance.

Array data from saline and alcohol-treated B6 and D2 mice was combined with that reported in the current study on ISS, ILS and the Lore5 congenic strains to show genetically convergent expression patterns across the PFC. Hierarchical clustering of the six distinct strains revealed coordinate expression patterns for *Ndr1* alongside multiple myelin-associated genes, including *Mbp* and *Plp1*, the two most abundant myelin genes within the CNS. Relative expression of this myelin-associated cluster was prominently correlated to the *Lore5* genotype and alcohol sensitivity (Figure 54A). Thus, the more alcohol behavioral sensitive strains (D2, ILS, and L.5S) exhibit lower basal myelin-associated expression, while the less behaviorally sensitive mice (B6, ISS, and S.5L) had higher relative expression of *Ndr1* and other myelin genes.

Analyzing basal gene expression across the PFC of the LXS RI panel (n = 42) further identified a network of myelin-associated genes associated with *Ndr1* (Figure 54B). The same 13 genes also strongly connected across the PFC of BXD RI mice (n = 29), and significantly correlated to acute alcohol behavioral sensitivity and alcohol drinking behavior (Table 6). Identification of differential *Ndr1* expression, as well as a network of myelin-associated genes linked to *Ndr1*, in mice selectively bred for the loss

of righting reflex behavior may implicate basal differences in a myelin gene network for this specific alcohol behavioral response (i.e. LORR behavior). *Ndr1* baseline expression in the PFC significantly correlated to other genes determined in our previous studies in mice and across species. The expression of the myelin gene claudin-11 (*Cldn11*) correlated to *Ndr1* (Pearson's $r = 0.657$, $p\text{-value} = 8.96 \times 10^{-7}$) in LXS PFC. Additionally, as shown in Figure 54C, expression of the myelin-associated gene network and *Ndr1* is correlated to PFC expression of the chloride intracellular ion channel 4 (*Clic4*) (Pearson's $r = 0.736$, $p\text{-value} = 4.21 \times 10^{-9}$). Differential expression of *Clic4* is associated with alcohol drinking behavior across the PFC of cynomolgus macaques and mice (Figure 15). Our laboratory has also demonstrated that over-expression of *Clic4* neuronal expression, using the adeno-associated virus type 2 (Srivastava et al., 1983), in mouse PFC decreases duration of alcohol-induced LORR (Bhandari et al., 2012). Coordinate expression of myelin-related genes with *Clic4* further suggests that differential basal expression of a myelin-associated gene network in PFC is a determinant of acute behavioral effects of alcohol as measured by duration in the loss of righting reflex behavior.

Acute Behavioral Sensitivity in a Demyelinated Mouse Model.

A network of myelin-associated genes in the PFC is coordinately regulated across species. Continued investigation within PFC across the BXD RI panel and progenitors, Fyn kinase knockout mice, and mice selectively bred for acute sensitivity to alcohol provided strong corroborating evidence for basal variation of a myelin-associated gene network as an underlying factor in acute behavioral sensitivity to alcohol. Among the

differing acute behavioral measures may be influenced by variation in myelin gene expression our data suggests basal variation in myelin contributes to acute functional tolerance to the sedative-hypnotic effects of acute alcohol (i.e. LORR). In order to extend these previous observations we used a demyelination mouse model to test the hypothesis that the baseline abundance of a myelin gene expression network is a crucial determinant in specifically mediating the sedative-hypnotic effects of acute alcohol exposure as determined by the LORR. Cuprizone is a copper chelating agent that selectively targets mature oligodendrocytes, causing demyelination, in adult C57BL/6 mice (Matsushima and Morell, 2001). Using the neurotoxicant cuprizone allows for the investigation of myelin in an adult animal model on a single genetic background, which are devoid of potential developmental compensation (Torkildsen et al., 2008).

C57BL/6J mice were chronically fed 0.2% (w/w) of cuprizone or normal rodent chow for a period of 5 weeks. A 5-week duration of cuprizone exposure is sufficient to facilitate regional differences for a reduction in myelin-associated gene expression (Hiremath et al., 1998, Gudi et al., 2009). Using quantitative real-time polymerase chain reaction (Q-rtPCR) (Wong and Medrano, 2005), the mRNA abundance of seven myelin genes were confirmed for decreased cuprizone-induced expression in PFC (Figure 55). This included decreased expression of the LORR quantitative trait gene *Ndr1* in response to cuprizone treatment. Exposure to 0.2% cuprizone did not alter expression of *Clic4*, which if over-expressed in PFC neurons lessens the duration for LORR. Immunostaining of coronal sections (30 μ m) with the myelin stain fluoromyelin (Wu et al., 2006) demonstrated a 20-30% decrease in width of the corpus callosum, and qualitative thinning of myelinated fibers in the corpus callosum (Figure 56). Coordinate

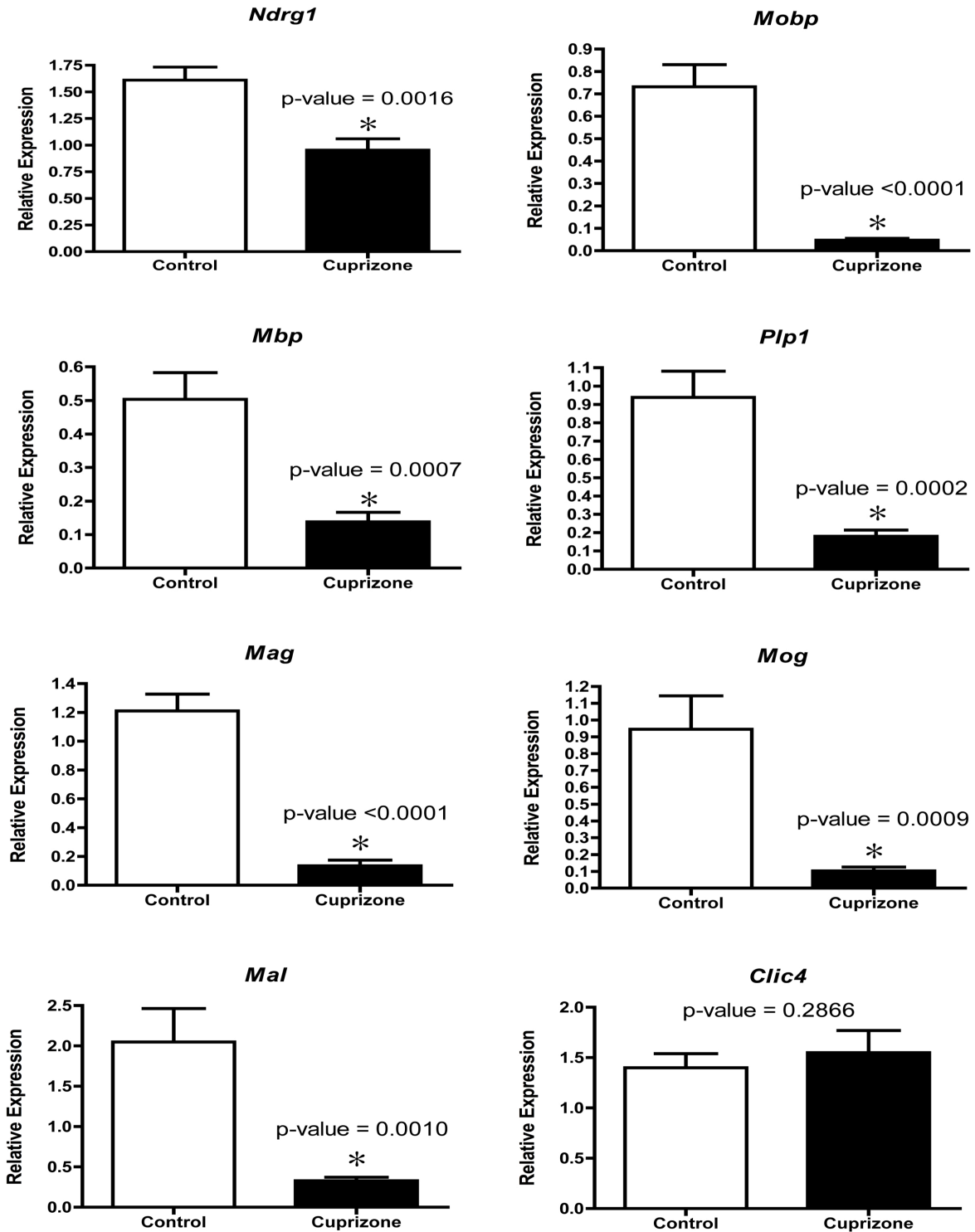


Figure 55. Validation of cuprizone model for myelin-associated gene expression. Quantitative real-time PCR analysis of *Ndr1*, *Mobp*, *Mbp*, *Plp1*, *Mag*, *Mog*, *Mal*, and *Clic4*, demonstrating a selective decrease for basal expression of the myelin gene network in mouse PFC.

