A ROLE FOR MPDZ IN ETHANOL WITHDRAWAL BUT NOT

BINGE-LIKE ETHANOL DRINKING

By

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LIST OF ABBREVIATIONS

°C – Degrees Celsius

1B-DID – One-bottle drinking in the dark

2BC-CA – Two-bottle choice continuous access

2B-DID – Two-bottle drinking in the dark

2DG – ^{14}C-2-deoxyglucose

^{3}H – Tritiated hydrogen

5HT$_{2A,B,C}$R – Serotonin receptor subtypes 2A, 2B, and 2C

AAV – Adeno-associated virus

ACSF – Artificial cerebrospinal fluid

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA – Analysis of variance

AP – Anterior-posterior

AUC – Area under the curve

AUD – Alcohol use disorder

B6 – C57BL/6J inbred mouse strain

BAC – Bacterial artificial chromosome

BAPTA – 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid

BCA – Bicinchoninic acid

BEC – Blood ethanol concentration

BLAST – Basic Local Alignment Search Tool

B$_{max}$ – Maximum number of binding sites

BXD RI – Recombinant inbred strain derived from a B6 female and D2 male

CaCl$_2$ – Calcium chloride

Cadm1 – Cell adhesion molecule 1

CaMKII – Calcium/calmodulin-dependent protein kinase

Cas 9 – Clustered, regularly interspaced, short palindromic repeats associated protein

cDNA – Complementary deoxyribonucleic acid

clSNr – Caudolateral substantia nigra pars reticulata

cm – Centimeter

CNS – Central nervous system

CPP – Conditioned place preference

CRISPR – Clustered, regularly interspaced, short palindromic repeats

D2 – DBA/2J inbred mouse strain

D_3R – Dopamine D3 receptor

DID – Drinking in the dark

DSM-V – *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition*

DV – Dorsal ventral

ES – Embryonic stem cell

EWD – Ethanol withdrawal

fmol – Femtomole

FHP – Family History Positive

FHN – Family History Negative

FVB – Friend leukemia virus B

g – gram

GABA – γ-aminobutyric acid

$GABA_AR$ – γ-aminobutyric acid type A receptor

$GABA_BR$ – γ-aminobutyric acid type B receptor

Gabbr1 – encodes R1 subunit of the γ-aminobutyric acid type B receptor

Gabbr2 – encodes R2 subunit of the γ-aminobutyric acid type B receptor

GABBR1 – R1 subunit of the γ-aminobutyric acid type B receptor

GABBR2 – R2 subunit of the γ-aminobutyric acid type B receptor

GAP – GTP-ase activating proteins

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GAT – γ-aminobutyric transporter

GFP – Green fluorescent protein

GIRK – G-protein inwardly rectifying potassium channel

GIRK1 – G-protein inwardly rectifying potassium channel type 1

GIRK2 – G-protein inwardly rectifying potassium channel type 2

GIRK2c – G-protein inwardly rectifying potassium channel type 2, isoform c

GIRK3 – G-protein inwardly rectifying potassium channel type 3

GlyT – Glycine transporter

g/kg – Gram per kilogram

G-protein – Guanine nucleotide-binding protein

HEPES – N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

h – Hour

Het – Heterozygote

HIC – Handling-induced convulsion

Hom – Homozygote

HPC – Hippocampus

HS – Heterogeneous stock

HS4 – Heterogeneous stock derived from four founder strains

Hz – Hertz

i.p. – Intraperitoneal

ITF – Immediate transcription factor

Kb – Kilobase

KCl – Potassium chloride

K_d – ligand concentration that binds to half the receptor sites (affinity)

kD - Kilodalton

Kcnj3 – Encodes G-protein inwardly rectifying potassium channel type 1

Kcnj6 – Encodes G-protein inwardly rectifying potassium channel type 2

Kcnj9 – Encodes G-protein inwardly rectifying potassium channel type 3

kg – Kilogram

Hz – Kilohertz

LCMD – Laser capture microdissection

$M\Omega$ – Megaohm

MT1R – Melatonin Receptor Type 1

mg – milligram

$MgCl_2$ – Magnesium chloride

min – Minute

ML – Medial-lateral

ml – milliliter

ml/kg – milliliter per kilogram

mm – millimeter

mM – millimolar

Mpdz$^{+/-}$ – *Mpdz* knockout heterozygote

Mpdz^{Tg} – *Mpdz* transgenic

mRNA – Messenger ribonucleic acid

MUPP1 – Multiple Post-synaptic density-95, Discs Large, and Zona Occludens protein 1

mV – Millivolts

nA – nanoamplitude

NaCl – Sodium chloride

$NaHCO_3$ – Sodium bicarbonate

NaH_2PO_4 – Monosodium phosphate

NG2 – Neural/glial antigen 2

nM – Nanomolar

NMDAR – N-methyl-D-aspartate receptors

NR2B – N-methyl-D-aspartate receptors subtype 2B

NS20Y – Neuroblastoma cell line

OR2AG1 – Olfactory receptor family 2, subfamily AG, member 1

PDZ – Postsynaptic density-95, Discs Large, and Zona Occludens

PTZ – Pentylenetetrazol

QPCR – Quantitative polymerase chain reaction

QTG – Quantitative trait gene

QTL – Quantitative trait locus

RI – Recombinant inbred

RNA – Ribonucleic acid

RNAi – Ribonucleic acid interference

SC – Scrambled Control

sec – Seconds

SEM – Standard error of the mean

SERT – Serotonin transporter

shRNA – Short-hairpin ribonucleic acid

sIPSC – Spontaneous inhibitory post-synaptic current

SN – Subtantia nigra

SNc – Substantia nigra pars compacta

SNr – Substantia nigra pars reticulata

SynGAP – Synaptic Ras GTPase-activating protein

Tris-HCl – Tris hydrochloride

TU – Titer units

µl – microliter

µm – Micrometers

µM – micromolar

v/v – Volume per volume

v/w – Volume per weight

WT – Wild-type

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ABSTRACT

Alcoholism is a debilitating disease, influenced by both biological (genetic) and environment factors. Unfortunately, the genetic determinants of risk remain largely unknown, hindering effective prevention and treatment of dependent individuals. Recent quantitative trait mapping studies have identified the *Mpdz* gene, which encodes the multiple Post-synaptic density-95, Discs Large, Zona Occludens protein 1 MUPP1, in predisposition to ethanol withdrawal (EWD) in mice. The aim of my dissertation was to assess the caudolateral substantia nigra pars reticulata (clSNr) as a region through which *Mpdz* affects EWD, and assess the potential role of the *Mpdz* gene in predisposition to binge-like ethanol drinking. Further, the aim of my dissertation was to assess the effect of MUPP1 on γ-aminobutyric acid type B receptor ($GABA_BR$) mediated responses, as an important step towards identifying how this gene may influence EWD and potentially binge-like ethanol drinking.

Although no animal model exactly duplicates clinically defined alcoholism, models for specific traits, such as risk for excessive intake and withdrawal, have proven beneficial for identifying potential genetic determinants of liability in humans. The handling induced convulsion (HIC) scale is a sensitive and quantitative measure of central nervous system (CNS) hyperexcitability, that can be used to assess EWD severity, and the drinking in the dark (DID) procedure is a validated model of binge-like ethanol drinking in mice. I implemented these procedures to assess two alcohol phenotypes that may be associated with the negative and positive reinforcement, respectively. Further, I used *Mpdz* targeted RNA interference (RNAi), as well as *Mpdz* heterozygote knockout ($Mpdz^{+/-}$) and *Mpdz* transgenic ($Mpdz^{Tg}$) mice to begin to elucidate the role of *Mpdz*/MUPP1 in influencing risk for EWD and binge-like ethanol drinking.

In Chapter 1, I introduce research identifying the *Mpdz* gene as a quantitative trait gene (QTG) in predisposition to EWD, with reduced expression predicted to be associated with an enhancement in EWD severity. Further, I introduce evidence supporting the clSNr as a region that may mediate the effects of *Mpdz* on EWD. Finally, I introduce evidence supporting an effect of MUPP1 on $GABA_BR$ function, and evidence for a role of both of these proteins in EWD and ethanol drinking phenotypes.

In Chapter 2, I describe how I used the method of RNAi to assess the role of *Mpdz* expression in the clSNr on predisposition to EWD. I found that reduced expression of *Mpdz* in the clSNr was associated with a significant enhancement in EWD severity, with no changes in baseline HICs or pentylenetetrazol-enhanced HICs. Consistent with previous work, these data provide further confirmation of *Mpdz* as a QTG for EWD, and demonstrated that its expression in the clSNr is involved.

In Chapter 3, I describe how I used behavioral, pharmacological, and molecular techniques to assess whether clSNr $GABA_BRs$ mediate EWD severity, and whether expression of *Mpdz*/MUPP1 was associated with an enhancement in $GABA_BR$-mediated responses both behaviorally and neurophysiologically. I demonstrated that activation of clSNr $GABA_BRs$ enhanced HICs and inhibition of these receptors during EWD significantly reduced EWD severity. This represents the first evidence that $GABA_BRs$ within the clSNr are involved in EWD, suggesting that $GABA_BRs$ in this region may play a role in mediating CNS hyperexcitability. Further, using novel $Mpdz^{+/-}$ and $Mpdz^{Tg}$ genetic models, I demonstrated an association between *Mpdz*/MUPP1 expression and $GABA_BR$-mediated HICs, with reduced expression associated with greater HICs and vice versa. Further, I demonstrated a greater enhancement in SNr $GABA_BR$ response to baclofen through G-protein inwardly rectifying potassium (GIRK) channels in $Mpdz^{+/-}$ compared to wild-type (WT) littermates. Using radioligand saturation binding and gene

expression analyses, no differences in $GABA_B R$ density, binding affinity, or expression in whole brain or the clSNr of $Mpdz^{+/-}$ and WT littermates were detected.

In Chapter 4, I describe how I used three variations of the DID procedure to assess the potential effect of *Mpdz* on predisposition to binge-like ethanol intake. No association between reduced *Mpdz*/MUPP1 expression and binge-like ethanol intake or ethanol preference was detected. Further, reduced *Mpdz*/MUPP1 expression was not associated with a difference in the effectiveness of baclofen to reduce binge-like ethanol drinking.

In Chapter 5, I discuss the interpretation of my findings, the relationship to the broader literature, and future directions of this research. Together, these experiments provided additional confirmation for a role of *Mpdz* in predisposition to EWD and demonstrated the clSNr to be involved. My data also demonstrated an association between reduced *Mpdz*/MUPP1 expression and heightened $GABA_B R$-mediated responses, but were unable to detect an association between *Mpdz*/MUPP1 expression and binge-like ethanol drinking. The results of this research may inform future studies of genetic susceptibility to alcohol use disorders in human clinical populations and response to pharmacotherapeutic treatments for dependent individuals.

Chapter 1: Introduction

This chapter is adapted from a book chapter that has been accepted for publication: Kruse, LC & Buck, KJ (expected publication date: June 2016). The role of the substantia nigra pars reticulata in sedative-hypnotic withdrawal. In V. Preedy (Ed.), *Neuropathology of Drug Addiction and Substance Misuse*. Oxford, United Kingdom: Elsevier.

General Introduction

Alcohol, a sedative-hypnotic drug, is widely used and abused for its euphoric and sedative properties. Alcohol use disorders (AUDs) are a debilitating affliction with tremendous health and monetary costs for the individual and society. In 2012, 5.1% of the global burden of disease was attributable to alcohol use, and about 3.3 million deaths were attributable to harmful alcohol use (WHO, 2014). In the United States alone, 65% of adults reported alcohol use on a yearly basis, with 8.5% characterized as having an AUD (Falk et al., 2008).

AUDs are heterogeneous, complex disorders, influenced by a host of biological (genetic) and environmental factors. Genetically determined risk for alcohol addiction has been demonstrated in humans in which twin, family, and adoption studies identify a significant genetic component, with alcoholism being 50-65% heritable (Ducci and Goldman, 2008; Palmer et al., 2012; Reich et al., 1999). Unfortunately, owing to its complexity, the genetic determinants of risk for AUDs remain largely unknown, hindering its prevention and the treatment of dependent individuals.

Alcohol is classified as a central nervous system (CNS) depressant and sedative-hypnotic that dysregulates inhibitory and excitatory neurotransmission by acting on γ-

aminobutyric acid (GABA) and glutamate receptors (Harris et al., 2008), respectively. Briefly, with short-term alcohol exposure, alcohol alters the release of neurotransmitters and may disrupt function of proteins such as receptors. Following repeated/chronic alcohol exposure, neuronal adaptations occur in an attempt to attenuate the inhibitory actions of alcohol and alcohol is thus required to maintain normal brain functioning (Finn and Crabbe, 1997; Valenzuela, 1997). When alcohol use is discontinued (withdrawn), the neuronal adaptations are unopposed and a rebound, stimulatory state occurs resulting in hyperexcitability of the CNS. This abnormal state can manifest as a variety of withdrawal signs, which define a preexisting state of physical dependence on alcohol (Bayard et al., 2004; Goldstein and Pal, 1971; Majchrowicz, 1975; Manasco et al., 2012; Schuckit et al., 1995). Physical dependence and associated withdrawal are believed to be powerful motivational factors that perpetuate alcohol use and abuse, and enhance vulnerability for relapse (Koob and LeMoal, 2001).

A hypothesis has been presented incorporating the criterion for clinical diagnosis of a substance use disorder to describe the cyclical nature of addiction and the underlying motivational mechanisms. The proposed model suggests that continued use and abuse of a substance, such as alcohol, may be perpetuated by three main elements including binge intoxication, withdrawal/negative affect, and preoccupation/anticipation with the substance (Koob and Le Moal, 2001; Koob and Volkow, 2010). This model is not static, as there is a progression overtime leading from initial drug use to addiction. In general, there is gradual shift from social to compulsive/uncontrollable use associated with the development of craving, tolerance, and withdrawal, as well as relapse following attempts to abstain, factors that are influenced by both positive and negative reinforcement. Withdrawal from alcohol is thought to be largely associated with negative reinforcement that can be defined as an increase in behavior in order to avoid aversive

stimuli (i.e., increased drug taking for alleviation of a drug-generated aversive state). Excessive/binge consumption of alcohol is thought to be largely associated with positive reinforcement and can be defined as an increase in a behavior in response to rewarding stimuli (i.e., increased drug taking in response to the drug's rewarding effects). My dissertation focused on withdrawal, binge intoxication, and the influence of genetic predisposing factors, to begin to understand the genetic and neurobiological bases influencing risk for AUDs.

Alcohol withdrawal in humans

Physical dependence can be defined as the manifestation of withdrawal symptoms after exposure to alcohol is suspended. In humans, the initial symptoms of withdrawal, which can be categorized as the acute/initial abstinence phase of the withdrawal syndrome, may be evident as early as three to six hours (h) following cessation of intake, and reflect a generalized state of increased CNS hyperexcitability associated with mild tremors, as well as the presence of headaches, nausea, anxiety, and insomnia, which typically abate within one to three days (Heilig et al., 2010). Withdrawal seizures may also occur in a small percentage of the patient population (approximately 5-10%) (Schuckit et al., 1995). In three to four days, enhanced autonomic nervous system activation resulting in increased sweating, blood pressure, body temperature, pulse, and heartbeat may also be apparent in a subset of dependent individuals (Finn and Crabbe, 1997; Schuckit et al., 1995). During this time more severe tremors (e.g. delirium tremens) may also occur. These symptoms typically abate within five days of alcohol cessation (Becker, 2008). Three to six weeks following cessation of intake, a subset of physically dependent individuals may enter the early abstinence phase where insomnia, anxiety, and depressed mood are present, but in the absence of

the above-mentioned symptoms of the acute phase (Heilig et al., 2010). Finally, the protracted abstinence phase may last for months and is characterized by anhedonia and anxiety as evidenced by a shift in affective processing during this period. Specifically, seemingly insignificant challenges can trigger negative affect and craving, and a lack of response to normally pleasurable events (Heilig et al., 2010). The severity of withdrawal symptoms experienced range from mild symptoms (e.g., following a single heavy binge episode) to more severe symptoms (e.g. following chronic alcohol intake) (Schuckit et al., 1995).

The incidence and severity of symptoms experienced during withdrawal are highly variable from person to person, and are a function of many factors including amount and pattern of alcohol use, pharmacokinetics, comorbid disease states, use of other drugs, age, and sex (Saitz, 1998; Schuckit et al., 1995). Further, these factors are also influenced by genetics. The elucidation of how these factors and the underlying mechanisms contribute to risk for dependence is important for the development of preventative and therapeutic treatments for AUDs.

Alcohol withdrawal in mice

No animal model can exactly duplicate clinically defined alcohol (ethanol) dependence, but robust behavioral models for specific traits, such as excessive intake and withdrawal, have proven beneficial for identifying potential genetic and neural determinants of risk in humans (Barkley-Levenson and Crabbe, 2012; Ehlers et al., 2010). Physiological dependence can be operationally defined as the manifestation of physical disturbances (withdrawal) after alcohol administration is suspended. Numerous models have been developed and widely used for the assessment of ethanol withdrawal (EWD) symptoms during all three phases of the clinical withdrawal process, including

increased CNS excitability, anxiety- and depression-like behaviors, anhedonia, hypothermia, gait and locomotor abnormalities, increased autonomic nervous system activation, tremors, and convulsions (Becker, 2008; Crabbe et al., 2012a; Crandall et al., 1989; Goldstein and Pal, 1971). Behaviors in animal models associated with negative affect (anxiety-like, depression-like, anhedonia) are more difficult to interpret, as affective states in animals may not directly model the human states and underlying motivations (Heilig et al., 2010). In addition, increased autonomic nervous system associated behaviors (increased blood pressure and heart rate) may not provide substantial information on the CNS mechanisms which underlie withdrawal. Therefore, the assessment of convulsions (i.e., CNS hyperexcitability), which are easily identifiable, highly quantitative, and occur across species (Goldstein and Pal, 1971; McCulley et al., 2012; Schuckit et al., 1995), is a useful measure of EWD in mice (Buck et al., 1997; Metten and Crabbe, 1994; Metten and Crabbe, 2005).

During the first few days of alcohol withdrawal (i.e., acute/initial abstinence phase), seizure thresholds are significantly lowered in humans or animal models (including mice). In mice, EWD convulsions can be spontaneous, although this is rare, but are more commonly experimenter-induced, and are a measure of the behavioral manifestation of heightened CNS excitability (e.g., hyperexcitability), a hallmark state of the initial abstinence phase of the alcohol withdrawal syndrome (Heilig et al., 2010; Kathmann et al., 1996). Common experimenter-induced methods in animals include handling induced convulsions (HICs), exposure to sensory stimuli (e.g., audiogenic stimulus), mild electric shock, and chemiconvulsant exposure (e.g., exposure to drugs that enhance CNS excitability), which can all be applied to measure reduced seizure threshold during withdrawal (Becker, 2008; Colombo et al., 2000; Goldstein and Pal, 1971; Kosobud and Crabbe, 1990). The HIC scale, first quantified and defined by Dr.

Dora Goldstein in the 1970s, has been most commonly used as it is a technically simple procedure, and highly correlated with additional facets of EWD including lethargy, tremor, tail lift, startle to noise, spontaneous convulsions, and death (Goldstein and Pal, 1971).

The HIC scale is a sensitive and quantitative measure of CNS hyperexcitability that has been highly validated for assessing EWD severity and genetic determinants in mice (Buck et al., 1997; Goldstein and Kakihana, 1975; Metten and Crabbe, 1994; Metten and Crabbe, 2005). The seven-point HIC scale (Table 1-1) is used to quantify the severity of clonic and tonic-clonic convulsions based on degree of experimenter manipulation. A detailed description of the values assigned to clonic and tonic-clonic convulsions can be found in Table 1-1 (Chen et al., 2011). Goldstein (1972) demonstrated that the HIC scale is dose-dependent, as intensity of withdrawal is associated with length of exposure to ethanol and concentration of ethanol in the blood. Building upon previous findings that withdrawal convulsions are apparent following a single (acute) administration (McQuarrie and Fingl, 1958), Goldstein also demonstrated that withdrawal convulsions are even evident following a single dose of ethanol (5 grams per kilogram [g/kg], intraperitoneal injection [i.p.]). Therefore, both acute and chronic withdrawal in mice can be assessed using the HIC scale.

A commonly used acute model of withdrawal assesses withdrawal following a single hypnotic dose of ethanol (4 g/kg, i.p.) (Buck et al., 1997; Chen et al., 2008; Kruse et al., 2014; Metten and Crabbe, 1994; Milner et al., 2015), and will be referred to as acute EWD throughout my dissertation. In chronic models, withdrawal is assessed after exposure to ethanol vapor for extended periods or intervals of time (Terdal and Crabbe, 1994), or prolonged exposure to a liquid diet containing differing concentrations of ethanol (Mead and Little, 1995). The acute model is advantageous in that the potential

issues of metabolic tolerance and prolonged stress induced by long-term, forced exposure, are mitigated, allowing focus on CNS mechanisms contributing to withdrawal, rather than metabolism or stress (Keith and Crabbe, 1992). Further, the acute model allows the assessment of genetic and neurobiological mechanisms that contribute to initial susceptibility to withdrawal prior to chronic ethanol exposure, identifying a baseline state that may influence risk for an AUD. Chronic models offer the advantage that withdrawal is more robust and may better reflect genetic/neuroadaptations that occur with long-term exposure to ethanol (Daniels & Buck, 2002).

Further, previous work has shown that genetic differences in acute withdrawal liability may be related to high or low risk for onset of an AUD. A longitudinal study of alcoholism in Family History Positive (FHP) and Family History Negative (FHN) males, showed a greater percentage of an AUD in FHP (28.6%) compared to FHN (10.8%) (Schuckit and Smith, 1996), and that lower response to alcohol on the descending limb of the blood alcohol concentration curve in FHP males was a predictor of future development of alcoholism (Newlin and Thomson, 1990; Schuckit and Smith, 1996). Interestingly, a study looking at withdrawal reactions to an acute dose of alcohol demonstrated that FHP males reported greater withdrawal symptoms (e.g., sweatiness, tachycardia, and shakiness) 3 – 18 h following ingestion of approximately 1 g/kg of alcohol, compared to FHN males (McCaul et al., 1991), and additional studies showed that sons of alcoholics also reported greater hangover symptoms compared to sons of nonalcoholics, with comparable reports of frequency/quantity of drinking (Newlin and Pretorius, 1990; Span and Earleywine, 1999). However, a longitudinal study assessing the development of an AUD in the same individuals assessed for acute withdrawal response is warranted to confirm this measurement as a predictor of development of an AUD. Caveats aside, the acute withdrawal model in mice may therefore be used to

elucidate the factors, such as genetics, that influence acute response to alcohol, which could be informative for high or low risk for future onset of an AUD.

Handling-induced convulsion (HIC) rating scale.

Symptom	Score
No convulsion or facial grimace after gentle 180° spin	0
A facial grimace is seen after gentle 180° spin	1
No convulsion when lifted by the tail, but a tonic convulsion is elicited by a gentle 180° spin	2
Tonic-clonic convulsion after a gentle 180° spin	3
Tonic convulsion upon lifting by the tail	4
Tonic-clonic convulsion when lifted by the tail, often with the onset delayed up to 1-2s	5
Severe, tonic-clonic convulsion when lifted by the tail, with a quick onset and long duration, often continuing for several seconds after the mouse is released	6
Severe, tonic-clonic convulsion elicited prior to lifting by the tail, with a quick onset and long duration	7

Table 1-1. HIC scale. The HIC scale is a seven-point scale used to measure CNS excitability, and has been validated as a sensitive and quantitative measure of chemiconvulsant and EWD induced hyperexcitability in mice. This scale measures experimenter-induced tonic and tonic-clonic convulsions which are produced by lifting a mouse by the tail, and gently spinning the mouse in a 180° arc to elicit a convulsion. Tonic convulsions manifest as muscle stiffness and rigidity, whereas clonic convulsions are characterized by muscle spasms and jerks (Chen et al., 2011).

Mapping genetic determinants of risk

As mentioned previously, risk for an AUD is influenced by genetic factors, with heritability estimated at approximately 50-65% (Ducci and Goldman, 2008). Mouse models have proven valuable for studying genetic contributions to complex behaviors such as AUDs, and particularly in mapping studies for the identification of chromosomal intervals or specific genes associated with ethanol-related phenotypes. Human studies have largely focused on the diagnosis and potential endophenotypes for AUDs (e.g., metabolism and brain waves) (Hines et al., 2005), with less known about genetic vulnerability to withdrawal possibly due to the complexity of the withdrawal syndrome. For this reason and others, robust animal models of withdrawal have been useful for the identification of genes that contribute to withdrawal risk. The significance of identifying genes involved in quantitative traits such as EWD in animal models lies in the conservation of genetic linkage to alcohol dependence phenotypes found to be syntenic in the human and mouse genomes. A quantitative trait is one that has measureable phenotypic variation within a population owing to underlying variability in genetic and/or environmental influences. One approach used to identify genes with significant effects on a phenotype (or quantitative trait) of interest is QTL mapping (Milner and Buck, 2010; Plomin and McClearn, 1993). A QTL can be defined as a chromosomal region (genetic locus) in which allelic variation differentially influences the observed phenotype. QTL mapping involves the identification of chromosomal regions where a marker allele(s) and phenotypic trait covary, and has been used to identify loci associated with predisposition to EWD in mice (Buck et al., 1997; Fehr et al., 2002; Shirley et al., 2004). Different mouse models commonly employed to study genetic contributions to ethanol-related phenotypes, as well as in QTL mapping studies, are described below.

Genetic mouse models

Genetic mouse models are useful tools for studying genetics because there is naturally occurring genetic variation among inbred strains (see below) facilitating the study of complex traits, tools for the manipulation of the mouse genome are readily available (e.g. ribonucleic acid interference [RNAi], gene knockout, gene overexpression), and short- or long-term selective breeding (see below) for behavioral traits can produce high risk and divergent genotypes for alcohol phenotypes of interest (Belknap et al., 1997; Buck et al., 1997; Crabbe et al., 2009; Hitzemann et al., 2009).

Inbred mice, F2 hybrids, and BXD RI strains. Inbred mice are genetically identical due to >20 generations of brother x sister mating (Silver, 1995), and hundreds of strains with unique genomes exist. Two of the most commonly used and highly characterized inbred mouse strains in alcohol research are DBA/2J (D2) and C57BL/6J (B6) mice. The D2 strain exhibits severe alcohol withdrawal convulsions (using both acute and chronic models), and low oral ethanol self-administration, whereas the B6 strain exhibits mild withdrawal convulsions and high (relatively) oral ethanol self-administration (Metten and Crabbe, 1994; Metten and Crabbe, 2005; Yoneyama et al., 2008). Inbred mice are advantageous in that they are genetically identical within strains so variation in a phenotype within a strain can be expected to be due to environmental variation and/or experimenter manipulation. Further, panels of inbred strains can be used to assess genetic correlations between phenotypes of interest (Metten and Crabbe, 1994; Metten and Crabbe, 2005). The D2 and B6 strains and populations derived from these two progenitors including F2 hybrids, recombinant inbred (RI) strains derived from a B6 and D2 (BXD RI), and lines selectively bred for EWD severity, have been useful towards the identification of genetic determinants of risk for EWD in quantitative trait locus (QTL, see below) mapping studies (Buck et al., 1997; Crabbe, 1998; Metten et al.,

2014). Briefly, F2 mice are generated by crossing B6 and D2 producing F1 offspring that are genetically uniform (Silver, 1995). Subsequent intercrossing of F1 mice produces F2 offspring that are genetically unique, containing a mix of each parental strain but in random configuration (Silver, 1995). BXD RI strains are generated by intercrossing F1 offspring followed by brother × sister mating for ≥20 generations to produce inbred mice with an essentially permanent set of recombination events, and three to four times more recombination events leading to increased genetic variability compared to F2 hybrid mice (Taylor, 1978; Silver, 1995). In total, including the recently developed expanded panel of BXD RI strains (Peirce et al., 2004), there are presently 76 BXD RI strains. BXD RIs are advantageous because genetic maps exist for most RI strain sets and data acquired are cumulative and can therefore be compared across laboratories and time. Overall, F2 mice and BXD RI strains provide additional genetic power, which has proven useful for co-varying genotype with phenotype in QTL mapping studies (Buck et al., 1997).

   Selectively bred mice. If a phenotypic trait is heritable, mice can be selectively bred for that trait. In general, beginning with a foundation population (such as F2 hybrids or genetically heterogeneous [HS] stock mice), mice are assessed for a phenotype of interest. For bidirectional selective breeding, the parental mice with the highest scores on the trait of interest serve as breeders for the "high" line, and mice with the lowest scores serve as breeders for the "low" line. Offspring are then assessed for the phenotype of interest in each subsequent generation, and the highest of the high line and lowest of the low line may be chosen to perpetuate the lines. Short- or long-term selective breeding is advantageous in that it can produce high risk and divergent genotypes for alcohol phenotypes of interest (e.g., acute or chronic EWD, ethanol consumption, or blood ethanol levels associated with consumption), correlated

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responses can be assessed, and heritability of the phenotype can be estimated (Belknap et al., 1997; Buck et al., 1997; Crabbe et al., 2009; Hitzemann et al., 2009).

Congenic mice. Congenic mice have also proven to be useful tools in QTL mapping. In general, congenic mice are typically created from a cross between a recipient inbred partner and a partner (commonly inbred but does not have to be) carrying the donor allele (i.e., allele of interest) (Silver, 1995). In each subsequent generation, offspring that receive the donor allele are backcrossed to the desired inbred strain mice, and by the tenth generation the congenic should be >99% identical to the background inbred strain and contain a chromosomal interval introgressed from the donor (Silver, 1995). Phenotypic differences between the congenic and background strain mice are expected to be influenced by genes contained within the introgressed interval. If the introgressed interval contains a gene or gene(s) that influence the phenotype being measured, it may be referred to as a QTL. However, this introgressed interval is typically large (10-35 Centimorgans or more) and may therefore contain hundreds of genes (Nadeau and Frankel, 2000). Interval-specific congenic strains (ISCS) are useful to further refine the QTL of interest, and are developed by backcrossing a conventional congenic strain to background strain mice. Offspring are then backcrossed to create mice that contain regions of progenitor allelic recombination within the starting congenic interval. These recombinant mice can then be backcrossed to produce multiple offspring with the same introgressed interval and are referred to as an interval-specific congenic line (ISCL). Intercrossing the ISCL creates an ISCS (Milner and Buck, 2010). In mapping studies, conventional congenic mice, ISCSs, and ISCLs can be used to isolate QTLs associated with a specific phenotype (discussed further below) (Fehr et al., 2002; Palmer et al., 2006; Shirley et al., 2004).

Knockout mice. Knockout and transgenic (see below) mice are commonly used to study target gene effects on a behavior. Further, following identification of a quantitative trait gene in mapping studies (QTG; see below), knockout and transgenic mouse models are commonly used to confirm the QTG. Knockout mice can be created through the mutation of a gene of interest through homologous recombination often in embryonic stem (ES) cells producing inactivation of the gene. The ES cell is then injected into a donor blastocyst, which is implanted into a pseudo-pregnant female of the recipient inbred strain that may or may not differ from the donor inbred strain, producing chimeric mice. To generate an inbred genetic background, offspring with germline transmission of the gene mutation are subsequently backcrossed until the genetic background is >98% of the inbred strain of interest, producing mice with complete (homozygous) or partial (heterozygous) knockout of the gene of interest (Hall et al., 2009). Conventional knockout models must be interpreted with caution however, as developmental compensation of the target gene or other gene products may occur. Further, passenger genes from the embryonic stem cell flanking the gene of interest may be present or the transgene may insert at any location causing disruption, and the resultant phenotype produced may not be related to the targeted gene, but another, unknown gene (Haruyama et al., 2009; Hummler et al., 1994; Picciotto and Wickman, 1998). Beyond conventional knockouts, a novel gene mutagenesis technique using the bacterial CRISPR (clustered, regularly interspace, short palindromic repeats)/Cas 9 (CRISPR associated protein) system has been developed as a simple and efficient method for generating knockouts and sequence specific knockouts. Briefly, CRISPR RNA-guided Cas9 nucleases use small base-pairing guide RNAs to target and cleave DNA in a sequence-specific manner and can be directly injected into zygotes (Wang et al., 2013). It is therefore advantageous over conventional knockout models in that it is

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more efficient, less costly, easier to generate complex genetic knockouts (i.e., multiple genes), and the complications with flanking genes from the ES cells are avoided.

Transgenic mice. In contrast to knockout mice, transgenic mice overexpress a particular gene of interest. One method for creating transgenic mice involves injecting a bacterial artificial chromosome containing the full-length gene of interest into a zygote to produce founder mice containing the transgene of interest. Ideally, two to three founder (F0) mice are then crossed with mice of the same background strain to create F1 mice to assess potential germline transmission of the gene. More than one founder mouse is ideally used in case germline transmission does not occur in one of the founders. To produce a transgenic with an inbred background, the founder can be backcrossed until the genetic background is >98% of the inbred strain of interest to generate both homozygote and heterozygote mice overexpressing the gene (Haruyama et al., 2009). Similar precautions must be taken with transgenic models as passenger genes may occur, or disruption of normal gene sequence(s) due to integration of the transgene could influence the phenotype of interest (Haruyama et al., 2009; Picciotto and Wickman, 1998).

Identification of Mpdz as a QTG

Using the informative mapping populations derived from B6 and D2 progenitor strains discussed above, in addition to lines selectively bred for high and low withdrawal, highly significant QTLs (all $p < 10^{-5}$) for predisposition to both acute and chronic EWD have been identified on mouse chromosomes 1, 4, 11, and 19 (Buck et al., 1997; Buck et al., 2002; Fehr et al., 2002; Kozell et al., 2009). A QTL on chromosome 4 is the focus of the work in my dissertation. Following the identification of a QTL on chromosome 4, a conventional congenic strain, ISCLs (heterozygotes), and ISCSs (homozygotes) were

useful in further narrowing down the QTL interval of interest, in order to begin to assess its potential broader roles and progress toward identification of the underlying QTGs (see below; Fehr et al., 2002; Shirley et al., 2004; Milner and Buck, 2010). Briefly, the conventional congenic model possessed a donor region from the B6 strain that spanned a highly significant QTL on chromosome 4 introgressed onto a D2 genetic background, and confirmed "capture" of an acute EWD QTL, with congenic mice demonstrating significantly more severe withdrawal compared to D2 background strain mice (Fehr et al., 2002). This conventional congenic was used as the point of departure to develop a series of ISCLs and ISCSs used to further isolate the chromosome 4 QTL (Fehr et al., 2002; Shirley et al., 2004).

QTL fine-mapping has been key to progress toward identification of one or more underlying QTGs within a QTL interval (Fehr et al., 2002; Shirley et al., 2004). Thus, to more finely map the chromosome 4 QTL, a panel of QTL ISCSs were created, and used successfully to finely map this acute EWD QTL to a small (1.8 megabase) interval. Detailed molecular analyses of the resident genes within the maximal 1.8 megabase interval identified a single QTG, *Mpdz* (Shirley et al., 2004). *Mpdz* encodes the multiple Post-synaptic density-95, Discs Large, Zona Occludens protein 1 MUPP1 (Simpson et al., 1999). This and other members of the PDZ domain protein family are scaffolding proteins thought to alter the rate and fidelity of the signal transduction pathways of the receptors and proteins with which they associate (Sheng and Sala, 2001). Allelic variation in *Mpdz* expression (Shirley et al., 2004) and coding sequence (Fehr et al., 2002) are significantly genetically correlated with EWD severity, either one or both of which may contribute to its effects on withdrawal.

In order to further assess *Mpdz* as a QTG for withdrawal, targeted *Mpdz*/MUPP1 genetic models were created, to confirm this finding. An *Mpdz* knockout heterozygote

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($Mpdz^{+/-}$) model was created on an inbred B6 background in which $Mpdz$ expression is significantly reduced by approximately half that in $Mpdz^{+/-}$ compared to WT littermates (Milner et al., 2015). In addition, two independent $Mpdz$ transgenic ($Mpdz^{Tg}$) mouse strains have been created on an inbred D2 background, in which $Mpdz$ expression is significantly increased by approximately 3-fold compared to nontransgenic (WT) littermates (Milner et al., 2015). Descriptions of the creation of the $Mpdz$ heterozygote knockout and transgenic models are detailed in Chapter 3 and have been published (Milner et al., 2015). As expected, $Mpdz^{+/-}$ and $Mpdz^{Tg}$ mice exhibited significantly more severe and less severe EWD HICs, respectively, compared to appropriate WT littermates, respectively (Milner et al., 2015). These data, in addition to inbred and congenic strain data, supported the hypothesis of an inverse relationship between $Mpdz$/MUPP1 expression and severity of EWD, and implicated $Mpdz$ in withdrawal risk.

A known limitation of using conventional knockout and transgenic models is the possibility that developmental compensation could occur, or that flanking genes rather than the target gene effects *per se* influence the phenotype of interest, limiting interpretation of the results. Fortunately, recent advances in molecular biology and neuroscience have developed the approach of RNAi, which allows the precise targeting and selective reduction of a target gene in a discrete anatomical location (Bahi and Dreyer, 2012). RNAi has therefore emerged as an effective technique for disentangling target gene effects from these potential confounds (see Chapter 2). Therefore, studies utilizing approaches to disentangle potential compensatory effects from target gene effects are important to rigorously confirm that $Mpdz$ is a QTG for predisposition to acute EWD.

PDZ domain proteins

PDZ domains, like those in MUPP1, are protein interaction domains that bind specific sequences located in the carboxyl (C)-terminus of target proteins. Three classes of PDZ domains associated with different C-terminus sequences exist (Sheng and Sala, 2001). In general, Class I PDZ domains recognize sequences S/T-X-V and S/T-X-L, Class II recognize ϕ-X-ϕ, and Class III recognize D-X-V, where amino acid residues are X (unspecified), S (serine), T (threonine), V (valine), L (leucine), ϕ (hydrophobic), and D (aspartic acid). However, specificity for binding of the PDZ domains to proteins is thought to involve more than just these residues. Proteins that contain PDZ domains are thought to function to alter CNS cell signaling through receptor/protein sorting, clustering, trafficking, stabilization, localization, and/or the formation of macromolecular complexes (Balasubramanian et al., 2007; Becamel et al., 2001; Guillaume et al., 2008; Krapivinsky et al., 2004; Rama et al., 2008; Sheng and Sala, 2001; Sitek et al., 2003). In addition to MUPP1, other PDZ-domain containing proteins such as PSD-95 and Homer, have been implicated in a range of ethanol behaviors including, ethanol consumption and preference, ethanol conditioned place preference, ethanol-induced sedation, ethanol-induced locomotor sensitization, and ethanol-induced neuroplasticity (Camp et al., 2011; Cozzoli et al., 2014; Szumlinksi et al., 2005).

MUPP1

MUPP1 is a unique PDZ domain-containing scaffolding protein that is expressed widely throughout the brain (Sitek et al., 2003). It includes 13 distinct PDZ binding domains (including Class I, II, and III domains), and associates with a highly diverse array of neuronal proteins and receptors (Figure 1-1) (Balasubramanian et al., 2007;

Baliova et al., 2014; Dooley et al., 2009; Fujita et al., 2012; Krapivinsky et al., 2004; Li et al., 2012; Sitek et al., 2003; Ullmer et al., 1998). In addition to its expression in the brain, MUPP1 is also expressed at tight junctions in epithelial cells and peripheral Schwann cells, kidney cells, and spermatozoa (Poliak et al., 2002; Sindic et al., 2009). Interestingly, MUPP1 contains no known catalytic domain in its protein sequence, and is therefore thought to function through the receptors and other proteins with which it complexes (Ullmer et al., 1998).

MUPP1 was initially cloned in a yeast two-hybrid screen with a C-terminus peptide of the serotonin-2C receptor (5-HT$_{2C}$R) as the bait (Ullmer et al., 1998). Subsequent studies have identified numerous additional interaction partners, including several thought to be involved in the actions of alcohol (Figure 1-1). Within the CNS, MUPP1 has been shown to associate directly with and/or functionally modulate diverse proteins including guanine nucleotide binding protein (G-protein) coupled receptors (Balasubramanian et al., 2007; Dooley et al., 2009; Griffon et al., 2003; Guillaume et al., 2008; Ullmer et al., 1998), ionotropic receptors (Krapivinsky et al., 2004), neurotransmitter transporters (Baliova et al., 2014), cell adhesion molecules (Cadm) (Fujita et al., 2012), calcium-calmodulin dependent protein kinases (CaMK) (Krapivinsky et al., 2004), GTP-ase activating proteins (GAP) (Krapivinsky et al., 2004), and gap junction proteins (Li et al., 2012).

MUPP1 may affect excitatory and/or inhibitory signaling by regulating the formation and function of macromolecular complexes. For instance, MUPP1 physically associates with synaptic GAP (SynGAP) and CaMKII to form a complex with the 2B subunit (NR2B) containing N-methyl-D-aspartate receptors (NMDAR), at excitatory synapses. Selective disruption of the SynGAP-MUPP1 interaction produced an increase in the number of synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

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(AMPA) receptor-containing clusters and responses, and enhanced excitatory postsynaptic currents in hippocampal slices (Krapivinsky et al., 2004; Rama et al., 2008). Less is known about the role of MUPP1 on inhibitory signaling. However, recent work by Fujita and colleagues (2012) demonstrated that MUPP1 associated with Cadm1 (cell adhesion molecule 1), was co-localized on dendrites of cultured Purkinje cells (likely at inhibitory synapses) and hippocampal neurons with Cadm1 and GABA type B receptors (GABA$_B$R), and was reduced in Cadm1 knockout mice. An associated increase in GABA$_B$R protein was evident in Cadm1 knockout mice, and the investigators proposed a model through which these proteins may form a ternary complex at inhibitory synapses to mediate inhibitory neurotransmission (Fujita et al., 2012). These are just two examples of how MUPP1's formation of macromolecular complexes may influence neuronal signaling, but many more likely exist since MUPP1 associates with a diverse array of proteins through its thirteen PDZ domains.

OR2AG1*
NG2
PDZ1

PDZ2 GAT1*

PDZ3 GAT3*
GlyT1*

PDZ4

Cadm1* PDZ5

PDZ6 D_3R*

PDZ7 GAT2*
GlyT2*

PDZ8

PDZ9

MT1R
Connexin36 PDZ10 $5\text{-}HT_{2A,B,C}R$
SERT

PDZ11

PDZ12

Tech* PDZ13 $GABA_BR$
SynGAPα/CaMKII/NR2B
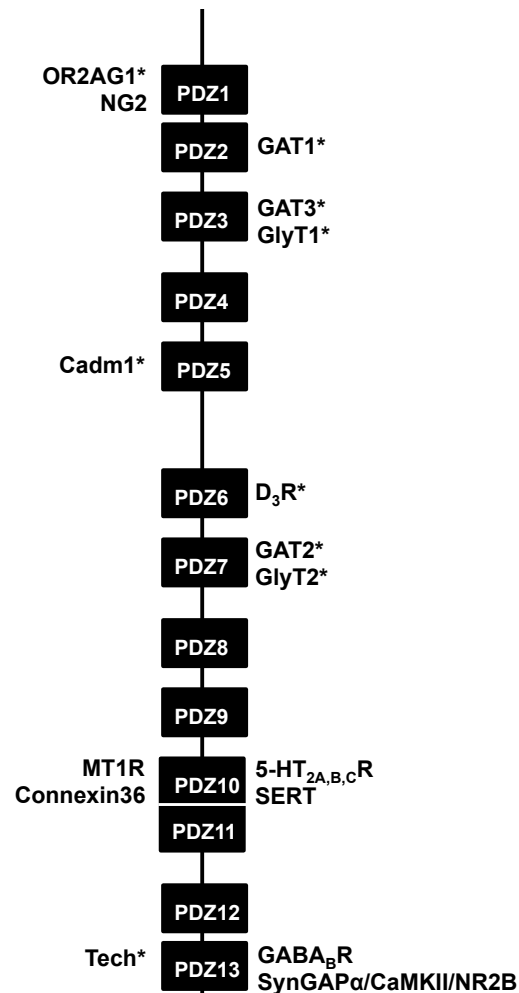
Figure 1-1. MUPP1 structure and known association partners. The 13 PDZ binding domains of MUPP1 directly associate with a variety of proteins. Proteins listed in the figure are a subset of known MUPP1 interacting partners with relevance in CNS signaling and function (Balasubramanian et al., 2007; Baliova et al., 2014; Barritt et al., 2000; Dooley et al., 2009; Fujita et al., 2012; Griffon et al., 2003; Guillaume et al., 2008; Krapivinsky et al., 2004; Li et al., 2012; Ullmer et al., 1998). Proteins and receptors listed on the right may be involved in the reward, reinforcement, and withdrawal associated with alcohol dependence. *Several partners associate with more than one PDZ domain including GAT1 which also interacts with PDZ3-5 and 7, GAT2 with PDZ13,

GAT3 with PDZ4 and 7, GlyT1 with PDZ10 and 13, GlyT2 with PDZ13, D3 receptors with PDZ7-13, ORG2AG1 with PDZ1-2, Tech with PDZ10 and 13, and Cadm1 with PDZ1-5. *Abbreviations:* 5-HT$_{2A, B, C}$R = serotonin 2 A, B, and C receptor subtypes; Cadm1 = Cell adhesion molecule 1; CaMKII = calcium/calmodulin-dependent protein kinase; Connexin36 = gap junction protein; D$_3$R = dopamine D3 receptor; GABA$_B$R = GABA type B receptor; GAT = GABA transporter; GlyT = glycine transporter; MT1R = melatonin receptor type 1; NG2 = Neural/glial antigen 2; NR2B = NMDA receptor subunit 2B; OR2AG1 = Olfactory receptor family 2, subfamily AG, member 1; SERT = serotonin transporter; SynGAPα = synaptic Ras GTPase-activating protein; Tech = Ras homolog gene family, member A guanine nucleotide exchange factor.

