The Roles of *Mpdz* and *Kcnj9* in Ethanol Withdrawal: Pleiotropic Effects and Potential Mechanisms

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CERTIFICATE OF APPROVAL

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$5HT_{2C}$ –	Serotonin 2C receptor
7TM –	Seven transmembrane-spanning
AA –	Aradonic acid
B6 –	C57BL/6J inbred mouse strain
aCSF –	Artificial cerebrospinal fluid
AMPA –	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AUC –	Area under the curve
BEC –	Blood ethanol concentration
Ca^{2+} --	Calcium
CaMKII –	Ca^{2+}/calmodulin-dependent protein kinase
CNS –	Central nervous system
D2 –	DBA/2J inbred mouse strain
D2.B6 –	Congenic strain with B6 QTL donor region on D2 background
DAG –	Diacyl glycerol
ELISA –	Enzyme-linked immunosorbent assay
EPSC –	Excitatory post-synaptic current
ES –	Embyonic stem cell
EZM –	Elevated zero-maze
GABA –	Gamma aminobutyric acid
$GABA_B$ –	Gamma aminobutyric acid B receptor
GDP –	Guanosine diphosphate
GIRK –	G protein-coupled, inwardly-rectifying
GTP –	Guanosine triphosphate
GPCR –	G protein-coupled receptor

HEK – Human embryonic kidney cell

HET – Heterozygote

HIC – Handling-induced convulsion

ICV – Intracerebroventricular

ISCL – Interval-specific congenic line

IP1 – *myo*-inositol monophosphate

IP3 – D-*myo*-inositol 1,4,5 triphosphate

K^+ -- Potassium

KO – Knockout

mRNA – messenger RNA

miRNA – microRNA

MPDZ – Multiple PDZ (PSD-95/Disc-large/Zonula occludens-1) domain protein

nM – Nanomolar

NMDA – N-methyl-D-aspartate

NPY – Neuropeptide Y

NR2B – Ionotropic glutamate receptor subunit

PA – Phosphatidic acid

PIP_2 – Phosphatidylinositol 4,5- bisphosphate

PKC – Protein kinase C

PLC – Phospholipase C

PLD – Phospholipase D

PSD-95 – Postsynaptic density protein 95

QTL – Quantitative trait locus

QTG – Quantitative trait gene

RISC – RNA-induced silencing complex

RNA – Ribonucleic acid

RNAi – RNA interference

SEM – Standard error of the mean

SNP – Single nucleotide polymorphism

TST – Tail suspension test

WT – Wildtype

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ABSTRACT

Identification of genetic components underlying alcohol dependence is one primary focus of alcohol research. Although a heritable component for the development of alcohol dependence is well established, specific genetic contributions to alcohol use disorders remain difficult to isolate. However, new strategies are being developed to identify discrete genes influencing ethanol behaviors in rodent models. Quantitative trait loci (QTL) mapping in mice has allowed for the identification of specific chromosomal segments harboring gene(s), called quantitative trait genes (QTGs), that influence ethanol withdrawal convulsion severity; and fine-mapping efforts have reduced these chromosomal regions to sizes amenable to discrete gene identification.

Our laboratory employed QTL mapping strategies to identify two high-quality QTGs for ethanol withdrawal severity on mouse chromosomes 1 and 4. *Mpdz*, located on chromosome 4, encodes the multiple PDZ domain (MPDZ) protein, which is present throughout the central nervous system and functions to influence intracellular receptor signaling. *Kcnj9*, located on chromosome 1, encodes a subunit of G protein-coupled inwardly-rectifying potassium (GIRK) channels, which are distributed throughout the central nervous system and influence multiple intracellular activities. Following the initial identification of these genes, murine knockout models were developed for both *Mpdz* and *Kcnj9*, allowing for a more precise examination of their effects on ethanol-related phenotypes. One goal of this dissertation was to examine multiple ethanol-related behaviors in *Mpdz* and *Kcnj9* knockout models, with the hypothesis that both *Mpdz* and *Kcnj9* genotype status would significantly influence the expression of ethanol withdrawal-induced convulsion severity, and that their influence would extend to other behaviors exhibited during acute ethanol withdrawal.

In chapters 2 and 3, *Mpdz* heterozygote knockout and *Kcnj9* knockout models were examined for convulsion severity, locomotor activity, anxiety-like behavior and

depression-like behavior during acute withdrawal. Results from these experiments confirm a role for both genes in ethanol withdrawal convulsion severity, and provide evidence for additional, quantifiable behaviors (i.e., reduced locomotor activity) apparent during withdrawal. Testing of these knockout models for other ethanol-related behaviors produced more ambiguous results, with ethanol withdrawal and genotype status independently influencing many of the behaviors examined. Although the results obtained within this dissertation do not provide strong evidence for a role of either *Mpdz* or *Kcnj9* in multiple acute withdrawal behaviors, they do suggest a role for these genes in other complex behaviors, warranting further investigation.

A second goal of this dissertation was to examine potential mechanisms of action by which *Mpdz* exerts its effects on withdrawal convulsion severity. Because $5HT_{2C}$ receptors have been previously implicated in a variety of ethanol-related behaviors, and have established interactions with MPDZ, chapter 4 examined the potential relationship between *MPDZ* expression and $5HT_{2C}$ receptor activity *in vitro*. In these experiments, a reduction in intracellular *MPDZ* was associated with an increase in $5HT_{2C}$ receptor activity (as measured through the IP3 pathway), providing initial evidence for a functional relationship between these protein partners. Chapter 5 examined this association *in vivo* by examining convulsion phenotypes associated with central administration of SB242084, a potent $5HT_{2C}$ receptor antagonist, to *Mpdz* knockout and DBA/2J inbred strain mice in ethanol withdrawal and control conditions. *Mpdz* knockout mice exhibited slightly higher SB242084-induced convulsions compared to wildtype littermates, providing additional, *in vivo* evidence for a functional relationship between MPDZ and $5HT_{2C}$ receptors. However, this association did not influence ethanol withdrawal convulsion severity, either in the *Mpdz* knockout mice or high withdrawal (i.e., DBA/2J) inbred strain mice. Although experimental limitations should be considered, these

results suggest that MPDZ's association with $5HT_{2C}$ receptors may not be a primary mechanism by which *Mpdz* exerts its behavioral effects.

Overall, the results of this dissertation provide additional evidence to support a role for both *Mpdz* and *Kcnj9* in ethanol withdrawal convulsion severity, although these genes' potential effects on other complex trait behaviors have yet to be fully elucidated. Additionally, results obtained in this dissertation provide evidence of a functional effect of MPDZ expression on $5HT_{2C}$ receptor activity *in vitro* and *in vivo*, although the influence of this interaction on ethanol withdrawal severity has not been confirmed.

CHAPTER 1: General Introduction

Alcohol (ethanol) use disorders constitute a serious worldwide problem, producing a host of detrimental consequences for affected individuals and their families, as well as society at large. Although signs and symptoms of an alcohol use disorder can vary greatly between affected individuals, the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) provides diagnostic criteria for two alcohol use disorders: alcohol abuse and alcohol dependence. Alcohol abuse is defined as a maladaptive pattern of repeated alcohol use despite recurrent adverse consequences. A diagnosis of alcohol abuse comprises one or more of the following criteria over a 12-month period: impairment in performing work- or family-related obligations, hazardous use (such as driving while intoxicated), legal problems or social/interpersonal problems related to use. Additionally, the individual cannot meet the criteria for alcohol dependence, which is defined as alcohol abuse combined with a physiological manifestation of use, such as tolerance (more alcohol needed to achieve 'high'), withdrawal symptoms, or an uncontrollable drive to drink (American Psychological Association [DSM-IV], 2000). Diagnosed cases of alcohol dependence are especially high for industrialized nations, with between 2-12% of the adult population in Western Europe and America meeting the criteria for alcohol dependence (Köhnke, 2008), and over 18 million Americans (8.5% of the population 18 years of age and older) estimated to meet the diagnostic criteria of an alcohol use disorder (Grant et al., 2004).

Alcohol dependence encompasses a cluster of physiological, cognitive and behavioral symptoms that vary considerably in both occurrence and severity within dependent populations. Although research delineating the biological and environmental contributions to this disorder has made significant advances in the past few decades, the complexity and variability with which addiction disorders are expressed, as well as the

diverse etiologies surrounding the development of these disorders, make them difficult to effectively identify and treat.

The risk for development of alcohol dependence arises from multiple and discrete environmental and genetic factors. Although genetic factors are not absolute in their propensity to increase risk for alcoholism, a preponderance of evidence for clear transmission of risk for alcoholism within families has long been recognized, and has become the focus of much research attempting to decipher heritable contributions to this disorder and more effectively diagnose and treat affected populations.

Genetic contributions to alcohol dependence

A genetic contribution to alcoholism was initially supported by adoption studies that demonstrated an increased risk for severe alcohol-related problems in children of alcoholics who were adopted out, even if raised without knowledge of their biological parents' substance use (Cadoret et al., 1980; Schuckit et al., 1972). Several large twin and nuclear family studies published in the 1990s substantiate the conclusion that alcoholism is over 50% heritable (Goldman, 1993; Goldman & Bergen, 1998; Prescott & Kendler, 1999; Reich et al., 1999).

Despite the sizable heritability component contributing to alcohol use disorders, specific genetic determinants of alcohol abuse and alcoholism remain largely unknown. To date, only a few genes have exhibited a consistent association with alcoholism or alcohol-related phenotypes. These include isoforms of the alcohol metabolizing enzymes *ADH1*, *ADH4, and ALDH2,* and neurotransmitter receptor subunits *GABRB1*, *GABRA2,* and *CHRM2* (reviewed in Dick & Foroud, 2003; Edenberg & Foroud, 2006; Kuo et al., 2008, 2009). Despite significant advances in candidate gene classification for alcohol-related traits in human populations, initial identification of these genes has relied heavily on *a priori* knowledge of physiological responses to alcohol and neurotransmitter

systems previously associated with this drug (Zhu & Zhou, 2007). Systematic approaches to gene discovery outside of these pathways are critical if novel genes and mechanisms underlying alcohol dependence are to be discovered and translated into improved treatment and prevention strategies. However, the many practical and ethical limitations involved with identification of novel genes in human subjects necessitate the use of animal models to examine various genetic and physiological aspects associated with this disorder.

Alcohol dependence and associated withdrawal symptoms

ln addition to the initial effects of alcohol, the continued use of this drug depends on multiple factors that profoundly affect both the pattern of alcohol use and the likelihood that alcohol use will progress from casual use to abuse and dependence in some individuals. One factor important to the diagnosis of alcohol dependence is the capacity of alcohol to produce physical and psychological changes that manifest a wide range of symptoms following alcohol's elimination from the body (i.e., withdrawal). Apparent withdrawal symptoms define a preexisting state of physical dependence on the drug, and these episodes can constitute a powerful motivational force that perpetuates alcohol use, and may contribute to relapse (Little et al., 2005). In alcoholic populations, the alcohol withdrawal syndrome is generally defined through the presence of one or more acute symptoms that range from psychological to physiological in nature (McKeon et al., 2008). Common symptoms reported during withdrawal include: feelings of anxiety and depression (Heilig et al., 2010), hyperalgesia (Gatch, 2009) and sleep disturbances (Brower et al., 2008; Malcolm et al., 2007). Additionally, withdrawal from alcohol can induce symptoms of central nervous system (CNS) hyperexcitability, including tremor, autonomic nervous system overactivity and, in more extreme cases, convulsions and delirium tremens that can be life-threatening (McKeon et al., 2008; Monte et al., 2010).

Although many symptoms are associated with alcohol withdrawal in affected populations, the CNS hyperexcitability observed during withdrawal has allowed for the creation of a robust phenotype beneficial to withdrawal-associated gene identification in mouse models.

Mouse models of alcohol withdrawal

A comprehensive understanding of genetic variation in humans and informative animal models is crucial to establish relationships between genotype and biological function (Collins et al., 2003). Although no animal model exactly duplicates clinically defined alcoholism, robust animal models for some aspects of the disorder (e.g., acute sensitivity, drinking/preference, tolerance, withdrawal) are useful to identify potential genetic determinants of liability in humans (Uhl et al., 2008).

The mouse model is particularly advantageous to the identification of genes influencing alcohol-related behaviors due to the relatively high homology between mouse and human genomes, and the increasingly sophisticated methodologies by which the murine genome can be manipulated and novel genetic models can be developed (Singh et al., 2007). Alcohol withdrawal behaviors are exhibited in rodent populations, many of which are directly relevant to clinical withdrawal symptoms, such as withdrawal-induced anxiety-like behavior or sleep disturbances (Brager et al., 2010; Veatch, 2006; Verleye et al., 2009; Wills et al., 2009). However, these behavioral phenotypes have not been widely reported during acute withdrawal in murine models, and may be significantly influenced by environment-specific conditions (Crabbe et al., 1999b, Wahlsten et al., 2003). To date the most effective behavioral model of alcohol withdrawal severity in mice is a convulsion phenotype which is exacerbated in mice withdrawing from acute or chronic ethanol exposure, called the handling-induced convulsion (HIC; Goldstein & Pal, 1971).

Alcohol withdrawal convulsions occur in all species studied, including humans (Friedman, 1980; Rathlev et al., 2006), and can provide a quantitative index of the ethanol withdrawal severity in mice (Goldstein & Pal, 1971). Dr. Dora Goldstein developed a system for inducing physical dependence on ethanol in mice by administering ethanol vapor continuously to animals confined in an inhalation chamber. She also described and quantified the characteristic HIC displayed by withdrawing mice (Goldstein & Pal, 1971). A HIC is assessed by lifting a mouse gently by the tail, spinning it in a 180º arc, and measuring the convulsion response, with the severity of the response being rated by an experimenter on a scale from 0 (no convulsion) to 7 (spontaneous convulsion; Crabbe et al., 1983a). HICs are assessed hourly between 2-12 hours after administration of a hypnotic dose of ethanol, and again at hours 24-25 to ensure that withdrawal HICs have abated. The HIC phenotype is easily measured and quantified, allowing for a standard measure of withdrawal severity that can be reliably assessed across mouse models and research laboratories. HIC severity after ethanol inhalation is dose- and duration-dependent (Goldstein, 1972), and there is a clear genetic contribution to individual differences in this trait (Goldstein, 1973). Crabbe et al. (1983a) subsequently showed that there are substantial genetically determined differences among inbred mouse strains and that these differences are pharmacodynamic rather than pharmacokinetic in nature. The establishment of this withdrawal phenotype as a quantifiable trait with a clear genetic influence has allowed for the identification of discrete genetic influences underlying withdrawal severity in mice.

Quantitative trait locus (QTL) mapping in rodents

A quantitative trait is one that has measurable and continuous phenotypic variation within a population owing to a continuous range of variability in genetic and/or environmental influences. A QTL is one such genetic locus in which allelic variation is

associated with the phenotypic variation (Hunter & Crawford, 2008). Generally, quantitative traits are multifactorial, meaning they are influenced by multiple genetic and environmental factors. QTLs are mapped as chromosomal intervals (figure 1) between two flanking genetic markers that harbor the gene(s) influencing the phenotype of interest. To successfully map a QTL, influence on a trait must be detected amid considerable "noise" from other QTLs and non-genetic sources of individual variation. This has been made feasible through the implementation of technologies to identify genetic polymorphisms throughout the genome and the development of statistical methods to map QTLs from specific genetic marker and phenotypic (i.e., trait) data (Doerge, 2002). The identification of the chromosomal regions where marker allelic and phenotypic differences co-vary implicates the presence of a QTL. Each QTL identifies the genomic location of a gene or genes (referred to as quantitative trait genes or QTGs) affecting the trait of interest. The power of this approach was first demonstrated in plants and later in rodents (Crabbe & Belknap, 1993; Plomin et al., 1991; Stuber, 1995; Tanksley et al., 1992), and has been used widely to identify genetic contributions to a variety of complex phenotypes (Almasy & Blangero, 2009; Hunter & Crawford, 2008; Johnson et al., 1992; Kliebenstein, 2009; Roff, 2007; Rothschild et al., 2007).

Over the past 20 years, numerous QTLs for alcohol-related behaviors have been detected in various mouse crosses with logarithm of odds (LOD) scores that achieve “highly significant” status (typically ~$p<10^{-4}$; Abiola et al., 2003). This accomplishment has contributed greatly to the now widely-held view that the genetic influence on most behavioral phenotypes involves the actions of multiple gene products, each with a moderate to small effect (many are responsible for less than 5% of the observed phenotypic variation; Flint et al., 2005). Mapping populations that have been used successfully to detect and map alcohol response QTLs in mice include F2 intercross populations, in which mice from two different inbred strains are mated to produce

progeny with unique genetic recombinations comprised of both progenitor genotypes spanning across the entire chromosome. Mice from F2 intercrosses can then be selectively mated to produce recombinant inbred (RI) strains, which are entire inbred lines consisting of a unique pattern of progenitor strain chromosomal regions. Because the RI lines are ultimately inbred, they can be tested both phenotypically and genetically to identify discrete QTLs for any number of complex traits. Other mapping populations used for QTL analysis include multiple inbred strain intercrosses called heterogeneous stock strains (usually 4 or 8 inbred strains are used), and can be used to create many different types of informative genetic models for a particular trait of interest (for reviews on QTL mapping populations, see Johnson et al., 1992; Plomin & McClearn, 1993). Strategies that increase the density of genetic recombinations within a mapping population provide higher mapping resolution, but require a significantly denser molecular marker map to achieve the statistical power required for QTL detection. For alcohol-related traits, initial detection of QTLs has most often employed RI and F2 populations, which can typically map QTLs to ~10-50 centimorgan (cM) regions, corresponding to ~20-100 megabases (Mb) in the mouse genome. For reviews on QTL mapping population development and discovery for alcohol-related traits, see Crabbe et al., 1998, 1999a; Phillips et al., 2002 and Spence et al., 2009.

Populations derived from the C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains have been widely examined for ethanol-related behaviors due to their phenotypic divergence in a wide variety of ethanol responses and the presence of genome-spanning genetic polymorphisms among mice derived from these two progenitor strains (Crabbe, 2002, but see Roberts et al., 2007). Crosses derived from these two strains have proven to be extremely useful to detect significant QTLs for alcohol withdrawal (Bennett, 2000; Buck et al., 1997, 2002; Crabbe et al., 1983b, 1994a; Philip et al., 2010), tolerance (Bennett et al., 2007; Crabbe et al., 1994; Kirstein et al., 2002), sensitization

(Phillips et al., 1995, 1998a), conditioned taste aversion (Risinger & Cunningham, 1998), and preference (Belknap et al., 1997; Bice et al., 2006, 2009; Phillips et al., 1998b; Rodriguez et al., 1995; Tabakoff et al., 2008).

The well documented difference between the B6 and D2 inbred mouse strains in susceptibility to withdrawal after chronic ethanol exposure (Crabbe et al., 1983b; Kakihana, 1979), or after a single sedative-hypnotic dose of alcohol (Metten et al., 1998a), has provided an excellent starting point for dissecting genetic influences involved in physical/physiological dependence on alcohol and to study how common allele variants influence genetic predisposition to physical dependence on alcohol. Initial genome-wide evaluations of chronic and acute ethanol withdrawal in populations derived from the D2 and B6 progenitor strains (including RI, F2, and short-term selectively bred lines for ethanol withdrawal severity) identified significant QTLs that influence genetic predisposition to alcohol withdrawal on chromosomes 1, 4, 11 and 19 (Buck et al., 1997, 2002). Using more than one mapping population to identify and verify specific QTLs is an approach that takes into consideration the consequences of type I (identification of a false QTL) and type II (failure to detect an actual QTL) errors (Belknap et al., 1996), providing independent validation for the QTL of interest and increasing the likelihood that detection and fine-mapping in other genetic populations (at least those containing B6 and D2 progenitor strains) will be successful.

Fine-Mapping and Quantitative Trait Gene (QTG) Identification

Despite the success of QTL mapping approaches, QTLs are typically coarsely mapped, with hundreds of potential candidate genes within the QTL region. Effective strategies to identify causal genes within a large QTL region are lacking, with the successes largely relying upon *a priori* knowledge of a gene's involvement in biological systems associated with the trait of interest (e.g., the identification of genes encoding for

receptors within the dopaminergic or serotonergic systems). Although some identified QTLs overlap genes or gene systems already implicated in ethanol behaviors, many ethanol-related QTLs do not contain genes that can be directly linked to drug-related neurotransmitter systems. For these cases, multiple strategies have been proposed to reduce the number of potential candidate genes within the QTL interval, including examination of polymorphisms between strains used for mapping and *in vitro* examination of gene function (Abiola et al., 2003).

Figure 1 outlines one of the most powerful strategies to map a QTL precisely employs the development of interval-specific congenic (ISC) strains (Darvasi, 1997). This approach is particularly time-and cost-efficient when a traditional congenic or consomic strain already exists (Bennett, 2000). Congenic and consomic strains are developed using marker-assisted backcross breeding strategies to isolate an entire chromosome (consomic) or a chromosomal segment (congenic) from one inbred strain onto the genetic background of a different inbred strain. After ~10 generations of backcrossing, the congenic strain has effectively isolated the QTL of interest by full introgression from a “donor” strain onto a “recipient” (or background) strain, where >98% of the genome is inbred background strain-derived outside the introgressed region (Darvasi, 1997). This breeding strategy allows researchers to examine the QTL effects without significant background genomic influences from the donor strain outside the QTL interval (Rogner & Avner, 2003). However, in some congenic strains, the introgressed interval is large (10-35 cM or more) and may contain a number candidate genes (Nadeau & Frankel, 2000). This issue is especially apparent in QTLs overlaying recombination ‘hotspots,’ specific chromosomal segments containing overlapping QTLs for a wide variety of traits (Mozhui et al., 2008) and influencing gene expression throughout the rest of the genome (Breitling et al., 2008).

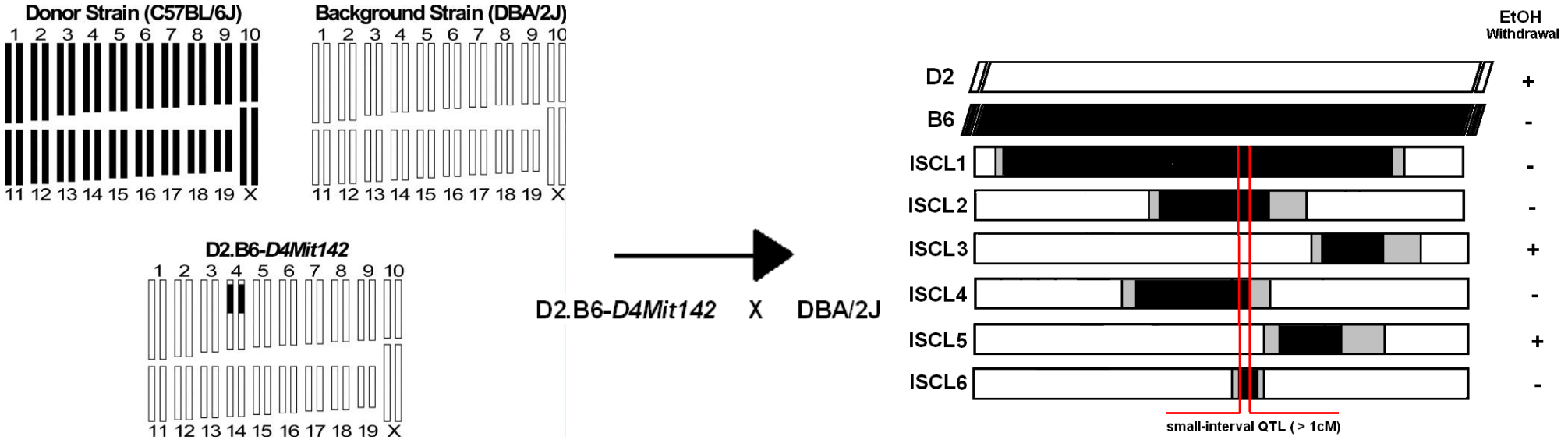
ISC strains further refine the QTL of interest and facilitate progression to discrete genes that are contributing to the phenotype under examination (Darvasi, 1997). ISC strains are developed by backcrossing a standard congenic (or consomic) to background strain mice to create N_1 generation mice. These mice are further backcrossed to create N_2 mice that may exhibit distinct regions of progenitor allelic recombination within the starting congenic interval. The recombinant N_2 mice are genotyped across the QTL to determine the boundaries of the individuals' allelic recombination within the region, and sample size required to effectively identify recombinants can be calculated using QTL effect size and the 95% confidence interval surrounding the QTL (Darvasi & Soller, 1997; Visscher & Goddard, 2004). These N_2 mice are backcrossed to produce multiple offspring carrying the same introgressed region (referred to as an ISC line), which carries one copy of the donor QTL and one copy of the background QTL. ISC line mice can then be intercrossed to create a finished ISC strain that homozygous for the donor region spanning the QTL of interest. Because each ISC strain contains a unique donor region within the QTL of interest, a panel of ISC strains can be used to isolate a much smaller interval of interest (~1 Mb) within the starting QTL region (Kozell et al., 2008; Shirley et al., 2004), thus facilitating QTG candidate identification and analyses (Denmark & Buck, 2008; Kozell et al., 2009; Shirley et al., 2004).

In addition to facilitating fine-mapping, congenic strains are useful to confirm QTLs, such as those involved in ethanol intoxication, withdrawal and consumption behaviors (Bennett et al., 2008; Fehr et al., 2002; Ferraro et al., 2005; Lorenzana et al., 2007; Ma et al., 2009). Congenic strains can also be useful to examine potential pleiotropic effects of QTLs, such as those that influence responses to multiple drugs (Palmer et al., 2006; Shirley et al., 2004), and assess interactions among QTLs (Burgio et al., 2007; Moison, 2007). ISC strains have been particularly useful to examine neural circuitry and mechanisms by which a QTL may affect behavior (Chen et al., 2008; Reilly, Milner et al.,

2008; Rogers et al., 2008). The utility of the ISC models to identify specific genes potentially underlying the phenotype of interest while simultaneously examining multiple behavioral and physiological influences of the QTL them especially attractive to study alcohol-related traits.

Despite advances in fine-mapping model development, identification of the QTGs underlying the phenotypic effects of QTLs remains a challenge in the translation of preclinical research (Flint et al., 2005). Successful strategies have relied upon evidence from several sources, and many have used congenic strains to their advantage (Belknap et al. 2001; Cicila et al. 2001; Liang et al. 2007; Shirley et al. 2004). Approximately 20 genes are widely accepted as QTGs for complex traits in mammals. In most cases, they were identified using a stepwise process that eliminated all other possibilities, involving positional cloning (fine-mapping), comparing the sequences and expression levels of the genes in the progenitor strains or congenic vs. background strains and, when feasible, *in vivo* confirmation. Discussions on various sources of proof that support the conclusion that a candidate gene is a QTG can be found in Belknap et al., 2001, Flint et al., 2005 and Phillips et al., 2002. Our laboratory has employed ISC models to identify high-quality QTG candidates for established QTLs on mouse chromosomes 1 and 4.

Figure 1. Development of congenic and interval-specific congenic (ISC) strains. Left figure represents chromosome 4 congenic strain, with QTL from B6 donor strain introgressed onto a D2 strain background. Backcross breeding between chromosome 4 congenic strain and D2 background strain produces progeny containing unique progenitor-derived loci within the QTL of interest (right figure). Genotypic and phenotypic analyses using this model can reduce the original QTL to a size amenable to individual gene identification.



Identification of Mpdz as an ethanol withdrawal QTG

Fine-mapping of an ethanol withdrawal QTL mapped to mouse chromosome 4 (*Alcw2*) used a traditional D2.B6 congenic strain (D2.B6-*D4Mit142*; D2 background strain.B6 donor strain) as the point of departure to develop a series of ISC mice (Fehr et al., 2002; Shirley et al., 2004). The development and phenotypic analyses of six ISC lines with distinct introgressed intervals narrowed the QTL to a maximal 1.8 Mb interval and was key toward identification of a gene within this interval with coding and/or expression differences indicative of a QTG (Shirley et al., 2004). Using public and private sequencing databases, the five genes within the *Alcw2* interval were identified and evaluated based on the following criteria: sequence difference between the two progenitor strains (B6 and D2) in the protein-coding region; differential gene and/or protein expression between D2.B6 congenic mice and D2 background strain mice or between the two progenitor strains in whole brain and brain regions implicated in convulsion or alcohol withdrawal behaviors. Only one gene (i.e., *Mpdz*) met these criteria and was identified as a promising QTG candidate to underlie the phenotypic effects of the chromosome 4 QTL. Further examination of *Mpdz* sequence variants among 15 standard inbred mouse strains revealed that haplotype differences among these strains produced three protein variants (MPDZ1-MPDZ3) and that variant status was significantly correlated with ethanol withdrawal severity (Fehr et al., 2002), suggesting that *Mpdz* allelic status is associated with ethanol withdrawal severity on multiple genetic backgrounds.

