Analysis of *Mpdz* as a candidate gene for alcohol dependence and associated phenotypes

by

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A DISSERTATION

Presented to the Department of Behavioral Neuroscience and the

Oregon Health & Science University

School of Medicine

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

May 2004

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

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

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Acknowledgements

This project would not have been completed without the help and guidance of several people. I am very grateful to my thesis advisor, Kari Buck, who has taught me how to think more deeply about problems and who has provided me with support and guidance the last 5 years; I admire her as a scientist and friend. John Crabbe, has given me much guidance and has taught me how to think more logically and clearly about scientific questions. Renee Shirley helped with the chronic alcohol withdrawal experiment in the chromosome 4 congenic and helped with design and interpretations. All the initial mapping of the alcohol withdrawal locus on chromosome 4 was done by Dr. Christoph Fehr, a former post-doctoral fellow in the laboratory, without whose work this project would have never been conceived. My office-mate and fellow graduate student, Heather Hood, who was also there to hear me out with problems and has provided me with much needed support. Aaron Janowsky and John Belknap, who were always available for questions and encouragement. Nikki Walter, who was great with questions and who would always listen to me ramble on with excitement over some new finding. Tara Macey, another fellow graduate student who was there for support and advice. Katherine Wolfrum who also gave me a lot of important advice and support. Sue Burkart-Kasch and Carrie McKinnon, who gave me support, advice and mice. Bill Woodward, who helped with interpretations of many of the molecular experiments. Finally, my parents to whom this thesis is dedicated to and who have always given me their unconditional support and encouragement.

Abstract

Alcoholism is a major health problem in the world. It is now accepted that alcoholism is a complex disease with multiple genetic and environmental factors. Physical dependence on alcohol can be a critical motivational force for continuation of the addiction cycle. Severity of alcohol withdrawal is a heritable trait. However, specific genes that increase or decrease risk have been difficult to ascertain in humans because of the heterogeneous nature of the disease and the complex interaction with environmental factors. Inbred mouse strains are an alternative model organism to investigate genetic components of alcohol dependence. The DBA/2J inbred mouse strain shows severe withdrawal following alcohol exposure, while the C57BL/6J shows milder withdrawal severity. Using crosses between these progenitor strains, we have mapped with a high degree of certainty a gene that influences differences in alcohol withdrawal severity. *Mpdz*, which encodes the Multiple PDZ domain protein, is the only gene with sequence and gene expression differences within the critical genomic interval on chromosome 4. Here we use a combination of molecular and behavioral methods to investigate the function of *Mpdz* on alcohol withdrawal severity and associated phenotypes.

A novel congenic strain possessing a unique genetic variant of *Mpdz* from the C57BL/6J donor strain, introgressed on a uniform DBA/2J background strain, shows less severe acute and chronic alcohol withdrawal compared to the DBA/2J background strain. This congenic strain also shows less severe convulsions compared to the DBA/2J strain after administration of strychnine and N-methyl-D-

aspartate, but not pentylenetetrazol. C57BL/6J donor strain mice show less severe convulsions compared to DBA/2J background strain mice following administration of a 5-HT_{2C} receptor antagonist. These data implicate *Mpdz* as a pleiotropic gene affecting convulsion severity from some, but not all, convulsion inducing agents.

DBA/2J strain mice show a selective activation of an extended limbic brain circuit during withdrawal from chronic alcohol exposure, while activation of an extended basal ganglia circuit is implicated in severe acute alcohol withdrawal. A potential mechanism whereby *Mpdz* affects genetic differences in alcohol withdrawal severity is implicated through its association with 5-HT_{2C} receptors. Congenic and DBA/2J background strain mice show differential responses to 5-HT_{2C} receptor agonist and antagonist during acute alcohol withdrawal. MPDZ is localized in critical brain regions associated with genetic differences in withdrawal severity. In addition, MPDZ is co-localized with 5-HT_{2C} receptors in this critical brain circuit.

Overall, these results suggest that *Mpdz* is a quantitative trait gene for alcohol dependence, that *Mpdz* has pleiotropic effects on some, but not all, handling-induced convulsions, and that *Mpdz* influences genetic differences in alcohol withdrawal severity, in part, through 5-HT_{2C} receptors. This work will be important for development of future hypotheses of the role of genetic variation in *Mpdz* on other addiction phenotypes and seizure disorders with emphasis on the phenomena of epistasis and pleiotropism.

Chapter I

Introduction

Alcohol Dependence

Alcohol is one of the oldest psychotropic agents known. Despite this, alcohol abuse continues to be a major social, economic and public health problem the world over. Alcoholism, like other psychiatric diseases, is complex in nature whereby multiple genetic and environmental factors interact over the lifespan to produce an alcoholic individual. Alcohol dependence is a key factor perpetuating the continued use of alcohol. The criteria for defining alcohol dependence according to the Diagnostic and Statistical Manual of Mental Disorders - Fourth Edition (DSM-IV) are the presence of any of the following 3 over a 1 year period: (1): tolerance (increase in drinking to achieve the same initial effect), (2): drinking more than intended, (3): unsuccessful attempts to cut down on use, (4): excessive time related to alcohol (obtaining or hangover), (5): impaired social or work activities due to alcohol, (6): use despite physical or psychological consequences, and (7): alcohol withdrawal signs or symptoms (DSM-IV, 1994). The last of these factors (i.e., alcohol withdrawal) is a very relevant feature of alcoholism in both the clinic and laboratory; and it is the focus of this work.

The alcohol withdrawal syndrome is a powerful motivating force in keeping an individual 'dependent' on alcohol; alcoholics continue to drink, in part, to avoid the aversive physical and psychological states that manifest during alcohol withdrawal (Koob and Le Moal, 2001). Withdrawal symptoms such as seizures

can be life threatening if left untreated and these symptoms become increasingly worse as an alcoholic goes through multiple withdrawals, a phenomenon termed alcohol withdrawal kindling (Ballenger and Post, 1978; Becker and Hale, 1993) Alcohol withdrawal seizures have been demonstrated in every species tested including humans (Friedman, 1980). In mice, alcohol withdrawal convulsions are an easily quantifiable measure of withdrawal severity. Because other withdrawal symptoms are highly correlated with convulsions during withdrawal (Crabbe, 1983; Kosobud and Crabbe, 1986) it is common practice to use convulsive activity as the primary index of withdrawal severity in the laboratory.

Himmelsbach and Homeostasis

The withdrawal syndrome consists of a host of biological responses which appear upon removal of alcohol. These include symptoms such as anxiety, headache, severe sweating, elevated blood pressure, hyper-arousal, restlessness, delusions, delirium tremens, and seizures (DSM-IV, 1994). Withdrawal signs are usually opposite in direction to the initial effects of the drug, which has brought forward theories of homeostasis. Over 60 years ago, C.K. Himmelsbach was the first to devise a theory of drug withdrawal centered around the idea of homeostasis (Himmelsbach, 1941). A person's initial experience with a drug produces primary drug effects (e.g., euphoria, sedation, etc.), which over time lessen in intensity due to adaptations, which try to oppose the primary drug effects, and function to maintain a steady state of equilibrium (homeostasis). This is referred to as tolerance. Once a person stops taking a drug these adaptations are unmasked producing the withdrawal syndrome consisting of

qualitatively opposite effects to the primary effects of the drug. Generally speaking, the compensatory mechanisms which operate during drug withdrawal are referred to as neuroadaptations. And, it is these neuroadaptations that are one key factor in changing a social drinker into an alcoholic (Lewohl et al., 2000; Mayfield et al., 2002).

Mechanisms of withdrawal: The role of genetic factors

While environmental factors (e.g., socio-economic factors, peer pressure, alcohol availability, etc.) are an important aspect of alcoholism, understanding the biological or genetic factors underlying this disease has gained the most attention from the research community. Genes control approximately 50-60 % of the population variance in alcohol dependence, which family, twin and adoption studies have convincingly demonstrated (McGue, 1999). This statistic, however, is only qualitatively informative because of the lack of knowledge about the actual genes which increase or decrease risk for alcoholism. Isolating specific alcoholism risk genes, (among the 30,000 or so genes in the human genome) is a daunting task due to several factors. There is substantial phenotypic heterogeneity in the manifestation of alcohol dependence (e.g., age of onset, drinking history, comorbid disorders), along with a complex interplay of genes and environment that make identification of specific genes almost impossible.

Still, a major goal of genetic research on alcoholism is the identification of some "marker" that could have predictive validity in determining if someone may be genetically susceptible to develop severe problems with the use of alcohol. Risk for onset of alcoholism is associated with genetic differences in acute

alcohol withdrawal severity. This acute withdrawal response is thought to be akin to a severe type of hangover and men who have biological relatives that have alcoholism report greater withdrawal responses after a large single dose of alcohol compared to sons of non-alcoholics (McCaul et al., 1991). Another study reported that sons of alcoholics report greater hangover symptoms compared to sons of nonalcoholics (Newlin and Pretorius, 1990). These studies show that acute alcohol withdrawal is potentially an important predictor of future development of alcohol problems. But many other risk factors for severe alcoholism have also been identified in recent years, which include low initial response to alcohol, genetic differences in alcohol metabolizing enzymes, and electrophysiological measures (Schuckit, 2000). However, the studies described herein focus primarily on an acute alcohol withdrawal model.

Animal models of alcohol withdrawal

Animal models of alcohol dependence/withdrawal offer an alternative method of identifying genes for alcoholism in humans. *Mus musculus*, the laboratory mouse, is used to study the genetics of alcohol related behaviors for a number of reasons. Many traits associated with alcoholism such as ethanol tolerance and physical dependence (withdrawal) can be demonstrated in mice. Mice and humans share extensively conserved chromosomal regions, so that when genes are identified in mice the chromosomal regions will almost always be known in humans (Pennacchio and Rubin, 2003). Environmental factors can be controlled more effectively in mice than in humans, which is necessary for investigating genetic effects. Confounds associated with genetic heterogeneity (i.e., a similar

phenotype being caused by different mutations) are reduced with the use of inbred strains of mice because each individual of the same sex within an inbred strain is genetically identical; differences between two inbred strains are genetic in origin, while differences observed between individuals within the same strain are environmentally controlled. Inbred mouse strains by definition are genetically stable over generations and across laboratories which lends itself to a stable and reliable measurements of behavioral responses. Finally and on a more practical level, many inbred mouse strains are commercially available for testing, which offers a rich genetic pool. However, there are some obvious disadvantages of using animal models. Alcoholism is a human disease and therefore no single animal model or inbred strain encompasses every aspect of it. At best, animal models can only model single behaviors associated with alcoholism. And practically speaking, inbred mouse strains are relatively expensive (Crabbe, 2002).

McClearn and Rodgers (1959) opened a new era in the genetics of alcoholism research with their pioneering studies on alcohol preference among inbred strains of mice. They were the first to demonstrate systematically a genetic difference in alcohol preference among inbred mouse strains. They found that at one end of the behavioral spectrum the C57BL strain voluntarily consumed large quantities of alcohol, whereas the DBA strain virtually avoided alcohol altogether. Other inbred mouse strains tested showed intermediate alcohol preference compared the C57BL and DBA strains. Since this work in 1959, the C57BL and DBA strains, and related strains have been shown to differ

on a battery of alcohol related phenotypes including alcohol tolerance and dependence (Crabbe, 1983). Multiple studies show that the B6 strain displays less severe alcohol withdrawal severity compared to the D2 strain after both acute and chronic treatments (Buck et al., 1997; Crabbe, 1998). Because of the large phenotypic difference in alcohol withdrawal severity between these two inbred strains, they are used as progenitor strains for many genetic mapping studies.

Mapping of Quantitative Traits

In contrast to discrete, large effect, Mendelian (qualitative) traits, quantitative traits continuously vary in a population and usually have a smaller affect on the phenotype. Height, weight and blood pressure are such examples of quantitative traits. A Quantitative Trait Locus (QTL) is a genetic locus with alleles that affect variation in a complex trait. Complex traits such as alcoholism are affected by several polymorphic genes (several QTL) as well as environmental factors. Furthermore, gene-gene interactions (Hood et al., 2001 Frankel and Schork, 1996) and gene-environment interactions, called epistasis, (Crabbe et al., 1999a) are becoming increasingly more studied in the field.

QTL are identified when phenotypic measures (e.g., alcohol withdrawal convulsions) significantly co-vary with genotypic status (allelic markers) at particular loci in the organism's genome. When QTL are first discovered, they are usually mapped only to broad chromosomal regions within the range of 10-30 centimorgans (cM). This imprecision in initial mapping of QTL is related to the number of recombination events in the mapping population; the smaller the QTL

effect size the larger the mapping population needed to attain a given map resolution. In general, success in any QTL mapping project is determined by how heritable the trait is, the genetic architecture of the trait (dominant, recessive or additive), and how many genes affect the trait (Abiola et al., 2003). Even with these limitations, remarkable success has been achieved in identifying QTL for a wide range of behaviors including behaviors associated with drug addiction (Crabbe et al., 1999b; Buck and Finn, 2001).

Strategies for verification of QTL

One accepted method for verification of a QTL (i.e., proof of a real phenotypic/genotypic association) is to create and test a congenic strain that contains the QTL interval introgressed on a uniform inbred background (Figure 1-1). Introgression of QTL regions is done by backcrossing mice heterozygous for the chromosomal segment where the QTL has mapped, defined by two or more microsatellite markers (Bennett, 2000). This congenic strain should show the acquisition of the phenotypic effect, compared to the background strain, thereby demonstrating 'capture' of the QTL within the introgressed region. For example, if the congenic strain possesses alleles from the resistant strain (say for resistance to hypertension) in the introgressed region, when compared to the background strain, the congenic strain should show a resistance to hypertension if the QTL is actually 'captured.' Furthermore, conventional congenic strains that capture the QTL effect can be used as a starting point for finer mapping of the QTL; interval specific congenic lines (ISCL) are one way to progress to finer map

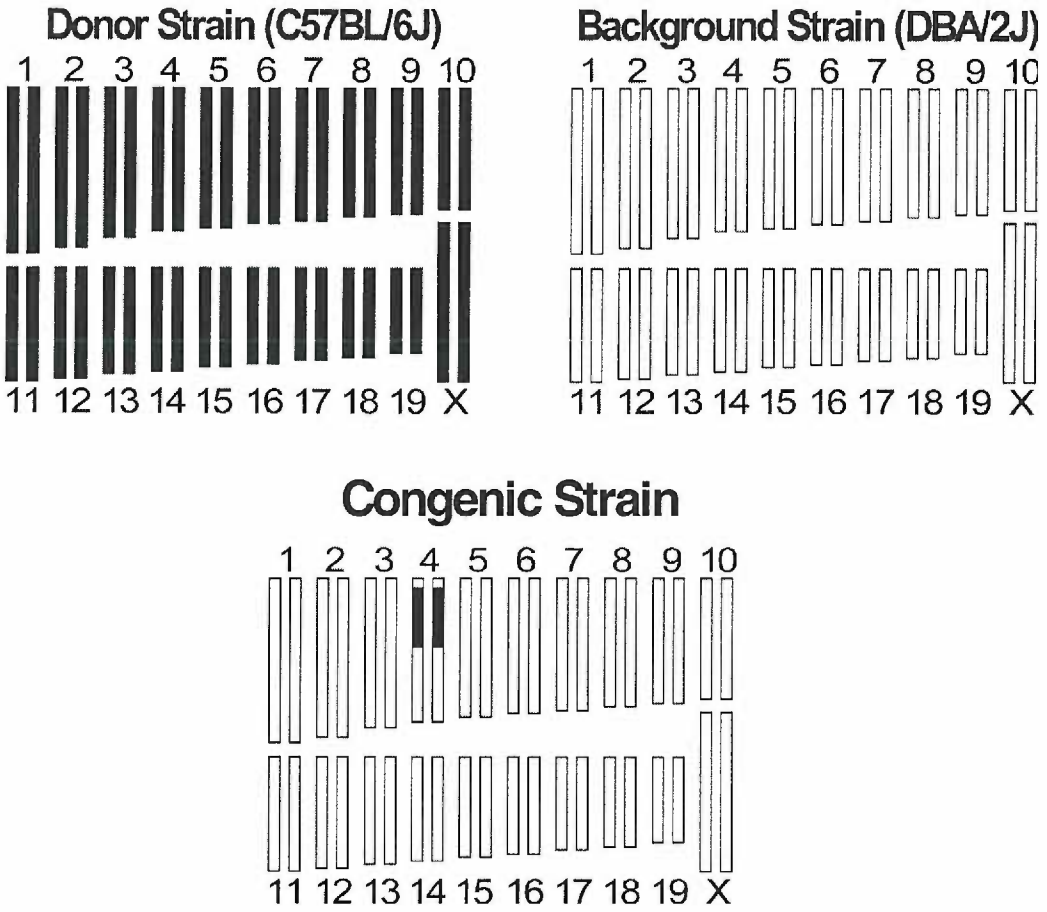

Figure 1-1: Development of Chromosome 4 congenic strain from donor (C57BL/6J; black chromosomes) and background (DBA/2J; white chromosomes) inbred mouse strains. The introgressed region shown in black in the congenic strain represents the genomic interval containing genetic material from the C57BL/6J donor strain. This interval contains *Mpdz*, along with only a handful of other genes The white region shows the genetic material from the DBA/2J background strain

resolution which has been used successively by our group to fine map an alcohol withdrawal QTL to a 1.5 Mb interval (Fehr et al., 2002; Shirley et al., 2004). However, a limitation of the congenic strain approach is that 'non-capture' of the QTL could be masked by background strain effects, a form of epistasis in which genes in the background strain interact with genes from the donor strain to mask a potential QTL effect.