MUPP1 and GABA_BRs

GABA_BRs have been implicated in the rewarding and reinforcing properties of alcohol, as well as EWD (Colombo et al., 2000; Colombo et al., 2002; Humeniuk et al., 1994; Vlachou and Markou, 2010). Interestingly, using fusion protein pull-down assays, the R2 subunit of the GABA_BR (GABBR2) was identified as an interacting partner with MUPP1. Immunohistochemical analyses in mice and rats also identify an overlap in the distribution patterns of MUPP1 and GABA_BRs in cultured neurons and brain (Balasubramanian et al., 2007; Fujita et al., 2012). Therefore, of particular interest to my dissertation was the potential influence of MUPP1 on GABA_BR function and associated behavior in order to begin to identify the effect of MUPP1 on GABA_BR function.

GABA_BRs are G-protein coupled seven transmembrane domain metabotropic receptors that mediate slow synaptic inhibition to hyperpolarize neurons when stimulated (Bowery, 1993; Devi, 2001). GABA_BRs are a heterodimeric assembly comprised of GABBR1 and GABBR2, the dimerization of which is necessary for functional GABA_BRs (Jones et al., 1998; Kaupmann et al., 1998). GABBR1 possesses the GABA binding site, and is limited to cellular vesicles in the absence of GABBR2 expression. GABBR2 binds $G\alpha_{i/o}$ and $G\beta\gamma$, and is required to transport GABBR1 to the cell surface, and can be expressed without GABBR1 on the cell surface but cannot bind GABA (Devi, 2001; Robbins et al., 2001). Activation of GABA_BRs dissociates the $G_{i/o}$-proteins from GABBR2, inhibits adenylyl cyclase through $\alpha_{i/o}$, and inhibits voltage gated calcium channels through $\beta\gamma$ on pre- and postsynaptic neurons, and stimulates postsynaptic G-protein inwardly rectifying potassium (GIRK) channels through $\beta\gamma$ (Bowery, 1993; Robbins et al., 2001).

Interestingly, disruption of the GABBR2-MUPP1 interaction *in vitro* through MUPP1-targeted RNAi and mutation of the GABBR2 C-terminus has been shown to

alter GABA$_B$R function by reducing receptor stability (i.e., reduced half-life assessed by pulse-chase metabolic labeling), as well as attenuating the duration of GABA$_B$R signaling (i.e., calcium mobilization) assessed through chimeric G$_{qi5}$ where this chimeric G-protein can link G$_i$ coupled GABA$_B$R to G$_q$-activated intracellular calcium mobilization, in COS-7 cells transfected with GABA$_B$Rs (Balasubramanian et al., 2007). These were the first data to implicate MUPP1 in GABA$_B$R function and suggest that disruption of this association can reduce GABA$_B$R function. However, it is widely known that GABA$_B$Rs in cultured neurons and particularly transfected cells, may function differently from native GABA$_B$Rs. In fact, in Cadm1 (cell adhesion molecule 1) knockout mice which also demonstrate reduced MUPP1, GABBR2 is increased in the cerebellum suggesting, albeit indirectly, that native GABA$_B$R function may instead be increased in animals with reduced MUPP1 (Fujita et al., 2012).

Behaviorally, the potential effect of *Mpdz* on GABA$_B$R-mediated HICs in response to baclofen, a highly selective GABA$_B$R agonist, has also been assessed. Previous work has shown that baclofen enhances convulsions (HICs) (Humeniuk et al., 1994; Mead and Little, 1995; Reilly et al., 2008). Interestingly, baclofen-enhanced HICs were found to be significantly greater in chromosome 4 congenic compared to D2 background strain mice (Shirley et al., 2004), but no genotype difference in baseline or pentylenetetrazol (PTZ)-enhanced HICs were detected (Reilly et al., 2008). These were the first data to assess the potential effect of *Mpdz* (i.e., through the chromosome 4 QTL spanning *Mpdz*) on a GABA$_B$R-mediated response behaviorally, but it is possible that other genes within the introgressed region could have influenced this phenotype. In Chapter 3, I employed behavioral, molecular, and neurophysiological techniques to more directly assess whether and how reduced MUPP1 expression may affect GABA$_B$R function and related behavior. I hypothesized that reduced MUPP1 expression would be

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associated with heightened GABA$_B$R-mediated HICs, and vice versa. I initially assessed this through systemic administration of baclofen utilizing both the $Mpdz^{+/-}$ and $Mpdz^{Tg}$ genetic models. Comparing $Mpdz^{+/-}$ and WT littermates, I also assessed the role of reduced MUPP1 expression on GABA$_B$R-mediated locomotor depression. The complementary use of both transgenic and knockout heterozygote models allowed a more direct comparison of the role of $Mpdz$ in GABA$_B$R-mediated hyperexcitability, and comparisons with the effect of $Mpdz$ on EWD (Milner et al., 2015). Further, radioligand receptor binding, gene expression, and neurophysiological analyses in $Mpdz^{+/-}$ and WT littermates were used to explore that hypothesis that reduced MUPP1 was associated with heightened GABA$_B$R function.

Neural circuitry associated with EWD

Approaches that have been used to elucidate the neural circuitry involved in EWD include immediate transcription factor (ITF) expression and focused lesions (Chen et al., 2008; Chen et al., 2009; Kozell et al., 2005). ITFs are deoxyribonucleic acid (DNA)-binding proteins that are thought to initiate gene transcription following the onset of a stimulus. In general, the induction of cFos, an immediate early gene, and other ITFs, is commonly used as a marker of neuronal activation associated with a phenotype of interest. Acute EWD-associated neural activation assessed at 7 h post-ethanol administration, the time of peak HIC severity, was apparent across numerous brain regions including the extended basal ganglia, extended amygdala, and regions of the cortex and hippocampus as identified using D2 and B6 mice (Kozell et al., 2005). However, strain differences in cFos induction associated with withdrawal were most apparent in the extended basal ganglia, and particularly in regions involved in limbic, rather than motor function. Greater activation was evident in D2 mice, which experience

greater EWD HICs than B6 mice. These regions included the SNr, subthalamic nucleus, ventral striatum (nucleus accumbens), and ventral pallidum. Activation of the extended basal ganglia, and particularly subregions associated with limbic function (Chen et al., 2008), appeared to be involved in genetic risk for acute EWD.

The SNr and its involvement in CNS hyperexcitability

The SNr, one of two major output pathways of the basal ganglia, integrates basal ganglia signaling and sends processed signals to select pathways. This region is primarily comprised of GABA projection neurons, with a minor subset of dopamine neurons, and the activity and function of the SNr is tightly regulated by two distinct pathways originating in the striatum referred to as the direct and indirect pathways (Figure 1-2) (Bolam et al., 2000). Through the direct pathway (striato-nigral pathway), inhibitory GABAergic neurons project from the striatum to the SNr, exerting direct inhibitory control over this region (Bolam et al., 2000). Through the indirect pathway (striato-pallidal-subthalamic-nigral pathway), striatal GABAergic neurons project to the lateral globus pallidus, which in turn sends GABAergic projections to the subthalamic nucleus, activating the SNr through excitatory glutamatergic projection neurons (Bolam et al., 2000). SNr activity is also influenced by serotonergic projections from the dorsal raphe nucleus (Corvaja et al., 1993) and GABAergic projections directly from the globus pallidus (Smith and Bolam, 1990), and is further modulated by GABA, serotonin, dopamine, and glutamate receptors (Eberle-Wang et al., 1997; Veliskova et al., 2001; Zhou et al., 2009). Intra-SNr axon collaterals are also involved in local feedback regulation to modulate SNr activity and function (Mailly et al., 2003). SNr GABA projection neurons inhibit the ventromedial thalamus, pedunculopontine tegmental nucleus, and superior colliculus, output structures of the basal ganglia (Bolam et al.,

2000; Deniau and Chevalier, 1992; Di Chiara et al., 1979; Kha et al., 2001). Reduced

GABA release from the SNr disinhibits output structures of the SNr, which has been

shown to produce anticonvulsant activity, and increased GABA release from the SNr is

thought to be proconvulsant (discussed further below).
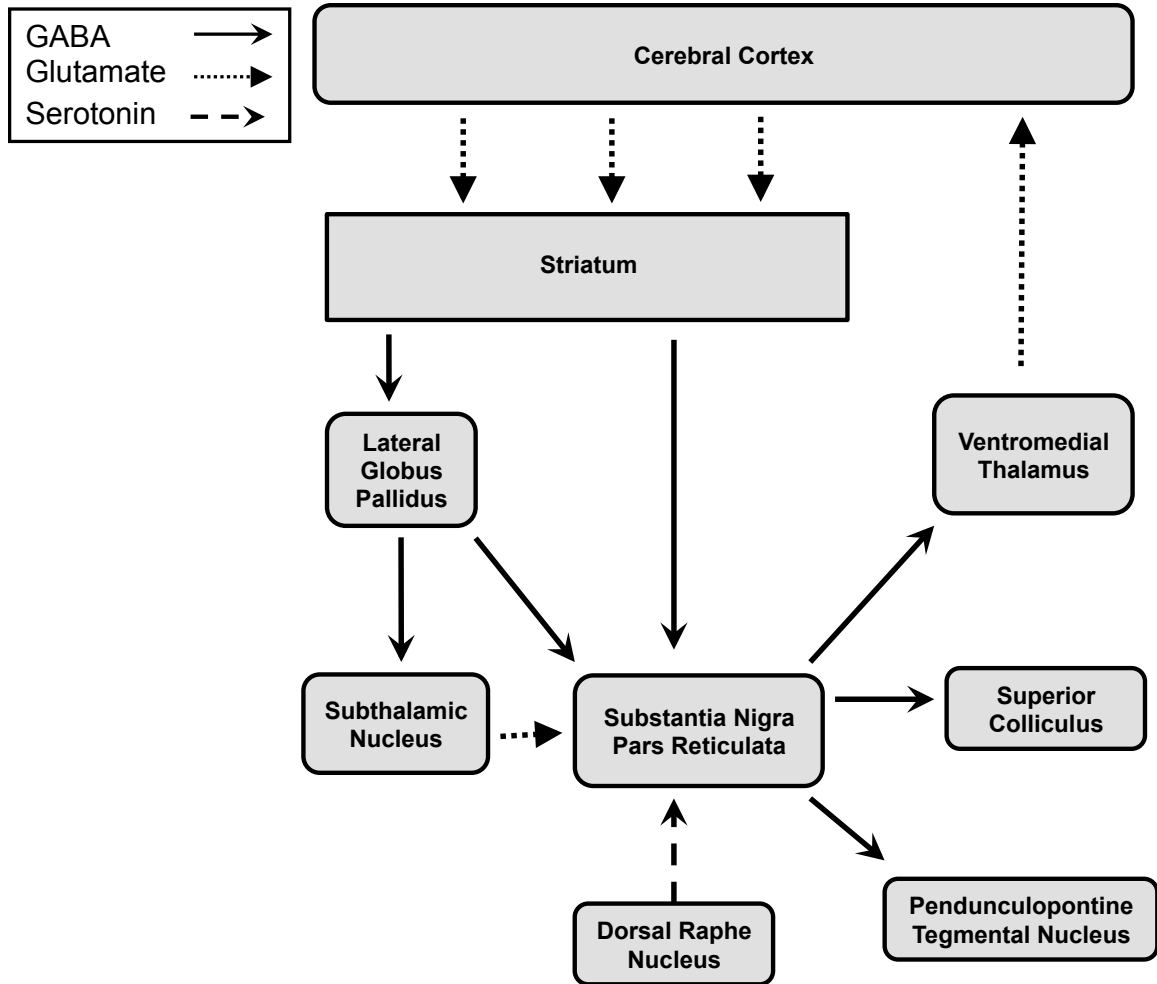
Figure 1-2. Input and output projections of the SNr. Activity of the SNr is controlled by the direct (striato-nigral) and indirect (striato-pallidal-subthalamic-nigral) pathways of the basal ganglia, as well as projections from the lateral globus pallidus and the dorsal raphe nucleus. The SNr projects to the ventromedial thalamus, superior colliculus, and pendunculopontine tegmental nucleus.

The SNr has long been implicated in mediating seizure activity. In the 1980-1990s, metabolic mapping studies using $[^{14}C]$-2-deoxyglucose (2DG) in rats were among the first to demonstrate substantial and consistent uptake of glucose (a marker of neuronal activation) in the SNr across several seizure models including seizures induced by kainic acid, pentetrazole, and bicuculline (Ben-Ari et al., 1981; Nehlig et al., 1992), and numerous pharmacological and lesion studies in rats since then also support this role of the SNr in seizures. In general, suppression of the SNr proper with $GABA_A$ agonists (e.g., muscimol) or benzodiazepines disinhibited output structures, and reduced (Deransart and Depaulis, 2002; Iadarola and Gale, 1982) limbic motor seizures and generalized convulsive seizures, but not audiogenic seizures (Maggio and Gale, 1989; Turksi et al., 1986). Further, lesioning the SN reduced $GABA_A$ receptor antagonist (bicuculline)-induced seizures, although no distinction between pars compacta and pars reticulata was made (Garant and Gale, 1983).

Interestingly, a functional dichotomy between the posterior (caudal) and anterior (rostral) regions of the SNr on seizure activity has also been identified. 2DG uptake studies showed that the posterior SNr was active during flurothyl-induced pre-clonic seizures and is therefore thought to be an early gateway for seizure initiation/propagation, whereas activation of the anterior subregion was apparent during the seizure and may be involved in seizure propagation or cessation (Shehab et al., 1996; Veliskova et al., 2005). Further, administration of a selective NMDAR antagonist into the posterior SNr was proconvulsant, whereas this antagonist produced anticonvulsant effects in the anterior SNr (Veliskova et al., 2001), and administration of muscimol to the anterior SNr has been shown to be anticonvulsant, and proconvulsant in the posterior SNr as assessed by flurothyl seizure threshold (Moshe et al., 1995). Further, Shehab and colleagues (1996) attempted to map regional specificity of the SNr

and found that the caudal (posterior) SNr was actually the most sensitive region for the efficacy of muscimol in suppressing electroshock tonic seizures. Finally, distribution of GABA type A receptor (GABA$_A$R) subunits and 5-HT$_{2C}$R receptors, appear to be greater in the caudal compared to the rostral SNr (Eberle-Wang et al., 1997; Nicholson et al., 1992). Although these studies provided some conflicting evidence as to the function of each region of the SNr, which could, in part, be due to differences in method of seizure induction, these data provided strong evidence that the anterior and posterior SNr are functionally distinct in their regulation of seizure phenotypes in rats.

Beyond seizures, the SNr has also been shown to be sensitive to sedative-hypnotics as intravenous ethanol, phenobarbital, and zolpidem all have been shown to depress SNr neuronal firing rates (Diana et al., 1993; Mereu and Gessa, 1985; Waszczak et al., 1986; Zhang et al., 2008). Recent studies in mice used electrolytic and ibotenic acid lesions of discrete subregions of the SNr as well as the subthalamic nucleus to behaviorally assess the role of the SNr in EWD. The rostrolateral SNr and caudolateral SNr (clSNr) were each targeted because both were implicated in acute EWD in a chromosome 4 QTL dependent manner (Chen et al., 2008), and previous work has shown a putative functional dichotomy of the SNr (Moshe et al., 1995; Shehab et al., 1996; Veliskova et al., 2001; Veliskova et al., 2005). Ablation of the clSNr, but not the rostrolateral SNr or subthalamic nucleus, significantly attenuated acute and repeated EWD HICs (Chen et al., 2011; Chen et al., 2008). These data confirmed that intrinsic cells of the clSNr, rather than fibers of passage, were crucially involved in EWD, and also expand understanding of functional differences between the caudal and rostral subregions of the SNr in mice. Further, baseline (pre-drug administration) and chemiconvulsant (i.e., PTZ-enhanced convulsions) were unaffected, indicating that lesions of the clSNr may affect EWD convulsions with some degree of specificity.

Importantly, to my knowledge, this study was the first to show a functional dichotomy of subregions of the SNr in mice, similar to the seizure work in rats.

Neural circuitry associated with EWD in an Mpdz dependent manner

In order to begin to ascertain what brain region(s) are involved in EWD in a QTL dependent manner, chromosome 4 QTL congenic and D2 background strain mice were compared (Chen and Buck, 2010; Chen et al., 2008). Discrete regions of the basal ganglia associated with limbic (rather than motor) functions exhibited acute EWD associated activation of cFos that was genotype (QTL) dependent (i.e., less activated in congenic compared to D2 background strain mice). This was particularly evident in the rostral, caudal, and dorsomedial regions of the SNr. These data were the first to implicate QTL actions in the SNr as mediating its effect on EWD. Notably, ITF mapping studies using additional QTL congenic models (for other withdrawal QTLs on chromosomes 1 and 11) also demonstrated genetic differences in withdrawal associated activation in the SNr, suggesting that this region may play an important genetic role in a final common pathway for acute EWD. As presented in Chapter 2, part of my research directly tested this role of *Mpdz* in the clSNr on EWD using RNAi. These data further confirmed *Mpdz* as a QTG for EWD. I hypothesized that reduced expression of *Mpdz* in the clSNr would be associated with an enhancement in acute EWD severity, without generally affecting seizure susceptibility. These data would be the first to confirm a QTG identified in QTL mapping studies in an ethanol related phenotype, and provide evidence of an associated neurobiological mechanism.

GABA_BRs and EWD

GABA$_A$Rs have long been implicated in the neurobiology of alcohol withdrawal seizures, as benzodiazepines continue to be a consistent and effective clinical treatment in suppressing withdrawal symptoms (Manasco et al., 2012; Mayo-Smith, 1997). Preclinical studies have shown that withdrawal seizures are reduced by activation of GABA$_A$R through systemic or site-specific (i.e., SN and SNr) administration of agonists or positive modulators (Frye et al., 1983; Gonzales and Hettinger, 1984; Tanchuck et al., 2013), but there has been less focus on the role of GABA$_B$Rs in withdrawal. In the mid-1990s, two concurrent studies showed that activation of GABA$_B$Rs (using baclofen) was proconvulsant in control animals and further potentiated EWD convulsions in dependent mice, whereas inhibiting these receptors suppressed EWD, and blocked this potentiation by baclofen (Humeniuk et al., 1994; Mead and Little, 1995). Beyond withdrawal convulsions, in preclinical studies baclofen reduced EWD symptoms associated with social anxiety and tremors (Colombo et al., 2000; File et al., 1991), and in clinical studies baclofen reduced severity of the alcohol withdrawal syndrome at lower doses, but high doses of baclofen actually produced convulsions and seizures (Addolorato et al., 2002; Leggio et al., 2010; Leung et al., 2006). These studies importantly identified GABA$_B$Rs as a putative neural substrate involved in mediating effects of the alcohol withdrawal syndrome.

Activation of pre- and postsynaptic SNr GABA$_B$Rs alters activity of GABA projection neurons (Chan et al., 1998; Floran et al., 1988; Misgeld et al., 1995; Rick and Lacey, 1994), which terminate in output structures (Figure 1-2). Although activation of SN/SNr GABA$_A$Rs has been shown to attenuate EWD severity (Frye et al., 1983; Gonzales and Hettinger, 1984; Tanchuck et al., 2013), the impact of direct activation or inhibition of SNr GABA$_B$Rs on EWD has not been explored. As presented in Chapter 3,

part of my research tested this directly using site-directed administration of a selective GABA$_B$R agonist (baclofen) and antagonist (CGP55845) into the clSNr of control and ethanol withdrawn mice. I hypothesized that clSNr GABA$_B$Rs are involved in the CNS hyperexcitability, and that inhibition of these receptors would attenuate the severity of EWD. Therefore, in Chapter 3 I sought to further examine the role of clSNr GABA$_B$Rs as a neurobiological substrate of EWD, and to build more evidence for a role of these receptors as a putative mechanism through which *Mpdz* expression in the clSNr influences EWD.

Binge alcohol drinking in humans

Withdrawal from alcohol is thought to negatively reinforce the cycle of addiction, whereas binge-intoxication is thought to be positively reinforce the cycle (Koob and LeMoal, 2001). A frequent element of human AUDs is the presence of detrimental patterns of excessive or binge drinking that produces sustained blood ethanol levels and behavioral intoxication, a pattern of behavior that may be a risk factor for future development of an AUD (Viner and Taylor, 2007). In humans, binge drinking is defined as consuming (approximately) ≥4 drinks in women and ≥5 drinks in men within an approximate 2 h timeframe resulting in blood alcohol concentrations of >0.8 milligram per milliter (mg/ml) or 0.08 g% (DHHS-NIH, 2004). Binge drinking may emerge prior to, and contribute to the development of AUDs (Viner and Taylor, 2007).

No single animal model can capture all features of clinically defined AUDs. Therefore, beyond the role of *Mpdz* in EWD, I was interested in elucidating the effect of this gene in binge-like ethanol drinking.

Animal models of ethanol drinking

Numerous animal models of ethanol drinking have been developed for modeling aspects of AUDs (Becker, 2013). One of the most frequently used procedures to assess ethanol consumption is the two-bottle choice continuous access (2BC-CA) procedure, where mice have 24 h access to one water bottle and one ethanol bottle (Belknap et al., 1997; Crabbe et al., 2011; Milner et al., 2015; Phillips et al., 2004; Yoneyama et al., 2008). Typically, the procedure begins with low concentrations of ethanol (3-6%), and progressively increases to higher concentrations (10-20%), with one to four days of access to each concentration.   A 2BC-CA procedure permits the assessment of preference for ethanol over water, which may indicate a motivation to consume ethanol over fluid in general. This has been one of the most commonly used procedures of ethanol self-administration in mice and therefore there exists a substantial amount of information on the underlying genetic and neurobiological mechanisms of this phenotype (Bahi and Dreyer, 2012; Belknap et al, 1993; Crabbe et al., 2011; Gabriel and Cunningham, 2005; Giardino et al., 2011; Metten et al., Grahame et al., 1999; Milner et al., 2015; Yoneyama et al., 2008). However, it is limited by the ambiguity of the pattern of ethanol intake over the 24 h access period and whether or not this pattern produces significant (above binge levels [>0.8 mg/ml]) and prolonged blood ethanol levels and associated behavioral intoxication (Dole & Gentry, 1984). The 2BC-CA procedure is thus limited in its clinical relevance. Therefore, approaches have been used for the development of a model for excessive/binge drinking, and greater potential for examining the underlying mechanisms and effective pharmacotherapies.

Dr. Justin Rhodes and Dr. John Crabbe (2005) developed a limited access drinking in the dark (DID) procedure as a model of binge-like ethanol drinking in mice. A commonly used DID experiment lasts for four days, with daily limited access (2 h – 4 h)

to a single drinking tube containing 20% ethanol during the animals dark cycle, when ingestive behaviors peak (Goldstein and Kakihana, 1977). Typically, no water choice is available during the limited access DID session. This model has been repeatedly validated as a "binge" model of ethanol consumption as some mice will consume to blood ethanol concentrations (BEC) >0.8 mg/ml and often >1.0 mg/ml, which have been shown to be associated with behavioral intoxication (Crabbe et al., 2009; Kasten et al., 2015; Kaur et al., 2012; Rhodes et al., 2005; Rhodes et al., 2007). Further, variants of this procedure have been used to provide additional information about aspects of consumption. The addition of a water bottle during the limited access session has also been used to offer a choice between water and ethanol, which permits the determination of preference for ethanol over water (Phillips et al., 2010), although BECs may be attenuated (Crabbe et al., 2009). Further, a recent study used a variation of the DID procedure where B6 mice had access to repeated cycles of DID (3, 6, and 10), resulting in increased ethanol consumption in subsequent access to 2BC-CA (Cox et al., 2013). Therefore, the DID procedure and its variants provide a valuable model for assessing the genetic and neurobiological influences on initial predisposition to binge-like ethanol drinking.

Ethanol consumption and EWD in mice

Several genetic mouse models have been used to examine shared gene effects on ethanol consumption and EWD. Specifically, a significant genetic correlation has been identified between EWD (HICs) and ethanol consumption in 2BC-CA (i.e., preference drinking) in mice, where genetic predisposition to high EWD HICs, may be associated with lower ethanol preference drinking, and vice versa (Hitzemann et al., 2009; Metten et al., 1998b), although this relationship may be influenced by and perhaps

limited to certain genetic backgrounds (Crabbe et al., 2013; Hitzemann et al., 2009; Metten et al., 1998b). Data supporting the genetic relationship between withdrawal and ethanol consumption in 2BC-CA was demonstrated in D2 and B6 mice and populations derived from these progenitor strains including the BXD RIs and lines selectively bred for differential acute EWD (Buck et al., 1997; Metten et al., 1998a) or 2BC-CA ethanol consumption (Metten et al., 1998b; Phillips et al., 1994). This relationship has also been detected in animals selected from HS stock mice, but appears to be more complex. Lines selectively bred for high versus low consumption from HS4 founders (derived from crossing the B6, D2, BALB/cJ, and LP/J inbred strains) showed low and high withdrawal, respectively, but animals selected for high versus low withdrawal bred from HS4 showed no difference in 2BC-CA ethanol consumption (Hitzemann et al., 2009). It should be noted that the genetic correlation between high consumption in 2BC-CA and low EWD (Metten et al., 1998b) appears inconsistent with clinical studies mentioned previously, which found reportedly greater response to acute withdrawal symptoms but no difference in quantity/frequency of drinking in children of alcoholics who are at-risk for the development of an AUD (Newlin and Pretorius, 1990; Span and Earleywine, 1999). This inconsistency may be due to the fact that, in mice, the relationship between acute EWD and drinking in 2BC-CA is a genetic correlation rather than a causal relationship. Further, as discussed above, this genetic correlation is not always consistent since mice selectively bred for high and low acute EWD did not differ in ethanol consumption (Hitzemann et al., 2009), a finding that may be more consistent with abovementioned clinical studies.

There is some evidence of shared gene effects on 2BC-CA preference drinking and limited access DID, but results are mixed. Significant correlations between these two drinking phenotypes were detected using a panel of inbred strains ($r = 0.70$)

(Rhodes et al., 2007; Crabbe et al., 2012b), as well as B6FVBF2 hybrid mice (r = 0.12-0.23) (Phillips et al., 2010), but mice selectively bred for high BECs attained using the DID paradigm actually consumed less ethanol at 20%, 30%, and 40% concentrations in 2BC-CA preference drinking compared to control mice, with no differences in consumption of lower ethanol concentrations between 3% and 20% (Crabbe et al., 2011). It should be noted that this association appears to be primarily driven by four strains of mice from the C57/C58 lineage (Dr. John Crabbe, personal communication), suggesting that the positive relationship between 2BC-CA and DID may be most apparent in B6 mice.

Mpdz and ethanol drinking

Recent QTL mapping using a dual-trait selectively bred line for high acute EWD and low ethanol consumption using 2BC-CA drinking and vice versa, has confirmed a chromosome 4 QTL in contributing to the genetic correlation between acute EWD HICs and preference drinking in mice (Metten et al., 2014). Although a significant genetic correlation between EWD and consumption using 2BC-CA has been recognized for some time, the specific QTG(s) that contribute to this relationship remain anonymous. However, recent data demonstrated that $Mpdz^{+/-}$ mice consume significantly less ethanol (6%, 10%, and 20%) in a 2BC-CA paradigm compared to WT littermates, indicating *Mpdz* as a gene that contributes to this genetic relationship between withdrawal and ethanol consumption (Milner et al., 2015), and identifying a role of *Mpdz* in predisposition to reduce ethanol consumption.

No single animal model of ethanol drinking can emulate all features of human AUDs, and therefore the use of several models, and particularly ones that model aspects of excessive or binge ethanol drinking, is crucial in defining the role of *Mpdz* in potential

risk for AUDs. Given that there is some evidence for a genetic correlation between 2BC-CA and DID, and that this relationship may be particularly apparent in mice from the C57/C58 lineage (including B6), in Chapter 4 I assessed the role of *Mpdz* in binge-like ethanol drinking and associated BECs, as well as ethanol preference associated with binge-like ethanol drinking in *Mpdz*$^{+/-}$ and WT littermates. Mice were assessed using several variants of the typical DID procedure including single and repeated access to DID, as well as choice between water and ethanol during limited access DID. I hypothesized that if the same relationship between *Mpdz* and 2BC-CA preference drinking exists for *Mpdz* and DID, that *Mpdz*$^{+/-}$ would demonstrate reduced binge-like ethanol drinking compared to WT littermates at both initial exposure to DID, as well as with repeated access, and that this would be associated with lower BECs. This data is important for identifying a potential role of the *Mpdz* gene in a clinically relevant drinking phenotype.

Beyond mice, the human homolog of *Mpdz* (*MPDZ*) has also been implicated in risk for an AUD. Specifically, recent human studies identified an association between single nucleotide polymorphisms in *MPDZ* and alcohol consumption, as well as *MPDZ* haplotype status and alcohol dependence without a history of withdrawal seizures (Karpyak et al., 2009; Karpyak et al., 2012; Tabakoff et al., 2009; see below). Further, a post-mortem study found *MPDZ* expression to be reduced in the hippocampus of alcoholics compared to non-alcoholics, although many other genes were also found to be differentially expressed (McClintick et al., 2013). As discussed above, a genetic relationship between high ethanol consumption in 2BC-CA and low EWD HICs correlated with *Mpdz* status may exist in mice (Metten et al., 2014; Milner et al., 2015). If this same relationship also exists in humans, one could predict an association with *MPDZ* (sequence and/or expression) in alcoholics with no history of withdrawal seizures,

but not in alcoholics with a history of severe withdrawal. This appears to be the case as one study established that *MPDZ* haplotype status was associated with a diagnosis of alcoholism in subjects with *no* history of severe withdrawal, but not in alcoholics with a history of withdrawal seizures and/or delirium tremens (Karpyak et al., 2009). This study also performed direct comparisons between *MPDZ* and *Mpdz* to assess potential homology in variability in human DNA sites to variability in sites in the mouse associated with EWD but failed to detect any identical sequence variations. The results of mouse studies on *Mpdz* have the potential to inform the human studies, as it is intriguing to speculate that allelic variation in *MPDZ* and associated differences in expression might plausibly contribute to this difference in clinical populations and/or potentially contribute to differential response to pharmacotherapeutic treatments to prevent relapse to abuse.

GABA$_B$Rs in alcohol drinking

Although the role of GABA$_B$Rs in EWD has not been extensively explored, there is substantial evidence implicating GABA$_B$Rs in the rewarding and reinforcing properties of alcohol in both humans and animals (Agabio and Colombo, 2014). In rats, acute or repeated administration of baclofen (both i.p. and intracranial) reduced the acquisition and maintenance of ethanol drinking, as well as relapse-like drinking (Colombo et al., 2000; Colombo et al., 2002; Colombo et al., 2003). In mice, baclofen (i.p. and intracranial) reduced the rewarding effects of alcohol as assessed by ethanol-induced locomotor activation and conditioned place preference, maintenance of ethanol drinking, and binge-like ethanol drinking (Agabio and Colombo, 2014; Bechtholt and Cunningham, 2005; Boehm et al., 2002; Broadbent and Harless, 1999; Kasten et al., 2015; Moore and Boehm, 2009; Tanchuck et al., 2010).

Based on strong evidence from preclinical studies supporting a role for GABA$_B$Rs in the reinforcing and rewarding properties of alcohol, baclofen is currently being assessed in clinical trials for its efficacy in reducing alcohol consumption, craving, the alcohol withdrawal syndrome, and relapse in dependent individuals (Addolorato et al., 2011; Agabio and Colombo, 2014; Phillips and Reed, 2014). Thus far, baclofen has been effective in reducing the number of drinks per day and craving for alcohol, as well as increasing the number of abstinent days in dependent individuals (Addolorato et al., 2000; Flannery et al., 2004). However, the results have been mixed as baclofen is efficacious in some alcoholics but not in others (Garbutt et al., 2010). This variability in baclofen, as well as other pharmacotherapies, as a putative treatment for AUDs may be, in part, influenced by genetic factors (Leggio et al., 2013; Roden and George, 2002).

As discussed previously, genetic risk can influence predisposition to development of an AUD. However, in addition, it is plausible that the same genes associated with genetic risk for an AUD may also influence response to pharmacotherapies in dependent individuals, resulting in increased or decreased effectiveness of the treatment. In both humans and mice *Mpdz* has been implicated in genetic risk for ethanol-related phenotypes, but no studies have looked at the role of *Mpdz* in influencing response to treatment. Given that *Mpdz* associates with numerous receptors, and may influence function of the GABA$_B$R, it is possible that *Mpdz* has effects on ethanol-related phenotypes, such as treatment, beyond initial predisposition. Further, based on evidence from a chromosome 4 congenic model suggesting that QTL status and *Mpdz* expression may be associated with altered GABA$_B$R-mediated responses, an intriguing idea has emerged that *MPDZ* (the human homolog of *Mpdz*) haplotype status and/or expression may affect the pharmacotherapeutic efficacy of baclofen to attenuate alcohol drinking, craving, and prolong abstinence. As presented in

Chapter 4, I therefore explored the hypothesis that the pharmacotherapeutic effect of baclofen to reduce binge-like drinking would be greater in $Mpdz^{+/-}$ compared to WT littermates based on previous behavioral work suggesting a potential association between greater $GABA_BR$-mediated responses with lower $Mpdz$/MUPP1 expression (Reilly et al., 2008). These data are beneficial in informing similarities and/or differences between genetic risk and treatment of AUDs and the associated genes.

Central focus of dissertation

The central focus of my dissertation was to provide further confirmation of the $Mpdz$ gene as a QTG for acute EWD, and to begin to identify associated circuitry. Further, the focus was to examine the role of $Mpdz$ in risk for binge-like ethanol drinking. In addition, elucidating the functional importance of potential MUPP1 interactions with its interacting partners is a crucial step towards identifying the relevance of this gene in AUDs, and specifically, the potential mechanism through which $Mpdz$ affects EWD and ethanol drinking. Therefore, my dissertation also sought to elucidate the potential association between altered MUPP1 expression with $GABA_BR$ mediated-behaviors and function, as an initial step towards building evidence for the hypothesis that these receptors may be one potential mechanism through which $Mpdz$ affects EWD. The following chapters therefore employed a combination of behavioral, genetic, pharmacological, and molecular techniques to critically evaluate the role of $Mpdz$ and associated neurobiological mechanisms in predisposition to EWD and binge-like ethanol drinking, as an important step towards understanding the genetic and neurobiological bases for human AUDs.

Chapter 2: Role of _Mpdz_ expression in the caudolateral substantia nigra pars reticulata on acute ethanol withdrawal

This chapter is adapted from the following publication:

Kruse LC, Walter N, and Buck KJ (2014). _Mpdz_ expression in the caudolateral substantia nigra pars reticulata is crucially involved in alcohol withdrawal. _Genes, Brain and Behavior_ 13, 769-776.

Introduction

Following the cessation of alcohol intake, withdrawal symptoms may occur which are the manifestation of physical dependence. Withdrawal is associated with negative reinforcement of the continued use and abuse of alcohol, and plays a role in perpetuation the dependence cycle (Dreumont and Cunningham, 2014; Koob and Le Moal, 2001; Koob and Volkow, 2010). Unfortunately, genetic determinants of risk for alcohol withdrawal remain largely unknown, hindering effective prevention and treatment of dependent individuals.

No animal model can exactly duplicate clinically defined alcoholism, but models for specific factors, such as risk for EWD episodes have proven invaluable in the identification of the potential genetic determinants of liability in humans. Using a robust behavioral model of acute withdrawal (HICs), positional cloning, and molecular analyses, _Mpdz_ has been identified as a putative QTG for EWD in mice, demonstrating allelic variation in sequence (structure) and expression, either one or both of which could contribute to the QTL phenotypic effect on predisposition to withdrawal (Fehr et al., 2002; Shirley et al., 2004). _Mpdz_ and its human homolog (_MPDZ_) encode MUPP1, a multiple PDZ domain containing protein (Simpson et al., 1999; Ullmer et al., 1998),

which plays an important regulatory role in signal transduction pathways of the receptors and proteins with which it complexes (Sheng and Sala, 2001).

Recent studies using conventional *Mpdz* knockout heterozygotes and *Mpdz* transgenic mouse models further support an inverse relationship between *Mpdz* expression and EWD severity (Milner et al., 2015), but cannot disentangle *Mpdz* actions from potential effects of developmental compensation and/or linked genes. Fortunately, RNAi has emerged as a powerful approach to disentangle target gene effects from these potential confounds (Bahi and Dreyer, 2012). Briefly, the method of viral-mediated (i.e., lentivirus as in the present work) RNAi involves the microinjection of a short-hairpin RNA (shRNA) into a discrete region of interest where it incorporates into the chromosome through viral transduction. Lentiviruses are advantageous in that they efficiently transduce dividing and non-dividing cells and stably integrate into the host genome without a decrease in transgene expression over time, ensuring stable knockdown of a gene of interest (Blomer et al., 1997; Manjunath et al., 2009). RNAi occurs post-transcriptionally where the shRNA is transcribed in the cell, subsequently processed into short interfering (si) RNA which pairs with and targets the RNA induced silencing complex to the complementary target mRNA, cleaving the mRNA thereby effectively inducing gene specific silencing (Manjunath et al., 2009). RNAi is advantageous for studying gene effects in discrete brain regions since RNAi spread beyond the microinjection site is minimal, thus limiting target gene knockdown to a select region to provide additional mechanistic information (Bahi and Dreyer, 2012). Success of the RNAi approach is therefore dependent upon targeting the appropriate brain region(s). In the present experiments, I discretely target the clSNr. Analyses looking at the induction of cFos during EWD have implicated the SNr as involved in genetically determined differences in EWD in a manner dependent upon chromosome 4 QTL status, and

bilateral chemical and electrolytic lesions of the clSNr, but not rostromedial SNr, significantly mitigate EWD using acute and repeated ethanol exposure models (Chen et al., 2011; Chen et al., 2008). These studies confirmed the clSNr itself (rather than fibers of passage) as intrinsically involved in EWD.

For these reasons, I used an RNAi approach to directly and rigorously test the hypothesis that reduced *Mpdz* expression in the clSNr results in more severe acute EWD, without generally affecting seizure susceptibility. In both humans and mice, spontaneous convulsions are rare, but seizure threshold for some stimuli is lowered during EWD. The present studies assessed acute EWD using the HIC scale, a sensitive and quantitative measure of EWD, which is highly correlated with other signs of EWD and is substantially genetically determined (Goldstein and Kakihana, 1975; Goldstein and Pal, 1971; Metten and Crabbe, 1994).

Materials and methods

Subjects

Male D2 strain mice (Jackson Laboratories) were acclimated to our vivarium for 1-2 weeks. Mice were 50-57 days old at the time of surgery and behaviorally assessed starting at 70-77 days of age. Colony and procedure rooms were maintained on a 12 h light/dark cycle with lights on from 06:00 to 18:00, and the temperature maintained at 21±1˚C. All mice received water and food (LabDiet 5001Rodent Diet) *ad libitum*. All procedures were approved by the Oregon Health & Science University and VA Medical Center Institutional Animal Care and Use Committees in accordance with United States Department of Agriculture and United States Public Health Service guidelines.

Drugs

Two-hundred proof ethanol was purchased from Decon Labs, Inc. (King of Prussia, PA). PTZ and meloxicam were purchased from Sigma Aldrich (St. Louis, MO). All drugs were dissolved in 0.9% physiological saline for systemic administration.

Mpdz RNAi in mouse NS20Y cells

Five Sigma MISSION shRNA clones for *Mpdz*, designed and developed by the RNAi Consortium at the Broad Institute of MIT and Harvard, were additionally analyzed using Basic Local Alignment Search Tool (BLAST) to ensure specificity to reduce endogenous *Mpdz* mRNA expression in mouse neuroblastoma (NS20Y) cells compared to a control (scrambled) shRNA clone*.* Plasmid DNA (1 µg) was transfected into 60-70% confluent NS20Y cells using lipofectamine. Following selection for shRNA transfected cells (in 2 µg/ml puromyocin for two weeks), the cells were harvested and total RNA isolated. Relative *Mpdz* mRNA expression was assessed using a quantitative polymerase chain reaction (QPCR) assay as in previous work (Shirley et al., 2004). Knockdown of *Mpdz* mRNA expression was calculated relative to a general scrambled control that does not target any gene sequence in the mouse genome. Unlike an empty control vector, the scrambled bases (Table 2-1, underlined/italicized) ensure the control shRNA is capable of engaging the RNA-induced silencing complex to activate that RNAi pathway, but does not target any gene.

Behavioral Procedures

Intra-cISNr surgery and lentivirus microinjection

Mice were anesthetized using isoflurane (induced at a flow rate of 1.5 liter/min oxygen, 4% isoflurane, and maintained at 0.9 liter/min oxygen, 2.5% isoflurane), injected

with meloxicam (10 mg/kg ip, in 0.9% saline), and placed in a mouse stereotaxic instrument (Kopf Instruments). The skull surface was exposed and burr holes drilled at the appropriate coordinates. The clSNr coordinates were based on the Mouse Brain Atlas (Paxinos and Franklin, 2001) and empirically determined for D2 mice as follows: anterior-posterior (AP) = -3.1, medial-lateral (ML) = ±1.7, and dorsal-ventral (DV) = -4.5. In order to use green fluorescent protein (GFP) to assess targeting and spread, the *Mpdz* shRNA and scrambled control were subcloned into the pLKO.1-hPGK-puro-CMV-TurboGFP vector, then packaged into lentivirus to a high titer (10^8 titer units/ml; Sigma). Microinjectors were created as in previous work (Lasek and Azouaou, 2010) by affixing a 33 gauge stainless steel hypodermic tube within a shorter 26 gauge stainless steel hypodermic tube, and were attached to polyethylene-20 tubing affixed to a 10 µl Hamilton syringe. Microinjectors were lowered bilaterally to the designated coordinates and 1 µl (up to 10^5 titer units) per side microinjected over 5 min using an infusion pump, and left in place for an additional 10 min. During the two days post-surgery the mice received additional meloxicam and were monitored for potential weight loss, pain, and distress. Behavioral testing began three weeks later. Two replications of the RNAi experiment were performed 4 months apart.

Baseline and EWD HICs

Physiological dependence is characterized by the appearance of physical disturbances (withdrawal) after alcohol administration is suspended. Alcohol withdrawal induced seizures are a distinct measure of CNS physiological dependence occurring in both humans, as well as rodents. McQuarrie & Fingl (1958), first reported that EWD is apparent in mice following a single hypnotic dose of ethanol, and was later shown to be genetically determined (Crabbe et al., 1991; Goldstein and Kakihana, 1975). The

detection and fine-mapping of *Mpdz* utilized this acute model of EWD (Buck et al., 1997; Fehr et al., 2002; Shirley et al., 2004), which was therefore used in this experiment. D2 mice were used because they demonstrate robust EWD using acute models (Chen et al., 2008), allowing detection of increased *or* decreased EWD severity with experimental manipulation. Individual mice and different genetic models can differ in baseline HIC scores (Metten and Crabbe, 1994). Therefore, three weeks after surgery, mice were scored twice (30 min apart) for baseline (pre-ethanol) HICs prior to EWD testing. Immediately thereafter, mice received a single hypnotic dose of ethanol (4 g/kg, i.p., 20% v/v dissolved in 0.9% physiological saline) and were scored hourly between 2 and 12 h, and at 24 and 25 h post-ethanol. In order to create an index of EWD that is independent of individual differences in baseline HIC scores and that reflects differences in withdrawal convulsion severity, post-ethanol HIC scores were corrected for the individual's average baseline HIC score as done previously (Chen et al., 2008). Individual EWD severity scores were calculated as the area under the curve (AUC), which is calculated as a sum of the corrected HIC scores 2-12 h post-ethanol as in our previous work (Chen et al., 2011).

PTZ-enhanced HICs

One week following EWD in replication 2, mice were assessed for HICs in response to PTZ (30 mg/kg i.p.), which blocks GABA$_A$ receptor-mediated transmission, to determine whether *Mpdz* expression in the clSNr generally affects seizure susceptibility. Previous studies show that the severity of PTZ-enhanced HICs is not influenced by prior testing for EWD or surgery (Chen et al., 2008). This dose was used because it enhances HIC intensity without inducing other convulsions (e.g., tonic hindlimb extensor) associated with higher doses. Mice were scored for HICs at 10 time-points from 1 to 65

min post-PTZ injection as in previous work (Chen et al., 2008). PTZ enhanced HIC scores were corrected for individual differences in baseline HIC scores to create an index of response to PTZ independent of variability in individual baseline (pre-PTZ) scores. The PTZ-enhanced HIC severity scores were calculated as AUC scores and in previous work (Chen et al., 2011).