Identification of Kcnj9 as an ethanol withdrawal QTG candidate

Fine-mapping of the ethanol withdrawal QTL on the distal region of mouse chromosome 1 (*Alcw1*) used a D2.B6 congenic strain as the point of departure for the development of a series of ISC lines. This QTL (syntenic to chromosomal region 1q21-

q32 in humans) is exceptionally gene-dense, with multiple QTLs detected for alcohol-related behaviors, including locomotor and anxiety-like behaviors (Demarest et al., 2001; Henderson et al., 2004; Hitzemann et al., 2002), ethanol consumption (Tarantino et al., 1998), and ethanol-induced hypothermia (Crabbe et al., 1994). This convergence of alcohol-related QTLs makes it tempting to speculate that the gene(s) underlying the influence of *Alcw1* on withdrawal may have pleiotropic effects on other behavioral responses to ethanol, and shows the cumulative power of QTL mapping to detect multiple potential effects of what could be the same gene(s). Using an ISC strain panel spanning this QTL, our laboratory identified two distinct QTLs within the starting *Alcw1* interval. The more proximal QTL affects ethanol (but not pentobarbital) withdrawal severity, and harbors QTG candidates involved in oxidative stress (Denmark & Buck, 2008; Kozell et al., 2008), while the more distal QTL exhibits influences on withdrawal HIC responding from multiple sedative-hypnotic drugs, including ethanol, pentobarbital and zolpidem (Kozell et al., 2009). The concurrent development of a novel knockout model of one gene in within this QTL, *Kcnj9*, has provided an excellent resource to more precisely examine the effects of this particular gene. The use of the *Kcnj9* knockout model has provided *in vivo* evidence for this gene's involvement in pentobarbital, zolpidem and ethanol withdrawal severity (see chapter 3 for details), making *Kcnj9* a plausible QTG to underlie the distal chromosome 1 QTL's phenotypic effects.

Confirmation models and potential mechanisms of action for QTGs

Knockout Models for Mpdz and Kcnj9

One useful strategy to assess a QTG's effects on a murine phenotype is to develop a null mutant mouse model, in which "knockout" of a target gene is achieved through homologous recombination replacing the endogenous allele with a nonfunctional version (Guan et al., 2010). This approach has been used extensively, with >1000

knockout animal models currently available, and many more being developed (see www.knockoutmouse.org). For this reason, after a QTG (or a high-quality QTG candidate) has been identified, analyses utilizing one or more knockout models are a common first approach to assess potential QTG effects *in vivo* (Boehm et al., 2006; Boyce-Rustay et al., 2006; Kozell et al., 2009; Morice et al., 2010; Thanos et al., 2005). For acute alcohol withdrawal HIC severity, an *Mpdz* knockout heterozygote model has been developed on both C57BL/6J (B6) and 129/S5 inbred strain backgrounds. Although a *Mpdz* knockout homozygote progeny do not survive to adulthood, the *Mpdz* knockout heterozygote model shows no overt abnormalities in naïve locomotor, gustatory, memory or physiological function (S. Grant, personal communication), making it suitable for ethanol withdrawal behavioral testing. *Kcnj9* knockout homozygotes have also been developed in our laboratory. *Kcnj9* knockouts have no overt behavioral abnormalities (L. Kozell, personal communication) and exhibit reduced sedative-hypnotic withdrawal associated HICs compared to wildtype littermates (Kozell et al., 2009), suggesting that this may be a suitable model to study *Kcnj9* effects on ethanol withdrawal phenotypes.

QTG Mechanisms of Action: Potential Involvement of G protein-coupled receptors

Multiple lines of evidence identify *Mpdz* as a QTG (and *Kcnj9* as a high-quality QTG candidate) for ethanol withdrawal, but the exact mechanisms of action for these ethanol withdrawal QTGs are unknown currently. Associations between the protein products of these two QTGs and members of the G protein-coupled receptor (GPCR) family warrant particular interest, as GPCRs are heavily implicated in drug and alcohol use behaviors (Hack & Christie, 2003; Le Foll et al., 2009; Maccioni & Colombo, 2009), and GPCRs are potential therapeutic targets for alcohol use disorders (Heilig & Egli, 2006; Nagy, 2004).

GPCRs are a large and diverse family of seven transmembrane receptors that have essential and varied actions in the CNS. Upon ligand binding, GPCRs undergo a conformation change, activating intracellular heterotrimeric G proteins to initiate a wide array of intracellular effects (Marinissen & Gutkind, 2001). GPCR activation catalyzes both the exchange of GDP for GTP and the dissociation of the G protein into the G_{α} and $G_{\beta\gamma}$ subunits. G_{α} binds to GTP, and the G_{α}-GTP and $G_{\beta\gamma}$ complexes diffuse through the cell, having a variety of intracellular effects, including activation/suppression of ion channels and modification of multiple signaling pathways (Vilardaga et al., 2009). Although $G_{\beta\gamma}$ has a number of important intracellular effects (Ford et al., 1998), the most well-studied effects induced by GPCR activity stem from G_{α}-GTP activation. Twenty different G_{α} isoforms exist, and can be grouped into four families: G_i, which functions to inhibit adenylate cyclase and reduce cAMP production; G_s, which activates adenylyl cyclase to stimulate cAMP production; $G_{q/11}$, which activates phospholipase C (PLC);and $G_{12/13}$, which functions in cell structure dynamics (figure 2; Cabrera-Vera et al., 2003). The diversity and variation at every step of GPCR activation denotes the inherent complexity and specificity of this class of receptors, as well as their importance in regulating neuronal function. Although the GPCR family interacts with an assortment of proteins, their interactions with two specific protein families (PDZ domain proteins and GIRK channels) are of particular interest for this dissertation as *Mpdz* and *Kcnj9* encode proteins within these families.

GPCRs and PDZ domain proteins

Members of the PDZ domain family (named for three prototypical proteins: postsynaptic density protein [PSD-95], discs-large septate junction protein [DLG], and epithelial tight-junction protein zona occludens protein [ZO-1]) are widely expressed throughout the body, where they serve an essential structural role in protein complex

assembly, often at the level of the plasma membrane (Hung & Sheng, 2002). To date, more than 200 PDZ domain proteins have been identified (Spaller, 2006), and the diversity of the proteins with which they physically associate (often called interaction partners) and intracellular effects makes them important regulators of neuronal function (Sierralta & Mendoza, 2004). These proteins often serve 'scaffolding' functions, maintaining a physical link between GPCRs and one or more intracellular effector systems (Hall & Lefkowitz, 2002). The result of many PDZ domain interactions is increased intracellular signal transduction speed and/or efficiency (Ranganathan & Ross, 1997).

Mpdz and its human homolog *(MPDZ)* encode the multi-PDZ domain protein (MPDZ, also called MUPP1) which contains 13 PDZ domains (Simpson et al., 1999; Ullmer et al., 1998). MPDZ has been identified in a wide variety of cell types, where it acts as a scaffold protein for various protein complexes at the plasma membrane (Dooley et al., 2009; Jones et al., 2009). MPDZ is thought to alter the rate and/or fidelity of signal transduction mediated by proteins with which it physically associates (Nourry et al., 2003; Sheng & Sala, 2001). These include GPCRs mediating inhibitory neurotransmission (5-$HT_{2A,2B,2C}$ receptors and $GABA_B$ receptors; Balasubramanian et al., 2007; Becamel et al., 2004; Jones et al., 2009), a tyrosine kinase receptor (cKIT; Mancini et al., 2000) and tight junction proteins (D'Atri & Citi, 2002). MPDZ interactions with $5HT_{2C}$ and $GABA_B$ receptors are of particular relevance to my dissertation, as both of these receptors exhibit actions throughout the CNS and have previously been implicated in ethanol withdrawal (Addolorato & Leggio, 2010; Knapp et al., 2007) and convulsion (Ong & Kerr, 2000; Sperk et al., 2004) phenotypes in clinical and preclinical models. The physical association between MPDZ and 5-HT_{2C} receptors has been confirmed using *in vitro* and *in vivo* models (Becamel *et al*., 2001. 2004; Parker et al., 2003; Sharma et al., 2007, Ullmer et al., 1998). Additionally, administration of a 5-

$HT_{2C/1B}$ receptor selective agonist (meta-chlorophenylpiperazine [mCPP]) attenuates ethanol withdrawal HIC severity (Chen & Buck, unpublished results), whereas a selective 5-HT_{2C} receptor antagonist (SB242084) exacerbates ethanol withdrawal severity (Buck et al., 2004). Moreover, SB242084-enhanced HICs are less severe in D2.B6 chromosome 4 congenic mice compared to D2 background strain mice (Reilly, Milner et al., 2008), suggesting that a gene(s) within the introgressed QTL region affects $5HT_{2C}$ receptor function. Taken together, these results implicate the $5HT_{2C}$ receptor association with MPDZ as a potential mechanism by which *Mpdz* influences ethanol withdrawal.

The $GABA_B$ receptor also associates with MPDZ, at least *in vitro* (Balasubramanian et al., 2007), and has been implicated in alcohol actions on the CNS. Electrophysiological studies in rats show that ethanol increases presynaptic $GABA_B$ autoreceptor activity in various brain regions, an effect that regulates postsynaptic sensitivity of GABAergic synapses to ethanol (Ariwodola & Weiner, 2004; Silberman et al., 2009). Additionally, baclofen, a selective $GABA_B$ receptor agonist, effectively reduces ethanol consumption in rodent models (Colombo et al., 2004) and can reduce alcohol craving and increase abstinence duration in alcoholic subjects (Addolorato et al., 2002a,b; Addolorato & Leggio, 2010). *In vitro* experiments show that disruption of the interaction between the $GABA_B$ receptor and MPDZ significantly decreases the duration of $GABA_B$ receptor-mediated intracellular signaling (Balasubramanian et al., 2007). Baclofen administration significantly increased acute alcohol withdrawal severity in D2, but not B6 mice (Milner & Buck, unpublished results). Moreover, D2.B6 chromosome 4 congenic mice show an attenuated baclofen enhanced HICs compared to D2 background strain mice (Reilly, Milner et al., 2008). Taken together, these results suggest that *Mpdz* effects on ethanol withdrawal may involve $GABA_B$ and/or $5HT_{2C}$ receptors.

GPCRs and GIRK channels

G protein-coupled, inwardly rectifying K^+ ion (GIRK) channels are tetramers present throughout the CNS, which function as inhibitory post-synaptic modulators of a variety GPCRs (Dascal, 1997). In the CNS, GIRK1, GIRK2, and GIRK3 show widespread and overlapping patterns of distribution (Saenz del Burgo et al., 2008), and form functional homotetramers (GIRK2) or heterotetramers (GIRK1/2, GIRK1/3, GIRK2/3 and possibly GIRK 1/2/3) that are activated by $G_{\beta\gamma}$ following stimulation of $G_{i/o}$-coupled GPCRs (Lüscher et al., 1997; Lüscher & Slesinger, 2010). The GIRK channel family has been implicated in drug and alcohol use behaviors (Kobayashi et al., 2004; Lomazzi et al., 2008) using knockout models developed for each GIRK channel subunit (Kobayashi & Ikeda, 2006).

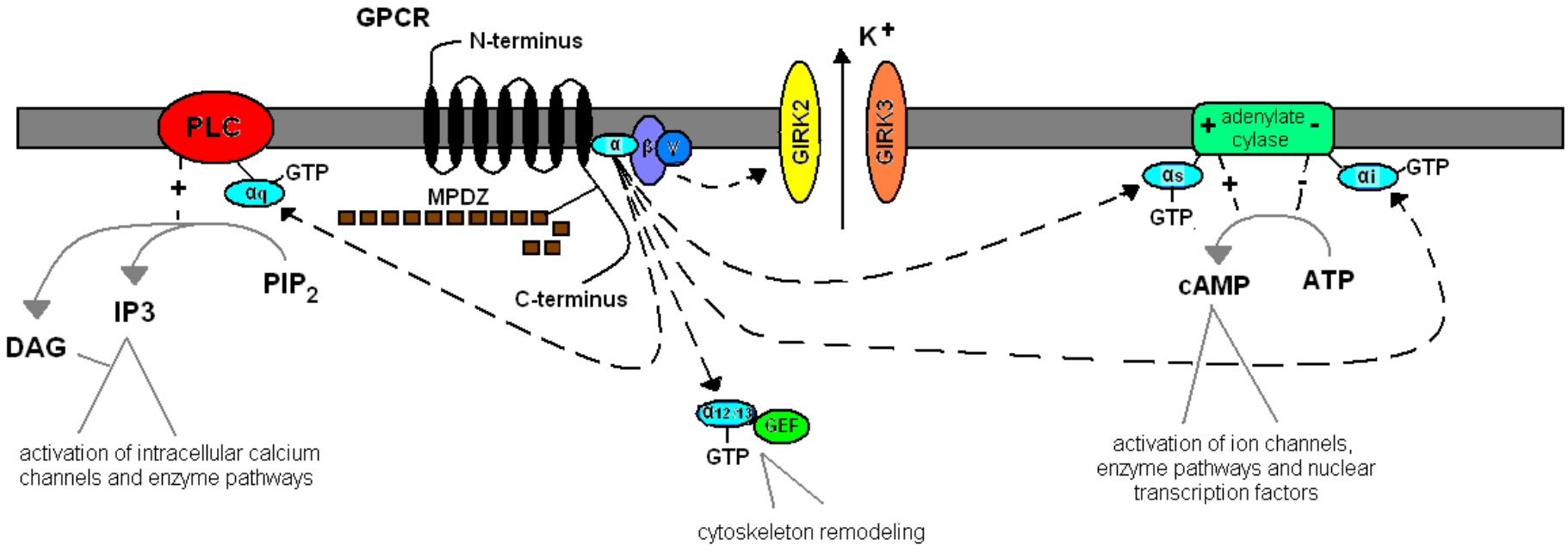
Kcnj9 and its human homolog (*KCNJ9*) encode a subunit of the GIRK channel family (GIRK3/Kir3.3). The GIRK3 protein is distributed throughout the CNS (Dascal, 1997) where it contributes to heteromeric GIRK channels (Cruz et al., 2004; Jelacic et al., 2000; Koyrakh et al., 2005; Labouebe et al., 2007). Although little is known about GIRK3 function in the brain, current evidence suggests roles for GIRK3 in channel formation (Koyrakh et al., 2005) and targeting for GIRK channel degradation (Ma et al., 2002). In hippocampal neurons, GIRK3 is thought to reduce accumulation of GIRK channels on the cell surface and reduce GIRK currents (Ma et al., 2002). In this context, reduced GIRK3 expression would be expected to increase GIRK channel currents and inhibit neuronal activation.

GIRK3-containing channels are activated by multiple GPCR types (Koyrakh et al., 2005; Lüscher & Slesinger, 2010). The association between GIRK3-containing channels and the $GABA_B$ receptor is of particular interest to ethanol withdrawal, as $GABA_B$ receptors modulate a variety of ethanol-related behaviors and can modify GABAergic signaling induced by ethanol exposure (Federici et al., 2009). $GABA_B$

receptor agonist actions in drug reward-related brain regions have been shown to be mediated through the GIRK3 channel and its association with a regulator of G-protein signaling (RGS) protein, whose expression is altered by chronic drug exposure (Lomazzi et al., 2008). Additionally, behavioral testing in GIRK3 knockout mice has established a role for GIRK3-containing channels in morphine withdrawal severity (Cruz et al., 2008) and analgesic response to α_2-adrenergic and cannabinoid receptor ligands (Smith et al., 2008). Taken together, these studies suggest that GIRK3 association with the $GABA_B$ receptor may be a mechanism by which *Kcnj9* influences ethanol-related behaviors including withdrawal.

Mpdz and *Kcnj9* encode proteins that are clearly different in form and function, and there is no current evidence to suggest that they act conjointly to influence a specific response, such as ethanol withdrawal-induced convulsions. However, their established influence on members of the GPCR family previously identified as influencing multiple ethanol-related behaviors (i.e., $GABA_B$ and $5HT_{2C}$ receptors) provides an excellent starting point by which to examine potential mechanisms of action for the QTG effects on ethanol withdrawal.

Figure 2. Schematic representation of GPCR activity in the cell. Solid lines denote a physical interaction between intracellular proteins, and dashed lines represent protein movement within the cell. Grey lines denote conversion actions mediated by protein interactions.



Dissertation Goals

The identification of potential candidate genes that underlie phenotypic effects on ethanol responses including withdrawal has been a major focus in our laboratory. Using multiple lines of converging evidence, we have identified *Mpdz* and *Kcnj9* as QTG candidates for ethanol withdrawal QTLs on mouse chromosomes 4 and 1, respectively. The overarching goals of my dissertation were to more rigorously assess the potential influence of these two QTGs on ethanol withdrawal convulsions and other ethanol-related behaviors, as well as assess potential mechanisms by which the most well established QTG (i.e., *Mpdz*) exerts its behavioral effects.

The goal of the first set of experiments was to test the influence of these two genes on ethanol withdrawal HIC severity *in vivo* using novel murine knockout models that have recently been developed in our laboratory or become available. Because many ethanol-related behaviors share underlying biological mechanisms (Chastain, 2006; Vengeliene et al., 2008) and may share underlying genetic contributions (Metten et al., 1998b), these knockout models were used to examine potential effects of these genes on other behaviors that are genetically correlated with ethanol withdrawal convulsions in mice (e.g., ethanol consumption) as well as additional measures of ethanol withdrawal (i.e., locomotor depression, anxiety-like behavior and depression-like behavior). Potential differences in ethanol elimination were also addressed. The hypothesis for this set of experiments was that *Mpdz* and/or *Kcnj9* knockout mice would show significantly altered ethanol withdrawal HIC severity compared to wildtype littermates, and that genotype-dependent differences could be observable in additional ethanol withdrawal and consumption behaviors.

I also examined potential mechanisms of action for *Mpdz* using *in vitro* and *in vivo* methodologies. *In vitro* methods (chapter 4) using RNA interference assessed the affect of reduced endogenous *MPDZ* expression on $5HT_{2C}$ receptor function. *In vivo*

methods (chapter 5) assessed the effect of central administration of a $5HT_{2C}$ receptor ligands on ethanol withdrawal convulsion severity using *Mpdz* knockout and D2 (high ethanol withdrawal) mice. The hypothesis for this set of experiments was that *Mpdz* expression levels would alter $5HT_{2C}$ receptor function *in vitro*, and that this association would influence ethanol withdrawal severity *in vivo*.

CHAPTER 2: Ethanol withdrawal and consumption in *Mpdz* mutant mice

Abstract

Recent positional cloning and expression analyses identify *Mpdz* as a quantitative trait gene (QTG) candidate for ethanol withdrawal in mice. Low expression of this gene and its protein product (multi-PDZ domain protein [MPDZ]) are associated with high withdrawal severity, but *in vivo* evidence for this relationship is lacking currently. Here, using *Mpdz* knockout models ($B6^{Mpdz+/-}$ and $129/S5^{Mpdz+/-}$; C57BL/6 and 129/S5 backgrounds, respectively), I assessed *Mpdz*'s effect on ethanol withdrawal and consumption. As expected, $B6^{Mpdz+/-}$ mice exhibited significantly ($p<0.05$) more severe withdrawal-associated convulsions than wildtype littermates. Taken together with previous results and recent analyses of an *Mpdz* overexpression transgenic model, my results confirm that *Mpdz* is a QTG for ethanol withdrawal convulsions. Additionally, $B6^{Mpdz+/-}$ showed a trend ($p<0.1$) for less ethanol consumption than wildtype littermates, suggesting that *Mpdz* expression may also contribute to the inverse genetic correlation existing between ethanol consumption and withdrawal severity. Using the $129/S5^{Mpdz+/-}$ model, a main effect of treatment was observed for withdrawal-associated convulsions, locomotor depression, anxiety-like and depression-like behaviors, but no differences between $129/S5^{Mpdz+/-}$ and wildtype littermates were detected. This suggests that *Mpdz* does not influence these withdrawal behaviors or that its influence is obscured on the 129/S5 background.

Introduction

Alcoholism and alcohol (ethanol) abuse affect up to 30% of Americans (Hasin et al., 2007), and are among the most highly heritable addictive disorders (Goldman et al., 1993). Withdrawal is a hallmark of alcohol physiological dependence, and constitutes a motivational force that perpetuates alcohol use/abuse and contributes to relapse (Little et al., 2005). Unfortunately, the complexities underlying genetic differences in risk for alcohol dependence/withdrawal remain largely unknown, hindering effective treatment and prevention.

Although no animal model duplicates clinically defined alcoholism, models for specific factors, including the withdrawal syndrome, are useful for identifying potential genetic determinants of liability in humans. One method to identify specific genetic factors relevant to alcohol dependence/withdrawal is quantitative trait locus (QTL) mapping. QTLs are chromosome sites containing alleles (genes) that affect a complex (quantitative) trait. Our laboratory previously identified a QTL on chromosome 4 with a large effect on alcohol withdrawal in mice (Buck et al., 1997; Fehr et al., 2002). We subsequently fine-mapped this QTL to a 1.8 Mb interval and identified *Mpdz* (which encodes the multi-PDZ domain protein, MPDZ; also called MUPP1) as a quantitative trait gene (QTG) candidate to underlie this QTL's phenotypic effects on alcohol withdrawal (Shirley et al., 2004). Inbred strain analyses and comparisons of congenic and background strains suggested that higher *Mpdz* expression is associated with less severe ethanol withdrawal (Fehr et al., 2002; Shirley et al., 2004), but *in vivo* evidence that *Mpdz* affects withdrawal convulsions and related behaviors is lacking currently.

Gene knockout and transgenic models are useful approaches to assess the behavioral effects of a QTG candidate *in vivo* (Abiola et al., 2003). In the present studies, I used two *Mpdz* knockout models ($B6^{Mpdz+/-}$ and $129/S5^{Mpdz+/-}$; on inbred C57BL/6 and 129/S5 genetic backgrounds, respectively) to assess the influence of *Mpdz*

on ethanol withdrawal behavior. The handling-induced convulsion (HIC) is a well-documented measure of ethanol withdrawal in mice that provides a quantitative and reproducible index of withdrawal severity (Goldstein & Pal, 1971; Metten et al., 1994, 1998a, 1999), and was the behavior on which identification of *Mpdz* was founded (Shirley et al., 2004). Some, but not all, signs of withdrawal are highly correlated with convulsive activity (Belknap et al., 1987; Kosobud & Crabbe, 1986). Additional quantifiable measures of ethanol withdrawal observed in mice include locomotor depression (Kliethermes et al., 2004; Sanders, 1980), anxiety-like behavior (Prediger et al., 2006; Verleye et al., 2009), and depression-like behaviors (Hirani et al., 2002). These are plausibly influenced by MPDZ, which physically associates with $GABA_B$ (Balasubramanian et al., 2007) and $5HT_{2C}$ (Becamel et al., 2001) receptors, both of which are implicated in ethanol withdrawal-associated behaviors (Humeniuk et al., 1994; Knapp et al. 2004, 2007; Mead & Little, 1995; Overstreet et al., 2006). Ethanol withdrawal severity is also genetically correlated with alcohol consumption/preference in independently tested groups of mice (Metten et al., 1998b), suggesting that ethanol withdrawal and consumption/preference may share specific genetic contributions (of which *Mpdz* may be one). Therefore, I assessed whether *Mpdz* exerts pleiotropic effects beyond withdrawal convulsions on these ethanol phenotypes.

Methods

Animals

Two *Mpdz* knockout models ($B6^{Mpdz+/-}$ and $129/S5^{Mpdz+/-}$) were recently developed by Dr. Seth Grant (Cambridge University, UK). Both were developed from a 129/Ola strain-derived embryonic stem (ES) cell line containing an insertional mutation by a gene-trapping vector (pGT1lxf, from Baygenomics [baygenomics.ucsf.edu]) of *Mpdz* resulting

in a disruption in the third PDZ domain of MPDZ. The insertional mutation was designed to create an in-frame fusion between the 5'-exons of the trapped gene and a reporter, ßgeo (a fusion of ß-galactosidase and neomycin phosphotransferase II), occurring within *Mpdz* introns 11-12. This yielded a fusion transcript containing *Mpdz* exons 1–11 and ßgeo. ES cells were microinjected into inbred B6 blastocysts to create chimeric mice. Chimeras were backcrossed to B6 or 129/S5 background strain mice to generate heterozygote knockouts for the *Mpdz* gene trap mutation on both inbred strain backgrounds. Although homozygote knockouts are not viable, knockout heterozygotes breed well and show no apparent abnormalities in physiological or baseline behavioral phenotypes (S. Grant, personal communication).

All of the knockout heterozygote and wildtype littermates used for behavioral testing (reciprocal knockout heterozygote x appropriate background strain crosses) were bred in our colonies at the VA animal facilities. Males and females were tested in approximately equal numbers. Mice were group-housed 2-5 per cage (28x17x11.5 cm lined with Bedicob® bedding) by sex. Mouse chow (Purina #5001) and water were available *ad libitum*. Procedure and colony rooms were kept at a temperature of 21 ± 1^{0}C. Lights were on in the colony from 0600-1800 hr. All procedures were approved by the VA and OHSU Institutional Animal Care and Use Committees in accordance with United States Department of Agriculture and United States Public Health Service guidelines.

Handling-induced convulsions (HICs)

Details of this procedure have been published (Metten et al., 1998a). $B6^{Mpdz+/-}$ and wildtype littermates (n=66 and 48 mice, respectively) or $129/S5^{Mpdz+/-}$ and wildtype littermates (n=90 and 81 mice, respectively) were used. Mice were scored twice for baseline (pre-ethanol) HICs 20 min apart followed by injection of a single hypnotic dose of ethanol (4 g/kg, i.p., 20% v/v in saline, at hr 0) or an equivalent volume of saline and

scored hourly for HICs from hr 2-12, and again at hr 24 and 25. In order to create an index of ethanol withdrawal that is independent of individual differences in baseline HIC scores, post-ethanol HIC scores were corrected for the individual's average baseline (pre-ethanol) HIC score as in previous work (Metten et al., 1998a). These data were used to compute the individual's ethanol withdrawal severity score, corresponding to the corrected area under the curve for hr 2-12 post-ethanol (Buck et al., 1997, Metten et al., 1998a).

Ethanol preference and consumption

Ethanol preference and consumption were measured using a method modified from Phillips et al. (2010). Naïve B6$^{Mpdz+/-}$ and wildtype littermates (n=39 and 44 mice, respectively) were used because B6 and B6-derived mice show high ethanol consumption and preference compared with other strains (Yoneyama et al., 2008). Mice were acclimated to individual housing and drinking from two 25 ml tubes (containing only tapwater) on days 1-2. On days 3-10, all mice were offered tapwater (in one tube) and ethanol (in the other tube; at two ascending concentrations of ethanol [3% and 10% v/v in tapwater]) for four consecutive days at each concentration. Consumption of ethanol and water were determined from reading drinking tubes every 24 hr. Potential side preferences were mitigated by switching ethanol and water tubes every two days (i.e., ethanol was on the right side for days 1-2 and on the left side for days 3-4). Evaporation and leakage were accounted for by placing drinking tubes in two empty cages, reading empty cage volume changes and subtracting these changes from individual consumption volumes. Subject weights were recorded on days 1, 3, 7 and 10 to calculate consumption data. Ethanol preference (ml ethanol/ml total fluid consumed) and consumption (g ethanol /kg body weight) were calculated for each individual as an

average across days 2 and 4 (for each concentration) to further mitigate potential side preference or novelty issues.

Homecage activity

Naïve 129/S5$^{Mpdz+/-}$ and wildtype littermates (n=36 and 44 mice, respectively) were assessed for locomotor activity in ethanol withdrawn and control (saline) animals. Following 1 hr of habituation to the procedure room, mice were administered ethanol (4 g/kg, i.p.) or saline at hr 0 and returned to their homecage for 8 hr. At hr 6 post-injection, the homecage top was removed and replaced with a 30x18 piece of plexiglass, during which locomotor behavior was video-recorded for 2 hr (from hr 6-8). Activity was later scored by an experimenter blind to treatment and genotype. To assess horizontal activity, the homecage was divided into four quadrants. An activity 'count' was reported when an individual fully entered (all 4 paws) a new quadrant. Vertical activity was scored as number of the rears (raised onto hindlimbs) exhibited. In order to minimize the number of mice used and avoid the potential effects of repeated ethanol administrations, this group of mice was tested for additional withdrawal behaviors (below).