Because there is no single 'gold-standard' for identification of candidate genes within a QTL interval, we have adopted a composite approach, whereby high resolution mapping of the QTL is combined with sequence and expression analysis of the candidate genes remaining within the interval (Shirley et al., 2004). However, some have argued that the most conclusive evidence for identifying the actual quantitative trait gene (QTG) is through allele replacement experiments, where the allele for the resistant strain (or vice versa) is replaced with the susceptible allele on a uniform genetic background (Glazier et al., 2002). 'Knock-in' of the alternative allele should show the predicted effect on the phenotype to prove the existence of the QTG.

Once a QTG is identified the functional mechanism by which it alters the phenotype is not usually known. In order to provide evidence for the existence of a QTG it must be demonstrated that: (1) there are genotype-dependent sequence and/or expression differences in the candidate gene (between the two progenitor strains or between congenic and background strain mice), (2) it is expressed in relevant brain regions, and (3) there is a mechanism whereby

genetic variation in the QTG affects the phenotype (Glazier et al., 2002; Abiola et al., 2003).

Mpdz is a candidate gene for alcohol withdrawal severity

Using the composite approach described above we mapped three significant loci for acute ethanol withdrawal in populations derived from crosses between the B6 and D2 progenitor strains, one of which is located on mouse chromosome 4 (*Alcw2)* (Buck et al., 1997). This QTL accounts for approximately 26% of the genetic variance in alcohol withdrawal severity. The 1-LOD confidence interval surrounding *Alcw2* was quite large (~ 35 cM) from the initial mapping effort. This confidence interval was estimated to contain about 1000 genes, with the possibility that any one or more genes within this interval could underlie the QTL association. Therefore, a major goal of our subsequent work was to attain high resolution mapping of *Alcw2* to reduce the number of candidate genes within this interval. This was achieved through strategies designed to reduce QTL regions to less than 1 cM (Darvasi, 1997, 1998). One of these strategies involves generation of interval-specific congenic lines (ISCLs), as mentioned above (Darvasi, 1997). We began by generating a conventional congenic strain, D2.B6-*D4Mit142*, for *Alcw2* and demonstrated 'capture' of the QTL effect by showing that the D2.B6-*D4Mit142* congenic strain exhibited significantly less acute ethanol withdrawal severity compared to the D2 background strain. We then created a series of ISCLs (ISCL1-5) to further narrow down the *Alcw2* interval. Identification of which ISCLs showed the QTL effect on phenotype scores and which did not identified the critical genomic

interval. We concluded from these analyses that the minimum critical interval is a 0.9 cM region spanning *D4Mit80* and *Mpdz* (37.7-38.6 cM). This region contained only 15 genes and/or predicted genes (Fehr et al., 2002). Through fine mapping we eliminated roughly 99 % of the possible candidate genes for this QTL, making a major step forward in the identification of the actual QTG.

Fehr et al. (2002) reported comparisons of coding region nucleotide sequences in 12 known genes in or near the *Alcw2* QTL interval. *Mpdz,* which encodes the Multiple PDZ domain protein (MPDZ), was the only gene that had single nucleotide polymorphisms between B6 and D2 progenitor strains, ten of which changed the protein sequence. Fehr et al., (2002) went on to sequence the *Mpdz* coding region in a panel of standard inbred mouse strains and found a significant correlation between *Mpdz* sequence variation and severity of acute ethanol withdrawal. Three protein variants (MPDZ1-3) were identified based on sequence analysis; strains that possessed the MPDZ-1 variant displayed the least severe ethanol withdrawal, strains with the MPDZ-2 variant had moderate withdrawal severity and strains with the MPDZ-3 variant had the most severe ethanol withdrawal. This suggested that MPDZ protein structure may be important for contributing to genetic differences in alcohol withdrawal severity. Thus, *Mpdz* was nominated as a promising candidate gene for this QTL.

We continued to look for recombination events within the QTL to narrow the interval and eliminate other candidates (Shirley et al., 2004). A sixth ISCL was generated and narrowed the QTL to only 1.5 Mb containing 5 known or predicted genes, including *Mpdz*. Expression of five genes in this interval was

confirmed and are shown in (table 1-1). In order to remain viable candidate genes, strain specific sequence and/or expression differences had to be demonstrated in appropriate tissue (i.e., brain). *Mpdz* remained the only gene with strain specific nucleotide differences in the coding region and was the only gene that showed brain specific expression differences between the two progenitor strains; B6 mice showed nearly a 1.8 ± 0.1 fold greater *Mpdz* mRNA expression compared to the D2 background strain. Chromosome 4 congenic strain mice also showed greater *Mpdz* mRNA expression (1.6 ± 0.1 fold change) compared to the D2 background strain. Moreover, differences in mRNA expression were reflected in MPDZ protein abundance, where congenic strain mice had 1.8 ± 0.1 fold greater MPDZ protein compared to the D2 background strain. The other candidate genes within the 1.5 Mb interval showed no strain specific differences in gene expression (table 1-1). However, one gene was not examined for expression because it encodes a potential ribosomal protein. Therefore this made it impossible to find sequence specific primers due to the common occurrence of this sequence to other ribosomal proteins. Finally, we also found that *Mpdz* gene expression is significantly correlated with acute alcohol withdrawal severity in a panel of standard inbred mouse strains, suggesting that differences in gene expression may be a potential mechanism by which *Mpdz* affects genetic differences in alcohol withdrawal severity. These data all point to the conclusion that *Mpdz* is a QTG underlying phenotypic differences in acute ethanol withdrawal severity (Shirley et al, 2004).

GenBank	Name	GenBank	Confirmed coding region SNPs (amino acid changes)	B6/D2 expression ratio (quantitative RT-PCR, mean +/- SEM)	Chromosome 4 congenic vs. background strain (quantitative RT-PCR mean +/- SEM)	Human homolog
NM_026821	-	C	0	1.0 +/- 0.1	nd	XM_210019
NM_010820	*Mpdz*	C	18 (10)	1.8 +/- 0.1	1.6 +/- 0.1	NM_003829
XM_161175	-	PE	0	nd	nd	none found
XM_196325	-	C	0	1.0 +/- 0.1	nd	none found
NM_008687	*Nfib*	C	0	1.0 +/- 0.1	nd	NM_005596

Table 1-1: *Mpdz* is the only gene with genotype-dependent sequence and expression differences. For the 5 confirmed candidate genes, GenBank annotations are C (confirmed) or PE (predicted with evidence). Quantitative RT-PCR expression results for whole brain compared B6 vs. D2 and congenic vs. background strain mice ($p < 0.005$, n = 6-12 independent samples per strain).

Characteristics and Functions of PDZ domain proteins

Over 10 years ago, PDZ domains were discovered in rat brain as ~ 90 amino acid repeats in three prototypical proteins (Cho et al., 1992). The name is derived from PSD-95, postsynaptic density protein, Disc-large, the Drosophila septate junction protein, ZO-1, the epithelial tight junction protein, (Sheng and Sala, 2001). These sequence repeats are of the form Gly-Leu-Gly-Phe and thus PDZ domains have also been referred to as GLGF repeats. PDZ domains are one of the most common motifs found in proteins and have been identified in all species so far including yeast. The human genome contains at least 400 PDZ domain proteins, *C. elegans* contains 92, and there are 131 known PDZ proteins in *Drosophila* (Nourry et al., 2003).

From an evolutionary standpoint, the common occurrence of the PDZ domain in so many proteins begs the question of 'why has this specific domain been conserved across species, and what is the functional importance of such a domain?' The first functional role for the PDZ domain was discovered when two of the PDZ domains of PSD-95 were found to bind in a sequence specific manner to the C-terminus peptide motif (-E-S/T-D/E-V) of the Shaker-type K^+ channel (Kim et al., 1995) and the NR2 subunit of NMDA receptors (Kornau et al., 1995; Niethammer et al., 1996). These initial findings paved the way for studies aimed at understanding PDZ domains as protein-protein interaction domains. Subsequent to this work functions ranging from regulation of epithelial polarity (Bilder, 2003; Bilder et al., 2003), signaling complex assembly (Harris and Lim, 2001; Garner et al., 2000), receptor trafficking (Xia et al., 2003),

modulation of receptor function (Bezprozvanny and Maximov, 2001), and downstream signaling events (Xia et al., 2003) have been attributed to PDZ domain proteins.

Three general groups of PDZ domain proteins have been classified based on number of domains and presence of other types of domains. Proteins such as GRIP and PAR-3 contain only PDZ domains and are grouped into one class. Membrane-associated guanylate kinases (MAGUKs) are a second class which comprise proteins such as PSD-95 and ZO-1 that contain one to three PDZ domains, one SH3 domain, and a guanylate kinase domain. The third class is proteins that contain PDZ domains and other protein domains such as ankyrin, LIM, and L27 domains. The Multiple PDZ-domain protein (MPDZ) is grouped in this class because it contains 13 PDZ domains and one L27 domain. Only PDZ domain proteins with SH3 (tyrosine kinase activity) and LIM (serine-threonine kinase activity) domains have intrinsic enzymatic activity and thus can activate signaling events directly.

When PDZ domain proteins were first discovered they were found to interact specifically with a short amino acid motif at the C-terminus of a variety of proteins. These short C-terminal motifs can also be classified into three groups: Class I motifs consist of the form (X-S/T-X-V* or X-S/T-X-L*); Class II motifs consist of the form (X-ϕ-X- ϕ*); and Class III motifs consist of the form X-D-X-V*), where (*) indicates the COOH group, (X) indicates any amino acid, and (ϕ) refers to a hyrdophobic residue (Sheng and Sala, 2001). However, PDZ domain proteins also interact with internal peptide motifs (Brenman et al., 1996) other

PDZ domain proteins to form dimers (Brenman et al., 1996), and plasma membrane lipids such a phosphatidylinositol 4,5-bisphosphate (PIP_2) (Zimmermann et al., 2002). Therefore it is becoming evident that PDZ domain proteins can interact with other peptide sequences beyond these three C-terminal motifs.

Overall, the functions of PDZ domain proteins that have been described are: (1): scaffolding type proteins, (2): regulators of signal transduction, possibility by controlling the rate and fidelity of signal processing, and (3): synaptic targeting of receptors. Given the functional roles and interaction capabilities of PDZ domain proteins described thus far, it is without doubt that they will be critical for regulating and integrating a host of molecular and cellular events, which makes them likely candidates for the study of complex genetic traits.

Function of The Multiple PDZ Domain Protein

Weiss and Javier (1997) fortuitously discovered MPDZ as the unidentified cellular protein that they called p220 (Weiss and Javier, 1997). When they disrupted the PDZ domain-binding motif of 9ORF1, a human viral protein, they found that it abolished its interaction with p220. Ullmer et al. (1998), using the yeast two hybrid technique with the C-terminal domain of the 5-HT_{2C} receptor as bait, isolated a full length cDNA clone encoding MPDZ, in which they called MUPP1 (the multiple PDZ domain protein). In their initial characterization, Ullmer et al. (1998) reported no catalytic domains, but found that *Mpdz* was abundant in brain and several peripheral tissues. They also mapped *Mpdz* to human

chromosome 9p24-p22. Simpson et al., (1999) subsequently identified, sequenced and mapped mouse *Mpdz* to chromosome 4. However, up until this time no functional role had been identified for *Mpdz.*

Lee et al. (2000) identified MPDZ as a cellular target for two viral oncoproteins, Adenovirus E4-ORF1 and High-Risk papillomavirus type 18 E6. They found differential effects of MPDZ on cellular proliferation depending on which oncoprotein it was bound to. From these studies Lee et al., (2000) concluded that MPDZ negatively regulates cellular proliferation and thus could be considered a tumor suppressor protein. However, other studies have not been as detailed. In most cases only evidence of binding of *Mpdz* to peptide sequences on the target protein has been reported and no precise function of *Mpdz* has been shown. Rather function is merely implied by what function the target protein mediates. Barritt et al. (2000) found that MPDZ binds to the membrane-spanning proteoglycan, NG2, and concluded that MPDZ might have a role in intercellular communication because of NG2's role in cell migration. MPDZ has also been shown to bind to the tyrosine kinase receptor, c-Kit, and the low affinity nerve growth factor receptor, p75 (Mancini et al., 2000). Both c-Kit and p75 are mediate cell growth and survival and thus MPDZ might participate in this function, directly or indirectly. Proteins that regulate phosphoinositide signaling, TAPP1 and TAPP2, also bind to MPDZ (Kimber et al., 2002). Because TAPP1 and TAPP2 mediate intracellular signals via growth factors and cytokines, this is additional evidence that MPDZ may participate in functions related to cell growth and survival. MPDZ binds to proteins (Claudin-1 and Claudin-8) that

regulate cellular polarization in epithelial cell tight junctions (Jeansonne et al., 2003; Hamazaki et al., 2002). The interaction of MPDZ with these tight-junction proteins suggests that MPDZ may have a role in cell polarity in neurons, but this has not been directly demonstrated.

Interaction partners for MPDZ that mediate neurotransmission have also been identified. Becamel et al. (2001) demonstrated that the C-terminal PDZ recognition motif of the 5-HT_{2C} receptor selectively interacts with PDZ domain 10 of MPDZ. Co-transfection of MPDZ with 5-HT_{2C} receptors in COS-7 cells induced intracellular and cell surface clustering of the two proteins, which suggests that MPDZ may function to recruit 5-HT_{2C} receptors to discrete cellular locations. *In situ* hybridization with an *Mpdz* specific riboprobe showed the highest expression of *Mpdz* in the cerebral cortex, hippocampus, and choroid plexus of rat brain (Becamel et al., 2001). The authors also provided evidence that MPDZ interacts with both 5-HT_{2A} and 5-HT_{2B} receptor subtypes *in vitro*. The selective interaction between the C-terminus of 5-HT_{2C} receptors and MPDZ *in vivo* was independently confirmed by Parker et al. (2003), who also demonstrated that agonist-induced phosphorylation of Ser^{458} within the PDZ recognition motif of the 5-HT_{2C} receptor regulates its interaction with MPDZ. The interaction of MPDZ with serotonin receptors implicates it in the broad spectrum of neuropsychiatric diseases associated with serotonin neurotransmission such as depression, anxiety, schizophrenia, eating disorders and addiction (Feldman, 1997). A major challenge will be to identify exactly how the binding of MPDZ to serotonin receptors regulates these disease states.

MPDZ also binds to proteins of brain glutamate and dopamine systems. Dakoji et al. (2003) found that MPDZ binds to stargazin, a transmembrane AMPA receptor regulatory protein, which regulates synaptic expression of AMPA receptor subunits. The stargazin/MPDZ interaction was only found *in vitro*, however (Dakoji et al., 2003). Using the yeast two hybrid system with the C-terminus of the dopamine D3 receptor as bait, MPDZ was identified as an interaction partner of this dopamine receptor (Griffon et al., 2003). In addition, MPDZ was found to interact with a member of the intracellular chloride channel family termed CICL6. CICL6 binds to the dopamine D2 receptor and thus MPDZ may indirectly regulate D2 receptors through this chloride channel. MPDZ's interactions with glutamate and dopamine receptor associated proteins highlight the ubiquitous nature of MPDZ's function, and suggest important protein networks that may be involved with cellular regulation of signal transduction and receptor trafficking.

Serotonin and Serotonin receptors

Central serotonin is a major neurotransmitter mediating feeding behavior, aggression, mood, perception, pain, and anxiety (Feldman, 1997). Serotonin neurons originate in hindbrain areas within the raphe nucleus, central gray, and surrounding reticular formation and project both rostrally and caudally through the central nervous system (Feldman, 1997). The descending (caudal serotonin system) consists of B1-B4 cell types in the medulla and caudal pons and send descending projections to the spinal cord; these projections regulate sensory, motor and autonomic functions. The ascending (rostral serotonin system)

consists of B5-B9 cell types in the raphe, rostral pons and mesencephalon and mediates a wide range of functions (Feldman, 1997). The effects of 5-hydroxytryptamine (5-HT or serotonin) are mediated by seven known receptors ($5\text{-}HT_1$-$5\text{-}HT_7$), with a total of 15 receptor subtypes (Roth et al., 1998). The synaptic availability of serotonin is regulated by an efficient reuptake system mediated by the sertonin transporter, while presynaptic autoreceptors such as $5\text{-}HT_{1B}$ receptors, also regulate release of serotonin from the presynaptic neuron. There are also somatodendritic autoreceptors such as the $5\text{-}HT_{1A}$ receptor, which suppress cell firing when activated.