Histology and Mpdz gene knockdown

Laser capture microdissection (LCMD). Within 2 h of the completion of behavioral testing, *Mpdz* shRNA and control animals were sacrificed by cervical dislocation and brains immediately removed and frozen (-80°C) until sectioning (coronal, 30 μm). Alternating slices containing the SNr were mounted on glass slides (for histology and GFP analyses), and polyethylene napthalate membrane glass slides (for LCMD), and stored (-80°C). Slides were dried for 5 min, then placed in 0.1% thionin for 5 seconds (sec), washed twice in diethylpyrocarbonate treated water for 15 sec, washed in ethanol (75%, 95%, and 100%, 30 sec each), and dried for 5 min. For histology, slides were examined using a fluorescent microscope (Leica Microsystems, 10x) to assess injection placement and the extent of GFP expression; this produced a template for the region of interest (i.e., the clSNr) for LCMD from an adjacent section. Using LCMD (Leica Microsystems, 6.3x) four clSNr tissue samples per side were isolated, combined, and stored in 50 μl RNAlater until processing for expression analyses.

QPCR. Total RNA was isolated from NS20Y cells using Qiagen RNeasy mini kit and from LCMD clSNr samples using the Ambion RNAqueous-Micro Kit. cDNA synthesis used a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), with preamplification (*in vivo* experiment only) using a TaqMan PreAmp Master Mix kit and probes for *Mpdz* and a control gene (*Reep5). Reep5* was used because it is a

highly stable reference gene expressed throughout the mouse brain (www.brain-map.org) (Kozell et al., 2009).   QPCR utilized *Mpdz* and *Reep5* TaqMan probes (Mm00447849_m1 and Mm00492230_m1, respectively), and was performed on an ABI Prism7500 thermal cycler using TaqMan Universal PCR Master Mix and standard reaction conditions.   A standard relative quantification method ($\Delta\Delta C_t$) was used which corrects for run to run variability by normalization of target and reference gene expression to calibrator sample expression (preamplified cDNA pooled from all samples) (Livak and Schmittgen, 2001). The calibrator was set to "1" and gene expression in clSNr samples was evaluated in reference to the calibrator.

Statistical analyses

The RNAi behavioral and *Mpdz* expression data were normally distributed (Shapiro-Wilks test, $p > 0.05$), so two-sample t-tests were used for comparisons of the *Mpdz* shRNA and control groups for EWD severity, baseline and PTZ enhanced HIC severities, and *Mpdz* mRNA expression.   Data were analyzed using SYSTAT 13 Statistical Software and are presented as mean ± SEM.   Throughout, the significance level was set at $p < 0.05$ (two-tailed).

Results

Mpdz RNAi efficacy in NS20Y cells

The results for five *Mpdz* shRNAs tested for their efficacies to affect endogenous *Mpdz* expression in mouse NS20Y cells compared to a scrambled control are summarized in Table 2-1. BLAST analyses confirmed that all plasmid sequences were unique to the *Mpdz* gene. The scrambled control used was a validated negative control that engages the RNAi pathway but does not target any sequence in the mouse

genome. The TRCN0000103482 plasmid demonstrated the highest knockdown of *Mpdz* expression (77%) relative to the scrambled control, and was therefore used for the subsequent *in vivo* RNAi experiments.

Plasmid	Targeted Sequence	*Mpdz* expression
Mpdz shRNA (TRCN0000103480)	CCGGCGCACCAGAATGAGTGTGTTTCTCGAG*AAACACACTCATTCTGGTGCG*TTTTTG	1.05
Mpdz shRNA (TRCN0000103481)	CCGGGCTCTGATAGATACACCTGATCTCGAG*ATCAGGTGTATCTATCAGAGC*TTTTTG	0.24
Mpdz shRNA (TRCN0000103482)	**CCGGGCCTTCAGGAATCTTTGTAAACTCGAG*TTTACAAAGATTCCTGAAGGC*TTTTTG**	**0.23**
Mpdz shRNA (TRCN0000103483)	CCGGGCATCTGAAATTCAGGGACTACTCGAG*TAGTCCCTGAATTTCAGATGC*TTTTTG	0.62
Mpdz shRNA (TRCN0000103484)	CCGGCATCTGAAATTCAGGGACTAACTCGAG*TTAGTCCCTGAATTTCAGATG*TTTTTG	0.50
Scrambled control (SCH002)	CCGGCAACAAGATGAAGAGCACCAACTCGAG*TTGGTGCTCTTCATCTTGTTG*TTTTT	1.00

Table 2-1. ***Mpdz* knockdown in NS20Y cells.** Five *Mpdz* shRNA plasmids were assessed for relative knockdown of *Mpdz* expression in NS20Y cells compared to scrambled control as assessed using QPCR. Each hairpin sequence is comprised of a 21 base stem (with the 21 underlined bases indicating the antisense and 21 italicized bases indicated the sense sequences), and a 6 base loop. The antisense bases hybridize to the *Mpdz* sequence in Exon 10. The bolded *Mpdz* shRNA indicates the sequence plasmid selected for the *in vivo* experiment.

Confirmation of *Mpdz* RNAi targeted expression in the clSNr

    Mpdz shRNA (TRCN0000103482) and a scrambled control shRNA were subcloned into pLKO.1-hPGK-puro-CMV and packaged in lentivirus to a high titer (10^8 titer units/ml). Figures 2-1a and 2-1b show representative photomicrographs of the clSNr microinjection site at 4x and 10x magnification, respectively, evidenced by GFP expression. Figure 2-1c shows a representative LCMD tissue section of the clSNr. Isolated clSNr tissue samples were subsequently used for *Mpdz* messenger RNA (mRNA) quantification using QPCR (Fig. 2-4; results discussed below). Figure 2-1d is a schematic illustration of the extent of the bilateral clSNr microinjection sites and GFP expression spread, which extended from -3.1 to -3.8 mm (AP) from Bregma, with occasional spread into the SN lateralis (Paxinos and Franklin, 2001).
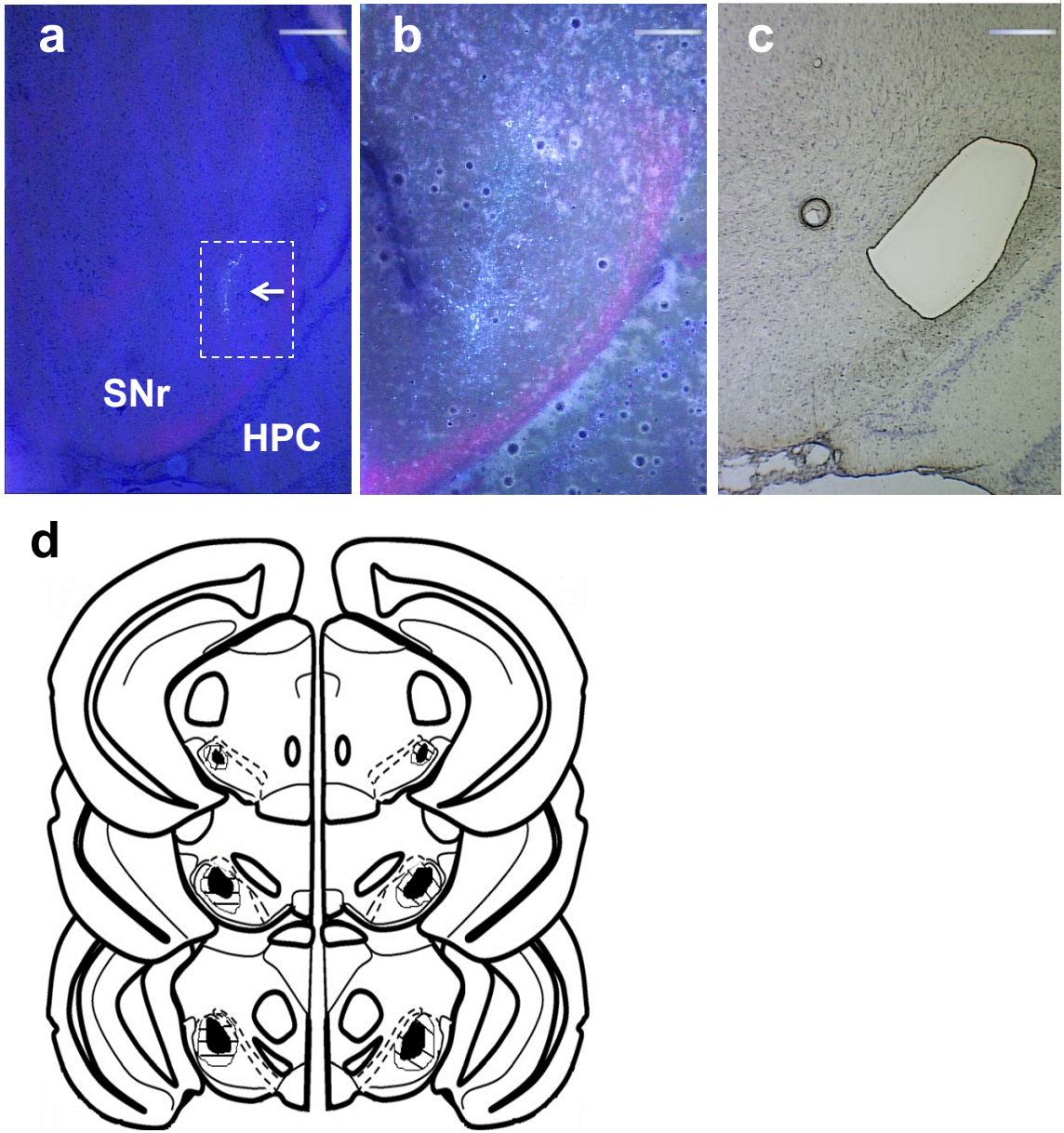
Figure 2-1. **Bilateral *Mpdz* RNAi targeting in the clSNr.** Representative photomicrograph showing fluorescence at the microinjection site (*arrow*) overlain a light photomicrograph to visualize structure and GFP expression at (a) 4X and (b) 10X (of the area outlined in white within panel a). (c) 4X Thionin stained light photomicrograph image with the clSNr dissected by LCMD. (d) Schematic diagram of a coronal brain section depicting microinjection sites and extent of GFP expression (indicating viral

transduction) in the clSNr. The darkened regions indicate the sites of clSNr microinjections from all confirmed bilateral hits, identified by GFP. The striped region indicates the extent of GFP spread (including the injection sites). Based on coordinates from the mouse brain atlas (Paxinos and Franklin, 2001), the confirmed microinjection sites and lentiviral spread ranged from -3.1 to -3.8 mm AP from Bregma in the clSNr. *Additional abbreviation*: HPC, hippocampus. Scale bars are located in the upper right hand corner of each panel.

Baseline and EWD HICs in *Mpdz* shRNA and control mice

Baseline (pre-ethanol) and EWD enhanced HICs were assessed in two studies conducted 4 months apart to attain sufficient animal numbers for analyses. Correct bilateral targeting of the clSNr was approximately 65%. Across both studies, for animals with confirmed bilateral targeting of the clSNr, *Mpdz* shRNA and control animals did not differ in baseline (pre-ethanol) HIC severity (0.5 ± 1.1 and 0.2 ± 0.6, respectively; p = 0.3). Statistical analyses confirmed that EWD was significantly more severe in *Mpdz* shRNA mice compared to scrambled controls (t_{28} = 3.0, p = 0.006, Fig. 2-2). Further using QPCR analyses, I demonstrated that this enhancement in EWD severity in *Mpdz* shRNA was associated with reduced expression of *Mpdz* (Fig. 2-2) in the clSNr, confirming *Mpdz* as a QTG for EWD risk, and identifying the clSNr as crucially involved.

In animals in which clSNr targeting was unilateral only, neither baseline HICs (0.4 ± 0.4 and 0.4 ± 0.7, respectively; p = 0.9) nor EWD severity (22.3 ± 7.4 and 24.3 ± 10.0, respectively, p = 0.5) differed between *Mpdz* shRNA and control animals (n = 14 and 20). This data was consistent with previous data showing that bilateral but not unilateral clSNr lesions mitigate EWD (Chen et al., 2011; Chen et al., 2008).
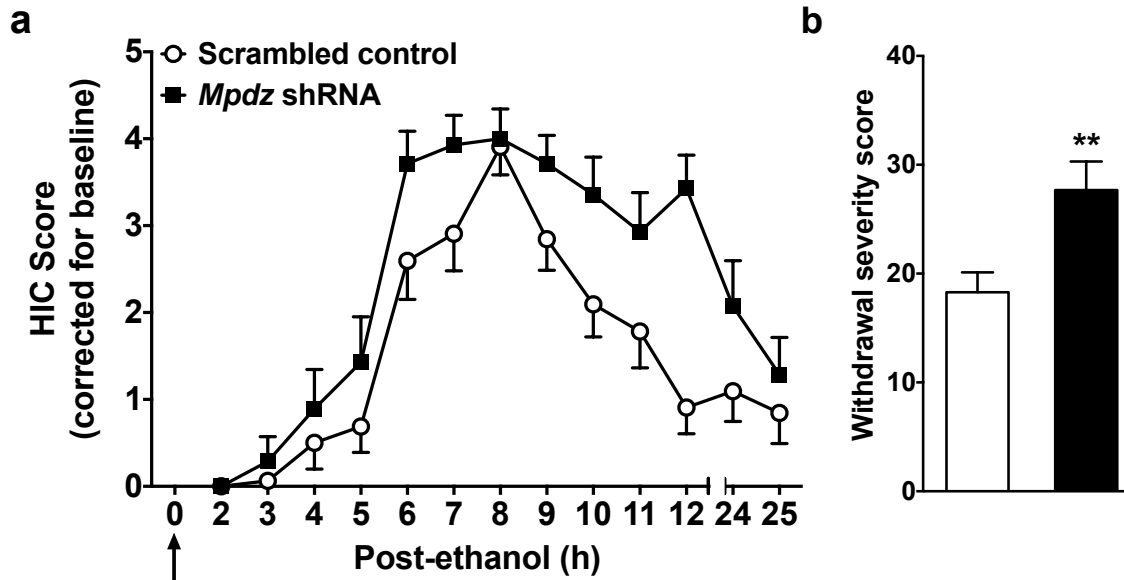
Figure 2-2. *Mpdz* **shRNA mice demonstrated more severe EWD compared to controls.** (a) HICs were assessed hourly from 2-12 h post-ethanol administration (*arrow*). Around 4 h post-ethanol, HIC scores increase above baseline, indicating a state of withdrawal hyperexcitability. (b) Individual withdrawal severity scores were calculated as the sum of the corrected HIC scores post-ethanol as in previous work (Chen et al., 2011). EWD was significantly more severe in *Mpdz* shRNA (n = 14) compared to control animals (n = 16). Data represent the mean HIC score values ± SEM (corrected for individual baseline scores) for mice with confirmed bilateral targeting of the cISNr.

PTZ-enhanced HICs in *Mpdz* shRNA and control mice

In order to assess whether *Mpdz* RNAi might have more general effects on enhanced HICs beyond EWD, the animals in the second study were assessed for PTZ-enhanced HICs. *Mpdz* shRNA and control animals with confirmed bilateral targeting of the clSNr did not differ in baseline (pre-PTZ) HIC scores (1.1 ± 1.5 and 1.4 ± 1.7, respectively, p = 0.7) or PTZ-enhanced HIC severity (p = 0.9; Fig. 2-3).
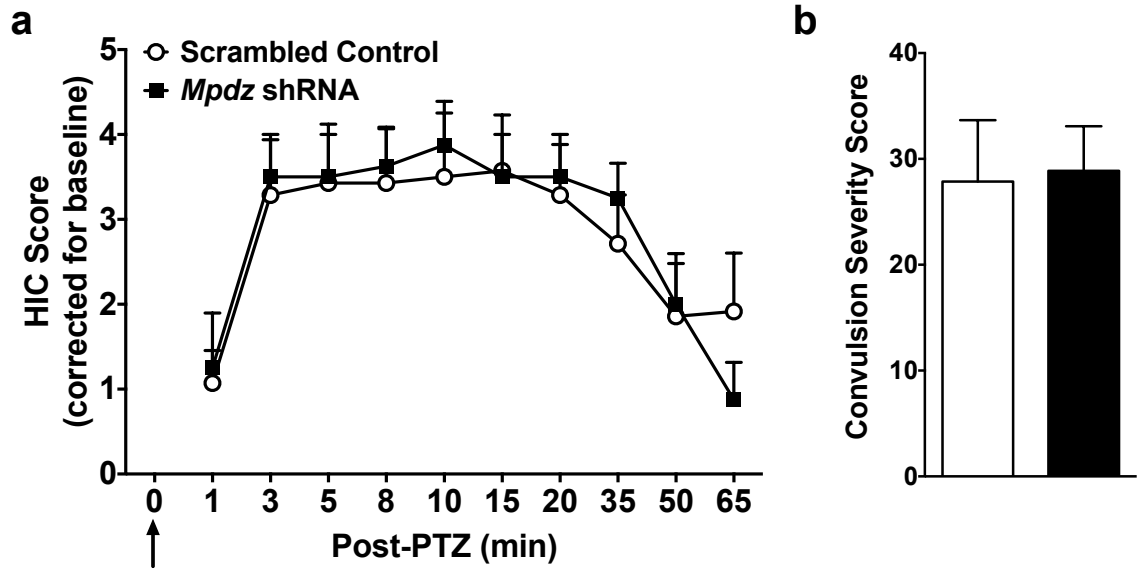
Figure 2-3. *Mpdz* **shRNA and control mice did not differ in PTZ-enhanced HICs.**
(a) HICs were assessed at 10 time-points from 1 to 65 min post-PTZ administration
(*arrow*). (b) Individual HIC severity scores were calculated as the sum of the corrected
HIC scores post-ethanol as in previous work (Chen *et al.*, 2011). PTZ-enhanced
convulsions did not differ between *Mpdz* shRNA (n = 8) compared to scrambled control
(n = 7) animals. Data represent the mean HIC score values ± SEM for mice with
confirmed bilateral targeting of the clSNr.

Mpdz expression in the clSNr of _Mpdz_ shRNA and control mice

As indicated in Figure 2-4, _Mpdz_ targeted RNAi significantly reduced _Mpdz_ mRNA expression in the clSNr compared to scrambled control animals. _Mpdz_ mRNA was reduced by 33% in the first replication of the RNAi experiment in _Mpdz_ shRNA compared to control animals (0.59 ± 0.04 and 0.88 ± 0.05, respectively, t_{28} = 4.2, p = 0.0002). A significant reduction in _Mpdz_ mRNA was also apparent in the second replication conducted approximately four months later in _Mpdz_ shRNA compared to control animals (1.15 ± 0.08 and 1.53 ± 0.07, respectively, t_{28}= 3.6, p = 0.001), and, not surprisingly, was somewhat smaller in magnitude (25% decrease) than in the first replication owing to a known reduction in titer over time.
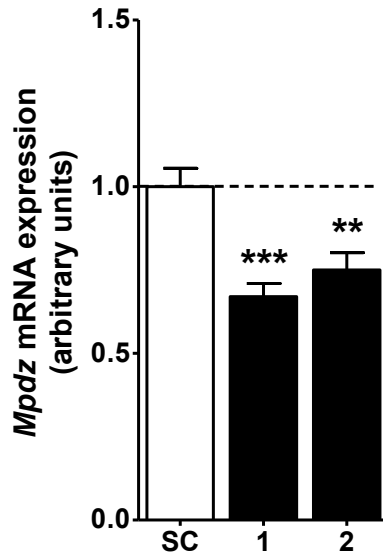
Figure 2-4. *Mpdz* **expression in the clSNr was reduced in** *Mpdz* **shRNA compared to scrambled control mice.** Data presented are for animals with confirmed bilateral targeting of the clSNr (Fig. 2-1d). *Mpdz* expression in LCMD clSNr of *Mpdz* shRNA animals was significantly reduced across two replications of the RNAi experiment conducted 4 months apart compared to scrambled control (SC) animals. Expression was reduced by approximately 33% in the first replication, and 25% in the second replication. **p < 0.01; ***p < 0.001 indicate a significant reduction compared to scrambled control.

Discussion

Naturally occurring genetic (allelic) variation affects *Mpdz/MPDZ* sequence and expression in mice (Fehr et al., 2002) and humans (Karpyak et al., 2009; Tabakoff et al., 2009), but the functional and behavioral consequences remain largely unknown. Association studies have implicated the mouse and human homologs in risk for alcoholism (Fehr et al., 2002; Karpyak et al., 2009; Shirley et al., 2004; Tabakoff et al., 2009). However, despite the recent creation of transgenic and knockout heterozygote mouse models (Milner et al., 2015), direct evidence that *Mpdz* affects these (or any) behavior has been lacking. In this chapter, I therefore demonstrated the first use of the *in vivo Mpdz* RNAi animal model, and provided the first direct evidence that *Mpdz* expression directly affects behavior. Specifically, I demonstrated that *Mpdz* targeted RNAi effectively reduced *Mpdz* expression in the clSNr, resulting in more severe EWD compared to control animals, with no change in baseline and PTZ-enhanced convulsions. These data provided further evidence that *Mpdz* is a QTG affecting predisposition to EWD.

The SNr is a main output region of the basal ganglia circuit and has been implicated in CNS hyperexcitability including EWD (Chen et al., 2008) and limbic seizures (Shehab et al., 1996; Veliskova et al., 2005). A growing body of literature has demonstrated that the caudal and rostral subregions of the SNr may be functionally distinct in their regulation of EWD as well as related limbic seizure phenotypes (Shehab et al., 1996; Veliskova et al., 2001; Veliskova et al., 2005). The caudal SNr, which is thought be active during pre-clonic seizures, may be involved in seizure initiation/propagation, and has been shown to be a particularly sensitive target for the suppression of tonic seizures. In contrast, the rostral subregion of the SNr has been shown to become activated while a seizure is occurring (Shehab et al., 1996; Veliskova et al., 2005).

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Interestingly, my studies found that intra-clSNr *Mpdz* RNAi substantially impacted EWD, but not baseline or PTZ-enhanced HICs, demonstrating that the clSNr, and particularly *Mpdz* expression within the clSNr, plays an important role in EWD but does not generally affect seizure susceptibility. Using LCMD to discretely isolate the clSNr allowed precise quantification of *Mpdz* mRNA expression in this region, but not protein content due to the low amount of tissue isolated. However, previous studies have demonstrated that reduced *Mpdz* mRNA expression is associated with a comparable reduction in MUPP1 protein expression in the brain (Shirley et al., 2004), and studies in Chapter 3 further corroborated this finding. On average, approximately 50-70% of the clSNr appeared to be transduced with the shRNA. These results are therefore consistent with and build upon previous data showing that clSNr (but not rostral SNr) lesions mitigated EWD but did not affect baseline or PTZ enhanced HICs (Chen et al., 2011; Chen et al., 2008), and further support a functional specificity of subregions of the SNr proper (Deniau and Chevalier, 1992; Shehab et al., 1996; Veliskova et al., 2005). Together, these studies indicate some specificity of the role of the clSNr in mediating EWD, rather than all HIC phenotypes. Further, the present work supports previous ITF studies which demonstrated greater c-Fos expression in the SNr during acute EWD in chromosome 4 congenic compared to noncongenic (i.e., D2 background strain) mice (Chen et al., 2008). These studies consistently demonstrated that the clSNr subregion plays a crucial role in mediating EWD convulsions, and confirmed previous studies that showed that *Mpdz* expression is inversely related to EWD severity.

RNAi mediated gene silencing through viral transduction is a well-established method for discretely silencing single genes of interest (Xia et al., 2002). Commonly used viruses for RNAi transfection include lenti- and adeno-associated viruses, the former of which was applied in my RNAi experiments. One possible limitation of

lentiviral-mediated transfection of shRNAs was the potential for insertional mutagenesis during integration into the chromosome or activation of innate immunity through the interferon response, both of which could produce off target gene effects influencing the phenotype of interest (Manjunath et al., 2009). However, this is unlikely as I clearly demonstrated that the effect of reduced *Mpdz* on EWD and PTZ-enhanced HICs were in line with previous work in *Mpdz* congenic, transgenic, and knockout heterozygote models (Milner et al., 2015; Reilly et al., 2008), and the behavioral experiments lasted less than one month, whereas these confounds are more of a concern with long-term transfection. Further, it was possible that the shRNA sequence alone produced off target gene effects. Again, this is unlikely because I confirmed that the effect of knockdown of *Mpdz* on EWD parallels previous research (Milner et al., 2015), and BLAST was used to ensure the sequence was unique to *Mpdz* and did not match any other gene sequences in the mouse genome. Therefore, RNAi mediated knockdown of *Mpdz* through lentiviral transfection was an efficient and successful technique for confirming *Mpdz* as a QTG for predisposition to EWD.

Beyond these experiments, no studies have examined the direct effect of *Mpdz* on behavior or associated neurocircuitry, but numerous *in vitro* studies have begun to identify MUPP1 association partners, providing necessary insight into protein function (Balasubramanian et al., 2007; Baliova et al., 2014; Griffon et al., 2003; Guillaume et al., 2008; Krapivinsky et al., 2004; Li et al., 2012; Ullmer et al., 1998). In this chapter, I provided clear evidence that reduced *Mpdz* expression in the clSNr is associated with an enhancement in EWD, but the mechanism (i.e., receptors and/or proteins) through which *Mpdz* affects EWD remains unclear. MUPP1 associates with GABA$_B$Rs, and these receptors are functionally impacted by MUPP1 (Balasubramanian et al., 2007), with recent studies demonstrating that the degree to which a selective GABA$_B$R agonist

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enhances HICs may be dependent upon QTL/*Mpdz* status (Reilly et al., 2008). Interestingly, GABA$_B$Rs are expressed both pre- and postsynaptically in the SNr and mediate inhibitory and excitatory signaling in this region (Rick and Lacey, 1994; Shen and Johnson, 1997), but their effect in EWD is unknown. Taken together with the knowledge that MUPP1 may mediate GABA$_B$R function (Balasubramanian et al., 2007; Reilly et al., 2008) which has been implicated in EWD (Humeniuk et al., 1994) and alcohol consumption (Colombo et al., 2002; Colombo et al., 2000), this interaction could be one potential mechanism contributing to the role of *Mpdz* in EWD and the dysregulation of clSNr signaling. Future studies will be important to begin to identify the functional importance of MUPP1 interactions with GABA$_B$Rs on EWD, as well as alcohol consumption to further elucidate the role of *Mpdz* in AUDs.

Although the exact mechanism is unknown, lower *Mpdz* expression may predispose to a dysregulation of normal clSNr inhibitory signaling onto output structures that becomes apparent during acute EWD, but not baseline or generalized CNS hyperexcitability, which presents as more severe withdrawal. Assessment of acute EWD is therefore important for identifying genetic and associated neurobiological factors that may predispose to future onset of AUDs.

Several recent clinical studies have identified a translational role for *Mpdz* in dependence. A post-mortem study identified a significant reduction of *MPDZ* expression in the hippocampus of dependent individuals, consistent with my work demonstrating that reduced expression was associated with enhanced EWD (McClintick et al., 2013). Further, recent association studies using human clinical populations also implicated *MPDZ* in alcohol consumption, as well as dependence (Gizer, 2011; Tabakoff et al., 2009) in a manner that may be dependent upon past alcohol withdrawal seizures (Karpyak et al., 2009). Recent preclinical studies have demonstrated that *Mpdz*^{+/-}

voluntarily consume less ethanol than WT littermates in 2BC-CA preference drinking, providing the first evidence that *Mpdz* may also influence ethanol consumption, preference, and/or reward (Milner et al., 2015). The independent identification of *Mpdz/MPDZ* in two different species as contributing to distinct phenotypes of AUDs supports a conserved and therefore possibly integral role of this gene in predisposition to alcohol dependence.

   To my knowledge, these studies were the first to employ RNAi techniques for targeted gene expression to advance the understanding of the important interplay between brain region specific gene expression and its influence on the severity of EWD. These studies provided clear evidence that *Mpdz* is a QTG for EWD, and demonstrated that its expression within the clSNr is crucially involved. Further, taken together with previous work (Chen et al., 2011; Chen et al., 2008), these studies support a critical role of the clSNr in modulation of EWD severity. Importantly, while the present RNAi studies were performed in D2 mice, evidence supporting the role of *Mpdz* in EWD is not limited to this strain. Recent work demonstrated that *Mpdz*^{+/-} (on a B6 background) also show increased EWD severity (Milner et al., 2015). Though this work contributed significantly to the understanding of the genetic determinants of withdrawal, results are based on a single measure of EWD, and future studies will be needed to assess the broader role of *Mpdz/*MUPP1 in additional measures of EWD (e.g., anxiety-like and depression-like behaviors), ethanol drinking and preference, and beyond to further understand the role of *Mpdz* in susceptibility for AUDs. The effect of *Mpdz* on risk for AUDs was of particular interest since drugs with known MUPP1 partners (e.g., GABA$_B$R and serotonin receptors) as an initial target are currently being used in clinical trials evaluating their efficacies to reduce craving, withdrawal, and relapse (Addolorato et al., 2011; Leggio et al., 2013). As more information becomes available on precise MUPP1 partner

interactions and the relevant neural circuit, the mechanism by which MUPP1 regulates EWD and its contribution to ethanol drinking, preference, and reward will become apparent and could be useful for identifying targets for new pharmacotherapies for AUDs.

Chapter 3: The role of GABA$_B$ receptors in ethanol withdrawal and MUPP1 effects

The data presented in this chapter are unpublished.

Introduction

MUPP1 is a unique PDZ-domain containing protein that physically associates/complexes with and influences the functioning of a highly diverse array of receptors and other proteins via its thirteen PDZ domains (Balasubramanian et al., 2007; Baliova et al., 2014; Dooley et al., 2009; Griffon et al., 2003; Krapivinsky et al., 2004; Ullmer et al., 1998). MUPP1 and other PDZ domain containing proteins are crucially involved in mediating function of the cell signaling pathways of the proteins and receptors with which they interact (Sheng and Sala, 2001; Simpson et al., 1999). Recent evidence has posited MUPP1 as an important regulator of G-protein coupled receptor function. Specifically, MUPP1 has been shown to alter G-protein receptor signaling through several mechanisms including altered coupling efficiency of G-proteins to their cognate receptor, plasma membrane localization, receptor signaling duration, receptor stability, and cell clustering (Balasubramanian et al., 2007; Becamel et al., 2001; Dooley et al., 2009; Guillaume et al., 2008), but the translational impact of these interactions is unknown.

The GABA$_B$R has been implicated in a wide range of CNS behaviors including (but not limited to) EWD, CNS hyperexcitability, the rewarding effects of ethanol, ethanol consumption, locomotor depression, and ethanol-induced locomotor stimulation (Bechtholt and Cunningham, 2005; Boehm et al., 2002; Colombo et al., 2002; Gianutsos and Moore, 1978; Humeniuk et al., 1994; Reilly et al., 2008). This receptor has been shown to associate with MUPP1 PDZ13 through the C-terminus of its R2 subunit

(GABBR2). Further, recent *in vitro* and behavioral work identified a putative effect of *Mpdz*/MUPP1 on GABA$_B$R function. In cultured cells transfected with the GABA$_B$R, disruption of the MUPP1-GABBR2 association reduced receptor stability (e.g., half-life) as well as duration of receptor signaling (e.g., increased decay of calcium signaling) (Balasubramanian et al., 2007). However, it is widely known that GABA$_B$Rs in cultured neurons and particularly transfected cells may function differently from native receptors. In fact, a recent study conducted in the brain actually demonstrated an association between reduced MUPP1 and enhanced GABBR2 expression as assessed using immunoreactivity and immunoblot analyses in the cerebellum of Cadm1 (cell adhesion molecule 1) knockout mice, suggesting GABA$_B$Rs may actually be increased with reduced MUPP1 (Fujita et al., 2012). This effect of MUPP1 may also translate to behavior as baclofen-enhanced HICs, a measure of CNS hyperexcitability mediated by GABA$_B$Rs, are affected in a chromosome 4 QTL dependent manner (Reilly et al., 2008). Together, these studies showed that the association of MUPP1 and GABA$_B$Rs has important implications for GABA$_B$R function and putatively associated behavior. However, a more direct assessment of the effect of MUPP1 on native GABA$_B$R function has not been previously performed, and no studies have examined the association between altered MUPP1 expression and GABA$_B$R-mediated behaviors. Thus, the functional and behavioral consequences of this association are currently unknown.

Genetic animal models commonly used to study the effect of target genes on behaviors include knockout, transgenic, and RNA interference models, which have proven invaluable for identifying genetic bases for complex disorders such as AUDs (Milner et al., 2015; Picciotto and Wickman, 1998; Thiele et al., 2004). Recently, the complementary use of *Mpdz* transgenic, knockout heterozygote, and RNAi genetic models confirmed *Mpdz* as a QTG for EWD, and demonstrate that lower expression of

Mpdz/MUPP1 is associated with increased risk for withdrawal (Kruse et al., 2014; Milner et al., 2015) (Chapter 2). Moreover, *Mpdz* expression within the clSNr is crucially involved. To my knowledge, these were the first studies to identify a *direct* association between *Mpdz* and behavior, but the specific mechanism through which *Mpdz*/MUPP1 affects any behavior, and particularly EWD, is unknown. GABA$_B$Rs are also expressed in the SNr (Bowery et al., 1987) both pre- and postsynaptically and mediate inhibitory and excitatory signaling in this region (Rick and Lacey, 1994; Shen and Johnson, 1997). Given that GABA$_B$Rs have been implicated in EWD (Humeniuk et al., 1994) and baclofen (a selective GABA$_B$R agonist) enhances HICs in a QTL/*Mpdz* dependent manner (Reilly et al., 2008), it is intriguing to speculate that mechanism of action of *Mpdz* in EWD (and perhaps beyond) may involve its effect on GABA$_B$R function.

Therefore, I examined the hypotheses that clSNr GABA$_B$Rs may mediate the severity of EWD, and that reduced expression of *Mpdz*/MUPP1 would be associated with heightened response to baclofen to begin to elucidate the potential effect of MUPP1 on this receptor. Using pharmacological manipulations of GABA$_B$R activity in the clSNr, I assessed the potential involvement of GABA$_B$Rs in the clSNr in EWD, a region in which *Mpdz* is involved in this phenotype (Kruse et al., 2014). Further, through the use of novel *Mpdz*$^{+/-}$ and *Mpdz*^{Tg} mouse models, I examined the hypothesis that altered MUPP1 expression may be associated with changes in a GABA$_B$R-mediated behavior related to CNS hyperexcitability. Finally, through the implementation of neurophysiological and molecular analyses in whole brain and SNr of *Mpdz*$^{+/-}$ mice, I began to elucidate the mechanism through which altered *Mpdz*/MUPP1 expression may be associated with changes in GABA$_B$R-mediated responses.

Materials and methods

Subjects

Male D2 mice from Jackson Laboratories (Bar Harbor, ME) were used to assess the role of clSNr GABA$_B$Rs in EWD. Upon arrival, D2 mice were acclimated to the vivarium for a minimum of one week, and were 60-67 days old at the time of surgery.

Male and female single gene knockout mice were created from a mouse ES cell line with an insertional mutation in MUPP1 (XG734, strain 129/Ola) in *Mpdz* intron 11-12, truncating MUPP1 after its third PDZ domain. The ES cell line was injected into B6 blastocysts to create chimeric mice which were backcrossed to B6 strain mice until the genetic background was estimated to be >98% B6 to generate the finished model B6-*Mpdz*$^{Gt(XG734)Byg(+/-)1KB}$ abbreviated as *Mpdz*$^{+/-}$ and WT littermates (Milner et al., 2015). These mice were used to assess the role of MUPP1 on GABA$_B$R function in behavioral, molecular, and neurophysiological analyses. Mice were approximately 60-90 days old for all experiments, except the neurophysiological analyses where mice were 22-32 days old. The experimental *Mpdz*$^{+/-}$ and WT littermates were bred in house, generated from *Mpdz*$^{+/-}$ X B6 backcross breeding as in our previous work (Milner et al., 2015).

Male and female *Mpdz* Transgenic mice were created from a B6 strain bacterial artificial chromosome (BAC) clone (RPCI-23-119B7 purchased from Research Genetics), which contained *Mpdz*. A linearized construct of the BAC clone was microinjected into B6D2F$_1$ zygotes. A founder mouse harboring the transgene was backcrossed to D2 strain mice until the genetic background was estimated to be ≥98% D2. Intercross breeding generated the finished homozygote model [D2-Tg(Rp23-11B7)^{1KB}] referred to hereafter as *Mpdz*^{Tg} and nontransgenic WT littermates, and further breeding was conducted in house as in our previous work (Milner et al., 2015). *Mpdz*^{Tg}

homozygotes, heterozygotes, and WT, 70-100 days old, were used to begin to assess the potential role of MUPP1 on baclofen-enhanced HICs.

All mice were housed in polycarbonate cages with *ad libitum* food (LabDiet 5001 Rodent Diet) and water in the colony and procedure rooms maintained on a 12 h light/dark cycle with lights on from 0600 to 1800, and temperature maintained at 21 ± 1˚C. All procedures were approved by the Oregon Health & Science University and VA Medical Center Institutional Animal Care and Use Committees in accordance with the United States Department of Agriculture and United States Public Health Service guidelines.

Reagents and Drugs

All reagents for the neurophysiology experiments were purchased from Sigma Chemicals (St. Louis, MO) except D-AP5 (D-(-)-2-amino-5-phosphonopentanoic acid), bicuculline, GABAzine (SR 95531 hydrobromide or 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide), kynurenic acid, and NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt) (all from Ascent Scientific, UK) and iodoacetic acid (Acros/Fisher). For quantitative western blots, MUPP1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary mouse monoclonal antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX) and alkaline-phosphatase conjugated goat anti-mouse secondary antibody was obtained from Biorad (Hercules, CA). For the radioligand receptor binding assay, [^{3}H]CGP54626 was purchased from American Radiolabeled Chemicals (St. Louis, MO) and baclofen was purchased from Tocris (Minneapolis, MN).

For the behavioral experiments, pharmaceutical grade baclofen and CGP55845 hydrochloride were purchased from Tocris, two-hundred proof ethanol was purchased

from Decon Laboratories, Inc. (King of Prussia, PA), and PTZ was purchased from Sigma Aldrich (St. Louis, MO). For systemic administration, baclofen, PTZ, and ethanol were dissolved in physiological saline (0.9%). For microinjections, baclofen was dissolved in artificial cerebral spinal fluid (ACSF) and CGP55845 was dissolved in ACSF plus 0.9% dimethyl sulfoxide. ACSF contained (in mM): 126 sodium chloride (NaCl), 21 NaHCO$_3$, 1 monosodium phosphate (NaH$_2$PO$_4$), 1 magnesium sulfate, 2.5 potassium chloride (KCl), 2.6 calcium chloride (CaCl$_2$), and 11 D-glucose (pH 7.4).

Procedures

clSNr cannulation surgery

Male D2 strain mice were anesthetized using isoflurane (induced at 1.5 liter/min oxygen and 4% isoflurane, and maintained at 0.9 liter/min oxygen containing 2.5% isoflurane), and were administered meloxicam (5 mg/kg, i.p., in physiological saline) immediately following induction to mitigate potential post-surgical pain. Mice were then placed in the stereotaxic apparatus (Kopf Instruments, Tujunga, CA). Ketac Conditioner (3M ESPE, St. Paul, MN) was applied to exposed skull surface then rinsed with saline prior to burr holes being drilled at the following bilateral clSNr coordinates in D2 mice as in previous work (Kruse et al., 2014): AP = -3.1, ML = ± 1.7, DV = -4.5. Guide cannulas were lowered simultaneously to 2 millimeters (mm) above the DV coordinates, followed by application of Ketac Fill Plus Applicap Cement to the skull to secure the guide cannula in place. Guide cannulas were fitted with stylets to prevent clogging prior to behavioral testing, which began one and a half weeks later. During the two days post-surgery mice received additional meloxicam injections and were monitored for potential weight loss, pain, and distress.

Intra-cISNr baclofen and CGP55845 in ethanol-withdrawn and control D2 mice

Prior to behavioral testing, cannulated mice were assessed twice (30 min apart) for baseline (pre-drug) HICs as in previous work (Chen et al., 2008; Kruse et al., 2014). Mice were then administered ethanol (4.0 g/kg, i.p., 20% v/v in physiological saline) or saline at 0 h, and scored for HICs hourly from 2-6 and 8-12 h post-injection. Immediately after HIC scoring at 6 h, mice were placed in a holding cage and microinjected with baclofen (20 ng/0.5 µl per side or 0.1 nanomole [nmol]/0.5 µl) or vehicle (ACSF) (final n = 4-5 per treatment group) with the injectors left in place for 1 min to allow sufficient diffusion into the tissue. All mice were then returned to home cages after the microinjection and scored for HICs every 20 min between 6-8 h. This was the first work assessing the effect of intra-cranial baclofen on HICs, so this dose of baclofen was chosen based upon previous work showing that microinjection of comparable doses was effective in eliciting behavioral effects (Boehm et al., 2002; Moore and Boehm, 2009; Zhou et al., 2005). Because HIC scores are typically highest approximately 6-8 h post-ethanol administration in D2 strain mice (Chen et al., 2011; Chen et al., 2008; Kruse et al., 2014), baclofen was microinjected immediately after the 6 h HIC assessment to assess the effect when EWD was highest and because the half-life of baclofen in blood is relatively short (120 min) (Mandema et al., 1992). In order to provide a sensitive measure of the effect of baclofen on HICs, and since previous work has shown that microinjections of baclofen may influence behavior for up to 2 h (Moore and Boehm, 2009; Zhou et al., 2005), HICs were assessed every 20 min for 2 h after baclofen (or vehicle) microinjection. A separate group of cannulated D2 mice (final n = 9-11 per treatment group) underwent the same procedure described above, except that mice were microinjected with CGP55845 (100 µmol/0.5 µl per side) or vehicle (ACSF

containing 0.9% dimethyl sulfoxide). This dose of CGP55845 was chosen as it has previously been shown to affect seizures when microinjected (Chen et al., 2004).

To create a withdrawal severity score independent of baseline scores, individual post-injection HIC scores were corrected for the individual's average baseline score as in previous work (Chen et al., 2008; Chen et al., 2011; Kruse et al., 2014). EWD associated HIC severity scores were then calculated as the sum of the corrected HIC scores from 6.2-12 h to assess the effect of baclofen administration on control HICs and EWD. For analyses, separate two-way ANOVAs were used with baseline HIC score, HIC severity score, or peak HIC score as the dependent variables, and systemic treatment (saline or ethanol) and intra-clSNr treatment (vehicle or baclofen; vehicle or CGP55845) as the independent variables.

Intra-clSNr CGP55845 and PTZ-enhanced HICs in D2 mice

The intra-clSNr CGP55845 study in control and ethanol withdrawn mice was conducted across two passes, and one week following intra-clSNr CGP55845 in the second pass the same mice were tested for the role of clSNr GABA$_B$Rs in PTZ-enhanced HICs. Mice were counterbalanced for intra-clSNr treatment. Previous studies showed that the severity of PTZ-enhanced HICs is not influenced by prior testing for EWD or surgery (Chen et al., 2008). The following time-course for assessment of PTZ-enhanced HICs was based on previous work (Chen et al., 2008; Kruse et al., 2014) but with the addition of several time-points as a more sensitive measurement of the effect of CGP55845 on PTZ-enhanced HICs. Mice were scored twice for baseline HICs, then administered 30 mg/kg dose of PTZ (i.p. at 0 min), and subsequently scored for HICs at 1, 3, 5, 8, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 65 min post-PTZ. This dose was used because it enhances HIC intensity without inducing other convulsions (e.g., tonic

hindlimb extensor) associated with higher doses. Immediately following HIC scoring at 10 min, mice were microinjected with CGP55845 (100 µmol/0.5 µl per side) or vehicle (ACSF containing 0.9% dimethyl sulfoxide). CGP55845 was microinjected at 10 min post-PTZ when HIC scores are high, for direct comparison with the effect of CGP54626 on EWD. PTZ-enhanced HIC severity scores were calculated as the sum of the corrected HIC scores for 15-65 min to assess the effect of CGP55845 compared to saline (final n = 8-12 per treatment group) on PTZ-enhanced HICs. Baseline HIC scores and HIC severity scores were analyzed using a two-sample t-test (two-tailed).

Histology for intra-clSNr procedures

Within one week of completion of the intra-clSNr experiments, mice were euthanized by cervical dislocation and decapitation. Brains were quickly removed (<1 min) and stored in ice-cold 4% paraformaldehyde for 24 h, then transferred to ice-cold 30% sucrose, where they remained until sliced coronally (30 µm sections) using a Leica Cryostat. The location of microinjector placement was assessed in brain slices containing the SNr by visually detecting tissue degradation under a microscope.