Elevated zero-maze

The elevated zero-maze (EZM) is a validated test for observation of anxiety-like behavior in rodents (Shepherd et al., 1994), and was assessed at a timepoint (hr 8) at which anxiety-like behavior has been reported post-ethanol (Verleye et al., 2009). The EZM (Flair Plastics; Portland, OR) is 45 cm high and consists of four equally-proportioned arms, forming a circle with an external diameter of 45 cm. The walls of the two closed arms are made of black acrylic 11 cm in height; the open arms have a small lip composed of C/B acrylic 3 mm in height. Animals were individually transferred 4 feet on the experimenter's hand (to mitigate the potential to elicit convulsions) from their

homecage to the EZM and allowed to explore the apparatus for 5 min under low-level illumination (20 lux). Behavior was video-recorded and later scored by an experimenter blind to genotype and treatment. The following behaviors were assessed: closed arm line crosses and rears, open arm line crosses and rears, transition line crosses between open and closed arms, head dips over the side of the open arms, stretched attends (extension and sudden retraction of the torso; Kliethermes et al., 2004), percent time spent in the open arms (a validated measure of anxiety-like behavior; Shepherd et al., 1994), urinations and boli produced (a purported measure of anxiety-like behavior; Hall et al., 1934; Milner & Crabbe, 2008).

Tail suspension test

The tail suspension test (TST) is validated test for depression-like behavior (Cryan et al., 2005), and was assessed at two time points (hr 12 and 24) at which depression-like behavior has been reported post-ethanol (Kokare et al., 2008). The TST apparatus (Flair Plastics, Portland, OR) consists of a gray polyvinyl chloride beam, 104 cm long and 54.5 cm high. Mice were removed from their homecage and transported 5 feet to the TST apparatus on the experimenter's hand. Using surgical tape, mice were suspended by taping the tail (3/4 of the way to tail tip) to the flat surface of the beam (63 cm above the floor) for 6 min. Behavior was video-recorded and scored by an experimenter blind to genotype and treatment. Percent (%) time immobile (i.e., no movement of head, limbs or torso) was assessed as a measure of depression-like behavior.

Blood ethanol concentrations

Naïve 129/S5$^{Mpdz+/-}$ and wildtype littermates (n=8 and 12, respectively) were injected with ethanol (4 g/kg, i.p.) and returned to their homecage. Tail blood samples

(20 µl) were drawn 45, 75, 240 and 600 min post-ethanol and collected into tubes containing 50 µl 5% zinc sulfate, 50 µl 0.3 M barium hydroxide and 300 µl water. Samples were mixed and centrifuged at 18,200 x g for 5 min. Ethanol-containing supernatants were analyzed on a model 6890 gas chromatograph (Agilent) to determine blood ethanol concentrations (BECs) as previously described (Terdal & Crabbe, 1994).

Data Analyses

Outliers were defined using the Grubb's outlier analysis and were removed prior to data analyses. Behaviors were analyzed using Systat® with analyses of variance (ANOVAs) to assess main effects of sex, genotype and treatment as well as interactions. Significant ($p<0.05$) results were subsequently assessed using Tukey's post hoc analyses. All p-values reported are from two-tailed analyses.

Results

Ethanol withdrawal HICs

HIC results for B6$^{Mpdz+/-}$ and wildtype littermates are shown in figure 3a. A main effect of sex was apparent for baseline HIC scores, with females exhibiting higher baseline HICs than males ($F_{(1,112)}=7.6$, $p=0.007$). A trend for a main effect of genotype was also detected, with B6$^{Mpdz+/-}$ exhibiting lower baseline HICs than wildtype littermates ($F_{(1,112)}=2.8$, $p= 0.09$).

For ethanol withdrawal severity scores (figure 3b), no main effect of sex or interactions involving sex were detected (all $F<0.7$, $p>0.4$), so data from both sexes were combined for subsequent analyses. A main effect of treatment was apparent, with ethanol withdrawn mice exhibiting more severe HICs than controls ($F_{(1,110)}=8.3$, $p=0.005$). A main effect of genotype was also observed, with B6$^{Mpdz+/-}$ exhibiting more

severe HICs than wildtype littermates ($F_{(1,110)}$=4.7, p=0.03). Although no genotype x treatment interaction was detected (F<0.8, p>0.4), the difference between genotypes in HIC scores was greater for ethanol withdrawn than for control mice, suggesting that *Mpdz* may influence both baseline and withdrawal associated convulsions.

Results for 129/S5$^{Mpdz+/-}$ and wildtype littermates are shown in figure 4a. No main effect of sex or interactions involving sex were detected (all F<1.9, p>0.2), so data for both sexes were combined. For baseline scores, no main effect of genotype or interactions involving genotype were detected (all F<0.9, p>0.3). Ethanol withdrawal severity scores (figure 4b) showed a main effect of treatment ($F_{(1,167)}$=89, p<0.001), but no main effect of genotype or genotype x treatment interaction (all F<0.5, p>0.5). Taken together, these results indicate that reductions in *Mpdz* expression increase ethanol withdrawal convulsions, and that this effect is dependent on genetic background.

Figure 3. Timecourse of ethanol withdrawal HICs in B6$^{Mpdz+/-}$ and wildtype (B6^{WT}) littermates. **(a)** B6$^{Mpdz+/-}$ (n=66) and B6^{WT} (n=48) mice were scored twice for baseline (pre-ethanol) HICs immediately before administration of 4 g/kg ethanol or saline (arrow indicates injection at hr 0), hourly from hr 2-12, and again at hr 24 and 25. Average baseline scores differed between B6$^{Mpdz+/-}$ and B6^{WT} (mean ± SEM = 0.2 ± 0.1 and 0.5 ± 0.2, respectively; p=0.04). HIC scores increase above baseline approximately 5 hr post-ethanol, indicating a state of withdrawal-associated hyperexcitability, which peaks ~7-8 hr post-ethanol. The data shown represent the mean ± SEM for individual post-ethanol HIC scores (corrected for individual baseline HIC scores). **(b)** Ethanol withdrawal severity (corrected area under the curve for 2-12 hr, mean ± SEM) was more severe in B6$^{Mpdz+/-}$ than in B6^{WT} littermates; *****p < 0.05.

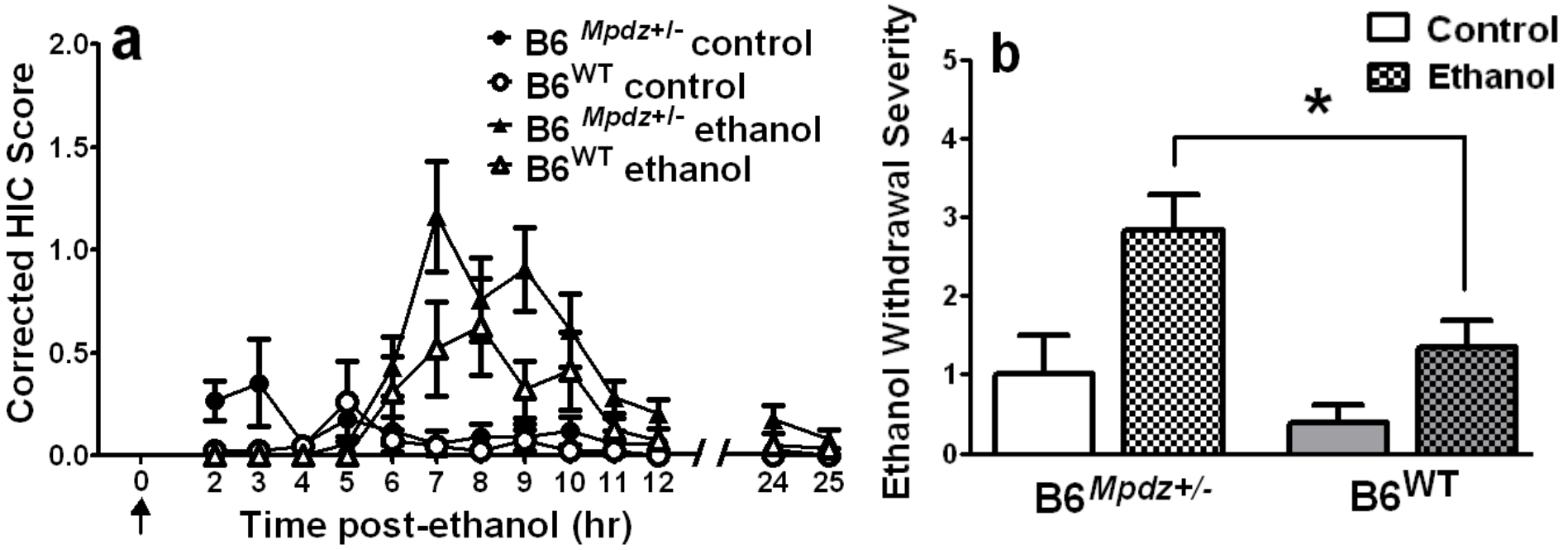
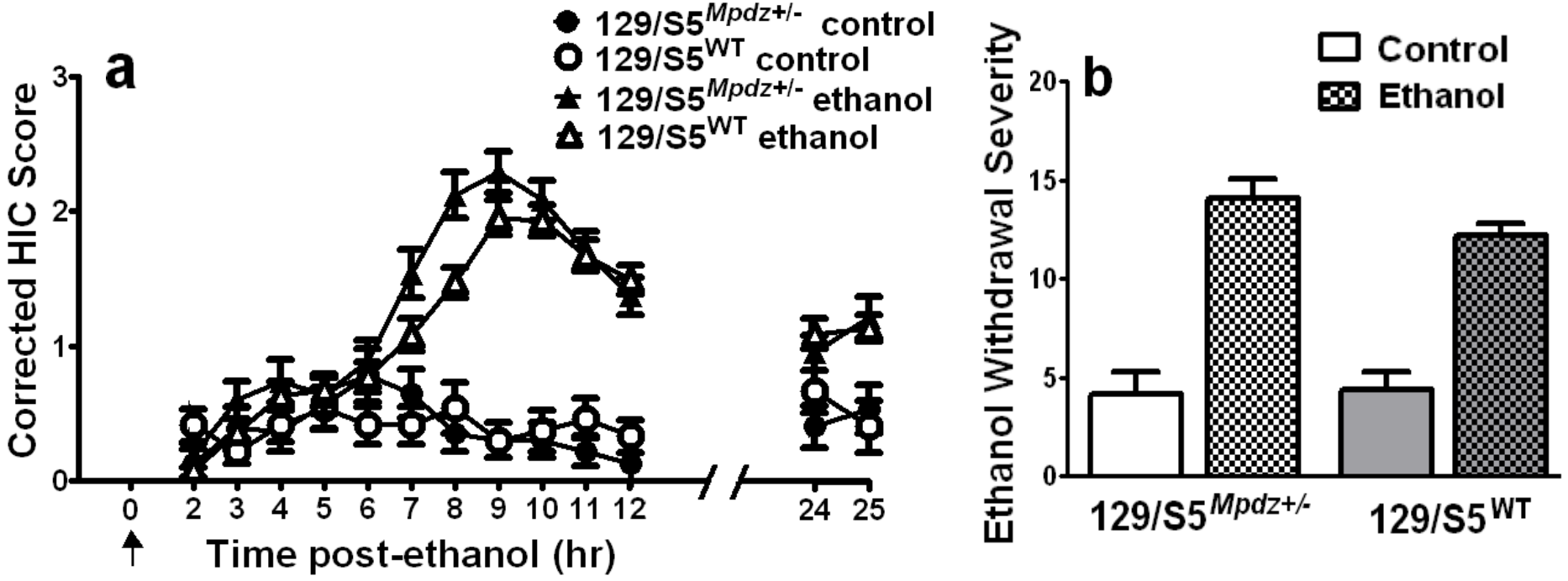


Figure 4. Timecourse of ethanol withdrawal HICs in 129/S5$^{Mpdz+/-}$ and wildtype (129/S5^{WT}) littermates. (a) 129/S5$^{Mpdz+/-}$ (n=90) and wildtype littermate (n=81) mice were scored twice for baseline (pre-ethanol) HICs immediately before administration of 4 g/kg ethanol or saline (arrow indicates injection at hr 0), hourly from hr 2-12, and again at hr 24 and 25. Average baseline scores did not differ between 129/S5$^{Mpdz+/-}$ and 129/S5^{WT} mice (mean ± SEM = 1.9 ± 0.1 and 2.0 ± 0.1, respectively; p>0.3). HIC scores increase above baseline around hr 7 post-ethanol, indicating a state of withdrawal associated hyperexcitability, and peak ~9 hr post-ethanol. The data shown represent the mean ± SEM for individual post-ethanol HIC scores (corrected for their baseline HIC score). **(b)** Ethanol withdrawal severity (corrected area under the curve for 2-12 hr post-ethanol) did not differ between 129/S5$^{Mpdz+/-}$ and 129/S5^{WT} mice.

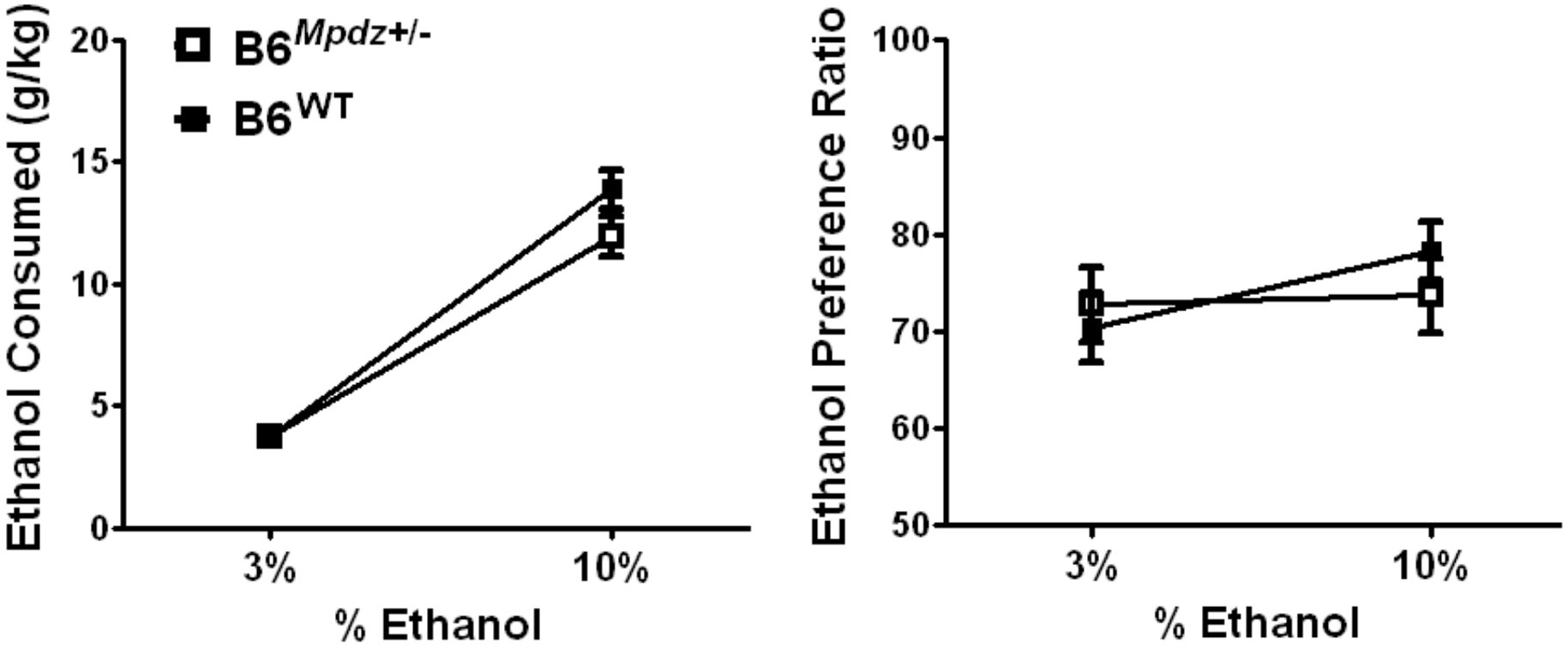


Ethanol consumption and preference

These analyses were performed in two passes four months apart, using B6$^{Mpdz+/-}$ and wildtype littermates. Water consumption did not differ between genotypes (all $F<1.4$, $p>0.2$). For the ethanol consumption/preference variables, an effect of pass was evident, with animals in pass 2, consuming approximately twice as much ethanol and exhibiting more ethanol preference than those in pass 1 ($F_{(1,75)}=8.7$, $p=0.004$). Although a pass x genotype interaction was apparent for 3% ethanol consumption ($F_{(1,75)}=4.3$, $p=0.04$), with B6$^{Mpdz+/-}$ consuming more ethanol than wildtype littermates in pass 1 (3.7 ± 0.4 and 2.8 ± 0.3 g/kg, respectively; $p=0.09$) and B6$^{Mpdz+/-}$ consuming less ethanol than wildtype littermates in pass 2 (3.8 ± 0.3 and 4.7 ± 0.2 g/kg, respectively; $p=0.01$), a pass x genotype interaction was not observed for the other ethanol variables (all $F<1.5$, $p>0.2$), so both passes were combined for subsequent analyses.

A main effect of sex was apparent for ethanol (3%) preference ($F_{(1,75)}=4.2$; $p=0.044$) and consumption ($F_{(1,75)}=9.5$; $p=0.003$), with females exhibiting higher ethanol preference (77.1 ± 3.4% and 66.1 ± 3.8%, respectively; $p=0.04$) and consumption (4.6 ± 0.2 and 2.9 ± 0.2 g/kg, respectively; $p<0.001$) than males. However, sex x genotype interactions were not detected (both $F<1.6$, $p>0.2$), so data for both sexes were combined for subsequent analyses. A trend for a main effect of genotype on ethanol (10%) consumption was detected, with B6$^{Mpdz+/-}$ consuming less ethanol than wildtype littermates ($F_{(1,79)}=3.5$, $p=0.065$; figure 5); genotype differences in 3% ethanol preference/consumption or 10% preference were not detected (all $F<0.8$, $p>0.3$).

Figure 5. Ethanol preference and consumption in B6$^{Mpdz+/-}$ and wildtype (B6^{WT}) littermates. B6$^{Mpdz+/-}$ (n=39) and B6^{WT} (n=44) mice were tested for ethanol consumption (g/kg) and preference (ml ethanol/ml total consumed) at two ascending concentrations (3% and 10% ethanol v/v in tapwater) for four days at each concentration. B6$^{Mpdz+/-}$ consumed less 10% ethanol than B6^{WT} mice (11.9 ± 0.8 and 14.1 ± 0.9 g/kg, respectively; $p<0.1$), but did not differ for ethanol preference. Data represent the mean ± SEM for days 2 and 4 at each ethanol concentration tested. In some cases the error bars are smaller than the symbol.



Homecage activity

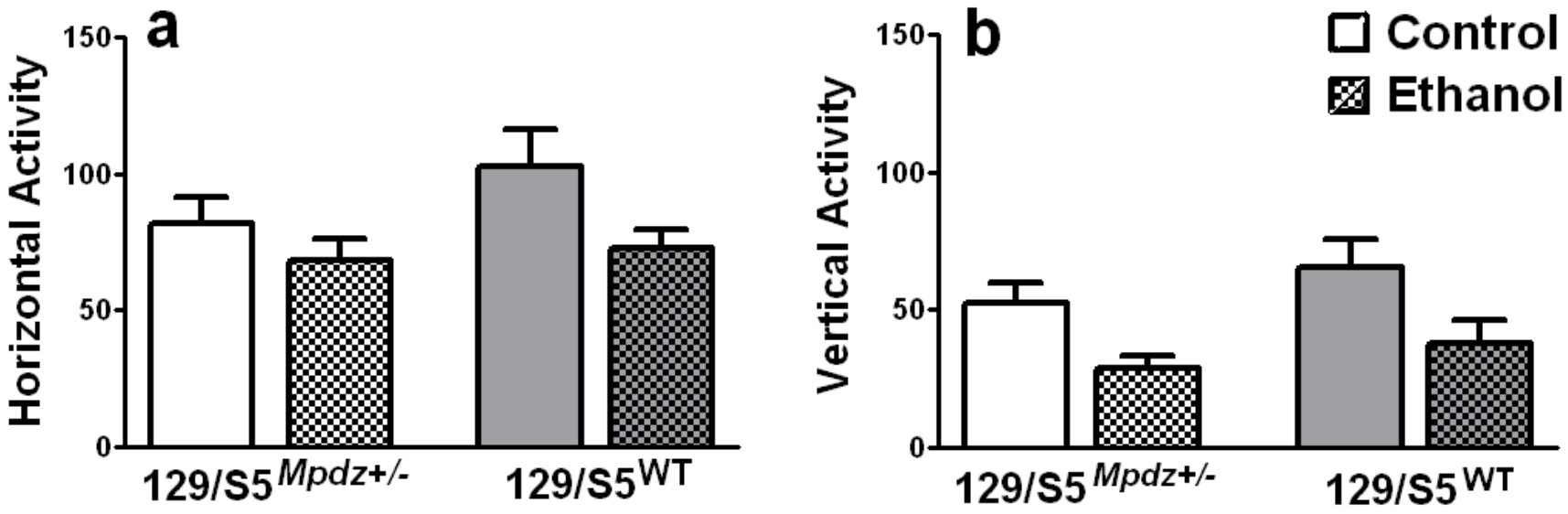
Using 129/S5$^{Mpdz+/-}$ and wildtype littermates, a main effect of sex was apparent, with females exhibiting more line crosses ($F_{(1,65)}$=6.5, p=0.013) and rears ($F_{(1,66)}$=4.6, p=0.035) than males; however, no sex x treatment or sex x genotype interactions were detected (both F<0.4, p>0.5), so data for both sexes were combined for subsequent analyses. A main effect of treatment was observed, with ethanol withdrawn mice exhibiting fewer line crosses ($F_{(1,65)}$=5.2, p=0.025) and rears ($F_{(1,66)}$=10.6, p=0.002) than controls (figure 6a-b). These results suggest that acute withdrawal is associated with locomotor depression, as has been reported in withdrawn mice following chronic ethanol exposure (Kliethermes et al., 2005). No main effect of genotype or genotype x treatment interaction were detected (both F<0.8, p>0.4), suggesting that *Mpdz* does not influence baseline locomotor activity or ethanol withdrawal-associated locomotor depression on this genetic background.

Elevated zero-maze (EZM)

Using 129/S5$^{Mpdz+/-}$ and wildtype littermates, no main effect of sex or interactions involving sex were detected (all F<1.8, p>0.2), so data for both sexes were combined for all subsequent analyses.

Activity in the closed arms. A trend for a main effect of treatment was detected, with ethanol withdrawn mice exhibiting fewer line crosses than controls ($F_{(1,66)}$=3.7, p=0.06; figure 7a). This indicates that withdrawal-associated locomotor depression may be a useful measure of acute ethanol withdrawal, as has been reported for using chronic withdrawal models (Kliethermes et al., 2004). However, no main effect of genotype or genotype x treatment interaction were detected (both F<1.2, p>0.3), suggesting that *Mpdz* does not influence baseline horizontal activity or ethanol withdrawal associated

Figure 6. Homecage activities in ethanol withdrawn and control mice. Data represent activity values (mean ± SEM) in ethanol withdrawn and control mice. **(a)** Horizontal activity (line crosses) and **(b)** vertical activity (rears) were recorded for 2 hr (hr 6-8) post-injection of 4 g/kg ethanol (i.p.) or saline. For both measures, ethanol withdrawn animals exhibited less homecage activity than control animals ($p<0.05$), but differences between 129/S5$^{Mpdz+/}$ (n=36) and wildtype littermates (129/S5^{WT}; n= 44) were not detected.



depression of horizontal activity, at least on this genetic background. No main effects of treatment or genotype, or a treatment x genotype interaction, were detected for rears (all $F<0.1$, $p>0.8$; figure 7e).

Head dips. A main effect of treatment was apparent, with ethanol withdrawn mice exhibiting fewer head dips than controls ($F_{(1,66)}=7.6$; $p=0.007$; figure 7d). This is consistent with previous reports indicating that head dips may be a sensitive index of acute and chronic ethanol withdrawal (Kliethermes et al., 2004; Milner et al., in preparation [see chapter 3]). However, no main effect of genotype or treatment x genotype interaction were detected (both $F<0.4$, $p>0.6$).

. *Stretched attends.* No main effect of treatment was detected ($F<0.6$, $p>0.5$). A trend for a main effect genotype was detected, with $129^{Mpdz+/-}$ exhibiting fewer stretched attends than wildtype littermates ($F_{(1,64)}=3.9$, $p=0.052$; figure 7h). However, no genotype x treatment interaction was detected ($F<0.6$, $p>0.5$), suggesting that *Mpdz* status may influence this behavior independent of ethanol withdrawal.

Activity in the open arms. A main effect of treatment was apparent for open arm line crosses, with ethanol withdrawn animals exhibiting fewer crosses than controls ($F_{(1,66)}=4.3$, $p=0.043$; figure 7b). A trend for a main effect of treatment was also detected for transition line crosses, with ethanol withdrawn animals exhibiting fewer transitions than controls ($F_{(1,66)}= 3.3$, $p= 0.074$; figure 7c). However, no main effect of genotype or genotype x treatment interaction was detected for either of these behaviors (both $F<1.7$, $p>0.2$). No main effects of treatment or genotype or treatment x genotype interaction were detected for open arm rears (all $F<0.5$, $p>0.5$; figure 7f). Taken together, these results suggest that *Mpdz* status does not influence activity in the EZM open arms.

Anxiety-like behavior (% time open arms). A trend for a main effect of treatment was apparent, with ethanol withdrawn mice exhibiting less anxiety-like behavior (a greater % time in the open arms) than controls ($F_{(1,66)}=3.9$, $p=0.053$; figure 7g). No main

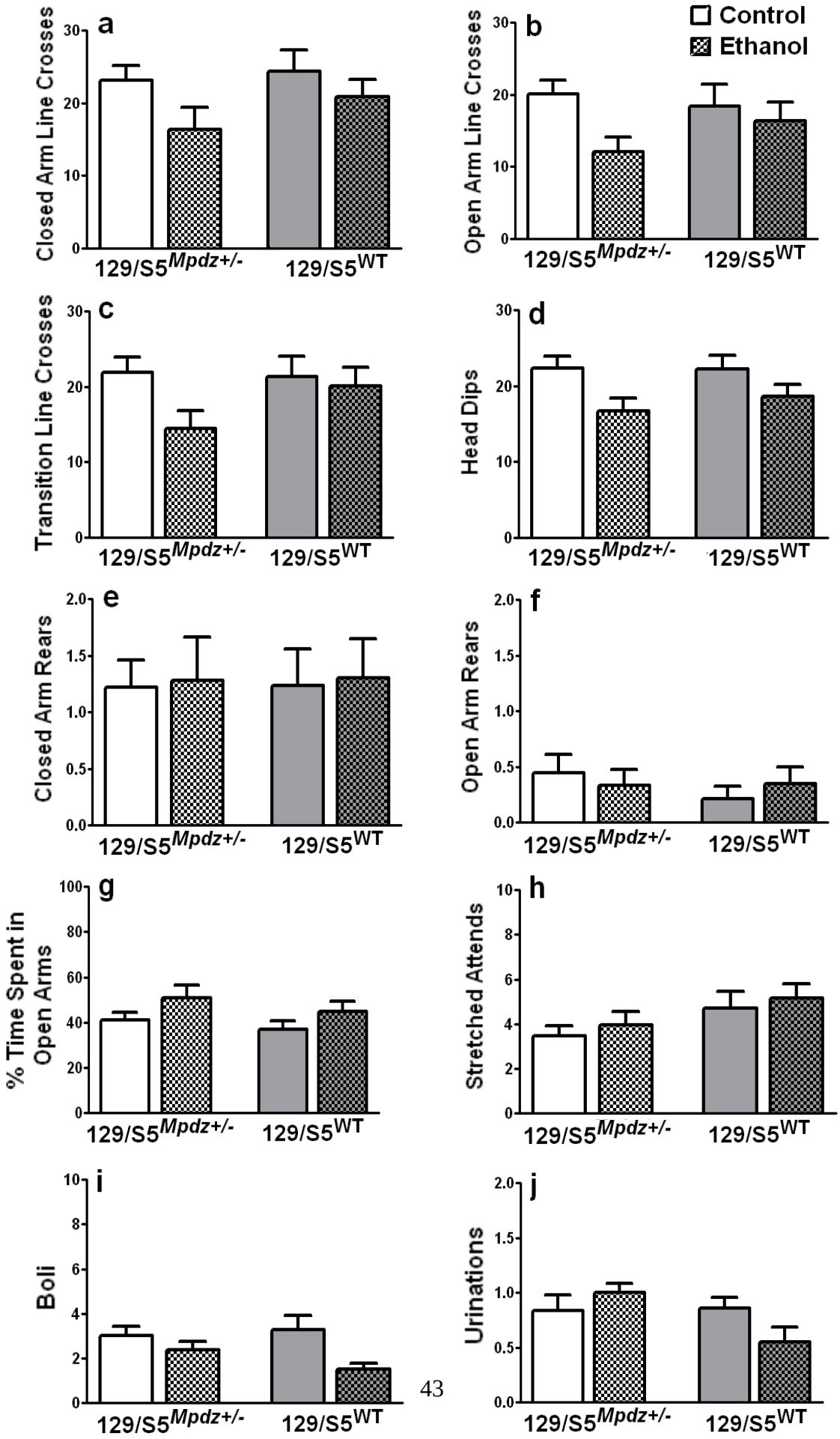
effect of genotype or genotype x treatment interaction was detected (both $F<1.2$, $p>0.3$), suggesting that *Mpdz* does not influence baseline or withdrawal-associated anxiety-like behavior in this model.