All of the 5-HT receptors, except the $5\text{-}HT_3$ receptor which is a ligand-gated channel, are G-protein coupled receptors (GPCR). Some of these receptors can exist as alternatively spliced variants ($5\text{-}HT_4$ and $5\text{-}HT_7$). RNA edited isoforms uniquely characterize $5\text{-}HT_{2C}$ receptors. These post-transcriptional events provide even a greater diversity within this already diverse family of receptors (Hoyer et al., 2002). Serotonin receptors are grouped into classes based on second messenger coupling and structural identity. $5\text{-}HT_1$ receptors (1A, 1B, and 1D subtypes) are negatively coupled to cAMP through the G-protein, Gi/0. $5\text{-}HT_4$ and $5\text{-}HT_7$ receptors are positively coupled to cAMP through the G-protein, Gs. $5\text{-}HT_2$ receptors (including 2A, 2B, and 2C subtypes) are positively coupled to phospholipase C (PLC) through the G-protein, Gq. $5\text{-}HT_3$ receptors are non-selective cation channels permeable to Na^+, Ca^{++}, and K^+. This range of second messenger coupling to G-proteins suggests the potential for phenotypic switching and crosstalk within and possibly between receptor

families (Hoyer et al., 2002). The traditional view of one receptor, one signal transduction pathway has become obscured by the findings that receptors can have multiple transduction pathways to which they are associated and 'switch' from one to another depending on the needs of the cell (Hill, 1998; Selbie and Hill, 1998). Crosstalk between different classes of receptors is also becoming more frequently studied (Hill, 1998; Selbie and Hill, 1998). For this reason molecules with interaction domains such as PDZ domains should prove to be critical for general integration of second messenger functions within the 5-HT family of receptors.

Hypothesis 1: Genetic variation in *Mpdz* affects strain differences in alcohol withdrawal severity via 5-HT_{2C} receptors

A list of proteins that bind to MPDZ and their functions are shown in table 1-2. A main focus of this thesis, however, was to examine the hypothesis that strain differences in acute alcohol withdrawal severity are due, in part, to genetic variation in *Mpdz* and its interaction with 5-HT_{2C} receptors. This hypothesis developed from several key observations in the literature. First and foremost, *Mpdz* was discovered based on its specific interaction with the C-terminus PDZ recognition motif of 5-HT_{2C} receptors (Parker et al., 2003). There is a great deal of evidence suggesting that 5-HT_{2C} receptors mediate the effects of a variety of convulsion types including chemical, electrical and sound-induced convulsions (Applegate and Tecott, 1998; Brennan et al., 1997). This point is important because of the alcohol withdrawal convulsion phenotype associated with *Mpdz.*

Protein	Function	Protein	Function
5-HT_2 receptor 2A 2B 2C	serotonin transmission (anxiety, appetite, mood)	c-Kit	cellular differentiation cell survival
Stargazin	AMPA receptor function	E4-ORF1 E6	cellular proliferation (viral oncoproteins)
ClCL-6 receptor	Neuronal Cl^- homeostasis	NG2	cell migration
D3 receptor	Dopamine transmission	Claudin-1 JAM	tight junction proteins (cellular polarization)
$GABA_B$ receptor	GABA transmission	TAPP1, -2	adaptor proteins cell signaling
		p75	nerve growth factor receptor cell growth and survival

Table 1-2: Proteins that interact with MPDZ. The names of the interacting proteins are shown along with their function. Of particular importance to this project is that MPDZ has a direct protein interaction with 5-HT_{2C} receptors. See text for references.

However, no one has reported a direct role for 5-HT_{2C} receptors in alcohol withdrawal convulsions, which was another novel area of investigation of this work. The majority of the literature on 5-HT_2 receptors and alcohol withdrawal deals with their role in regulating withdrawal induced anxiety and other symptoms (LeMarquand et al., 1994a, b). For example, antagonists at 5-HT_2 receptors reduce ethanol-withdrawal related anxiety and reduce drinking and alcohol preference (Meert, 1994). In addition, Pandey et al. (1996) reported strain differences in accumulation of second messengers coupled to the 5-HT_{2C} receptor in alcohol preferring versus nonpreferring rats; 5-HT_{2C} receptor inositol phosphate accumulation was greater in the alcohol-preferring compared to the non-preferring strain. Therefore, 5-HT_{2C} receptors may also have a role in genetic susceptibility to alcohol consumption. On a more anatomical basis, the 5-HT_{2C} receptor was chosen for further study because it is the most abundant 5-HT_2 receptor subtype in the brain and it is exclusively expressed in brain tissue with no peripheral distribution (Abramowski et al., 1995; Clemett et al., 2000). Given this evidence and the defined interaction between MPDZ and 5-HT_{2C} receptors (Becamel et al., 2001; Parker et al., 2003) this represented a plausible mechanism by which genetic variation in *Mpdz* regulates genotype-dependent differences in alcohol withdrawal severity.

Evidence for Pleiotropic Effects of *Mpdz*

In the initial identification of *Mpdz* as a candidate gene for alcohol withdrawal liability it was also found that this locus on chromosome 4 regulates pentobarbital withdrawal convulsions (Fehr et al., 2002). Subsequently, Fehr et

al. (2004) reported potential pleiotropic effects of *Mpdz* on convulsions induced through the lateral tail vein by chemiconvulsants that work on glutamatergic and GABAergic neurotransmission. The authors of that paper assessed genetic correlations in 9 standard inbred strains between MPDZ protein structure and sensitivity to various chemiconvulsants. The most significant correlations were obtained for kainic acid and pentylenetratrazole (PTZ) convulsions.

It is also known that other behavioral phenotypes map to or near the same genomic location as *Mpdz*. Ferroaro et al. (2002) mapped QTL that affect sensitivity to PTZ induced convulsions and found a suggestive QTL on mid-chromosome 4 (Ferraro et al., 1999). An audiogenic seizure locus is also found on mid-chromosome 4 in crosses between B6 and D2 mice, but this phenotype is age-dependent (Neumann and Collins, 1991). Two independent studies from our group mapped chronic ethanol withdrawal QTL to the same region on chromosome 4; one study was conducted in B6D2 F2 mice exposed to alcohol vapor for 3 days (Buck et al., 2002), while the other study was conducted in the inbred Withdrawal Seizure-Prone (WSP) and Withdrawal Seizure-Resistant (WSR) selectively bred strains (Bergeson et al., 2003). Together these results argue that mid mouse chromosome 4 harbors a gene which pleiotropicallly regulates several related convulsion phenotypes.

Hypothesis 2: *Mpdz* has pleiotropic effects on chemically modulated convulsions

Because of the structural and functional nature of PDZ domains, proteins such as MPDZ containing 13 of these interaction domains are ideally situated to integrate a wide range of neurochemical pathways leading to the expression and

regulation of potentially related behavioral phenotypes. Another major aim of the present work was to test this hypothesis by measuring handling-induced convulsions (HIC) after administration of several convulsant agents with well-defined mechanisms of action as well as testing the influence of the mid region of chromosome 4 on chronic alcohol withdrawal convulsions. Comparisons between a novel congenic strain, which possess genetic material from the B6 donor strain covering the QTL interval for alcohol withdrawal, introgressed on a uniform ~98% pure genetic background from the D2 strain, would elucidate the influence of *Mpdz* on multiple pharmacologically distinct convulsions.

Convulsants were chosen that alter the major neurotransmitter systems in the brain including serotonergic, glycinergic, glutamatergic, and GABAergic neurotransmission. In addition, all these neurotransmitter systems have been associated with regulation of some form of seizure phenotype. Increasing synaptic availability of serotonin via blockade of the serotonin transporter, for example, is known to suppress electrically induced seizures in a dose-dependent manner in rodents (Dailey et al., 1992; Prendiville and Gale, 1993; Pasini et al., 1996). And interestingly, central serotonergic systems are associated with both depression and epilepsy; epilepsy is more common among people who suffer from depression and vice versa (Jobe, 2003). 5-HT_{2C} receptors have been implicated in the anticonvulsant effects of serotonin because when a 5-$HT_{2C/B}$ receptor agonist, mCPP, is directly injected into the substantia nigra in rat brain it blocks focal seizures evoked electrically from area temptas (Pasini et al., 1996).

The role of 5-HT_{2C} receptors in seizures is further confirmed because 5-HT_{2C} receptor null mutant mice have an epilepsy phenotype (Tecott et al., 1995).

There is a huge literature on the brain GABAergic system and seizure disorders (Wong et al., 2003). The GABAergic system is the primary inhibitory system of the brain and thus it has a direct role in regulating neuronal excitability. Indeed, the most common treatment for epilepsy is administration of $GABA_A$ receptor agonists such as benzodiazepines (Wong et al., 2003). Competitive and non-competitive antagonists at $GABA_A$ receptors, such a pentlyenetretrazol (PTZ) decrease seizure thresholds in a number of experimental models (Ferraro et al., 1999). Alcohol withdrawal seizures are also intimately regulated by $GABA_A$ receptors, which has been a major focus of thirty years of research on the effects of alcohol on the brain (Reilly et al., 2001; Grobin et al., 1998). Glycine receptors are also inhibitory. But their role in convulsions is not as well characterized as that for the GABA system, principally because until recently only scant evidence has been put forth for the presence of glycine receptors in the brain (these receptors were thought to be primarily localized to the hindbrain and spinal chord) (Laube et al., 2002). However, the glycine receptor antagonist, strychnine, is a well known convulsant drug and mutations in the alpha 1 subunit of the glycine receptor leads to movement disorders like hyperekplexia (Andrew and Owen, 1997). Also, at least some of the acute *in vivo* effects of ethanol are influenced by the glycine receptor (Mihic, 1999).

The glutamate system is the principal excitatory neurotransmitter system in the brain and thus also has a critical role in the balance between excitation and

inhibition of neural networks. N-methyl-D-aspartate (NMDA) receptors mediate many of the effects of endogenous glutamate, but NMDA receptor activation is first dependent on activation of another class of glutamate receptors, AMPA receptors, which are required for removal of an intrinsic Mg^{++} block of the NMDA receptor channel (Hardingham and Bading, 2003). There is a well-defined link between activation of NMDA receptors and synaptic plasticity and long-term potentiation (Carroll and Zukin, 2002). Thus these receptors have been the focus of much work on correlates of learning and memory and other forms of synaptic plasticity such as neuroadaptations to drugs of abuse (Krystal et al., 2003a; Reilly et al., 2001) in addition to their critical role in seizure regulation. There is ample evidence for involvement of each of the above neurotransmitter systems in convulsions and the second major hypothesis of this work is that genetic variation in MPDZ regulates handling-induced convulsions modulated by some of these neurotransmitter systems.

The Immediate Early Gene, *cfos,* and Alcohol withdrawal

The immediate early gene, *cfos*, which encodes the inducible transcription factor (cFOS) is responsible for orchestrating and initiating a number of cellular events that leads to expression of various neuronal genes. *cfos* is rapidly induced after various stimuli; hence it is named as an immediate early gene where it can be detected as soon as 5 to 10 minutes after induction (Herdegen and Leah, 1998). The peak protein levels of cFOS are typically observed 1 to 3 hours after induction and gradually disappear from the cell nucleus by 4 to 6 hours after treatment (Kovacs, 1998).

cFOS is a member of the Fos family of transcription factors which also includes FosB, Fra-1 and Fra-2. cFOS heterodimerizes with members of the Jun family of transcription factors (c-jun, JunB, and JunD) to form an activator protein-1 (AP-1) complex which then binds DNA and regulates transcription (Kovacs, 1998). Fos induction is generally believed to represent functional activity of neurons (Sagar et al., 1988). For example, it has been shown that Fos expression is dependent on temporal activation of action potentials. Fos proteins are negatively correlated with the burst-interval patterns of action potentials (Fields et al., 1997). Thus, Fos expression has been used in a number of circumstances as a means to map brain regions associated with challenges with various stimuli including seizures (Zhang et al., 2002) and stress (Kovacs, 1998).

cFOS in particular has two features which make it useful for brain mapping. cFOS has very low basal levels of expression (Hughes et al., 1992) and it can be induced by a wide range of trans-synaptic/transcriptional stimulation (Kovacs, 1998). In addition, cFOS is rapidly induced by acute challenges, which has particular advantages for certain studies (Kovacs, 1998). However, cFOS as a mapping tool has limitations such as it is not ideal for measuring net inhibition of brain regions and the identity of the genes being induced by *cfos* are not known (Kovacs, 1998). In addition, because *cfos* is rapidly induced after acute challenges it may not be ideal for studying long term changes in brain activation caused by various stimuli. For example, Fra-1, which consists of fosB and ΔFosB is more suited to study the effects of chronic and repeated stimuli (Kovacs, 1998).

Given the strengths and weaknesses of using cFOS expression as a brain mapping tool, there has been considerable success in using its expression to map brain regions associated with tolerance and dependence to drugs (Herdegen and Leah, 1998). Psychostimulants, antidepressants, morphine and ethanol all have been shown to induce expression of Fos in the brain (Herdegen and Leah, 1998). Of key importance to the present thesis is that cFOS is induced during withdrawal from alcohol (Ryabinin, 2000). A common finding in these studies is that *cfos* expression is induced in limbic regions such as regions of the hippocampus, amygdala, septum, and bed nucleus of the stria terminalus for example (Knapp et al., 1998, 2001; Moy et al., 2001; Ryabinin et al., 1997; Morales et al., 1998; Dave et al., 1990).

Hypothesis 3: Withdrawal following chronic ethanol exposure activates differentbrain regions and circuits in C57BL/6J versus DBA/2J inbred mouse strains

Very few studies have examined the effects of genetic variation on cFos expression (Ryabinin et al., 1999; Hitzemann and Hitzemann, 1997). Even fewer studies have examined the effects of genetic variation on alcohol-withdrawal induced cFos expression (Olive et al., 2001). B6 and D2 mouse strains show substantial differences in withdrawal severity following chronic alcohol exposure (Crabbe, 1998); D2 inbred strain mice display severe withdrawal, while B6 mice show less severe withdrawal from alcohol. Withdrawal from chronic alcohol in these two inbred strains is known to induce genotype-specific patterns of gene expression in the brain (Daniels and Buck, 2002). It is reasonable to hypothesize

that strain differences in alcohol withdrawal severity are associated with genotype-dependent patterns of brain activation. Thus, the third major aim of this thesis was to identify brain regions and circuits activated during withdrawal from chronic alcohol in B6 and D2 inbred mouse strains using expression of cFOS as a mapping tool.

Chapter II

A Mechanism by which the Multiple PDZ Domain Protein Mediates Genetic Differences in Ethanol Withdrawal Severity Via 5-HT_{2C} receptors

Abstract

We previously fine mapped a quantitative trait locus (QTL) responsible for 26% of the genetic variance in acute ethanol withdrawal severity among mice derived from the C57BL/6J (B6) and DBA/2J (D2) mouse strains and identified ,*Mpdz,* which encodes the multiple PDZ domain protein (MPDZ), as the quantitative trait gene underlying this QTL. The purpose of this investigation was to define a mechanism whereby genetic variation in *Mpdz* affects alcohol withdrawal severity. 5-HT_2 receptors are implicated in ethanol withdrawal and alcoholics show a hyposensitivity of the serotoninergic system. Because MPDZ directly interacts with 5-HT_{2C} receptors, we hypothesized that genetic variation in *Mpdz* and its interaction with 5-HT_{2C} receptors may be a mechanism affecting individual differences in ethanol withdrawal severity. A novel congenic strain that captures the chromosome 4 QTL and possesses a unique genetic variant of *Mpdz* from the B6 donor strain introgressed on a D2 background strain shows significantly greater reduction in ethanol withdrawal convulsions after administration of a 5-$HT_{2C/B}$ receptor agonist (mCPP) compared to D2 background strain mice. This congenic strain also shows less severe exacerbation of ethanol withdrawal convulsions after administration of a selective 5-HT_{2C} receptor antagonist (SB-242084). Using standard immunohistochemical techniques we find that MPDZ

is localized in B6 and D2 mouse brain in critical brain regions that regulate alcohol withdrawal. By using confocal microscopy immunofluorescence we also show that MPDZ and 5-HT_{2C} receptors are co-localized in brain regions implicated in ethanol withdrawal. Congenic and D2 mice also appear to differ in co-localization and subcellular localization of MPDZ and 5-HT_{2C} receptors. Differences in co-localization between congenic and D2 mice may reflect differences in overall protein abundance, which was confirmed by semi-quantitative Western blot analysis for MPDZ. Neither coding region sequence or protein expression for the 5-HT_{2C} receptor differed between congenic versus D2 background strain mice or B6 donor strain mice. These results suggest a plausible mechanism by which genetic variation in *Mpdz* affects individual differences in ethanol withdrawal severity through 5-HT_{2C} receptors. These are the first studies of a quantitative trait gene mechanism and offers a heuristic model for the study of other addiction related genes.