Baclofen-enhanced HICs

The HIC scale is used to assess changes in centrally mediated neuronal hyperexcitability in response to EWD or chemiconvulsant drug administration. GABA$_B$R-mediated HICs were indexed on a scale from zero to seven (Table 1) as in previous work (Reilly et al., 2008). Female and male *Mpdz*$^{+/-}$ and WT littermates (n = 3-7 genotype/sex/treatment for both doses), as well as female and male *Mpdz*^{Tg} and WT littermates (n = 4-8/genotype/sex), were assessed for GABA$_B$R-mediated HICs (i.e., baclofen-enhanced HICs). Following 1 h acclimation to the testing room, mice were

scored twice (30 min apart) for baseline HICs. Half of $Mpdz^{+/-}$ and WT littermates were then administered baclofen (5 mg/kg, i.p.) and the other half received saline (at time 0) as a control. One week later, half of the $Mpdz^{+/-}$ and WT littermates were then administered baclofen (10 mg/kg, i.p.) and the other half received saline (at time 0). Baclofen and saline injections were counter-balanced across week. These doses were chosen based on previous work (Reilly et al., 2008). Two doses of baclofen were assessed in the $Mpdz^{+/-}$ and WT littermates because B6 background mice are not highly susceptible to chemiconvulsant HICs, and therefore a higher dose (10 mg/kg) was tested in order to elicit a larger response (Kosobud and Crabbe, 1990). All of the $Mpdz^{Tg}$ and WT littermates received 5 mg/kg (i.p.) baclofen. Only one dose of baclofen was assessed in $Mpdz^{Tg}$ homozygotes, heterozygotes, and WT littermates since D2 background mice are highly susceptible to chemiconvulsant induced HICs (Kosobud and Crabbe, 1990; Reilly et al., 2008). All mice were then assessed for HICs at 15, 30, 45, 60, 75, 90, 120, 150, and 180 min post-baclofen administration based on previous work (Reilly et al., 2008).

In order to account for potential individual and genetic model differences in baseline HIC scores and to create a HIC severity score independent of baseline, individual post-injection HIC scores were corrected for the individual's average baseline. Baclofen-enhanced HIC severity scores were then calculated as the sum of the corrected HIC scores to create an overall AUC score for overall HIC severity as in previous work (Reilly et al., 2008). Comparisons of the effect of 5 mg/kg or 10 mg/kg baclofen in $Mpdz^{+/-}$ and WT littermates were analyzed separately using a three-way ANOVA with HIC severity score as the dependent variable and genotype, sex, and treatment (baclofen or saline) as the independent variables. The two doses of baclofen were analyzed separately because they were assessed in different weeks so the mice

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received different treatments. Further, I was not interested in comparing responses across doses. For comparisons of *Mpdz^Tg* and WT littermates, data were analyzed using a two-way ANOVA with HIC severity score as the dependent variable and genotype (*Mpdz^Tg* homozygotes, heterozygotes, and WT littermates) and sex as the independent variables.

Locomotor activity

Female and male *Mpdz^+/-* and WT littermates (n = 3-6/genotype/sex/treatment) were compared for locomotor depression in response to four doses of baclofen using a standard three-day locomotor activity paradigm (Kruse et al., 2012). Eight automated locomotor activity monitors (AccuScan Instruments Inc., Columbus, OH) were used as in previous work (Kruse et al., 2012). These consist of a 40 x 40 x 30 centimeter (cm) sound-attenuating clear acrylic test chambers enclosed in an Environmental Control Chambers constructed from PVC/lexan, and were equipped with a fan for background noise and ventilation. Chambers were illuminated with a 3.3 Watt incandescent light bulb during activity testing. Locomotor activity data was determined by eight pairs of intersecting photocell beams (two cm above the chamber floor) evenly spaced along the walls of the chambers. Beam breaks were used by VERSADAT software to determine total horizontal distance travelled (in cm). On Days 1 and 2, habituation and baseline days, respectively, mice were administered saline (i.p.) immediately prior to placement in the activity chambers for 30 min. On Day 3, mice were pseudo-randomly divided into five treatment groups based on Day 2 (baseline) locomotor activity levels, and received a 0 (saline), 1, 2, 4, or 10 mg/kg (i.p.) dose of baclofen immediately prior to placement in the activity chambers for 30 min. Further, the range of doses was chosen to assess the

effect of *Mpdz* on doses that did and did not induce locomotor depression, based on previous work (Gianutsos and Moore, 1978; Villas Boas et al., 2012).

Locomotor activity data were analyzed using a three-way ANOVA with the number of horizontal beam breaks (in cm) during the total 30 min locomotor activity session used as the dependent variable, and genotype, sex, and baclofen dose (0, 1, 2, 4, and 10 mg/kg) used as the independent variables.

Quantitative western blots.

MUPP1 protein expression was compared in female and male *Mpdz$^{+/-}$* and WT littermates (n = 6 per genotype). Protein was isolated by homogenizing individual frozen whole brains from adult female and male *Mpdz$^{+/-}$* and WT mice in 10 millimolar [mM] Tris-hydrochloride (Tris-HCl), 150 mM sodium chloride (NaCl), and 0.5% Nonidet P-40 buffer containing protease inhibitors using a polytron (setting 6) for 10-15 sec; then lysed (1 h on ice) and centrifuged at 13,000 x *g* (10 min at 4˚C). The pellet was then discarded and the supernatant was used for the following steps. Each lysate was quantified using a bicinchoninic acid (BCA) protein assay with bovine serum albumin as the standard. Individual samples were diluted to approximately 40 μg protein/15 μl, boiled for 15 min in Laemmli buffer (with 5% β-mercaptethanol), loaded onto a 7.5% Tris-HCl polyacrylamide gel for electrophoresis (104 volts for 2 h), and transferred to a polyvinylidene fluoride membrane (18 h at 4˚C). Membranes were blocked in Tris-buffered saline with 0.1% Tween-20 buffer containing 5% nonfat dry milk for 1 h at 4˚C. All antibodies were diluted in blocking buffer and incubated for 1 h, except the MUPP1 primary antibody (18 h at 4˚C). Primary mouse monoclonal antibodies were used for the detection of MUPP1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and diluted at 1:2000 and 1:50,000, respectively. An alkaline-phosphatase conjugated goat

anti-mouse secondary antibody was used at a dilution of 1:3000 (Biorad, Hercules, CA). Membranes were washed prior to and following antibody incubation in Tris-buffered saline. To detect the secondary antibodies, membranes were incubated in ECF substrate for 5 min. MUPP1 expression was determined relative to GAPDH expression. For analyses, because the western blot data were not normally distributed (Shapiro Wilks; $p < 0.05$), Mann-Whitney U comparisons of the mean values were used to compare MUPP1 protein expression between $Mpdz^{+/-}$ and WT littermates. Samples sizes were 1-5 mice/genotype/sex so analyses were collapsed on sex.

Neurophysiology

$Mpdz^{+/-}$ and WT littermates were compared using neurophysiology for the response of SNr GABAergic neurons to baclofen application. Naïve animals (22-23 days old) of both sexes were tested. Neurophysiological responses to approximately half maximal (3 μM) and maximally effective (30 μM) concentrations of baclofen were tested. Spontaneous inhibitory postsynaptic currents (sIPSC) and macroscopic outward currents in response to bath application of baclofen to brain slices containing the SNr were measured. The following neurophysiological analyses were conducted by Dr. Olena Kolokushtina (postdoctoral fellow) in collaboration with Dr. David Rossi.

Forebrain slices. Forebrain slices were freshly prepared as in previous work (Rossi and Hamann, 1998). Briefly, $Mpdz^{+/-}$ and WT mice (22-32 days old) were anaesthetized with isoflurane and euthanized by decapitation. The whole brain was rapidly isolated and immersed in ice-cold ACSF containing (in mM): 124 NaCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2.5 KCl, 2.5 $CaCl_2$, 2 magnesium chloride ($MgCl_2$), 10 D-glucose, and bubbled with 95% oxygen and 5% carbon dioxide (pH 7.4). The forebrain was isolated and mounted in a slicing chamber filled with ice-cold ACSF, and sliced

coronally (225 μm) using a vibrating tissue slicer. Slices containing the SNr were incubated in ACSF (33 ± 1°C) for 1 h after dissection and then held at 22-23°C until use. Kynurenic acid (1 mM) was included in the dissection, incubation and holding solutions (to mitigate potential excitotoxic damage) but not the experimental solutions. All experiments were conducted on slices within 8 h of dissection, and most within 5-6 h of dissection. No parameter analyzed showed any significant difference across time after dissection.

Whole cell recordings. As in previous work (Rossi and Hamann, 1998), slices were placed in a submersion chamber on an upright microscope, and viewed with an Olympus 60X (0.9 numerical aperture) water immersion objective with differential interference contrast and infrared optics. Slices were perfused with ACSF (22-23ºC) at a rate of 7 milliliters (ml)/min. Drugs were dissolved in ACSF and applied by bath perfusion. Whole-cell recordings were made from the somata of visually identified GABAergic neurons (based on numerical predominance and size) in the SNr. Patch pipettes were constructed from thick-walled borosilicate glass capillaries and filled with an internal solution containing (in mM): 125 K^+-gluconate, 10 NaCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), 10 BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid), 2 MgATP, and 0.3 Na_2GTP. Internal solutions were pH adjusted to 7.2 with potassium hydroxide. Electrode resistance was 2.3-2.8 MΩ. Cells were rejected if access resistance was greater than 10 MΩ, or if the access resistance (monitored with –5 mV voltage steps) changed by more than 20% during the course of an experiment. Spontaneous synaptic currents were acquired at 20 kilohertz (KHz) after being filtered at 10 KHz. For analysis, spontaneous synaptic currents were digitally filtered at 2-5 KHz and analyzed with pClamp software (Axon Instruments). Spontaneous synaptic currents are defined as current deflections that

have an amplitude (measured from the mean current) two times greater than the peak to peak amplitude of the current noise, and which have at least a 3 fold slower decay than rise time (to avoid inclusion of channel openings or momentary seal loss). Macroscopic current responses were quantified as the mean current amplitude during a 50 ms stretch of steady state current relative to an identical stretch of current during the control period prior to drug application.

Repeated measures two-way ANOVA was used for comparisons of sIPSC frequency with genotype as the between-subjects factor and treatment (baseline, 3 μM baclofen, 30 μM baclofen) as the repeated measure. Repeated measures two-way ANOVA was also used for comparisons of macroscopic current with genotype as the between-subjects and dose (3 μM and 30 μM) as the repeated measure.

Radioligand receptor binding.

[^{3}H]CGP54626 direct saturation binding was compared in female and male _Mpdz$^{+/-}$_ and WT whole brain. The following methods were adapted from previous work (Green et al., 2000; Queva et al., 2003).

Brain homogenate preparation. _Mpdz$^{+/-}$_ and WT littermates (n = 3-5/genotype/sex) were euthanized by cervical dislocation, brains were rapidly removed, and placed in 1 ml of 0.32 M sucrose buffer on ice. All centrifugations were at 4°C. Individual whole brains were homogenized in a total volume of 2.5 ml of 0.32 M sucrose buffer and centrifuged for 10 min at 1000 × _g_. The supernatant was kept on ice, and the remaining P1 pellet was resuspended in 1 ml buffer, and centrifuged for 10 min at 1,000 × _g_. Supernatants from both spins were then combined and centrifuged at 20,000 × _g_ for 20 min, the P2 pellet was resuspended in 20 weight/volume (w/v) ice-cold water, and centrifuged at 8,000 × _g_ for 20 min. The supernatant was collected and centrifuged at

45,000 × *g* for 20 min, then resuspended, centrifuged at 45,000 × *g* for 30 min, and the resulting pellet was frozen at -80°C for a minimum of 18 h. These homogenization and centrifugation steps were performed to lyse the tissue and washout endogenous GABA.

[^{3}H]CGP54626 binding. Pellets were thawed on ice, resuspended in 20 volumes of suspension-assay buffer (50 mM Tris-HCl containing 2.5 mM $CaCl_2$ buffer, pH 7.4), incubated for 20 min at 20°C, and centrifuged at 7,000 × *g* at 18°C for 10 min. This was repeated three subsequent times, with incubation for 15 min, to further remove endogenous GABA. The final pellet was resuspended in buffer to a final concentration of 50-80 μg protein/50 μl. [^{3}H]CGP54626 binding (0.3 – 17 nM final concentration) was assessed (in duplicate) in a final volume of 250 μl at 20 ± 1°C for 30 min. [^{3}H]CGP54626, a selective $GABA_BR$ antagonist, was used because it is a selective high affinity ligand for $GABA_BRs$ (Bischoff et al., 1999; Green et al., 2000). Nonspecific binding was defined using 500 µM baclofen. Baclofen was used because it is a selective $GABA_BR$ agonist (Bischoff et al., 1999). The reaction was terminated by rapid filtration rinsing once with ice-cold saline (0.9%) using a 96-well Tomtec cell harvester (Hamden, CT). Bound radioactivity was measured using a PerkinElmer MicroBeta TriLux scintillation counter (Waltham, MA). The BCA protein assay was used for protein determination as in previous work (Shirley et al., 2004). Nonspecific binding values were subtracted from total binding to define specific [^{3}H]CGP54626 binding. Scatchard analyses were performed to determine $GABA_BR$ density (B_{max}) and affinity (K_d), and were compared between *Mpdz*$^{+/-}$ and WT using two-way ANOVA with genotype and sex as the independent variables.

Gene expression in whole brain and clSNr

Gene expression of *Gabbr1, Gabbr2, Kcnj3, Kcnj6, and Kcnj9* was assessed in whole brain (n = 4-6/genotype/sex for each gene) and clSNr of *Mpdz*^{+/-} and WT littermates (n = 4-6/genotype/sex for each gene). *Mpdz* gene expression was also assessed in the clSNr of *Mpdz*^{+/-} and WT, since whole brain expression has previously been assessed (Milner et al., 2015).

Histology and LCMD. Female and male *Mpdz*^{+/-} and WT mice were euthanized by cervical dislocation, individual brains dissected, the two hemispheres divided and flash frozen in liquid nitrogen and stored individually at -80°C. One hemisphere was used for total RNA isolation (below), and the other for LCMD. The half brains were sectioned coronally (30 µm) and the slices containing the SNr were mounted on polyethylene napthalate membrane glass slides, and placed on dry ice. Slides were then dried for 5 min, placed in 0.1% thionin for 5 sec, washed twice in diethylpyrocarbonate treated water for 15 sec, washed in ethanol (75%, 95%, and 100%, 30 sec each), and dried for 5 min. Using LCMD (Leica Microsystems, 6.3x) eight clSNr tissue samples per animal were isolated, combined, and stored in 50 µl RNAlater until processing for expression analyses, as in previous work (Kruse et al., 2014).

QPCR. Total RNA was isolated from half brains using Trizol reagent (Invitrogen), and from LCMD clSNr samples using Ambion RNAqueous-Micro Kit (Grand Island, NY, USA). cDNA synthesis used a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY, USA), with preamplification (clSNr samples only) using a TaqMan PreAmp Master Mix kit and probes for *Mpdz*, *Gabbr2, Gabbr1, Kcnj9, Kcnj6, Kcnj3,* and *Reep5.* QPCR utilized *Gabbr2* (Mm01352554_m1), *Gabbr1* (Mm00444578_m1), *Mpdz* (Mm00447849_m1)*, Kcnj9* (INT)*, Kcnj6* (Mm00440070_m1)*, Kcnj3* (Mm00434618_m1)*,* and a reference gene, *Reep5* (Mm00492230_m1), and was

performed on an ABI Prism 7500 thermal cycler using TaqMan Universal PCR Master Mix (Life Technologies) and standard reaction conditions. *Reep5* was used because it is a highly stable reference gene expressed throughout the mouse brains (www.brain-map/org) (Kozell et al., 2009; Kruse et al., 2014). A standard relative quantification ($\Delta\Delta C_t$) method (Livak and Schmittgen, 2001) normalized to a calibrator sample was used as in previous work (Kruse et al., 2014). The calibrator was set to "1", and gene expression was evaluated in reference to the calibrator. For QPCR analyses in both whole brain and clSNr, expression of individual genes was compared between *Mpdz*^{+/-} and WT littermates using a two-way ANOVA with genotype and sex as the independent variables.

General Statistical Analyses

Individual statistical analyses are described above for each experiment. Tukey's HSD post-hoc analyses were used to follow up significant main effects and interactions. For all experiments, significance was set at $p < 0.05$, and full F- and t-statistics are presented if p-values were < 0.2, otherwise only the p-value is indicated. Unless otherwise indicated, data were normally distributed (Shapiro Wilks $p > 0.05$). Data were analyzed using SYSTAT 13 Statistical Software and are presented as mean ± SEM.

Results

Intra-clSNr baclofen in ethanol-withdrawn and control D2 mice

The effect of clSNr $GABA_BR$ activation, through site-directed microinjections of baclofen, was assessed in acute ethanol-withdrawn and control (saline-treated) mice. The purpose of this experiment was to determine if clSNr $GABA_BRs$ are involved in CNS hyperexcitability, and if they influence EWD severity. The results and statistical analyses

reported were based on the mice with confirmed bilateral targeting of the clSNr (Fig. 3-1a). Baseline HIC scores for each treatment group were as follows: saline-vehicle (3.0 ± 1.0), saline-baclofen (2.2 ± 0.8), ethanol-vehicle (2.5 ± 0.7), and ethanol-baclofen (1.9 ± 0.7). No main effects of systemic treatment ($p = 0.6$) or intra-clSNr treatment ($p = 0.4$), was detected; nor was an interaction ($p = 0.9$) on baseline HICs scores. Taken together, this indicates that baseline scores were similar across treatment groups.

As expected, a main effect of ethanol treatment on HIC severity score was evident, with HICs significantly more severe in ethanol withdrawn compared to saline control animals ($F_{1,14} = 16.7$, $p = 0.001$; Fig. 3-1b). Further, a significant main effect of baclofen on HIC severity score was also evident ($F_{1,14} = 4.9$, $p = 0.04$), with HICs significantly more severe following microinjection of baclofen into the clSNr compared to vehicle treated mice, but no significant interaction was detected ($p = 0.9$) indicating the increase in HIC scores following intra-clSNr baclofen was evident in both ethanol withdrawn and control mice (Fig. 3-1b). These data indicated that activation of GABA$_B$Rs in the clSNr is proconvulsant, and that clSNr GABA$_B$Rs influence CNS hyperexcitability. Further, peak HIC scores (single highest HIC score post-baclofen; corrected for baseline) were also assessed, to determine if intra-clSNr baclofen compared to vehicle further enhanced peak HICs in ethanol withdrawn mice. Although main effects of both systemic treatment ($F_{1,14} = 5.7$, $p = 0.03$) and intra-clSNr treatment ($F_{1,14} = 15.7$, $p = 0.001$) were detected on peak HIC score, there was no significant interaction ($p = 0.5$). Finally, in animals in which clSNr targeting was unilateral only, there was no difference in EWD HIC severity scores between mice treated with vehicle or baclofen into the clSNr ($p > 0.05$).
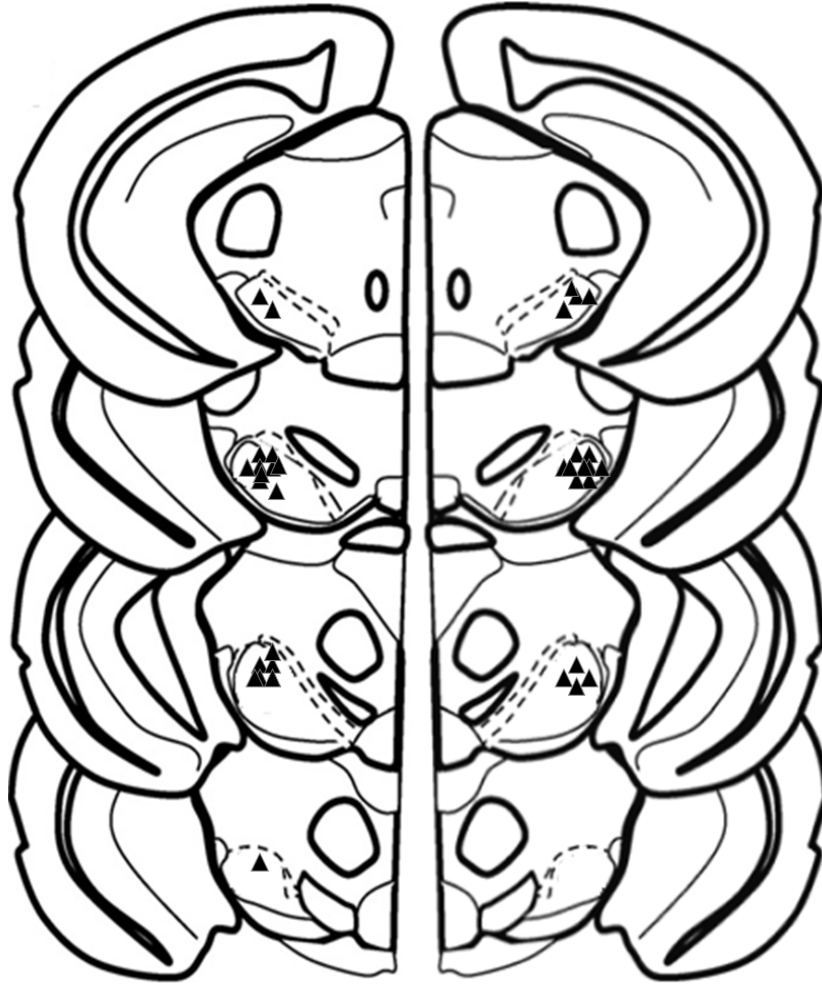
Figure 3-1a. Microinjection placements for intra-clSNr baclofen administration. Triangles indicate bilateral microinjection sites per animal (N = 18) of baclofen or vehicle in the clSNr. Only bilateral placements verified within the area of interest and therefore used for analyses are shown (~55% hit rate). Guide cannula were secured 2 mm above the clSNr, and microinjectors were lowered 2 mm below the end of the cannula for drug administration. Correct targeting of microinjections into the clSNr was identified through tissue degradation. The four panels illustrated extend from -3.1 to -3.9 mm AP from Bregma according to the stereotaxic atlas of Paxinos and Franklin (2001).
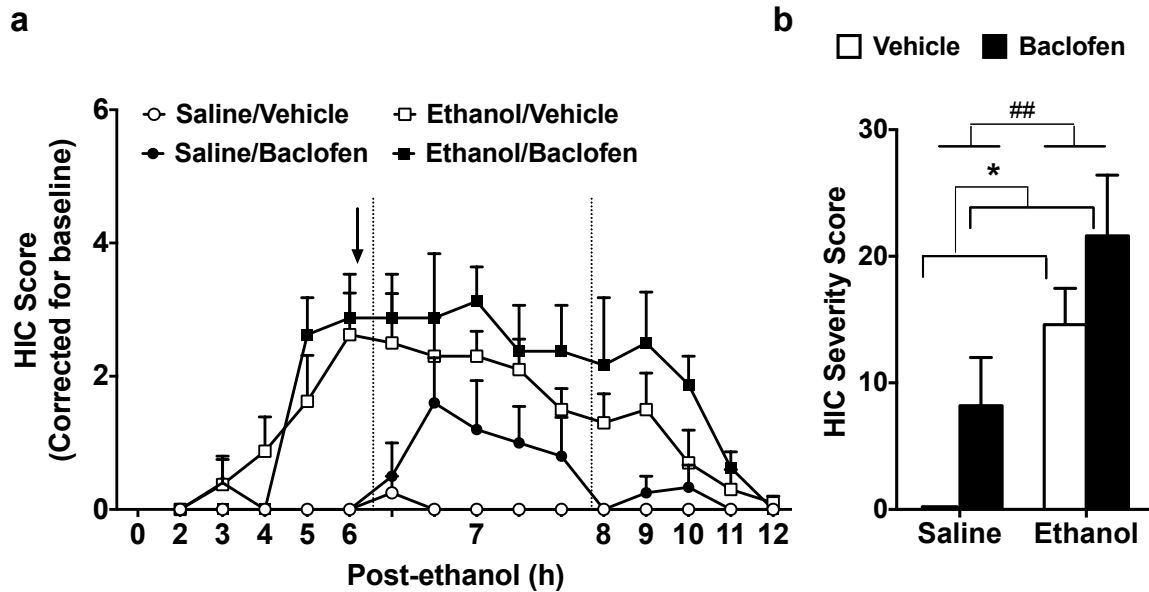
Figure 3-1b. Intra-clSNr baclofen enhanced HICs in ethanol-withdrawn and control mice. (a) D2 mice (n = 4-5 per treatment group) received ethanol (4 g/kg, i.p.) or saline at 0 h and were subsequently assessed for HICs from 2-6 and 8-12 h post-injection. Immediately after HIC scoring at 6 h, mice received a baclofen (20 ng/0.5 ul per side) or vehicle (ACSF) microinjection (*arrow*) and were assessed for HICs every 20 min until 8 h, as indicated by the time-points located between the dashed lines. (b) HIC severity scores were calculated as the sum of the corrected HIC scores post-baclofen or vehicle microinjection from 6.2 -12 h. HICs were significantly greater in mice treated with 4 g/kg ethanol compared to saline indicating a state of ethanol withdrawal (##$p < 0.01$). Intra-clSNr baclofen enhanced HICs in ethanol-withdrawn and control (saline-treated) animals compared to intra-clSNr vehicle (*$p < 0.05$). Data are presented as mean ± SEM. *$p < 0.05$ for baclofen compared to vehicle. ##$p < 0.01$ for ethanol-withdrawn compared to saline control.

Intra-clSNr CGP55845 in ethanol-withdrawn and control D2 mice

A selective GABA$_B$R antagonist (CGP55845) was microinjected into the clSNr at 6 h post-ethanol (or post-saline) administration to further assess the involvement of clSNr GABA$_B$Rs in ethanol, with the hypothesis that inhibition of GABA$_B$Rs within this region would attenuate the severity of EWD. The results and statistical analyses reported were based on mice with confirmed bilateral targeting of the clSNr (Fig. 3-2a). Baseline HIC scores for the four treatment groups were as follows: saline-vehicle (2.0 ± 0.6), saline-CGP55845 (1.8 ± 0.6), ethanol-vehicle (2.2 ± 0.6), and ethanol-CGP55845 (1.5 ± 0.5). No significant main effects on baseline HIC scores of systemic treatment (p = 0.8), intra-clSNr treatment (p = 0.5), or an interaction (p = 0.4) were detected, suggesting baseline score were similar across groups.

As expected, a main effect of ethanol treatment on HIC severity score was evident, with HICs significantly more severe in ethanol withdrawn compared to saline control animals ($F_{1,34}$ = 27.8, p < 0.001). Further, a significant main effect of intra-clSNr inhibition of GABA$_B$Rs on HIC severity was also evident ($F_{1,34}$ = 4.6, p = 0.04), with CGP55845 significantly reducing the HIC severity score compared to vehicle, and a significant interaction was also detected ($F_{1,23}$ = 5.1, p = 0.03; Fig. 3.2-b). Consistent with the involvement of GABA$_B$Rs in EWD, Tukey's post-hoc analyses confirmed that EWD was significantly reduced by intra-clSNr CGP55845 compared to vehicle (p = 0.02; Fig. 3-2b), but no effect of CGP55845 in saline control mice compared to vehicle was detected (p = 0.9; Fig. 3-2b) most likely because of a floor effect. Interestingly, post-hoc analyses further showed that HIC severity was significantly greater in ethanol-withdrawn mice treated with intra-clSNr vehicle compared to saline controls (p < 0.001), but ethanol-withdrawn mice treated with intra-clSNr CGP55845 did not statistically differ from saline controls (p = 0.2; Fig. 3-2b), further confirming that inhibition of GABA$_B$Rs

reduced EWD severity. Further, in animals in which clSNr targeting was unilateral only,

there was no difference in EWD HIC severity scores between mice treated with vehicle

or CGP55845 into the clSNr (p >0.05).
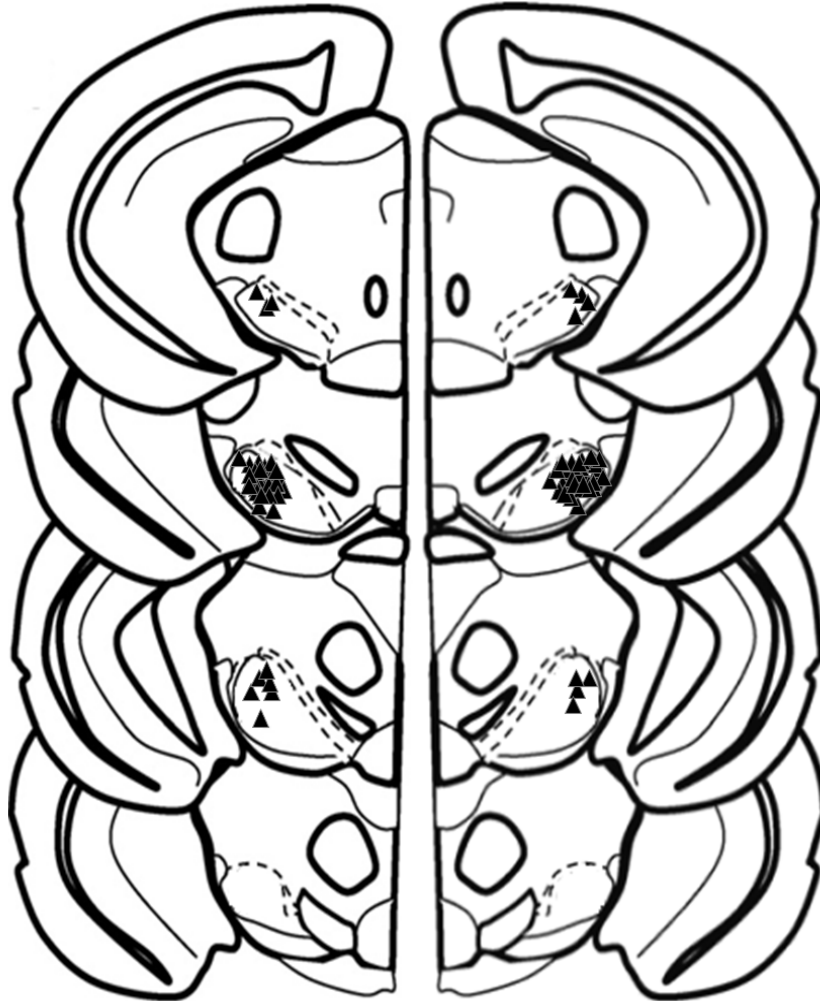
Figure 3-2a. Microinjection placements for intra-CGP55845 administration. Triangles indicate bilateral microinjection sites per animal (N = 38) of CGP55845 or vehicle in the clSNr. Only bilateral placements verified within the area of interest and therefore used for analyses are shown (~75% hit rate). Guide cannula was secured 2 mm above the clSNr, and microinjectors were lowered 2 mm below the end of the cannula for drug administration. Correct targeting of microinjections into the clSNr was identified through tissue degradation. The four panels illustrated extend from -3.1 to -3.9 mm AP from Bregma according to the stereotaxic atlas of Paxinos and Franklin (2001).
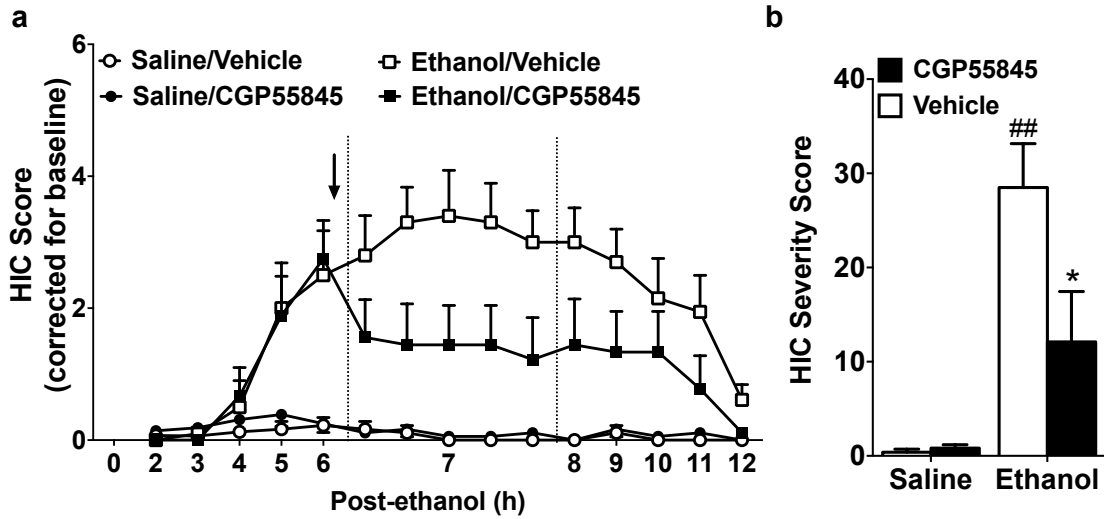
Figure 3-2b. Intra-cISNr CGP55845 attenuated EWD. (a) D2 mice (n = 9-11 per treatment group) received ethanol (4 g/kg, i.p.) or saline at 0 h and were subsequently assessed for HICs from 2-6 and 8-12 h post-injection. Immediately after HIC scoring at 6 h, mice received a CGP55845 (100 μmol/0.5 ul per side) or vehicle (ACSF containing 0.9% dimethyl sulfoxide) microinjection (*arrow*) and were assessed for HICs every 20 min until 8 h as indicated by the time-points located between the dashed lines. (b) HIC severity scores were calculated as the sum of the corrected HIC scores post-CGP55845 or vehicle microinjection from 6.2-12 h. EWD severity was significantly reduced by administration of a $GABA_BR$ antagonist into the cISNr (*$p < 0.05$). Post-hoc analyses confirmed that the ethanol-vehicle group significantly differed from both saline controls (##$p < 0.001$). Data are presented as mean ± SEM. *$p < 0.05$ for ethanol-CGP55845 compared to ethanol-vehicle. ##$p < 0.001$ for ethanol-vehicle compared to saline-vehicle and saline-CGP55845 control mice.

Intra-clSNr CGP55845 and PTZ-enhanced HICs in D2 mice.

I have demonstrated that inhibition of clSNr GABA$_B$Rs during EWD reduced the severity of withdrawal. To determine if the involvement of GABA$_B$Rs in the clSNr was more specific to EWD HICs, or extended to other HIC phenotypes, CGP55845 was also microinjected into the clSNr during PTZ-enhanced hyperexcitability. The results and statistical analyses reported were based on the mice with confirmed bilateral targeting of the clSNr (Fig. 3-3a). No differences in baseline HIC scores between the vehicle (0.2 ± 0.1) and CGP55845 (0.3 ± 0.2) treated animals were detected (p = 0.3). Unlike the effect of CGP55845 on reducing EWD, microinjection of CGP55845 into the clSNr did not alter the severity of PTZ-enhanced HICs (p = 0.4; Fig. 3-3b). In animals in which clSNr targeting was unilateral only, there was no effect of intra-clSNr treatment on PTZ-enhanced HICs (p > 0.05).

Taken together, these experiments demonstrated an involvement of clSNr GABA$_B$Rs in mediating EWD severity, and inhibition of these receptors did not affect PTZ-enhanced HICs. The involvement of GABA$_B$Rs in the clSNr during EWD suggests that if *Mpdz*/MUPP1 affects GABA$_B$R function in this region, GABA$_B$Rs may therefore be a mechanistic target through which clSNr *Mpdz* expression influences EWD. Therefore, identifying the effect of *Mpdz* on this receptor and associated behaviors may be useful towards determining the potential system through which *Mpdz* influences CNS hyperexcitability phenotypes such as EWD. The following experiments were therefore employed to determine if the potential effect of reduced *Mpdz*/MUPP1 expression was associated with heightened GABA$_B$R-mediated responses using both behavioral and neurophysiological analyses.
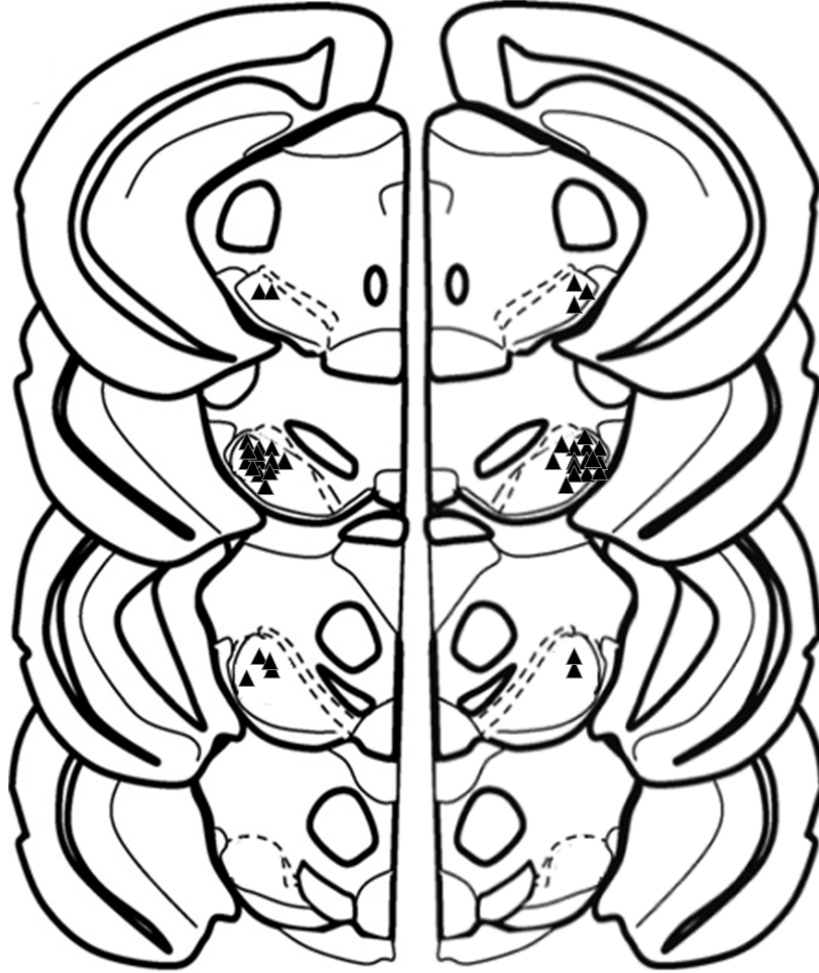
Figure 3-3a. Microinjection placements for intra-clSNr CGP55845 administration.
Triangles indicate bilateral microinjection sites per animal (N = 20) of CGP55846 or
vehicle in the clSNr. Only bilateral placements verified within the area of interest and
therefore used for analyses are shown (~75% hit rate). Guide cannula was secured 2
mm above the clSNr, and microinjectors were lowered 2 mm below the end of the
cannula for drug administration. Correct targeting of microinjections into the clSNr was
identified through tissue degradation. The four panels illustrated extend from -3.1 to -
3.9 mm AP from Bregma according to the stereotaxic atlas of Paxinos and Franklin

(2001).
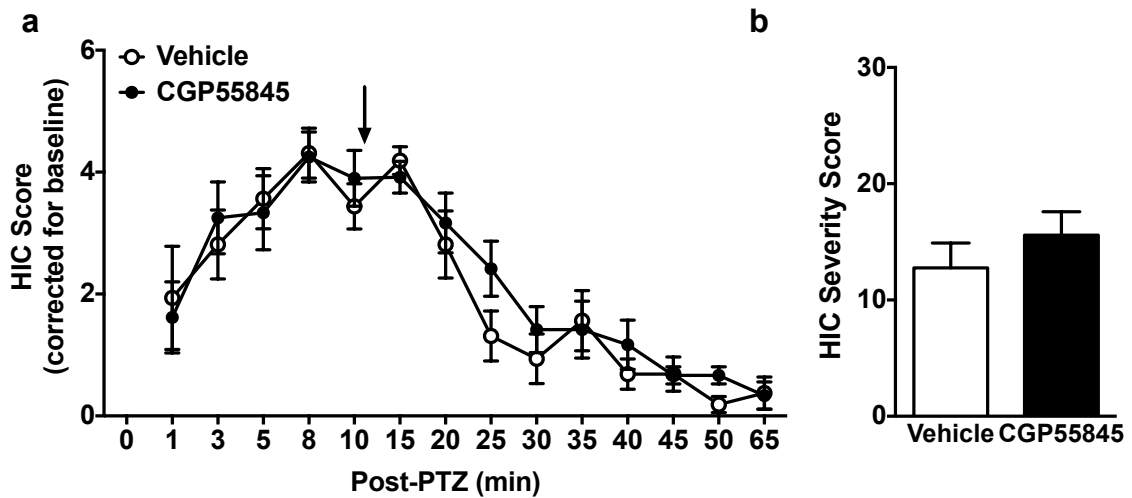
Figure 3-3b. Intra-cISNr CGP55845 did not affect PTZ-enhanced HICs. (a) D2 mice (n = 8-12 per treatment group) were administered PTZ (30 mg/kg, i.p.) at 0 h and subsequently assessed for HICs at 14 time-points post-PTZ administration. Immediately after HIC scoring at 10 min, mice received a CGP55845 (100 µmol/0.5 µl per side) or vehicle (ACSF containing 0.9% dimethyl-sulfoxide) microinjection (*arrow)* and were subsequently assessed for HICs until 65 min. (b) HIC severity scores were calculated as the sum of the corrected HIC scores post-CGP55845 or vehicle microinjection from 15 – 65 min. Inhibition of cISNr GABA$_B$Rs did not alter PTZ-enhanced HIC severity. Data are presented as mean ± SEM.

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MUPP1 expression in *Mpdz*$^{+/-}$ and WT

Although previous work has demonstrated a reduction of *Mpdz* mRNA expression in whole brain of *Mpdz*$^{+/-}$ compared to WT littermates, it was unknown if this translated to a comparable reduction in protein expression (Milner et al., 2015). Here, using semi-quantitative western blot analysis, I performed the first assessment of MUPP1 protein expression in *Mpdz*$^{+/-}$ and WT whole brain. MUPP1 protein expression was lower by approximately 55 ± 1% in *Mpdz*$^{+/-}$ compared to WT ($U_{1,12}$ = 4.0, p = 0.03; Fig. 3-4). This reduction was comparable to that for *Mpdz* mRNA expression in whole brain of *Mpdz*$^{+/-}$ (53 ± 1%) compared to WT (Milner et al., 2015).
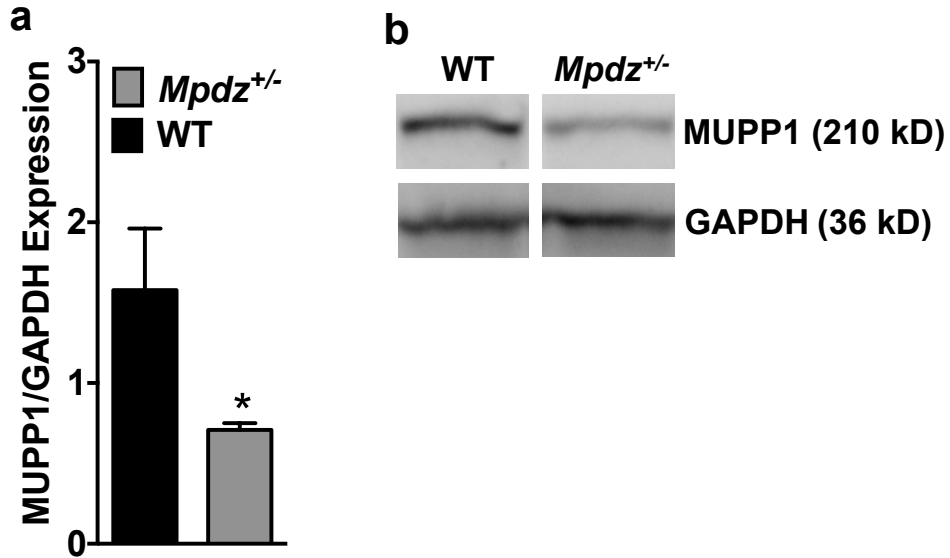
Figure 3-4. MUPP1 protein expression in whole brain is reduced in *Mpdz^+/-*^ compared to WT mice. (a) MUPP1 expression is reduced by 55 ± 1% in *Mpdz^+/-*^ compared to WT (n = 6 per genotype; *p < 0.05). (b) Representative bands from western blots of MUPP1 and control GAPDH expression in *Mpdz^+/-*^ and WT. MUPP1 was detected at 210 kilodalton (kD) and GAPDH was detected at 36 kD. Asterisk indicates a main effect of genotype on protein expression (*p < 0.05).

Baclofen-enhanced HICs in *Mpdz$^{+/-}$* and *Mpdz^{Tg}* mice

Baclofen-enhanced HICs are a measure of CNS hyperexcitability mediated by GABA$_B$Rs (Humeniuk et al., 1994; Reilly et al., 2008). In order to determine if altered MUPP1 expression was associated with a change in GABA$_B$R function, both *Mpdz$^{+/-}$* and *Mpdz^{Tg}* mice were compared to respective WT littermates for baclofen-enhanced HICs. For 5 mg/kg baclofen no main effects of genotype (p = 0.2), sex (F$_{1,41}$ = 3.4, p = 0.07), or treatment (p = 0.3), and no interactions (for all p > 0.2) were detected on baseline HIC scores (Fig. 3-5a). Similarly, for 10 mg/kg baclofen there were no main effects of genotype (p = 0.6), sex (F$_{1,40}$ = 3.3, p = 0.08), or treatment (p = 0.3) on baseline HICs. There was a significant treatment × sex interaction (F$_{1,40}$ = 8.1, p = 0.01), but no other interactions (p > 0.1) on baseline HIC scores. Post hoc analyses indicated that females treated with vehicle had greater HIC scores compared to males treated with vehicle (p = 0.01), and data were therefore collapsed on sex given no differences in genotype.