Boli. A main effect of treatment was apparent (figure 7i), with ethanol withdrawn mice exhibiting fewer boli than controls ($F_{(1,66)}=8.3$, $p=0.005$). A main effect of sex was also detected, with females producing more boli than males ($F_{(1,62)}=15.8$, $p<0.001$). However, no main effect of genotype or genotype x treatment x sex interaction was detected (both $F<2.1$, $p>0.2$), suggesting that *Mpdz* genotype and sex variables do not influence baseline or withdrawal-associated boli production.

Urinations. No main effect of treatment was evident ($F<0.3$, $p>0.6$). A trend for a small effect of genotype was detected, with $129^{Mpdz+/-}$ producing more urinations than wildtype littermates ($F_{(1,66)}=3.0$; $p=0.09$; figure 7j). A trend for a genotype x treatment interaction was also detected ($F_{(1,66)}=3.7$; $p=0.06$), with $129^{Mpdz+/-}$ exhibiting more urinations than wildtype littermates during ethanol withdrawal (1.0 ± 0.1 and 0.5 ± 0.1, respectively; $p=0.01$) but not in controls (both 0.8 ± 0.1; $p=0.9$). These results suggest that anxiety-like behaviors (withdrawal-associated or baseline) are not influenced by *Mpdz* status, at least on the 129/S5 genetic background.

Taken together, results in the EZM suggest that mice exhibit acute ethanol withdrawal associated locomotor depression, as an effect of treatment was apparent for closed and open arm activities. Anxiety-like behavior (% time spent in open arms, boli) was less apparent during ethanol withdrawal, a finding in contrast with previous literature (Kliethermes et al., 2004). These results may have been influenced by abnormalities in the 129/S5 genetic background, which has not been previously characterized for this phenotype.

Figure 7a-j. Activity and anxiety-like behaviors in the EZM using ethanol withdrawn and control mice. 129/S5$^{Mpdz+/-}$ (n=36) and wildtype littermates (129/S5^{WT}; n=44) were tested in the EZM at hr 8 post-injection of 4 g/kg ethanol (i.p.) or saline. Data represent activity or anxiety-like behavior values (mean ± SEM). **(a)** Closed arm, **(b)** open arm and **(c)** transition line crosses, as well as **(d)** head dips were lower in ethanol withdrawn compared to control mice, but no effect of *Mpdz* genotype was detected. **(e)** Closed arm and **(f)** open arm rears were not affected during withdrawal or by genotype. **(h)** Stretched attends were less frequent in 129/S5$^{Mpdz+/-}$ than 129/S5^{WT}, but were not influenced by withdrawal. **(g)** Anxiety-like behavior (% time spent in the open arms) and **(i)** boli produced were lower in withdrawn animals than controls, but no effect of genotype was detected. 129/S5$^{Mpdz+/-}$ produced more **(j)** urinations than 129/S5^{WT} during ethanol withdrawal.


a
Closed Arm Line Crosses
30
20
10
0
129/S5Mpdz+/-
129/S5WT
b
Control
Ethanol
Open Arm Line Crosses
c
Transition Line Crosses
d
Head Dips
e
Closed Arm Rears
2.0
1.5
1.0
0.5
0.0
f
Open Arm Rears
g
% Time Spent in Open Arms
100
80
60
40
20
h
Stretched Attends
10
8
6
4
2
i
Boli
j
Urinations

Tail suspension test

129$^{Mpdz+/-}$ and wildtype littermates were tested. Although precautions were taken to avoid eliciting convulsions, convulsions resembling HICs were observed in 10/70 (14%) of the mice tested, so the incidence of convulsions was analyzed as a potential confounding factor. Initial analyses did not detect a main effect of or interactions involving seizures at hr 12 (all $F<1.6$, $p>0.2$) or hr 24 (all $F<1.7$, $p>0.2$). Additionally, no correlation was observed between seizures and % time immobile (all $p>0.3$). Therefore, all of the mice tested in the TST were included in the subsequent analyses.

No main effect of sex or sex interactions were detected (all $F<1.7$, $p>0.2$), so data from both sexes were combined. A main effect of treatment was apparent at both timepoints assessed, with ethanol withdrawn mice exhibiting less % time immobile than controls at hr 12 ($F_{(1,66)}=5.3$, $p=0.02$) and hr 24 ($F_{(1,66)}=8.7$, $p=0.004$) post-injection (figure 8). However, no main effect of genotype or genotype x treatment interaction was detected ($F<0.1$, $p>0.7$), suggesting that *Mpdz* status does not influence baseline or withdrawal-associated depression-like behavior in this model.

Summary of behavioral results

Table 1 provides a summary of the expected and observed direction of effect for acute withdrawal behaviors in the *Mpdz* knockout models. As expected, B6$^{Mpdz+/-}$ exhibited lower withdrawal-associated convulsions than wildtype littermates, confirming *Mpdz* as a QTG for ethanol withdrawal convulsion severity. A trend for a reduction in ethanol consumption was also observed in B6$^{Mpdz+/-}$ compared to wildtype littermates, suggesting that *Mpdz* may influence ethanol consumption in the two-bottle preference test. 129/S5$^{Mpdz+/-}$ did not differ from wildtype littermates on any of the acute ethanol withdrawal-associated behaviors tested, suggesting that *Mpdz* does not influence ethanol withdrawal on this genetic background.

Figure 8. Depression-like behavior in ethanol withdrawn and control mice. 129/S5$^{Mpdz+/-}$ (n=36) and wildtype littermates (129/S5^{WT}; n=44) were assessed for depression-like behavior (i.e., greater % time immobile) at hr 12 and 24 post-ethanol (4 g/kg, i/p) or saline. Data reported are the % time immobile (mean ± SEM). Ethanol withdrawn mice (patterned bars) exhibited less depression-like behavior than controls (solid bars) at both timepoints.

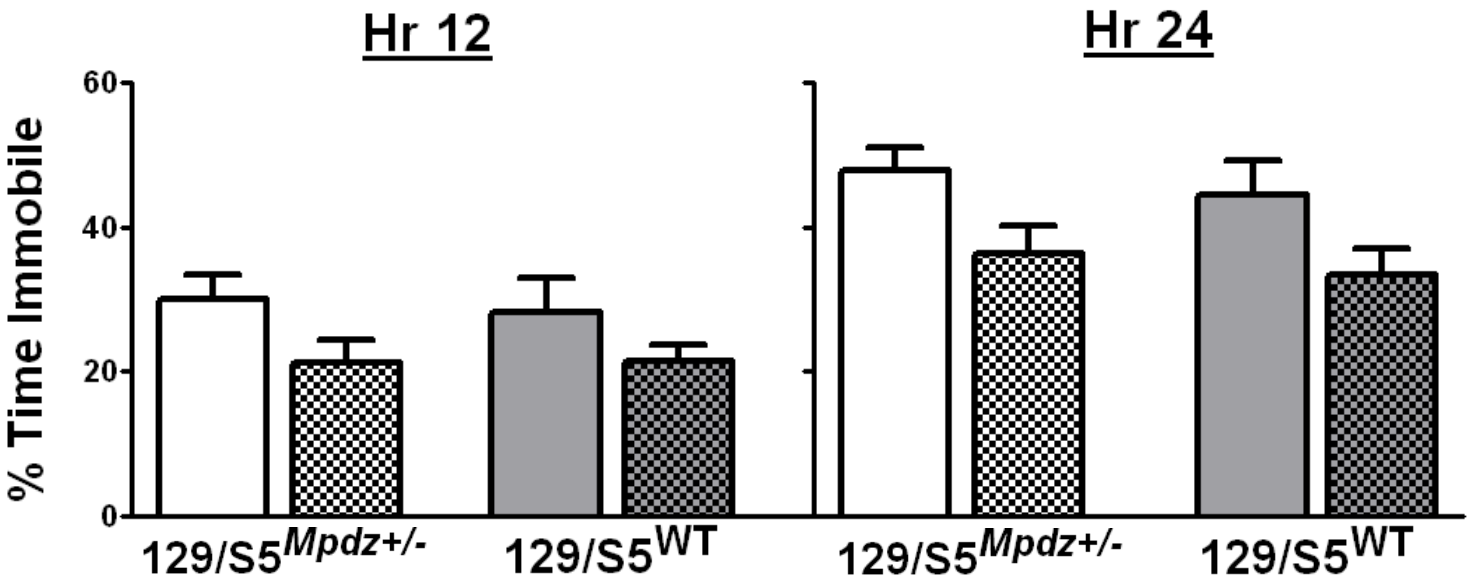


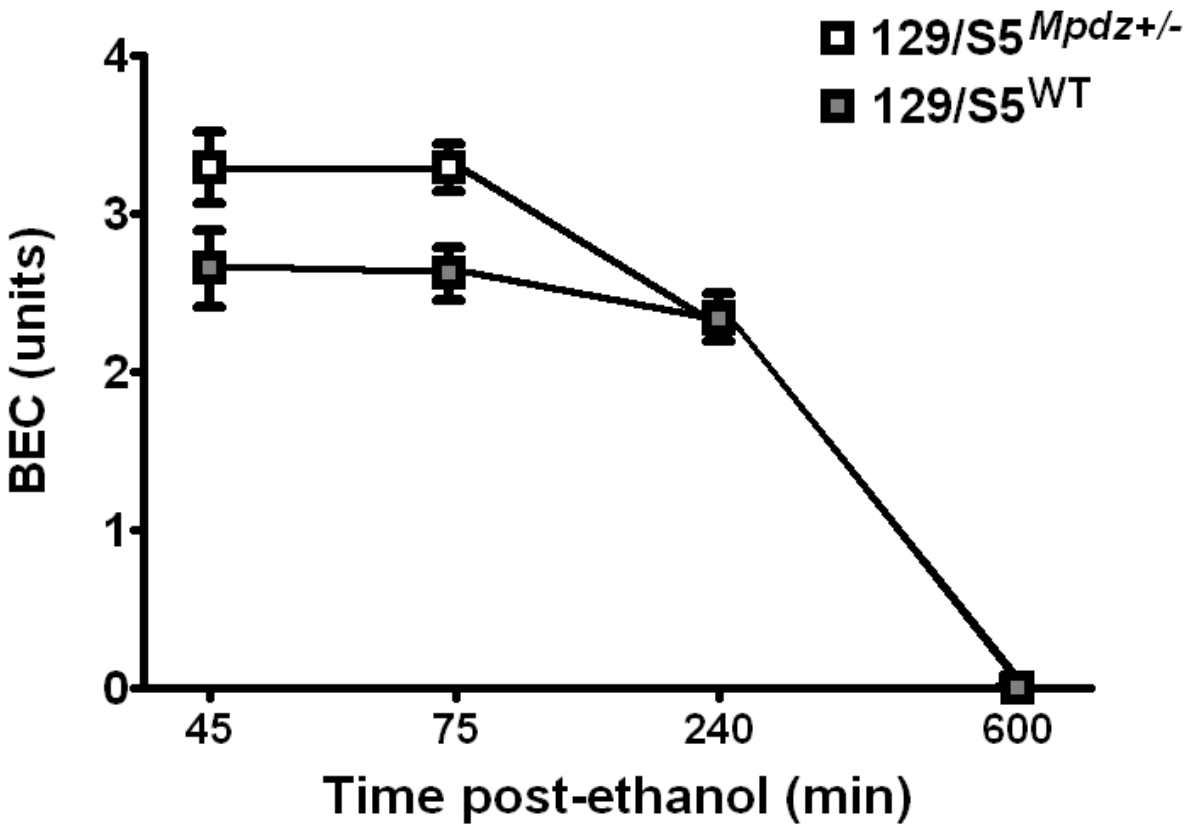
Table 1. Summary of behavioral results in *Mpdz* knockout mice. Outline of the expected (Exp) and observed (Obs) effects associated with acute ethanol withdrawal (WD), and associated with *Mpdz* expression in knockout ($B6^{Mpdz+/-}$ or $129/S5^{Mpdz+/-}$) compared to wildtype littermates on the phenotypes tested. ↑ indicates an increase in behavioral phenotypic scores, ↓ indicates a decrease in phenotypic scores, ─ indicates no change in the phenotype. N/A= data not collected

Phenotype	Exp WD	Obs WD	Exp KO	Obs KO
HIC severity ($B6^{Mpdz+/-}$)	↑	↑	↑	↑
HIC severity ($129/S5^{Mpdz+/-}$)	↑	↑	↑	─
Ethanol consumption ($B6^{Mpdz+/-}$)	N/A	N/A	↓	↓
Homecage locomotor activity ($129/S5^{Mpdz+/-}$)	↓	↓	↑	─
Locomotor activity in the EZM (line crosses, head dips; $129/S5^{Mpdz+/-}$)	↓	↓	↑	─
Anxiety-like behavior in the EZM (% time in open arms; $129/S5^{Mpdz+/-}$)	↓	↑	↑	─
Boli and urinations ($129/S5^{Mpdz+/-}$)	↑	↓	↑	─
Depression-like behavior in the TST (% time immobile; $129/S5^{Mpdz+/-}$)	↑	↓	↑	─

Blood ethanol concentrations

BECs were negligible at 600 min (10 hr) post-ethanol, so this timepoint was excluded from our analyses. Although a main effect of sex was apparent, with males exhibiting higher BECs than females ($F_{(1,15)}$=8.3, p=0.01), no interactions involving sex were detected (all F<1.8, p>0.2), so data from both sexes were collapsed for subsequent analyses. A repeated-measures analysis revealed main effects of genotype ($F_{(1,17)}$=6.5, p=0.02) and time post-ethanol ($F_{(2,34)}$=7.8; p=0.002), with 129/S5$^{Mpdz+/-}$ exhibiting higher BECs than wildtype littermates at 45 min and 75 min, but not at 240 min post-ethanol (figure 9). These data suggest that 129/S5$^{Mpdz+/-}$ and wildtype mice differ in initial ethanol elimination rates following acute administration.

Figure 9. Blood ethanol concentrations (BECs) in 129/S5$^{Mpdz+/-}$ and wildtype (129/S5^{WT}) mice. BECs (mean ± SEM) were assessed at 45, 75, 240 and 600 min post-administration of a hypnotic dose of ethanol (4 g/kg, i.p.). 129/S5$^{Mpdz+/-}$ (n=8) exhibited higher BECs than 129/S5^{WT} (n=12) littermates at 45 and 75 min post-ethanol ($p<0.05$), but not 240 or 600 min post-ethanol.



Discussion

The present studies provide the first evidence that *Mpdz* influences alcohol withdrawal behavior *in vivo* and, taken together with other supporting evidence (Shirley et al., 2004; Milner, Shirley et al., in preparation), confirm *Mpdz* as a QTG for ethanol withdrawal. Our analyses using novel B6$^{Mpdz+/-}$ mice indicate that reduced *Mpdz* expression is related to more severe ethanol withdrawal. The reciprocal is also evident, such that *Mpdz* transgenic mice with increased *Mpdz* expression (developed using a bacterial artificial chromosome in which *Mpdz* is the only full-length gene) display significantly less severe alcohol withdrawal than nontransgenic littermates (Milner, Shirley et al., in preparation). Moreover, the combination of BAC transgenic and KO allows us to evaluate the effects of increasing and reducing the dosage of the gene: low *Mpdz* expression is related to more severe alcohol withdrawal and high *Mpdz* expression to mild withdrawal.

Given the strong converging evidence that *Mpdz* is a QTG for ethanol withdrawal, it is somewhat surprising that differential ethanol withdrawal convulsions were not detected between 129/S5$^{Mpdz+/-}$ and wildtype mice. However, there are at least five plausible reasons why an effect of *Mpdz* knockdown is apparent on the B6 background, but not in the 129/S5 background. First, the chromosome 4 QTL for which *Mpdz* is a QTG candidate was identified using B6D2-derived mapping populations (Buck et al., 1997); however, QTL analyses for ethanol withdrawal have not including 129/S5 mice, it does not necessarily follow that this QTL will be evident on other genetic backgrounds. Second, 129/S5 mice have not been assessed for *Mpdz* or HIC status, but analyses of the related 129/J strain demonstrate that it expresses a different MPDZ variant and differs in *Mpdz* expression compared with B6 or D2 strain mice (Fehr et al., 2002), allowing the possibility that *Mpdz* structure and/or expression in 129-derived strains may not influence ethanol withdrawal. Third, it is possible that the 129/S5 *Mpdz*

allele exerts dominance (not apparent in B6- or D2-derived strains) that mitigates *Mpdz*'s effect in this knockout model. Although homozygote knockouts do not survive to testing age, examination of *Mpdz* expression in knockout homozygotes and heterozygotes compared to wildtype littermates in appropriate brain regions and/or cell populations can address this issue. Fourth, because 129/S5-derived mice were found to exhibit higher baseline HICs and a substantially delayed timecourse for withdrawal compared to B6- and D2-derived mice, it is possible that *Mpdz*'s influence on ethanol withdrawal convulsions is mitigated by one or both of these factors. Fifth, a linked gene might mitigate *Mpdz*'s effect in $129^{Mpdz+/-}$ mice (e.g., via an epistatic interaction with one or more 129/S5 background genes; see general discussion for details). Although it is possible that a gene linked to *Mpdz* may contribute to differential ethanol withdrawal in the $B6^{Mpdz+/-}$ model, this is unlikely given that increased *Mpdz* expression in BAC transgenic mice results in significantly less severe ethanol withdrawal compared to wildtype littermates. The fact that increased *Mpdz* expression is associated with significantly less severe ethanol withdrawal in BAC transgenic mice (D2 background) and vice versa in the $B6^{Mpdz+/-}$ model, is consistent with this QTL being robust in B6 and D2-derived mice but not in 129/S5 derived mice.

$B6^{Mpdz+/-}$ exhibited a trend to consume less ethanol than wildtype littermates in preliminary analyses, suggesting a broader role for *Mpdz* beyond withdrawal. Previous studies have established a negative genetic correlation between ethanol withdrawal convulsion severity and ethanol consumption/preference (Metten et al., 1998b), suggesting some genetic overlap between these behaviors. My results suggest that *Mpdz* may contribute to this genetic relationship. Notably, a recent human study found that variation in the human homolog (*MPDZ*) is associated with alcohol consumption behaviors in Caucasian men (Tabakoff et al., 2009).

Specific genetic associations between withdrawal convulsant behaviors and other withdrawal behaviors are not well-established, and it is possible that *Mpdz* exerts pleiotropic effects on ethanol-withdrawal. In the 129/S5$^{Mpdz+/-}$ model, acute ethanol withdrawal was associated with locomotor depression in familiar (homecage) and novel (EZM) environments. Taken together with other studies (Doremus-Fitzwater & Spear, 2007; Kliethermes et al., 2005), my results demonstrate that ethanol withdrawal-associated locomotor depression is robust and quantifiable, but *Mpdz* expression differences did not influence the withdrawal behaviors tested. Given that the 129/S5$^{Mpdz+/-}$ did not differ from wildtype littermates in ethanol withdrawal convulsion severity, it is not surprising that no effect on other withdrawal behavior was detected using this model.

Preliminary evidence from our laboratory indicates that ethanol withdrawal increases depression-like behavior in the TST, and to a lesser degree in D2.B6 chromosome 4 congenic compared with background strain (D2) mice (Milner & Buck, unpublished results). Notably, a QTL centered on *Mpdz* has been reported for depression-like behavior in the TST using B6-derived mice (Liu et al., 2007). However, in 129/S5-derived mice, ethanol withdrawal was associated with reduced depression-like behavior in 129/S5-derived mice, which is opposite to the direction of effect observed in rodent models and in humans (Kokare et al., 2008; McKeon et al., 2008). Ethanol withdrawn 129/S5-derived mice also showed reduced anxiety-like behavior, which is opposite to the direction of effect observed in rodent models and in humans (Heilig et al., 2010; Verleye et al., 2009). This is further evidence that 129/S5-derived mice may exhibit atypical ethanol withdrawal, and that *Mpdz* effects on withdrawal may be confounded on this genetic background.

It is possible that convulsions activity influenced depression-like behavior in the TST, since I had limited power to detect effects of HICs on behavior in this task. Future

studies will be needed that assess withdrawal-associated depression-like behavior in other validated models where HICs are not as likely (e.g., forced swim test; Petite-Demouliere et al., 2005). Additionally, it is possible that withdrawal-associated anxiety-like and depression-like behaviors may not be observable in 129/S5-derived mice at the timepoints tested due to strain-specific factors that influence the timecourse of withdrawal (e.g., ethanol metabolism kinetics). Both withdrawal-induced motor impairment and anxiogenic-like behaviors have been observed up to 36-48 hr post-ethanol (Farook et al., 2007; Jansone et al., 2009; Philibin et al., 2008), so testing at additional timepoints may unveil anxiety-like and depression-like behaviors in 129/S5-derived mice. Finally, *Mpdz* may influence additional ethanol withdrawal-associated behaviors not tested in the current study. One potentially-relevant phenotype is hippocampal-associated memory, which is affected in ethanol withdrawn rodents (Obernier et al., 2002), given that MPDZ is central to protein complexes moderating synaptic plasticity in hippocampal neurons (Krapivinsky et al., 2004). Future studies should examine the potential role of *Mpdz* on ethanol phenotypes influenced by receptor systems associated with MPDZ to identify other pleiotropic effects of this gene *in vivo*.

CHAPTER 3: Ethanol withdrawal behaviors in *Kcnj9* knockout mice

Lauren C. Milner and Kari J. Buck

This manuscript has been prepared for submission to *Genes, Brain and Behavior*

Abstract

Using positional cloning and gene expression analyses, our laboratory previously identified *Kcnj9* as a quantitative trait gene (QTG) candidate for sedative-hypnotic withdrawal convulsions in mice. Here, using a *Kcnj9* knockout model, I assessed this gene's role in ethanol withdrawal convulsions, and its potential pleiotropic effects on additional withdrawal behaviors. Handling-induced convulsions, locomotor activity (in the homecage and the elevated-zero maze), anxiety-like behavior (in the elevated zero maze), and depression-like behavior (in the tail suspension test) were assessed following a single, hypnotic dose of ethanol (4 g/kg, i.p.) or saline. As expected, ethanol withdrawal convulsions were significantly ($p<0.5$) less severe in *Kcnj9* knockout mice than heterozygote and wildtype littermates. Significant acute withdrawal-associated anxiety-like behavior and locomotor depression (horizontal and vertical activities, head dips) were all significantly associated with acute ethanol withdrawal, while depression-like behavior was not. Significant main effects of genotype were apparent for depression-like behavior and vertical activity in the EZM, with trends ($p\sim0.1$) for anxiety-like behavior and horizontal activity in the EZM. Trends for genotype x treatment interactions were also detected for head dips in the EZM, and horizontal and vertical activities in the homecage. Overall, these results are consistent with the conclusion that *Kcnj9* is a QTG for ethanol withdrawal convulsions, and suggest that it has a broader role in withdrawal beyond convulsions as well as depression-like and anxiety-like behaviors.

Introduction

Alcohol (ethanol) abuse and dependence have devastating impacts to individual and societal health, and are responsible for 4.6% of premature deaths worldwide (Rehm et al. 2009). Alcohol withdrawal episodes are a hallmark of physical dependence (Hershon, 1977) and can be a motivating factor in continued alcohol use/abuse and relapse (Little et al., 2005). Alcohol withdrawal symptoms in humans range from psychological (e.g., anxiety and depression) to physiological (e.g., seizures and motor activity changes) in nature (Hersh et al., 1997; McKeon et al., 2008). Genetic contributions to withdrawal symptoms have been reported in alcoholic populations (Karpyak et al., 2009, 2010; Wetherill et al., 2008), and identification of genetic contributions may improve treatment of ethanol withdrawal and prevention of alcohol abuse in at risk individuals (Crabbe & Phillips, 2004; Haile et al., 2008).

Although no animal model entirely duplicates alcoholism, animal models for specific factors, such as the withdrawal syndrome, have been developed. Alcohol withdrawal convulsions occur in all species studied, including humans (Friedman, 1980) and the handling-induced convulsion (HIC) provide a quantitative index of withdrawal severity in mice (Goldstein & Pal, 1971). Our laboratory recently detected and fine-mapped a quantitative trait locus (QTL) on chromosome 1 with large effects on acute and chronic ethanol withdrawal convulsions (Buck et al., 1997, 2002). Positional cloning and expression analyses identified *Kcnj9* as a quantitative trait gene (QTG) candidate for this QTL and suggested that reduced *Kcnj9* expression is associated with less severe withdrawal convulsions (Kozell et al., 2009). *Kcnj9* encodes a subunit member of the G-protein coupled inwardly-rectifying potassium channel (GIRK3/Kir3.3; Lesage et al., 1995). GIRK3 is widely expressed in the brain, where it forms heteromeric GIRK1/3 or GIRK2/3 (and potentially GIRK1/2/3) channels (Koyrakh et al., 2005; Labouebe et al.,

2007; Lüscher & Slesinger, 2010; Torrecilla et al., 2002). GIRK channels are direct targets of ethanol action (Aryal et al., 2009; Chiou et al., 2002) and are implicated in ethanol consumption and analgesic responses (Blednov et al., 2001; Ikeda et al., 2002), although it's role in ethanol withdrawal is unknown currently.

Gene knockout and transgenic models are useful approaches to assess the behavioral effects of a QTG candidate *in vivo* (Abiola et al., 2003). Here, I tested a recently developed *Kcnj9* knockout model to assess this gene's involvement in ethanol withdrawal convulsions. Because some (but not all) signs of withdrawal are highly correlated with convulsive activity (Belknap & Laursen, 1987; Kosobud & Crabbe, 1986), I also assessed potential pleiotropic effects of *Kcnj9* on ethanol withdrawal-associated locomotor depression (Kliethermes et al., 2004, 2005), anxiety-like (Verleye et al., 2009) and depression-like (Hirani et al., 2002) behaviors. My results are consistent with the conclusion that *Kcnj9* is a QTG for ethanol withdrawal with effects that extend beyond withdrawal convulsions.

Methods

Animals

The *Kcnj9* knockout strain was developed and bred in our colony at the Veterinary Medical Unit of the Portland VA Medical Center (Kozell et al., 2009). A *Kcnj9* knockout heterozygote intercross yielded the knockout, heterozygote and wildtype littermates used in the current studies. Males and females were tested in approximately equal numbers. Mice were group-housed 2-5 per cage (28x17x11.5 cm lined with Bedicob® bedding) by sex. Mouse chow (Purina #5001) and water were available *ad libitum*. Procedure and colony rooms were kept at 21 ± 1^{0}C. Lights were on in the colony from 0600-1800 hr. All procedures were approved by the VA and OHSU Institutional

Animal Care and Use Committees in accordance with United States Department of Agriculture and United States Public Health Service guidelines.

Handling-induced convulsions (HICs)

McQuarrie and Fingl (1958) first demonstrated a state of withdrawal-associated CNS hyperexcitability following a single hypnotic dose of ethanol. Details of the acute ethanol withdrawal handling-induced convulsion (HIC) procedure used here have been published (Metten et al. 1994). To assess ethanol withdrawal severity, *Kcnj9* knockout, heterozygote and wildtype mice (n=33-102 per genotype) were scored twice for baseline HICs 20 min apart, followed by administration of a single hypnotic dose of ethanol (4 g/kg, i.p., 20% v/v) or an equivalent volume of vehicle (saline) and were scored hourly from 2-12 hr post-injection. In order to create an index of ethanol withdrawal that is independent of individual differences in baseline HIC scores, all post-ethanol HIC scores were corrected for the individual's baseline score as in previous work (Reilly, Milner et al., 2008). The corrected HIC scores were used to compute the individual's ethanol withdrawal severity score, corresponding to the area under the curve (AUC) over the full timecourse post-ethanol.

Homecage activity

A separate group of *Kcnj9* knockout, heterozygote and wildtype littermates (n=36-51 mice per genotype) were assessed for locomotor activity in the homecage using ethanol withdrawn and control mice. Following 1 hr of habituation to the procedure room, mice were administered ethanol (4 g/kg, i.p.) or saline (at hr 0), then returned to their homecage for 8 hr. At hr 6, the homecage top was removed and replaced with a 30x18 piece of plexiglass, during which locomotor behavior was video-recorded for 2 hr (hr 6-8 post-injection). Activity was scored by an experimenter blind to treatment and

genotype. To assess horizontal activity, the homecage was divided into four quadrants. An activity 'count' was recorded when an individual fully entered (all 4 paws) a new quadrant. Vertical activity was scored as number of the rears (raised onto hindlimbs) exhibited. In order to minimize the number of mice used and avoid the potential effects of repeated ethanol administrations, these animals were tested for additional withdrawal behaviors (below).