INTRODUCTION

Alcoholism is a complex disease under the control of multiple genetic and environmental factors. Evidence for a genetic contribution to alcoholism has come from several large twin and adoption studies, which show that alcoholism is 40-60% genetic (McGue, 1999). Evidence also exists for genetic heterogeneity in alcoholism and the influence of multiple genes (Schuckit, 1994), which has made identification of specific genes elusive.

Genetic mouse models for specific alcohol traits such as the withdrawal syndrome have proven to be an alternative to dissecting alcoholism genes in the

human condition (Crabbe et al., 1999b), principally because inbred mouse strains, for example, offer the advantage of controlled environment and reduced genetic heterogeneity. Identification of candidate genes in mouse models is a major step towards unraveling the complexity of alcoholism because of the high homology between the mouse and human genomes; once genes for susceptibility to alcohol related behaviors are identified in mice they will almost always have a human homologue that can be tested.

In our initial mapping studies we found evidence for a quantitative trait locus (QTL) on chromosome 4, which contains a gene(s) that influences ethanol withdrawal liability in mice. This QTL is responsible for 26% of the genetic variance in acute ethanol withdrawal severity among mice derived from the C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains (Buck et al., 1997). In subsequent work we fine-mapped the chromosome 4 QTL to a 1.5 Mb region which contained only 5 expressed genes (Fehr et al., 2002; Shirley et al., 2004). *Mpdz,* which encodes the multiple PDZ domain protein (MPDZ), is the quantitative trait gene (QTG). The two progenitor strains, B6 and D2, used to map this locus differ in MPDZ protein structure and ethanol withdrawal severity in a panel of standard inbred strains is genetically correlated with MPDZ protein status (Fehr et al., 2002). *Mpdz* gene expression is also genetically correlated with ethanol withdrawal severity in the same panel of standard inbred strains (Shirley et al., 2004). We have developed a novel congenic strain that captures the chromosome 4 QTL for acute ethanol withdrawal and acute pentobarbital withdrawal. This congenic strain has genetic material from the B6 donor strain

spanning the QTL interval transferred on a uniform D2 background strain. This congenic strain was invaluable for confirming that *Mpdz* is the QTG.

What is the mechanism by which genetic variation in *Mpdz* affects individual differences in alcohol withdrawal severity? MPDZ directly interacts with 5-HT_2 receptors and may regulate receptor trafficking and the speed and efficiency of 5-HT_2 receptor mediated signal transduction (Becamel et al., 2001; Parker et al., 2003). Because 5-HT_2 receptors are implicated in ethanol withdrawal behaviors such as anxiety and convulsions (LeMarquand et al., 1994a, b) and 5-HT_{2C} null mutant mice show increased susceptibility to chemical, electrical and sound-induced seizures (Brennan et al., 1997; Applegate and Tecott, 1998), we hypothesized that genetic variation in MPDZ and its interaction with 5-HT_{2C} receptors may contribute to individual differences in ethanol withdrawal severity. Here, we show that chromosome 4 congenic and D2 mice differ in behavioral responses to 5-HT_{2C} receptor agents during alcohol withdrawal and that MPDZ is localized and co-localized with 5-HT_{2C} receptors in critical brain regions associated with genetic differences in alcohol withdrawal severity. These results provide a novel functional mechanism by which genetic variation in *Mpdz* contributes to individual differences in alcohol withdrawal susceptibility through serotonin 5-HT_{2C} receptors.

MATERIALS AND METHODS

Animals. Adult male C57BL/6J (B6) and DBA/2J (D2) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Upon arrival at the AALAC-accredited animal facility at the Portland VA Medical Center, mice were

housed in groups of 4 per cage under a 12 h light/dark cycle. Mice were acclimated to the colony room for at least 2 weeks after arrival to control for effects of shipping stress. Male and female chromosome 4 congenic mice were bred at the Portland VA Medical Center in the Portland Alcohol Research Center Animal Production core. A separate group of male and female D2 background strain mice were also bred in parallel to the congenic mice. Food (Purina lab chow) and water were freely available at all times. Colony and procedure rooms were kept at an ambient temperature of 22 ^{0}C. All mice were between 60-100 days old at time of testing and were housed in groups of 4 in wire mesh cages with corn cobb bedding. All procedures with mice were conducted in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals* and animal procedures were approved by the Portland VA Instutional Animal Care and Use Committee.

Drugs. Ethanol was purchased from Aaper Co. (Shelbyville, KY) and was administered by i.p. injection at a dose of 4 g/kg (20 % v/v; in saline). *m*-chlorophenylpiperazine (mCPP) was purchased from Sigma (St. Louis, MO) and was administered by i.p. injection at doses of 1, 5, 7 or 10 mg/kg (in 0.9 % saline) in combination with 4 g/kg ethanol. SB-242084 was purchased from Sigma (St. Louis, MO) and administered in combination with ethanol (4 g/kg) by i.p. injection at a dose of 40 mg/kg in 8 % β-cyclodextrin and 25 mM citric acid.

Modulation of acute ethanol withdrawal by a 5-HT$_{2C}$ receptor agonists and antagonists. The handling-induced convulsion (HIC) was used to index ethanol withdrawal severity as previously described (Kosobud and Crabbe, 1986)

(Goldstein and Pal, 1971). The HIC scoring scale has been published elsewhere (Finn and Crabbe, 1999). Briefly, this procedure involves lifting the mouse by the tail, gently spinning it 180 degrees if necessary and rating convulsion severity based on a 7 point scale. A score of zero indicates no convulsion after tail lift and spin. HIC scores ranging from 1 to 3 require the gentle spin to elicit a tonic or clonic convulsion, whereas convulsions elicited by merely lifting the mouse by the tail are scored as 4-6. A score of 7 indicates a spontaneous convulsion prior to tail lift. For all behavioral experiments, two baseline (pre-drug) HIC scores were measured in mice 20 minutes apart and 30 minutes prior to i.p. injection of drug. Drugs were administered in combination with ethanol to avoid the confound of multiple injections. Pilot experiments indicated that more than one injection affected HIC scores, and would therefore have confounded interpretation of such data. Ethanol (4 g/kg) or ethanol plus mCPP (1, 5, or 10 mg/kg), a 5-$HT_{2C/B}$ receptor agonist, was injected (i.p.) in D2 strain mice. Post-drug HIC was measured beginning at hour 2, and then hourly through hour 12. In a separate experiment, chromosome 4 congenic and D2 strain mice were injected with ethanol (4 g/kg, i.p.) or ethanol plus 7 mg/kg mCPP after baseline HIC was measured. Post-drug HIC was measured beginning at hour 2, and then hourly through hour 12. In the next experiment, chromosome 4 congenic and D2 strain mice were injected (i.p.) with ethanol (4 g/kg) or ethanol plus 40 mg/kg SB-242084 (i.p.), a 5-HT_{2C} receptor antagonist, after baseline HIC was measured. Post-drug HIC was measured beginning at hour 2, then hourly through hour 12, and at hour 24 and 25 the next day.

Area under the withdrawal curve (AUC) was calculated as an overall measure of withdrawal severity by summing the HIC scores beginning with time points in which average drug-treated scores exceeded baseline HIC scores. There were no genotype-dependent baseline HIC differences, so AUC reflects the raw data uncorrected for baseline HIC. To analyze the effects of the drug alone on modulation of ethanol withdrawal HIC, and to correct for strain differences in ethanol withdrawal HIC, AUC data were expressed as a percent change from each strain's ethanol-alone group. For the dose-response experiment with mCPP, however, AUC is shown for each group because only D2 strain mice were tested.

Brain localization of MPDZ by immunohistochemistry. Ethanol-naïve B6 and D2 mice were perfused through the heart under ketamine (60 mg/kg; Fort Dodge laboratories, Fort Dodge, IA) and xylazine (8 mg/kg; Miles, Inc., Shawnee Mission, KS) anesthesia with 50 ml of 0.9 % saline and then with 50 ml of 4% paraformaldehyde (in 0.1 M phosphate buffer (PB)), pH = 7.4. Brains were then removed and placed in 4% paraformaldehyde to post-fix for 2 hours. Brains where then placed in fresh 30% sucrose (in 0.1 M PB) for 2 days. Brains were frozen on dry ice and sliced coronally at 30 μm sections. Slices were collected and stored in 10 mM phosphate buffered saline (PBS), pH = 7.4.

Brain slices were incubated in 3% H_2O_2 for 15 minutes at room temperature and then rinsed in PBS for 30 minutes. Slices were blocked for 1 hour in 3% normal rabbit serum containing 0.25% Triton-X 100 in 10 mM PBS. Slices were incubated in primary antibody against MPDZ (Upstate Biotechnology,

NY) at 4°C for 72 hours on a rotating shaker. Optimal antibody concentrations were determined experimentally with a primary antibody titration curve (1:250 – 1:2000). Optimal staining was observed at a dilution of 1:500. Slices were washed again in 10 mM PBS for 30 minutes and then incubated for 90 minutes at room temperature with secondary antibody (rabbit anti-sheep IgG) diluted 1:200 containing 3% normal rabbit serum and 0.25% Triton-X 100. Brain slices were then incubated with a with horseradish peroxidase avidin-biotin complex (ABC) solution for 90 minutes at room temperature. Slices were washed again in 10 mM PBS for 30 minutes. Slices were then incubated for 5 minutes in 0.1 M Tris, pH = 7.4 at room temperature. Visualization of MPDZ antibody staining was done by incubating slices in the substrate, diaminobenzidine (50 mg/100ml), containing 0.01 % nickel ammonium sulfate and 0.035% hydrogen peroxide for 30 minutes at room temperature. Brian slices were mounted onto slides, dehydrated, and cover-slipped in Permount.

MPDZ positive cells were detected using an Olympus BX60 light microscope. Images were captured at 10 X magnification through a Spot Insight digital color camera. The mouse atlas by Paxinos and Franklin (1997) was used to identify brain regions (Paxinos, 1997). Common landmarks (e.g., anterior commissure) were identified on the tissue sections to ensure comparable brain regions.

Brain regional co-localization of MPDZ with 5-HT_{2C} receptors using confocal microscopy immunofluorescence. Chromosome 4 congenic and D2 background strain mice were killed by cervical dislocation and brains were

extracted and fixed in 4 % paraformaldehyde (in 0.1 M phosphate buffer) for 24 hours and then in 30 % sucrose (in 0.1 M phosphate buffer) for 2 days. Brains were then frozen on dry ice and coronally sectioned at 30 μm on a sliding microtome and placed in 10 mM phosphate buffered saline (PBS). Brain slices were mounted on a glass microscope slide and washed in 10 mM PBS. Brain slices were incubated in 10 % normal donkey serum at room temperature for 1 hr with shaking. Slices were co-incubated for 72 hours at 4 degrees with primary antibody against MPDZ (polyclonal sheep anti-human MPDZ, corresponding to residues 926-1676; Upstate Biotechnology, Lake Placid, NY) diluted 1:250 in 10 mM PBS and primary antibody against 5-HT_{2C} receptor (polyclonal rabbit anti-human 5-HT_{2C} receptor, corresponding to residues 374-458; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:20 in 10 mM PBS. Slices were washed in 10 mM PBS (3 x 10 min) and then co-incubated for 24 hours at 4 degrees with secondary antibodies; for MPDZ the secondary antibody was a donkey anti-sheep IgG conjugated to a Rhodamine Red-X fluorophore (Jackson ImmunoResearch, West Grove, PA), for the 5-HT_{2C} receptor the secondary antibody was a donkey anti-rabbit IgG conjugated to a Oregon Green Alexa 488 flurorophore (Molecular Probes, Eugene, OR). Optimal antibody concentrations and incubation times were determined in pilot experiments. Slices were wash again in 10 mM PBS (3 x 10 min), and then incubated in slow fade equilibrium buffer for 5 minutes at room temperature. Slow Fade (Molecular Probes, Eugene, OR) was then applied to the slices to preserve the fluorescent signal and then slides were cover-slipped and sealed with nail polish.

Confocal laser microscopy was used to examine double-label immuno-fluoroscence images to determine co-localization and sub-cellular localization of MPDZ and 5-HT_{2C} receptors in brain slices from Chromosome 4 congenic and D2 background strain mice. Methods were similar to that of Grove et al. (2000). The Leica Corporation (Germany) TSC SP confocal system consisted of a Leica Corporation RBE inverted microscope equipped with an Ar laser producing light at 488 nm (for visualization of the Oregon Green Alexa Fluro 488 signal) and a Kr laser producing light at 568 nm (for visualization of the Rhodamine Red-X signal). For each experiment, flurophore signals were checked individually for bleed-through to the apposing detector. Adjusting laser intensity and detector window width eliminated all bleed through. A series of continuous (16 confocal sections) optical sections at 0.5 μm intervals along the z-axis of the tissue section was scanned for each fluorescent signal. The signals were obtained for each flurophore on one series of optical sections and stored separately as a series of 512 x 512 pixel images. The confocal images (40X) are presented as one z-plane confocal optical section (0.5 μm section) to demonstrate specific co-localization in a single neuron. Higher power images (200X) were processed to examine sub-cellular localization of proteins within a single cell.

MPDZ and 5-HT_{2C} receptor Western immunoblotting. Ethanol-naïve chromosome 4 congenic and D2 mice were killed by cervical dislocation and whole brain was quickly extracted, placed in tubes and frozen in liquid N_2. Brain tissue was stored at –70 ^{0} C until time of assay. Brain samples were homogenized in buffer containing 10 mM Tris-HCl, 150 mM NaCl, 0.5 % NP-40,

and complete protease inhibitors (Roche, Mannheim, Germany) using a polytron, setting 6 for 10-15 seconds. Tissue samples were then lysed on ice for 1 hr. Samples were centrifuged at 13,000 x g for 10 minutes at 4 ^{0} C. Supernatants were collected and used to determine protein concentrations with the bicinchonicnic acid (BCA) protein kit (Pierce, Rockford, IL). Total protein concentrations ranged from 29.2 – 47.8 μg/μl and were aliquoted and frozen at –70 ^{0} C until time of assay. A sheep polyclonal antibody against human MPDZ was purchased from Upstate Biotechnology (Upstate, NY). This antibody recognized a 210 kDa band as expected for the MPDZ protein. Pilot experiments were performed to determine the linear range for protein using the MPDZ antibody and it was found that 40 μg protein was in the linear range. Experiments were also performed to determine the optimal incubation time for the MPDZ antibody. Brain tissue samples were incubated with the MPDZ antibody for 1, 2, 4, 16, and 24 hours. It was found that a 24 hr incubation with the MPDZ antibody was saturating and was used in subsequent experiments. Forty micrograms of total protein from the whole brain of chromosome 4 congenic and D2 mice was mixed with 1X lammillee buffer and samples were boiled for 5 minutes to denature the proteins. For quantitative purposes, all samples were run on duplicate gels. Samples were loaded on 7.5 % Tris-HCl gels (Biorad, Hercules, CA) and separated at 150 V. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore; Billerica, MA) for 16 hr. PVDF membranes containing proteins were blocked for 1 hr at room temperature in blocking buffer containing 1 % Polyvinylpyrrolidone, 2 % nonfat dry milk, 3 %

bovine serum albumin and 0.5 % Tween-20. Membranes were then incubated with the MPDZ primary antibody diluted 1:1000 for 24 hrs at 4 ^{0} C. Membranes were washed in tris-buffered saline (TBS) containing Tween-20 and then incubated with an alkaline-phosphatase donkey anti-sheep secondary antibody (Jackson ImmunoResearch, PA) diluted 1:20,000 for 1 hr. Membranes were washed again in TBS containing Tween-20 and incubated in ECF substrate (Amersham-Pharmacia, Piscataway, NJ) for 6 minutes. Membranes were scanned on a Molecular Dynamics Typhoon 9410 variable mode imager (Amersham-Pharmacia, Piscataway, NJ) to visualize chemifluorescence. Images of membranes were digitally captured and protein bands were quantified using ImagQuant 5.2 software. GAPDH was used as an internal standard to control for subtle variations in protein loading. A monoclonal antibody against GAPDH was purchased from Chemicon (Temecula,CA). Procedures for GAPDH immunostaining were identical to that used for detection of MPDZ protein except where noted below. Membranes were incubated for 1 hr in blocking buffer and then in GAPDH primary antibody at a dilution of 1: 50, 000 for 1 hr. Membranes were washed in TBS containing Tween-20 and then incubated with an alkaline-phosphatase goat anti-mouse secondary antibody (Jackson ImmunoResearch, PA) diluted 1:100,000 for 1 hr.