The 5 mg/kg dose of baclofen enhanced HIC scores compared to saline treated animals (F$_{1,41}$ = 8.8, p = 0.005), however, there was no main effect of genotype (p = 0.4; Fig. 3-5b) or sex (p = 0.6), and no significant interactions between factors (all p > 0.4). The lack of difference between *Mpdz$^{+/-}$* and WT littermates may be due to dose and/or the relatively high variability in baclofen enhanced HIC scores for both *Mpdz$^{+/-}$* and WT (5.6 ± 2.0 and 2.8 ± 1.0, respectively) that was evident. Since B6 background mice are not highly susceptible to either withdrawal or chemiconvulsant enhanced HICs, it is possible the 5 mg/kg dose was not a high enough dose (Kosobud and Crabbe, 1990; Metten and Crabbe, 1994). Therefore, one week later, a higher dose of baclofen (10 mg/kg) was assessed. As expected, a three-way ANOVA indicated that this dose of

baclofen significantly enhanced HICs compared to saline ($F_{1,40}$ = 22.1, p < 0.0001). Further, there was a main effect of genotype ($F_{1,40}$ = 5.2, p = 0.028), as well as a significant genotype × treatment interaction ($F_{1,40}$ = 5.0, p= 0.03; Fig. 3-5b), but no main effect of sex ($F_{1,40}$ = 3.6, p = 0.07) and no significant genotype and/or treatment interactions with sex (p > 0.1). Tukey's post-hoc analyses confirmed that baclofen-enhanced HIC severity was significantly *greater* in *Mpdz*$^{+/-}$ compared to WT littermates (p = 0.02), with no genotype difference detected in saline control animals (p = 0.9). Further, WT littermates treated with baclofen did not significantly differ in HIC severity scores from WT littermates treated with saline (p = 0.4), whereas the *Mpdz*$^{+/-}$ treated with baclofen did significantly differ from the *Mpdz*$^{+/-}$ treated with saline in HIC severity score (p < 0.0001). These data demonstrated that *Mpdz*$^{+/-}$ were more susceptible to baclofen-enhanced HICs compared to WT, similar to increased susceptibility for EWD HICs (Milner et al., 2015), and indicate that the effect of *Mpdz* on CNS hyperexcitability may be mediated, at least in part, through GABA$_B$Rs.

In addition to *Mpdz*$^{+/-}$, *Mpdz*^{Tg} and WT littermates were also used to assess the association between MUPP1 expression on baclofen-enhanced HICs. No main effects of genotype (p = 0.3) or sex (p = 0.7), or an interaction between the two (p = 0.8) were detected on baseline HIC scores. Further, no main effect of sex (p = 0.3) or genotype × sex interaction (p = 0.3) were detected on baclofen-enhanced HICs. For baclofen (5 mg/kg) enhanced HICs, as expected, there was a significant main effect of genotype ($F_{2,30}$ = 6.2, p = 0.006), with post-hoc analyses confirming that baclofen-enhanced HICs in *Mpdz*^{Tg} homozygotes were significantly reduced compared to WT littermates (p = 0.004; Fig. 3-6b). HICs in *Mpdz*^{Tg} heterozygotes were intermediate and did not statistically differ from homozygotes (p = 0.25) or WT littermates (p = 0.16).

Together, these data utilizing two complementary *Mpdz* genetic models provided the first evidence for a genetic relationship between MUPP1 expression and a native $GABA_BR$-mediated response, with reduced MUPP1 expression associated with heightened response to baclofen, and vice versa.
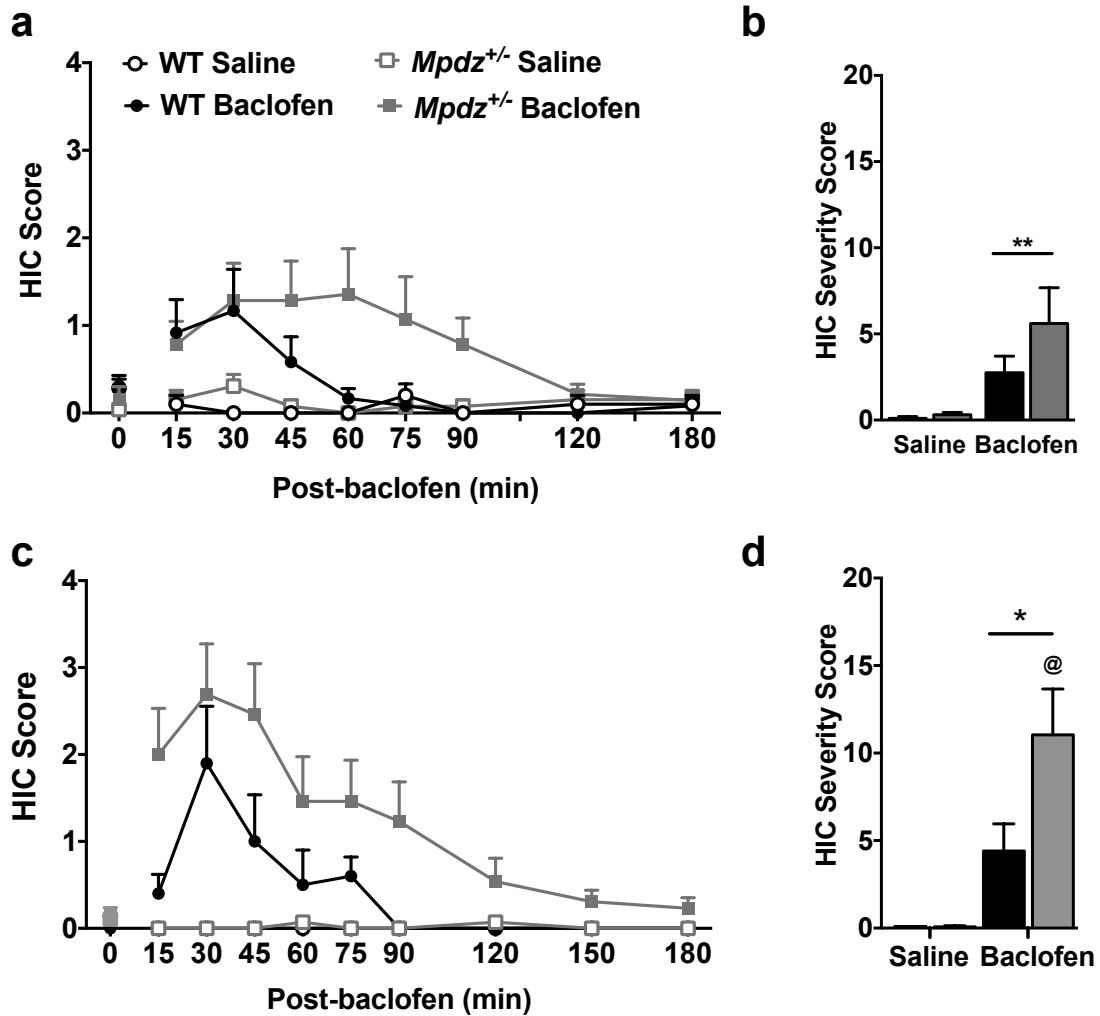
Figure 3-5. Baclofen-enhanced HICs (10 mg/kg) were greater in *Mpdz*^{+/-} compared to WT mice. (a) *Mpdz*^{+/-} and WT littermates (n = 10-14/genotype/treatment group) were assessed twice for baseline HIC scores, and subsequently administered saline or 5 mg/kg baclofen and assessed for HICs at the indicated time-points. (b) Baclofen (5 mgkg) significantly enhanced HICs in both *Mpdz*^{+/-} and WT mice (**$p < 0.01$), but baclofen-enhanced HIC severity did not differ between *Mpdz*^{+/-} and WT mice. One week later, (c) *Mpdz*^{+/-} and WT mice (n = 11-14/genotype/treatment group) were assessed for baseline HIC scores and subsequently administered saline or 10 mg/kg baclofen and

assessed for HICs at the indicated time-points. (d) Baclofen-enhanced HICs were evident in both *Mpdz*$^{+/-}$ and WT mice (*p < 0.05), but were significantly greater in *Mpdz*$^{+/-}$ mice (@p < 0.05). Average baseline HIC scores are shown at 0 h. Presented HICs scores from 15 min – 180 min were corrected for baseline HIC scores. *p < 0.05 indicates a main effect of baclofen compared to saline, @ indicates baclofen-enhanced HICs were greater in *Mpdz*$^{+/-}$ compared to WT littermates, **p< 0.01 indicates a main effect of baclofen compared to saline. Data are shown collapsed on sex.
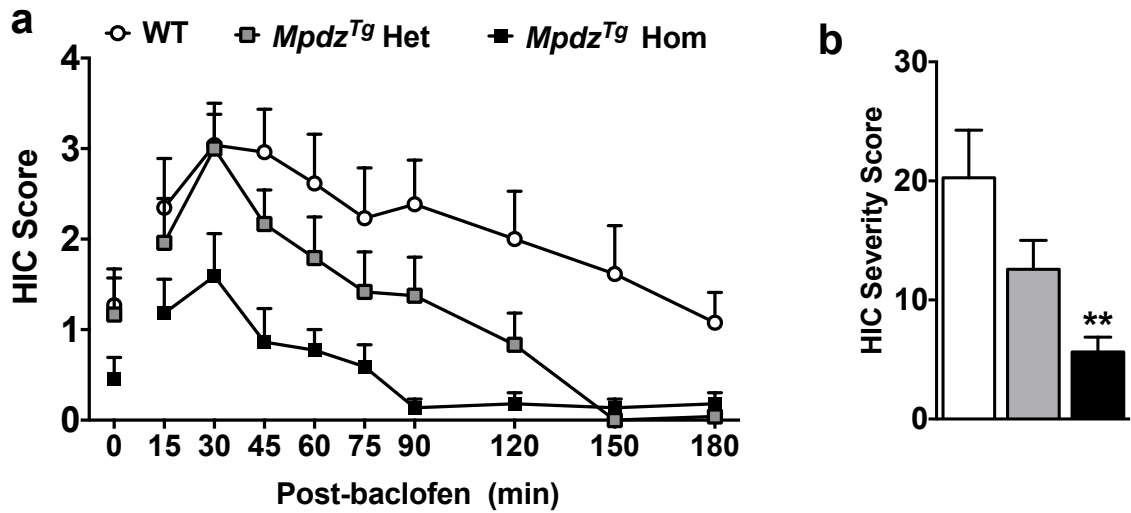
Figure 3-6. **Baclofen-enhanced HICs were lower in *Mpdz^Tg* homozygotes compared to WT mice.** (a) *Mpdz^Tg* homozygotes, heterozygotes, and WT mice (n = 11-13/genotype) were assessed twice for baseline HICs, which did not statistically differ (p > 0.2). Mice then were administered baclofen (5 mg/kg) at 0 h (*arrow*), and HICs were assessed at the indicated time-points. (b) Baclofen-enhanced HICs were significantly reduced in *Mpdz^Tg* homozyotes compared to WT mice (**p < 0.01). HIC scores were intermediate in *Mpdz^Tg* heterozygotes. Average baseline HIC scores are shown at 0 h. Presented HICs scores from 15 min – 180 min were corrected for baseline HIC scores. **p < 0.01 indicates reduced baclofen-enhanced HICs in *Mpdz^Tg* homozygotes compared to WT littermates. Data are shown collapsed on sex.

Locomotor activity in response to baclofen in *Mpdz*^{+/-} and WT mice

In order to assess whether differential response to baclofen in *Mpdz*$^{+/-}$ and WT littermates would extend to phenotypes beyond HICs, *Mpdz*$^{+/-}$ and WT littermates were compared for the effect of baclofen on locomotor activity across 4 doses (1, 2, 4, 10 mg/kg), as higher doses of baclofen are known to induce locomotor depression. No main effects of genotype or sex were detected on habituation day (Day 1; for both $p > 0.4$). Baseline locomotor activity values (Day 2) were used to pseudo-randomly assign mice to the five baclofen treatment groups, and no main effects of genotype, sex, or treatment group, or any interactions between factors (for all $p > 0.2$) were detected on baseline activity. No main effect of sex ($F_{1,67} = 2.8$, $p = 0.1$) or any interactions with genotype and/or treatment (all $p > 0.7$) were detected on locomotor activity on Day 3 (Fig. 3-7). Further, on Day 3, as expected, there was a main effect of baclofen on locomotor activity compared to saline ($F_{4,67} = 6.9$, $p < 0.0001$), with Tukey's post hoc analyses indicating that 4 and 10 mg/kg baclofen significantly reduced locomotor activity compared to saline controls (both $p < 0.01$). However, no main effect of genotype was detected ($p = 0.9$), indicating baclofen response was comparable at all doses between *Mpdz*$^{+/-}$ and WT littermates. These data indicated that the heightened response to baclofen in *Mpdz*$^{+/-}$ compared to WT littermates may not generalize to other baclofen-mediated behaviors.
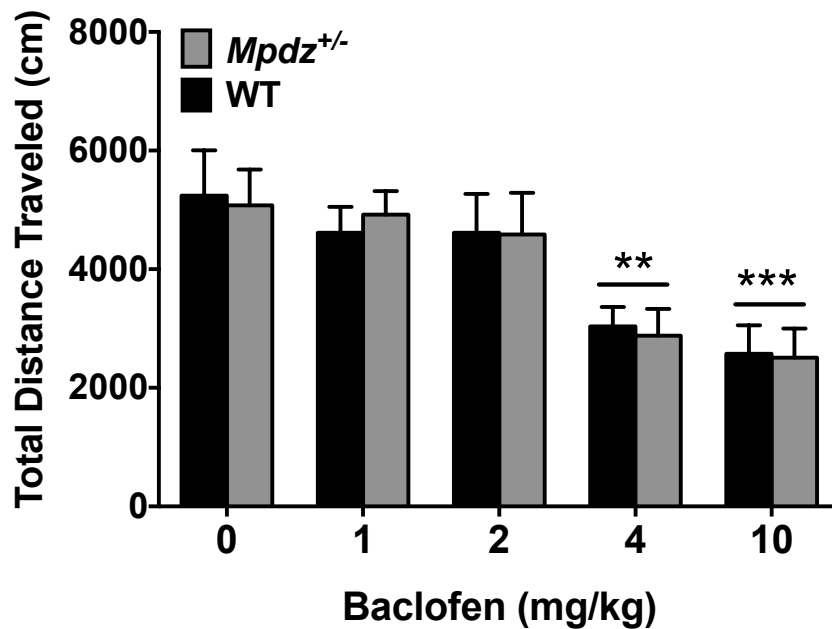
Figure 3-7. *Mpdz*$^{+/-}$ **and WT mice did not differ in locomotor activity in response to baclofen.** *Mpdz*$^{+/-}$ and WT littermates (n = 6-12/genotype/treatment group) did not differ in locomotor activity following administration of saline (0, i.p.) or baclofen (1, 2, 4, or 10 mg/kg, i.p.) on Day 3, as determined by total horizontal distance travelled in 30 min. Only the 4 mg/kg and 10 mg/kg doses of baclofen produced locomotor depression. **p < 0.01, ***p < 0.001 for comparisons to saline (0) controls. Data are shown collapsed on sex.

GABA$_B$R response in SNr GABAergic neurons of *Mpdz$^{+/-}$* and WT

The clSNr is involved in mediating *Mpdz* and GABA$_B$R effects on EWD (Kruse et al., 2014; Fig. 3-2). Although MUPP1 expression affects GABA$_B$R function in a heterologous cell system (Balasubramanian et al., 2007), evidence that MUPP1 expression affects native GABA$_B$R actions is currently lacking. Here, *Mpdz$^{+/-}$* and WT mice were compared to assess the potential effect of MUPP1 expression on native GABA$_B$R function in SNr neurons. Using voltage-clamp recordings from SNr GABAergic neurons in *Mpdz$^{+/-}$* and WT brain slices, the response to baclofen was assessed to determine if reduced MUPP1 expression was associated with a change in GABA$_B$R-mediated responses in the SNr. GABAergic neurons were of particular interest because the SNr is largely comprised of these neurons, and they are the main output projection of this region therefore playing a crucial role in inhibitory signaling (Bolam et al., 2000).

Whole cell recordings were made from the somata of visually identified GABAergic neurons, based on numerical predominance and size in the SNr (Richards et al., 1997). Voltage-clamped (V$_h$ = -50 mV) GABAergic neurons in *Mpdz$^{+/-}$* and WT had comparable mean input resistances of 539 ± 92 and 422 ± 40 MΩ, respectively, which did not differ statistically (p = 0.3). Baseline (pre-baclofen) sIPSC frequencies were not significantly different between *Mpdz$^{+/-}$* and WT (0.18 ± 0.04 and 0.21 ± 0.04 Hz, respectively, p = 0.6). Repeated measures ANOVA identified a main effect of baclofen (F$_{2,34}$ = 27.0, p < 0.0001), with post-hoc analyses confirming that 3 µM and 30 µM (p < 0.0001) baclofen powerfully reduced the frequency of sIPSC in *Mpdz$^{+/-}$* and WT neurons compared to baseline (Fig. 3-8a). No effect of genotype (F$_{1,17}$ = 0.5, p = 0.5; Fig. 3-8a) nor a genotype × treatment interaction (p = 0.8) were detected. Together, these data indicated that the reduction of sIPSC frequency in response to baclofen in SNr GABAergic neurons did not differ between *Mpdz$^{+/-}$* and WT for either baclofen dose

tested: 3 µM (63 ± 0.2% and 65 ± 0.7% reduction, respectively) and 30 µM (86 ± 0.7%

and 73 ± 0.8% reduction, respectively) (Fig. 3-8a). Potentially confounding interpretation

of these data, washout of baclofen resulted in a partial recovery of sIPSC frequency

compared to 30 µM values in WT (washout = 0.10 ± 0.02 Hz; 30 µM = 0.05 ± 0.01 Hz; t_{22}

= 2.6, p = 0.02) but not $Mpdz^{+/-}$ (washout = 0.03 ± 0.01 Hz; 30 µM = 0.02 ± 0.01 Hz; t_{12} =

0.7, p = 0.4). sIPSC is a measure of presynaptic vesicular GABA release, and therefore

data indicated that the potential effect of reduced MUPP1 expression was not associated

with a change in presynaptic $GABA_BR$ induced reduction of vesicular GABA release,

though interpretation is limited by the washout analyses.

Bath application of baclofen induces a macroscopic outward G-protein inwardly

rectifying potassium (GIRK) channel current that allows for the measurement of the

effect of postsynaptic $GABA_BR$-mediated responses to baclofen (Padgett and Slesinger,

2010). Repeated measures ANOVA indicated that the baclofen-enhanced postsynaptic

current was of significantly greater magnitude in $Mpdz^{+/-}$ SNr GABAergic neurons than in

WT ($F_{1,31}$ = 8.0, p = 0.0008; Fig. 3-8b), and a main effect of baclofen was also detected

($F_{1,31}$ = 26.0, p < 0.0001) with greater currents in response to 30 µM compared to 3 µM .

No genotype × treatment interaction (p = 0.6) was detected. Thus, as has been reported

previously (Floran et al., 1988; Rick and Lacey, 1994), activation of postsynaptic

$GABA_BRs$ induced an inhibitory current mediated by GIRKs. Therefore, these data

demonstrated that $Mpdz^{+/-}$ mice demonstrated heightened postsynaptic $GABA_BR$-GIRK

currents in response to baclofen compared to WT littermates.

a

b
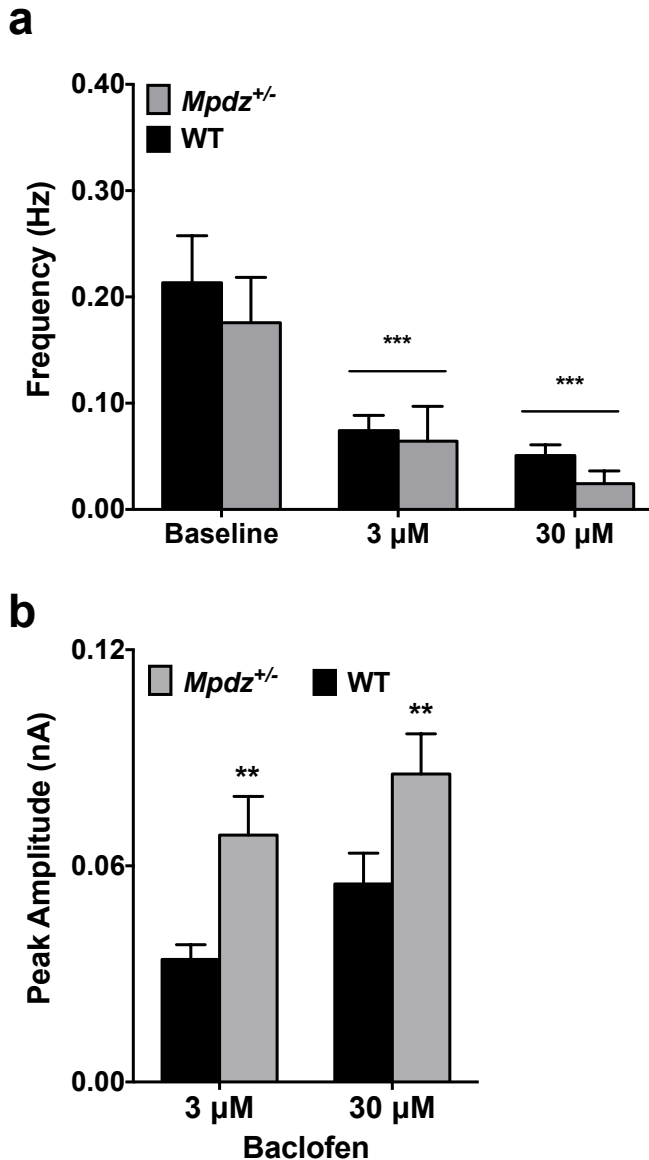
Figure 3-8. The response of *Mpdz*$^{+/-}$ and WT SNr GABAergic neurons to baclofen.
(a) SNr neurons from *Mpdz*$^{+/-}$ (n = 7 cells) and WT mice (n = 12 cells) did not differ in baseline (pre-baclofen) sIPSC frequency prior to bath application of baclofen. Baclofen (3 μM and 30 μM) comparably suppressed sIPSC frequency in both *Mpdz*$^{+/-}$ and WT compared to baseline (***$p < 0.0001$). (b) In SNr GABAergic neurons, bath application of baclofen induced an outward (hyperpolarizing) current which was of greater magnitude in *Mpdz*$^{+/-}$ (n = 12 cells) compared to WT (n = 22 cells) at both 3 μM and 30

µM concentrations.   ***$p < 0.001$ indicates a main effect of baclofen, and **$p < 0.01$ indicates a main effect of genotype.

GABA$_B$R density and binding affinity in *Mpdz$^{+/-}$* and WT

Here, to begin to test the hypothesis that heightened behavioral and neurophysiological responses to baclofen in *Mpdz$^{+/-}$* compared to WT might be related to differential GABA$_B$R density and/or affinity between these genetic models, [^{3}H]CGP54626 direct scatchard analysis was used as previously described (Queva et al., 2003) to assess GABA$_B$R binding in whole brain.

No main effect of sex or interaction with genotype on B$_{max}$ or K$_d$ (for all $p > 0.3$) was detected (Fig. 3-9). Further, *Mpdz$^{+/-}$* and WT did not differ in B$_{max}$ (885 ± 29 and 878 ± 21 femtomoles [fmol]/mg protein, respectively; $p = 0.9$) or K$_d$ (1.24 ± 0.03 and 1.15 ± 0.05 nM, respectively; $p = 0.3$) (Fig. 3-9). Thus, based on whole brain analyses at least, *Mpdz$^{+/-}$* and WT did not differ in GABA$_B$R density or affinity. However, a limitation of this experiment was that a potential significant difference in GABA$_B$R density and/or affinity might not have been detected if it is limited to a small brain region(s) or discrete population(s) of cells.
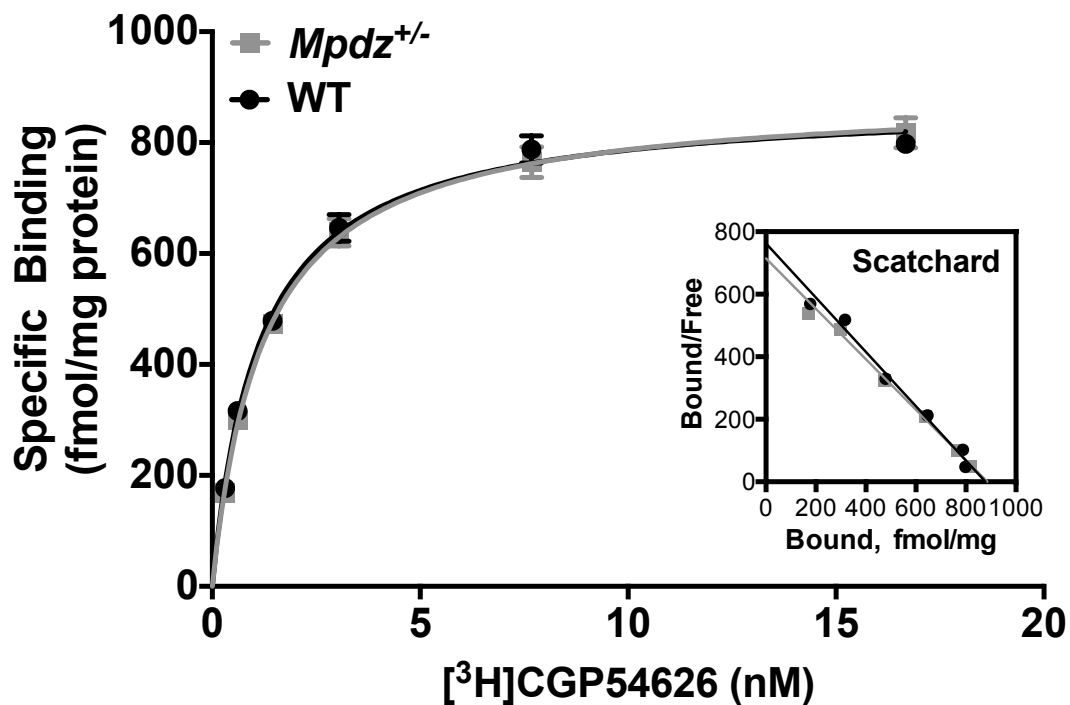
Figure 3-9. [³H]CGP54626 binding in *Mpdz*⁺ᐟ⁻ and WT whole brain. Data represents the mean values ± SEM based on individual *Mpdz*⁺ᐟ⁻ and WT mice (n = 7-8/genotype). *Inset:* Scatchard analysis indicated no difference between *Mpdz*⁺ᐟ⁻ and WT in B_{max} (885 ± 29 and 878 ± 21 fmol/mg protein, respectively) or K_d (1.24 ± 0.03 and 1.15 ± 0.05 nM, respectively) (both $p > 0.3$). Data are shown collapsed on sex.

GABA$_B$R and GIRK channel subunit gene expression in whole brain of *Mpdz$^{+/-}$* and WT

QPCR analyses were conducted in parallel to the saturation binding analyses as an additional method for addressing the hypothesis that the heightened behavioral and neurophysiological responses to baclofen in *Mpdz$^{+/-}$* compared to WT might be related to changes in the GABA$_B$R. Specifically, I hypothesized that differential expression of *Gabbr1* and *Gabbr2* in whole brains and clSNr would be evident between *Mpdz$^{+/-}$* and WT littermates. For *Gabbr1,* no main effects of sex ($F_{1,14}$ = 3.7, p = 0.07), genotype (p = 0.4), or interaction ($F_{1,14}$ = 3.8, p = 0.07) were detected. Similarly for *Gabbr2*, no main effect of sex ($F_{1,15}$ = 3.1, p = 0.1), genotype ($F_{1,15}$ = 2.9, p = 0.1), or interaction (p = 0.2) was detected.

Because neurophysiology analyses detected heightened GABA$_B$R outward currents mediated by GIRK channels, I also assessed gene expression of *Kcnj3*, *Kcnj6*, and *Kcnj9*, which encode the GIRK1, GIRK2, and GIRK3 subunits. For all three genes, separate two-way ANOVAs indicated that there were no main effects of sex (for all p > 0.5) or genotype (for all p > 0.3), and no significant interactions for *Kcnj6* ($F_{1,16}$ = 3.8, p = 0.07), or *Kcnj9* ($F_{1,16}$ = 3.3, p = 0.09). There was a significant genotype × sex interaction for *Kcnj3* ($F_{1,14}$ = 5.6, p = 0.04) but post-hoc analyses indicated no significant effects (all p > 0.15). Therefore, *Mpdz$^{+/-}$* and WT did not differ in gene expression of *Gabbr1*, *Gabbr2*, *Kcnj3, Kcnj6,* or *Kcnj9* in whole brain.

GABA$_B$R and GIRK subunit mRNA content in LCMD-clSNr of *Mpdz$^{+/-}$* and WT

A limitation of assessing expression in whole brain is that region-specific changes may be obscured, and therefore analyses in discrete regions may be crucial towards elucidating specific changes in gene expression. Therefore, with data pointing

to the crucial involvement of the clSNr in EWD in an *Mpdz* dependent manner, and analyses indicating clSNr GABA$_B$Rs may be involved in EWD, I also used LCMD-QPCR to quantify expression of *Gabbr1*, *Gabbr2*, *Kcnj3*, *Kcnj6*, *Kcnj9*, and *Mpdz* mRNA content specifically in the clSNr of *Mpdz$^{+/-}$* and WT.

There were no main effects of sex (for all p > 0.2) or significant interactions with genotype (for all p > 0.3) on expression of *Gabbr1*, *Gabbr2*, *Kcnj3*, *Kcnj6*, *Kcnj9*, or *Mpdz*. As expected, *Mpdz* mRNA in *Mpdz$^{+/-}$* was reduced by approximately half (46 ± 0.7%) compared to WT ($F_{1,16}$ = 21.6, p < 0.0001; Fig. 3-10b) in the clSNr. This is consistent with previous work showing reduced *Mpdz* mRNA (Milner et al., 2015) and MUPP1 protein in *Mpdz$^{+/-}$* compared to WT in whole brain. *Mpdz$^{+/-}$* and WT did not differ for *Gabbr1* or *Gabbr2* mRNA expression (both p > 0.3). Neither did they differ for *Kcnj3* or *Kcnj9* expression (both p > 0.4). However, *Kcnj6* mRNA was significantly greater by approximately 40 ± 0.3% in *Mpdz$^{+/-}$* compared to WT ($F_{1,14}$ = 12.8, p = 0.003; Fig. 3-10b) in the clSNr. This surprising result for *Kcnj6* raises the possibility that the neurophysiology finding of heightened SNr GABA$_B$R outward currents mediated through GIRK channels apparent in *Mpdz$^{+/-}$* compared to WT might be due to a potential effect of MUPP1 on GIRK channels rather than directly on the GABA$_B$R. Together with the neurophysiology results, this is the first evidence suggesting MUPP1 may affect GABA$_B$R-GIRK signaling through *Kcnj6*/GIRK2 expression.
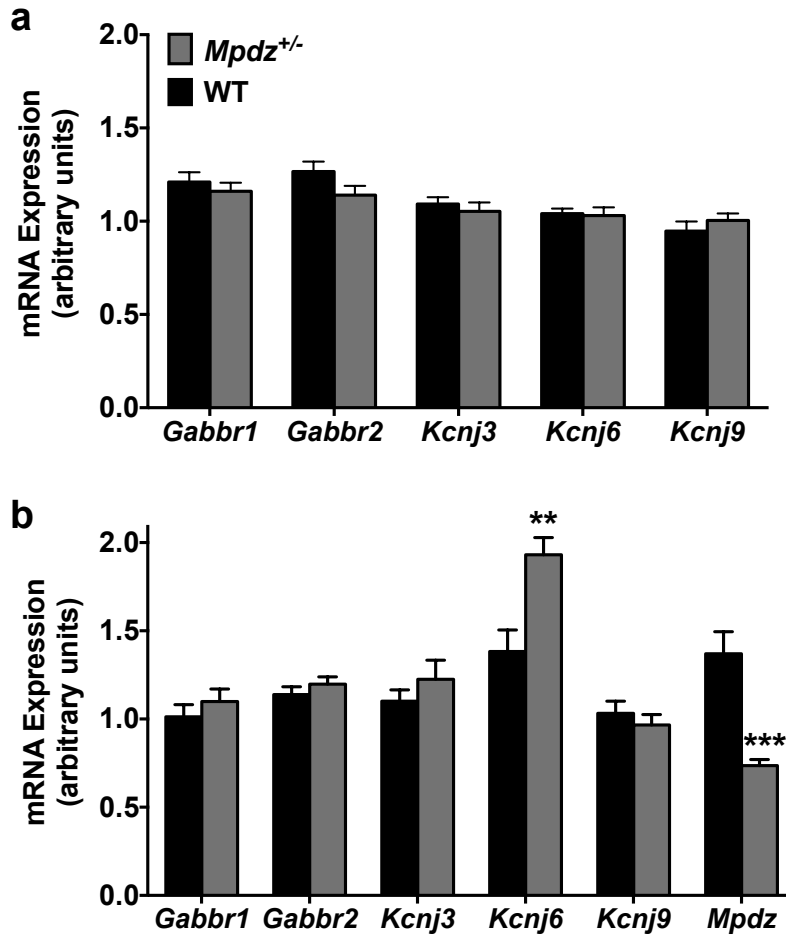
Figure 3-10. Gene expression in *Mpdz*^{+/-} and WT in (a) whole brain and (b) LCMD-cISNr. Using *Mpdz*^{+/-} and WT mice (n = 8-11/genotype for each gene), one hemisphere was used for whole brain assessment of gene expression and the other for gene expression discretely in the cISNr. (a) Whole brain QPCR detected no differences between *Mpdz*^{+/-} and WT for *Gabbr1*, *Gabbr2*, *Kcnj3*, *Kcnj6*, or *Kcnj9* (all $p > 0.05$). (b) As expected, QPCR using LCMD-cISNr detected a significant reduction in *Mpdz* mRNA expression (***$p < 0.001$). No difference between *Mpdz*^{+/-} and WT for *Gabbr1*, *Gabbr2*, *Kcnj3*, or *Kcnj9* (all $p > 0.3$, NS) mRNA expression was detected. However, *Kcnj6* mRNA expression was greater in *Mpdz*^{+/-} compared to WT (**$p < 0.01$). Data are presented as mean ± SEM and are shown collapsed on sex. **$p < 0.01$ and ***$p < 0.001$ indicate significant differences in *Mpdz*^{+/-} compared to WT.

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Discussion

  MUPP1 complexes and associates with a diverse array of proteins, and therefore likely plays a significant, but complex, role in behavior through these associations (Baliova et al., 2014; Simpson et al., 1999; Ullmer et al., 1998). Elucidating the functional effect of MUPP1 on its interacting partners and subsequent influence on associated behaviors is important for determining the importance of this gene in behavior, and particularly, its relevance in AUDs (Gizer, 2011; Karpyak et al., 2009; Tabakoff et al., 2009). Using a combination of behavioral, molecular, and neurophysiological analyses, my results provided the first evidence that inhibition of $GABA_B Rs$ in the clSNr attenuated the severity of EWD, a region where *Mpdz* expression is crucially involved in predisposition to EWD, and that this region may mediate CNS hyperexcitability (i.e., HICs) through $GABA_B Rs$. Further, I showed that some baclofen-mediated responses were heightened both behaviorally and neurophysiologically in *Mpdz*$^{+/-}$, and my data were the first to implicate MUPP1 expression as putatively involved in $GABA_B R$-GIRK signaling, putatively through an effect on *Kcnj6* (GIRK2) rather than the $GABA_B R$ itself. Together, these data support the conclusion that MUPP1 is may play an important regulatory role in $GABA_B R$ control over CNS hyperexcitability and $GABA_B R$-mediated inhibition in the SNr that may be mediated through $GABA_B R$-GIRK signaling.

  $GABA_B Rs$ have been implicated in the rewarding and reinforcing properties of ethanol (Bechtholt and Cunningham, 2005; Boehm et al., 2002; Colombo et al., 2002), but few studies have looked at the impact of these receptors in EWD. Activation of $GABA_B Rs$ through systemic administration of baclofen has been shown to enhance convulsions, and administration of $GABA_B R$ antagonists reduces chronic EWD convulsions and also blocks the proconvulsant effects of systemic baclofen (Humeniuk et al., 1994; Mead and Little, 1995; Reilly et al., 2008), but the neurocircuitry associated

with these effects is less known. I confirmed that selective GABA$_B$R activation in the clSNr enhanced HICs, providing evidence that the clSNr may play a role in proconvulsant effects of baclofen. My studies were also the first to demonstrate involvement of clSNr GABA$_B$Rs in EWD, without an effect on PTZ-enhanced HICs, which builds upon previous work showing that lesioning the clSNr (Chen et al., 2008) and RNAi-reduced expression of *Mpdz* in the clSNr both affected EWD without affecting PTZ HICs (Kruse et al., 2014). GABA$_B$R agonists (e.g. baclofen) and antagonists have been shown to be both proconvulsant (Humeniuk et al., 1994; Reilly et al., 2008; Vergnes et al., 1997) and anticonvulsant (Ault et al., 1986; Humeniuk et al., 1994; Vergnes et al., 1997) in different seizure models, indicating that GABA$_B$R activation/inhibition on neuronal excitability is highly complex, and suggesting that different brain regions/cellular mechanisms influence different seizure types. I therefore provided evidence that within the clSNr, activation of GABA$_B$Rs is proconvulsant, and inhibition is anticonvulsant under certain conditions. The clSNr has been shown to be involved in acute EWD (Chen et al., 2011; Chen et al., 2008), and in EWD in an *Mpdz* dependent manner (Kruse et al., 2014), and here I provided compelling evidence for a role of clSNr GABA$_B$Rs in two CNS hyperexcitability phenotypes (e.g., baclofen-enhanced HICs and EWD), both of which are enhanced with an associated reduction in MUPP1 expression (Milner et al., 2015).

Following confirmation that clSNr GABA$_B$Rs were involved in EWD, the next step in building evidence for these receptors as a putative mechanistic target of *Mpdz* was to determine if GABA$_B$R-mediated behaviors differed between *Mpdz*$^{+/-}$ and WT mice. Interestingly, a significant genetic association between MUPP1 expression and response to baclofen related to CNS hyperexcitability was detected, but not with locomotor depression, two behaviors influenced by CNS GABA$_B$Rs, although likely mediated by

different brain regions such as the ventral tegmental area (Boehm et al., 2002) and possibly the clSNr, respectively. Thus, the finding that $Mpdz^{+/-}$ exhibited increased response to baclofen effects on HICs, the same direction of effect as EWD, but not locomotor activity may point to MUPP1 effects on GABA$_B$R function in some, but not all, brain regions.

Although baclofen may preferentially activate presynaptic GABA$_B$Rs at low doses (Abellan et al., 2000), the doses used in the present study activated both pre- and postsynaptic GABA$_B$Rs (Abellan et al., 2000; Misgeld et al., 1995). The neurophysiology data suggests that reduced MUPP1 expression was associated with heightened postsynaptic SNr GABA$_B$R response to baclofen, but was without a detectable effect on presynaptic, as the magnitude of GIRK-mediated outward currents was greater in $Mpdz^{+/-}$ than WT with no difference in sIPSC frequency. These data are consistent with evidence that MUPP1 may be largely, if not exclusively localized postsynaptically (Estevez et al., 2008; Krapivinsky et al., 2004). Future studies using semi-quantitative autoradiography to simultaneously assess multiple brain regions and/or neurophysiological analyses to assess discrete cell populations will be useful to determine to what extent MUPP1 effects (e.g., on GABA$_B$R-GIRK function) might be limited to discrete brain regions and/or cell populations. Taken together, since both EWD and GABA$_B$R-mediated HICs are enhanced in $Mpdz^{+/-}$, the former of which involves the clSNr (Chen et al., 2011; Chen et al., 2008) and the latter of which may involve the clSNr, it is intriguing to speculate that the effect of $Mpdz$ on EWD risk may be mediated through increased signaling of the GABA$_B$R, potentially through its association with GIRK channels.

Although my results detected no evidence that the behavior or neurophysiology findings were associated with changes in GABA$_B$R density, binding affinity, or gene

expression, these studies were not without limitations, which are discussed in detail below. However, the unexpected finding of an increase in *Kcnj6* mRNA expression, which encodes GIRK2, was detected in the clSNr of *Mpdz*$^{+/-}$ compared to WT, with no difference in whole brain. One possible explanation is that enhanced GABA$_B$R function is indeed due to a direct effect of MUPP1 expression on the GABA$_B$R or the MUPP1-GABBR2 association, but the techniques I used were not sufficiently sensitive to detect a potential difference. For instance, MUPP1 could affect the coupling efficiency of the GABA$_B$R to intracellular G-proteins, particularly since the R2 subunit associates with α$_{i/o}$ and βγ G-proteins (Robbins et al., 2001). This has been previously demonstrated for MUPP1 effects on the melatonin 1 receptor (MT1R) interaction where disruption of the MUPP1-MT1R association reduced the coupling of G$_i$ to the MT1R as well as G$_i$-mediated signaling in cultured cells, without affecting receptor trafficking, subcellular localization, or degradation (Guillaume et al., 2008). This was the first evidence demonstrating that MUPP1 was capable of altering GPCR signaling by affecting stabilization of the coupling of G-proteins to their cognate receptor. Relevant to my work, lower G-protein coupling stability with reduced MUPP1 would not predict the apparent enhancement in GABA$_B$R-mediated responses evidenced in my studies. However, if reduced MUPP1 results in altered coupling of the heterotrimeric α$_{i/o}$/βγ complex coupling to GABBR2, then activation of the receptor could result in changes in the βγ dimer-mediated activation of GIRK channels and/or inhibition of voltage gated calcium channels. Consistent with this possibility is my finding of heightened SNr GABA$_B$R-GIRK currents (a postsynaptic response) but not GABA$_B$R sIPSCs (a presynaptic response) in *Mpdz*$^{+/-}$ compared to WT, as GIRK channels are localized postsynaptically (Luscher et al., 1997).

Another possible explanation is that the change in GABA$_B$R-mediated responses is through a separate protein or effector (e.g., GIRK2) that couples with the GABA$_B$R forming a complex with MUPP1 to affect GABA$_B$R-GIRK mediated signaling (Luscher et al., 1997; Slesinger et al., 1997). It is plausible that MUPP1 alters GABA$_B$R-mediated signaling. MUPP1 may associate and/or complex with another protein or effector outside of GABBR2 that is involved in GABA$_B$R signaling. For instance, postsynaptic GABA$_B$Rs couple to GIRK2 channels (Luscher et al., 1997; Slesinger et al., 1997), and I found an increase of *Kcnj6* (GIRK2) expression in the clSNr in *Mpdz*$^{+/-}$ mice. Thus, it is possible that increased expression of *Kcnj6* (and thus potentially GIRK2 protein), is involved in the heightened GABA$_B$R activated GIRK currents in *Mpdz*$^{+/-}$, as previous studies have shown that reduced expression of GIRK2 reduced GABA$_B$R-GIRK signaling (Koyrakh et al., 2005), so increased GIRK2 would therefore be expected to increase signaling. Interestingly, the C-terminus of GIRK2 (isoform c, GIRK2c) contains a PDZ binding motif comprised of four amino acids (–ESKV) that is predicted to associate with the PDZ4 domain of MUPP1 in mice (Hui et al., 2013) (http://webservice.baderlab.org/domains/POW/). Further the –ESKV motif also associates with sorting nexin-27, another PDZ-domain containing protein, which is a regulator of GABA$_B$R-activated GIRK currents in dopamine neurons (Munoz and Slesinger, 2014). Therefore, MUPP1 could act similarly to sorting nexin-27 in regulating GABA$_B$R-activated GIRK currents. However, owing to the lack of commercially available, high quality GIRK2 antibody, it remains to be determined whether or not the differential *Kcnj6* expression translates to comparable differences in GIRK2 protein and/or GIRK channel expression. Overall, there is a clear heightened response of GABA$_B$Rs to baclofen in *Mpdz*$^{+/-}$ mice associated with behavioral changes in CNS

excitability, as well as for $GABA_BR$-GIRK currents in the SNr, but future studies are required to elucidate the distinct mechanism.

Several limitations of these experiments need to be addressed. First, I assessed total specific binding in the radioligand binding analyses, so I could not distinguish between the effect of *Mpdz* specifically on $GABA_B$ surface receptors versus intracellular or postsynaptic versus presynaptic. Given that MUPP1 may be primarily postsynaptically localized (Estevez et al., 2008; Krapivinsky et al., 2004), it is plausible that the assessment of total binding (pre- and postsynaptic) obscured any potential effects of MUPP1 on postsynaptic $GABA_BR$ density and/or affinity, and therefore future studies assessing this distinction are necessary. Further, future studies are necessary to distinguish surface expression of $GABA_BRs$ from intracellular (e.g., using a biotinylation approach) to directly assess the potential effect of MUPP1 expression on $GABA_BRs$ expressed at the surface. Another limitation is that binding was assessed in whole brain, which might have obscured potential differences limited to discrete brain regions and/or cell populations. Thus, reduced MUPP1 could be associated with altered $GABA_BR$ binding in more discrete regions such as the clSNr, but owing to the size of the clSNr, discrete isolation of this region or even the SNr proper for saturation binding analyses would be technically challenging. Finally, a limitation of the intra-clSNr baclofen and CGP55845 microinjection experiments was the use of only one dose for each compound. Since intra-clSNr baclofen did not further enhance peak HIC scores in ethanol-withdrawn mice, it is possible that higher doses are required to detect this effect. Further it is possible that higher doses of CGP55845 could have affected PTZ-enhanced HICs, so future work will be needed to test whether or not inhibition of $GABA_BRs$ alters PTZ HIC severity. This is unlikely, however, given that this dose substantially attenuated EWD HICs, without any detectable effect in PTZ-enhanced HICs, but future studies are

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necessary to better define the role of GABA$_B$Rs in both phenotypes and to determine if the effect of CGP55845 on EWD is dose-dependent.