Elevated zero-maze

Withdrawal-associated locomotor depression and anxiety-like behavior were evaluated in the elevated zero-maze (EZM) at hr 8, a timepoint when ethanol withdrawal-associated anxiety-like behavior is observed (Verleye et al., 2009). The EZM (Flair Plastics; Portland, OR) is 45 cm high and consists of four equally-proportioned arms, forming a circle with an external diameter of 45 cm. The walls of the two closed arms are made of black acrylic 11 cm in height; the open arms have a small lip composed of C/B acrylic 3 mm in height. Animals were individually transferred 4 feet (on the experimenter's hand, to avoid eliciting convulsions) from their homecage to the EZM, where their behavior was video-recorded for 5 min under low-level illumination (20 lux) and scored by an experimenter blind to genotype and treatment. The following locomotor activities behaviors were assessed: closed arm line crosses and rears, open arm line crosses and rears, head dips (over the side of the open arms), and stretched attends (extension and sudden retraction of the torso; Kliethermes et al., 2004); as well as the percent time spent in the open arms (a validated measure of anxiety-like behavior; Shepherd et al., 1994), urinations and boli produced (also a purported measure of anxiety-like behavior; Hall et al., 1934, Milner & Crabbe, 2008).

Tail suspension test

The tail suspension test (TST) is validated test of depression-like behavior in rodents (Cryan et al., 2005), and was performed at two time points (hr 12 and 24) at which ethanol withdrawal associated depression-like behavior has been reported (Kokare et al., 2008). The apparatus (Flair Plastics, Portland, OR) consists of a gray polyvinyl chloride beam 104 cm long and 54.5 cm high. Mice were transported 5 feet from their homecage to the TST apparatus on the experimenter's hand. Using surgical tape, mice were suspended by taping the tail (3/4 of the way to tail tip) to the flat surface of the beam (63 cm above the floor) for 6 min. Behavior was video-recorded and scored by an experimenter blind to genotype and treatment. Percent time immobile (i.e., no movement of head, limbs or torso) was assessed as a measure of depression-like behavior.

Blood ethanol concentrations

Naïve *Kcnj9* knockout, heterozygote and wildtype littermates (n=8-9 per genotype) were injected with ethanol (4 g/kg, i.p.) and returned to their homecage. Tail blood samples (20 µl) were drawn 45, 75, 240 and 600 min post-ethanol and collected into tubes containing 50 µl 5% zinc sulfate, 50 µl 0.3 M barium hydroxide and 300 µl water. Samples were mixed and centrifuged at 18,200 x g for 5 min. Ethanol-containing supernatants were analyzed on a model 6890 gas chromatograph (Agilent) to determine blood ethanol concentrations (BECs) as previously described (Terdal & Crabbe, 1994).

Genotyping

DNA was extracted from tail biopsy or ear punch tissue using the PuregeneDNAisolation kit (Genera Biosystems, Victoria, Australia) according to the manufacturer's instructions. *Kcnj9* knockout, heterozygote, and wildtype littermates

were differentiated using a PCR-based assay with a common forward primer (G3com) and two reverse primers (G3WT and C3KO) as in previous work (Kozell et al., 2009). Knockout and wildtype mice produce 500 and 645 bp PCR products, respectively. Heterozygote mice produce both PCR products. All PCRs are performed using QIAGEN HotStar under standard conditions with a 55°C annealing temperature. The primer sequences are as follows: G3com (GATACTAGACTAGCGTAACTCTGGAT), G3WT(GATAAAGAGCACAGACTGGGTGTCG),
and G3KO (CAAAGCTGAGACATCTCTTTGGCTCTG).

Data Analyses

Outliers were defined using the Grubb's outlier analysis and were removed before subsequent data analyses. Behaviors were analyzed using Systat® statistical software program with analyses of variance (ANOVAs) to assess main effects of sex, genotype and treatment as well as interactions. Significant ($p<0.05$) results were subsequently assessed using Tukey post hoc analyses. All p-values given are two-tailed.

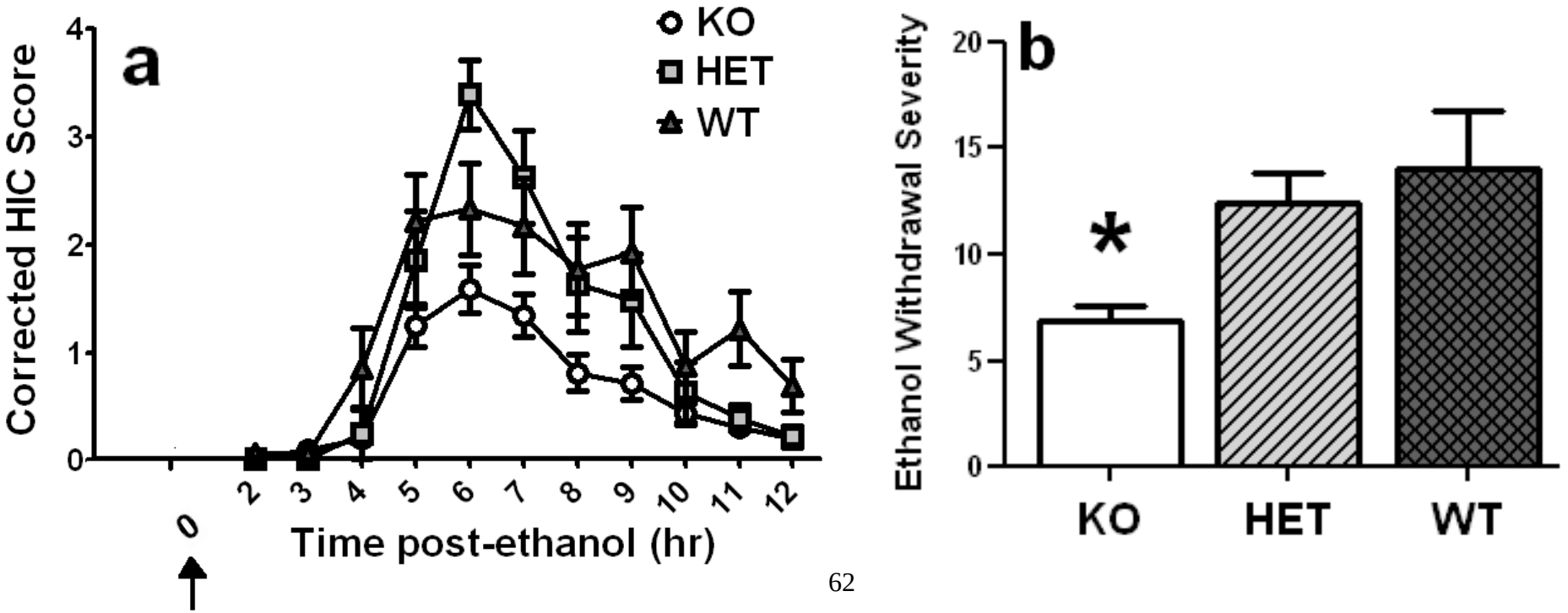
Results

Withdrawal convulsions

A main effect of treatment was observed across genotypes, with ethanol withdrawn mice exhibiting more severe withdrawal convulsions than controls ($F_{(1,217)}=69$, $p<0.001$). No effect of genotype was detected in baseline or control (saline) HICs (both $F<0.1$, $p>0.8$), so only ethanol withdrawn mice were used for subsequent analyses. A main effect of sex was apparent, with females exhibiting less severe withdrawal than males ($F_{(1,169)}=4.4$, $p=0.04$), which is typical in rodent models (Kosobud & Crabbe, 1986;

Reilly et al., 2009). However, no sex x genotype interaction was detected ($F<0.2$, $p>0.8$), so data from both sexes were combined for subsequent analyses. A main effect of genotype was apparent (figure 10; $F_{(2,172)}=9.5$, $p<0.001$), with *Kcnj9* knockout mice exhibiting less severe withdrawal-associated convulsions than heterozygote and wildtype littermates (withdrawal severity score = 8.7 ± 0.8, 13.8 ± 1.2 and 14.7 ± 1.7, respectively; $p<0.007$). Taken together with our previous work (Kozell et al., 2009), these data identify *Kcnj9* as a high-quality QTG candidate for ethanol withdrawal.

Figure 10. Timecourse for acute ethanol withdrawal convulsions. **(a)** Mice were scored twice for baseline (pre-ethanol) HICs immediately before administration of 4 g/kg ethanol (arrow indicates ethanol i.p. injection at hr 0) and hourly up to 12 hr post-ethanol. Average baseline HIC scores did not differ between *Kcnj9* knockout (KO), knockout heterozygote (HET) and wildtype (WT) littermates (mean ± SEM = 0.1 ± 0.3, 0.1 ± 0.4 and 0.1 ± 0.3, n= 101, 32 and 39, respectively; $F<0.2$, $p>0.8$). HIC scores increased above baseline approximately 4 hr post-ethanol indicating a state of hyperexcitability, and peaked approximately hr 6 post-ethanol. Data represent individual HIC scores (mean ± SEM) corrected for individual baseline HIC scores. **(b)** Data represent the ethanol withdrawal severity scores (mean ± SEM) indexed as the corrected area under the curve for hr 2-12 post-ethanol as in previous work (Buck et al., 1997). *Kcnj9* KO mice exhibited less severe (*$p<0.05$) ethanol withdrawal severity than both HET and WT littermates.

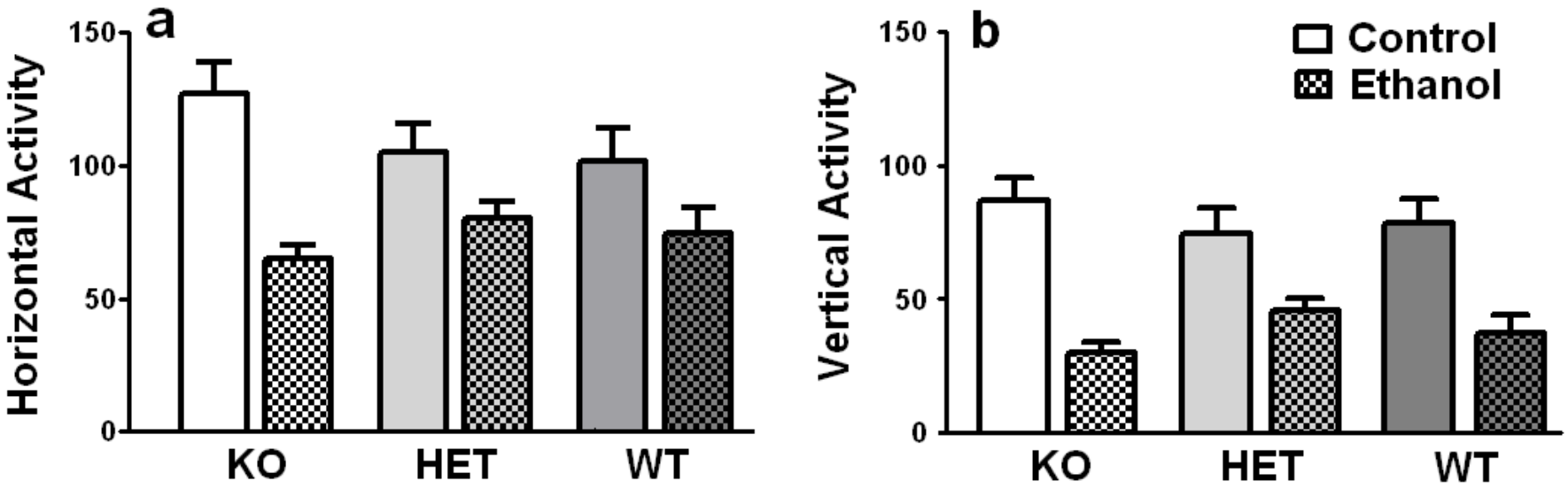


Homecage activity

A trend for a main effect of sex was observed for vertical activity, with females exhibiting more vertical activity than males ($F_{(1,123)}$=2.3, p=0.13); however, no interactions with sex were detected (all F<0.7, p>0.4), so data from both sexes were combined for subsequent analyses. A main effect of treatment was observed for horizontal (line crosses, $F_{(1,132)}$=29, p<0.001; figure 11a) and vertical (rears, $F_{(1,131)}$=61, p<0.001; figure 11b) activities, with ethanol withdrawn mice exhibiting less horizontal and vertical activity than controls. These results are in accordance with previous literature reporting withdrawal-associated locomotor depression following chronic ethanol exposure (Kliethermes et al., 2005), and provide evidence for an additional, quantifiable behavior (beyond HICs) associated with acute ethanol withdrawal.

Trends for genotype x treatment interactions were detected for horizontal and vertical activities ($F_{(2,132)}$=2.9 and $F_{(2,131)}$=2.5, respectively, both p<0.1). *Kcnj9* knockout mice exhibited more withdrawal-associated locomotor depression than heterozygote or wildtype littermates (line crosses= 65 ± 5, 80 ± 6 and 75 ± 9, respectively, p=0.22; rears= 30 ± 4, 46 ± 4 and 37 ± 7, respectively, p=0.05). This may be due, at least in part, to higher baseline activities in *Kcnj9* knockouts compared to heterozygote and wildtype mice, although this did not reach statistical significance (F<1.4, p>0.3).

Figure 11. Homecage activities using ethanol withdrawn and control mice. Data represent activity values (mean ± SEM) for *Kcnj9* knockout (KO), knockout heterozygote (HET) and wildtype (WT) littermates (n=48, 53 and 36, respectively). **(a)** Horizontal activity (line crosses) and **(b)** vertical activity (rears) were recorded for 2 hr (hr 6-8) post-injection of ethanol (4 g/kg, i.p.) or saline. Ethanol withdrawn mice (patterned bars) exhibited significantly ($p<0.05$) less homecage activity than saline control mice (solid bars).



Elevated zero-maze

Unless otherwise noted, no main effect of sex or interactions involving sex were detected (all $F<1.8$, $p>0.2$), so data for males and females were analyzed together.

Activity (closed arms). A main effect of treatment was apparent, with ethanol withdrawn mice exhibiting less horizontal (line crosses) and vertical (rears) activity than controls ($F_{(1,132)}=14.7$, $p<0.001$ and $F_{(1,130)}=18.9$, $p<0.001$, respectively; figure 12a,b). This indicates that changes in horizontal and vertical activity in a novel environment can be useful measures of ethanol withdrawal in the acute model, as they are in chronic models (Doremus-Fitzwater & Spear, 2007). A main effect of genotype was apparent for vertical activity ($F_{(2,130)}=3.2$, $p=0.04$), with *Kcnj9* knockouts and heterozygotes exhibiting more rears than wildtype littermates (7.3 ± 0.7, 7.6 ± 0.6 and 4.9 ± 0.8, respectively, $p=0.03$) and a trend for a main effect of genotype on horizontal activity ($F_{(2,130)}=1.7$, $p=0.18$), with knockouts and heterozygotes making more line crosses than wildtype littermates (26.4 ± 1.6, 25.5 ± 1.2 and 21.8 ± 1.7, respectively, $p=0.12$). However, no genotype x treatment interactions were detected (all $F<1.3$, $p>0.3$), suggesting that *Kcnj9* status influences EZM closed arm activity in ethanol withdrawn and control mice.

Activity (head dips). A main effect of treatment was apparent, with ethanol withdrawn animals exhibiting fewer head dips than controls ($F_{(2,131)}=18.6$, $p<0.001$; figure 12e). This is consistent with other data indicating that head dips are a sensitive index of ethanol withdrawal (Kleithermes et al., 2005; Chapter 2). A trend for a treatment x genotype interaction was also detected ($F_{(2,131)}=3.0$, $p=0.053$), with ethanol withdrawn *Kcnj9* knockout and heterozygote mice exhibiting more head dips than wildtype littermates (10.8 ± 1.2, 13.0 ± 1.0 and 7.5 ± 0.9, respectively, $p=0.003$). These data suggest that *Kcnj9* status may affect withdrawal-associated head dip activity.

Activity (stretched attends). A trend for a main effect of treatment was detected, with ethanol withdrawn mice exhibiting fewer stretched attends than controls ($F_{(2,131)}=2.1$,

p=0.15; figure 12f); however, no main effect of genotype or treatment x genotype interactions were detected (both F<0.4, p>0.7), suggesting that *Kcnj9* status does not influence this behavior.

Activity (open arms). A trend for a main effect of treatment was detected for vertical activity, with ethanol withdrawn mice exhibiting fewer rears than controls ($F_{(2,134)}$=3.3, p=0.07; figure 12d). No main effect of genotype or genotype x treatment interaction was detected (both F<2.2, p>0.2).

Trends for a main effects of treatment ($F_{(2,131)}$=2.6, p=0.10) and genotype ($F_{(2,131)}$=3.0, p=0.055) were detected for horizontal activity (figure 12c), with ethanol withdrawn mice exhibiting fewer line crosses than controls (5.6 ± 0.7 and 7.6 ± 1.1, respectively, p=0.1), and *Kcnj9* knockout and heterozygote mice exhibiting more line crosses than wildtype littermates (7.5 ± 1.1, 7.1 ± 1.1 and 3.9 ± 0.9, respectively, p=0.04). However, no genotype x treatment interaction was detected (F<0.1, p<0.9), suggesting that *Kcnj9* status influences this behavior in both ethanol withdrawn and control mice.

Anxiety-like behavior (% time in the open arms). A three-way ANOVA (genotype x treatment x sex) identified a main effect of sex ($F_{(1,126)}$= .1, p=0.009) and a sex x treatment interaction ($F_{(1,126)}$=4.3, p=0.04), with control (but not ethanol withdrawn) females exhibiting greater anxiety-like behavior (lower % time in the open arms) than males. Data was therefore separated by sex for subsequent analyses. In males, a main effect of treatment was apparent, with ethanol withdrawn mice exhibiting greater anxiety-like behavior than controls ($F_{(1,64)}$=8.5, p=0.005). These results demonstrate withdrawal-associated anxiety-like behavior using the acute model, as has been observed in chronic models (Kliethermes et al., 2004; Verleye et al., 2009).

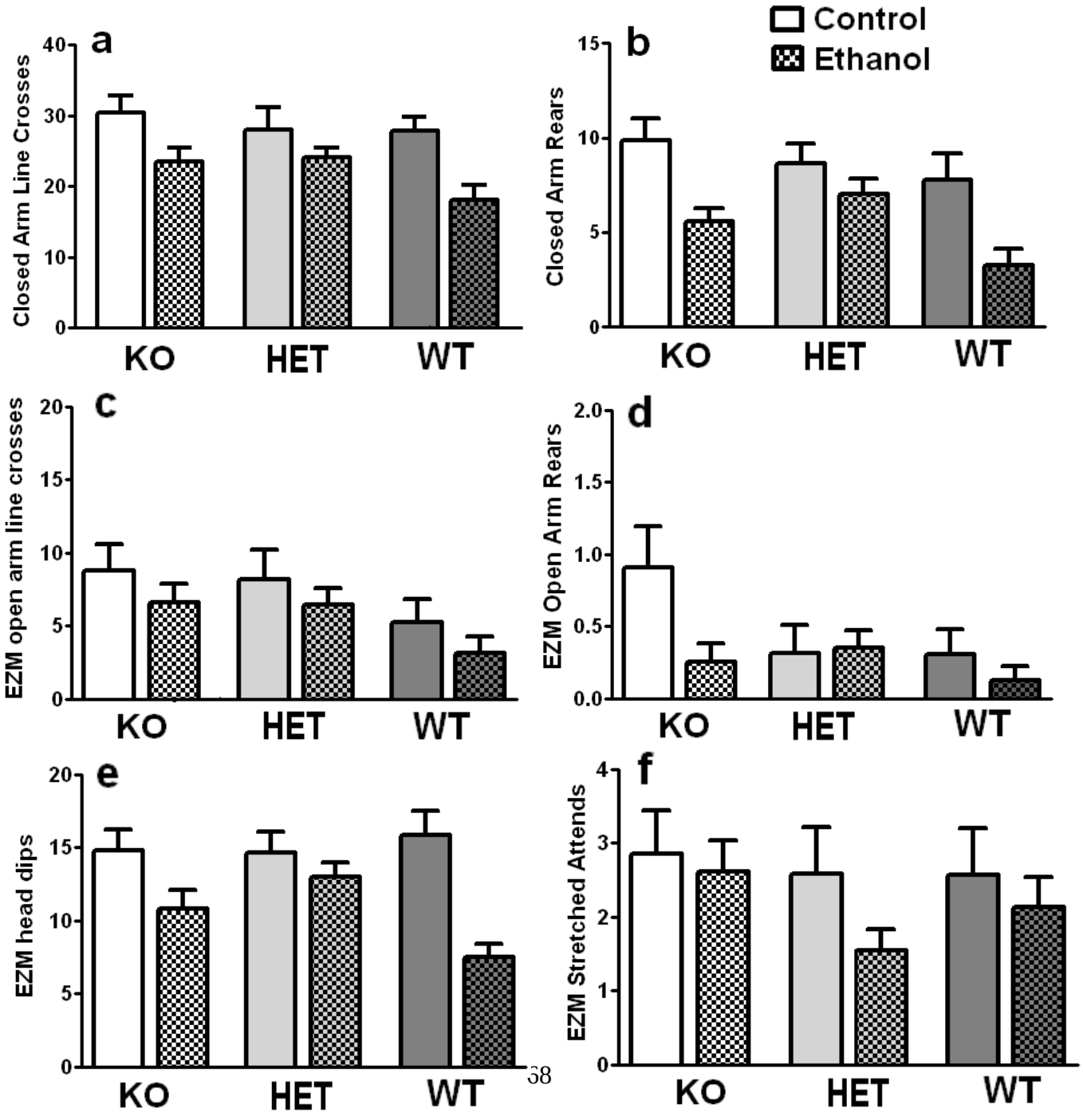
A trend for a main effect of genotype was detected in males ($F_{(2,64)}$=2.1, p=0.13; figure 13a), with knockout mice exhibiting less anxiety-like behavior than wildtype

littermates (% time in open arm = 19.1 ± 3.3 and 11.6 ± 2.7, respectively, p=0.15). However, a genotype x treatment interaction was not detected (F<0.9, p>0.4), suggesting that *Kcnj9* status influences anxiety-like behavior in both ethanol withdrawn and control mice. In females, no main effects of treatment or genotype or interactions were detected (figure 14b; all F<1.2, p>0.2), indicating that the treatment and genotype effects observed are sex-limited.

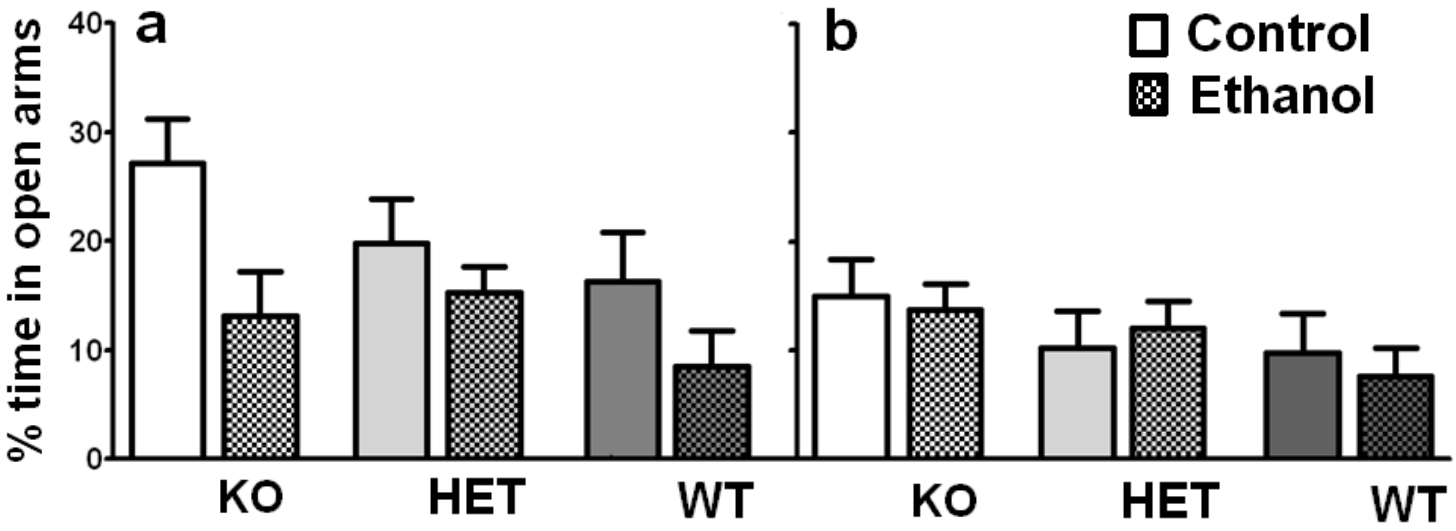
Boli. A genotype x sex interaction was apparent ($F_{(2,125)}$=3.2; p=0.046), so males and females were analyzed separately. No main effect of treatment or interactions involving treatment were detected (both F<0.3, p>0.6), so acute withdrawal-associated anxiety-like behavior was not observed. A main effect of genotype was apparent in males ($F_{(2,63)}$=7.4; p=0.001; figure 14a), with *Kcnj9* heterozygotes producing more boli than knockout or wildtype littermates (4.9 ± 0.4, 2.2 ± 0.4 and 3.1 ± 0.6 respectively, p=0.001). No main effects of treatment or genotype were detected in females (all F<0.7, p>0.5; figure 14b), suggesting that *Kcnj9* effects on this phenotype may be sex-limited.

Urinations. A trend for a main effect of sex was detected, with females producing more urinations than males ($F_{(1,126)}$=2.0, p=0.11); however, no main effects of sex or interactions involving sex were detected (all F<0.3, p>0.7), so data from both sexes were combined for subsequent analyses. A main effect of genotype was apparent for urinations ($F_{(2,126)}$=5.0; p=0.009), with *Kcnj9* heterozygotes producing more urinations than knockout and wildtype littermates (0.8 ± 0.1, 0.6 ± 0.1 and 0.4 ± 0.1, respectively, p=0.03). A trend for a genotype x treatment interaction was detected ($F_{(2,132)}$=2.6, p=0.076), with the effect of genotype being more apparent in control animals than in ethanol withdrawn animals (figure 15c).

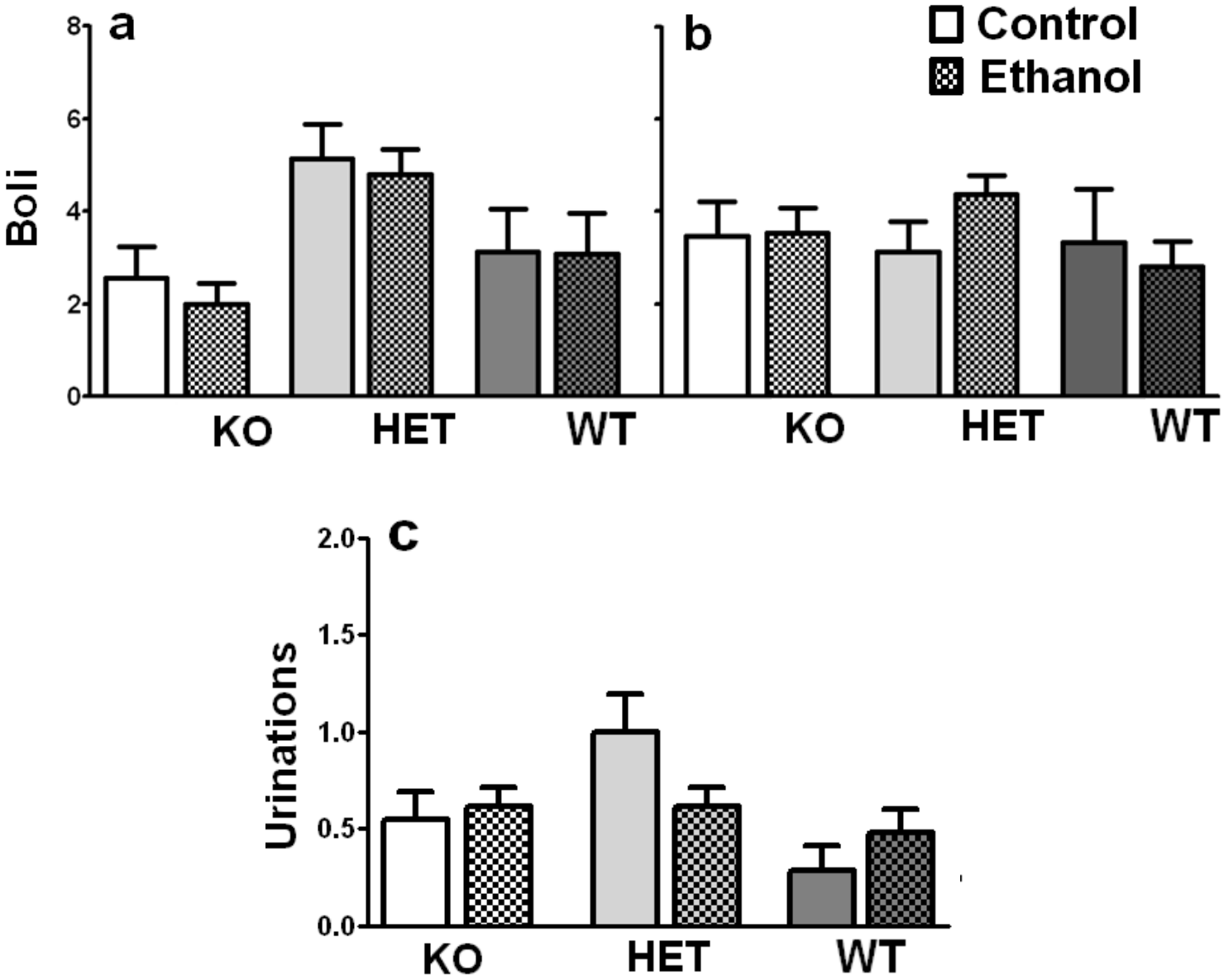
Figures 12a-f. Behaviors in the EZM in ethanol withdrawn and control mice. (a,b) Closed arm line crosses and rears, **(c,d)** open arm line crosses and rears, **(e)** head dips, and **(f)** stretched attends in ethanol withdrawn (hr 8 post-ethanol, 4 g/kg, i.p.) and control (saline) animals. Data represent mean values ± SEM in *Kcnj9* KO, HET and WT littermates (n=48, 51 and 36, respectively). Withdrawal-associated locomotor depression was observed in the closed and open arms across genotypes.