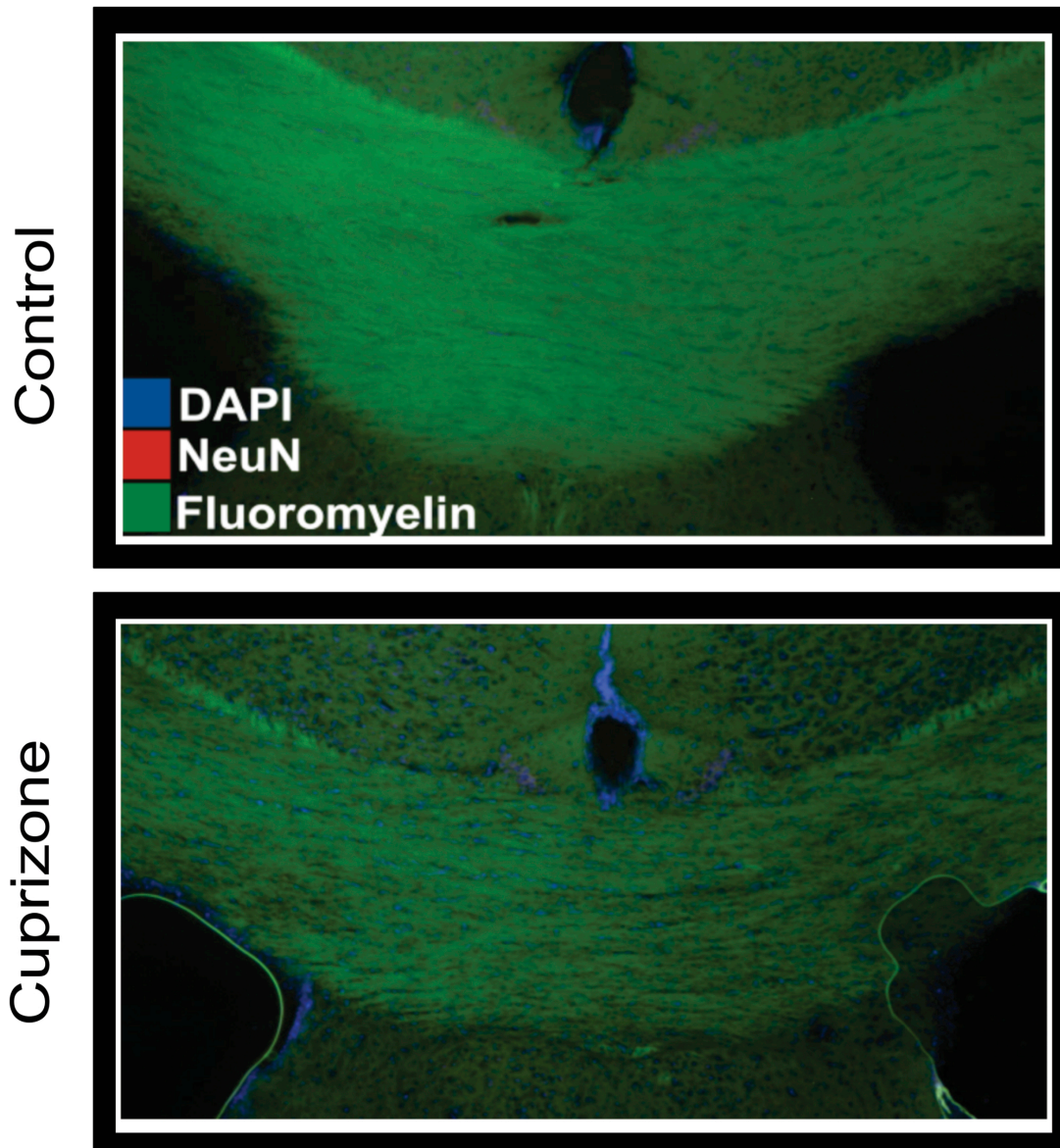


Figure 56. Validation of cuprizone model for decreased CNS white matter. Immunostaining using Invitrogen BrainStain imaging kit for DAPI (blue), NeuN (red), and fluoromyelin (green) showing decreased myelin content in the corpus callosum of mice fed a cuprizone containing diet.

decreases in myelin within the corpus callosum, and decreases in myelin mRNA abundance for seven different genes in the PFC suggests cuprizone is causing reduced expression of the entire myelin-associated gene network. Importantly, cuprizone induced down-regulation of myelin genes occurs in the PFC of rodents, but not other brain regions such as the hippocampus and striatum (Gregg et al., 2009). Decreased expression of myelin-related transcripts, resulting from a cuprizone containing diet, is associated with impairments in PFC-mediated behaviors (Gregg et al., 2009).

Assessment of Anxiety-Like Behavior. The light-dark box test, based on the natural preference of rodents for the dark compartment, was used to assess anxiety-like or anxiogenic-like behavior of cuprizone fed mice (Bourin and Hascoet, 2003). Alcohol is a powerful anxiolytic agent in mice and humans, associated with dysfunction of the PFC (Costall et al., 1988, Koob, 2006). There was no effect of acute alcohol [$F(1, 28) = 0.007$, p -value = 0.933], cuprizone exposure [$F(1,28) = 0.065$, p -value = 0.358], or interaction of acute alcohol and cuprizone [$F(1,28) = 0.873$, p -value = 0.801] (Figure 57A). Mice treated with acute alcohol showed an increase in percent distance traveled in the light [$F(1,28) = 55.40$, p -value < 0.0001] (Figure 57B) and percent time spent in the light [$F(1,28) = 49.33$, p -value < 0.0001] (Figure 57C). Saline treated mice spent ~15-20% of their time in the light-compartment, compared to alcohol treated animals spending ~50-60% of their time in the light-compartment, suggesting a general anxiolytic effect of acute alcohol administration. Cuprizone had no effect on percent distance traveled [$F(1,28) = 0.0765$, p -value = 0.784] or percent time in the light-compartment [$F(1,28) = 0.333$, p -value = 0.569]. Additionally, cuprizone showed not

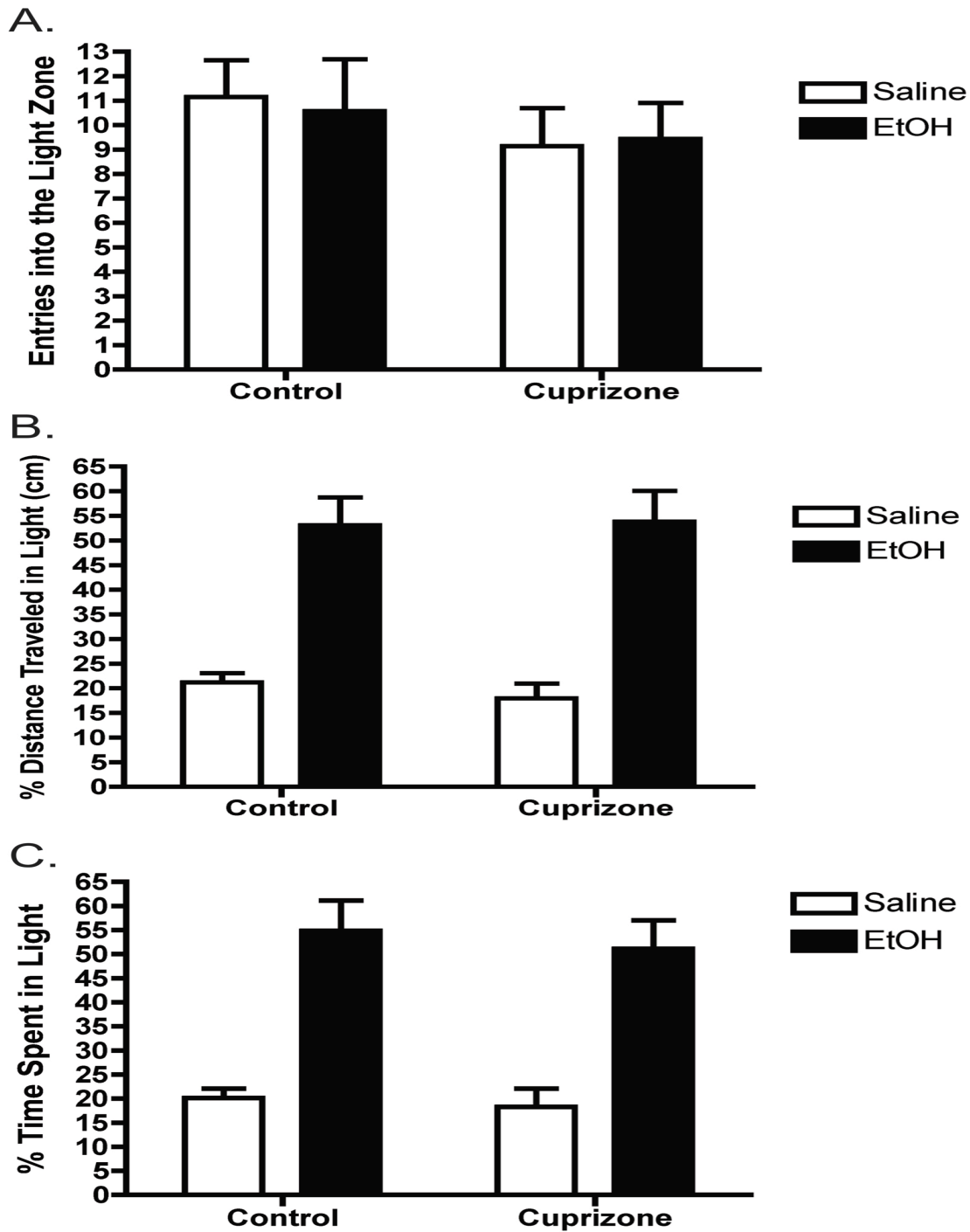


Figure 57. Cuprizone effect on anxiety-like behavior. Light dark box analysis for (A) entries into the light zone, (B) percent distance traveled in the light (PDT), and (C) percent time spent in the light (PTS) for saline and alcohol in cuprizone mice and controls. Main effect of alcohol treatment on PDT [$F(1,28) = 55.40$, p -value < 0.0001] and PTS [$F(1,28) = 49.33$, p -value < 0.0001]. Data Mean \pm SE.

interaction with alcohol for percent distance traveled in the light [$F(1,28) = 0.194$, p -value = 0.663] or percent time in the light-compartment [$F(1,28) = 0.0374$, p -value = 0.848]. Thus, decreased myelin gene expression due to cuprizone exposure has no effect on basal anxiety or alcohol-induced anxiolytic behavior.