5-HT$_{2C}$ receptor DNA sequence analysis. PCR products from the 5-HT$_{2C}$ receptor coding region were gel purified and both strands sequenced with gene specific primers from cDNA obtained from B6 and D2 mouse whole brain. Primer sequences were as follows:

F1: 5'-GCATAGGCCAATGAACACCT-3'

R1: 5'-ACCGGCTATGCTCAATAGGA-3'

F2: 5'-CCTGTCTCTGCTTGCAATTCT-3',

R2: 5'-TTGTTGATAGCTTGCATGGTG-3'

F3: 5'-GGGTGATGAGGAAGAGAACG-3',

R3: 5'-GGACTTTCCTACAAGAGATTTCC-3'.

BigDye terminator (v3.1) sequencing was performed in house on an ABI373 fluorescent sequencer as per protocol (Foster City, CA). XL (extra long) gel stretch reads consistently reached 600 bp allowing double coverage of the PCR products when sequenced with each primer. 64 samples were run per XL gel. Sequence results were aligned and edited in Sequencher (Gene Codes, Ann Arbor, MI), a DNA assembly program.

Statistical Analysis. Dose-response curves were analyzed by ANOVA with dose as a factor and AUC as the dependent variable. Post-hoc Tukey's tests were conducted where appropriate. For behavioral experiments and Western immunoblotting comparing congenic and D2 strain mice, t-test were used with alpha set at 0.05.

RESULTS

Figure (2-1) shows negative control experiments for MPDZ and 5-HT_{2C} receptor antibodies from cells and/or tissue types that do not endogenously express these proteins. Figure (2-1A) shows MPDZ detection in mouse cerebral cortex (CX), but not in COS-7 cells which do not endogenously express MPDZ (Becamel et al., 2002). Figure 2-1B shows 5-HT_{2C} receptor detection in mouse cerebral

cortex (CX), but not in COS-7 cells or mouse liver (L), where 5-HT_{2C} receptor protein is not expressed (Becamel et al., 2001; Clemett et al., 2000).

We set out to test the hypothesis that 5-HT_{2C} receptor agonists and antagonists modulate, specifically, ethanol withdrawal convulsions in our mouse populations. Figure 2-2A shows modulation of ethanol withdrawal convulsions in D2 mice after administration of mCPP, a 5-$HT_{2C/B}$ receptor agonist. D2 mice treated with ethanol (4 g/kg, i.p.) show characteristic severe ethanol withdrawal convulsions. In contrast, D2 mice treated with a combination of ethanol (4 g/kg; i.p.) and mCPP (5 mg/kg or 10 mg/kg; i.p.) show a reduction in ethanol withdrawal HIC. Baseline HIC was not different between strains. Figure 2-2B shows AUC, an overall measure of withdrawal severity, for D2 mice treated with ethanol alone or ethanol plus 1, 5, or 10 mg/kg mCPP. ANOVA indicated a significant effect of dose on AUC [$F(3,186) = 6.7$, $p < 0.0001$. Post-hoc analysis showed that D2 mice treated with ethanol + 5 mg/kg mCPP had a significant

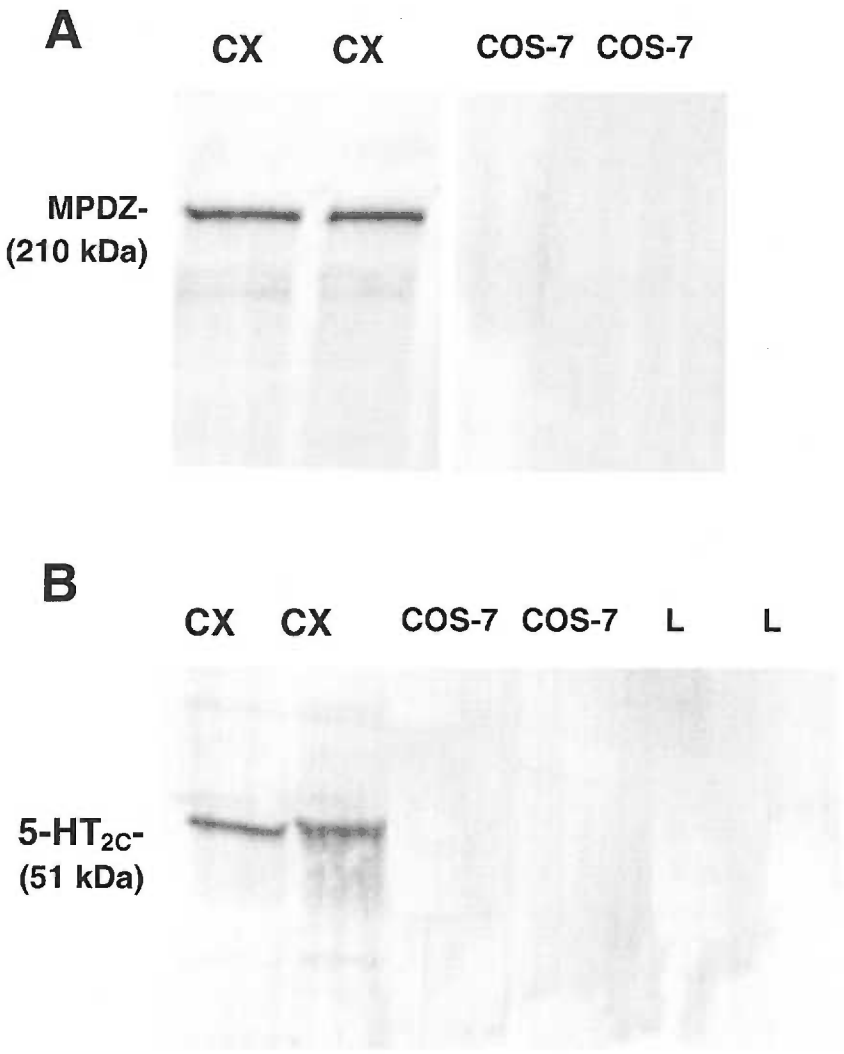

Figure 2-1: Negative controls for Western immunoblotting. (**A**): Forty micrograms of protein from mouse cerebral cortex (CX) or COS-7 cells was loaded in each lane. This Western blot shows the specificity of the MPDZ antibody because it detects a band of about 210 kDa corresponding to mouse MPDZ in the cortex but not in COS-7 cells which have been previously found not to express this protein endogenously. (**B**): Forty micrograms of protein from mouse cerebral cortex (CX), COS-7 cells or mouse liver (L) was loaded in each lane. This Western blot shows the specificity of the 5-HT_{2C} receptor antibody because it detects a band of about 51 kDa corresponding to mouse 5-HT_{2C} receptor in the cortex but not in COS-7 cells or liver which have been previously found not to express this protein endogenously.

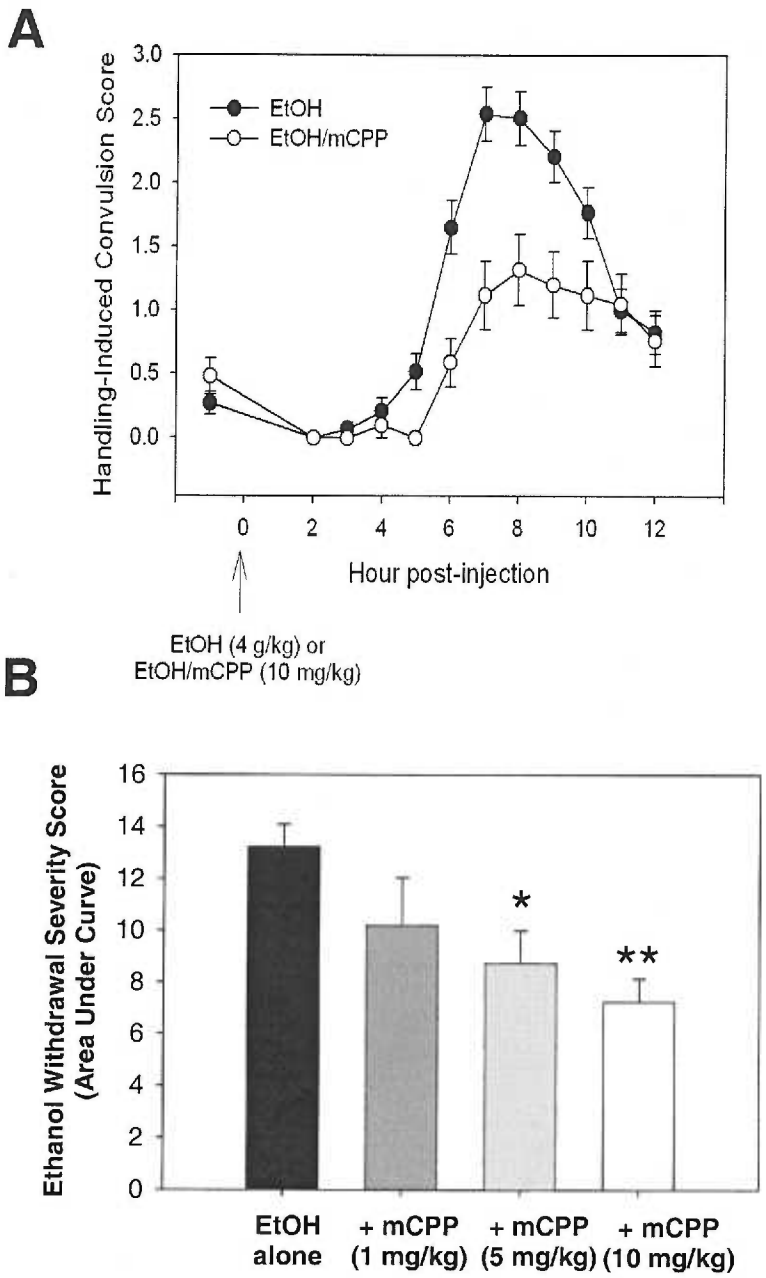

Figure 2-2: mCPP dose-dependently decreases ethanol withdrawal convulsions in D2 mice. **(A)**: Time course of ethanol withdrawal convulsions measured using handling-induced convulsion (HIC). Baseline HICs were taken twice, 30 minutes prior to i.p. injection (hr 0, arrowhead) of ethanol (4 g/kg) or ethanol plus mCPP (10 mg/kg). D2 mice injected with ethanol alone exhibit increases, then decreases in HIC which waxes and wanes over the 12 hour testing period. In contrast, D2 mice treated with ethanol plus 10 mg/kg mCPP show a reduction in ethanol withdrawal convulsions over this time course. **(B):** Area under the ethanol withdrawal curves shown as an overall measure of withdrawal severity. Bars represent the mean ± SEM for mice treated with ethanol alone or ethanol plus 1, 5, or 10 mg/kg mCPP. Mice treated with ethanol plus 5 mg/kg mCPP show a significant reduction in ethanol withdrawal severity (* $p < 0.05$) as well as mice treated with ethanol plus 10 mg/kg (** $p < 0.0001$) compared to the ethanol alone group. Data presented are from 20-82 mice per treatment group.

reduction in AUC ($p < 0.05$) as well as those mice treated with ethanol + 10 mg/kg mCPP ($p < 0.0001$) compared to the ethanol alone group.

To address the influence of genetic variation in *Mpdz* on 5-HT_{2C} receptor modulation of ethanol withdrawal convulsions, we compared chromosome 4 congenic mice to D2 background strain mice for mCPP modulation of ethanol withdrawal severity. Figure 2-3A shows the time course of ethanol withdrawal convulsions in congenic and D2 mice treated with ethanol alone (4 g/kg, i.p.) or ethanol plus 7 mg/kg mCPP (i.p.). Congenic strain mice show a greater reduction in ethanol withdrawal severity compared to D2 background strain mice after administration of ethanol plus mCPP. AUC was calculated for mice treated with ethanol alone and with ethanol plus mCPP. Figure 2-3B shows the percent change from each strain's ethanol alone treated group. Congenic mice show a significantly greater reduction in ethanol withdrawal convulsions after mCPP compared to D2 strain mice ($t = 4.3$; $df = 31$ $p < 0.0001$). This result suggests that genetic variation in *Mpdz* may differentially affect responses to 5-HT_{2C} receptor agonists during ethanol withdrawal.

To study the influence of genetic variation in *Mpdz* on 5-HT_{2C} receptor modulation of ethanol withdrawal severity further, we administered a highly selective 5-HT_{2C} receptor antagonist, SB-242084, during ethanol withdrawal (Kennett et al., 1997). Figure 2-4A shows the time course of ethanol withdrawal convulsions in congenic and D2 mice treated with ethanol alone (4 g/kg, i.p.) or ethanol plus SB-242084 (40 mg/kg, i.p.). After administration of the 5-HT_{2C} receptor antagonist, SB-242084, D2 mice show a much greater increase in

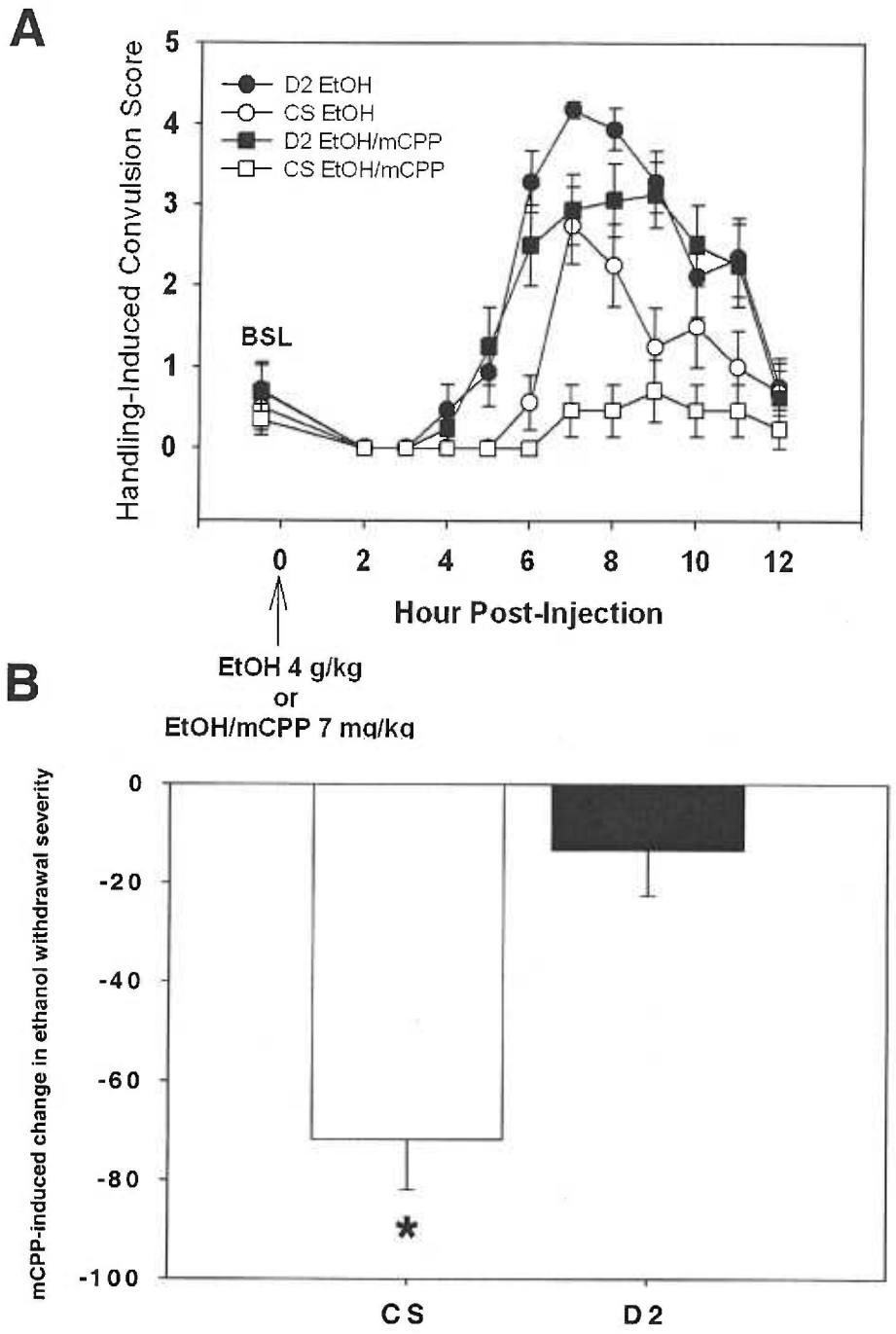

Figure 2-3: Chromosome 4 congenic mice show a greater reduction in ethanol withdrawal convulsions after mCPP compared to D2 background strain mice. **(A):** Time course of ethanol withdrawal convulsions in congenic strain (CS) and background (D2) strain mice. Baseline HICs were taken twice, 30 minutes prior to i.p. injection (hr 0, arrowhead) of ethanol (4 g/kg) or ethanol plus mCPP (7 mg/kg). The reduction in ethanol withdrawal convulsions by mCPP is greater in congenic versus D2 background strain mice. **(B):** Area under the ethanol withdrawal curves (AUC) shown as an overall measure of withdrawal severity. Bars represent the mean ± SEM for AUC expressed as a percent change of ethanol plus mCPP groups versus ethanol alone groups. Congenic strain mice show a more significant reduction in ethanol withdrawal convulsions after mCPP compared to D2 strain mice (* $p < 0.0001$). Data presented are from 16-18 mice per strain and treatment group.