Previous *in vitro* work has implicated a role of MUPP1 on the function of both excitatory and inhibitory neurotransmission (Fujita et al., 2012; Krapivinsky et al., 2004; Rama et al., 2008), but my work is the first to identify a specific brain region where this relationship is evident and a plausible translational impact on behavior using *Mpdz* genetic models. However, when using conventional knockout and transgenic genetic models, the results must be interpreted with caution as the possibility of developmental compensation, flanking genes, and/or insertion of the transgene disrupting other genes affecting the phenotype of interest is possible (Haruyama et al., 2009; Hummler et al., 1994; Picciotto and Wickman, 1998). Future studies using RNAi to directly assess the effect of *Mpdz*/MUPP1 on GABA$_B$R-mediated behaviors and function are necessary. However, the present results are likely specific to MUPP1 since I previously confirmed that the effect of *Mpdz* on EWD and PTZ-enhanced HICs in the *Mpdz*$^{+/-}$ and *Mpdz*^{Tg} models was consistent with the effect of *Mpdz* on both of these behaviors using *Mpdz* targeted RNAi, and the present results were consistent with the predicted effects of MUPP1 (Chapter 2; Kruse et al., 2014). Considering these results together with Chapter 2, it is intriguing to speculate that lower *Mpdz*/MUPP1 expression may result in altered of signaling of the clSNr involving heightened GABA$_B$R response evident during acute EWD, but future studies are required to directly examine this hypothesis.

Chapter 4: The role of *Mpdz* on binge-like ethanol drinking

The data presented in this chapter are unpublished.

Introduction

      A frequent element of human AUDs is repeated bouts of heavy drinking episodes leading to intoxication, a pattern that may contribute to the transition to dependence and positively reinforce the cycle of addiction in dependent individuals (Koob and Le Moal, 2001; Koob and Volkow, 2010; Viner and Taylor, 2007). This detrimental pattern of drinking may therefore be a risk factor for future onset of an AUD. Maximum number of drinks per day has been identified as a potential phenotype in genetic risk for an AUD, is heritable (~50%), and is closely related to excessive alcohol consumption and diagnosis with an AUD (Grant et al., 2009; Kendler et al., 2010; Saccone et al., 2000). Further, the effectiveness of some pharmacotherapies to reduce alcohol consumption may also be genetically determined (Leggio et al., 2013). This could explain the variability in effectiveness of potential pharmacotherapies such as baclofen that are currently in clinical trials for treating AUDs (Addolorato et al., 2000; Garbutt et al., 2010; Leggio et al., 2010; Roden and George, 2002). For instance, one trial found that baclofen was more effective in reducing alcohol consumption in individuals with the long/long variant genotype of the serotonin transporter promoter region (5-HTTLPR) compared to individuals with the short/long variant, as well as in individuals with a specific polymorphism of the D4 dopamine receptor (Leggio et al., 2013). Thus, excessive/binge intake may be an important phenotype for studying genetic risk for an AUD and response to pharmacotherapies.

Different animal models of ethanol drinking in rodents have been developed to model various aspects of AUDs. One of the most commonly used procedures of ethanol consumption is 2BC-CA that allows the determination of possible preference for ethanol over water. However, this model is limited in that rodents rarely voluntarily consume ethanol in a manner that produces significant elevation in blood ethanol levels for sustained periods of time (Dole & Gentry, 1984). Therefore, animal models of excessive/binge ethanol drinking associated with high BECs are crucial for understanding the neurobiological and genetic underpinnings that influence the maladaptive behavior of excessive/binge drinking in humans. In the DID procedure, B6 mice can consume sufficiently large amounts of ethanol to produce BECs associated with intoxication (i.e., >0.8 mg/ml) when given limited access to a single bottle of ethanol (1B-DID) (Crabbe et al., 2009; Rhodes et al., 2005; Rhodes et al., 2007). DID is also advantageous for assessing the effectiveness of pharmacotherapies to reduce ethanol intake and intoxication, as the limited access period gives a clear time-frame for when to administer treatment and measure response (Kasten et al., 2015; Moore and Boehm, 2009). Variations of the typical DID procedure have also been used to provide additional information about particular aspects of ethanol consumption. Offering the choice of water (2B-DID) during the limited access session is advantageous in that mice have the choice between consuming ethanol or water (Phillips et al., 2010). Further, testing repeated cycles of DID (discussed in detail in Chapter 1) may be used as a potential model for studying neurobiological or genetic factors associated with long term ethanol exposure.

Mpdz$^{+/-}$ mice consume less ethanol than WT littermates in 2BC-CA (Milner et al., 2015), and there has been some evidence suggesting overlapping genetic influences on 2BC-CA and DID, although it may be strongest in C57/C58 substrains (Rhodes et al.,

2007; Crabbe et al., 2012b). No single animal model can capture all features of human AUDs, so the use of multiple models to examine subsets of the clinical condition is necessary. The role of *Mpdz*/MUPP1 in risk for binge-like ethanol intake was therefore assessed using a modified DID paradigm. Further, given that reduced *Mpdz*/MUPP1 was associated with heightened $GABA_BR$-mediated responses to baclofen (Chapter 3), the effectiveness of baclofen to reduce drinking may be enhanced in $Mpdz^{+/-}$ compared to WT littermates. Specifically, I assessed the effect of *Mpdz* on binge-like ethanol intake following single and repeated DID and associated BECs, ethanol preference, and effectiveness of baclofen to reduce binge-like drinking.

Materials and Methods

Subjects

Adult (60-90 day old) female and male $Mpdz^{+/-}$ and WT littermates were bred and maintained in house as described previously (Milner et al., 2015) (Chapter 3). Colony rooms were on a 12 h reverse light/dark cycle with lights on at 21:00, and the temperature maintained at 21 ± 1˚C. Animals were housed in polycarbonate cages and received *ad libitum* water and food (LabDiet 5001Rodent Diet), except during select times in Experiments 15 and 16 (below). All procedures were approved by the Oregon Health & Science University and VA Medical Center Institutional Animal Care and Use Committees in accordance with United States Department of Agriculture and United States Public Health Service guidelines.

Drugs

R(+)-baclofen hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in 0.9% physiological saline. Two-hundred proof ethanol was purchased

from Decon Labs, Inc. (King of Prussia, PA) and diluted in tap water to the appropriate concentrations.

General DID procedures

Prior to DID in the following experiments, $Mpdz^{+/-}$ and WT mice were acclimated to the procedure room, a reversed light-dark cycle, and isolate housing. Three weeks prior to the start of the experiments, the group-housed mice (two to four per cage by sex) were moved from the colony room to the procedure room. Two weeks prior to the start of the experiments, mice were switched to a reverse light/dark cycle on a 12/12 h schedule with lights on at 21:00. Finally, one week prior to the start of the experiments, mice were individually housed, and acclimated to a single water bottle with a sipper tube. A repeated DID procedure (Cox et al., 2013) adapted from the original DID procedure by Rhodes and colleagues (2005) was used. Ethanol (20% v/v, unless indicated otherwise as in the 2 h 1B-DID experiment) was presented 3 h into the dark cycle in a 10 ml stoppered Falcon disposable clear polystyrene serological pipet (Fisher Scientific) fit with a sipper tube containing a ball bearing. Mice were weighed prior to presentation of the drinking tube(s). Amount of ethanol intake was recorded in ml. Drinking tubes in an empty cage served as a control for ethanol spillage due to experimenter handling and/or leaky sippers, and average fluid loss (ml) from the control tubes was deducted from each animal's intake. Different variations of the typical DID procedure (described below) were employed to examine how different parameters of DID (i.e., initial versus repeated exposure, voluntary versus forced access, multiple ethanol concentrations, etc.) may be influenced by $Mpdz$ expression.

			"2 h 1B-DID"				
Week	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
1	2 h 0% ethanol	2 h 3% ethanol	2 h 6% ethanol	2 h 10% ethanol	2 h 20% ethanol	--	--
2	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol BEC	--	--
3	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	--	--
4	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol saline (i.p.)	2 h 20% ethanol 10 mg/kg baclofen or saline (i.p.)	--	--
5	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol saline (i.p.)	2 h 20% ethanol 5 mg/kg baclofen or saline (i.p.)	--	--
6	2 h Water	2 h Water	2 h Water	2 h Water	2 h Water 5 mg/kg baclofen or saline (i.p.)	--	--

Table 4-1. Summary of the methods for 2 h 1B-DID. This table summarizes the methods for the assessment of ethanol intake in 2 h 1B-DID across Weeks 1-5. Throughout, 2 h indicates that a single tube of ethanol was presented for 2 h. In Week 1, the 0%, 3%, 6%, 10%, and 20% indicate the concentration of ethanol available during the 2 h period, otherwise in Weeks 2-5 20% (v/v) ethanol was presented during the 2 h period. BEC indicates that retro-orbital sinus blood samples were collected immediately after ethanol tubes were removed at the end of the 2 h DID period. Saline on Day 4 indicates that all mice were administered saline (i.p.) 15 min prior to the 2 h DID period. On Day 5 (test day), 10 mg/kg (Week 4) and 5 mg/kg (Week 5) baclofen indicates the dose of baclofen (i.p.) administered to half of the mice 15 min prior to the 2 h DID period, whereas the other half received saline as indicated. Water indicates that a water tube, rather than an ethanol tube, was presented during the 2 h DID period.

2 h 1B-DID

For a summary of methods, see Table 4.1. Female and male *Mpdz$^{+/-}$* and WT littermates (n = 9-17/genotype/sex) were compared for ethanol drinking in single and repeated 2 h 1B-DID, for five consecutive days per week across a total of five weeks. To more easily differentiate this paradigm from the 1B-DID and 2B-DID experiments, I refer to this experiment as 2 h 1B-DID rather than binge-like ethanol drinking since only 2 h drinking was assessed, and on average mice do not attain BECs of >0.8 mg/ml, but do consume large amounts in this limited access timeframe (Moore and Boehm, 2009; Moore et al., 2007; Rhodes et al., 2007). This model was used to determine if *Mpdz* affects ethanol intake resulting in BECs just below, but not above the binge threshold. In Week 1, ethanol concentration was increased daily (Days 1-5) as follows: 0%, 3%, 6%, 10%, and 20% (v/v), to assess 2 h 1B-DID at concentrations for which *Mpdz$^{+/-}$* and WT differed in ethanol consumption using a 2BC-CA paradigm (Milner et al., 2015). Further, as 20% ethanol may have aversive gustatory effects, an escalation to 20% was used in an attempt to attenuate this aversion. Following Week 1, on Days 1-5 in Weeks 2-5, mice were presented with a single tube containing 20% (v/v) ethanol for 2 h. The effectiveness of baclofen to reduce intake was assessed in Weeks 4 and 5 (described in detail below). Retro-orbital sinus blood samples were collected immediately after 2 h ethanol exposure on Day 5 in Week 2 to determine if *Mpdz$^{+/-}$* and WT littermates differed in BECs associated with 2 h ethanol intake, and BEC values determined (*see BECs below*).

Ethanol intake was analyzed using three-way repeated measures ANOVA with genotype and sex as the between-subjects factor, and concentration of ethanol (Week 1) or day of ethanol access (Weeks 2-3) as the repeated measure. Week 1 was analyzed separately from Weeks 2 and 3 to compare the effect of *Mpdz* on different

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concentrations of ethanol. BEC values and body weight were analyzed using a two-way ANOVA with genotype and sex as the independent variables. BEC values and ethanol intake were compared using a linear regression with BEC regressed on ethanol intake.

Baclofen on 2 h 1B-DID ethanol intake. For a summary of the methods, see Table 4.1. On Day 5 in Week 4 and Week 5, *Mpdz*$^{+/-}$ and WT littermates (n = 4-9/genotype/sex/treatment group) were tested for the effect of baclofen to reduce ethanol intake in 2 h 1B-DID. Baclofen was assessed after several weeks of DID to model the effectiveness of baclofen after repeated exposure to ethanol. All mice received saline (i.p.) 15 min prior to 2 h 1B-DID on Day 4 in Weeks 4 and 5, for acclimation to potential stress induced by handling and injections. On Day 5 in Week 4, 15 min prior to ethanol access for 2 h, half of the *Mpdz*$^{+/-}$ and WT littermates were administered saline (i.p.) and the other half received 10 mg/kg baclofen (i.p.). On Day 5 of Week 5, 15 min prior to ethanol access for 2 h, half of the *Mpdz*$^{+/-}$ and WT littermates received saline (i.p.) and the other half received 5 mg/kg baclofen. Mice that received baclofen in Week 4 were administered saline in Week 5, and vice versa, to reduce the potential confound of repeated baclofen testing. Baclofen was administered 15 min prior to ethanol access based on previous work (Boehm et al., 2002; Colombo et al., 2000) and to minimize access to ethanol during initial stress associated with the injection. Baclofen has a half-life in the blood of approximately 2 h (Mandema et al., 1992) so it was expected to be effective throughout the full 2 h drinking period. These doses were selected because they have previously been shown to effectively reduce ethanol consumption across different drinking models in rats and B6 mice (Kasten et al., 2015; Tanchuck et al., 2011). In Week 6, *Mpdz*$^{+/-}$ and WT littermates were tested for 2 h water intake, in which a water tube, rather than an ethanol tube, was presented for 2 h on Days 1-5. On Day 4 of Week 6 mice received saline 15 min prior to water DID. On Day 5 of Week 6, half of

the *Mpdz*$^{+/-}$ and WT littermates received saline (i.p.) and the other half received 5 mg/kg baclofen 15 min prior to 2 h water intake to determine if baclofen generally reduced fluid intake, or was specific to ethanol.

Ethanol intake (Week 4 and Week 5) and water intake (Week 6) on Days 1-4 were analyzed separately using three-way repeated measures ANOVA with genotype and sex as the between-subjects factor and day of DID access as the repeated measure. The effect of baclofen on ethanol intake (Weeks 4 and 5) and water intake (Week 6) on Day 5 was analyzed separately in each week using a three-way ANOVA with genotype, sex, and treatment (baclofen or saline) as the independent variables. Day 5 was the test day for the effect of baclofen (compared to saline) and was therefore analyzed separately from Days 1-4.

"1B-DID"

Week	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	4 h 20% ethanol BEC	--	--	--
2	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	4 h 20% ethanol	--	--	--
3	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	4 h 20% ethanol	--	--	--
4	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	4 h 20% ethanol	--	--	--
5	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	4 h 20% ethanol	--	--	--
6	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	4 h 20% ethanol	--	--	--
7	2 h 20% ethanol	2 h 20% ethanol	2 h 20% ethanol	4 h 20% ethanol BEC	--	--	--

Table 4-2. Summary of the methods 1B-DID. This table summarizes the methods for the assessment of binge-like ethanol intake across Weeks 1-7 of 1B-DID. Throughout, mice had access to a single ethanol tube containing a 20% (v/v) ethanol concentration. 2 h indicates that a single ethanol tube was presented for 2 h. 4 h indicates that a single ethanol tube was presented for 4 h.   BEC indicates that retro-orbital sinus bloods samples were collected immediately after ethanol tubes were removed at the end of the 4 h DID period.

1B-DID

For a summary of methods, see Table 4.2. *Mpdz*$^{+/-}$ and WT littermates (females only; n = 13-14 per genotype) were assessed for binge-like ethanol intake and associated intoxication with single and repeated exposure (7 weeks) using a typical 4-day DID paradigm. The purpose of this experiment was to assess the effect of *Mpdz* on initial binge-like ethanol intake, as well as repeated binge-like ethanol intake (Cox et al., 2013). Briefly, mice had access to a single tube of ethanol for 2 h on Days 1-3, and for 4 h on Day 4 per week, across Weeks 1-7 of 1B-DID. Retro-orbital sinus bloods samples were collected immediately after 4 h DID on Day 4 in Weeks 1 and 7, and BEC values were determined (*see BECs*).

2 h ethanol intake (2 h, Days 1-3) and 4 h ethanol intake (Day 4) across Weeks 1-7 were analyzed separately using two-way repeated measures ANOVA with genotype as the between subjects, and day of access (2 h data; 21 days) or week (4 h data; 7 weeks) as the within subjects independent variables. As the 4 h ethanol intake data were my direct measure of binge-like ethanol drinking, Day 4 (4 h) data were analyzed separately from 2 h data. Genotype differences in BEC values and body weight were analyzed using a two-sample t-test. BEC values and ethanol intake were compared using a linear regression with BEC regressed on ethanol intake.

"2B-DID"

Week	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1	2 h 20% ethanol and water	2 h 20% ethanol and water	2 h 20% ethanol and water	4 h 20% ethanol and water	--	--	--
2	2 h 20% ethanol and water	2 h 20% ethanol and water	2 h 20% ethanol and water	4 h 20% ethanol and water	--	--	--
3	2 h 20% ethanol and water	2 h 20% ethanol and water	2 h 20% ethanol and water	4 h 20% ethanol and water	--	--	--
4	2 h 20% ethanol and water	2 h 20% ethanol and water	2 h 20% ethanol and water saline (i.p.)	4 h 20% ethanol and water 2.5 mg/kg baclofen or saline (i.p.)	--	--	--
5	2 h 20% ethanol and water	2 h 20% ethanol and water	2 h 20% ethanol and water saline (i.p.)	4 h 20% ethanol and water 1.25 mg/kg baclofen or saline (i.p.)	--	--	--

Table 4-3. Summary of the methods for 2B-DID. This table summarizes the methods for the assessment of binge-like ethanol intake in 2B-DID across Weeks 1-5. Throughout, mice had access to one ethanol (20% v/v) and one water tube containing tap water during DID. 2 h indicates that an ethanol and water tube ethanol were presented for 2 h. 4 h indicates that ethanol and water tubes were presented for 4 h. Saline on Day 3 indicates that all mice were administered saline (i.p.) 15 min prior to the 2 h DID period. On Day 4, 2.5 mg/kg (Week 4) and 1.25 mg/kg (Week 5) baclofen indicate the dose of baclofen (i.p.) administered to half of the mice 15 min prior to the 4 h DID period, whereas the other half received saline.

2B-DID

Female and male *Mpdz*$^{+/-}$ and WT littermates (n = 9-12/genotype/sex) were compared using a 2B-DID paradigm in order to assess ethanol intake with a choice between ethanol and water, as well as ethanol preference, in a binge-like ethanol drinking model. Binge-like ethanol intake in single and repeated DID was again tested to determine the effect of *Mpdz* on initial intake versus intake associated with repeated exposure (Cox et al., 2013). Mice had 2 h access to one ethanol and one water tube on Days 1-3, and 4 h access on Day 4 per week, across Weeks 1-5. To account for any development of side preference, ethanol and water tubes were alternated between left and right sides of the cages daily. In addition to ethanol intake, total fluid intake (water and ethanol) was also assessed. In Weeks 4 and 5 (described in detail below), the effect of baclofen on binge-like ethanol intake and preference was assessed.

2 h ethanol intake, total fluid intake, preference, and water intake (Days 1-3), as well as 4 h ethanol intake, total fluid intake, preference and water intake (Day 4) across Weeks 1-3 were analyzed separately using repeated measures three-way ANOVA with genotype and sex as the between-subjects, and day of access (2 h data; 9 days) or week (4 h data; 3 weeks) as the repeated measure. The 4 h ethanol intake data were my direct measure of binge-like ethanol drinking and were therefore analyzed separately from the 2 h data and independently for Weeks 1-3. Preference ratio was determined by dividing amount of ethanol intake (ml) by total fluid intake (ml), and a one-sample t-test (sample means compared to 0.5 which is neutral preference) was used to analyze preference for ethanol over water within each genotype. A value of 0.5 indicates no preference. A two-way ANOVA with genotype and sex as the independent variables was used to analyze differences in body weight.

Baclofen on binge-like ethanol intake (2B-DID). On Day 4 in Week 4 and Week 5, the effect of baclofen to decrease binge-like ethanol intake and ethanol preference was assessed in *Mpdz⁺ᐟ⁻* and WT littermates (Week 4: n = 4-6/genotype/sex/treatment and Week 5: n = 3-6/genotype/sex/treatment). To minimize stress associated with handling and injections, on Day 3 in Week 4 and Week 5, all mice received saline (i.p.) 15 min prior to 2B-DID. On Day 4 in Week 4, mice received saline (i.p.) or 2.5 g/kg baclofen (i.p.) 15 min prior to access to one ethanol tube and one water tube for 4 h (Table 4.3). On Day 4 in Week 5, mice received saline (i.p.) or 1.25 g/kg baclofen 15 min prior to 4 h 2B-DID. These lower doses were selected because they are devoid of locomotor effects and were shown to reduce drinking in a free choice (water versus ethanol) paradigm (Villas Boas et al., 2012). Mice that received baclofen in Week 4 were administered saline in Week 5, and vice versa, to reduce the potential confound of repeated baclofen testing.

The effect of baclofen compared to saline on ethanol intake, total fluid intake, and water intake in the first 2 h, second 2 h, and total 4 h periods of DID on Day 4 in Weeks 4 and 5 was assessed using three-way ANOVA with genotype, sex, and treatment (baclofen or saline) as the independent variables. Differences in 4 h preference were also assessed using a three-way ANOVA. The effect of 2.5 mg/kg (Week 4) and 1.25 mg/kg (Week 5) baclofen on intake and preference were analyzed separately since the Week 5 group had an additional week of DID and therefore the two groups did not receive the same treatment and could not be accurately compared.

BEC

Retro-orbital sinus blood samples were analyzed according to previous work (Rustay and Crabbe, 2004). Blood samples (20 µl) were collected using a capillary tube

and immediately placed into tubes containing 50 µl of ice-cold zinc sulfate. 300 µl of ice-cold distilled water and 50 µl of ice-cold barium hydroxide were added to the samples, which were then vortexed and centrifuged at 12,400 g for 5 min. Approximately 400 µl of the supernatant was removed and frozen until analysis using gas chromatography (Model 6890N; Agilent Technologies, Santa Clara, CA) and compared to a standard ethanol concentration curve (0.2369 to 4.932 mg/ml).

General Statistical Analyses

Individual statistical analyses are described above for each experiment. Total amount of ethanol intake was analyzed in g/kg body weight to account for individual differences in weight, and total fluid intake and water intake were analyzed as milliliter per kilogram (ml/kg) body weight. Unless otherwise indicated, Tukey's HSD post-hoc analyses were used to follow up significant main effects and interactions. All analyses were conducted in SYSTAT 13 Statistical Software. Degrees of freedom were adjusted by SYSTAT 13 for missing values in repeated measures analyses. Data are presented as mean ± SEM. Significance was set at $p < 0.05$ unless otherwise indicated. Full F- and t-statistics are presented for p-values < 0.2, otherwise only the p-value is indicated. All data were normally distributed (Shapiro-wilks test of normality; $p > 0.05$).

Results

Ethanol intake in *Mpdz*$^{+/-}$ and WT mice (2 h 1B-DID)

Separate ANOVAs for body weight in Week 1 and Week 5 indicated no main effect of genotype (for both $p > 0.3$) or a genotype × sex interaction (for both $p > 0.3$), although there was a main effect of sex ($F_{1,47} = 76$, $p < 0.0001$ and $F_{1,47} = 73$, $p < 0.0001$, respectively) with males weighing more. *Mpdz*$^{+/-}$ and WT did not differ in body

weight at the start (22.1 ± 0.5 and 22.5 ± 1.7 g, respectively) or end of the experiment (23.9 ± 0.6 and 24.0 ± 0.5 g, respectively). $Mpdz^{+/-}$ and WT littermates had access to increasing concentrations of ethanol for 2 h on Days 1-5 in Week 1, and 20% ethanol for 2 h on Days 1-5 in Weeks 2-3. For Week 1, repeated measures ANOVA indicated a main effect of ethanol concentration ($F_{4,164}$ = 91.6, p < 0.0001), a main effect of sex ($F_{1,41}$ = 8.7 , p = 0.005) with females consuming more than males, but no main effect of genotype (p = 0.4), and no significant interactions (for all p > 0.4). Post-hoc analyses confirmed that intake differed between all concentrations (p < 0.0001) except 0% and 3% (p = 0.9), or 6% and 10% (p = 0.1). These data indicated that $Mpdz^{+/-}$ and WT littermates did not differ in 2 h intake of water (0%), or 3%, 6%, 10%, or 20% ethanol concentrations (Fig. 4-1a), suggesting concentration of ethanol in the lack of effect of $Mpdz$ on 2 h 1B-DID intake.

$Mpdz^{+/-}$ and WT littermates had access to 20% ethanol across Weeks 2 and 3. Repeated measures ANOVA indicated a main effect of sex ($F_{1,45}$ = 36.7, p < 0.0001) with females consuming more than males, but no main effects of genotype (p = 0.9; Fig. 4-1a) or day of ethanol intake (p = 0.7), and a trend towards a sex × day ($F_{9,405}$ = 2.0, p = 0.053) interaction, but no other interactions (all p > 0.3). Therefore, consistent with Week 1, $Mpdz^{+/-}$ and WT littermates did not differ in 2 h ethanol intake across Weeks 2 and 3 (Fig. 4-1a).

BECs were assessed at the end of Week 2, and $Mpdz^{+/-}$ and WT littermates did not differ in BEC values (p = 0.4; Fig. 4-1b), and there was no effect of sex (p = 0.6). A significant genotype × sex interaction was detected (p = 0.02), but Tukey's HSD post-hoc analyses failed to reveal any significant effects (all p > 0.15) so data were collapsed on sex. On average, ethanol intake was not sufficient to attain BECs above the binge threshold (>0.8 mg/ml), as BECs were 0.69 ± 0.08 mg/ml and 0.70 ± 0.07 mg/ml for

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Mpdz^{+/-} and WT mice, respectively. Individual BEC values were significantly correlated (Fig. 4-1c) with ethanol intake for *Mpdz*^{+/-} ($r(21) = 0.73$; $p = 0.0001$) and WT littermates ($r(15) = 0.49$; $p = 0.04$).
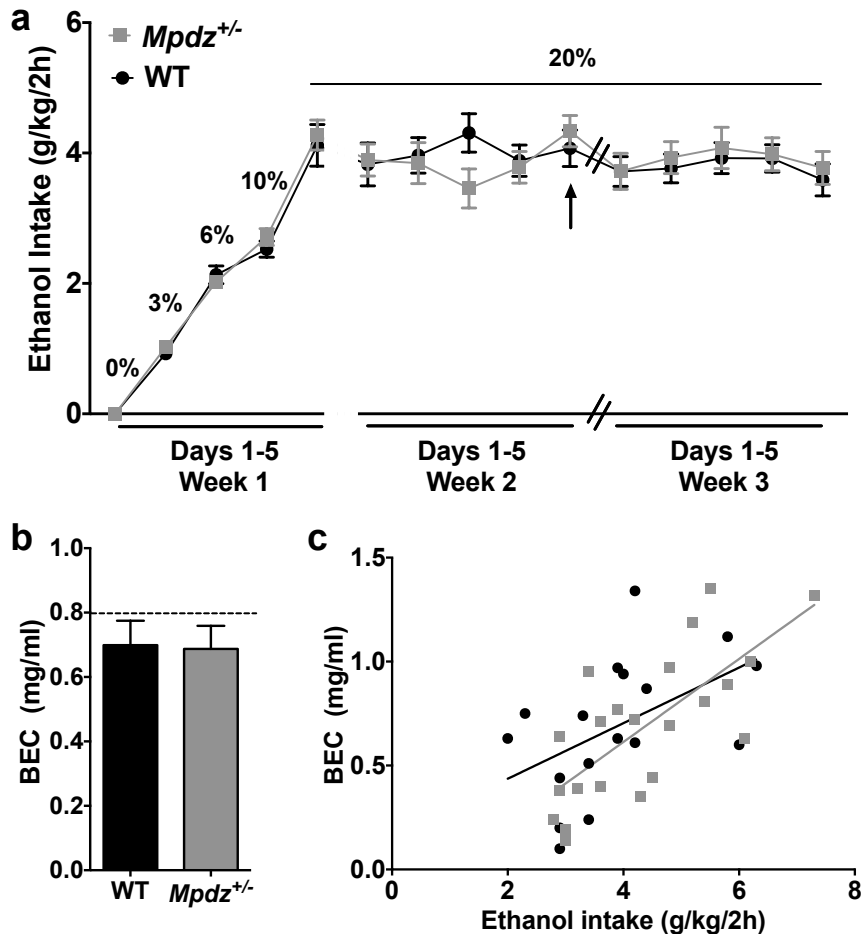
Figure 4-1. *Mpdz*^{+/-} and WT mice did not differ in 2 h 1B-DID. *Mpdz*^{+/-} and WT (n = 23-28/genotype) mice had 2 h access to ethanol on Days 1-5 across Weeks 1-5, with baclofen pretreatment on Day 5 in Weeks 4 and 5 (Weeks 4 and 5 data presented in Fig. 4-2). (a) In Week 1 *Mpdz*^{+/-} and WT littermates did not differ in 2 h intake of water (0%) or 3%, 6%, 10%, or 20% ethanol concentrations. Similarly, across Weeks 2 and 3, *Mpdz*^{+/-} and WT mice did not differ in 2 h intake of 20% ethanol. (b) Neither *Mpdz*^{+/-} nor

WT littermates attained BECs above the binge threshold (>0.8 mg/ml; *dashed line*) and nor did they differ in BEC values taken at the end of the Day 5 in Week 2. c) 2 h ethanol intake was significantly correlated with BEC values for *Mpdz*^{+/-} (r = 0.73; p = 0.001) and WT (r = 0.49; p = 0.04).   Data are presented as mean ± SEM (a,b) and are shown collapsed on sex.

Effect of baclofen on decreasing ethanol intake (2 h 1B-DID) in Mpdz^{+/-} *and WT mice*

Separate repeated measures ANOVAs indicated a main effect of sex on 2 h ethanol intake across Days 1-4 in Week 4 ($F_{1,45}$ = 33.2, p < 0.0001), as well as Days 1-4 in Week 5 ($F_{1,44}$ = 20.7, p < 0.0001) with females consuming more than males, but no main effect of genotype (for both weeks p = 0.8) and no genotype × sex interactions (for both weeks p > 0.2) were detected. Ethanol intake remained consistent across Days 1-4 in Week 4 (p = 0.4), but a main effect of day on ethanol intake was detected in Week 5 ($F_{3,138}$ = 22.4, p < 0.0001) although there was no interaction with genotype and/or sex (all p > 0.5). Therefore, *Mpdz*^{+/-} and WT littermates did not differ in 2 h ethanol intake across Days 1-4 in Week 4 (Fig. 4-2a) or Week 5 (Fig. 4-2c).

In Weeks 4 and 5, the effectiveness of baclofen (10 mg/kg and 5 mg/kg, respectively) to reduce ethanol drinking in *Mpdz*^{+/-} and WT littermates was assessed. Three-way ANOVA indicated that baclofen (10 mg/kg) significantly decreased 2 h ethanol intake ($F_{1,42}$ = 48.4, p < 0.0001; Fig. 4-2b) with no main effect of genotype (p = 0.6) and a trend towards a sex effect ($F_{1,42}$ = 4.1, p = 0.054), but no significant interactions between factors (for all p > 0.2). Similar results were obtained using a lower dose of baclofen (5 mg/kg; Fig. 4-2d) with a main effect of treatment ($F_{1,43}$ = 16.1, p < 0.0001), a main effect of sex ($F_{1,43}$ = 7.9, p < 0.0001), but no main effect of genotype ($F_{1,43}$ = 2.8, p = 0.1), and no significant interactions between factors (for all p > 0.3). Consistently, these data indicated that both 5 mg/kg and 10 mg/kg doses of baclofen

effectively reduced 2 h ethanol intake, but $Mpdz^{+/-}$ and WT littermates did not differ in response to baclofen to reduce drinking.

To determine the selectivity of baclofen on ethanol compared to general fluid intake, in Week 6, $Mpdz^{+/-}$ and WT littermates were assessed for water intake across Days 1-4 with 2 h access to a water tube, and the effect of 5 mg/kg baclofen on water intake was assessed on Day 5. For water intake on Days 1-4, repeated measures ANOVA indicated a main effect of sex ($F_{1,32}$ = 10.5, p = 0.003) with females consuming more than males and a main effect of day ($F_{3,96}$ = 3.3, p = 0.03), but no main effect of genotype (p = 6), and no significant interactions between factors (for all p > 0.2), indicating no difference in water intake between $Mpdz^{+/-}$ and WT littermates (Fig. 4-3a). On Day 5, baclofen decreased 2 h water intake ($F_{1,36}$ = 9.9, p =0.003), but no main effects of genotype (p = 0.8) or sex ($F_{1,36}$ = 3.8, p = 0.06), and no significant interactions between factors (for all p > 0.3) were detected. The effect of 10 mg/kg baclofen on water intake was not assessed since the 5 mg/kg dose was associated with a clear reduction, indicating a higher dose would also be expected to reduce water intake. Therefore, although 5 mg/kg and 10 mg/kg baclofen were effective in reducing ethanol intake in a 2h 1B-DID paradigm, these higher doses of baclofen may not be specific to ethanol intake, as 5 mg/kg baclofen also generalized to water intake. It is likely that this lack of specificity is related to the locomotor depressant effects of higher doses of baclofen (Gianutsos and Moore, 1978; Villas Boas et al., 2012), although previous studies reported no effect on water intake with higher doses of baclofen, but the 5 mg/kg dose has been shown to reduce sucrose operant self-administration (Colombo et al., 2000; Kasten et al., 2015; Tanchuck et al., 2011).
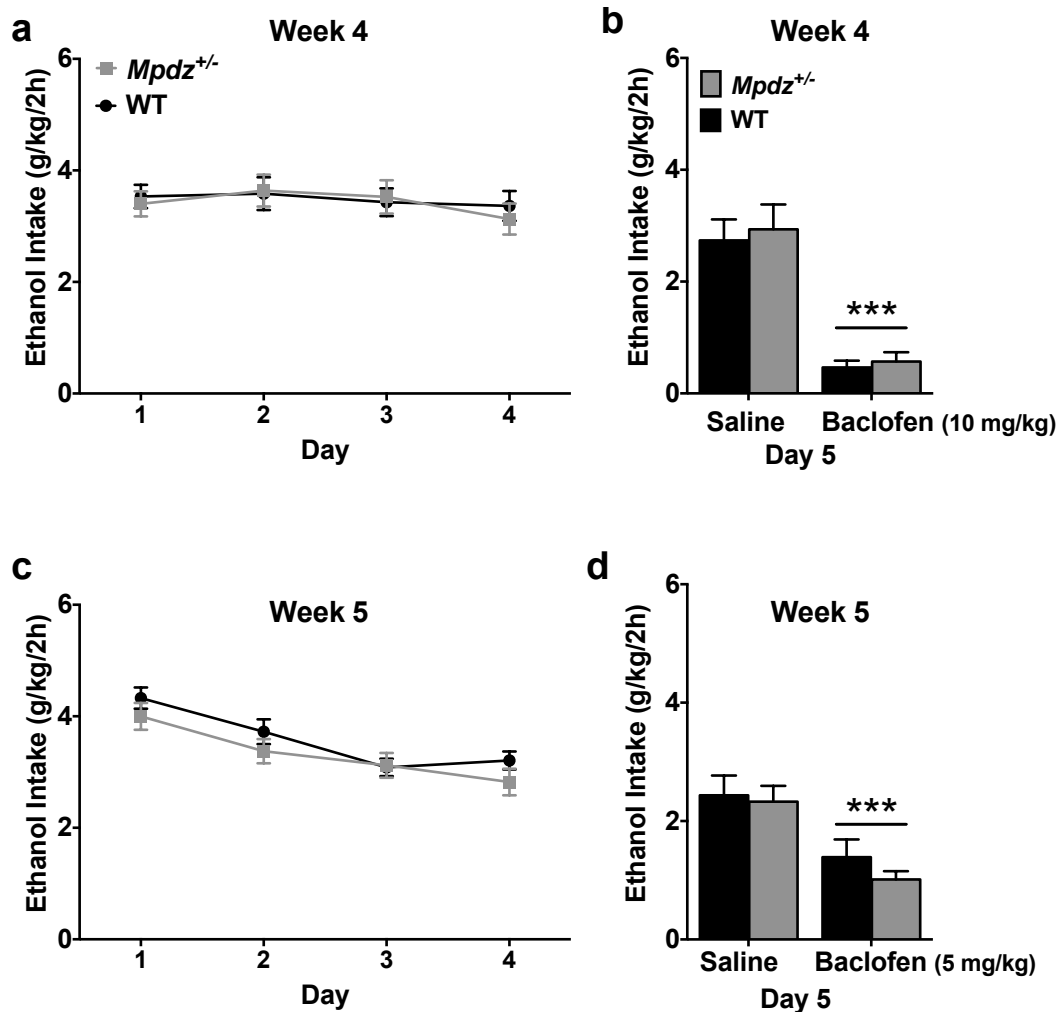
Figure 4-2. Baclofen administration reduced ethanol intake comparably in _Mpdz_[+/-] and WT mice (2 h 1B-DID). _Mpdz_[+/-] and WT littermates (n = 10-14/genotype/treatment) had 2 h access to ethanol on Days 1-5 in Weeks 4 and 5, with baclofen or saline administered on Day 5 in both weeks. _Mpdz_[+/-] and WT littermates did not differ in 2 h ethanol intake across Days 1-4 in (a) Week 4 or (c) Week 5. Systemic administration of (b) 10 mg/kg and (d) 5 mg/kg baclofen 15 min prior to ethanol access significantly reduced 2 h ethanol intake in both _Mpdz_[+/-] and WT mice (***$p < 0.001$) compared to saline treated mice, with no genotype difference in effectiveness of baclofen on reducing intake. Data are presented as mean ± SEM and are shown collapsed on sex. ***$p < 0.001$ indicate a main effect of baclofen on ethanol intake.
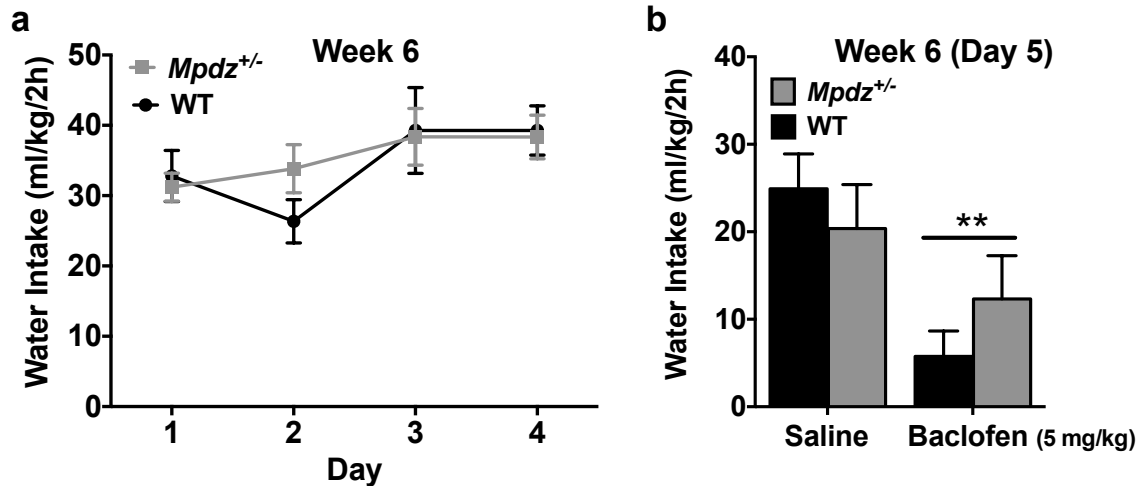
Figure 4-3. Baclofen (5 mg/kg) administration reduced water intake comparably in *Mpdz*$^{+/-}$ and WT mice (2 h 1B-DID). *Mpdz*$^{+/-}$ and WT mice were assessed for the effect of baclofen (5 mg/kg) on 2 h water intake. (a) *Mpdz*$^{+/-}$ and WT littermates did not differ in 2 h water intake across Days 1-4 in Week 6. (b) On Day 5, administration of baclofen (5 mg/kg) prior to water access reduced 2 h water intake (**$p < 0.01$) similarly in *Mpdz*$^{+/-}$ and WT littermates (n = 4-9/genotype/sex/treatment group). **$p < 0.01$ indicates a main effect of baclofen on water intake. Data are presented as mean ± SEM and collapsed on sex.

Binge-like ethanol intake in *Mpdz*$^{+/-}$ and WT mice (1B-DID)

Mpdz$^{+/-}$ and WT littermates were assessed for differences in binge-like ethanol drinking with single and repeated 1B-DID exposure. The purpose of this experiment was to determine if *Mpdz* predisposes to either initial binge-like ethanol intake or intake following repeated exposure. Only females were used in the analyses for this experiment due to a dearth of *Mpdz*$^{+/-}$ males in this cohort. *Mpdz*$^{+/-}$ and WT littermates did not differ in body weight at the beginning (19.5 ± 0.2 and 19.6 ± 0.3, respectively; p = 0.9) or end (21.8 ± 0.2 and 22.0 ± 0.3 g, respectively; p = 0.6) of the seven weeks of 1B-DID.

Repeated measures ANOVA on 2 h ethanol intake (Days 1-3 across Weeks 1-7) indicated no main effect of genotype (p = 0.5), but a main effect of day of ethanol intake ($F_{20.400}$ = 4.4, p < 0.001), although no interaction with genotype was apparent (p = 0.8). Therefore, *Mpdz*$^{+/-}$ and WT littermates did not differ in the 2 h ethanol intake (Days 1-3) across Weeks 1-7 (Fig. 4-4a). In order to assess the effect of *Mpdz* on binge-like ethanol drinking, 4 h intake (Day 4) was compared between *Mpdz*$^{+/-}$ and WT littermates across Weeks 1-7. Repeated measures ANOVA on 4 h ethanol intake indicated a trend towards a main effect of genotype ($F_{1,25}$ = 2.8, p = 0.08; Fig. 4-4b) and a significant main effect of Week ($F_{6,150}$ = 6.1, p < 0.0001), but no interaction (p = 0.7). Therefore, these data did not detect an effect of *Mpdz* on initial binge-like ethanol drinking or drinking following repeated DID.