Assessment of Locomotor Behavior. Acute 2 g/kg of alcohol may enable altered locomotor behavior depending on the genetic background of mice; however, 2 g/kg (i.p) of acute alcohol has no effect on locomotor activation in C57BL/6J mice (Phillips et al., 1995). Non-specific effects on initial exploratory or locomotor behavior may confound interpretation of anxiety-like responses and other behavioral measures. Reducing the basal amount of myelin-associated gene expression with cuprizone did not change baseline locomotor behavior [$F(1,28) = 2.22$, p -value = 0.148] or interact with alcohol for distance traveled [$F(1,28) = 1.49$, p -value = 0.233] (Figure 58A). Lack of differences in total distance traveled suggests a cuprizone-induced demyelination phenotype had no competing effects on locomotor behavior. Acute alcohol also did not alter acute locomotor activity [$F(1,28) = 1.80$, p -value = 0.191], reconfirming previous results from our laboratory and others for C57BL/6J mice. Average velocity, however, was diminished for cuprizone mice and controls [$F(1,28) = 97.1$, p -value < 0.0001] following acute alcohol (Figure 58B). Cuprizone did not alter mean velocity [$F(1,28) = 0.347$, p -value = 0.560] or interact with alcohol to change the average velocity [$F(1,28) = 0.397$, p -value = 0.534], suggesting a main effect of alcohol for this behavioral measure. The number of ambulatory episodes did not change due to cuprizone exposure [$F(1,28) = 0.559$, p -value = 0.461], acute alcohol treatment [$F(1,28) = 1.13$,

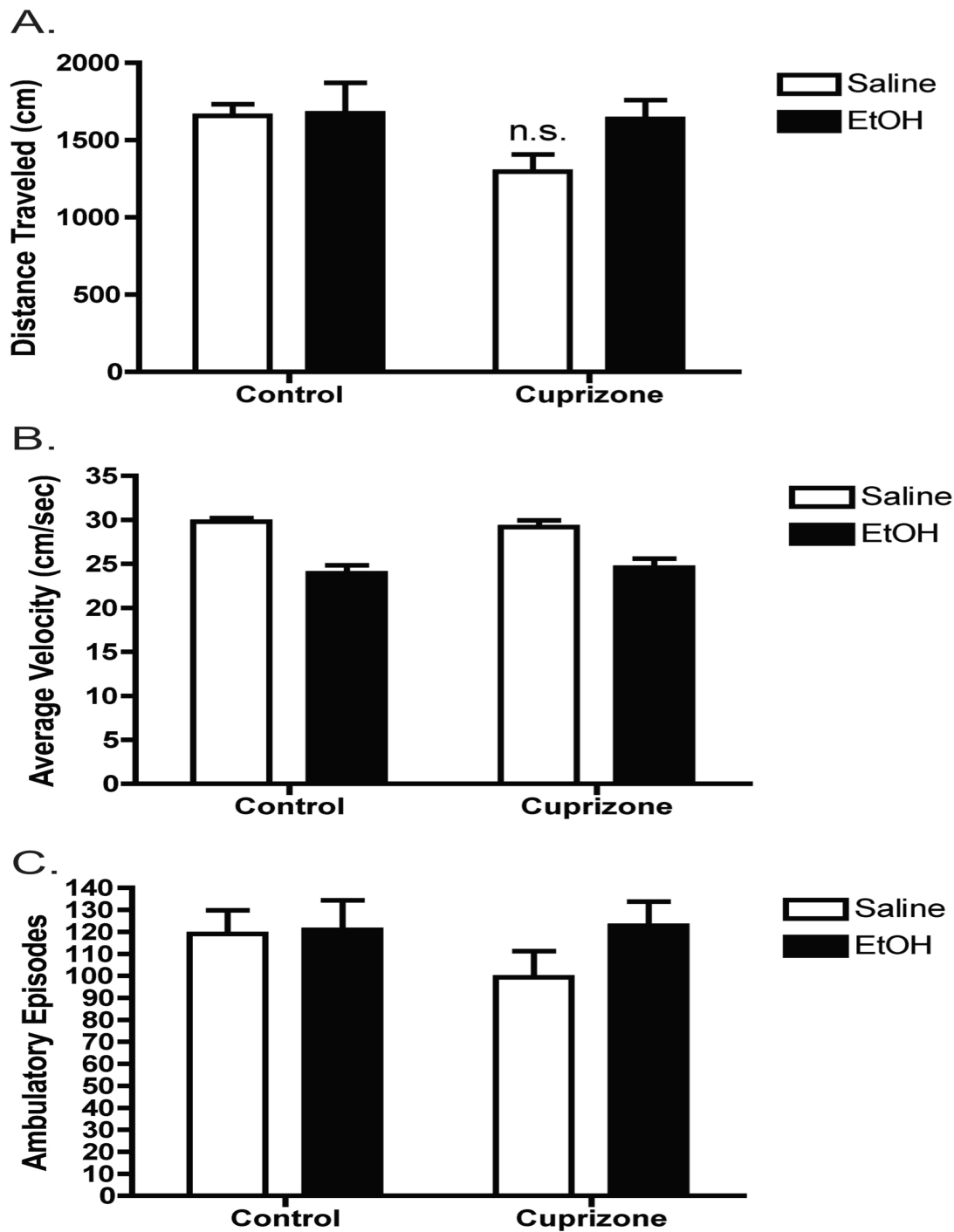


Figure 58. Cuprizone effect on locomotor behavior. (A) Distance traveled, (B) average velocity, and (C) ambulatory episodes following 2 g/kg (i.p.) alcohol administration for controls and cuprizone exposure. Main effect of alcohol administration on average velocity [$F(1,28) = 97.1$, p -value < 0.0001], but no effect on general locomotor function. Data Mean \pm SE.

p-value = 0.297], or interaction of the two factors [$F(1,28) = 0.820$, p-value = 0.373] (Figure 58C).

Mice were also monitored for jumping behavior, number of vertical counts, and number of abnormal repetitive behaviors or stereotypy. Acute 2g/kg of alcohol markedly decreased jumping behavior [$F(1,28) = 19.05$, p-value = 0.0002] (Figure 59A) and the total number of vertical movements [$F(1,28) = 97.13$, p-value < 0.0001] (Figure 59B). Cuprizone did not alter the number of jumps [$F(1,28) = 0.027$, p-value = 0.870], vertical counts [$F(1,28) = 0.347$, p-value = 0.560], or interact with acute alcohol actions on the number of jumps [$F(1,28) = 0.003$, p-value = 0.957] and vertical movements [$F(1,28) = 0.397$, p-value = 0.534]. Abnormal brain function associated with psychiatric disorders may be apparent by divergent stereotypy behavior (Garner, 2005). Stereotypy counts were reduced in response to acute alcohol administration [$F(1,28) = 24.55$, p-value < 0.0001] (Figure 59C). Decreasing myelin gene expression, using the neurotoxicant cuprizone, did not alter the number of repetitive movements [$F(1,28) = 3.33$, p-value = 0.079], or modify alcohol-induced stereotypy counts [$F(1,28) = 0.850$, p-value = 0.364]. Although acute alcohol lowered the average velocity, number of jumps, vertical counts, and stereotypy counts, there were no differences or interaction effect due to a cuprizone containing diet on general locomotor-related behavioral measures. The absence of altered locomotor responses may suggest that reduced myelin-associated gene expression does not confound the results witnessed for anxiety-like behavior or other quantitative traits associated with overt locomotor behavior.

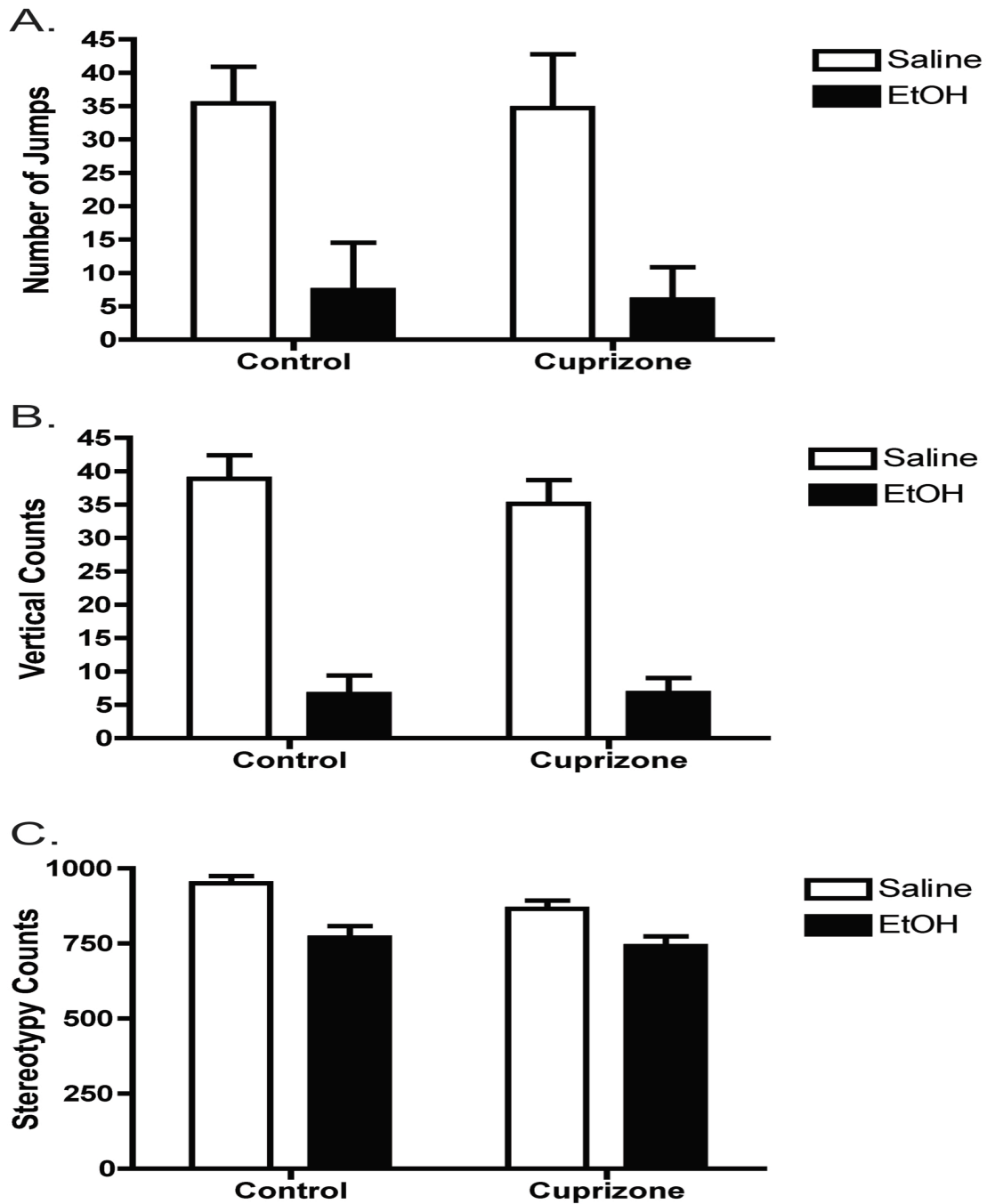


Figure 59. Cuprizone effect on additional motor-oriented behaviors. Cuprizone was insensitive to behavioral effects on (A) jumping activity, (B) number of vertical movements, or (C) stereotypy counts; demonstrating a main effect of alcohol administration on jumping [$F(1,28) = 19.05$, p -value < 0.0002], vertical counts [$F(1,28) = 97.13$, p -value < 0.0001], and stereotypy [$F(1,28) = 24.55$, p -value < 0.0001]. Data Mean \pm SE.