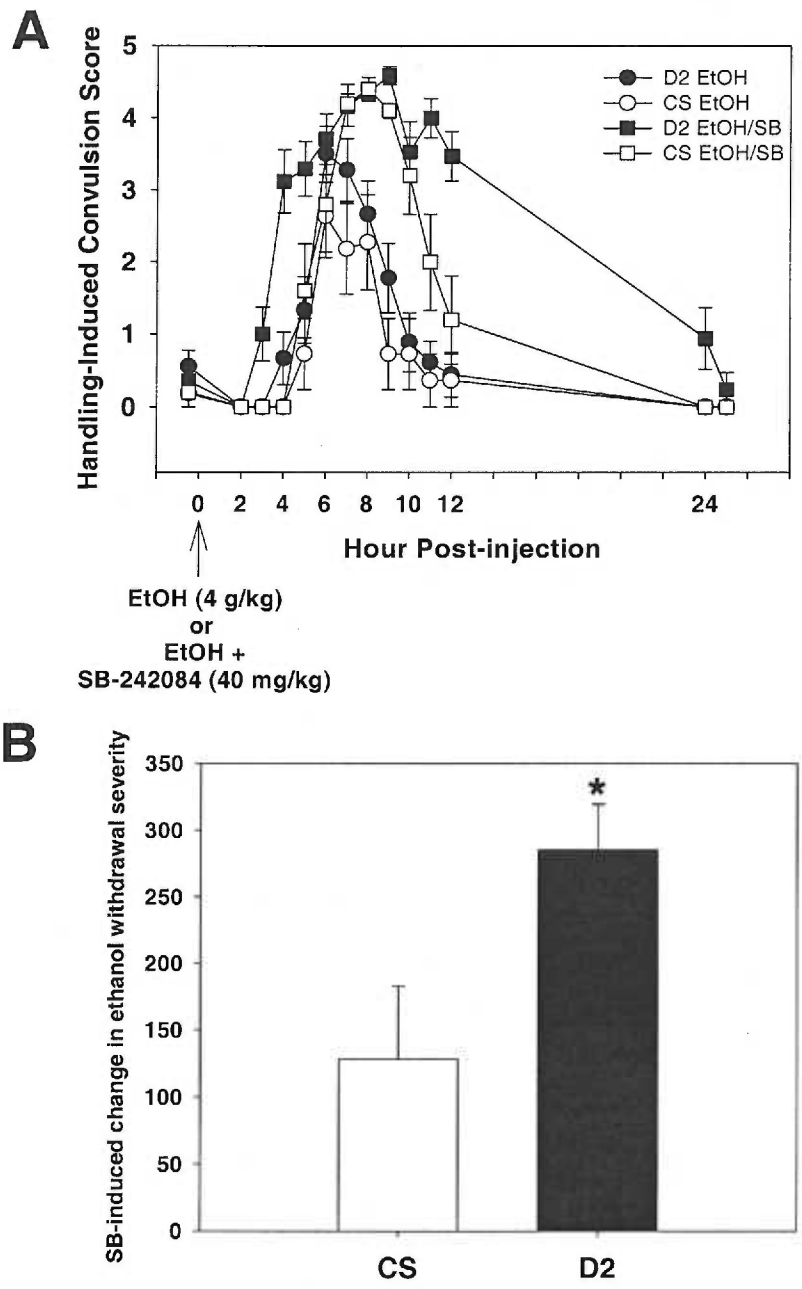

Figure 2-4: Background (D2) strain mice show a greater increase in ethanol withdrawal convulsions after SB-242084 compared to congenic (CS) strain mice **(A):** Time course of ethanol withdrawal convulsions in background (D2) and CS mice. Baseline HICs were taken twice 30 minutes prior to i.p. injection (hr 0, arrowhead) of ethanol (4 g/kg) or ethanol plus SB-242084 (40 mg/kg). D2 mice show a greater exacerbation of ethanol withdrawal convulsions after SB-242084 compared to congenic strain mice. **(B):** Area under the ethanol withdrawal curves (AUC) shown as an overall measure of withdrawal severity. Bars represent the mean ± SEM for AUC expressed as a percent change of ethanol plus SB-242084 groups versus ethanol alone groups. D2 strain mice show a more significant increase in ethanol withdrawal convulsions after SB-242084 compared to congenic strain mice (* $p < 0.0001$). Data presented are from 17-18 mice per strain and treatment condition.

ethanol withdrawal severity compared to congenic strain mice. This effect is shown in Figure 2-4B, where AUC was computed for ethanol alone and ethanol plus SB-242084 groups and then AUC was expressed as a percent change from each strain's ethanol-alone group. SB-242084 produces a significantly greater increase in ethanol withdrawal convulsions in D2 compared to congenic strain mice ($t = 9.6$; $df = 24$, $p < 0.0001$).

Using standard immunohistochemistry techniques we compared B6 and D2 mice for MPDZ cell staining in several critical brain regions involved with acute alcohol withdrawal. Figure 2-5 shows results from the substantia nigra, subthalamic nucleus, and medial globus pallidus in both B6 and D2 mice. MPDZ is present in all regions in B6 and D2 strains. B6 mice appear to have a higher abundance of MPDZ in all three regions (Figure 2-5 A, C, and E).

MPDZ and 5-HT_{2C} receptors interact *in vivo* (Becamel et al., 2001). We find co-localization of MPDZ with 5-HT_{2C} receptors in D2 mouse brain in brain regions we have identified as being critical for genetic differences in ethanol withdrawal severity (Buck et al, in preparation). These critical brain regions are part of an extended basal ganglia circuit which includes the substantia nigra pars reticulata, subthalamic nucleus, medial globus pallidus, and frontal cortical regions such as the cingulate and prelimbic cortices. Figure 2-6 shows co-localization of MPDZ with 5-HT_{2C} receptors in some of these brain regions in D2 strain mice including the substantia nigra, medical globus pallidus, and cingulate cortex. Co-localization of MPDZ with 5-HT_{2C} receptors is observed most prominently around the plasma membrane, indicating that MPDZ may act to

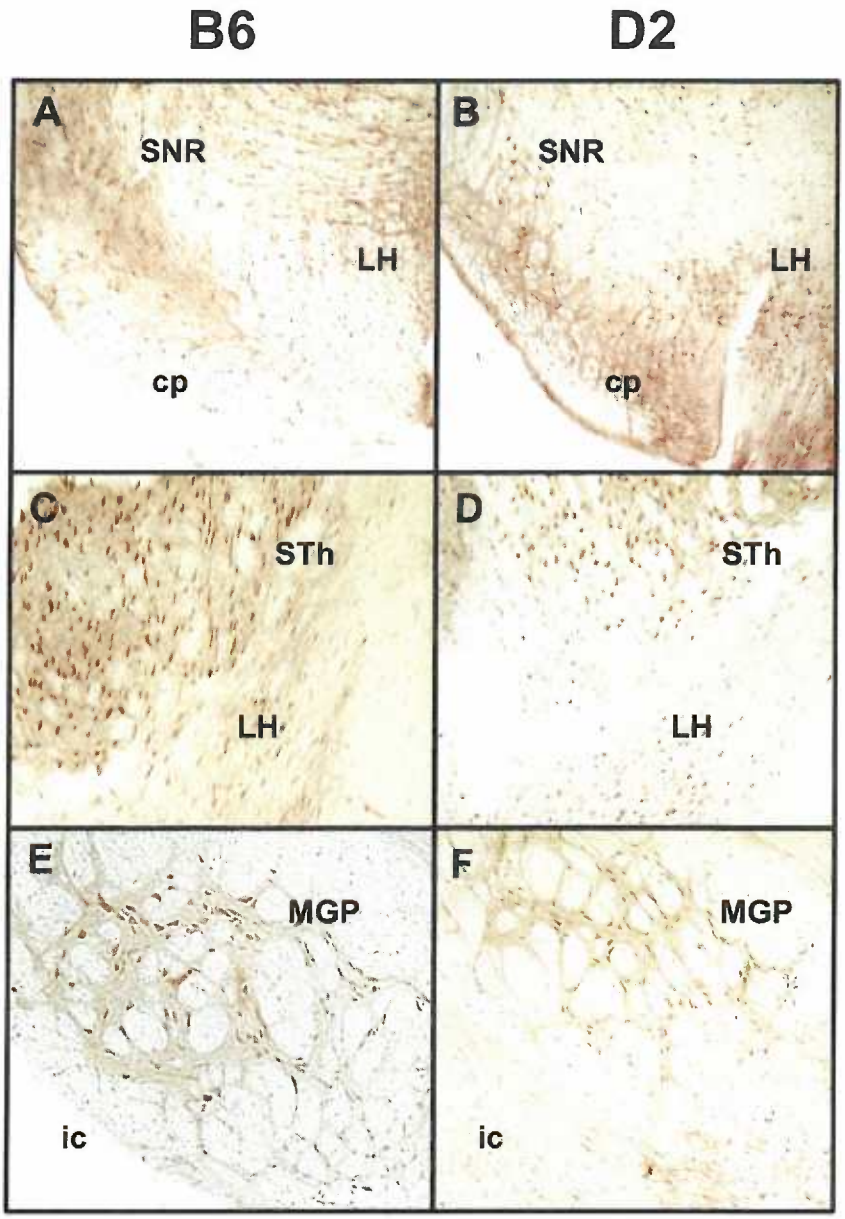

Figure 2-5: Brain localization of MPDZ by immunohistochemistry. Immunohistochemical staining for MPDZ positive cells was performed on 30 μm coronal brain sections from B6 and D2 inbred mouse strains. Figure **A**, **C**, and **D** represents sections from B6 mouse brain. Figures **B**, **D**, **F** represents sections from D2 mouse brain. Photomicrographs (10X) are representative examples from at least two independent experiments. Abbreviations: SNR (Substantia Nigra reticular part); cp (cerebral peduncle, basal part); LH (lateral hypothalamic area); STh (subthalamic nucleus); MGP (medial globus pallidus); ic (internal capsule).

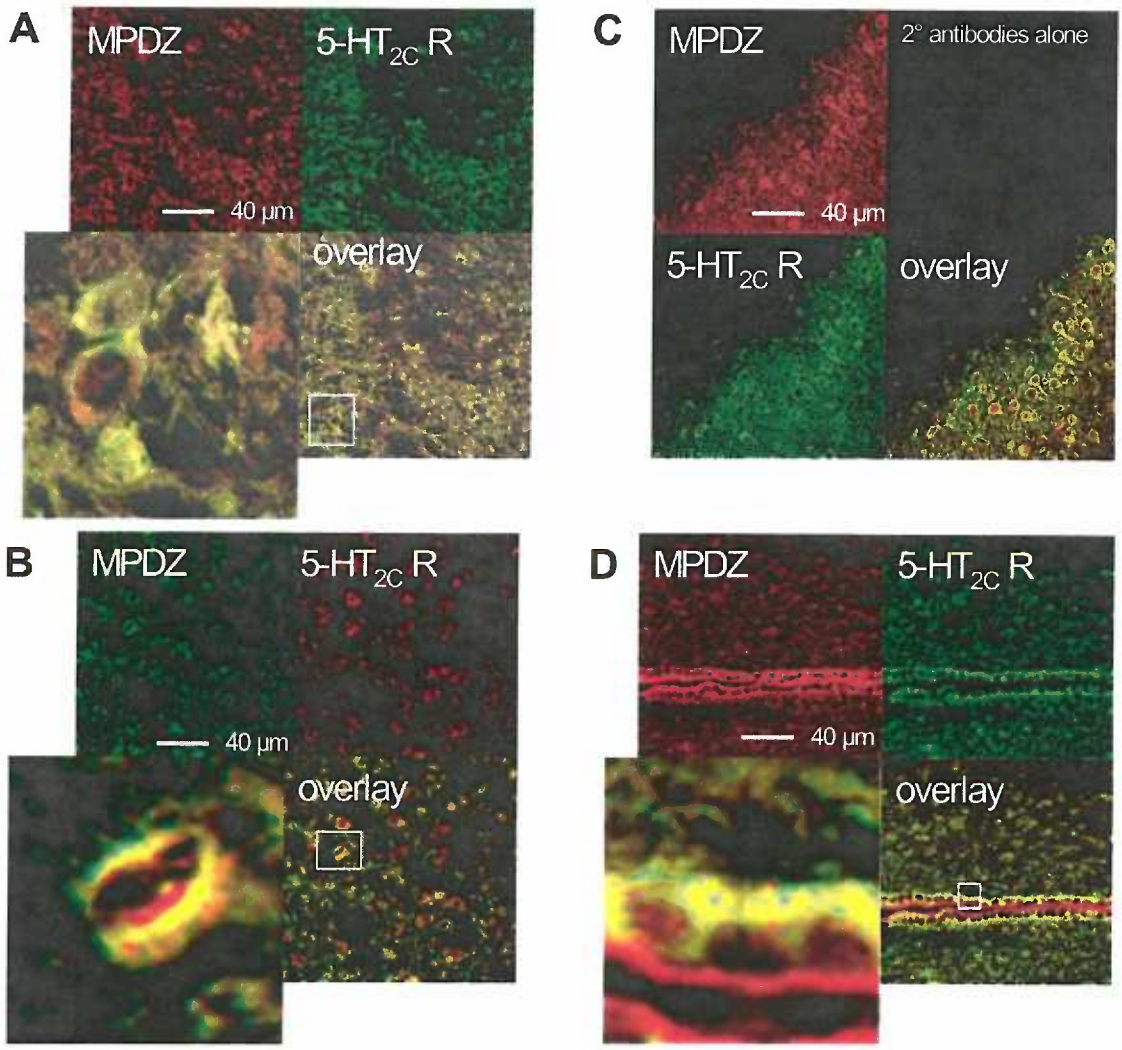

Figure 2-6: MPDZ and 5-HT_{2C} receptors are co-localized in D2 mouse brain regions implicated in genetic differences in ethanol withdrawal. Red staining shows MPDZ protein, while Green staining indicates 5-HT_{2C} receptor localization. Each image is a 0.5 µm optical confocal section obtained at 40X magnification. Co-localization is indicated in the overlay image, where the red signal image was overlayed on the green signal image and the appearance of yellow indicates where the two proteins are co-localized in the cell. Larger images in **A**, **B**, and **D**, are a magnification of the area highlighted by the white box in the overlay images. (**A**): Co-localization of MPDZ with 5-HT_{2C} receptor in mouse substantia nigra. (**B**): Co-localization of MPDZ with 5-HT_{2C} receptor in mouse medial globus pallidus. (**C**): Co-localization of MPDZ with 5-HT_{2C} receptor in mouse cingulate cortex. Black image shows a control experiment performed where tissue slices were incubated with both secondary antibodies alone to show the amount of non-specific staining (**D**): Co-localization of MPDZ with 5-HT_{2C} receptor in mouse choroid plexus. Each image is representative of two independent experiments.

stabilize or cluster 5-HT_{2C} receptors at the cell surface as previous studies suggest (Becamel et al., 2001). We also observed significant co-localization of MPDZ with 5-HT_{2C} receptors in the choroid plexus, a region that is highly enriched with 5-HT_{2C} receptors (Clemett et al., 2000). Figure 2-7 shows comparison of MPDZ and 5-HT_{2C} receptor co-localization in congenic versus D2 strain mice in the cingulate cortex. Congenic strain mice differ from D2 background strain in subcellular localization of MPDZ and 5-HT_{2C} receptors. Both MPDZ and 5-HT_{2C} receptors are expressed more closely to the plasma membrane in congenic mice, while in D2 mice MPDZ and 5-HT_{2C} receptors are more diffusely distributed throughout the neuron. This result suggests potential genetic differences in 5-HT_{2C} receptor trafficking.

We used semi-quantitative Western immunoblotting to examine overall protein abundance for these proteins. In whole brain homogenates, congenic mice show a nearly 1.8 fold greater abundance of MPDZ compared to D2 strain mice (Figure 2-8). Congenic strain mice do not differ from D2 background strain mice in abundance of the internal protein standard, GAPDH. Figure 2-8B shows that MPDZ abundance is significantly greater in congenic mice versus D2 strain mice ($t = 2.6$, $df = 8$, $p < 0.05$). We also performed semi-quantitative Western blot analysis on 5-HT_{2C} receptor protein abundance in congenic mice compared to D2 strain mice (Figure 2-9). There was no significant difference between congenic mice compared to D2 strain mice in 5-HT_{2C} receptor protein abundance.