Figures 13a-b. EZM anxiety-like behavior in ethanol withdrawn and control mice. Data represent the percent time spent in the open arms (mean ± SEM) at hr 8 post-injection of ethanol (4 g/kg, i.p.) or saline. **(a)** Males: Percent time spent in the open arms was decreased in ethanol withdrawn compared to control animals ($p<0.05$). A trend for was detected for lower percent time in the open arms in *Kcnj9* KO than HET and WT mice (n=21, 29, 20, respectively; $p<0.1$). **(b)** Females: No treatment or genotype differences were detected.



Figures 14a-c. Boli and urinations in the EZM. Data represent the number of boli or urinations (mean ± SEM) at hr 8 post-injection of ethanol (4 g/kg, i.p.) or saline. **(a)** Males: More boli were produced by *Kcnj9* KO heterozygotes (HET) than knockout (KO) and wildtype (WT) littermates across treatment groups. **(b)** Females: No effects of treatment or genotype on the number of boli produced were detected. **(c)** Control HET mice produced more urinations than KO and WT littermates.



Tail suspension test (TST)

No main effect of sex or sex interactions were detected % time immobile (all $F<1$, $p>0.26$), so data from both sexes were combined. A repeated-measures ANOVA demonstrated main effects of genotype ($F_{(2,129)}=9.1$, $p<0.001$) and time ($F_{(1,\ 129)}= 52$, $p<0.001$), as well as a genotype x time interaction ($F_{(2,129)}=4.9$, $p=0.009$), with greater % time was spent immobile at 24 hr than at 12 hr (figure 15a,b). *Kcnj9* knockout mice exhibited less depression-like behavior (lower % time immobile) than heterozygote and wildtype littermates at hr 12 (1.6 ± 0.5, 3.1 ± 0.6 and 4.6 ± 0.9, respectively, $p=0.019$) and hr 24 (4.8 ± 0.9, 7.9 ± 1.4 and 14.4 ± 2.3, respectively, $p<0.001$). However, no main effect of treatment or treatment x genotype interaction was detected (both $F<0.7$, $p>0.39$). Taken together, these results suggest that reduced *Kcnj9* expression is associated with less depression-like behavior in ethanol withdrawn and control animals.

Summary of behavioral results

Table 2 provides a summary of the expected and observed direction of effect for acute withdrawal behaviors in this *Kcnj9* knockout model. As expected, *Kcnj9* knockout mice exhibited lower withdrawal-associated HICs than heterozygote and wildtype littermates. This supports the conclusion that *Kcnj9* is a QTG for ethanol withdrawal convulsions. These results also identify additional measures of acute ethanol withdrawal. Ethanol withdrawal-associated anxiety-like behavior was apparent in the EZM, and was less severe in knockout mice compared to wildtype littermates. Acute ethanol withdrawal-associated locomotor depression was also apparent, and was less severe in knockout mice in a novel environment but not the homecage. *Kcnj9* knockout mice also demonstrated less depression-like behavior than heterozygote and wildtype littermates across treatment groups.

Figure 15a-b. TST depression-like behavior in ethanol withdrawn and control animals. Mice were scored for depression-like behavior **(a)** hr 12 and **(b)** hr 24 post-injection of ethanol (4 g/kg, i.p.) or saline. Data represent the % time spent immobile (mean ± SEM). *Kcnj9* knockout (KO) and KO heterozygote (HET) mice exhibited significantly ($p<0.05$) lower percent time immobile than wildtype littermates (WT) across treatment groups.

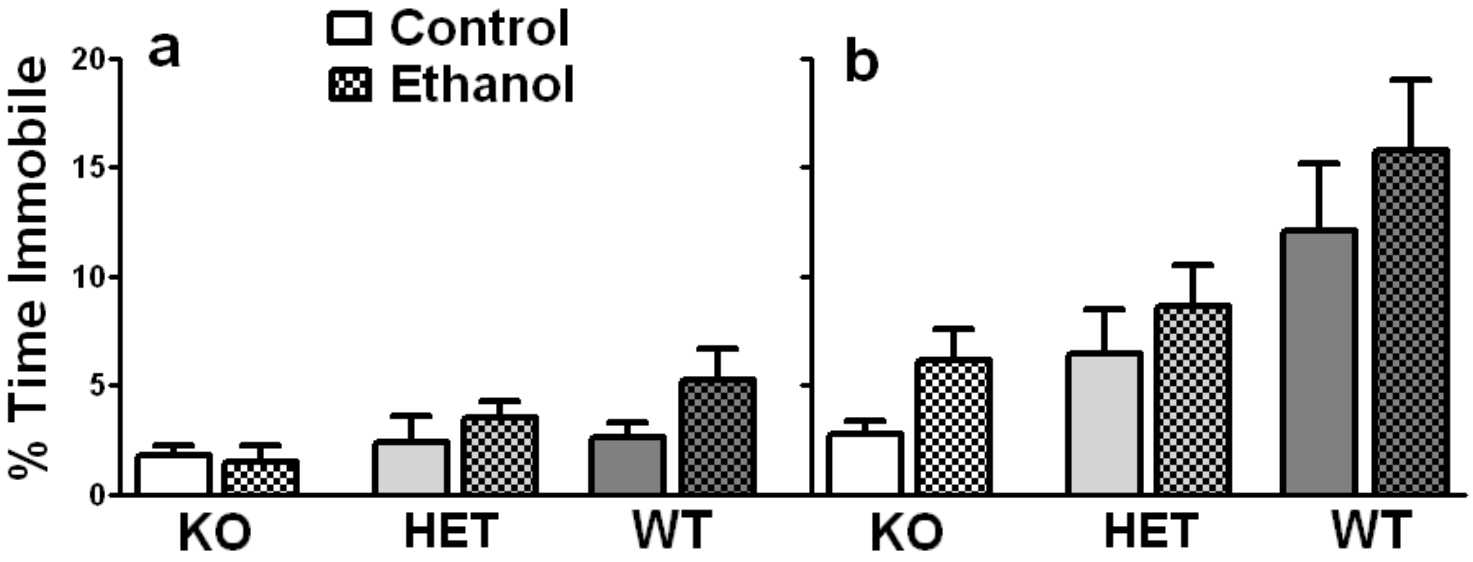


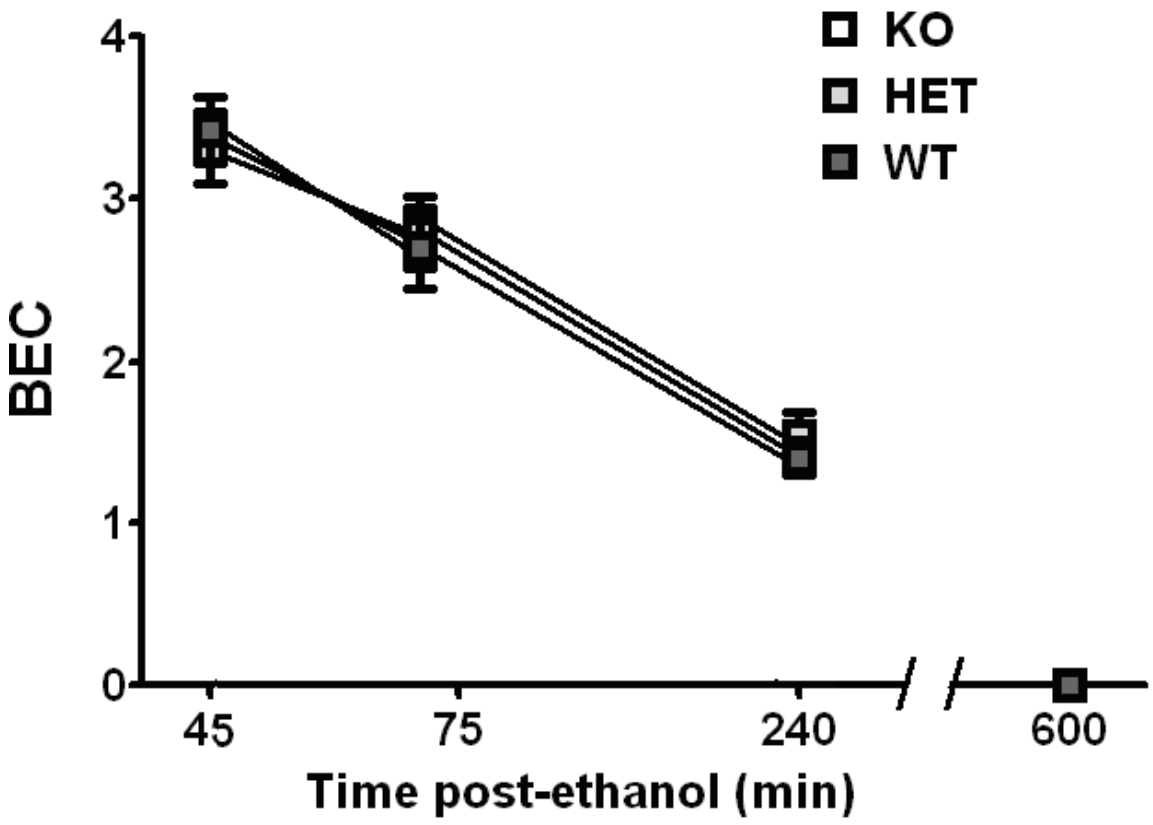
Table 2. Summary of behavioral results. Outline of the expected (Exp) and observed (Obs) effects associated with acute ethanol withdrawal (WD), and/or associated with *Kcnj9* knockout (KO) status (compared to heterozygote or wildtype littermates) on the phenotypes tested. ↑ indicates an increase in behavioral phenotypic scores, ↓ indicates a decrease in phenotypic scores, ─ indicates no change in the phenotype.

Phenotype	Exp WD	Obs WD	Exp KO	Obs KO
HIC severity	↑	↑	↓	↓
Activity in the homecage (line crosses, rears)	↓	↓	↓	↑
Activity in the EZM (line crosses, head dips)	↓	↓	↓	↓
Anxiety-like behavior (% time in open arms in EZM)	↑	↑	↓	↓
Boli	↑	─	↓	─
Urinations	↑	─	↓	↑
Depression-like behavior (% time immobile in TST)	↑	─	↓	↓

Blood ethanol concentrations (BECs)

BEC values were zero (not detectable) at 600 min (10 hr) post-ethanol, so this timepoint was omitted from subsequent analyses. A repeated-measures analysis indicated main effects of sex ($F_{(1,19)}$=8.5, p=0.009) and time ($F_{(2,38)}$=90, p<0.001), with males exhibiting higher BECs than females across both genotypes and all timepoints tested. No sex interactions were detected (both F<2.1, p>0.15), so data from both sexes were combined for subsequent analyses. A repeated-measures analysis detected no main effect of genotype or genotype x time interaction (both F<0.2, p>0.9). Thus, genotype-dependent differences in behavior are not related to differential ethanol pharmacokinetics (figure 16).

Figure 16. Blood ethanol concentrations (BECs) in *Kcnj9* knockout (KO), heterozygote (HET) and wildtype (WT) mice. BECs (mean ± SEM) were assessed at 45, 75, 240 and 600 min post-administration of a hypnotic dose of ethanol (4 g/kg, i.p.). No effect of genotype on BECs was detected.



Discussion

Our laboratory previously identified *Kcnj9* as a QTG candidate to underlie the phenotypic effects of a QTL on chromosome 1 with large effects on barbiturate withdrawal (Buck et al., 1999) and ethanol withdrawal following acute or chronic administration (Buck et al., 1997, 2002). The present studies were performed in order to assess the role of *Kcnj9* on ethanol withdrawal convulsions and its potential pleiotropic effects on additional ethanol withdrawal behaviors. My results provide the first evidence that *Kcnj9* affects ethanol withdrawal convulsions *in vivo*, Additionally, my results indicate that locomotor depression (reduced horizontal and vertical activity in the homecage and EZM, reduced head dips in the EZM) and anxiety-like behavior are also useful measures of acute ethanol withdrawal; and that depression-like behavior is impacted in *Kcnj9* knockout mice. Taken together, my results identify *Kcnj9* as a high-quality QTG candidate for ethanol withdrawal convulsions and beyond.

Withdrawal-associated locomotor depression and anxiety-like behaviors are apparent following chronic ethanol exposure in mice (Kliethermes et al., 2004, 2005). The present studies demonstrate that the acute model also results in withdrawal-associated locomotor depression and increased anxiety-like behavior. Moreover, *Kcnj9* knockout and heterozygote mice showed a trend for lower anxiety-like behavior than wildtype littermates across treatment groups, suggesting a role for this gene in anxiety-like behavior. However, this measure of anxiety-like behavior may be confounded by locomotor activity. Previous studies observed high intra-test correlations between activity and anxiety-like behaviors (Kliethermes, 2005; Milner & Crabbe, 2008), and our results are no exception, with high correlations among activity and anxiety-like variables apparent within and across genotypes (data not shown). Boli produced is another purported measure of anxiety-like behavior or 'emotionality' (Hall, 1934), but was lower in *Kcnj9* knockout and wildtype mice compared to heterozygotes. Moreover, no effect of

ethanol withdrawal was observed on boli produced. Future studies utilizing other tests of anxiety-like behavior that are not thought to be activity-dependent (e.g., ultrasonic vocalizations, marble burying) may be useful to clarify the potential relationship between *Kcnj9* expression and anxiety-like behavior in ethanol withdrawn and control animals.

These results are the first to indicate a role for *Kcnj9* in depression-like behavior. Depression can be a significant factor in risk for and/or severity of alcohol dependence (Sintov et al., 2009), and is frequently comorbid in alcohol dependent individuals (Corcos et al., 2008; Davis et al., 2008). Future studies will be needed using other validated models of depression-like behavior (e.g., Porsoult's forced swim test) to more fully assess the role of *Kcnj9* in depression-like behaviors in ethanol withdrawn and control animals.

GIRK channel interaction with $G_{i/o}$ protein-coupled receptors (Doupnik, 2008), including $GABA_B$ receptors, suggests possible mechanisms by which *Kcnj9* may affect the behaviors tested here. $GABA_B$ receptors influence ethanol-related behaviors (Castelli et al., 2005; Maccioni et al., 2008; Stromberg, 2004); and baclofen, a selective $GABA_B$ receptor agonist, suppresses ethanol withdrawal in rodents (Colombo et al., 2004; Knapp et al., 2007) and human alcoholics (Addolorato & Leggio, 2010; Leggio et al., 2010). Ethanol enhances $GABA_B$ receptor-mediated inhibitory postsynaptic transmission in midbrain neurons by facilitating GIRK channels (Federici et al., 2009) and enhances baclofen-evoked GIRK currents in cerebellar granule cells (Kobayashi et al., 1999; Lewohl et al., 1999). This evidence suggests that ethanol's effects are mediated, at least in part, through $GABA_B$ receptor activation of GIRK currents. $GABA_B$ receptors are also associated with anxiety and depression in rodent and human populations (Cryan & Slattery, 2010; Partyka et al., 2007; Pilc & Nowak, 2005), and administration of $GABA_B$ antagonists increases anxiety-like behavior and reduces depression-like behavior in rodents (Nowak et al., 2006; Ong & Kerr, 2000). Although

outside of the scope of this dissertation, further examination of the role of the $GABA_B$/GIRK3 channel association in ethanol-related anxiety-like and depression-like behaviors is warranted. Future studies will be needed using pharmacological (e.g., baclofen effects on ethanol withdrawal and baseline behaviors in *Kcnj9* knockout models) and electrophysiological approaches (i.e., $GABA_B$ receptor function in *Kcnj9* knockout model) to elucidate the potential role of $GABA_B$ receptors in mediating the effects of *Kcnj9*/GIRK3 on ethanol withdrawal, as well as anxiety-like and depression-like behaviors.

GIRK channels are also activated by opioid receptor stimulation (Ikeda et al., 2002), and GIRK3 knockout mice show attenuated acute responses to morphine (Marker et al., 2004), and reduced morphine withdrawal severity (Cruz et al., 2008). The opioid receptor system is activated by acute exposure to ethanol (Drews & Zimmer, 2010) and opioid receptor antagonists reduce ethanol withdrawal severity in rodents (Beadles-Bohling & Wiren, 2006; Ghozland et al., 2005) and humans (Haile et al., 2008). The potential contribution of opioid receptors in mediating the effects of *Kcnj9*/GIRK3 on ethanol withdrawal, as well as anxiety-like and depression-like behaviors, should be considered in future studies.

In summary, my results identify *Kcnj9* as a high-quality QTG candidate for ethanol withdrawal, and suggest that this gene has with pleiotropic effects beyond withdrawal convulsions. Future studies in our laboratory will focus on testing additional ethanol behaviors (e.g., ethanol consumption/preference, withdrawal-induced drinking) that are genetically correlated with ethanol withdrawal convulsion severity, as well as potential mechanisms of action by which GIRK3 may affect ethanol withdrawal and related behaviors.

CHAPTER 4: *MPDZ* expression affects 5-HT_{2C} receptor function

Abstract

The physical association between the multi-PDZ domain protein (MPDZ) and serotonin $5HT_{2C}$ receptors is thought to regulate this receptor's function, but direct evidence is lacking. Using RNA interference, I assessed the impact of reduced MPDZ gene (*MPDZ)* expression on $5HT_{2C}$ receptor function in a human embryonic kidney (HEK) cell line that stably expresses the human 5-HT_{2C} receptor and endogenously expresses *MPDZ*. RNA interference using a microRNA (miRNA) approach reduced MPDZ expression by 40% compared to control cells (transfected with no miRNA and untransfected). $5HT_{2C}$ receptor function was assessed using accumulation of myo-inositol monophosphate (IP1) as a measure of $5HT_{2C}$ receptor activation of the D-*myo*-inositol 1,4,5 triphosphate (IP3) pathway. No differences were detected in basal IP1 accumulation between miRNA-transfected and control cells. 5-HT (100 nM) stimulated IP1 accumulation was increased in miRNA transfected cells by 55% compared to control cells, and was completely blocked by a selective 5-HT_{2C} receptor antagonist (1 μM SB242084). My results provide the first evidence that MPDZ impacts 5-HT_{2C} receptor function, and demonstrate that reduced *MPDZ* expression is associated with increased 5-HT_{2C} receptor function in the IP3/IP1 pathway.

Introduction

Members of the PDZ domain family (named for three prototypical proteins: postsynaptic density protein [PSD-95], discs-large septate junction protein [DLG], and epithelial tight-junction protein zona occludens protein [ZO-1]) have emerged as central organizers of protein complexes at the plasma membrane (Kim & Sheng, 2004), and function to enhance the rate and fidelity of signal transduction in specific pathways (Fanning & Anderson, 1999; Sheng & Sala 2001; Tsunoda et al., 1997). The multiple PDZ domain (MPDZ, also called MUPP1) protein is the largest known protein in the PDZ domain family (Ullmer et al., 1998). MPDZ is located postsynaptically, where it can associate with membrane-bound receptors to influence receptor function (Balasubramanian et al., 2007; Krapivinsky et al., 2004).

A physical association between MPDZ and $5HT_{2C}$ receptor ($5HT_{2C}R$) is well documented. *MPDZ* (the gene encoding MPDZ) was initially identified in a yeast two-hybrid screen using the C-terminus of the $5HT_{2C}R$ as bait (Ullmer et al., 1998). Subsequent work found that MPDZ induced clustering of cell surface $5HT_{2C}Rs$, and that $5HT_{2C}R$ activation induced a conformational change in MPDZ (Becamel et al., 2001). Serotonin-induced $5HT_{2C}R$ phosphorylation disrupts its association with MPDZ (Parker et al., 2003). Despite the abundant evidence for MPDZ-$5HT_{2C}R$ association, the role of MPDZ on $5HT_{2C}R$ function has yet to be established.

The present studies were designed to assess the effect of MPDZ expression on $5HT_{2C}R$ function. RNA interference (RNAi) is a rigorous approach to reduce endogenous expression of a target gene/protein (Gao & Zhang, 2007; Kurreck, 2009). Here, we used a microRNA (miRNA) approach to reduce endogenous *MPDZ* expression in a cell line that stably expresses human $5HT_{2C}Rs$ (Schlag et al., 2004). miRNAs are short sequences (19-23 base pairs) complementary to the 3' untranslated region of a target gene. When introduced into a cell, miRNA is incorporated into the RNA-induced

silencing complex (RISC), which uses the miRNA to find endogenous complementary endogenous mRNA strands and cleave them, thus preventing the target gene from being translated into its protein.

$5HT_{2C}$Rs affect a number of signaling systems, including the D-*myo*-inositol 1,4,5 triphosphate (IP3) pathway, which is activated by $G_{\alpha q}$-coupled receptors (e.g., $5HT_{2C}$Rs) and has a wide range of downstream effects (figure 21; reviewed in Berridge, 2009). Other important G-protein pathways have been identified (see discussion for details), but the IP3 pathway was chosen for initial *in vitro* investigation due to its robust activation by $5HT_{2C}$Rs and well-studied intracellular effects. Here, we used an enzyme-linked immunosorbent assay (ELISA) measuring inositol monophosphate (IP1) to assess $5HT_{2C}$R-mediated stimulated activation of the IP3 pathway. IP1 is a product obtained by partial dephosphorylation of IP3, and its accumulation can be quantified as an indirect measure of G protein-coupled receptor (GPCR)/G_q activation (Trinquet et al., 2006). My results demonstrate for the first time that *MPDZ* expression affects $5HT_{2C}$R activation, as measured through the IP3 pathway.

Methods

RNA interference

A miRNA construct was designed by Invitrogen (BLOCK-it Pol II miR RNAi, Carlsbad, CA) based on the mouse target sequence (in boldface): 5'-TGCTG**TAGAAATGACAGCAGAGCTGA** GTTTTGG CCACTGACTGACTCAGCT CTTGTCATTTCTA-3'. This construct was subcloned into a lentiviral compatible vector (iLenti GFP, Capital Biosciences, Rockville, MD) and contains an H1 promoter to drive miRNA expression and a SV40 promoter to drive expression of the Neo-resistance gene for positive selection. Human embryonic kidney (HEK) cells stably expressing human

$5HT_{2C}$Rs ($5HT_{2C}$R-HEK) were grown to ~70% confluence in 6-well plates (BD Falcon, Franklin Lakes, NJ). Transfection with either the miRNA construct or a control construct (containing all elements except the miRNA sequence) was performed using the transfection agent Lipofectamine 2000® (Invitrogen, Carlsbad, CA) according to manufacturer's instructions: 10 µl (1 µg) of construct was added to 40 µl Dulbecco's modified eagle medium (DMEM) and, in a separate tube, 3 µl lipofectamine was added to 47 µl DMEM. Tubes were continuously inverted for 5 min. The plasmids were then combined with the lipofectamine (100 µl) and incubated at room temperature for 20 min before being added to $5HT_{2C}$R-HEK cells in 2 ml DMEM with fetal bovine serum (FBS). The cells were incubated for 24 hr at 37°C in 5% CO_2. After 24 hr, the DMEM/FBS was replaced with DMEM/FBS/penicillin-streptomycin and incubated for another 24 hr under the same conditions. To serve as an additional control, one plate of $5HT_{2C}$R-HEK cells underwent the same general transfection procedure, but without transfection of a plasmid.

MPDZ expression

At 48 hr post-transfection, miRNA and control $5HT_{2C}$R-HEK cells were harvested in 5 ml of sterile-filtered DMEM and centrifuged at 2000 x g to pellet the cells. Total RNA was isolated from each well with the RNeasy Kit as per protocol (Qiagen, Valencia, CA). 1 µg of total RNA was reverse transcribed using random hexamers (High Capacity cDNA Archive kit; Applied Biosystems, Foster City, CA). For each candidate, *MPDZ* relative expression was measured using a validated TaqMan assay (Hs00187106_m1, Applied Biosystems,), which spans an intron as a control against contaminating genomic DNA. Reactions (20 µl) were performed in an ABI Prism 7500 Thermal Cycler using 2-Step PCR Master Mix . Crossing point values (C_t) for target gene expression levels were determined by the standard TaqMan software package and normalized to a reference

gene (*REEP5*: *Hs01075582_m1*) expression. The comparative ($\Delta\Delta C_t$) method (Livak & Schmittgen, 2001) was used for relative quantification of *MPDZ* and *REEP5* expression.

IP1 accumulation

Methods for IP1 accumulation in this study have been previously published (Trinquet et al., 2006). $5HT_{2C}$R-HEK cells expressing miRNA or control plasmids, as well as untransfected cells, were grown to 80% confluence in 150 mm diameter plates (Fisher Scientific, Pittsburgh, PA) in DMEM/FBS in which the FBS was charcoal-stripped and heat-inactivated to remove endogenous steroids and neurotransmitters that may influence $5HT_{2C}$R expression *in vitro* (Saucier et al., 1998). Cells were then plated (~400,000/well in a 24-well plate) in 0.5 ml charcoal-stripped DMEM for 24 hr. Unless otherwise noted, all reagents were provided in the IP1 accumulation assay (IP-One Assay Kit; Cisbio Bioassay, Bedford, MA). First, cells were incubated (37°C, 5% CO_2) with 0.5 ml DMEM for 1 hr, then incubated in 100 µl 1X stimulation buffer alone, stimulation buffer containing 100 nM serotonin (5HT; Sigma-Aldrich, St. Louis, MO), or containing 1 µM SB242084 (10 min pre-incubation; Tocris Biosciences, Ellisville, MO) + 100 nM 5HT for 1 hr (37°C, 5% CO_2). 50 µl of lysis buffer was added to each well for 30 min before a 50 µl sample from each well was transferred to ELISA plates. 25 µl IP1-horseradish-peroxidase (HRP) conjugate and 25 µl anti-IP1 monoclonal antibody were added to each standard or sample and incubated at room temperature for 3 hr before being washed 6 times with milliQ water containing 0.15% Tween 20 (250 µl/well for each wash). 100 µl of the HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB) was then added to each standard/sample and incubated at room temperature in the dark for 30 min. The reaction was terminated with stop solution (100 µl/well) and absorbance was analyzed using a Benchmarkplus® microplate reader (Biorad Laboratories, Hercules, CA) at 420 nm (initial reads) and 650 nm (error reads). Absorbance values at 650 nm

were subtracted from absorbance values at 420 nm for each well and averaged between duplicates. Analyses of these data were performed using Prism® graphing software. Standard concentrations (0.17-5,000 nM IP1) were fit to a nonlinear curve, and sample values were extrapolated using the standard concentration curve. The extrapolated values for each sample (Xs) were log-transformed, first by 10^X and then by $(1e^9)*X$, to calculate IP1 accumulation (in nmol). The transformed variables were analyzed using two-sample t-tests from the Systat® statistical software program ($\alpha = 0.05$).

Results

MPDZ expression

Endogenous *MPDZ* expression did not differ between control transfected (no miRNA) and nontransfected control cells, indicating that transfection itself did not influence endogenous *MPDZ* expression. *MPDZ* expression in miRNA-transfected cells was substantially reduced (38%) compared to control cells.