Assessment of Gaiting Behavior. Ataxia and fine-motor coordination were determined using the footprint test, also referred to as gait analysis, (Crawley, 1999) for mice on normal rodent chow and food supplemented with cuprizone. Pronounced loss of CNS myelin can impair fine-motor control in mice (Jensen et al., 1993, Emery et al., 2009). Stride length, sway length, and stance length were recorded from paw tracings for individual mice (Figure 60A). Cuprizone did not alter stride length [$t(10) = 0.323$, p -value = 0.753] (Figure 60B), stance length [$t(10) = 0.097$, p -value = 0.924] (Figure 60C), or sway length [$t(10) = 0.164$, p -value = 0.873] (Figure 60D). The near equivalent gait analysis measurements indicates decreased CNS myelin, resulting from 5 weeks of cuprizone exposure does not interfere with coordinated fine-motor control.

Handling-Induced Convulsion Behavior. A state of CNS hyper-excitability follows acute high-dose and chronic exposure to alcohol, causing convulsions in withdrawn mice (Mc and Fingl, 1958). Damage to the myelin sheath can facilitate CNS mediated convulsing activity (You et al., 2011). A blinded observer scored handling-induced convulsions (HIC) in cuprizone fed mice and controls for baseline HIC activity, and subsequent a single exposure of 4-g/kg intraperitoneal alcohol. Cuprizone did not change baseline susceptibility to HIC [$t(30) = 0.361$, 0.533] (Figure 61A). Video recording of HIC every hour from 2-8 hours post alcohol exposure lead to a time-dependent an increase in HIC behavior [$F(6, 84) = 7.29$, p -value < 0.0001] (Figure 61B), but showed no interactions with cuprizone diet [$F(6, 84) = 0.938$, p -value = 0.472]. HiC scores typically reach a maximum threshold approximately 7-hours post alcohol exposure (Belknap et al., 1993, Roberts et al., 1995). Collapsing the data for

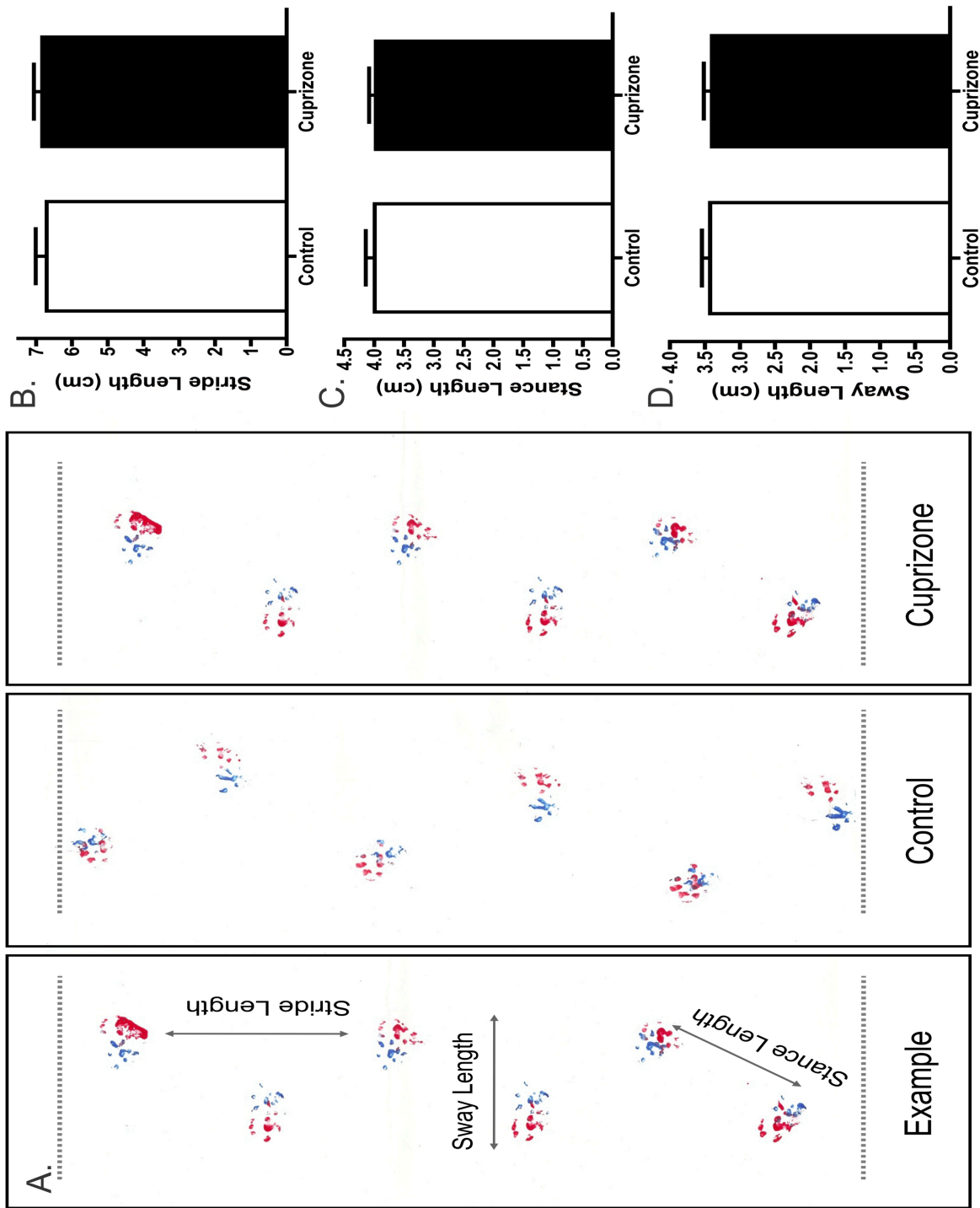


Figure 60. Cuprizone effect on gaiting behavior. (A) Gait analysis was conducted to determine potential effects on fine-motor coordination, demonstrating no effect on (B) stride length $t(10) = 0.323$, $p\text{-value} = 0.753$, (C) stance length $t(10) = 0.097$, $p\text{-value} = 0.924$, or (D) sway length $t(10) = 0.164$, $p\text{-value} = 0.873$, suggesting no adverse effects of cuprizone on mouse gaiting behavior. Data Mean \pm SE.

area under the curve (Buck et al., 1997) from 2-8 hours revealed no differences between cuprizone and controls for alcohol-induced withdrawal behavior [$t(14) = 0.476$, p -value = 0.641] (Figure 61C). Genetic variation and divergent expression of the quantitative trait gene multiple PDZ domain protein (*Mpdz*) is a major determinant of withdrawal episodes from alcohol and other drugs of abuse (Fehr et al., 2002, Fehr et al., 2004, Shirley et al., 2004). Expression of *Mpdz* is associated with myelin gene expression in PFC (Kerns et al., 2005). Q-rtPCR demonstrated *Mpdz* expression was not altered by acute 4 g/kg of alcohol or a cuprizone containing diet [Interaction effect: $F(1,28) = 0.006$, p -value = 0.940] (Figure 61D). Thus, cuprizone does not cause altered HIC behavior following an acute high-dose alcohol exposure or alter the expression of a QTG for HIC activity. Animals also did not differ for HIC behavior following 24 hours of alcohol withdrawal (data not shown).

Loss of Righting Reflex Behavior. Microarray analysis of the PFC from C57BL/6J, DBA/2J, Fyn kinase knockout mice, Inbred Long-Sleep, Inbred Short-Sleep, and *Lore5* congenic mice suggested an inverse relationship between myelin-associated gene expression and alcohol-induced loss of righting reflex behavior. Cuprizone exposure led to a significant decrease of expression in a myelin-associated gene network within PFC (Figure 55). The reduction in myelin gene expression did not affect the onset for the LORR following a sedative-hypnotic dose of acute alcohol administration [$t(18) = 0.600$, p -value = 0.556] (Figure 62A). Both cuprizone fed animals and controls acquired the LORR behavior with a mean onset time approximately equal to 85 seconds. Animals treated with saline did not acquire the LORR behavior at any time-point. Cuprizone mice