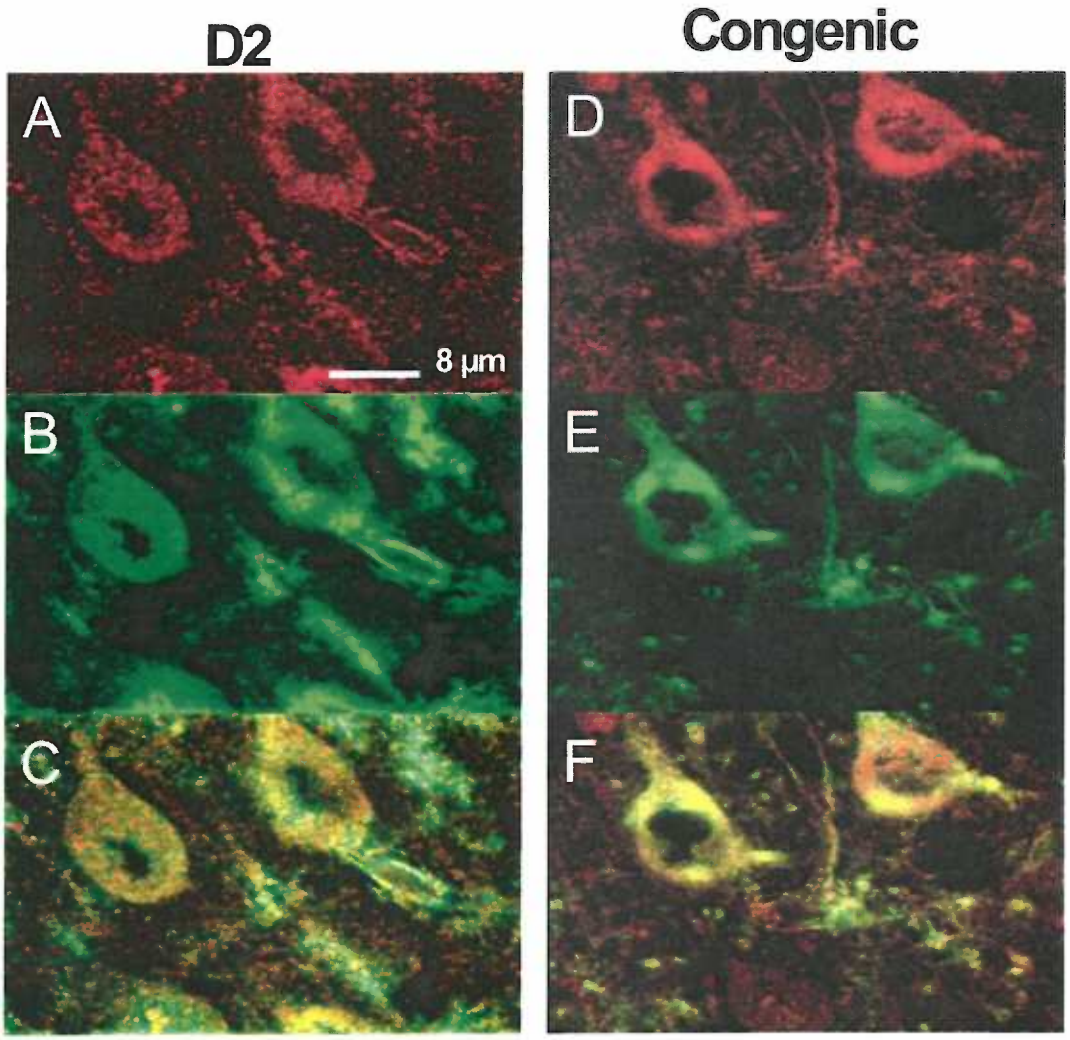

Figure 2-7: MPDZ and 5-HT_{2C} receptors are differentially co-localized in cingulate cortex and show different patterns of sub-cellular localization in congenic versus D2 background strain mice. Each image is a 0.5 µm optical confocal section obtained at 200X magnification. (**A**), (**B**), and (**C**): D2 strain mice, where (**A**) shows MPDZ immunolabeling with a red flurophore, (**B**): shows 5-HT_{2C} receptor immunolabeling with a green flurophore, and (**C**): shows the overaly of (**A**) and (**B**), where yellow indicates co-localization. (**D**), (**E**), and (**F**): congenic mice, where (D) shows MPDZ immunolabeling with a red flurophore, (**E**): shows 5-HT_{2C} receptor immunolabeling with a green flurophore, and (**F**): shows the overlay of (**D**) and (**E**), where yellow indicates co-localization.

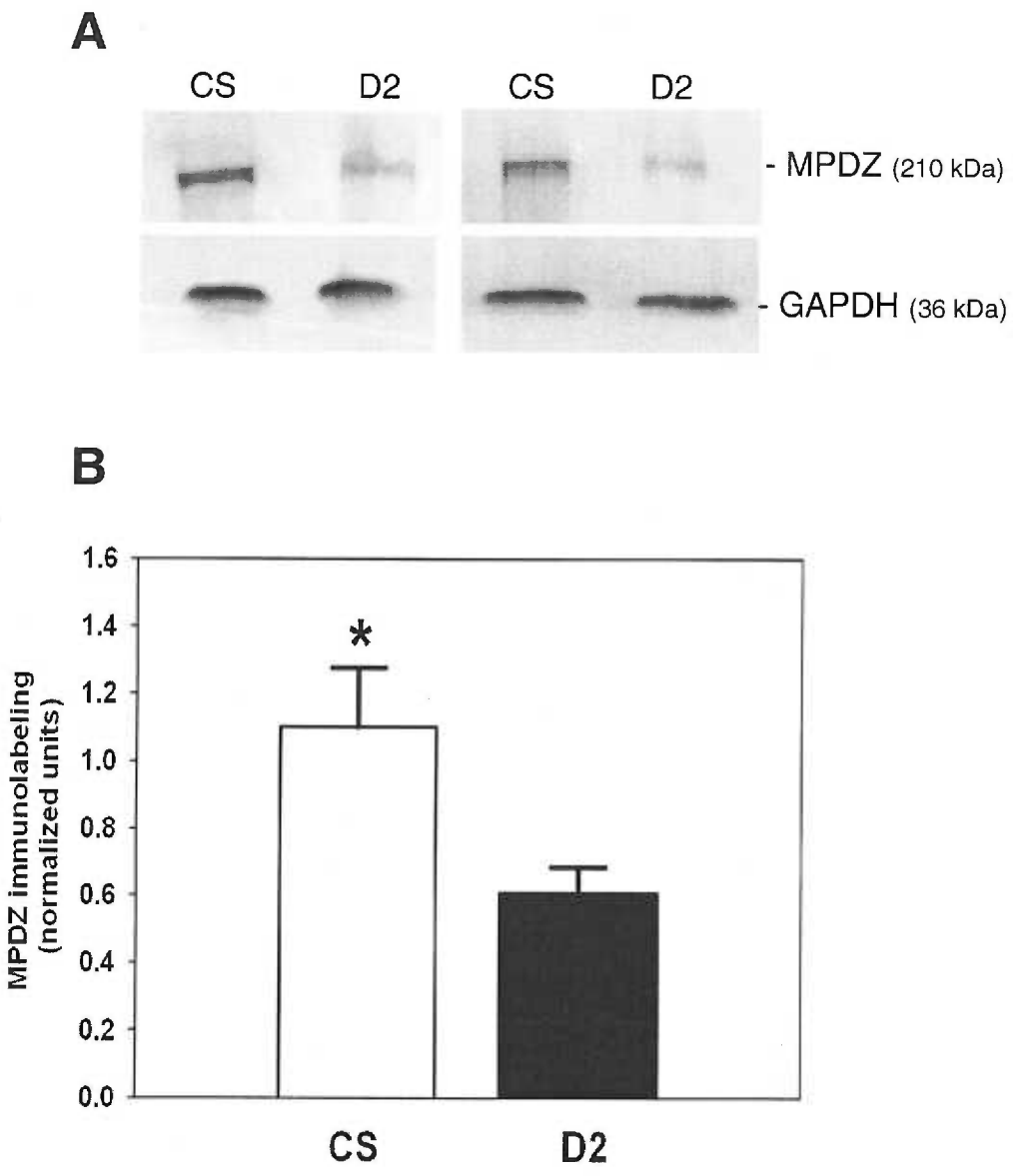

Figure 2-8: MPDZ protein abundance is higher in congenic (CS) versus D2 background strain mice. Whole brain homogenates from CS and D2 mice were separated by SDS-PAGE and then transferred to PVDF membrane for Western immunoblotting analysis. (**A**): Representative Western blot showing that a polyclonal sheep anti-human MPDZ primary antibody (Upstate Biotechnology, Lake Placid, NY) recognized a 210 kDa protein as expected for mouse MPDZ. Also shown is staining with a monoclonal GAPDH primary antibody (Chemicon, Temcula, CA) which recognized a 36 kDa protein as expected for mouse GAPDH. GAPDH was used as a protein loading control to normalize MPDZ protein staining for potential subtle differences in protein loading. (**B**): MPDZ immunolabeling expressed as a ratio between the MPDZ signal and the GAPDH signal. CS mice show nearly a 1.8 fold increase in MPDZ protein abundance compared to D2 mice (* $p < 0.05$). Bars represent the mean ± SEM for 5 mice per strain run in duplicate.

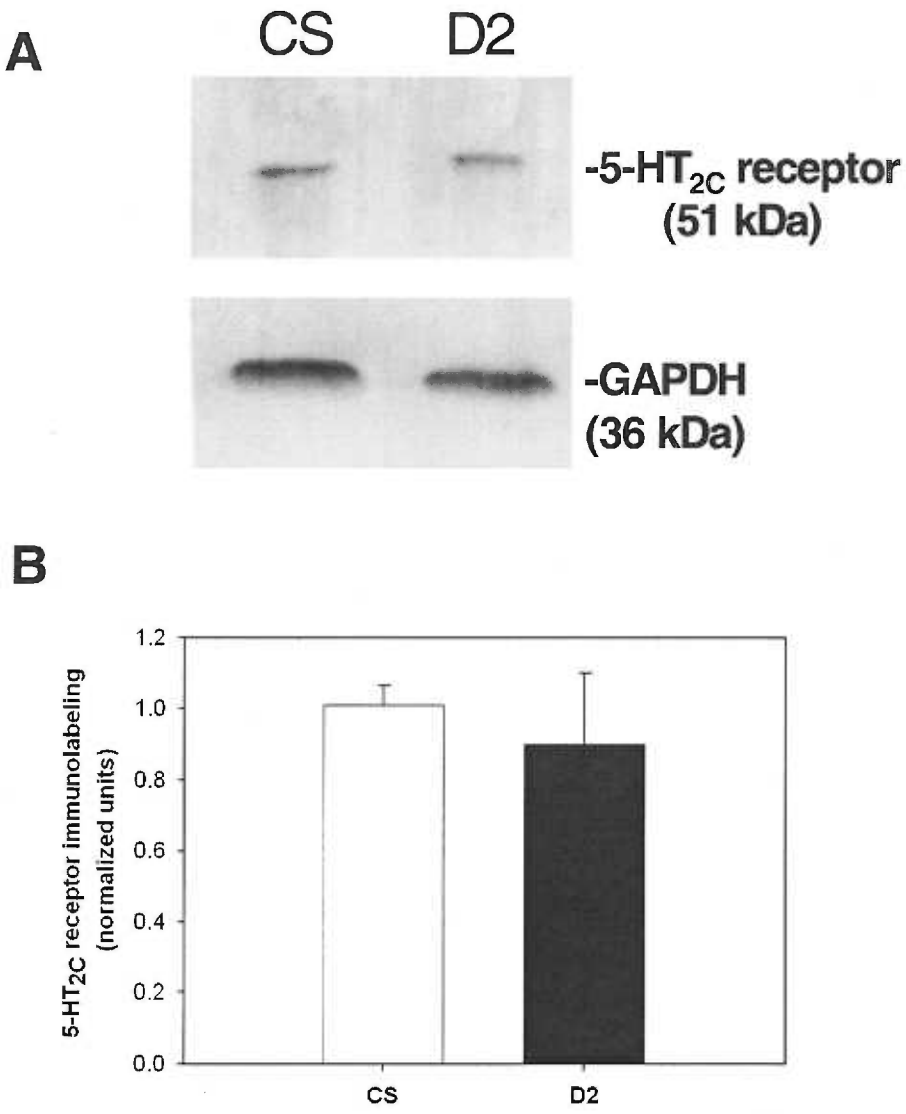

Figure 2-9: 5-HT_{2C} receptor protein abundance does not differ between congenic (CS) versus D2 background strain mice. Whole brain homogenates from CS and D2 mice were separated by SDS-PAGE and then transferred to PVDF membrane for Western immunoblotting analysis. (**A**): Representative Western blot showing that a polyclonal rabbit anti-human MPDZ primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) recognized a 51 kDa protein as expected for mouse the 5-HT_{2C} receptor. Also shown is staining with a monoclonal GAPDH primary antibody (Chemicon, Temcula, CA) which recognized a 36 kDa protein as expected for mouse GAPDH. GAPDH was used as a protein loading control to normalize MPDZ protein staining for potential subtle differences in protein loading. (**B**): 5-HT_{2C} receptor immunolabeling expressed as a ratio between the 5-HT_{2C} receptor signal and the GAPDH signal. Bars represent the mean ± SEM for 5 mice per strain run in duplicate.

5-HT_{2C} receptors possess a class I PDZ recognition motif (-X-S/T-X-V*) at the C-terminus of the protein (Becamel et al., 2001). We sequenced the entire coding region of the 5-HT_{2C} receptor in the B6 and D2 mouse strains. There were no differences in nucleotide sequence between B6 and D2 strains. Both B6 and D2 mice share identical amino acids in the PDZ recognition motif at the C-terminus (Figure 2-10).

DISCUSSION

The major findings of this investigation are that: (1) a 5-HT_{2C} receptor agonist and antagonist modulate ethanol withdrawal convulsions, (2) genetic variation in *Mpdz* influences modulation of ethanol withdrawal convulsions through 5-HT_{2C} receptors, and (3) MPDZ is localized and co-localized with 5-HT_{2C} receptors in critical brain regions associated with strain differences in ethanol withdrawal severity in our mouse populations. These conclusions collectively point to a novel mechanism by MPDZ regulates individual differences in ethanol withdrawal severity through its association with 5-HT_{2C} receptors. These are the first studies taking an established quantitative trait gene (QTG) underlying an addiction QTL to a functional mechanism.

An important insight that we have gained from these studies is that the function of the 5-HT_{2C} receptor is altered depending on the genetic variant of MPDZ to which it binds to. We show that both 5-HT_{2C} receptor agonist and antagonists responses are different in the congenic compared to D2 background strain mice. During ethanol withdrawal, handling-induced convulsions are significantly more reduced in the congenic than the D2 background strain after

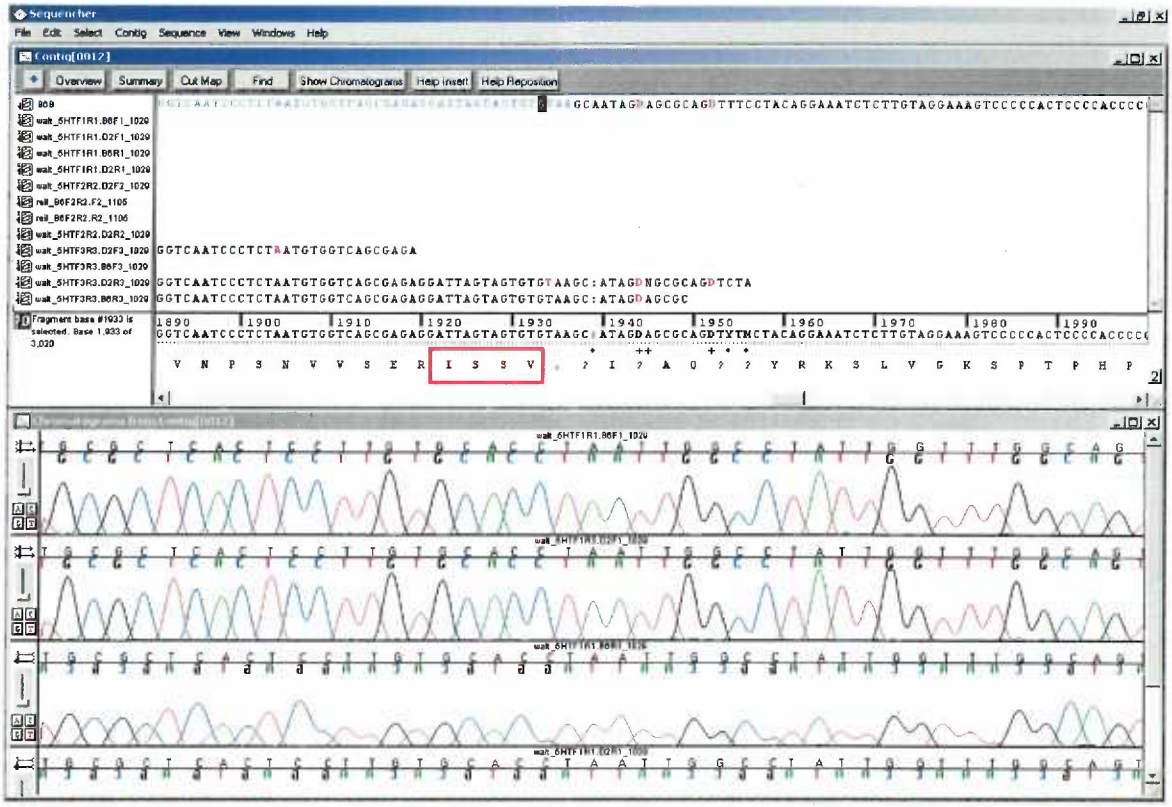

Figure 2-10: 5-HT_{2C} receptor nucleotide coding region sequence comparison between B6 and D2 mouse strains. The extreme carboxy-terminal region of the 5-HT_{2C} receptor is shown for B6 and D2 strains. There is no sequence difference in the PDZ recognition motif between B6 and D2 mice (enclosed in red box).

administration of a 5-HT_{2C} receptor mixed agonist (mCPP), whereas ethanol withdrawal convulsions are significantly more enhanced in the D2 background strain than in the congenic strain mice after administration of a highly selective 5-HT_{2C} receptor antagonist (SB-242084). It is interesting to note that the progenitor strains used to create the congenic, B6 and D2, differ in serotonergic tone in brain (Schlesinger, 1969). But this genetic difference in serotonergic tone between the progenitor strains is likely to contribute only a very small portion of the overall difference observed in responses to 5-HT_{2C} receptor agonist and antagonist because the congenic strain is >98% from the D2 background strain. Therefore, this supports the idea that allelic variation in *Mpdz*, and not overall differences in serotonergic tone, mediates the effects of 5-HT_{2C} receptor agents. Western blot analysis of 5-HT_{2C} receptor protein abundance also supports this idea because congenic strain mice do not differ from D2 background strain mice in protein abundance for this receptor.