BEC associated with 4 h binge-like ethanol intake was also compared between *Mpdz*$^{+/-}$ and WT littermates in Week 1 and Week 7 of 1B-DID. Consistent with ethanol intake, *Mpdz*$^{+/-}$ and WT littermates did not differ in BEC in Week 1 (p = 0.5; Fig. 4-5a). Unexpectedly, although there was no difference in 4 h ethanol intake in Week 7, slightly higher BEC values were apparent in *Mpdz*$^{+/-}$ (t_{25} = 2.3, p = 0.03; Fig. 4-5c) compared to

WT littermates in Week 7. However, 4 h ethanol intake was significantly correlated with BEC values for *Mpdz*$^{+/-}$ in Week 1 (*r(9)* = 0.82, p = 0.002; Fig. 4-5b), but not Week 7 (*r(12)* = 0.06; p = 0.8; Fig. 4-5d), and correlated for WT littermates in Week 1 (*r(11)* = 0.71, p = 0.007; Fig. 4-5b) and Week 7 (*r(11)* = 0.76, p = 0.002; Fig. 4-5d). Upon further exploration of the 4 h intake period in Week 7, *Mpdz*$^{+/-}$ showed a trend (t_{25} = 2.0, p = 0.05) toward greater ethanol intake than WT littermates in the first 2 h of ethanol access (5.1 ± 0.2 and 4.5 ± 0.2 g/kg, respectively), but not the second 2 h period (4.9 ± 0.3 and 4.9 ± 0.3, p = 0.9, respectively) or across total 4 h (Fig. 4-5b). Therefore, the higher BEC values in Week 7 in *Mpdz*$^{+/-}$ could be a consequence of a lack of correlation with ethanol intake and a trend towards a higher increase in the first 2 h of intake in *Mpdz*$^{+/-}$.

a

b
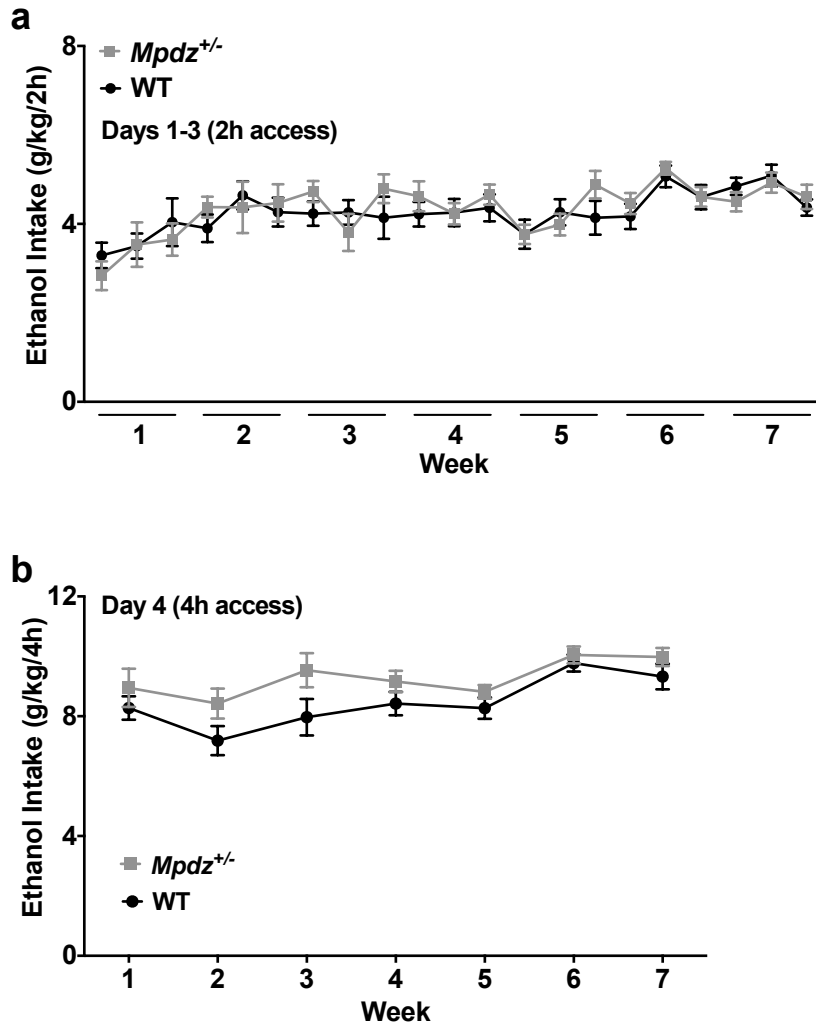
Figure 4-4. *Mpdz*^{+/-} **and WT mice did not differ in binge-like ethanol intake (1B-DID).** *Mpdz*^{+/-} and WT mice (n = 13-14/genotype) had 2 h limited access to ethanol (20% v/v) on Days 1-3 (a) and 4 h access on Day 4 (b) in Weeks 1-7. (a) On Days 1-3 per week, *Mpdz*^{+/-} and WT mice did not differ in 2 h ethanol intake across Weeks 1-7. (b) *Mpdz*^{+/-} and WT mice did not differ in 4 h binge-like ethanol intake on Day 4 across Weeks 1-7. Data are presented as mean ± SEM.
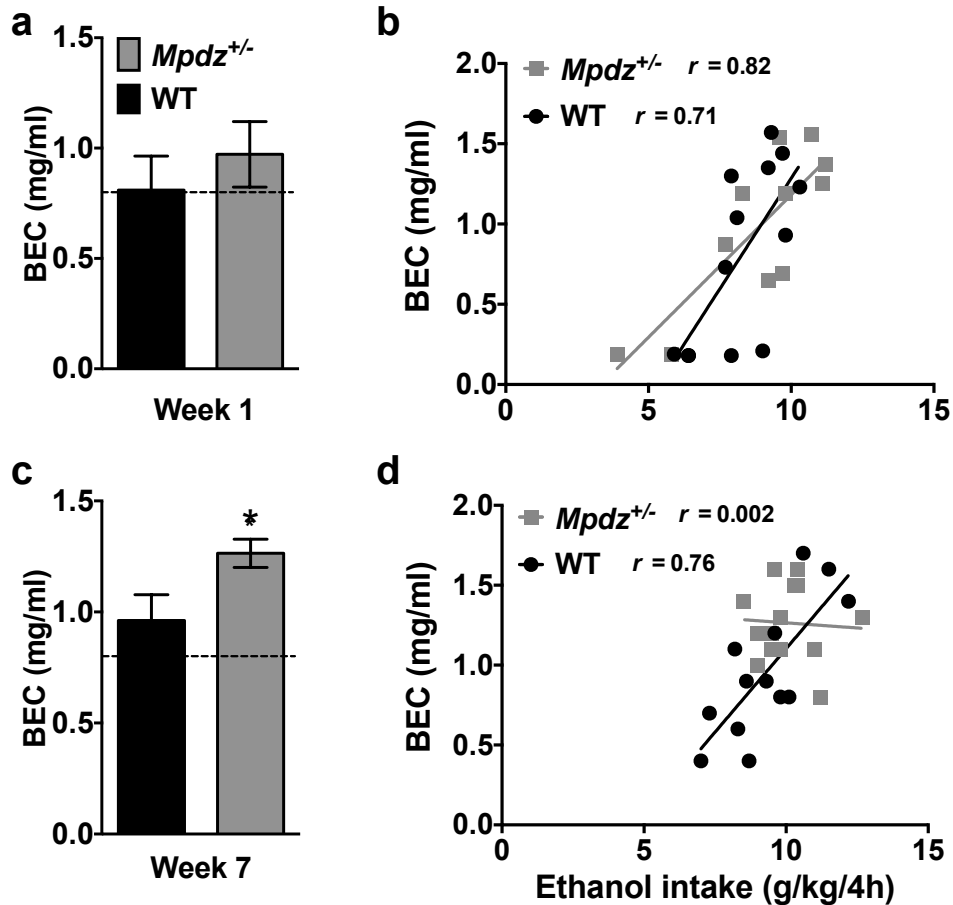
Figure 4-5. Binge-like ethanol intake and correlated BEC values in Week 1 and Week 7 (1B-DID). (a) Following 4 h ethanol intake in Week 1 on Day 4, *Mpdz*+/- and WT littermates both attained BEC values at or above binge threshold (>0.8 mg/ml; *dashed line*), but did not differ in BEC. (b) Significant correlations between 4 h ethanol intake and BEC values in both *Mpdz*+/- (*r* = 0.82; p =0.002) and WT mice (*r* = 0.71; p = 0.007) were apparent in Week 1 (Day 4). (c) In Week 7 on Day 4, following 4 h ethanol intake, BEC values were higher in *Mpdz*+/- compared to WT littermates (*p < 0.05). (d) No significant correlation between 4 h ethanol intake and BEC values in *Mpdz*+/- (*r* = 0.06; p = 0.8) was detected, but a significant correlation was apparent in WT (*r* = 0.76; p = 0.002). N = 11-14 per genotype. Data are presented as mean ± SEM (a,c). *p < 0.05 indicates higher BEC in *Mpdz*+/-.

Binge-like ethanol intake and preference in *Mpdz*$^{+/-}$ and WT mice (2B-DID)

 Mpdz$^{+/-}$ and WT littermates were also assessed for binge-like ethanol intake in the 2B-DID procedure. Separate ANOVAs for body weight in Week 1 and Week 5 indicated no main effect of genotype (for both p > 0.8), a main effect of sex ($F_{1,40}$ = 289, p < 0.0001 and $F_{1,40}$ = 170, p < 0.0001, respectively) with males weighing more, but no interaction (for both p > 0.7). Therefore, *Mpdz*$^{+/-}$ and WT did not differ in body weight at the beginning of the experiment (22.0 ± 0.8 and 21.8 ± 0.7, respectively) or end of the experiment (23.6 ± 0.7 and 23.4 ± 0.6). In addition to ethanol intake, the combined intake of water and ethanol was also assessed and presented as total fluid intake. Repeated measures ANOVA on 2 h ethanol intake and 2 h total fluid intake across Weeks 1-3 indicated a main effect of sex ($F_{1,38}$ = 19.6, p < 0.0001 and $F_{1,24}$ = 38.1, p < 0.0001, respectively) with females consuming more than males, and a main effect of day ($F_{8,304}$ = 3.5, p = 0.001 and $F_{8,192}$ = 3.5, p = 0.001, respectively), but no main effect of genotype (p = 0.5 and $F_{1,24}$ = 2.8, p = 0.07, respectively; Fig. 4-6). For both 2 h ethanol intake and total fluid intake, no significant interactions between factors were detected (for all p > 0.2). Further, *Mpdz*$^{+/-}$ and WT did not differ in 2 h preference (data not shown) for ethanol across Weeks 1-3 (p = 0.9), although there was a main effect of sex ($F_{1,24}$ = 5.9, p = 0.02) and day ($F_{8,192}$ = 9.6, p < 0.0001), but no interactions between factors (for all p > 0.1). Therefore, *Mpdz*$^{+/-}$ and WT littermates did not differ in 2 h ethanol intake (Fig. 4-6a) or 2 h total fluid intake (Fig. 4-6b) across Weeks 1-3 of 2B-DID.

 Binge-like ethanol intake (4 h) was assessed on Day 4 across Weeks 1-3. Separate repeated measures ANOVAs for 4 h ethanol intake and 4 h total fluid intake indicated main effects of sex ($F_{1,35}$ = 21.5, p < 0.0001 and $F_{1,35}$ = 9.7, p = 0.004, respectively) and week ($F_{2,70}$ = 8.7, p < 0.0001 and $F_{2,70}$ = 21.5, p < 0.0001,

respectively), and a trend towards a sex × week interaction for 4 h ethanol intake ($F_{2,70}$ = 3.0, p = 0.06), but no other interactions between genotype, sex, and/or week for ethanol or total fluid intake (p > 0.15) were detected. No main effects of genotype for 4 h ethanol or total fluid intake (for both p > 0.6) were detected, indicating that $Mpdz^{+/-}$ and WT littermates did not differ in binge-like ethanol intake or total amount of fluid consumed in 2B-DID. 2B-DID is advantageous in that it permits the assessment of preference for ethanol over water. For 4 h ethanol preference across Weeks 1-3 no main effects of genotype, sex, or week (for all p > 0.3), and no significant interactions (for all p > 0.1) were detected (Fig. 4-7b). Although $Mpdz^{+/-}$ and WT littermates did not differ in ethanol preference, one-sample t-tests confirmed that both $Mpdz^{+/-}$ and WT littermates preferred ethanol over water (preference ratio > 0.5) in Week 1 (t_{19} = 14.1, p < 0.0001 and t_{20} = 16.9, p < 0.0001, respectively), Week 2 (t_{20} = 11.8, p < 0.0001 and t_{22} = 16.1, p < 0.0001, respectively), and Week 3 (t_{20} = 21.8, p < 0.0001 and t_{20} = 18.9, p < 0.0001, respectively) (Fig. 4-7b). Taken together, these data demonstrated that $Mpdz^{+/-}$ and WT mice did not differ in binge-like ethanol intake (4 h) or preference either upon initial or repeated exposure to DID.

BEC values were not assessed in this experiment, so it is unknown if mice achieved BECs above the binge threshold. However, on average, $Mpdz^{+/-}$ consumed between 6.4 ± 0.4 to 8.1 ± 0.3 g/kg and WT mice consumed between 7.2 ± 0.4 to 8.1 ± 0.4 g/kg ethanol in the 4 h sessions across Weeks 1-3, intake levels which reliably produce BEC values >0.8 mg/ml in 1B-DID (Fig. 4.5; Rhodes et al., 2005). However, it should be noted that average 4 h intake in 2B-DID was lower than 1B-DID.

Similar to the ethanol drinking data, $Mpdz^{+/-}$ and WT littermates did not differ in 2 h intake of water across Weeks 1-3 of drinking ($F_{1,24}$ = 2.8, p = 0.07; Fig. 4-8a), but there were main effects of sex (females > males; $F_{1,24}$ = 5.5, p = 0.03) and day of water

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intake ($F_{8,192}$ = 6.3, p < 0.0001).   There was a significant genotype × sex × day

interaction ($F_{8,192}$ = 2.8, p = 0.01), but no other interactions between factors (for all p >

0.6).   Data are presented collapsed on sex as post hoc analyses indicated no

observable differences between groups (Fig. 4-8a).   Similarly, *Mpdz*^{+/-} and WT

littermates did not differ in 4 h water intake (p = 0.8; Fig. 4-8b) across Weeks 1-3, and no

main effects of sex or week, or any interactions between factors were detected (for all p

> 0.2).
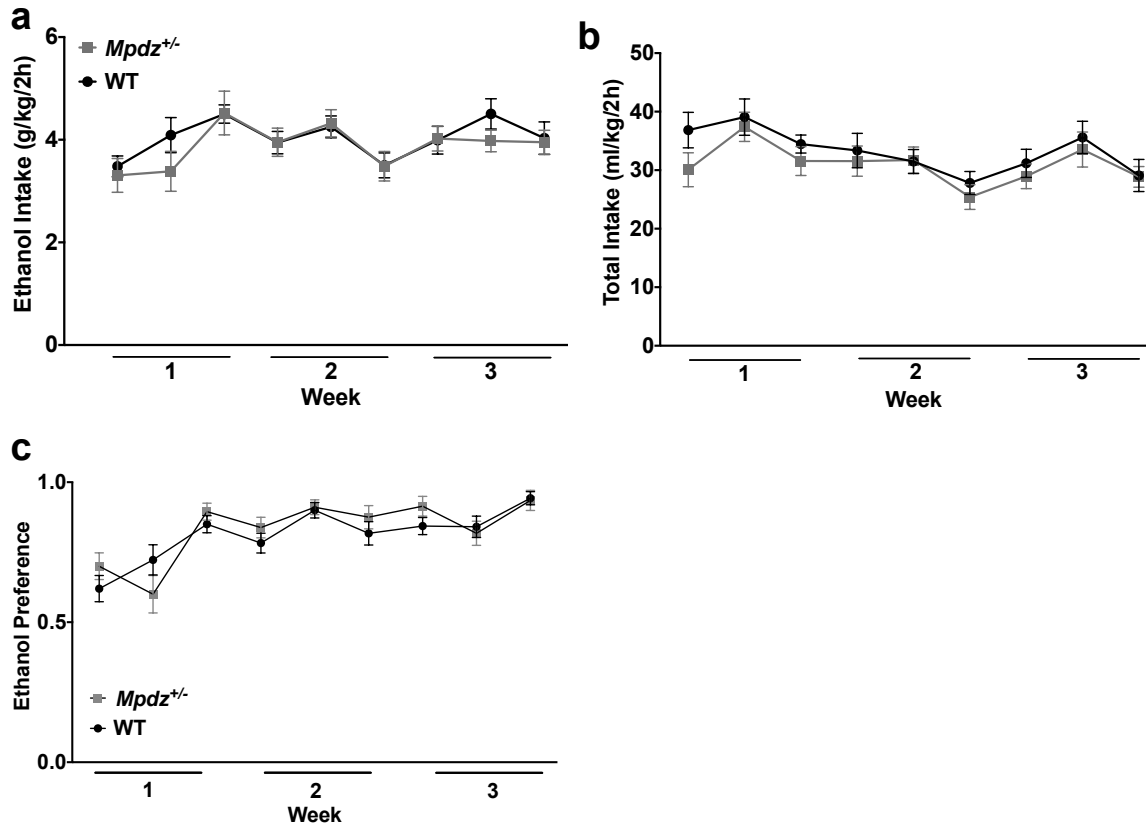
Figure 4-6. *Mpdz*+/- **and WT mice did not differ in 2 h ethanol intake (2B-DID).**

Mpdz+/- and WT mice had 2 h access to one ethanol tube (20% v/v) and one water tube on Days 1-3 across Weeks 1-3. *Mpdz*+/- and WT littermates (n = 21-23/genotype) did not differ in (a) 2 h ethanol intake, (b) 2 h total fluid intake, or (c) 2 h ethanol preference across three weeks of the 2B-DID paradigm. Data are presented as mean ± SEM and are collapsed on sex.
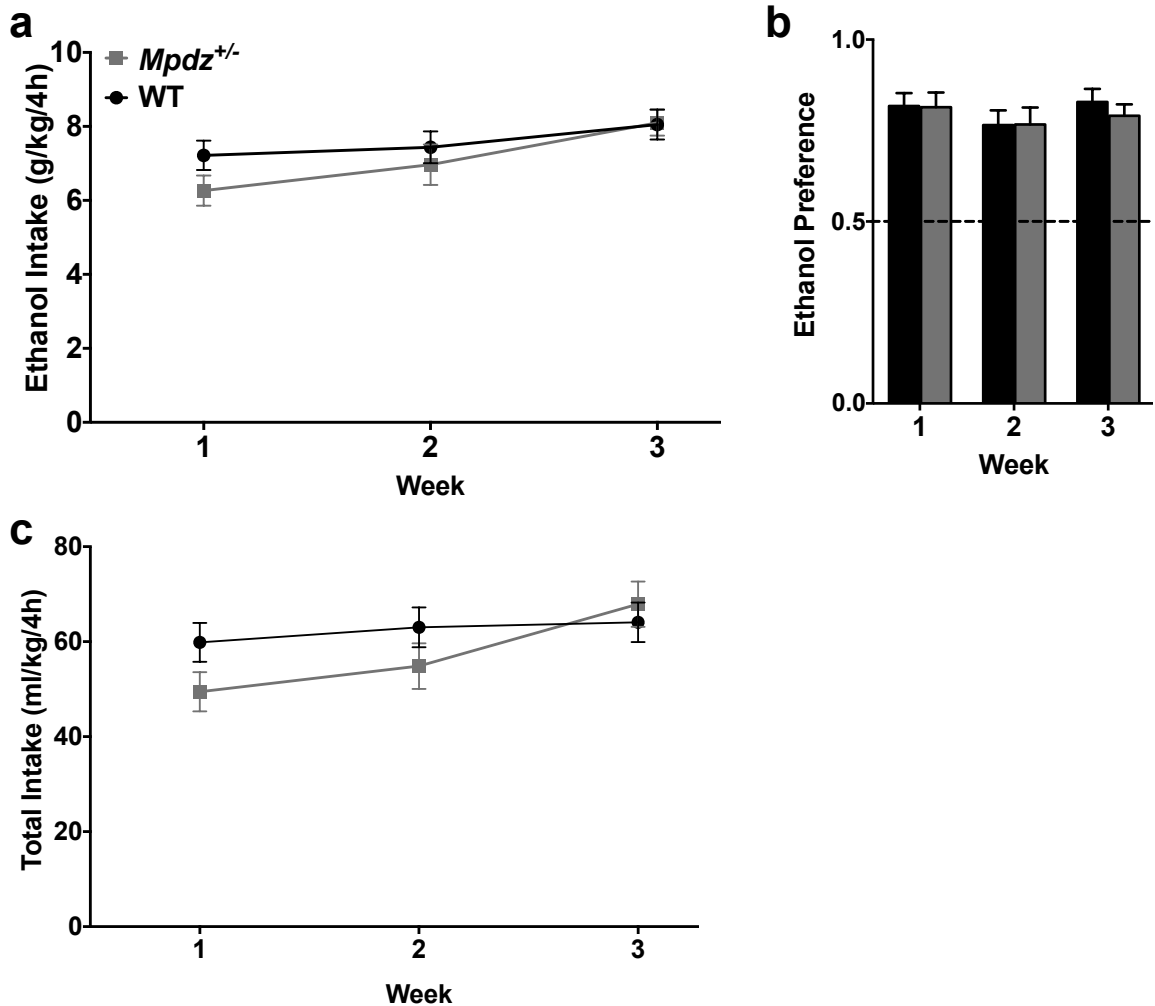
Figure 4-7. *Mpdz$^{+/-}$* **and WT mice did not differ in binge-like ethanol intake or preference (2B-DID).** *Mpdz$^{+/-}$* and WT had 4 h access to one ethanol tube (20% v/v) and one water tube on Day 4 across Weeks 1-3. *Mpdz$^{+/-}$* and WT littermates (n = 21-23/genotype) did not differ in 4 h ethanol intake (a) or 4 h total fluid intake (c) across Weeks 1-3 of DID. (b) Both *Mpdz$^{+/-}$* and WT mice preferred ethanol to water (preference ratio > 0.5; for both $p < 0.0001$), with no genotype difference in preference across Weeks 1-3. Data are presented as mean ± SEM, and are collapsed on sex.

a

b
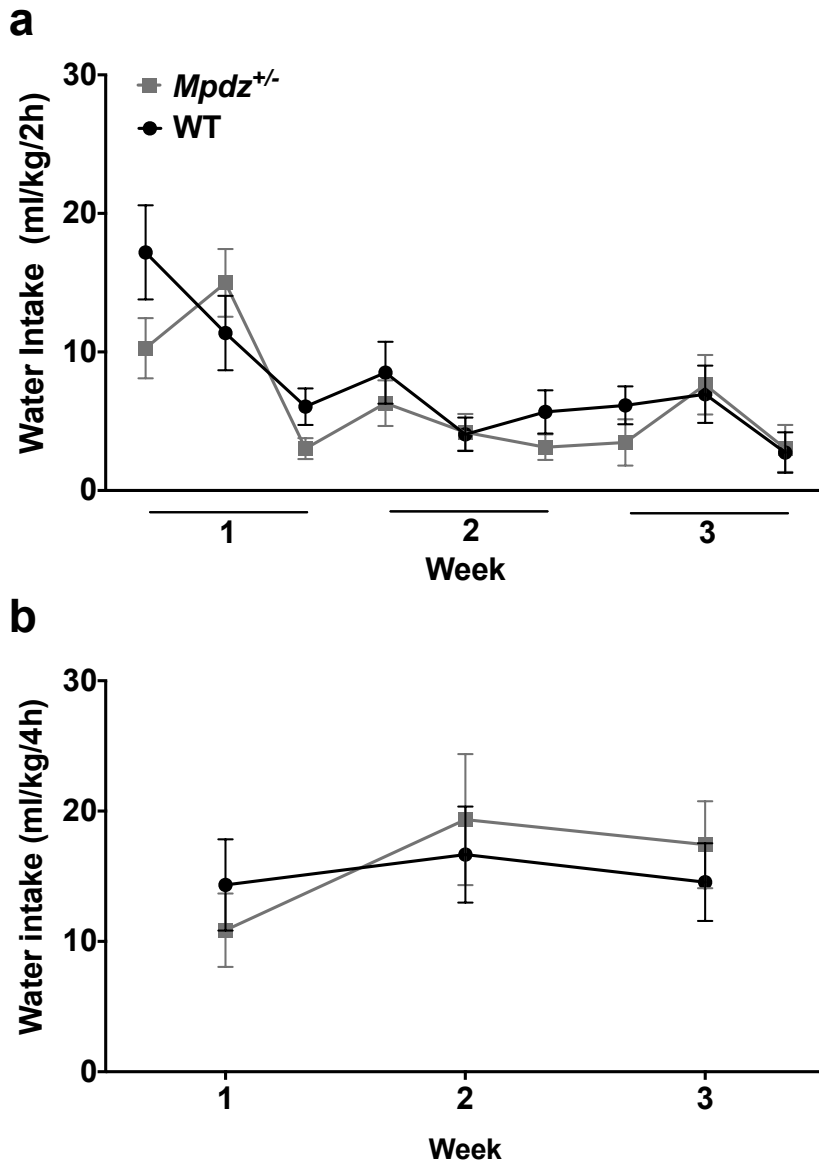
Figure 4-8. *Mpdz*^{+/-} **and WT mice did not differ in water intake (2B-DID).** *Mpdz*^{+/-} and WT had 2 h access on Days 1-3 and 4 h access on Day 4 to one ethanol tube (20% v/v) and one water tube on Day 4 across Weeks 1-3. (a) 2 h water intake (Days 1-3) and (b) 4 h water (Day 4) intake in ml/kg are presented for Weeks 1-3. *Mpdz*^{+/-} and WT littermates (n = 21-23/genotype) did not differ in 2 h or 4 h water intake. Data are presented as mean ± SEM and are shown collapsed on sex.

The effect of baclofen on decreasing binge-like ethanol intake and preference in Mpdz$^{+/-}$

and WT mice (2B-DID).

Following three weeks of drinking, *Mpdz$^{+/-}$* and WT were assessed for response

to baclofen to reduce 4 h binge-like ethanol drinking at doses devoid of locomotor effects

(Villas Boas et al., 2012).   Because reduced MUPP1 is associated with heightened

GABA$_B$R function (Chapter 3), I hypothesized that if GABA$_B$R function significantly

affects binge-like ethanol drinking it might do so to a greater degree in *Mpdz$^{+/-}$* compared

to WT littermates.   Two doses of baclofen, 2.5 mg/kg and 1.25 mg/kg, were used to

identify doses that would effectively reduce ethanol intake without inducing locomotor

depression or reducing water intake (Villas Boas et al., 2012).   Total fluid intake (water

and ethanol) and water intake were also assessed.

On Day 4 in Week 4, ANOVAs indicated a main effect of sex on 4 h ethanol

intake and 4 h total fluid intake ($F_{1,36}$ = 37.3, p < 0.0001 and $F_{1,32}$ = 20.4, p < 0.0001,

respectively), as well as the first 2 h period ($F_{1,36}$ = 18.3, p < 0.0001 and $F_{1,32}$ = 7.9, p =

0.008, respectively), and second 2 h period ($F_{1,35}$ = 26.6, p < 0.0001 and $F_{1,32}$ = 25.2, p <

0.0001, respectively) of DID with females consuming more than males, but no

interactions with genotype and/or treatment (for all p > 0.2).   Baclofen (2.5 mg/kg)

significantly reduced 4 h ethanol intake ($F_{1,36}$ = 12.7, p = 0.001; Fig. 4-9a) and 4 h total

fluid intake ($F_{1,32}$ = 11.3, p = 0.002; Fig. 4-9b).   This was driven by the effectiveness of

baclofen on ethanol intake and total fluid intake in the first 2 h period ($F_{1,36}$ = 10.9, p =

0.002 and $F_{1,32}$ = 8.7, p = 0.006, respectively; Fig. 4-9), as well as the second 2 h period

($F_{1,35}$ = 6.1, p = 0.02 and $F_{1,32}$ = 7.4, p = 0.01, respectively; Fig. 4-9).   However,

throughout, no differences between *Mpdz$^{+/-}$* and WT littermates were detected in ethanol

intake (Fig. 4-9a) or total fluid intake (Fig. 4-9b) in the 4 h, first 2 h, or second 2 h

periods, with no genotype × treatment interactions (for all p > 0.2).

For 4 h ethanol preference, there was a trend towards a main effect of sex ($F_{1,34}$ = 3.7, p = 0.06), but no main effect of genotype (p = 0.4) or treatment (p = 0.7), and no significant interactions between any of the factors (for all p > 0.2). Therefore, even though 2.5 mg/kg baclofen significantly decreased ethanol intake, it did not alter 4 h ethanol preference (Fig. 4-9c), and although both $Mpdz^{+/-}$ and WT littermates preferred ethanol over water following saline (t_8 = 2.5, p = 0.04 and t_{10} = 2.3, p = 0.04, respectively) or baclofen (t_{10} = 2.8, p = 0.02 and t_{11} = 2.2, p = 0.04, respectively) treatment, they did not differ in preference for ethanol in the 4 h DID session (Fig 4-9c). Further, the effect of baclofen was specific to ethanol intake, as baclofen did not alter 4 h water intake ($F_{1,35}$ = 2.8, p = 0.1) or water intake in the first 2 h (p = 0.3) or the second 2 h periods ($F_{1,35}$ = 3.0, p = 0.1), and no interactions with genotype and/or sex were detected (for all p > 0.1) (Fig. 4-11a). There were no main effects of sex or genotype in water intake in the 4 h or first 2 h periods (for all p > 0.2), but there was a trend towards a main effect of sex in the second 2 h period ($F_{1,35}$ = 4.0, p = 0.053) and a significant genotype × sex interaction ($F_{1,35}$ = 4.4, p = 0.04) with post hoc analyses revealing that female and male $Mpdz^{+/-}$ significantly differed in water intake (p = 0.04). However, data are still presented collapsed on sex since no differences between genotypes on water intake were detected.

BEC values were not assessed in this experiment, so it is unknown if reduced ethanol intake was reflected in BEC values below the binge intoxication threshold. However, both $Mpdz^{+/-}$ and WT mice consumed only 3.7 ± 0.7 g/kg and 3.7 ± 0.9 g/kg ethanol, respectively, in 4 h following baclofen pretreatment (2.5 mg/kg), intake levels that on average are not sufficient to produce BECs >0.8 mg/ml, as evidenced in the 2 h 1B-DID experiment (Fig. 4-1b,c).

A lower dose of baclofen (1.25 mg/kg) was assessed on Day 4 in Week 5. ANOVAs indicated a main effect of sex on 4 h ethanol intake ($F_{1,26}$ = 27.6, p < 0.0001) and 4 h total fluid intake ($F_{1,24}$ = 10.5, p = 0.003), as well as a main effect of sex in the first 2 h of ethanol intake ($F_{1,27}$ = 19.0, p < 0.0001) with a trend towards a main effect of sex in total fluid ($F_{1,24}$ = 3.6, p = 0.07), and significant effects in the second 2 h periods ($F_{1,27}$ = 17.4, p < 0.0001 and $F_{1,24}$ = 9.4, p = 0.005, respectively), with females consuming more than males. However, no significant interactions of sex with genotype (all p > 0.6), treatment (all p > 0.7), or genotype and treatment (all p > 0.2) in the 4 h, first 2 h, and second 2 h periods for ethanol and total fluid intake were detected. There was a main effect of 1.25 mg/kg baclofen in reducing the first 2 h period of ethanol intake ($F_{1,27}$ = 7.3, p = 0.01; Fig. 4.10a), but not total fluid intake ($F_{1,24}$ = 2.9, p = 0.1; Fig. 4.10b). Further, there was no effect of baclofen in the second 2 h period (for both p > 0.3), and overall 1.25 mg/kg baclofen was insufficient in reducing 4 h binge-like ethanol intake and 4 h total fluid intake ($F_{1,26}$ = 2.8, p = 0.1 and p = 0.6, respectively). Throughout, no differences between *Mpdz*^{+/-} and WT littermates were detected in ethanol intake (Fig. 4-10a) in the 4 h, first 2 h, or second 2 h periods (for all p > 0.5), and no differences in total fluid intake (Fig. 4-10b) in the 4 h ($F_{1,24}$ = 1.9, p = 0.18), first 2 h (p = 0.4), or second 2 h period (p = 0.2) were detected. No significant genotype × treatment interactions for ethanol or total fluid intake for 4 h, first 2 h, or second 2 h periods were detected (for all p > 0.1).

For the lower dose of baclofen (1.25 mg/kg), both *Mpdz*^{+/-} and WT littermates preferred ethanol over water following saline (t_7 = 5.1, p = 0.001 and t_8 = 9.1, p < 0.0001, respectively) or baclofen (t_5 = 10.2, p = 0.002 and t_7 = 8.9, p = 0.001, respectively) treatment. However, for 4 h ethanol preference there were no main effects of treatment (p = 0.8), sex (p = 0.6), or genotype ($F_{1,23}$ = 2.2, p = 0.15). There was a trend towards a

significant genotype × sex × treatment interaction ($F_{1,23}$ = 4.2, p = 0.05), but post hoc analyses failed to reveal significant effects. Further, there were no main effects of treatment or sex on water intake in the 4 h, first 2 h, or the second 2 h periods (for all p > 0.2; Fig. 4-11b), and *Mpdz*^{+/-} and WT littermates did not differ in water intake in the first 2 h or second 2 h periods (for both p > 0.2), but there was a main effect of genotype on 4 h intake ($F_{1,26}$ = 6.3 p = 0.02; Fig. 4-11b). No significant interactions were detected between treatment, sex, and/or genotype.

Together, these data indicated that 2.5 mg/kg but not 1.25 mg/kg baclofen significantly reduced 4 h binge-like ethanol drinking comparably in *Mpdz*^{+/-} and WT littermates, with no effect on ethanol preference. Together, these data did not detect an effect of lower MUPP1 expression on the effectiveness of baclofen to reduce binge-like ethanol drinking.
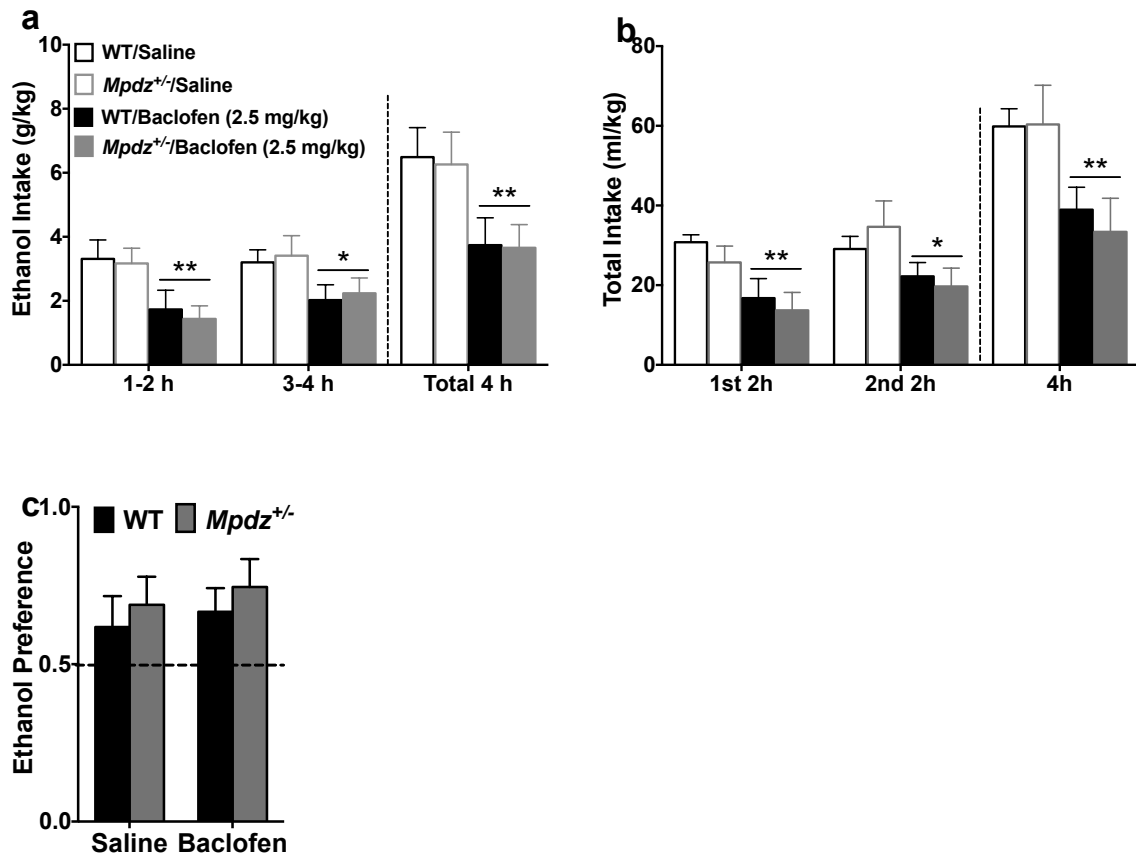
Figure 4-9. Baclofen (2.5 mg/kg) reduced binge-like ethanol intake comparably in *Mpdz*+/- and WT mice (2B-DID). On the Day 4 of Week 4, *Mpdz*+/- and WT received 2.5 mg/kg (i.p.) baclofen 15 min prior to 4 h DID. Baclofen (2.5 mg/kg) reduced (a) ethanol intake and (b) total fluid intake in the first 2 h (**$p < 0.01$) and second 2 h of DID (*$p < 0.05$), as well as in the total 4 h of DID (**$p < 0.01$) comparably in both *Mpdz*+/- and WT mice (n = 9-12/genotype/treatment group). No genotype differences in the effect of baclofen on ethanol intake were detected. (c) *Mpdz*+/- and WT mice both comparably preferred ethanol to water (preference ratio > 0.5, $p < 0.05$; *dashed line*), and baclofen administration did not affect ethanol preference. Data are presented as mean ± SEM. **$p < 0.01$ and * $p < 0.05$ comparing baclofen to saline treatment on intake. Data are shown collapsed on sex.

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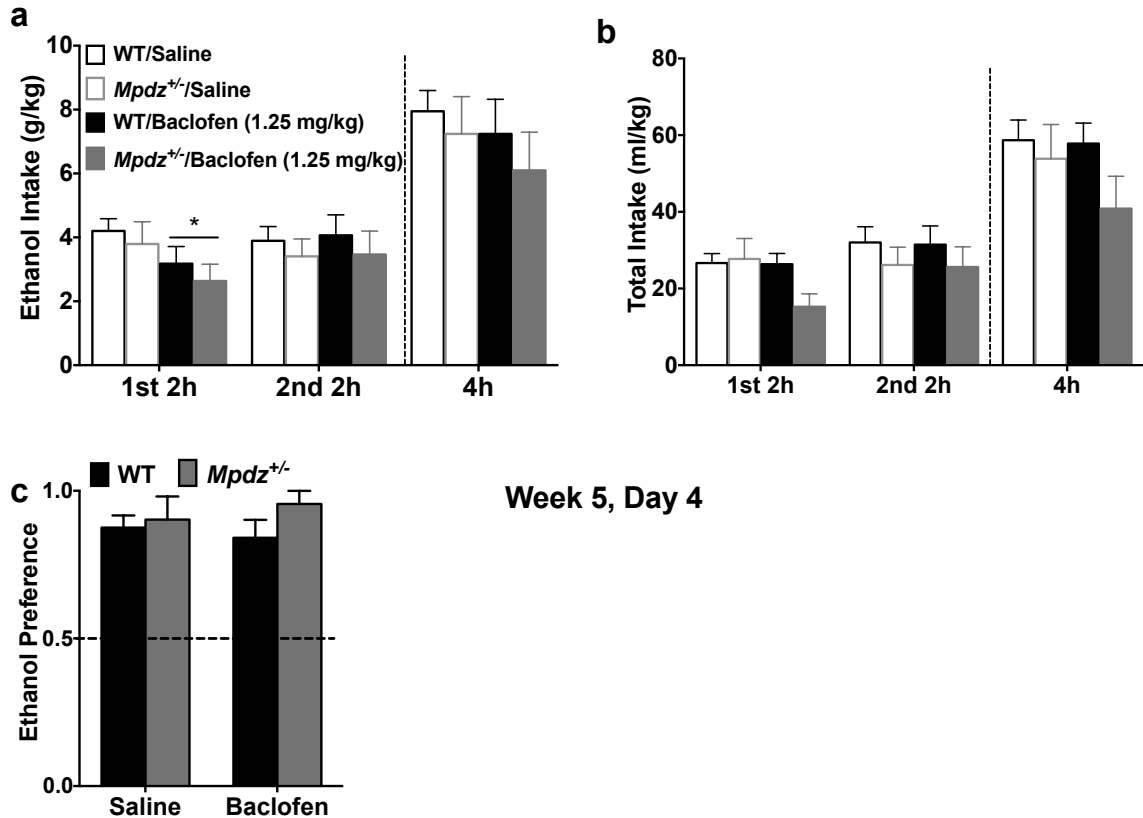
Figure 4-10. Baclofen (1.25 mg/kg) did not reduce binge-like ethanol intake in *Mpdz*^{+/-} and WT mice (2B-DID). On Day 4 in Week 5, $Mpdz^{+/-}$ and WT littermates (n = 8-10/genotype/treatment group) received 1.25 mg/kg (i.p.) baclofen 15 min prior to 4 h DID. Baclofen (1.25 mg/kg) reduced (a) ethanol intake in the first 2 h period of 4 h DID, but not (b) total fluid intake, or intake in the second 2 h or total 4 h periods of ethanol access. No genotype differences in ethanol intake or total fluid intake were detected. (c) $Mpdz^{+/-}$ and WT both comparably preferred ethanol to water (preference ratio > 0.5, $p < 0.0001$; *dashed line*), and baclofen administration did not affect ethanol preference. Data are presented as mean ± SEM. * $p = 0.05$ indicates a main effect of baclofen on ethanol intake. Data are shown collapsed on sex.
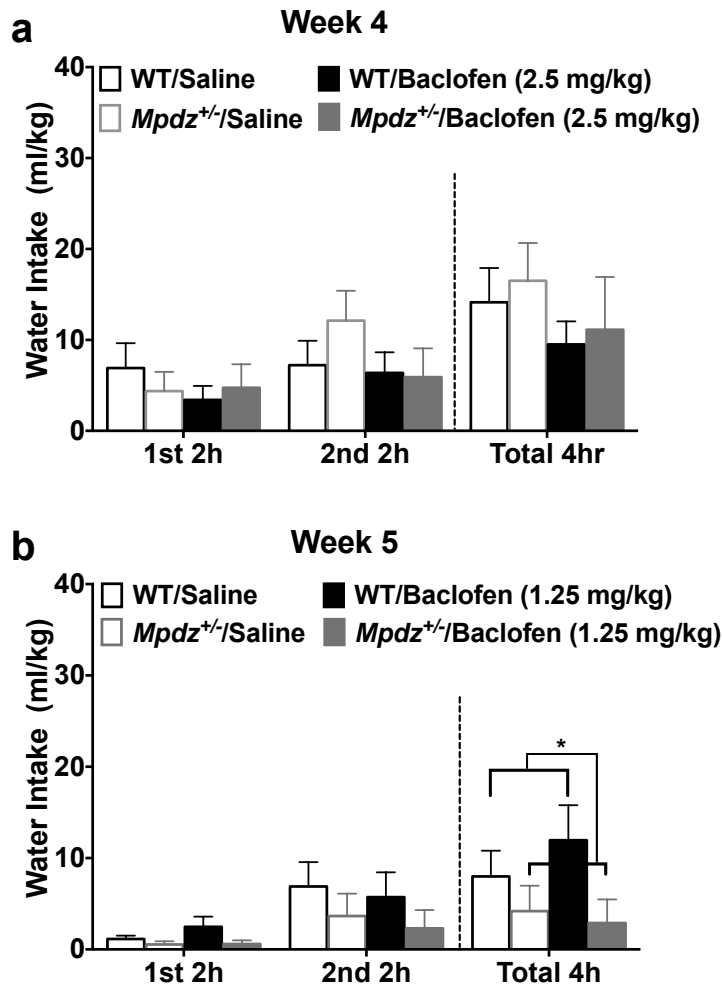
Figure 4-11. Baclofen (2.5 mg/kg and 1.25 mg/kg) did not alter water intake (2B-DID). (a) On Day 4 in Week 4, although baclofen (2.5 mg/kg, i.p.) significantly reduced ethanol intake, this dose did not affect water intake. (b) Consistently, on Day 4 in Week 5, a lower dose of baclofen (1.25 mg/kg, i.p.) did not alter water intake, but 4 h water intake was lower in $Mpdz^{+/-}$ compared to WT (*$p < 0.05$). Data are presented as mean ± SEM. Data are shown collapsed on sex.

Discussion

To provide a more complete understanding of the genetic risk for AUDs the use of multiple preclinical models that assess distinct features of AUDs is necessary. The role of *Mpdz* in predisposition to withdrawal is clear (Chapter 2) (Kruse et al., 2014; Milner et al., 2015). In the present Chapter I set out to define the role of *Mpdz* beyond withdrawal to binge-like intoxication, a feature of alcohol abuse and dependence thought to be associated with positive reinforcement of the dependence cycle (Koob and Le Moal, 2001; Koob and Volkow, 2010). I hypothesized that the potential effect of reduced MUPP1 expression would be associated with reduced binge-like ethanol intake. However, in comparisons between *Mpdz*^{+/-} and WT littermates, using three different variations of the standard DID procedure, I consistently found no evidence that *Mpdz* expression contributes to risk for binge-like ethanol drinking or associated BECs, suggesting *Mpdz* may not predispose to this alcohol phenotype associated with the positive and/or rewarding components of AUDs. However, a limitation of the present experiments was the use of only one genetic model to assess the effect of *Mpdz* on risk for binge-like drinking, which is discussed in detail below. Importantly, my studies demonstrated that binge-like ethanol drinking was reduced by baclofen at a concentration not associated with locomotor depression or generalized effects on fluid (i.e., water) intake. The effectiveness of baclofen was not affected by MUPP1 expression, however, which has important clinical implications suggesting baclofen may be an effective pharmacotherapy for AUDs in humans regardless of *MPDZ* haplotype status (Karpyak et al., 2009; Tabakoff et al., 2009).

Mpdz^{+/-} consume less ethanol in 2BC-CA preference drinking compared to WT littermates (Milner et al., 2015), and if this relationship remained consistent for binge-like ethanol drinking, I hypothesized that *Mpdz*^{+/-} would also demonstrate reduced ethanol

intake and associated BECs using a DID procedure. However, in this chapter, I presented evidence to the contrary, even when using parameters in DID that were comparable to 2BC-CA, including access to increasing ethanol concentrations, intake producing BEC values below the binge threshold, repeated ethanol exposure, and availability of a water tube. These data importantly demonstrated that the inconsistency between my results with *Mpdz*$^{+/-}$ and WT mice in DID compared to the 2BC-CA results (Milner et al., 2015), cannot be attributed to differences in ethanol concentration, length of ethanol exposure, or forced versus voluntary drinking of ethanol. Rather, this inconsistency was plausibly due to inherent differences in the preclinical models and the phenotypes they measure. Overall, these data did not detect evidence for a prominent role of *Mpdz* in risk for initial binge-like ethanol drinking or repeated binge-like ethanol drinking.