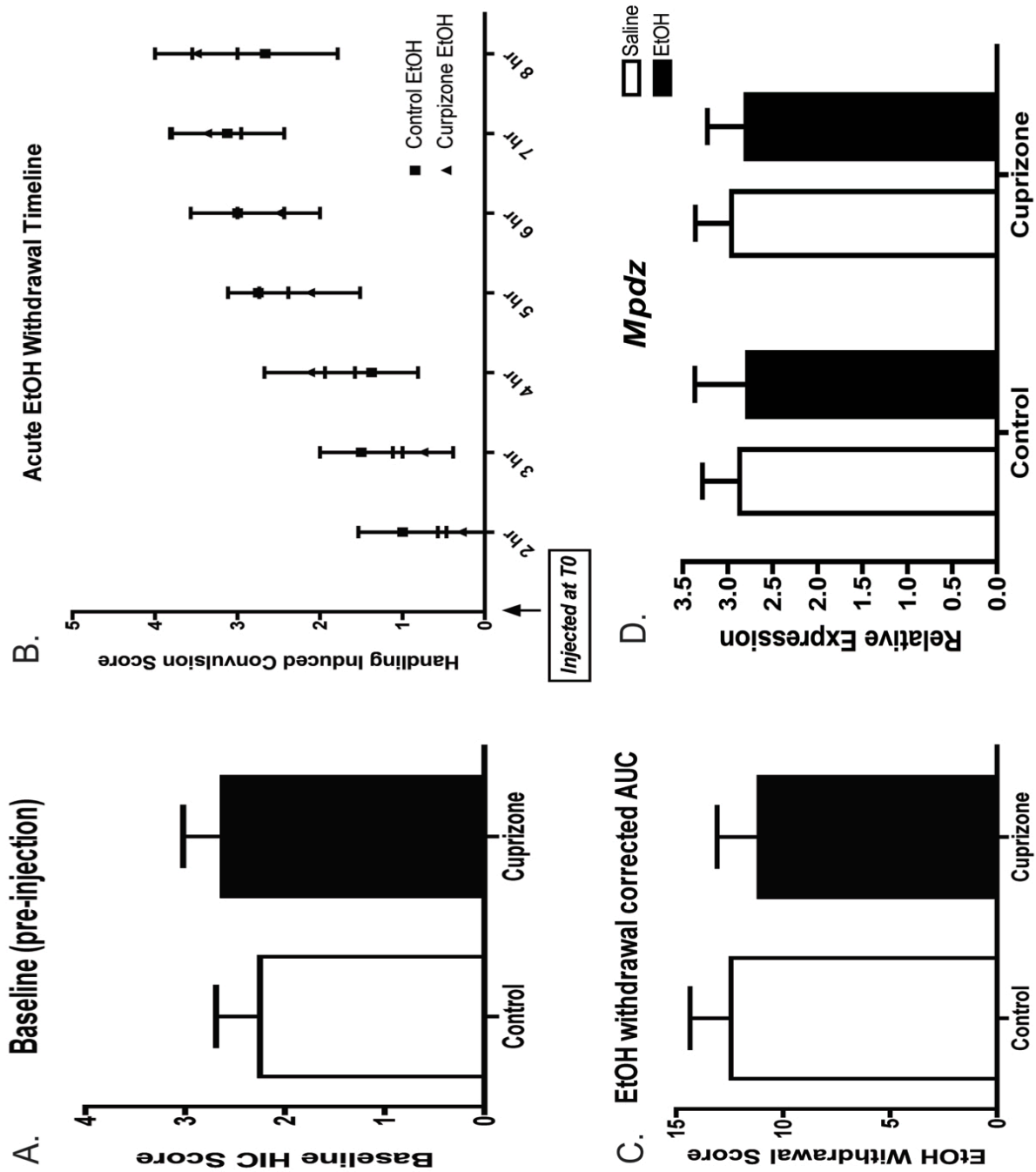


Figure 61. Cuprizone effect on handling induced convulsion (HiC) behavior. Blinded observation of HIC activity revealed cuprizone had no effect on (A) baseline HIC $t(30) = 0.361, 0.533$, (B) alcohol-withdrawal HIC time-course [$F(6, 84) = 7.29$, p -value < 0.0001], or (C) area under the curved (AUC) for alcohol-induced withdrawal scores $t(14) = 0.476$, p -value = 0.641, suggesting no adverse effects of cuprizone on CNS hyperexcitability. Cuprizone also did not effect expression of the HIC quantitative trait gene *Mpdz* in PFC [$F(1, 28) = 0.006$, p -value = 0.940]. Data Mean \pm SE.

had longer sleep time following 4 g/kg of alcohol exposure, with greater duration in their LORR behavior compared to controls [$t(18) = 3.44$, $p\text{-value} = 0.0029$] (Figure 62B). Control mice had a mean duration of LORR equal to 42.4 minutes, compared to a mean duration for the LORR of 60.7 minutes in cuprizone-exposed mice. Altered LORR behavior was not explained by non-specific effects on alcohol metabolism, with a main-effect of time between cuprizone and controls [$F(4,35) = 24.69$, $p\text{-value} < 0.0001$]. Differences in the LORR behavior are also not explained by confounding effects of cuprizone on motor function due to the lack of significant effects of cuprizone on locomotor-related behavior or gaiting behavior. The increased duration of the LORR suggests reduced myelin gene expression is associated with reduced acute-functional tolerance to the sedative-hypnotic effects of acute alcohol administration.

Discussion

Using whole-genome oligonucleotide microarrays, expression profiling was applied to aid in the identification of candidate QTGs underlying the *Lore5* QTL spanning a portion mouse chromosome 15. The *Lore5* QTL influences the duration in the loss of righting reflex (LORR) behavior, a measure of acute neurosensitivity and development of acute tolerance to a sedative-hypnotic dose of alcohol (Tabakoff and Ritzmann, 1979). Localizing the causal genetic variant or variants underneath the plethora of complex trait QTLs for alcohol and other drugs of abuse has been frustratingly elusive, with only a limited number of QTGs having been conclusively identified (Phillips et al., 2008, Spence et al., 2009). High-throughput methods of measuring differential expression and sequence variation have accelerated the identification of genetics

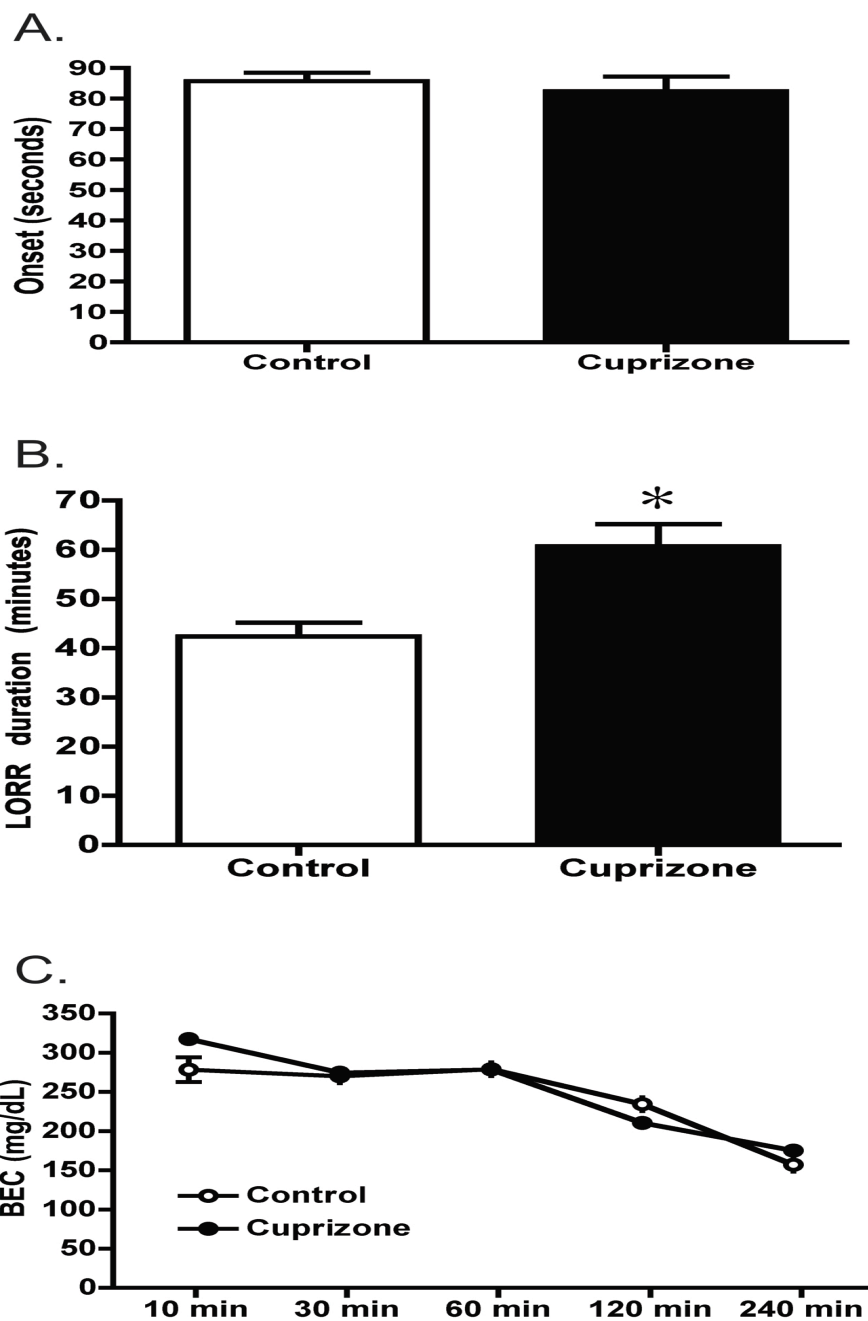


Figure 62. Cuprizone effect on LORR behavior and alcohol metabolism. Cuprizone treatment did not effect alcohol-induced (4 g/kg i.p.) (A) time of onset for LORR $t(18) = 0.600$, p -value = 0.556, but greater mean duration in LORR behavior compared to controls $*t(18) = 3.44$, p -value = 0.0029, confirming effects related to expression of *Ndr1*. Cuprizone also did not have off-target effects with a main effect of time (C) alcohol metabolism [$F(4,35) = 24.69$, p -value < 0.0001], suggesting a neurobiological effect of cuprizone on LORR. Data Mean \pm SE.

factors shaping the manifestation of complex traits. Although the precise brain-region or physiological system underlying the LORR is unknown, the PFC is an important point of convergence in neural processing and motor execution. Comparing an unbiased genomic profile for mice selectively bred for differentially sensitivity to the LORR, including interval specific congenics for a known behavioral QTL, our analysis has uncovered a restricted amount of gene expression patterns related to the *Lore5* QTL. Pairing together genetics, genomics, and network- based bioinformatics approaches demonstrated the LORR is driven through a network of myelin-associated genes anchored by a genetic variant in N-myc downstream-regulated gene 1 (*Ndrg1*) beneath the *Lore5* interval.

Genomic evaluation of reciprocal congenics and inbred mice used in this study combined with recent high-density genotyping of the ISS and ILS strains, limited the *Lore5* interval to two regions of Chr 15 (56.99-72.64 and 94.71-94.71 Mb). Dissection of this Chr 15 region assumes genes located within the IBD region are not genetically divergent between selected strains. A recent SNP analysis of 198 genetically distinct strains of mice, including ILS and ISS mice, further demonstrated a large portion of the *Lore5* QTL is IBD. C57BL/6 mice, having a very dense database of publicly available genetic markers, also possessed a very similar haplotype block structure compared to ILS and ISS mice across the IBD interval. Although undetected mutations may still exist within the IBD region of the *Lore5* QTL, our *in silico* analysis by and large suggests causal genetic variants rest within 56.99-72.64 or 94.71-94.71 Mb on Chr 15. Additionally, no genes within the IBD region of the *Lore5* QTL, or the more distal region 94.71-94.71 Mb, revealed any cis-eQTLs across the LXS RI population. Expression of

Ndr1 within both the prefrontal cortex and hippocampus demonstrated a cis-eQTL, suggesting the expression of *Ndr1* is linked to recombination events occurring between genetic offspring of ILS and ISS progenitors. The existence of a cis-eQTL aligned with a phenotypic QTL advocates regulatory variation corresponding to the presence of disease or intermediate phenotypes linked to disease (Gilad et al., 2008, Hansen et al., 2008, Williams et al., 2009).