IP1 accumulation

Control transfected (no miRNA) and nontransfected control cells no did not differ in baseline or 5HT-stimulated IP1 accumulation (all $t<1.8$, $p>0.2$). Therefore, in order to increase the statistical power of my analyses, these results were combined for subsequent comparisons to 'control' cells. Baseline IP1 accumulation ($t<1.4$, $p>0.2$; figure 17a) did not differ between miRNA-transfected and control cells. Using 100 µM 5HT (maximally effective to stimulate IP1 accumulation; A. Eshleman, personal communication), miRNA-transfected cells showed increased IP1 accumulation compared to control cells ($t(9)=-2.6$, $p=0.03$; figure 17b). Pre-incubation with 1 µM SB242084, a highly selective $5HT_{2C}$ receptor antagonist (Bromidge et al., 1997),

completed blocked 5HT-stimulated IP1 accumulation (figure 17c), confirming that 5HT-stimulated IP1 accumulation is mediated by $5HT_{2C}$Rs.

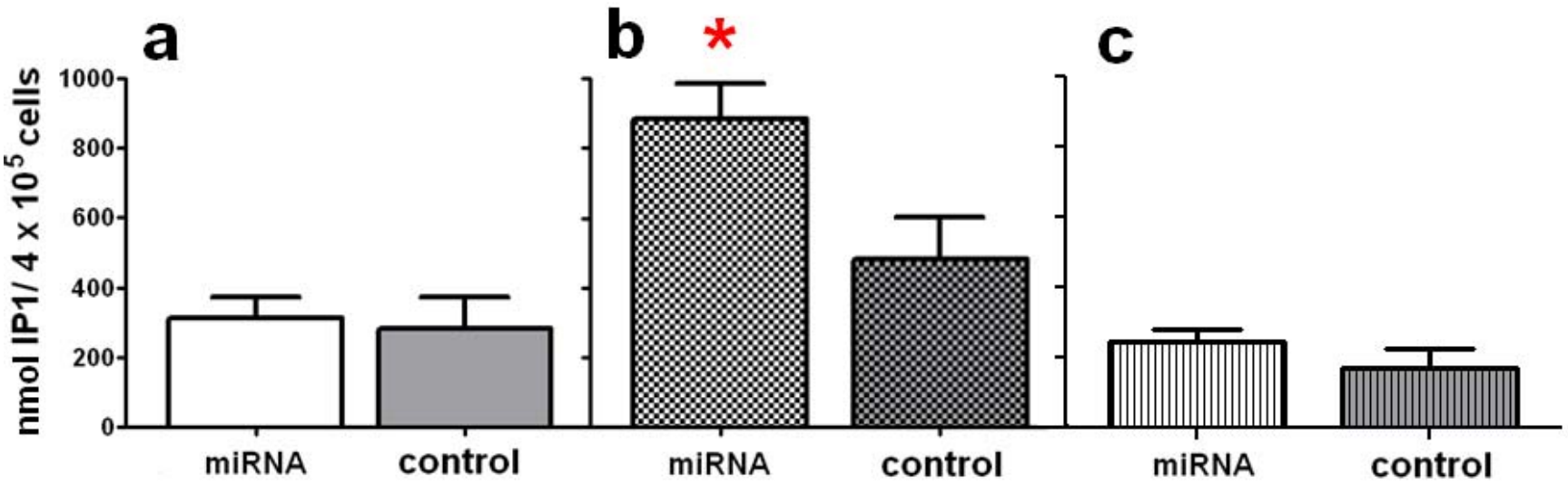
Discussion

These results demonstrate that even a modest reduction in *MPDZ* expression results in markedly increased $5HT_{2C}$R-mediated IP1 accumulation. To my knowledge, these results provide the first evidence that *MPDZ* expression affects $5HT_{2C}$R function.

$5HT_{2C}$Rs can activate multiple signaling systems, including the phospholipase A_2 (PLA_2) pathway, which stimulates arachidonic acid release; and the phospholipase D (PLD) pathway, which stimulates diacylglycerol (to increase protein kinase C production); and phosphatidic acid, which functions in lipid signaling (figure 18; reviewed in Klein, 2005; Millan et al., 2008). Although beyond the scope of this dissertation, future studies should examine the potential affect of *MPDZ* expression on additional $5HT_{2C}$R-activated second messenger systems.

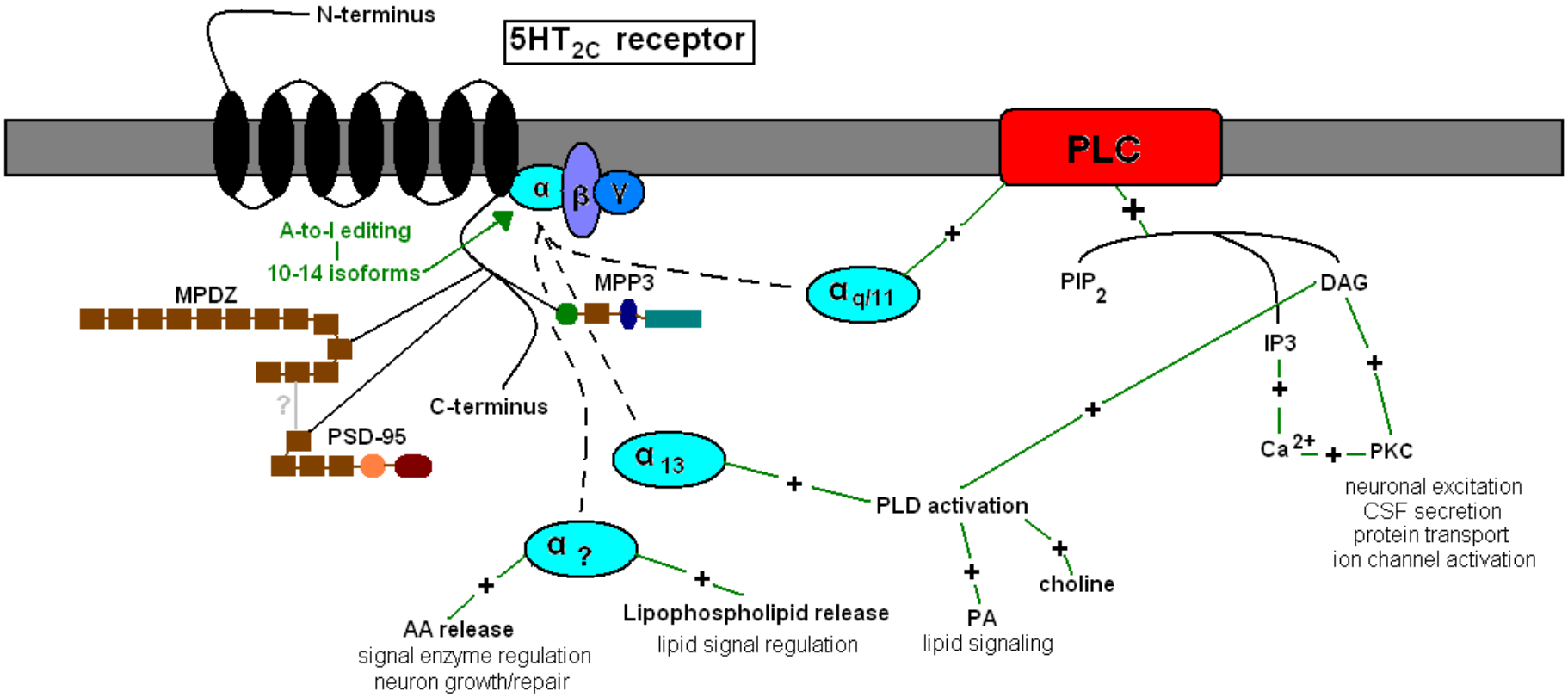
MPDZ may also interact with other $5HT_{2C}$R-associated proteins to dually modify downstream signaling. The association of MPDZ and $5HT_{2C}$Rs occurs within a multiprotein complex, where the $5HT_{2C}$R C-terminus has multiple protein binding partners mediating a variety of intracellular effects (Funk et al., 2009; Jones et al., 2009; Krapivinsky et al., 2004). In addition to MPDZ, other PDZ domain proteins interact with the $5HT_{2C}$R and can have opposite effects on its desensitization kinetics (Gavarini et al., 2004), allowing for the possibility that MPDZ and other PDZ domain proteins act to fine-tune $5HT_{2C}$R function.

Figure 17a-c. IP1 accumulation in $5HT_{2C}$R-HEK cells. Data represent IP1 accumulation (mean ± SEM from four independent assays) under the following conditions: (a) baseline (no 5HT), (b) 100 μM 5HT, and (c) 1 μM SB242084 + 100 μM 5HT. *p<0.05.



The diversity of $5HT_{2C}R$ isoforms may also influence downstream effects of the MPDZ/$5HT_{2C}$ receptor association. Although all the members of the $5HT_2R$ family exhibit substantial overlap in molecular structure and amino acid sequence (Leysen, 2004), the $5HT_{2C}R$ is the only known seven transmembrane domain receptor that undergoes post-transcriptional editing, resulting in multiple receptor isoforms with unique signaling properties. Adenosine-to-ionosine (A to I) editing at the second intracellular domain of the $5HT_{2C}R$ can result in 24 potential $5HT_{2C}R$ isoforms, of which 10-14 have been identified in rodent and human populations (Berg et al., 2008; Millan et al., 2008). Recent evidence suggests that different $5HT_{2C}R$ isoforms may preferentially couple to specific G protein subunits, thereby specifying downstream messenger activation in different neuronal populations (Marion et al., 2004; Wang et al., 2000), and may also influence constitutive activity levels apparent in the unedited isoform (Berg et al., 2008; Niswender et al., 1999). Brain region-specific (Werry et al., 2008) and strain-specific (Hackler et al., 2006) expression of these isoforms have been reported, so future studies examining mRNA expression of unedited and edited $5HT_{2C}R$ isoforms in miRNA-transfected $5HT_{2C}R$ cells may help to further elucidate the relationship between *MPDZ* expression changes and corresponding $5HT_{2C}R$ function.

Figure 18. Schematic representation of $5HT_{2C}R$ activation pathways. Diagram represents postsynaptic activity of the $5HT_{2C}R$. Solid black lines represent physical interactions, dashed lines represent protein movement within the cell, and green lines denote protein activation effects on secondary effector systems. All abbreviations can be found in the list of abbreviations (pages vi-ix).



These studies contribute significantly to progress in understanding the mechanism by which MPDZ may affect behavior (Liu et al., 2007; Shirley et al., 2004; Tabakoff et al., 2009), but there are some limitations. Although the host cells and transfected $5HT_{2C}R$ are human in origin, I used an miRNA directed at the mouse *Mpdz* homolog in order for future studies to assess its function *in vivo* (see general discussion for details). It is plausible that using a human MPDZ miRNA would reduce endogenous *MPDZ* expression to an even greater degree. Second, my results use a heterologous $5HT_{2C}R$ expression system and future studies will be needed using native preparations. Third, my results are based on a single measure of $5HT_{2C}R$ function that, while an important first step, will need to be expanded in future studies assessing other $5HT_{2C}R$-affected signaling pathways. Finally, it is possible that differential IP1 accumulation is also influenced by differences in $5HT_{2C}$ receptor density. This seems unlikely given that all of the cells used were derived from the same cell line stably transfected with the human $5HT_{2C}R$, but should be assessed in future studies using a selective $5HT_{2C}R$ ligand.

CHAPTER 5: Pharmacological assessment of $5HT_{2C}$ receptor function during acute ethanol withdrawal in *Mpdz* knockout and DBA/2J strain mice

Abstract

Mpdz (encoding the multi-PDZ domain protein, MPDZ) is implicated as a gene contributing to variation in predisposition to ethanol withdrawal convulsions in mice. MPDZ physically associates with and is thought to regulate the function of $5HT_{2C}$ receptors, which influence ethanol-related and convulsion phenotypes in humans and animal models. The present studies examined the effects of a $5HT_{2C}$ receptor ligand on handling-induced convulsions (HICs) in ethanol withdrawn and control mice. *Mpdz* knockout ($B6^{Mpdz+/-)}$ and wildtype littermates as well as DBA/2J strain mice were administered a hypnotic dose of ethanol (4 g/kg, i.p.) or saline (hr 0). At hr 7, a selective $5HT_{2C}$ receptor antagonist (0.01 µg SB242084) or vehicle (artificial cerebrospinal fluid) was administered intracerebroventricularly. Animals were assessed for HICs hourly from hr 2-12, and every 20 min between hr 7-9. *Mpdz* knockout mice displayed more severe SB242082-enhanced convulsions than wildtype littermates, supporting the conclusion that *Mpdz* expression impacts $5HT_{2C}$ receptor-mediated behavior; however, ethanol withdrawal-associated convulsions were not apparent. DBA/2J strain (high withdrawal) mice were also tested, but SB242084 did not affect HIC severity in control or ethanol withdrawn animals. These results provide *in vivo* evidence for a functional association between *Mpdz* expression and $5HT_{2C}$ receptor function, although this association's potential influence on ethanol withdrawal convulsion severity remains unknown.

Introduction

Recently, our laboratory identified *Mpdz* on chromosome 4 (Shirley et al., 2004) as a high-quality quantitative trait gene (QTG) candidates to affect alcohol withdrawal convulsion severity in mice. *Mpdz* encodes for the multi-PDZ domain protein (MPDZ; also called MUPP1), which is widely-expressed throughout the body (Sitek et al., 2003) and acts at the cell membrane to organize multiprotein complexes involved in cell signaling (Ackermann et al., 2009; Ebnet et al., 2004; Sindic et al., 2009). MPDZ has been established to physically associate with inhibitory receptors implicated in ethanol- and convulsion phenotypes that represent plausible mechanisms of action by which MPDZ can exert its effects on behavior and withdrawal.

MPDZ physically associates with $5HT_{2C}$ receptors ($5HT_{2C}$Rs) *in vitro* and *in vivo* (Becamel et al., 2001, 2004; Parker et al., 2003; Sharma et al., 2007). $5HT_{2C}$Rs mediate ethanol's actions on GABA release in withdrawal-relevant brain regions (Theile et al., 2009), and the human *5HT2C* gene is associated with alcohol use disorders in affected populations (Yasseen et al., 2010). Previous analyses indicate that central administration of *meta*-Chlorophenylpiperazine (mCPP), an agonist at $5HT_1$ and $5HT_2$ receptors (Kahn et al., 1990), attenuates ethanol withdrawal convulsions in mice (Chen & Buck, unpublished results); and peripheral administration of SB242084, a selective $5HT_{2C}$R antagonist, both elicits convulsions and enhances convulsions elicited by chemiconvulsant drugs (Morita et al., 2005; Reilly, Milner et al., 2008). Here, I assessed the effects of intracerebroventricularly (ICV)-administered SB242084 on handing-induced convulsions (HICs) using ethanol withdrawn and control mice. *Mpdz* knockout ($B6^{Mpdz+/-}$; see chapter 2 for details on this model on an inbred C57BL/6 [B6] genetic background) and wildtype littermates were tested in order to assess the role of *Mpdz* expression on $5HT_{2C}$R-mediated enhancement of HICs; and DBA/2J (D2) mice were tested because they display SB242084-enhanced HICs (Reilly, Milner et al., 2008) and

exhibit robust acute ethanol withdrawal (Crabbe et al., 1983). Taken together, these studies suggest that manipulation reduced *Mpdz* expression is associated with more pronounced $5HT_{2C}$R-mediated HIC responses, although this association did not influence ethanol withdrawal convulsion severity.

Methods

Animals

$B6^{Mpdz+/-}$ and wildtype littermates were bred in our colony at the VA animal facility. D2 strain males (50-60 days old) were purchased from Jackson Laboratories (Sacramento, CA) and were housed in our facilities at the VA for 2 weeks prior to surgery. Mice were group-housed 2-5 per cage (28x17x11.5 cm lined with Bedicob® bedding) by sex. Mouse chow (Purina #5001) and water were available *ad libitum*. Procedure and colony rooms were kept at a temperature of 21 ± 1^0C. Lights were on in the colony from 0600-1800 hr. All procedures were approved by the VA and OHSU Institutional Animal Care and Use Committees in accordance with United States Department of Agriculture and United States Public Health Service guidelines.

Cannula implantation surgery

Animals were anesthetized with isoflurane and placed into a stereotaxic apparatus (Cartesian Instruments, Stereotaxic Alignment System; David Kopf Inc., Tujunga, CA). A midline incision was made on the scalp to expose the skull. A dental drill was used to drill a small hole through the skull (0.46 mm posterior from bregma, 1.20 mm lateral (left side) from midsaggital suture, DV- 2.0 mm deep from skull surface according to Paxinos and Franklin [2001]) and a 25 gauge stainless steel cannula (PlasticsOne, Inc., Roanoke, VA) was lowered to sit directly above the lateral ventricle.

The cannula was fastened to the skull using three stainless steel screws and cranioplastic cement (Plastics One, Inc., Roanoke, VA). Following surgery, each mouse was administered ketorolac (3 mg/kg in 0.3 ml saline, s.c.) to reduce pain and swelling, and placed in a holding cage for recovery (5-10 min) before being returned to its homecage. All mice were allowed at least 7 days to recover before experimental testing.

Handling-induced convulsions

Methods for HIC scoring have been previously published (Reilly, Milner et al., 2008). B6$^{Mpdz+/-}$ or wildtype mice were assigned to one of four treatment groups (ethanol/SB242084, ethanol/vehicle, saline/SB242084 or saline/vehicle; n=3-6/group). Mice were scored twice for baseline (pre-ethanol) HICs before being administered a hypnotic dose of ethanol (4 g/kg, 20% v/v, i.p.) or an equivalent volume of saline (at hr 0) and returned to their homecage. At hr 7, animals received SB242084 (Tocris Biosciences, Ellisville, MI; 0.02 µg in 1 µl vehicle) or vehicle (artificial cerebrospinal fluid [aCSF], 126 mM NaCl, 2.5 mM KCl, 2.4 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 20 mM $NaHCO_3$, and 10 mM glucose, also containing 0.5 mM β-cyclodextrin and 0.25 mM citric acid to increase the solubility of SB242084). These were administered ICV (over 1 min) using a 32 gauge stainless steel injector (PlasticsOne Inc., Roanoke, VA) and microinjection pump (CMA microdialysis, North Chelmsford, MA). Injectors remained in place for 30 sec following the infusion, after which the animal was returned to its homecage. HIC scores were recorded hourly from hr 2-12 (and every 20 min between hr 7-10), and at hr 24 and 25. Following the experiment, brains were removed and flash frozen in liquid nitrogen. Brains were sliced on a cryostat (30 µM sections) and checked for injection tracks to confirm cannula placement.

D2 mice (n=40) were tested using the methods above except that a lower concentration of SB242084 (0.01 µg) in a smaller volume of vehicle (0.4 µl) was used, and only males were tested.

In order to create an index of ethanol withdrawal response that is independent of individual differences in baseline HIC scores and reflects differences in withdrawal convulsion severity, post-ethanol HIC scores were corrected for the individual's average baseline HIC score as in previous work (Metten et al., 1998a; Buck et al 1997). Ethanol withdrawal severity scores were calculated as the area under the curve (AUC; the summed, corrected HIC scores) over the full timecourse post-ethanol.

Data analyses

Outliers were detected using Grubb's outlier analysis, and removed before performing subsequent analyses. Behavioral results were analyzed in the Systat® statistical software program with analyses of variance (ANOVAs). Significant ($p < 0.05$) effects of genotype, ethanol (treatment) and SB242084 (drug) were followed up using Tukey's post-hoc analyses.

Results

SB enhancement of HICs in B6$^{Mpdz+/-}$ and wildtype littermates

Initial analyses did not detect main effects of sex or interactions involving sex (all $F<0.7$, $p>0.8$), so data from males and females were combined. No main effects of genotype ($F<1.4$, $p>0.2$), treatment (ethanol withdrawn or saline control; $F<0.3$, $p>0.6$), or drug (SB242084 or vehicle; $F<0.1$, $p>0.9$), or significant interactions among these variables were detected (all $F<1.46$, $p>0.2$; figure 19). Analyses of withdrawal summary scores for the 2 hr immediately following SB242084 administration (hr 7:20, 7:40, 8:00,

8:20, 8:40; 9:00, 9:20 and 9:40) detected a trend for a main effect of genotype ($F_{(1,26)}$= 3.2, p<0.1), with B6$^{Mpdz+/-}$ exhibiting more severe HICs than wildtype littermates. Trends for genotype x drug and treatment x drug interactions were also detected (both F>1.9, p<0.2). In saline control animals, B6$^{Mpdz+/-}$ showed a trend for more severe SB242084-enhanced convulsions than wildtype littermates (2.3 ± 1 and 1 ± 0.5, respectively; p=0.18). This difference was not apparent in the ethanol withdrawn animals. Although preliminary, these results are consistent with the conclusion that *Mpdz* expression affects $5HT_{2C}R$ function *in vivo*. This was apparent in control animals. However, acute ethanol withdrawal is not robust in B6-derived models, and no effect of SB242084 on withdrawal associated convulsions was detected.

SB242084 enhancement of HICs in D2 mice

As expected, a main effect of treatment was apparent, with withdrawn mice exhibiting significantly more severe HICs compared to saline controls ($F_{(1,52)}$=58, p<0.001; figure 20), across groups. No main effects of drug or treatment x drug interactions were observed (all F<1.2, p>0.3). Analyses on withdrawal summary scores for the 2 hr immediately following drug administration (hr 7:20, 7:40, 8:00, 8:20, 8:40; 9:00, 9:20 and 9:40) also did not detect a main effect of drug on HIC severity during acute ethanol withdrawal (F<1.6, p>0.2).

Figure 19. Timecourse of ethanol withdrawal HICs following ICV-infused SB242084 or vehicle in B6$^{Mpdz+/-}$ and wildtype littermates. B6$^{Mpdz+/-}$ and wildtype littermate (B6^{WT}) mice of both sexes were scored twice for baseline HICs immediately before administration of saline **(a)** or 4 g/kg i.p. ethanol **(c)**, and hourly from hr 2-7. AVB represents to the average baseline HIC score. At hr 7, SB242084 (0.02 µg in 1 µl) or 1 µl vehicle (aCSF) was infused into the lateral ventricle over 60 sec. HICs were scored every 20 min from hr 7-9, hourly from hr 9-12, and at hr 24 and 25. Data represent mean HIC scores ± SEM (n=3-6 per group). HIC severity scores were calculated as the area under the curve (AUC, corrected for baseline HIC scores) for hr 2-12 (mean ± SEM). SB242084-enhanced HICs were more apparent in B6$^{Mpdz+/-}$ than in wildtype littermates in control animals **(b)**, but not in ethanol withdrawn animals **(d)**. #p<0.1.

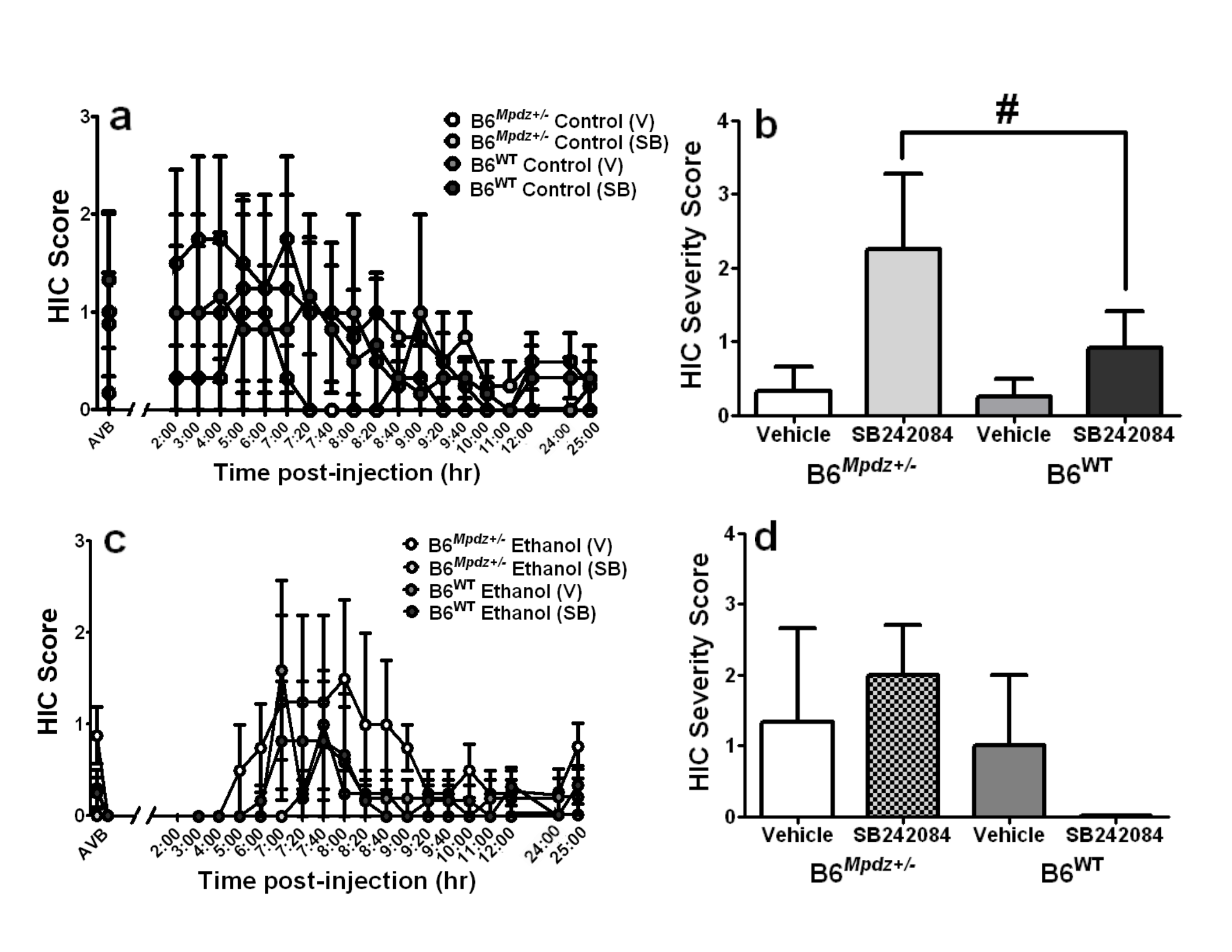
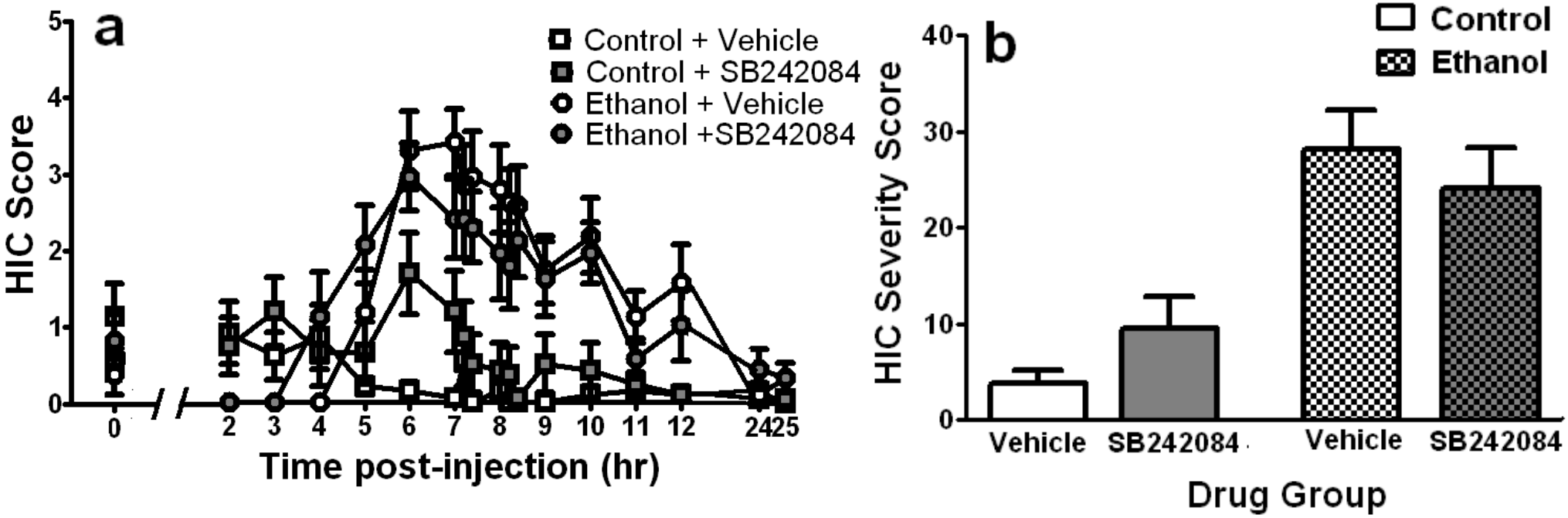

a
HIC Score
Time post-injection (hr)
B6Mpdz+/- Control (V)
B6Mpdz+/- Control (SB)
B6WT Control (V)
B6WT Control (SB)
AVB
2:00
3:00
4:00
5:00
6:00
7:00
7:20
7:40
8:00
8:20
8:40
9:00
9:20
9:40
10:00
11:00
12:00
24:00
25:00
b
HIC Severity Score
#
Vehicle
SB242084
Vehicle
SB242084
B6Mpdz+/-
B6WT
c
HIC Score
Time post-injection (hr)
B6Mpdz+/- Ethanol (V)
B6Mpdz+/- Ethanol (SB)
B6WT Ethanol (V)
B6WT Ethanol (SB)
d
HIC Severity Score
Vehicle
SB242084
Vehicle
SB242084
B6Mpdz+/-
B6WT

Figure 20. Baseline and ethanol withdrawal-associated HICs in D2 strain mice before and after ICV administration SB242084 or vehicle. (a) Male mice (n=40) were scored twice for baseline HICs immediately before administration of 4 g/kg i.p. ethanol or an equivalent volume of saline, and hourly from hr 2-7. AVB refers to the average baseline HIC score. At hr 7, SB242084 (0.01 µg in 0.4 µl vehicle) or 0.4 µl vehicle (aCSF) was infused into the lateral ventricle over 60 sec. HICs were then scored every 20 min between hr 7-9, hourly between hr 9-12, and at hr 24 and 25. Data represent mean HIC scores ± SEM. Square symbols represent saline control mice, and circles represent ethanol withdrawn mice. **(b)** HIC severity scores were calculated as the area under the curve (AUC, corrected for baseline HIC scores) for hr 2-12 (mean ± SEM). Across drug groups, HICs were more severe in ethanol withdrawn mice (patterned bars) compared to controls (solid bars).


a
HIC Score
Time post-injection (hr)
Control + Vehicle
Control + SB242084
Ethanol + Vehicle
Ethanol +SB242084
b
HIC Severity Score
Control
Ethanol
Vehicle
SB242084
Vehicle
SB242084
Drug Group

Discussion

Mpdz has emerged as a confirmed QTG for the ethanol withdrawal convulsion QTL (*Alcw2*) on mouse chromosome 4 (Chapter 3; Shirley et al., 2004), but the mechanism by which *Mpdz* affects ethanol withdrawal remains to be elucidated. $5HT_{2C}$Rs are implicated as contributing to HICs, and comparisons of congenic and background strain mice implicate $5HT_{2C}$Rs as involved in mediating *Alcw2* effects on CNS excitability (e.g., HICs; Reilly, Milner et al., 2008). Preliminary analyses of peripherally administered SB242084 in $B6^{Mpdz+/-}$ mice and wildtype littermates suggests that lower *Mpdz*/MPDZ expression may be associated with more severe HICs (Milner & Buck, unpublished results) and the ICV administration of SB242084 results obtained here provide additional evidence for this relationship. $5HT_{2C}$Rs are implicated in ethanol phenotypes (Overstreet et al., 2007; Pandey et al., 1996), including behaviors exhibited during withdrawal (Knapp et al., 2007), making them a plausible mechanism of action for *Mpdz*'s effects on ethanol withdrawal convulsions.