The lack of detectable role of *Mpdz* in risk for binge-like ethanol drinking could be explained by several limitations of the present experiments. First of all, I only assessed the effect of *Mpdz* using one genetic model on a single genetic background, and it is plausible that modifier loci not present on a B6 background, but present on another, are necessary to observe an effect of *Mpdz* on binge-like ethanol drinking. Therefore, reducing *Mpdz* expression on a different genetic background could produce the hypothesized phenotype (Coleman and Hummel, 1973; Doetschman, 2009). Additionally, allelic variation in the *Mpdz* sequence contributing to altered MUPP1 function and/or expression may influence binge-like ethanol drinking, similar to how *MPDZ* haplotype status is associated with alcohol consumption and dependence in clinical populations (Gizer, 2011; Karpyak et al., 2009; Tabakoff et al., 2009). The recent development of a novel gene mutagenesis technique using CRISPR/Cas 9 systems has been identified as a simple and efficient method for generating knockouts and sequence

specific knockouts (Wang et al., 2013).   This technique could therefore be applied in future studies to address both of these limitations.   Further, it is possible that other models of binge-like drinking, particularly ones associated with intermittent periods of acute or chronic withdrawal (i.e., chronic intermittent ethanol exposure) (Becker and Lopez, 2004; Lopez and Becker, 2005) are necessary to observe an effect of *Mpdz*, due to the known role of this gene in withdrawal (Kruse et al., 2014; Milner et al., 2015). Finally, a multivariate analysis on genetic mutations and ethanol-related phenotypes demonstrated that changes in DID appear to be substantially resistant to genetic mutation with only 6% of the single gene mutants tested differing from WT, whereas other models such as 2BC-CA were sensitive to changes with 51% of mutants differing from WT (Blednov et al., 2012), a finding that could also explain the lack of effect of *Mpdz* on DID.

The role of shared genes effects on ethanol intake in 2BC-CA preference drinking and DID remains to be elucidated.   Rhodes and colleagues in 2007 demonstrated a significant genetic correlation between 20% ethanol intake in DID and 10% ethanol intake in 2BC-CA drinking (r = 0.70) from two separate studies across seven standard inbred strains, and more recent analyses across 23 standard inbred strains corroborated this finding (Belknap et al., 1993; Crabbe et al., 2012b).   However, when four strains of mice from the C57/C58 lineage (including B6 mice) were removed from analyses, a significant correlation was no longer evident, suggesting this genetic correlation may be primarily driven by mice from the C57/C58 lineage (Dr. John Crabbe, personal communication).   Beyond inbred strains, selectively bred mice have also been assessed for an genetic association between these two phenotypes. Mice selected from an outbred stock (HS/Ibg) and bred for high 2BC-CA ethanol preference drinking showed greater ethanol intake in limited access 2BC drinking (Grahame et al., 1999)

and binge-like intake in a variant of DID, compared to the divergent line selected for low preference drinking (unpublished results discussed in Crabbe et al., 2011), suggesting shared gene effects for both phenotypes. However, HDID mice created from a genetically heterogeneous stock (HS/Npt; originally derived from 8 inbred strains including B6 and D2) and selected for BECs attained in 4 h DID, did not differ from controls in 2BC-CA preference drinking at concentrations from 3-20% (Crabbe et al., 2011). Further, mice selectively bred for high EWD following chronic ethanol exposure (Withdrawal Seizure Prone; WSP) showed increased ethanol intake in DID compared to the divergently selected line (Withdrawal Seizure Resistant; WSR), but an inconsistent association with 2BC-CA preference drinking (Crabbe et al., 2013). Therefore, some genetic overlap between 2BC-CA and DID is apparent although it appears to be strongly driven by mice from a specific lineage, but there is also evidence to support distinct genetic determinants influencing each phenotype. *Mpdz*, therefore, appears to be one such gene contributing to these phenotypes as genetically distinguishable, which is another possible explanation for the disparate effect of *Mpdz* in 2BC-CA preference drinking and DID.

An advantage of using 2B-DID in addition to 1B-DID was the ability to assess the choice between ethanol and water in a binge-like drinking model. The finding that similar amounts of ethanol were consumed in both 1B-DID and 2B-DID paradigms confirmed that even with the availability of water, mice will still choose to consume ethanol to levels previously shown to produce BECs above the binge-threshold in 1B-DID (Rhodes et al., 2007), thereby further validating the 1B-DID model. However, future studies confirming that ethanol intake was associated with BEC values above the binge-threshold in 2B-DID are necessary. Further, 2B-DID allowed the assessment of ethanol preference in a binge-like drinking model, although *Mpdz*$^{+/-}$ and WT littermates did not

differ in this phenotype. Unexpectedly, although baclofen (2.5 mg/kg) reduced binge-like ethanol drinking it did not alter ethanol preference. My work is not the first to demonstrate a disparity between ethanol drinking and preference, as others have shown that differences in ethanol drinking are not always reflected in the preference ratio in relation to genetic, neurobiological, and/or pharmacological manipulations, indicating that interpretation of the preference ratio is not always clear (Belknap et al., 1993; Gabriel and Cunningham, 2005; Giardino et al., 2011; Milner et al., 2015). For instance, $Mpdz^{+/-}$ and WT littermates differed in ethanol consumption in 2BC-CA, but not preference for 6%, 10%, or 20% ethanol concentrations (Milner et al., 2015). To even further complicate the interpretation, $Mpdz^{+/-}$ and WT littermates strongly preferred 20% ethanol during binge-like ethanol intake in 2B-DID (preference ratio was approximately 0.75-0.85), but not in 2BC-CA (preference ratio was approximately 0.35-0.40; Milner et al, 2015), suggesting pre-and post-absorptive factors such as gustation/olfaction and metabolism, respectively, (Belknap et al., 1977) may strongly modulate this phenotype. It can be presumed that the preference ratio attempts to measure reward or reward seeking (i.e., choosing or "preferring" to consume ethanol over water), but is limited by other interpretations such as novelty, pre-absorptive effects, or nonspecific effects on water intake (Gabriel and Cunningham, 2005). Further, there are other models such as conditioned place preference that are more highly validated for determining measures of reward or reward seeking (Cunningham et al., 2006). Therefore, amount of ethanol consumed and BEC attained in these drinking paradigms are likely more consistent and valuable phenotypes for determining the genetic and neurobiological bases influencing risk for AUDs, as opposed to preference ratio.

Previous work has demonstrated that administration of a high dose (10 mg/kg) of baclofen significantly reduced ethanol drinking using a DID paradigm (Kasten et al.,

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2015). A main limitation of this work was that this concentration of baclofen has been shown to induce locomotor depression (Gianutsos and Moore, 1978; Villas Boas et al., 2012), making it difficult to disentangle the effect of baclofen on reducing the hedonic aspects of drinking from more general locomotor effects. Using a dose of baclofen (2.5 mg/kg) that does not produce locomotor depression observed with higher doses (Gianutsos and Moore, 1978; Villas Boas et al., 2012) (Chapter 3), my studies demonstrated that binge-like ethanol drinking was selectively decreased by baclofen, without generally affecting water intake, but no difference in response between $Mpdz^{+/-}$ and WT mice was detected. The effect of baclofen was tested following repeated weeks of binge-like ethanol drinking as opposed to single exposure for translational value to human AUDs. It is plausible that $Mpdz$ expression could influence the effectiveness of baclofen to reduce initial binge-like ethanol drinking, when neuroadaptations that likely become apparent following repeated binge episodes (i.e., repeated ethanol exposure; Valenzuela, 1997) have yet to occur. Future studies assessing the potential influence of MUPP1 on the effect of baclofen during first exposure to binge-like ethanol drinking are necessary to determine response to baclofen is altered prior to repeated ethanol exposure to further define the influence of MUPP1 on GABA$_B$R function and associated ethanol phenotypes.

Further, the finding that reduced MUPP1 expression was not associated with a change in effectiveness of baclofen to reduce drinking builds upon my results from Chapter 3, which demonstrated an association between reduced MUPP1 expression and heightened response to baclofen on HICs but not locomotor depression, indicating that the effect of MUPP1 expression on GABA$_B$R function may be more specific to behaviors associated with CNS hyperexcitability (e.g., GABA$_B$R-mediated HICs and EWD) rather than generalizing to all GABA$_B$R-mediated behaviors. Further, since I

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tested comparable doses of baclofen (4-5 mg/kg and 10 mg/kg) across all three experiments (e.g., HICs, locomotor activity, and ethanol intake), these findings cannot be explained by a difference in dosage, lending more support to the selectivity of this interaction on CNS hyperexcitability phenotypes. However, the effect of baclofen to reduce 2BC-CA drinking in $Mpdz^{+/-}$ and WT littermates has not been assessed, so future studies assessing other GABA$_B$R-mediated behaviors, and specifically those associated with ethanol, are necessary to confirm this hypothesis.

The present experiments were unable to detect a role of $Mpdz$ in predisposition to binge-like ethanol drinking and intoxication, a phenotype associated with positive reinforcement (Koob and Volkow, 2010), although the limitations discussed above need to be considered. Importantly, future studies directly assessing the role of $Mpdz$ in the rewarding (e.g. conditioned place preference) and reinforcing (e.g. operant self-administration) properties of ethanol, are necessary to further elucidate the role of $Mpdz$ in genetic predisposition to AUDs and how $Mpdz$ may influence positive reinforcement. Further, although the effectiveness of baclofen was not dependent upon MUPP1 expression, preliminary data has indicated that a selective 5-HT$_{2C}$R agonist was actually more effective at reducing binge-like ethanol drinking in $Mpdz^{+/-}$ compared to WT littermates (Kruse, unpublished), suggesting that beyond baclofen, $Mpdz$ may influence the effectiveness of other pharmacotherapeutic treatments targeting interaction partners of MUPP1 beyond the GABA$_B$R (Becamel et al., 2001; Leggio et al., 2013; Ullmer et al., 1998). Overall, preclinical and clinical studies have independently identified $Mpdz$ as a gene influencing risk for AUDs although for different ethanol phenotypes, implicating this gene as an effective screening tool and for the development of new pharmacotherapies for the treatment of alcohol abuse and dependence.

Chapter 5: Overall Discussion

Overview

The overarching goal of my dissertation was to explore the role of *Mpdz* in EWD and binge-like ethanol drinking, and to begin to examine the functional importance of MUPP1 interactions with the $GABA_BR$. I first used RNAi to provide additional evidence for *Mpdz* as a QTG for EWD and to assess the role of the clSNr in this relationship. I then explored the role of clSNr $GABA_BRs$ in EWD, and investigated the association between altered MUPP1 expression and $GABA_BR$-mediated responses. Finally, I examined the role of *Mpdz* in risk for binge-like ethanol drinking and the effect of reduced MUPP1 on the effectiveness of a $GABA_BR$ agonist to reduce binge-like drinking. Together, these studies aimed to define the role of *Mpdz* in alcohol phenotypes associated with both negatively (withdrawal) and positively (binge intoxication) reinforcing components of AUDs, and to begin to elucidate the underlying neurobiological mechanisms as an important step toward the identification of genetic determinants of liability in humans.

	Experiment	Results
Chapter 2	EWD HICs	**EWD severity was greater in mice with reduced *Mpdz* expression in the cISNr compared to scrambled control mice.**
	PTZ-enhanced HICs	*Mpdz shRNA mice did not differ from scrambled control mice in PTZ-enhanced HICs.*
Chapter 3	Intra-nigral baclofen on HICs	**Microinjection of baclofen into the cISNr enhanced HICs in both ethanol withdrawn and control D2 mice.**
	Intra-nigral CGP55845 on HICs	**Microinjection of CGP55845 into the cISNr reduced EWD severity in D2 mice.** *Microinjection of CGP55845 did not alter severity of PTZ-enhanced HICs.*
	Baclofen-enhanced HICs	***Mpdz^{+/-}* had greater baclofen enhanced HICs compared to WT littermates.** ***Mpdz^{Tg}* homozygotes had lower baclofen enhanced HICs compared to WT littermates.**
	Baclofen on locomotor activity	*Mpdz^{+/-} did not differ from WT littermates in locomotor activity following 0, 1, 2, 4, or 10 mg/kg baclofen.*
	SNr GABA_BR response to baclofen	*Mpdz^{+/-} did not differ from WT in the effect of baclofen to reduce sIPSC frequency in GABAergic neurons.* ***Mpdz^{+/-}* had heightened postsynaptic GABA_BR-GIRK currents in response to baclofen compared to WT.**

Chapter 3	GABA$_B$R mRNA expression in whole brain	*Mpdz$^{+/-}$ did not differ from WT in whole brain gene expression of Gabbr1, Gabbr2, Kcnj3, Kcnj6, or Kcnj9.*
	GABA$_B$R mRNA expression in clSNr	**Mpdz$^{+/-}$ had reduced Mpdz expression and greater Kcnj6 expression in the clSNr compared to WT, but not Gabbr1, Gabbr2, Kcnj3 or Kcnj9 expression.**
Chapter 4	2 h 1B-DID	*Mpdz$^{+/-}$ did not differ from WT littermates in ethanol intake in 2 h 1B-DID across three weeks of DID, nor did they differ in BEC.* *Mpdz$^{+/-}$ did not differ from WT littermates in the effect of baclofen (10 mg/kg or 5 mg/kg) to reduce ethanol intake in 2 h 1B-DID.*
	1B-DID	*Mpdz$^{+/-}$ did not differ from WT littermates in binge-like ethanol intake in 1B-DID across seven weeks.* *Mpdz$^{+/-}$ did not differ from WT littermates in BEC in Week 1.*
	2B-DID	*Mpdz$^{+/-}$ did not differ from WT littermates in binge-like ethanol intake in 2B-DID or preference for ethanol across three weeks of DID.* *Mpdz$^{+/-}$ did not differ from WT littermates in response to the effect of baclofen (2.5 mg/kg) to reduce binge-like ethanol drinking.*

Table 5-1. Summary of results from Chapters 2, 3, and 4. Bolded cells indicate

significant effects and italicized cells indicate a lack of significant effects.

Summary of Major Findings

In Chapter 2, I used RNAi to directly assess the impact of reduced *Mpdz* in the clSNr on EWD (Koob and Le Moal, 2001; Koob and Volkow, 2010). I found that administration of an shRNA uniquely targeted to silence *Mpdz* gene expression was highly efficacious in reducing *Mpdz* mRNA expression in the clSNr of D2 mice. This reduction of *Mpdz* was associated with a significant enhancement in acute EWD severity, but no change in baseline HICs or PTZ-enhanced HICs. These data provided additional confirmation for *Mpdz* as a QTG for EWD, and identified the clSNr as a region crucially involved in mediating the effect of *Mpdz* on withdrawal. Finally, a primary finding of this chapter was that *Mpdz* selectively affected withdrawal without generally affecting seizure susceptibility, indicating a specificity of the actions of *Mpdz* in the clSNr.

In Chapter 3, to my knowledge, I provided the first evidence for the involvement of clSNr $GABA_B$Rs in EWD and $GABA_B$R-mediated HICs. Specifically, using pharmacological manipulations of the clSNr, I showed that activation of $GABA_B$Rs significantly enhanced HICs in control and ethanol withdrawn mice, and inhibition of these receptors attenuated the severity of EWD, without altering baseline or PTZ-enhanced HICs. In addition, I demonstrated that reduced MUPP1 expression was associated with heightened $GABA_B$R-mediated responses using comparisons between $Mpdz^{+/-}$ and WT mice. I found that reduced MUPP1 expression was associated with greater baclofen-enhanced HICs, and vice versa, with no effect on locomotor activity, suggesting that altered MUPP1 expression may only influence some $GABA_B$R-mediated behaviors. In collaboration with Dr. David Rossi, greater postsynaptic SNr $GABA_B$R outward currents induced by baclofen, known to be mediated by GIRK channels were demonstrated in $Mpdz^{+/-}$ compared to WT littermates. I then used radioligand saturation binding and QPCR analyses to assess the hypothesis that these behavioral and

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functional increases in $Mpdz^{+/-}$ compared to WT were associated with increased GABA$_B$R binding and expression in $Mpdz^{+/-}$. Contrary to my hypotheses, no differences between $Mpdz^{+/-}$ and WT littermates in GABA$_B$R density or binding affinity (whole brain), or expression (whole brain and clSNr) were detected. Unexpectedly, increased $Kcnj6$ (which encodes GIRK2) mRNA expression in the clSNr of $Mpdz^{+/-}$ compared to WT littermates was detected. This chapter identified a role of GABA$_B$Rs in EWD, and demonstrated heightened response to baclofen in both behavioral and neurophysiological comparisons of $Mpdz^{+/-}$ to WT littermates. Beginning to elucidate the functional importance of potential MUPP1 interactions with GABA$_B$Rs is an important step towards identifying a potential mechanism through which $Mpdz$ affects EWD.

In Chapter 4, in comparisons between $Mpdz^{+/-}$ and WT littermates using three different variations of the standard DID procedure, I detected no evidence of an effect of $Mpdz$ in predisposition to binge-like ethanol intake or ethanol preference. Specifically, no differences in 2 h intake of 3, 6, 10, or 20% ethanol, or BECs following 2 h access to 20% ethanol were detected between $Mpdz^{+/-}$ and WT littermates. Further, no differences in binge-like ethanol intake (4 h) with either single or repeated DID exposure, were apparent between $Mpdz^{+/-}$ and WT littermates, consistent with no differences in associated BECs. Further, with the presence of both a water and ethanol tube during limited access DID (2B-DID), I found no evidence of differences in binge-like ethanol intake or preference between $Mpdz^{+/-}$ and WT littermates. Finally, my studies demonstrated that binge-like ethanol drinking was reduced by baclofen at a dose not associated with locomotor depression or generalized effects on fluid (i.e., water) intake, but no difference was detected in the effectiveness of baclofen to reduce drinking between $Mpdz^{+/-}$ and WT littermates. These data added further support to findings from Chapter 3 indicating that the increased baclofen responses associated with lower

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MUPP1 might only affect select behaviors, possibly those associated with CNS hyperexcitability.

		Genetic Model			
	D2 and B6	**Chromosome 4 QTL Congenic and D2 Background Strain**	**$Mpdz^{+/-}$ and WT**	**$Mpdz^{TG}$ and WT**	**$Mpdz$ shRNA and Scrambled Control**
***Mpdz* Expression**	D2 < B6[A]	D2 < Congenic[A]	$Mpdz^{+/-}$ < WT[E]	WT < $Mpdz^{Tg}$ [E]	***Mpdz* shRNA < Control[F] (Chapter 2)**
EWD (HIC)	D2 > B6[A,B]	D2 > Congenic[B]	$Mpdz^{+/-}$ > WT[E]	WT > $Mpdz^{Tg}$ [E]	***Mpdz* shRNA > Control[F] (Chapter 2)**
EWD associated c-Fos in SNr	D2 > B6[H]	D2 > Congenic[G*]	———	———	———
PTZ Enhanced HICs	———	D2 = Congenic[D]	$Mpdz^{+/-}$ = WT[E]	WT = $Mpdz^{Tg}$ [E]	***Mpdz* shRNA = Control[F] (Chapter 2)**
Baclofen-Enhanced HICs	———	D2 > Congenic[D]	**$Mpdz^{+/-}$ > WT (Chapter 3)**	**WT > $Mpdz^{Tg}$ (Chapter 3)**	———
2BC-CA	D2 < B6[C^]	———	$Mpdz^{+/-}$ < WT[E]	———	———
1B-DID	D2 < B6[I^]	———	**$Mpdz^{+/-}$ = WT (Chapter 4)**	———	———

Table 5-2. *Mpdz* genetic model comparisons and ethanol-associated behaviors. This table provides a summary of current evidence from both my dissertation and the literature for a role of *Mpdz* in a range of ethanol-associated and $GABA_BR$-behaviors as related to level of *Mpdz* expression. For all comparisons, < or > is shown indicating a significant difference ($p < 0.05$) between genotypes, otherwise = is shown indicating no difference between genotypes. Bolded cells indicate results presented in my dissertation. ^Indicates data were part of an inbred strain correlation study, so no direct

statistical comparison between genotypes was made. *Greater c-Fos activation in the caudal SNr of D2 compared to chromosome 4 congenic was also evident. A) Shirley et al., 2004; B) Fehr et al., 2002; C) Yoneyama et al., 2008; D) Reilly et al, 2008; E) Milner et al., 2015; F) Kruse et al., 2014; G) Chen et al., 2008; H) Kozell et al., 2005. I) Rhodes et al., 2007.

The role of *Mpdz* in predisposition to EWD but not binge-like ethanol drinking

A review of the literature has posited MUPP1 as a dynamic regulator of cell signaling through a diverse array of receptors and proteins (Balasubramanian et al., 2007; Baliova et al., 2014; Becamel et al., 2001; Dooley et al., 2009; Griffon et al., 2003; Guillaume et al., 2008; Krapivinsky et al., 2004; Li et al., 2012; Poliak et al., 2002; Rama et al., 2008; Ullmer et al., 1998) but the physiological and behavioral consequences of MUPP1 have not been widely explored. In particular, MUPP1 has been shown to be highly concentrated at tight junctions in the peripheral (Poliak et al., 2002) and central nervous system (Li et al., 2012), and may be involved in the formation of macromolecular complexes to affect electrical signaling (Hamazaki et al., 2002; Jeansonne et al., 2003). *In vitro* studies have implicated MUPP1 in macromolecular complex formation, trafficking, receptor clustering and signaling, and G-protein coupling (Balasubramanian et al., 2007; Guillaume et al., 2008; Krapivinsky et al., 2004; Parker et al., 2003), but only a few studies have more directly investigated MUPP1 in the CNS, mostly identifying interaction partners and expression patterns using cultured brain cells. In these studies, neurons were primarily isolated from the hippocampus, cerebral cortex, and cerebellum, and MUPP1 was demonstrated to be largely expressed on the plasma membrane of cell bodies (Balasubramanian et al., 2007) and on distal and proximal

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dendrites (Estevez et al., 2008; Fujita et al., 2012; Sitek et al., 2003), two sites to which GABA$_B$Rs localize (Breton and Stuart, 2012; Fujita et al., 2012). In addition, MUPP1 is thought to be primarily located postsynaptically in neurons and particularly enriched at postsynaptic densities (Estevez et al., 2008; Krapivinsky et al., 2004). Previous work exploring MUPP1 function has identified an intriguing role of MUPP1 in receptor signaling (Balasubramanian et al., 2007; Becamel et al., 2001; Guillaume et al., 2008; Krapivinsky et al., 2004; Rama et al., 2008), and the work in my dissertation began to characterize the behavioral and associated neurobiological relevance of *Mpdz*/MUPP1.

Table 5-2 presents a summary of current work associated with *Mpdz*. Using three distinct *Mpdz* targeted genetic models as well as chromosome 4 congenic and informative inbred strain (D2 and B6) comparisons, a negative genetic association between level of *Mpdz* expression and acute EWD severity has been demonstrated (Fehr et al., 2002; Kruse et al., 2014; Milner et al., 2015; Shirley et al., 2004). My dissertation work confirmed *Mpdz* as a proven QTG for predisposition to EWD withdrawal, but found no detectable effect of *Mpdz* on binge-like ethanol drinking. The results of my research build upon recent association studies in human clinical populations that have also implicated *MPDZ* in AUDs, as significant associations between *MPDZ* haplotype status or single nucleotide polymorphisms in *MPDZ* and alcohol dependent individuals or alcohol consumption, respectively, were identified (Karpyak et al., 2009; Tabakoff et al., 2009). In humans, symptoms experienced during withdrawal contribute to the transition to and perpetuation of dependence, as well as relapse in abstinent alcoholics, with withdrawal implicated as a component of negative reinforcement of the dependence cycle (Koob and Le Moal, 2001; Koob and Volkow, 2010). It is widely accepted that a dysregulation of excitatory and/or inhibitory neurotransmission is apparent during the acute withdrawal/initial abstinence phase in

humans, as well as mice, in which there may be a general net effect of CNS hyperexcitability during withdrawal (Valenzuela, 1997). The manifestation of CNS hyperexcitability was modeled through the assessment of HICs during acute EWD in my dissertation. With a reduction of *Mpdz* in the clSNr there was a clear enhancement of this dysregulation (i.e., as assessed by HICs) providing evidence that *Mpdz* influences an ethanol phenotype that may be associated with negative reinforcement (Dreumont and Cunningham, 2014). As I assessed a single measure of EWD, future work investigating the contribution of *Mpdz* to additional translational withdrawal symptoms (e.g., anxiety, depression, anhedonia, insomnia) using other animal models of withdrawal (discussed in Chapter 1) in all three phases of the alcohol withdrawal syndrome (acute/initial abstinence, early abstinence, and protracted abstinence) (Heilig et al., 2010) are necessary to further define the importance of *Mpdz* in predisposition to AUDs, and in particular.

Of particular significance of the present experiments was the confirmation of the effect of *Mpdz* on predisposition to acute EWD. Specifically, predisposition to greater EWD severity was associated with reduced *Mpdz*/MUPP1 expression (and presumably function), which could occur in the human population through variants of the *MPDZ* gene. As discussed in Chapter 1, greater acute withdrawal symptoms were reported in subjects with a family history of alcohol dependence (McCaul et al., 1991; Newlin and Pretorius, 1990; Span and Earleywine, 1999), a population at-risk for the development of alcohol dependence later in life (Schuckit and Smith, 1996). Therefore, at a genetic level, I confirmed that reduced expression of *Mpdz* predisposes to more severe acute EWD. This information could be used clinically to identify at-risk populations based on *MPDZ* haplotype status through response to the acute/initial withdrawal effects of alcohol.

Further, my work proves important in beginning to elucidate the genetic and neurobiological substrates involved in acute EWD. Therefore, at a neurobiological level, I showed that reduced expression of *Mpdz*/MUPP1 discretely in the clSNr produced an increase in EWD severity, which is presumably a result of greater activation within the clSNr and increased inhibition (i.e., GABA release) of output structures (Bolam et al., 2000; Deniau and Chevalier, 1992), since activation of this region is apparent during EWD and is therefore expected to be proconvulsant (Chen et al., 2011; Chen et al., 2008). Further, I demonstrated that the potential effect of reduced *Mpdz*/MUPP1 expression was associated with heightened GABA$_B$R-sensitive HICs, as well as heightened GABA$_B$R-mediated responses through GIRK channels in the SNr, demonstrating a clear change at both the behavioral and neurophysiological level in function of a receptor system implicated in AUDs (Agabio and Colombo, 2014). Further, my studies were the first to demonstrate a clear involvement of clSNr GABA$_B$Rs in influencing EWD severity. Based on these data, it is therefore intriguing to speculate that one mechanism through which *Mpdz* influences predisposition to EWD may be through the GABA$_B$R. Specifically, reduced expression of *Mpdz* in the clSNr may lead to increased GABA$_B$R function either directly through the MUPP1-GABBR2 association or indirectly through an effector associated with GABA$_B$R signaling (i.e., GIRK2; discussed in detail in Chapter 3), which manifests during withdrawal resulting in greater acute EWD severity (discussed further below). Further, it is intriguing to speculate that this predisposition of reduced *Mpdz*/MUPP1 to (presumably) heightened functioning of the clSNr and associated GABA$_B$Rs may prime this region to become more susceptible to further dysregulation with repeated/chronic ethanol exposure, to influence chronic withdrawal.

Whereas withdrawal may be associated with negative reinforcement of AUDs, excessive ethanol drinking or binge intoxication is thought to be associated with positive reinforcement (Koob and Le Moal, 2001; Koob and Volkow, 2010). My experiments using DID to examine binge-like intake failed to detect an association between reduced MUPP1 expression and binge-like ethanol drinking or with preference to consume ethanol over water. Technical limitations of these findings were discussed in Chapter 4. Given the proven role of *Mpdz* in predisposition to EWD, it is plausible that the induction of withdrawal in between or prior to limited access periods is necessary to observe an effect of *Mpdz* on binge-like drinking. B6 background mice are not highly susceptible to EWD HICs (Metten and Crabbe, 1994; Metten and Crabbe, 2005; Milner et al., 2015), and it is unlikely that meaningful withdrawal occurred following 4 h binge-like drinking in the *Mpdz*$^{+/-}$ and WT littermates. For instance, previous work has shown that even up to 10 weeks of repeated DID failed to induce substantial withdrawal HICs assessed approximately 24 h after the final 4 h limited access period in B6 mice (Cox et al., 2013), although it is unclear if HICs would have been evident if assessed within several hours following the end of DID. Another study using HDID mice did detect withdrawal HICs up to 6 h post-binge-like ethanol drinking, but withdrawal was mild, suggesting it is unlikely that substantial withdrawal was evident post-binge-like drinking in B6 background mice (Crabbe et al., 2014). Therefore, I propose that using a model such as chronic intermittent exposure (Becker and Lopez, 2004) where mice undergo bouts of chronic ethanol exposure (i.e., vapor or liquid diet) and withdrawal in between periods of voluntary ethanol drinking (or variations of this model with acute or repeated withdrawal since it is unknown if *Mpdz* predisposes to chronic EWD) may uncover an effect of *Mpdz* on binge-like ethanol drinking.

Specifically, in the chronic intermittent exposure model, ethanol consumption in a limited access session is significantly increased following intermittent bouts of chronic withdrawal (Becker and Lopez, 2004; Lopez and Becker, 2005). Therefore, one could predict increased binge-like ethanol consumption in $Mpdz^{+/-}$ mice which experience more severe withdrawal compared to WT littermates, an interpretation that is consistent with the negative reinforcement hypothesis of withdrawal (Koob and Le Moal, 2001). This interpretation is also consistent with a clinical study that identified reduced expression of $MPDZ$ in the hippocampus of alcoholics, as well as studies showing that children of alcoholics who reportedly experienced greater acute withdrawal symptoms may be at-risk for future development of alcoholism (McCaul et al., 1991; Newlin and Pretorius, 1990; Schuckit and Smith, 1996; Span and Earleywine, 1999). These models and interpretations are not without limitations, but are a necessary step towards further elucidation of the role of $Mpdz$ in predisposition to binge-like drinking and other alcohol phenotypes. Although it is clear that $Mpdz$ expression predisposes to acute EWD, how this initial predisposition may relate to chronic ethanol exposure remains to be determined and these models could be useful towards this end.

In addition to EWD and binge-like ethanol drinking, I also assessed the potential effect of MUPP1 on the $GABA_BR$ and associated behaviors. In total, I assessed the association of reduced MUPP1 expression with three behaviors mediated by $GABA_BRs$ including baclofen-enhanced HICs, baclofen-induced locomotor depression, and baclofen-reduced binge-like ethanol drinking. Although reduced MUPP1 expression was associated with a heightened response to baclofen to enhance HICs, the same direction of effect as $Mpdz$ on EWD HICs, expression did not influence the effect of baclofen associated with reducing binge-like ethanol drinking or locomotor activity. Given the differential expression of MUPP1 (Sitek et al., 2003) and distribution of $GABA_BRs$

(Bowery et al., 1987) throughout the brain, an intriguing explanation is that the potential effect of MUPP1 on GABA$_B$R-mediated responses may be limited to certain brain regions or certain cell populations, as baclofen HICs may be mediated through the clSNr but other brains regions have been implicated in the effects of baclofen on ethanol consumption (e.g., ventral tegmental area, nucleus accumbens, and ventral pallidum) (Kemppainen et al., 2012; Moore and Boehm, 2009; Wilden et al., 2014) and locomotor depression (e.g., ventral tegmental area) (Boehm et al., 2002). The clSNr was of particular interest in my studies based on the crucial role of *Mpdz* in the region of EWD, so future studies examining the potential effect of MUPP1 expression on GABA$_B$R-mediated responses in other brains regions and on other behaviors are necessary to confirm the specificity of this effect.

Mpdz, the clSNr, and GABA$_B$Rs associated with EWD

Since the SNr was first identified as a critical region in controlling the initiation, propagation, and/or cessation of CNS hyperexcitability states (Ben-Ari et al., 1981; Deransart and Depaulis, 2002; Garant and Gale, 1983; Iadarola and Gale, 1982; Nehlig et al., 1992), numerous studies have recognized a complex heterogeneity of this region in both function and structure (Chen et al., 2008; Deniau and Chevalier, 1992; Mailly et al., 2003). As described in Chapter 1, a functional dichotomy between the rostral (anterior) and caudal (posterior) SNr has been identified in mice and rats (Chen et al., 2008; Moshe et al., 1995; Shehab et al., 1996; Veliskova et al., 2001; Veliskova et al., 2005; Veliskova and Moshe, 2006). My work builds on this heterogeneity of the SNr by demonstrating a selective involvement of the caudolateral subregion of the SNr in EWD in an *Mpdz* and GABA$_B$R dependent manner, without generally effecting seizure

susceptibility (e.g., PTZ-enhanced HICs; Kruse et al., 2014). This may be consistent with results from a series of studies by Deniau and colleagues that elucidated the heterogeneous structure of the SNr by identifying an onion-like structural organization, where afferent projections from striatal regions synapsed onto distinct subnuclei, and were conserved in the efferent projections to the ventromedial thalamus, superior colliculus, and pedunculopontine tegmental nucleus, as well as locally through an axon collateral network (Deniau and Chevalier, 1992; Deniau et al., 1996; Mailly et al., 2003). Within the SNr exists a dense array of axon collaterals that branch from what will be referred to as 'parent' projection neurons and synapse onto adjacent projection neurons thereby inhibiting them. This network is thought to alter the synchrony of the discharge rate of SNr cells innervating the same targets, which could, at least in part, explain the complex signaling of this region (Mailly et al., 2003). This heterogeneity within the SNr may contribute importantly to MUPP1 effects on $GABA_BR$ function in the clSNr, as well as the effect of *Mpdz* and $GABA_BRs$ in the clSNr on HICs, which is discussed in detail below (Figure 5-1).

In general, the SNr exerts a tonic inhibitory control on output structures of the basal ganglia including the ventromedial thalamus, superior colliculus, and pedunculopontine tegmental nucleus (Bolam et al., 2000; Deniau and Chevalier, 1992; Di Chiara et al., 1979; Kha et al., 2001). This tonic inhibition is interrupted by striatal activity, leading to phasic activation of the SNr that is expected to further inhibit output structures (proconvulsant), or phasic inhibition of the SNr that is expected to disinhibit output structures (anticonvulsant) (Veliskova and Moshe, 2006). However, this simplified model does not take into account the differential function of discrete subregions of the SNr or the local axon collateral network. For instance, two separate studies demonstrated that (presumed) inhibition of the caudal SNr through activation of

GABA$_A$Rs with muscimol and antagonism of glutamate receptors with AP7 actually resulted in proconvulsant activity, but when measuring flurothyl seizure threshold both of these drugs were anticonvulsant in the rostral SNr (Moshe et al., 1995; Veliskova et al., 2001). My work was consistent with these findings of proconvulsant behaviors in response to presumed caudal SNr inhibition, as I showed that GABA$_B$R activation in the clSNr enhanced HICs and inhibition reduced EWD severity. However, in terms of the simplified model of the SNr in which inhibition is expected to be anticonvulsant and activation to be proconvulsant, my results appear inconsistent, but may be explained by the local axon collateral network. Specifically, I propose that inhibition of parent projection neurons by activation of GABA$_B$Rs would result in disinhibition of adjacent projection neurons through reduced GABA release from parent neuron axon collaterals. In turn, this would be expected to increase GABA release from the adjacent neuron on output structures producing increased HICs (Figure 5-1). Thus, during EWD, antagonism of GABA$_B$Rs on the parent neuron would disinhibit the parent projection neurons, allowing inhibition of adjacent projection neurons through axon collaterals, inhibiting GABA release from adjacent neurons onto output structures resulting in reduced HICs (Figure 5-1). There are limitations to this proposed model however, as it does not take into account the effect of GABA$_B$Rs on the adjacent neuron or GABAergic interneurons, but there is evidence supporting differential distribution of receptors throughout the SNr that could explain this (Eberle-Wang et al., 1997; Nicholson et al., 1992). Intra-nigral signaling is highly heterogeneous and complex, but one consistent finding is that the overall output of GABA release is proconvulsant (Chen et al., 2011; Chen et al., 2008; Depaulis et al., 1990; Okada et al., 1989; Veliskova and Moshe, 2006). Thus, there is likely a dynamic interplay between intra-clSNr signaling of neuron populations influenced by axon collaterals, GABA$_B$R distribution (as well as other

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receptors), and differential afferent projections/strength of input signals onto these populations, that coalesce to produce the behavioral effects demonstrated in my studies.

Based on my data, I previously speculated that reduced *Mpdz* expression in the clSNr may enhance EWD through heightened function of clSNr $GABA_BR$-GIRK currents, the mechanism of which fits with the proposed model described above. Specifically, during EWD I propose that $GABA_BRs$ are activated, and thus heightened $GABA_BR$-mediated inhibition of clSNr parent projection neurons with reduced *Mpdz* would be expected to be more efficient in inhibiting the parent neuron or a larger number of parent neurons, thereby disinhibiting adjacent projection neurons allowing a greater release of GABA to inhibit output structures, resulting in more severe HICs (Figure 5-1). Although this proposed axon collateral model is not without limitations, my data demonstrated a clear behavioral output of greater HICs in response to clSNr $GABA_BR$ activation, a reduction in EWD with clSNr $GABA_BR$ inhibition, and enhanced EWD with lower clSNr *Mpdz*, and this model begins to describe a mechanism through which these behaviors may occur. My work therefore adds to the growing literature supporting the SNr as a complex and highly heterogeneous region, and helps to further define the role of the caudolateral subregion in mediating select CNS excitability phenotypes (i.e., EWD and $GABA_BR$-mediated HICs, but not PTZ-enhanced HICs) as influenced by underlying genetic and neural mechanisms.

During EWD *or* with GABA$_B$R agonist

clSNr

GABA

Disinhibition of
adjacent
projection neurons

Output
Structures

↑ ↑ Increased GABA release
Increased HICs

During EWD + GABA$_B$R antagonist

clSNr

GABA

Inhibition of
adjacent
projection neurons

Output
Structures

↓ ↓ Reduced GABA release
Reduced HICs

During EWD *or* with GABA$_B$R agonist

↓ MUPP1

↑ GABA$_B$R-GIRK
currents

clSNr

GABA

Greater disinhibition
Of adjacent
projection neurons

Output
Structures

↑ ↑ ↑ Increased GABA release
Increased HICs

Key

◆
‖ active
GABA$_B$R

‖ inactive
GABA$_B$R

Figure 5-1. Proposed model of MUPP1 and GABA$_B$R effects in the clSNr on HICs.

Specific details and relevant background information of this proposed model are discussed in the text (pages 176-178). Briefly, (*top left*) I hypothesize that during EWD or in response to a GABA$_B$R agonist (e.g., baclofen), GABA$_B$Rs located on a parent GABAergic neuron in the clSNr are activated causing reduced GABA release and disinhibition of an adjacent GABAergic neuron. This results in increased GABA release on output structures from the adjacent neuron and increased EWD or GABA$_B$R-mediated HICs. (*top right*) When an antagonist is administered during EWD, the parent neuron is disinhibited increasing GABA release onto the adjacent projection neuron effectively inhibiting it and reducing release of GABA onto output structures leading to a reduction in EWD HICs. (*bottom left*) With reduced MUPP1 expression, greater GABA$_B$R-GIRK currents are evident in the parent neuron leading to increased inhibition of this neuron and reduced release of GABA onto the adjacent neuron. Greater disinhibition of the adjacent neuron results in increased GABA release onto output structures leading to enhanced EWD HICs or GABA$_B$R-mediated HICs. Potential output structures include the ventromedial thalamus, superior colliculus, and pedunculopontine tegmental nucleus. *Not pictured:* Other receptors located on both the parent and adjacent neurons may also contribute to excitation and inhibition of the neurons. Further, GABA release from the parent neuron onto output structures must also be taken into consideration, and future studies are required to determine if this model may distinguish between selective activity during EWD compared to chemiconvulsants. With these caveats in mind, I hypothesize that a dynamic interplay between parent and adjacent neurons with an overall net effect of greater GABA release from populations of adjacent in the clSNr leads to increased EWD HICs. I hypothesize that this mechanism is enhanced with reduced MUPP1 expression.

This proposed model for clSNr *Mpdz* and GABA$_B$R involvement in EWD is based on the speculation that *Mpdz* functions, at least in part, through the GABA$_B$R to influence EWD. However, as my data did not directly test this, it is possible that my findings of heightened GABA$_B$R-mediated responses with reduced *Mpdz* are not related to EWD, and instead other receptors may mediate the effects of *Mpdz* on EWD, such as the 5-HT$_{2C}$R and/or the NR2B subunit of the NMDA receptor (Becamel et al., 2001; Krapivinsky et al., 2004; Ullmer et al., 1998). For instance, preliminary data from the Buck Laboratory has demonstrated similar enhancements in 5-HT$_{2C}$R-mediated responses with reduced *Mpdz* including greater HICs in response to systemic administration of a selective 5-HT$_{2C}$R antagonist, as well as a greater number of SNr neurons responding to serotonin as assessed in neurophysiological analyses using *Mpdz*$^{+/-}$ compared to WT littermates (unpublished). Further, published data in chromosome 4 congenic mice demonstrated reduced 5-HT$_{2C}$R-mediated HICs compared to D2 background strain mice (Reilly et al., 2008), the same direction of effect as GABA$_B$R-mediated HICs and EWD (Milner et al., 2015). Further, MUPP1 is thought to associate with NR2B-containing NMDA receptors, which are expressed in the SNr (Suarez et al., 2010), through the SynGAP/CaMKII complex, an association that has been shown to be involved in excitatory signaling (Krapivinsky et al., 2004; Rama et al., 2008). Therefore, *Mpdz* may also regulate SNr activity through glutamate projections from the subthalamic nucleus, although previous work demonstrated no effect of lesions of the subthalamic nucleus on acute EWD (Chen et al., 2008). Overall, I provided evidence that the GABA$_B$R may be one potential mechanism mediating the effect of *Mpdz* on predisposition to EWD, but future studies are required to directly assess this hypothesis and the role of other MUPP1 interacting partners.

Conclusions

Alcohol abuse and dependence are highly complex psychiatric disorders. To provide a more complete understanding of the genetic and neurobiological bases influencing risk for AUDs as an important step towards the identification of effective prevention of and treatment for dependence in humans, the use of preclinical models has proven useful. Through the use of two validated paradigms that measure discrete alcohol phenotypes, and a combination of behavioral, genetic, pharmacological, and molecular techniques, in my dissertation I further confirmed the role of *Mpdz* in EWD risk and began to elucidate the potential underlying neurobiological mechanisms, but found no association with binge-like ethanol drinking. *Mpdz* affected withdrawal through expression in the clSNr, a functionally distinct subregion of the heterogeneous SNr proper. Further, I assessed the potential effect of MUPP1 on the GABA$_B$R as an important step in elucidating how *Mpdz* may influence EWD and other ethanol-related phenotypes. I found that lower *Mpdz*/MUPP1 expression was associated with heightened baclofen-enhanced HICs and heightened postsynaptic GABA$_B$R-GIRK signaling in the SNr. Further investigation of the genetic influence of *Mpdz* on discrete alcohol phenotypes could provide further insight into risk for AUDs and the development of pharmacotherapeutic treatments for dependent individuals.

Future Directions

The present studies employed acute withdrawal HICs as a behavioral model of EWD in mice, and identified a region through which *Mpdz* predisposes to EWD. However, the effect of *Mpdz* on withdrawal following chronic ethanol exposure, and how this region may become further dysregulated with repeated exposure and become

recruited during chronic withdrawal, has not been investigated. Interestingly, a mapping study using two independent lines selected for chronic withdrawal including the WSP, WSR, and an inbred WSP/WSR F2 intercross and the Colorado HW (high withdrawal), LW (low withdrawal), and an inbred HW/LW intercross, identified a QTL on mid-chromosome 4 for predisposition to chronic EWD, a similar QTL identified in the acute EWD mapping studies (Bergeson et al., 2003; Buck et al., 1997; Fehr et al., 2002). Another study using F_2 also identified a suggestive QTL on chromosome 4 (Buck et al., 2002). Further, chromosome 4 congenic mice demonstrate less severe EWD HICs following chronic ethanol exposure compared to D2 background strain mice (Buck, unpublished), the same direction of effect as for acute EWD (Fehr et al., 2002). Thus the genetic predisposition of *Mpdz* to withdrawal may extend beyond acute to chronic EWD, and it would therefore be intriguing to examine the involvement of the clSNr and GABA$_B$Rs in this phenotype. Further, as I did not directly assess the hypothesis that *Mpdz*/MUPP1 mediates EWD through the its effect on the GABA$_B$R, but provided evidence that altered MUPP1 does impact GABA$_B$R function, future studies directly assessing this hypothesis are necessary. Relatedly, studies examining the specific mechanism through which MUPP1 enhances postsynaptic GABA$_B$R signaling in the SNr and whether or not this relationship exists in other brain regions associated with ethanol-related phenotypes are necessary. Finally, in general, future work assessing the contribution of *Mpdz* to withdrawal symptoms associated with other features of the acute/initial abstinence phase of withdrawal (i.e., tremors, anxiety, insomnia), and particularly the early abstinence (i.e., depression and negative affective states) and protracted abstinence (i.e., anhedonia and increased response to stressful events) phases of withdrawal (Heilig et al., 2010), are necessary to further define a distinctive framework for the integral role of *Mpdz* in predisposition to dependence.

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