Several investigations of ILS and ISS mice, and their descendents, have delineated several genetic loci contributing to the LORR and other alcohol-related behavioral traits. Similar to other genetic inquires of substance abuse and other complex traits; progress has been slow towards uncovering the genetic components underlying the LORR (Crabbe, 2002, Flint and Mackay, 2009). Our genetical-genomics analysis of the *Lore5* QTL for both basal and alcohol-response gene expression strongly supports a role for *Ndr1* in mediating acute sensitivity to the LORR. Differences in baseline expression for *Ndr1* were initially confirmed through Northern blot analysis of ILS, ISS, L.5S, and S.5L mouse PFC. PFC expression of *Ndr1* under baseline conditions also correlates to the duration in LORR across a heterogeneous stock of mice, further implicating *Ndr1* as a probable QTG for the sedative-hypnotic properties of alcohol. Combined with the cis-eQTL inquiry of LXS RI mice, these data suggest that *Ndr1* gene regulatory sequence variation between genotypes may cause differential expression of the gene and contributes to the observed behavioral differences in response to high-dose alcohol administration.

The precise function of *Ndr1* is uncertain, but *Ndr1* has been implicated in multiple biological processes. Variation in the expression of *Ndr1* may serve as an

early indicator in different cancerous growths (Chua et al., 2007, Inagaki et al., 2009, Strzelczyk et al., 2009). Cell growth and differentiation related transcription factors myelocytomatosis oncogene (*Myc*), p53 gene product (*p53*), and hypoxia inducible factor-1 alpha (*Hif1a*) serve as upstream regulators of *Ndr1* (Ellen et al., 2008). Functioning within the CNS, *Ndr1* has an uncharacterized role in neuronal-glia cell differentiation and myelination (Kalaydjieva et al., 2000, Berger et al., 2004). Over stimulation of the proto-oncogene *Myc* inhibits the expression of *Ndr1*, leading to escalating hypomyelination (Jensen et al., 1998). Deficiencies or mutations in *Ndr1* are known to produce a progressive demyelination phenotype (Kalaydjieva et al., 2000, Okuda et al., 2004), suggesting that *Ndr1* expression is an important regulator for a subgroup of myelin genes. The expression pattern for *Ndr1* clusters with a group of myelin genes that also have basal expression patterns associated with the Lore5 genotype and behavioral sensitivity. Basal expression for a network of myelin-related genes strongly correlates to *Ndr1* expression within PFC across two different recombinant inbred panels of mice. The myelin gene network, including *Ndr1*, significantly correlates to acute sensitivity and alcohol consumption within the PFC of BXD mice. Furthermore, decreasing the expression of *Ndr1* and the myelin gene network with the neurotoxicant cuprizone reliably altered the predicted phenotype (i.e. LORR), without significantly impairing gross motor function.

Cross-species network analysis of gene expression determined alcohol-drinking behavior is significantly associated with myelin-related gene expression across mice, monkeys, and humans within the PFC. CNS myelin integrity is severely compromised in the frontal systems of alcoholics (Pfefferbaum et al., 2009), and is down-regulated in

prefrontal cortex of alcoholics (Lewohl et al., 2000). In addition to alcoholism, myelin dysfunction occurs in a myriad of substance abuse and neuropsychiatric disorders (Lim and Helpert, 2002). Myelin-related transcripts are also down regulated in schizophrenia and bipolar disorder (Davis and Haroutunian, 2003, Tkachev et al., 2003).

Commonalities between alcoholism and other psychiatric conditions for abnormal myelin gene expression and structure may suggest comorbid pathophysiology related to oligodendrocytes dysfunction underlying CNS mediated diseases. Although aberrant myelin expression clearly occurs in alcohol dependence from chronic heavy alcohol drinking behavior, our results point towards a novel role of basal myelin abundance in neurobehavioral functions related to acute alcohol exposure.

Myelin gene discrepancies within the CNS during critical developmental periods may confer vulnerability to neuropsychiatric conditions (Harris et al., 2009). Subtle variations in myelin are associated with subtle differences in cognitive and behavioral performance (Perrin et al., 2008, Paus, 2010). Oligodendrocytes, the myelin forming cells of the CNS, express growth factors that support neuronal function and survival (Du and Dreyfus, 2002). Myelination influences structural and functional plasticity within neuronal populations (Woodhoo et al., 2009, Raiker et al., 2010), which may suggest dynamic interactions among CNS cell types and consequential behavioral processes. The precise function of some myelin-related genes is still uncertain (Montague et al., 2006); however, NDRG1 is proposed to regulate cholesterol transport and associated intracellular trafficking mechanisms (Hinds et al., 2004, Hunter et al., 2005). The composition of myelin is fundamentally different from the cell membrane, enriched with myelin structural proteins, glycosphingolipids, and cholesterol (Gielen et al., 2006).

Thus, NDRG1 may play a central role in the variation of myelination through regulating protein-lipid interactions and trafficking to site of neuronal ensheathment.

Our work suggests that *Ndr1* and a myelin-related gene network may play a fundamental role in alcohol-induced loss of righting reflex. Additional evidence from our previous microarray studies further suggests that variation in a myelin-associated gene network within the PFC is an underlying aspect of acute alcohol sensitivity and long-term drinking behavior. Continued investigation of *Ndr1* is necessary to understand the role of *Ndr1* in the regulation of myelination and alcohol behavioral phenotypes; however, *Ndr1* may function as a QTG for the sedative-hypnotics effects of alcohol. Acting within the framework of a genetical-genomics network, we propose *Ndr1* is an important molecular endophenotype for long-term risk of developing an alcohol use disorder.

CHAPTER 7:

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Myelin is a major constituent of the vertebrate nervous system, accounting for nearly half of the total volume within the human brain (Filley, 2010). Insulating neuronal axons, myelin facilitates the saltatory conduction of action potentials along nodes of Ranvier to permit efficient communication throughout different brain regions (Zoupi et al., 2011). The vast neuronal architecture of the human brain relies on up to 176,000 km of myelinated fibers to properly function (Marnier et al., 2003). Although the multi-lamellar structure of myelin sheaths are comprised largely of lipids, CNS myelin is associated with approximately 342 proteins (Jahn et al., 2009). Major, as well as minor, alterations in the amount of CNS myelin can impair cognitive behavior (Bartzokis et al., 2007, Johansen-Berg, 2010).

Chronic alcohol abuse leads to myelin deficits, particularly in frontal regions, associated with cognitive and motor function impairments (Pfefferbaum et al., 2009). The maximum amount of daily alcohol consumed negatively correlates to the degree of myelin deficiency (Krill et al., 1997), with pronounced loss occurring in the prefrontal cortex white matter (Harper and Matsumoto, 2005). Myelin gene expression is significantly down regulated in the prefrontal cortex of alcoholics (Lewohl et al., 2000, Mayfield et al., 2002). Interestingly, deficits of myelin are also observed in the frontal-limbic systems, including the prefrontal cortex, for major depressive disorder (Ouyang et al., 2011, Tham et al., 2011), substance abusers without comorbid alcohol problems (Schlaepfer et al., 2006), schizophrenia (Katsel et al., 2005, Regenold et al., 2007), as

well as other psychiatric and neurological disorders such as autism spectrum disorder (Fields, 2008, Zikopoulos and Barbas, 2010).

Convergent evidence in myelin abnormalities from multiple CNS-related disorders may suggest a common underlying mechanism in disease; however, the extent to which myelin affects differing behavioral systems is unclear. Additionally, aberrant myelin expression may be a common secondary factor caused by different primary mechanisms for separate neuropsychiatric or neurological diseases. Characterizing intermediate phenotypes for both behavioral and molecular processes may assist in defining key aspects of disease (Meyer-Lindenberg and Weinberger, 2006). Divergence in the coordinate regulation of gene expression is one potential molecular endophenotype leading to behavioral perturbations (Gould and Gottesman, 2006). The advent of microarray technologies has led to a high-throughput methodology for an unbiased, parallel assessment of gene expression within complex systems (Sobek et al., 2006). A previous microarray study from our laboratory determined basal and alcohol-responsive brain-region specific gene expression patterns in mesocorticolimbic system of C57BL/6J and DBA/2J mice (Kerns et al., 2005); two strains of mice that differ in acute alcohol sensitivity and long-term drinking behavior. Variation in acute behavioral responses to alcohol is an indicator of the long-term risk of heavy-drinking behavior in humans and animal models (Schuckit, 1994, Metten et al., 1998b). Myelin-associated gene expression was differentially regulated by acute alcohol exposure and under baseline conditions in the PFC between C57BL/6J and DBA/2J mice (Figure 6). Contrasting gene expression profiles in myelin-related genes pointed towards a possible role of myelin within PFC for acute alcohol sensitivity and self-administration. Based on

these observations in mice and previous findings from postmortem alcoholic brain tissue we hypothesized that the expression of a myelin-associated gene network is an important determinant of alcohol behavioral phenotypes.

Gene expression data was obtained for chronic alcohol drinking female and male cynomolgus macaques, rhesus macaques, humans, and a panel of BXD recombinant inbred mice. Applying weighted gene co-expression network analysis (WGCNA) to each of these five datasets revealed a statistically conserved co-expression pattern for myelin-associated gene expression with the PFC across species (Figure 35). The myelin gene network significantly correlated to alcohol drinking behavior in mice and rhesus macaques, which sufficiently varied in alcohol intake. The amount of alcohol consumed for human samples was unavailable. Focusing on rhesus macaques as a surrogate for the neurobiology of chronic alcohol exposure in human brain revealed a set of ten gene expression networks associated with average daily alcohol intake spanning one year of self-administration behavior (Figure 23). This core group of ten alcohol drinking-related gene networks may be important for future targeted inquiries of genetic or genomic aspects of chronic alcohol exposure. Combined, the networks were over-represented in binding sites for several key microRNAs, including miR-9, miR-212, and miR-219. Alcohol up-regulates miR-9 and contributes to mechanisms in alcohol tolerance (Pietrzykowski et al., 2008). Signaling events regulated by miR-212 confer vulnerability to cocaine addiction (Hollander et al., 2010, Im et al., 2010), but has yet to be directly studied in relation alcohol abuse. Inhibition of miR-219 impairs NMDA receptor transmission and associated behaviors (Kocerha et al., 2009). Oligodendrocytes rely on miR-219 for developmental control of myelination (Dugas et

al., 2010), suggesting microRNAs may mediate different actions in differing CNS cell types. The role of miR-219 in alcohol drinking behavior and acute behavioral responses has also yet to be investigated.