Although reduced *Mpdz* expression is associated with increased $5HT_{2C}$R-mediated convulsions in control mice, this was not apparent in ethanol treated mice. However, ethanol withdrawal *per se* was not apparent, which is not surprising given that B6-derived mice exhibit little or no acute ethanol withdrawal associated HICs (Buck et al., 1997). Therefore, I examined the effects of SB242084 in DBA/2J (high withdrawal) strain mice (Crabbe et al., 1983a). Although ethanol withdrawal was evident in D2 mice, no effect of SB242084 was detected. Additionally, SB242084 did not increase baseline HIC scores, as was observed in $B6^{Mpdz+/-}$ when a 2-fold higher dose of SB242084 was used. These results are surprising given that peripheral administration of SB242084 and intranigral administration of mCPP both induce HICs in D2-derived mice (Chen & Buck, unpublished results; Reilly, Milner et al., 2008). Taken together with my results in $B6^{Mpdz+/-}$ mice, this suggests that ICV administration of SB242084 may not have resulted

in sufficient access to the appropriate brain region(s) to affect baseline and/or withdrawal-associated convulsions. Future studies will be needed (e.g., using different vehicles, larger doses or infusion volumes, and/or site-directed administration into brain regions implicated in mediating the effects of *Mpdz* on withdrawal; Chen et al., 2008) that may improve SB242084 solubility and delivery in D2 mice.

It should also be considered that MPDZ associates with additional proteins that may also influence ethanol-related behaviors (Heydecke et al., 2006; Nguyen et al., 2003; Parker et al., 2003). For example, MPDZ associates with NR2B subunit-containing NMDA receptors, which are implicated in ethanol consumption (Incerti et al., 2010; Henniger et al., 2003; Wang et al., 2007), and are potential treatment targets for alcohol withdrawal and dependence (Nagy, 2004). Future studies examining NMDA receptor in the B6$^{Mpdz+/-}$ knockout model may also identify a role for these receptors in *Mpdz*'s influence on ethanol withdrawal.

CHAPTER 6: General Discussion

Summary of results

The identification of a QTG (or QTG candidate) often brings challenges, such as the development and examination of appropriate behavioral models, strategies to determine relevant molecular associations, and identification of relevant brain regions. However, this type of information is essential to understanding how QTGs influence ethanol-related phenotypes, with the hope of using this information to create more effective treatment and prevention strategies for alcoholic populations.

One goal of this dissertation was to more thoroughly examine ethanol withdrawal convulsions for two high-quality QTG candidates, *Mpdz* and *Kcnj9*, previously implicated in ethanol withdrawal convulsion severity in mice. In chapters 2 and 3, novel knockout models for *Mpdz* and *Kcnj9* were employed to provide evidence for these QTGs' effects on ethanol withdrawal-associated convulsion behavior *in vivo.* In chapter 2, my results obtained using the B6$^{Mpdz+/-}$ model, taken together with results using *Mpdz* overexpressing transgenic mice (Milner, Shirley et al., in preparation), provide compelling evidence that *Mpdz* is a QTG affecting ethanol withdrawal convulsion behavior. However, effects of *Mpdz* were not detected in the 129/S5$^{Mpdz+/-}$ model, possibly due to the 129/S5 background strain issues noted in chapter 2. In chapter 3, my results support the conclusion that *Kcnj9* is a QTG for ethanol withdrawal severity in D2-derived mice.

A second goal of this dissertation was to examine other ethanol-related behaviors in these models to identify potential pleiotropic effects of *Mpdz* and *Kcnj9* beyond withdrawal convulsions. Acute ethanol withdrawal was associated with reduced locomotor activity in both studies, expanding upon results using chronic withdrawal models (Kliethermes et al., 2004). In the 129/S5$^{Mpdz+/-}$ model, ethanol withdrawn mice

exhibited lower anxiety-like and depression-like behavior than controls, results contrary to evidence suggesting that ethanol withdrawal increases these behaviors in rodents (Kokare et al., 2008). Again, the assessment of ethanol withdrawal using the 129/S5 background strain likely confounded the results observed. Interestingly, B6$^{Mpdz+/-}$ mice consumed slightly less ethanol than wildtype littermates, suggesting that *Mpdz* may exert pleiotropic effects on ethanol responses, if not necessarily on other ethanol withdrawal responses. A negative genetic association between consumption and withdrawal behaviors has been reported (Metten et al., 1998), so future studies using B6$^{Mpdz+/-}$ mice will expand on the ethanol consumption data reported in this dissertation.

In addition to locomotor depression, D2-derived *Kcnj9* knockout model mice exhibited increased anxiety-like behavior during acute ethanol withdrawal, a finding consistent with chronic models (Verleye et al., 2009). Furthermore, *Kcnj9* influenced locomotor activity in the homecage and elevated zero-maze in both ethanol withdrawn and saline control mice, suggesting that this gene has effects on behaviors independent of ethanol withdrawal. *Kcnj9* also influenced depression-like behavior independent of ethanol withdrawal, with lower *Kcnj9* expression associated with less depression-like behavior. Depression is frequently reported as comorbid with (Cornelius et al., 2003; Schuckit, 2006), and shares underling biological and genetic contributions with (Baigent, 2005) substance abuse disorders, so *Kcnj9*'s influence on this phenotype suggests that alterations in *Kcnj9* expression may influence complex traits (e.g., depression-like behavior) associated with risk for alcohol dependence development in certain individuals. In sum, these results suggest that both *Mpdz* and *Kcnj9* may exert effects on behavioral responses to ethanol and other complex behaviors beyond ethanol withdrawal convulsion severity.

The last goal of this dissertation was to test the relationship between MPDZ and $5HT_{2C}$ receptor as a possible mechanism of action by which *Mpdz* exerts its effects on

ethanol withdrawal convulsion severity. In chapter 4, I assessed changes $5HT_{2C}$ receptor function (through the IP3 pathway) associated with differential *MPDZ* expression *in vitro*. In chapter 5, I tested convulsion responses to a centrally-administered $5HT_{2C}$ receptor ligand in knockout and inbred mouse models in order to assess potential influences of this receptor on ethanol withdrawal behavior *in vivo.* My results suggest a functional relationship between MPDZ and $5HT_{2C}$ receptors; as lower *MPDZ*/*Mpdz* expression was associated with higher agonist-stimulated $5HT_{2C}$ receptor activity *in vitro* (chapter 4) and higher $5HT_{2C}$ receptor-mediated convulsions *in vivo* (chapter 5). However, an effect of this association on ethanol withdrawal convulsions was not detected. Recent evidence from our laboratory suggests that intranigral stimulation of $5HT_{2C}$ receptors (using mCPP) reduces ethanol withdrawal severity, suggesting that ICV administration may have been an ineffective method of administration or that administration of SB242084 (a $5HT_{2C}$ receptor antagonist) is insufficient to observe $5HT_{2C}$ receptor-mediated activity during acute ethanol withdrawal. Future studies examining brain region-specific (i.e., intranigral) SB242084 administration, or examining ICV administration of a $5HT_{2C}$ receptor agonist, during acute ethanol withdrawal may identify a role for this receptor in ethanol withdrawal severity. However, it is also possible that the association between MPDZ and $5HT_{2C}$ receptors does not significantly influence this phenotype, and other potential mechanisms for *Mpdz*'s effects on ethanol withdrawal should be noted.

Alternative mechanisms for *Mpdz*

Another mechanism by which MPDZ may influence withdrawal severity is via its associations within an N-methyl-D-aspartate (NMDA) receptor complex. In hippocampal neurons, Krapivinsky et al. (2004) report evidence a multiprotein complex, containing the

MPDZ and the ionotropic glutamate receptor subunit 2B (NR2B) subunit of the NMDA receptor, that colocalizes with two regulatory proteins (a GTPase activating protein, SynGAP, and a Ca^{2+}/calmodulin-dependent protein kinase, CAMKII) to regulate α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor response. In these cells, NMDA-mediated activation Ca^{2+} influx results in a disruption between the MPDZ-SynGAP interaction, allowing for SynGAP phosphorylation and ultimately resulting in increased frequency of AMPA receptor response and clustering. This MPDZ-containing complex is of particular interest, as the NR2B subunit is inhibited by acute ethanol exposure (Wirkner et al., 1999), and chronic ethanol exposure leads to significant changes in NR2B subunit expression in the postsynaptic membrane (Nagy, 2004), and in blood cells of alcohol-withdrawing patients (Biermann et al., 2009). Furthermore, sensitivity to NMDA-elicited seizures was reduced during chronic ethanol withdrawal in mice (Finn & Crabbe, 1999), and changes in NR2B expression in ethanol withdrawn mice are associated with changes in neuronal hyperexcitability, possibly contributing to withdrawal-induced seizures (Clapp et al., 2010).

Mpdz's effects on ethanol withdrawal severity may also be mediated through MPDZ physical associations with proteins involved in synaptic plasticity at the dendrite. Dendritic spines are specialized structures that receive the majority of central excitatory synaptic input. Chronic ethanol exposure can induce morphological changes in these structures (Zhou et al., 2007), resulting in alterations in synaptic plasticity associated with addictive and drug seeking behaviors (Chandler, 2003). Changes in dendritic morphology have also been implicated in the expression of convulsant behaviors (Carpenter-Hyland & Chandler, 2006; Oberheim et al., 2008), although changes associated drug withdrawal-induced convulsions have not yet been examined. In cortical neurons, MPDZ associates with $5HT_{2A}$ receptors, where MPDZ functions to localize $5HT_{2A}$ receptors. Transient activation of $5HT_{2A}$ receptors results in a transient

increase in dendritic spine size, and activates second messenger pathways involved in dendritic spine remodeling (Jones et al., 2009). It is therefore plausible that *Mpdz*'s effect on ethanol withdrawal-induced neuronal hyperexcitability (behavioral expressed as HICs) is mediated through its association with one or more proteins that regulate excitatory transmission at the dendritic spine. Although beyond the scope of this dissertation, the examination of MPDZ's association with NMDA and $5HT_{2A}$ receptors may provide additional mechanisms by which *Mpdz* exerts its behavioral effects *in vivo*.

Potential mechanisms for *Kcnj9*

Although studies addressing the mechanism by which *Kcnj9*/GIRK3 affects ethanol withdrawal were beyond the scope of this dissertation, I discuss potential mechanisms of action here. Certainly, future studies should examine the association between GIRK3 and $GABA_B$ receptors as a potential mechanism by which this gene influences withdrawal severity. Activation of GIRK3-containing channels by $GABA_B$ receptors is evident (Lomazzi et al., 2006), and is a plausible mechanism by which *Kcnj9* influences sedative-hypnotic drug withdrawal (Kozell et al., 2009). Baclofen, a selective $GABA_B$ receptor agonist, increases convulsion severity during ethanol withdrawal (Humeniuk et al., 1994).

GIRK3 knockout models also provide evidence for its association with other receptors that regulate drug and alcohol behaviors. The most striking example is activation of GIRK3-containing channels by opioid receptors, with GIRK3 knockout mice showing attenuated hypothermic and analgesic responses to morphine (Marker et al., 2004), as well as a reduction in morphine withdrawal severity (Cruz et al., 2008). The modulation of alcohol's effects by opioid receptors is well established (Gianoulakis, 2009). Naltrexone, a widely-employed mu-opioid receptor antagonist, may help alleviate

alcohol withdrawal symptoms in clinical populations (Haile et al., 2008) and administration of opioid receptor ligands can alter ethanol withdrawal convulsion severity in rodent models (Beadles-Bohling & Wiren, 2006; Ghozland et al., 2005), suggesting a possible role for opioid receptors in ethanol withdrawal phenotypes influenced by *Kcnj9*. Future studies examining opioid receptor function in *Kcnj9* knockout mice should be performed to identify (or exclude) opioid receptors as mechanisms of action for *Kcnj9*'s behavioral effects.

Dissertation limitations and considerations

Knockout model limitations

Despite the strengths of knockout approaches, it has limitations, one of which is particularly relevant to QTG identification. Embryonic stem (ES) cell lines used to generate targeted knockout models are most commonly derived from 129 substrains (see Simpson et al., 1997), although some successes have been reported using ES cells derived from B6 strain mice (Seong et al., 2004). 129 substrains can be challenging to work with due to the divergent genotypes (and physiological phenotypes) present (Threadgill et al., 1997). Additionally, the substrain used for knockout development may not exhibit phenotypes relevant or sufficient to study the behavioral trait of interest. Therefore, after gene-targeting using 129 ES cells, knockout mice are often backcrossed to a different background strain (as was done for the B6$^{Mpd+/-}$ and D2-derived *Kcnj9* knockout models used in this dissertation). However, due to recombination restriction, the region immediately flanking the target gene will inevitably contain 129 ES-derived genes, so phenotypic differences between knockout and background strain mice may be due to the target (e.g., QTG candidate) gene, a 129-derived passenger gene or genes, or both (Su et al., 2009). This issue can be addressed, at least in part, by examining the

other linked QTG candidates for differential expression and/or sequence variation between knockout and wildtype littermates (Kozell et al., 2009) or by comparison of the knockout strain with a co-developed 129.B6 co-isogenic strain spanning the QTL of interest (Crusio, 2004). The major drawback to these solutions, however, is that simultaneous development of the multiple control lines required can be both time and cost-prohibitive for the examination of a single QTG.

Additionally, target gene knockout can lead to embryonic lethality or developmental abnormalities. Compensatory mechanisms may be established during development that confounds interpretation (Picciotto & Wickman, 1998). To circumvent these issues, complementary (and potentially superior) genetic models can be examined (e.g., conditional knockout models, transgenic models with increased target gene expression). Conditional knockout models offer the advantage of temporally and/or spatially restricted knockout of the gene, thus potentially mitigating compensatory and developmental confounds (Kühn & Schwenk, 2003), but development of these models can also be lengthy and costly.

RNA interference (RNAi) is increasingly used to reduce endogenous target gene expression in adult animals by preventing translation of endogenous mRNA from the gene of interest into a functional protein (Lu et al., 2005). This method is particularly attractive because it results in target gene expression changes without the 129 passenger gene or developmental compensation issues associated with other genetic models. Commonly employed methods of RNAi administration are achieved through the use of lentiviral vectors (Singer & Verma, 2008), which stably reduce targeted gene expression levels for a least one month post-transfection (Van den Haute et al., 2003). The viral infection also spreads very little from the site of the injection, so altered gene expression can be limited to a precise brain region, thereby reducing endogenous protein levels of the gene of interest in a targeted (i.e., region-specific) manner. The

RNAi approach is being used to assess target gene effects on behavior, including the role of brain-derived neurotrophic factor (BDNF) in the dorsolateral striatum (Jeanblanc et al., 2009), mu-opioid receptors in the ventral tegmental area (Lasek et al., 2007), and α4 subunit-containing $GABA_A$ receptors in the nucleus accumbens (Rewal et al., 2009) on alcohol consumption and preference behaviors in mice. Our laboratory is currently testing a lentiviral-mediated miRNA construct targeted against *Mpdz* to examine this QTG's influence on alcohol withdrawal in brain regions implicated in alcohol withdrawal HIC severity (see below).

Epistatic and environmental considerations

An important consideration that must be taken into account when examining a discrete genetic influence on an alcohol-related trait is the likely possibility that the QTL's influence on trait expression is modified by other genetic and environmental influences within the genome. In QTL mapping, influence of one gene upon the expression or function of another (i.e., epistatsis) can substantially alter a phenotype of interest. When investigating the influence of a particular QTL or gene on a specific inbred strain background, epistatic influence from background genes can often confound result interpretation of genes with small effect sizes (Crabbe, 2002), highlighting the importance of using multiple inbred strain (or inbred strain-derived) models to thoroughly examine potential QTGs. In some cases, researchers are identifying regions of potential epistatic interaction for drug-related behaviors even before a candidate gene has been identified (Phillips et al., 2002). Genome-wide scans performed in mapping populations have identified QTL epistatic interactions on sedative drug withdrawal severity (Hood et al., 2001) and ethanol consumption (Bachmanov et al., 2002). Although epistasis has the potential to obscure the influence of relevant, small effect QTLs for alcohol-related behaviors, developing strategies to identify these types of interactions can also provide

information about relevant loci outside the primary region of interest that may influence phenotypic expression and ultimately provide a more accurate picture of a QTL's effect across heterogeneous populations.

Another important consideration influencing phenotypic variation is environmental influence. Gene (or QTL) x environment interactions can have a negative effect in complex trait research, as inconsistencies between testing environments can influence behavioral responses in these strains despite experimenter attempts for consistency (Crabbe et al., 1999b) and can be a confounding factor during initial discovery or replication of genetic findings when using specific mouse models (Wahlsten, 2001). However, laboratory environment influences do not generally obscure large phenotypic differences between strains (Wahlsten et al., 2003), suggesting that the QTLs with substantial effect sizes discovered for ethanol-related traits are not likely to be significantly confounded by small variations in testing environment. Additionally, as gene x environment interactions occur frequently in human populations for all behavioral traits, often with significant behavioral outcomes (Caspi et al., 2003; Laucht et al., 2009), researchers are beginning to use rodent models to explicitly examine these types of interactions. The influence of stress on alcohol-related behaviors has been well-documented for many mammals (Campbell et al., 2009) and work is currently being performed using rodent and primate models to examine the relationship between stress exposure and discrete genetic factors known to influence alcohol-associated behaviors (reviewed in Stacey et al., 2009). Sher et al. (2010) outline two additional environmental factors (early alcohol exposure and social environment) that are studied using various genetic rodent models to dissect genetic and environmental influences. Although gene x environmental interactions can be potentially confounding to stable expression of a QTL phenotype, the ability to manipulate and regulate genetic and environmental factors associated with alcohol use disorders allows preclinical researchers an additional

avenue to examine the complexity of genetic and environmental influence on alcohol behaviors in order to more accurately model the human condition and provide more detailed mechanistic/risk information relating to discrete QTGs associated with alcohol behaviors in rodents.

Human relevance of QTGs identified in mice

The human relevance of mouse QTG data depends on using robust animal models and on the high degree of homology between the human and mouse genomes at the gene level (Gregory et al., 2002). Moreover, QTG identification is likely to identify genes for regulatory or rate-limiting proteins that are important therapeutic targets for populations affected by alcohol use disorders (Korstanje & Paigen, 2002).

The QTGs identified for alcohol responses in mice can also play an important role in human subjects. One of the most striking examples of overlap is the distal region of chromosome 1, where alcohol traits have been identified in both human (Bierut et al., 2004; Nurnberger et al., 2001; Reich et al., 1998) and mouse (Buck et al., 2002; Denmark & Buck, 2008; Kozell et al., 2008) models. Ehlers et al. (2010) recently reported on this region, showing that multiple QTLs for human alcohol and tobacco dependence are syntenic with mouse QTLs for alcohol and pentobarbital withdrawal severity, suggesting that high-quality QTGs identified using mouse models may be directly relevant to substance abuse risk in human populations.

MPDZ is present in humans (maps to chromosome 9p24-p22) and may also contribute to risk for alcoholism in some clinical populations. At least three QTLs on human chromosome 9 are detected in mapping studies for alcoholism and associated endophenotypes (Bergen et al., 2003; Daw et al., 2005; Long et al., 1998; Ma et al., 2003; Saunders et al., 2009; Williams et al., 2005), although most are still suggestive

associations and mapped to relatively large regions. At least two of these studies identify markers on chromosome 9p22-p21 associated with alcoholism (Long et al., 1998; Williams et al., 2005), making *MPDZ* a viable candidate gene for these loci detected in human studies. Furthermore, recent studies in human populations have identified *MPDZ* as a potential candidate gene associated with alcohol consumption behaviors (Tabakoff et al., 2009) and risk of dependence (Karpyak et al., 2009), although association with withdrawal symptoms was not observed. These human association studies provide evidence that specific genes associated with alcohol behaviors in rodent populations may be directly relevant to alcohol risk factors in clinical populations.

Ultimately, I view the purpose of QTL research in mice as the identification of genetic targets for humans. In some cases the QTG will be the same in mouse and man (Mogil et al., 2003). More likely, QTL research will identify a relevant gene network. A comprehensive understanding of genetic variation, both in humans and animal models, is crucial to establish relationships between genotype and biological function (Collins et al., 2003).

Future directions

Examination of Mpdz and Kcnj9 using lentiviral-mediated RNAi

As noted above, lentiviral-mediated RNAi delivery is an exceptionally promising method to study QTG effects in mammalian models. In our laboratory, preliminary work has begun to assess behavioral effects of lentiviral vectors containing RNAi against *Mpdz* and *Kcnj9* in mice. Using RNAi is especially attractive in the examination of *Mpdz* effects *in vivo*, as it allows for regionally-specific *Mpdz* reduction in the substantia nigra, a region of the basal ganglia that has been identified as mediating the *Alcw2* QTL's

effect on ethanol withdrawal (Chen et al., 2008). Achieving QTG expression changes in a reasonable timeframe (i.e., months instead of years to develop), and in a region-specific manner, will allow our laboratory to more directly assess the relevant brain circuitry associated with QTG effects on ethanol phenotypes.

Examination of other QTG-relevant phenotypes

The lack of *Mpdz*-dependent effects observed for ethanol withdrawal behaviors other than the HIC phenotype suggests that this QTG's influence on ethanol withdrawal may be restricted to withdrawal convulsion severity. However, its potential influence on ethanol consumption behaviors suggests that *Mpdz* may exert pleiotropic effects on ethanol-related behaviors not expressed during acute withdrawal. Future experiments should examine ethanol-related behaviors that are associated with MPDZ interaction partners. For example, MPDZ's association with the NR2B subunit of the NMDA receptor may influence a number of ethanol-related behaviors influenced by NMDA receptor activity, including ethanol-induced conditioned place preference (Gremel & Cunningham, 2010), ethanol self-administration (Raeder et al., 2008) and ethanol sedation (Sharko & Hodge, 2008). Examination of NMDA receptor-influenced ethanol behaviors may reveal additional pleiotropic effects of *Mpdz*.

Similarly, GIRK3's association with the opioid receptor system, a receptor system heavily involved in analgesic responses, suggests that *Kcnj9* may influence ethanol-related analgesic effects (i.e., ethanol-induced analgesia or withdrawal-induced hyperanalgesia). *Kcnj9*'s influence on basal depression-like behavior provides additional avenues for further exploration of this gene's effects on ethanol-related phenotypes. Comorbid depression-like behavior and high alcohol consumption have been observed in rodent models (Rezvani et al., 2007; Tizabi et al., 2009), suggesting common underlying biological mechanisms for these behaviors. If *Kcnj9* influences basal levels

of depression-like behavior, it may influence other complex behaviors that are associated with alcohol use disorders. Future studies examining the *Kcnj9* knockout model in multiple depression-like behavior tasks (e.g., Porsolt's forced swim task) should be undertaken to better define this gene's potential role in this behavior, as well as this behavior's potential relationship to ethanol-related phenotypes.

Examination of other QTG-relevant mechanisms

Due to MPDZ's participation in multiprotein complexes associated with a variety of receptor systems implicated in alcohol-related behaviors, a broader examination of these pathways is warranted. $B6^{Mpdz+/-}$ and wildtype brain tissue could be examined for NR2B subunit expression differences and/or NR2B-MPDZ colocalization differences in brain regions relevant to withdrawal HIC severity. Additionally, miRNA-induced reduction of *Mpdz* in cells expressing functional, NR2B-containing NMDA receptors could more thoroughly examine the intracellular effects of this interaction, both in naïve and ethanol-exposed conditions. Lastly, pharmacological investigations using the *Mpdz* knockout model could be undertaken to examine the potential behavioral effects of NR2B-MPDZ interaction during ethanol withdrawal.

A more through examination of the activation of GIRK3-containing channels by ethanol-induced opioid receptor stimulation is also warranted. Future experiments examining potential differences in opioid receptor expression and function between the *Kcnj9* knockouts and wildtype littermates, either through pharmacological or molecular methods, should be performed to determine if mu-opioid activation of GIRK3-containing channels influence ethanol's behavioral effects. Additionally, downstream intracellular effects of this interaction (i.e., changes in $G_{i/o}$ protein-lined effector systems) could be examined using RNAi-mediated *Kcnj9* knockdown *in vitro*.

General conclusions

The identification and examination of ethanol-related QTGs in mouse models is an important contributing factor to understanding the complex role of genetic contributions to alcohol dependence and withdrawal states in affected populations. The identification of *Mpdz* and *Kcnj9* as two high-quality QTGs underlying ethanol withdrawal severity, and the subsequent development of novel *Mpdz* and *Kcnj9* knockout mouse strains, has allowed for a more thorough examination of these genes' effects on ethanol withdrawal behaviors and receptor systems through which these genes may be exerting their withdrawal effects. In this dissertation, behavioral, pharmacological and molecular methods were utilized to more fully examine both *Mpdz* and *Kcnj9* in the context of ethanol withdrawal. The results obtained provide additional support for these genes' effects on withdrawal convulsion severity, although gene effects on other ethanol withdrawal behaviors were not consistently observed. Additionally, evidence for a functional interaction was observed between MPDZ and $5HT_{2C}$ receptors *in vitro* and *in vivo*, but this association did not influence ethanol withdrawal severity, at least in the models tested.

Although complex, the data collected in this dissertation provides one avenue by which researchers identifying novel QTGs for ethanol-related behaviors can begin to examine the QTG's interaction within the mammalian nervous system, and to identify the physiological mechanisms by which genes exert their phenotypic effects. Clearly, this process is not perfect, as the results collected in both the behavioral and pharmacological experiments were somewhat ambiguous, and a clear mechanism of effect for these genes was not fully identified. However, as the number of QTGs identified for ethanol-related traits will undoubtedly grow in the coming years, strategies to determine their effects must be undertaken in order to determine their relevance to

risk for development and potential treatment of alcohol use disorders in clinical populations.

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