MicroRNAs play key roles in CNS plasticity and behavior (Dreyer, 2010, Bredy et al., 2011). Directly profiling expression of microRNAs in the PFC of controls and alcohol-exposed subjects will provide another layer of experimental data to supplement the gene expression results obtained across species. Coordinate regulation of microRNAs and mRNA with shared functional roles may help define transcriptional networks operating in response to acute and chronic alcohol. Profiling the expression pattern of microRNAs from intact brain tissue, rather than isolated cell-types, may be essential given the diverse expression of microRNAs among different brain regions and CNS cell types (Cao et al., 2006, Kapsimali et al., 2007). Comparing microRNA networks across species may further help narrow large inventories of microRNA to a limited conserved set of microRNAs. Elucidating specific microRNA focal points sharing expression patterns and sequence homology will be important given the diversity of microRNAs across differing species (Berezikov et al., 2006, Bak et al., 2008). Variation in microRNAs and microRNA networks may serve as modifiers of gene expression networks acting on complex traits (Bandiera et al., 2010, Su et al., 2011). Our analysis suggests differential expression of miR-219 may be a critical point of intersection for alcohol behavioral traits through downstream effects on NMDA receptor function and coordinate expression of myelin-associated gene expression.

The myelin-associated gene network within the rhesus macaque and BXD RI mouse PFC datasets significantly correlated to alcohol intake; however, coordinate

expression was positively, and not negatively, associated with drinking behavior. Multiple studies conducted on alcoholic brain tissue have ascertained an inverse association between alcohol consumption and myelin brain volume (Harper and Kril, 1990, Harper, 2007). The discrepancies between our preclinical models and the effects on myelin gene expression in humans may be due to the timeline of alcohol intake and patterns of alcohol self-administration. In view of our previous analysis of C57BL/6J and DBA/2J mouse PFC (Kerns et al., 2005), a positive correlation to alcohol drinking behavior in mice and non-human primates also put forth the possibility that higher basal myelin gene expression was related to increased alcohol intake patterns and decreased sensitivity to acute alcohol-related behaviors. The conservation of a myelin gene network across species and a compilation of previous alcohol behavioral genetic studies conducted on BXD RI strains allowed us to test the hypothesis that basal variation of myelin-related gene expression in PFC was an underlying factor in alcohol behavioral phenotypes. Myelin was strongly conserved across 71 different strains of mice within PFC (Figure 40&41). The expression and connectivity of the PFC myelin network was unlike available data from nucleus accumbens and ventral tegmental area across the BXD RI panel (Figure 42). Most importantly, the PFC myelin network, as well as individual myelin genes, was positively correlated to alcohol drinking behavior and inversely associated with acute sensitivity. Future studies on BXD RI mice selected for extreme differences in myelin gene expression could be used to verify the behavioral results from our *in silico* analysis. In addition to behavioral phenotypes, the myelin gene network may be regulated through correlated transcripts. For example, the progesterone receptor (*Pgr*) is significantly correlated to the first principal component of

the myelin gene expression network. Progesterone is neurosteroid known to promote myelination and myelin repair (Schumacher et al., 2012). The progesterone metabolite allopregnanolone, and other endogenous neurosteroids, may exert differences in the positive (i.e. reward) and negative (i.e. withdrawal) effects of alcohol (Finn et al., 2010). Thus, further investigation into interaction of the progesterone receptor and the myelin gene network may reveal novel insights into neurobiology of short and long-term alcohol behavioral phenotypes.

The alcohol drinking model employed for the BXD RI mice used for our chronic alcohol exposure studies were performed using an alcohol vapor intermittent access model originally developed for C57BL/6J mice (Becker and Lopez, 2004, Lopez and Becker, 2005). Differences in genetic background may impart differential sensitivity to the effects of alcohol on myelin-associated gene expression. C57BL/6J mice are insensitive to acute alcohol regulation of myelin gene expression in PFC, as was evident for DBA/2J mice. Additionally, due to the robust metabolism of alcohol in mice the alcohol vapor model uses the alcohol dehydrogenase inhibitor pyrazole (Blomstrand et al., 1979) to establish consistent blood and brain alcohol concentrations during vapor exposure. Pyrazole has off target effects, acting as a non-competitive antagonist for NMDA receptors (Pereira et al., 1992). Antagonists of NMDA receptors possess neuroprotective effects within the CNS (Choi et al., 1988, Choi and Rothman, 1990). Brain damaged caused by chronic alcohol exposure and withdrawal partially the result of excitotoxicity related to increased NMDA receptor function (Lovinger, 1993). Excitotoxicity as result of glutamate receptor malfunction also causes damage to oligodendrocytes and myelin (Matute et al., 2007). Although air controls also receive

pyrazole injections, in the absence of adverse side effects pyrazole may be masking the neurotoxic effects of chronic alcohol exposure in mice. Using alternative drinking models, such as two-bottle choice intermittent access (Wise, 1973, Simms et al., 2008) or the liquid diet technique (Lieber and DeCarli, 1989), on a variety of genetic backgrounds may help refine an animal model for reductions in CNS myelin gene expression. Selection of the proper genetic background may be essential due to differential regulation of alcohol between strains (Kerns et al., 2005), and some strains of mice have pre-existent missing or incomplete major white matter fiber tracts (Livy and Wahlsten, 1991).

Although our analyses of chronic alcohol exposure across species did not recapitulate an overall down-regulation of myelin gene expression as seen with postmortem brain tissue of alcoholics, the network approach did demonstrate a conserved myelin gene expression network associated with drinking behavior. The myelin network was further conserved in alcohol naïve mice, but suggested a novel hypothesis for the role of myelin variation as a predisposing element in acute alcohol sensitivity. Literature association analysis identified the non-receptor tyrosine kinase Fyn as a potential signaling mechanism regulating differences in myelin gene expression (Figure 44). Previous work has shown Fyn kinase knockout mice lack acute tolerance to a sedative hypnotic dose as determined by the loss of righting reflex (LORR) behavior (Miyakawa et al., 1997, Boehm et al., 2003). Our microarray analysis of Fyn kinase knockout mice revealed a significant decrease in multiple myelin-associated transcripts with the NAC and PFC (Figure 46), suggesting a developmental defect in myelin expression for Fyn knockout animals. Extending the Fyn knockout

profiling results to BXD and LXS PFC gene expression discovered a set of 50 genes sharing robust correlated expression and protein-protein interactions (Figure 48& 50) also correlated to the LORR behavior (Figure 49). Probing the Fyn-dependent networks through knockout mice or viral-vector approaches, may identify new molecular factors regulating the sedative-hypnotic properties of acute alcohol and myelin-associated gene expression. Although the basal Fyn-dependent network was over-represented for genes regulating myelin expression, no myelin structural proteins were consistently correlated to *Fyn* expression across all of the microarray studies. The lack in reproducible correlation may be due to the developmental deficits occurring in the Fyn kinase knockout mouse. All of the PFC samples for BXD, LXS and Fyn knockout mice were harvested from adult animals. Myelin mRNA levels peak within the first three weeks of development (Campagnoni and Macklin, 1988), consistent with the time-course of Fyn kinase on myelinogenesis (Lu et al., 2005). If our studies had focused on earlier developmental time-points corresponding with the regulation of myelinogenesis a correlation between *Fyn* and myelin genes may have been detected. Regardless, the decrease in basal abundance for myelin-associated gene expression within the PFC of Fyn knockout mice is consistent with an inverse relationship to acute alcohol sensitivity. The reproducible LORR behavioral phenotype in Fyn knockout animals further narrowed our attention to the potential of myelin-related gene expression as a determinant for a specific alcohol behavioral response, the LORR.

Derived from a multi-generational cross of eight founder strains, genetically inbred long sleep (ILS) and inbred short sleep mice (ISS) were established for divergent sensitivity to alcohol-induced LORR (McClearn, 1970), a measure of acute tolerance in

mice (Ponomarev and Crabbe, 2004). LXS mice, using ILS and ISS mice as progenitors, are one of the largest panels of recombinant inbred mice created to determine the genetic influences underlying susceptibility to the LORR behavior (Bennett et al., 2006). Mapping phenotypic variation for LORR provisionally determined seven quantitative trait loci (QTL) for this specific behavioral trait within the LXS reference population (Markel et al., 1996). Four of the seven QTL (chromosomes 1, 2, 11, and 15) were later confirmed and captured within interval-specific congenic strains, allowing for fine-mapping the LORR genetic loci (Bennett et al., 2008). Through collaboration with these investigators we conducted a genetical-genomic inquiry (Jansen and Nap, 2001) of ILS, ISS, L.5S, and S.5L mice to determine potential quantitative trait genes (QTGs) residing within the chromosome 15 interval (*Lore5*) for the LORR. Genetic variation paired with differential expression identified the myelin-related gene *Ndr1* as a high priority candidate for the LORR. Expression of *Ndr1* was confirmed for the *Lore5* genotype, and showed a significant correlation to the duration of LORR across a heterogeneous stock of mice (Figure 53). The function of *Ndr1* is largely uncertain, but is suggested to be involved in cholesterol transport and intracellular trafficking. Polymorphisms and genetic null mutations for *Ndr1* lead to a demyelination phenotype (Okuda et al., 2004, Echaniz-Laguna et al., 2007). Expression of *Ndr1* was correlated to other myelin-associated genes in every genomic profiling experiment conducted herein, and ranked as one of the top five genes associated with alcohol drinking across species (Figure 35). Further investigation of *Ndr1* will be invaluable in determining the role of *Ndr1* in the LORR and alcohol drinking behavior. Our laboratory is currently in the process of generating an *Ndr1* conditional knockout

mouse that will be used for directly testing alcohol behavioral phenotypes and the underlying neurobiology linked with *Ndr1*.

Alternative splicing could be an essential component of downstream functional consequences for *Ndr1*. A mutation in NDRG1 resulting in skipping of exon 9 is associated with demyelination in humans (Hunter et al., 2003). The role of alternative splicing for *Ndr1* in animal models has yet to be explored. Reintroduction of the short form of *Mbp* into shiverer mice, which normally lack all forms of *Mbp*, largely recovers myelin structural and behavioral abnormalities (Kimura et al., 1989). Expression of *Fyn* was associated with the RNA binding protein quaking (*Qk*), which regulates alternative splicing of myelin-associated genes (Wu et al., 2002, Zhao et al., 2010a). Determining uniquely spliced forms of *Ndr1* and other myelin-associated genes using next-generation sequencing technologies (Metzker, 2010), tiling or exon arrays (Johnson et al., 2003, Soreq et al., 2011), or other methodologies may assist in pinpointing distinct myelin gene products associated with the development of complex traits.

Studying the mechanisms specifically regulated by *Ndr1* could delineate how differential expression of *Ndr1* affects myelin gene expression and structural variation of the myelin sheath. Cell culture systems permit the dissection of specific *processes* not easily attainable *in vivo* (Gibbons and Dragunow, 2010). Although traditional culture methods are typically inadequate for discerning the final stages of myelinogenesis *in vitro*, newer specialized coculture systems can clarify substrates required for adhesion, ensheathment, wrapping, and compaction (Watkins et al., 2008, Emery et al., 2009). Expression of *Ndr1* could be silenced in culture using available siRNA constructs and then determine potential dysregulation of coordinately regulated myelin transcripts and

formation of the myelin sheath. Suppressing *NdrG1* in culture could also be used to test the consequences of alcohol exposure. A previous proteomics analysis has shown NDRG1 is predominantly phosphorylated by PKA and interacts with molecular chaperone proteins such as GPR94 (Tu et al., 2007). Alcohol acutely regulates the expression of GPR94 and other molecular chaperones (Miles et al., 1994, Wilke et al., 2000). Increased expression of molecular chaperones is a suggested protective mechanism against glutamate excitotoxicity (Lowenstein et al., 1991), which may be related to the maladaptive effects of chronic alcohol within the CNS. Depletion of cholesterol, a major constituent of the myelin sheath, is another protective mechanism against glutamate receptor induced excitotoxicity (Ponce et al., 2010). Cholesterol depletion also causes a reduction in NDRG1 (Ponce et al., 2010). The relationship of NDRG1 with cholesterol and molecular chaperones may suggest a functional association to NMDA receptor mediated excitotoxicity due to chronic alcohol exposure. Specialized CNS coculture systems for myelination could unravel a mechanistic role of NDRG1 and myelin gene expression related to NMDA receptor signaling processes in development and response to alcohol exposure.

Decreasing myelin-associated gene expression using the neurotoxicant cuprizone complemented our previous studies, showing myelin deficits increased sensitivity to the sedative-hypnotic effects of acute alcohol. Increased duration of the LORR was not due to adverse effects on motor function or altered alcohol metabolism. Notwithstanding the potential for cuprizone to exert global reductions in CNS myelin beyond just the PFC, cuprizone treatment showed down-regulation of the myelin gene network in an adult animal altered the predicted alcohol behavioral phenotype. Further behavioral

phenotypes could certainly be investigated using the cuprizone model of demyelination. Two-bottle choice drinking behavior and the stationary dowel test are two behavioral models that may be of immediate interest due to their relation to our studies on myelin-associated gene expression. Alcohol consumption is generally inversely correlated to sensitivity to the loss of righting reflex behavior. Studies on the Fyn kinase knockout mice demonstrate an increased sensitivity to the LORR is associated with decreased alcohol drinking behavior (Boehm et al., 2003). Fyn kinase knockout mice also demonstrate similar behavioral responses to the LORR and stationary dowel test (Boehm et al., 2003). The stationary dowel test is commonly used to test acute functional tolerance to sedative-hypnotic effects of acute alcohol administration (Erwin and Deitrich, 1996, Kirstein et al., 2002). Thus, assessing vulnerability to alcohol drinking behavior and sensitivity for the stationary towel, due to cuprizone exposure, may extend our observations for the behavioral pharmacology of alcohol exposure related to variation in myelin-associated gene expression. However, different behavioral phenotypes may capture different aspects of complex behavioral domains (Crabbe et al., 2005) and be caused by multiple genetic factors not captured in a single animal model (Crabbe et al., 1994b).

The cuprizone model of demyelination also has the added advantage of undergoing remyelination following the removal of cuprizone and re-administration of normal rodent chow (Morell et al., 1998, Jurevics et al., 2002). Although cuprizone could have unforeseen neurobiological effects, future work could evaluate alcohol behavioral phenotypes during different stages of demyelination and remyelination. Operating on a C57BL/6J background the cuprizone model also permits the usage of genetic knockout

animals, which are traditionally established on a C57BL/6J background, to determine potential interactions of a selected gene(s) and myelin on alcohol behavioral phenotypes. Soluble drugs may be added to an animal's drinking water to probe other mechanisms related to myelin gene expression in cuprizone exposed mice. For example, addition of the atypical antipsychotic quetiapine to an animal's drinking water alleviates myelin abnormalities due to a concomitant cuprizone containing diet (Zhang et al., 2008). The versatility of the cuprizone model could thus allow a variety of genetic and pharmacological approaches to investigate the neurobiology of myelin in relation to alcoholism and associated behavioral phenotypes in a preclinical animal model.

Human brain imaging, or imaging genetics, is an available platform for probing intermediate phenotypes related to the function of PFC networks and neuropsychiatric disease (Tan et al., 2009). Several techniques (i.e. magnetic resonance imaging, diffusion tensor imaging, fractional anisotropy) are available for measuring myelin content/structure in the human brain. Although substantial heterogeneity exists among the brains of differing individuals, large-scale three-dimensional mapping strategies can be combined with behavioral and genetic profiling to determine maladaptive brain structure underlying disease (Thompson et al., 2002, Van Essen et al., 2012). Overall brain and white matter volume is highly heritable in humans (Hulshoff Pol et al., 2006, Schmitt et al., 2007). A single nucleotide polymorphism in Fyn kinase is associated with alcohol dependence and related endophenotypes (Schumann et al., 2003, Pastor et al., 2009); however, it is unknown whether this polymorphism in Fyn kinase is also associated with myelin variation in alcohol dependent individuals. Additionally, it is uncertain whether polymorphisms directly within *NDRG1*, or other myelin-related genes,

are associated with structural variation and alcoholism. Identifying the intersection of genetic components involving myelinogenesis and acute behavioral responses to alcohol may be important for distinguishing genetic risk factors for the development of an alcohol use disorder.

Through a system-based approach, using gene expression networks as an intermediate phenotype, the work presented herein suggests myelin-associated gene expression is a quantitative trait gene network linked to alcohol behaviors. Acute and chronic alcohol exposure causes changes in myelin-related gene expression associated with CNS plasticity; however, our analyses suggest a novel relationship of basal variation in myelin gene expression within prefrontal cortex as a determinant for alcohol behavioral phenotypes across species (Figure 63). Combining genetical-genomics inquiries on myelin gene expression with future studies on structural variation of myelin can facilitate a translational approach for understanding the neurobiology of alcohol abuse and dependence (Badea et al., 2009, Poot et al., 2011). Although additional experimental evidence will further refine the relationship between myelin gene expression and alcohol-related phenotypes, our work may warrant a longitudinal analysis of myelin in human populations as an underlying component in the risk of developing an alcohol use disorder. Continuing investigation of the myelin gene network may provide unique insights into the susceptibility for neuropsychiatric disease, and be an avenue to therapeutic interventions.

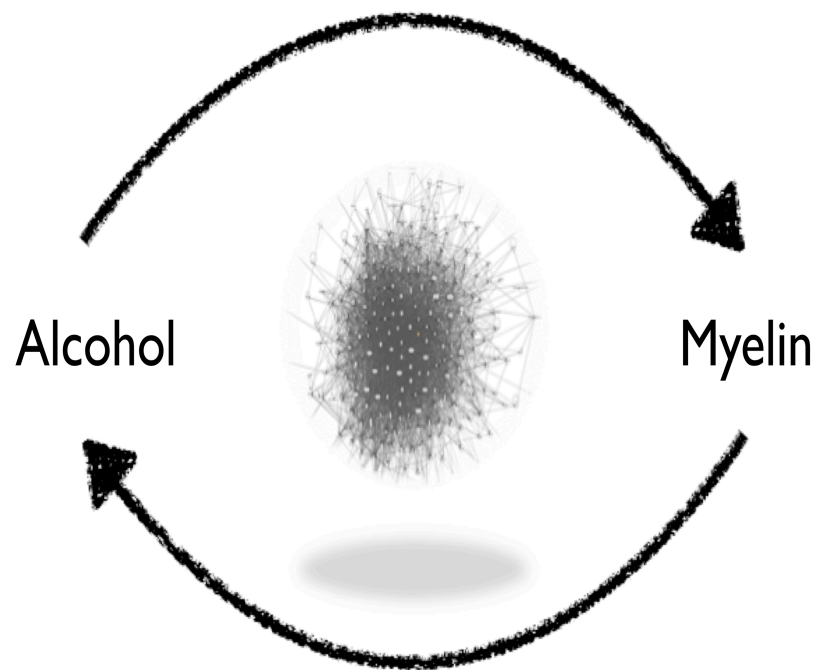


Figure 63. Myelin and Alcoholism. Prior works suggests acute and chronic alcohol exposure alters myelin-associated gene expression in PFC, which may be related to short- and long-term CNS plasticity. However, our analyses suggest basal variation in myelin-associated gene expression may be a quantitative trait gene network (QTGN) for alcohol behavioral phenotypes, especially as it relates to the loss of righting reflex behavior.

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VITA

Sean Patrick Farris was born on July 21, 1983 in Dayton, Ohio. He graduated with a Bachelor of Science in Forensic Science during the month of December 2005 from Eastern Kentucky University in Richmond, Kentucky; following completion of an internship with the Pinellas County Crime Laboratory in Largo, Florida and directed research by Dr. Darrin L. Smith at Eastern Kentucky University. In August 2006 he began graduate school at the Medical College of Virginia Campus at Virginia Commonwealth University in Richmond, Virginia where he went on to join the laboratory of Dr. Michael F. Miles in the Spring of 2007. Upon acceptance of this dissertation, he will be awarded a Doctorate of Philosophy in Pharmacology and Toxicology. While attending graduate school he has received multiple honors including a Student Merit Achievement Award from the Research Society on Alcoholism, C.C. Clayton Award for Outstanding Scholarly Achievement, Phi Kappa Phi Award for Outstanding Scholarly Achievement, and the Lauren A. Woods Award for the top doctoral student in the Department of Pharmacology and Toxicology. During the course of his graduate student career he was supported by a Ruth L. Kirschstein National Research Service Award for Individual Predoctoral Fellows (F31 AA018615) from the National Institute of Alcohol Abuse and Alcoholism.