Aside from the genetic implications of this work, we demonstrate that 5-HT_{2C} receptors regulate ethanol withdrawal convulsions, which has not been shown previously. However, 5-HT_{2C} receptors have been implicated in regulating susceptibility to a variety of seizure types including chemical seizures induced by flurothyl, seizures induced by electrical kindling of the olfactory bulb or seizures induced by electroshock to the cornea (Brennan et al., 1997; Applegate and Tecott, 1998). Null mutation of the mouse 5-HT_{2C} receptor produces an increase in all these types of seizures. Our results with 5-HT_{2C} receptor agonist and antagonist agree with the results obtained with 5-HT_{2C} receptor knockout mice

(Tecott et al., 1995) whereby antagonism of the 5-HT_{2C} receptor produces an exacerbation of ethanol withdrawal convulsions and agonism of 5-HT_{2C} receptors produces reduction of withdrawal convulsions. Previous studies also find that 5-HT_{2C} receptors regulate genetic susceptibility to high ethanol consumption (Pandey et al., 1996) as well as an involvement in some ethanol withdrawal symptoms such as anxiety (LeMarquand et al., 1994a, b). Our studies add to this work by showing that 5-HT_{2C} receptors have a role in ethanol withdrawal convulsions. That serotonin receptors can modulate alcohol withdrawal convulsions is a novel finding because of the more traditional view that alcohol withdrawal convulsions are mediated through GABAergic and glutamatgeric neurotransmission (Reilly et al., 2001).

Our results also suggest treatments for alcohol dependence via drugs that affect 5-HT_{2C} receptors. It is commonly observed that patients with alcohol dependence show comorbid depression (Cornelius et al., 2003). Previous studies show that 5-HT_{2C} receptors are responsible for some of the discriminative stimulus properties (Dekeyne and Millan, 2003) of serotonin specific reuptake inhibitors (SSRIs), and agents that work at 5-HT_{2C} receptors show efficacy as antidepressants in some models of depression (Dekeyne et al., 2000). The results of the present study suggest that the efficacy of antidepressants such as SSRIs and 5-HT_{2C} receptor agents in the treatment of alcohol dependence may be due to an alteration in alcohol withdrawal symptoms such as convulsions or tremor (Janiri et al., 1998). Previous studies also support this hypothesis; the

5-$HT_{2C/B}$ receptor agonist used in the present study (mCPP) is the active metabolite of trazodone (Rotzinger et al., 1998), an atypical antidepressant, which decreases craving for alcohol (Janiri et al., 1998) and reduces insomnia and ratings of depression in alcohol dependent patients (Le Bon et al., 2003). Furthermore, alcoholics show a blunted neuroendocrine response to mCPP challenges (George et al., 1997; Krystal et al., 1996) and activation of a basal ganglia circuit by mCPP is significantly reduced in alcoholics compared to controls (Hommer et al., 1997). These results suggest that a hyposensitivity of the serotonergic system is associated with alcohol dependence and our results support this idea because of the lower response to mCPP during severe withdrawal in D2 strain mice. Our results suggest that the serotonergic hyposensitivity observed in alcoholics may result from genetic variation in *Mpdz.* Future studies will be needed to address this hypothesis.

A previous study showed that *Mpdz* mRNA is found with high abundance in cerebral cortical layers, the hippocampus, the granular layer of the dentate gyrus, and the choroid plexus of the rat (Becamel et al., 2001). Our immunohistochemical analysis shows that MPDZ protein is localized in critical brain regions associated with strain differences in alcohol withdrawal between B6 and D2 mouse brain. These regions include, the substantia nigra, subthalamic nucleus, lateral hypothalamus, and medial globus pallidus. However, MPDZ protein is also localized in other brain regions not implicated in genetic differences in acute alcohol withdrawal severity (e.g., hippocampus, amygdala). Our results with a commercially available antibody for MPDZ are in good

agreement with one report of MPDZ protein localization in B6 mouse brain using a different antibody (Sitek et al., 2003). Collectively, these results confirm the ubiquitous brain expression profile of both mRNA and protein for MPDZ. The results of the present study also suggest that B6 and D2 strain mice may differ in MPDZ protein abundance in these regions.

Previous *in situ* and immunohistochemical studies also show ubiquitous expression of 5-HT_{2C} receptors in the mammalian brain. The region with the highest 5-HT_{2C} receptor mRNA expression in all studies is the choroid plexus followed by regions of the cortex, hippocampus, extended amygdala, ventral striatum, and regions of the basal ganglia such as the substantia nigra (Pasqualetti et al., 1999; Lopez-Gimenez et al., 2001). Several studies have used 5-HT_{2C} specific antibodies to study brain regional localization of 5-HT_{2C} receptor protein. Similar to *in situ* hybridization studies, immunohistochemical analysis shows high levels of 5-HT_{2C} receptor protein in choroid plexus, cortical regions (piriform and cingulate), septum, hypothalamus, striatum and substantia nigra (Clemett et al., 2000; Abramowski et al., 1995; Sharma et al., 1997). Taken together with results from *Mpdz* localization studies, MPDZ and 5-HT_{2C} receptors appear to be co-distributed in the same brain regions (see table 2-1).

Using expression of the immediate early gene *cfos,* we have mapped the brain circuits associated with genetic differences in acute ethanol withdrawal severity in our mouse populations (Buck et al., in preparation). These results suggest that an extended basal ganglia circuit is significantly more activated in the D2 strain, which displays severe ethanol withdrawal severity. Brain regions

REGION	*Mpdz* mRNA	MPDZ protein	5-HT_{2C} mRNA	5-HT_{2C} protein	Protein Co-localization
Cortex	X	X	X	X	X
Hipp	X	X	X	X	nd
Chor. Pl.	X	X	X	X	X
SN	nd	X	X	X	X
STh	nd	X	X	X	X
LH	nd	X	X	X	nd
MGP	nd	X	X	X	X
AMG	nd	X	X	X	X
Septum	nd	X	X	X	X
Hypoth	nd	X	X	X	nd
Striatum	nd	X	X	X	nd

Table 2-1: Selected brain distribution pattern for *Mpdz* mRNA and protein and 5-HT_{2C} receptor mRNA and protein. Co-localization of MPDZ and 5-HT_{2C} receptors is also shown. An x in a row indicates that mRNA or protein has been localized in that region, nd (not determined). ***Abbreviations:*** Hipp (hippocampus), Chor. Pl. (Choroid Plexus); SN (substantia nigra); STh (subthalamic nucleus); LH (lateral hypothalamus); MGP (medial globus pallidus); AMG (amygdala); Hypoth (hypothalamus).

which show significant FOS expression include: substantia nigra pars reticulata, subthalamic nucleus, medial golbus pallidus, ventral pallidum, and cingulate cortex. If the association between MPDZ and 5-HT_{2C} receptors influences genetic differences in ethanol withdrawal severity, they should show brain-regional co-localization in critical circuits that underlie withdrawal severity. In the present study, we show that MPDZ and 5-HT_{2C} receptors are co-localized in the substantia nigra, medial globus pallidus, and cingulate cortex, regions that are within the extended basal ganglia circuit. We also find significant co-localization of MPDZ with 5-HT_{2C} receptors in the choroid plexus, as expected from previous studies that find a high abundance of 5-HT_{2C} receptors in this region (Clemett et al., 2000). However, the choroid plexus is not a region implicated in the extended basal ganglia circuit. We also found co-localization in other regions of the brain such as the amygdala. These results suggest that the association between MPDZ and 5-HT_{2C} receptors may not be exclusive to regulation of alcohol withdrawal convulsions. Co-localization of MPDZ with 5-HT_{2C} receptors might mediate other phenotypes supporting the already prescribed role of *Mpdz* as a pleiotropic gene (Fehr et al., 2004). Our results extend the work of Sitek et al., (2003) by providing direct evidence that MPDZ and 5-HT_{2C} receptors are co-localized in the same cell. The results of Sitek et al. (2003) only suggested that MPDZ and 5-HT_{2C} receptors might be localized in the same brain regions, but did not measure 5-HT_{2C} receptor expression in their study. Here, using confocal microscopy immunofluroscence, we show specific co-localization of MPDZ and 5-HT_{2C} receptors within the same neuron.

The basal ganglia is a group of subcortical brain regions which receives a substantial amount of serotonergic innervation from the dorsal raphe (Feldman, 1997). Therefore, it is not surprising that we find an abundance of co-localization between MPDZ and 5-HT_{2C} receptors in these regions. In fact, a previous study demonstrated that 5-HT_{2C} receptors are the most prominent serotonin receptor subtype in the basal ganglia (Wolf and Schutz, 1997). The extended basal ganglia circuit is classically implicated in the control of motor behavior (Hauber, 1998) and seizures (Deransart et al., 1998). Activation of 5-HT_{2C} receptors in the substantia nigra reduces incidence of limbic motor seizures evoked from area tempestas (Pasini et al., 1996) demonstrating that 5-HT_{2C} receptors specifically regulate seizures within the basal ganglia. Our behavioral studies with 5-HT_{2C} receptor agents coupled with results from our co-localization studies offer a new mechanism in which the association of 5-HT_{2C} receptors with genetic variants of MPDZ in the basal ganglia, regulates the severity of convulsions during ethanol withdrawal.

One of the first functional MPDZ studies, showed that it affects clustering of 5-HT_{2C} receptors at specific cellular and subcellular localizations *in vitro* (Becamel et al., 2001). That study suggested a role for MPDZ in 5-HT_{2C} receptor trafficking, which is a hallmark function of other PDZ domain containing proteins (Sheng and Sala, 2001). When we compared our congenic strain to the D2 background strain, we found a different pattern of subcellular localization of MPDZ and 5-HT_{2C} receptors in neurons from the cingulate cortex. In congenic mice, MPDZ and 5-HT_{2C} receptors are more localized to the plasma membrane,

whereas in D2 background strain mice we find that the two proteins have a diffuse distribution throughout the cell. These results suggest potential differences in 5-HT_{2C} receptor trafficking between congenic mice versus D2 background strain mice, and implicate genetic variation in *Mpdz* as a factor which affects this trafficking. This finding could indicate that in congenic strain mice, 5-HT_{2C} receptors are more efficiently trafficked to the cell membrane compared to D2 mice as a result of association with different allelic variants of MPDZ. Because the MPDZ variant in congenic mice produces less severe alcohol withdrawal convulsions and because activation of 5-HT_{2C} receptors reduces withdrawal severity, having an *Mpdz* variant which enables more efficient trafficking of 5-HT_{2C} receptors would produce, in effect, more functional 5-HT_{2C} receptors to be activated by synaptic 5-HT. Therefore, genetic differences in the efficiency of 5-HT_{2C} receptor trafficking by MPDZ is one likely mechanism affecting the intensity of alcohol withdrawal in congenic versus D2 strain mice. This hypothesis awaits further confirmation with additional studies using biotinylation assays for detection of plasma membrane expression of 5-HT_{2C} receptors in congenic versus D2 background strain mice.

Additional mechanisms which mediate differences in alcohol withdrawal severity are also possible. Because the 5-HT_{2C} receptor and MPDZ interaction is dynamically regulated by receptor phosphorlaytion within the carboxy terminal PDZ recognition motif (Parker et al., 2003), it is quite possible that differences in phosphorlaytion of the 5-HT_{2C} receptor between congenic and D2 strain mice may have an influence on alcohol withdrawal severity. Deletion of the carboxy-

terminus PDZ recognition motif of 5-HT_{2C} receptors delays resensitization of receptor responses (Backstrom et al., 2000) and therefore congenic versus D2 background strain mice could also differ in resensitization state of the receptor by virtue of differences in interaction with MPDZ. Our sequence results show that B6 and D2 mice do not differ in amino acid sequence for the 5-HT_{2C} receptor. Furthermore, there are no polymorphisms between B6 and D2 mouse strains in PDZ domain 10 of *Mpdz* (Fehr et al., 2002), which is the domain that interacts with the carboxy-terminus of the 5-HT_{2C} receptor (Becamel et al., 2001) Parker et al., 2003). This suggests that congenic and D2 strain mice potentially have similar sequence specific interactions between MPDZ and 5-HT_{2C} receptors. However, it is important to keep in mind that this interpretation does not take into account the way protein folding affects this interaction. Because MPDZ contains ten amino acid differences between B6 and D2 mice (Fehr et al., 2002), it can be hypothesized that any one of these changes could potentially affect the sequence specific interaction between MPDZ and 5-HT_{2C} receptors due to the way protein folding effects the interaction. Finally, for some PDZ domain containing proteins such as PSD-95, palmitoylation regulates synaptic strength and activity dependent plasticity (El-Husseini et al., 2000). This is another potential mechanism that could affect genetic differences in alcohol withdrawal severity between congenic and D2 background strain mice if MPDZ is differentially palmitoylated.

A caveat of the present studies is the specificity of the serotonergic drugs used in the behavioral experiments. mCPP is generally considered a non-selective 5-HT receptor drug. However, mCPP has the highest affinity for the 5-HT_{2C} receptor subtype, where it is a partial agonist (Sanders-Bush and Breeding, 1990). It also binds to 5-HT_3 and 5-HT_{2A} receptors where it acts as an antagonist. In addition, mCPP binds to 5-HT_{1B}, 5-HT_7 and 5-HT_6 receptor subtypes, but with an affinity more than an order of magnitude lower than its affinity for the 5-HT_{2C} receptor. (Hoyer, 1988). Despite its nonspecific pharmacology, many of its behavioral effects have been demonstrated to be specific to mCPP's activation of 5-HT_{2C} receptors through blockade of behavioral responses by more selective 5-HT_{2C} receptor antagonists (Lucki et al., 1989; Kennett et al., 1989, 1994, 1997). On the other hand, SB-242084 is defined as one of the first 'selective' 5-HT_{2C} receptor antagonists, demonstrating nearly 100 to 150-fold selectivity over the 5-HT_{2A} and 5-HT_{2B} receptor subtypes as well as a 100-fold selectivity over a range of other 5-HT, dopamine, and adrenergic receptors (Kennett et al., 1997). Our results with SB-242084 on withdrawal convulsions are consistent with the seizure phenotype of mice with genetic knockout of the 5-HT_{2C} receptor (Tecott et al., 1995). Null mutant 5-HT_{2C} receptor mice show an increase in seizure susceptiblity induced by chemical, electrical and sound stimuli (Brennan et al., 1997; Applegate and Tecott, 1998). This indicates that SB-242084 is likely to be targeting the 5-HT_{2C} receptor. Because of the greater selectivity of SB-242084 for 5-HT_{2C} receptors and the consistency of the results between mCPP and SB-242084 on withdrawal convulsions, this

argues that these drugs, at the doses tested, are within a range that is selective for 5-HT_{2C} receptors. In addition, we tested ethanol-naïve D2 and congenic strain mice with several doses of mCPP (1, 7, and 21 mg/kg) and measured handling convulsions. Low doses similar to the ones used in the present study reduced handling-induced convulsions as expected for activation of 5-HT_{2C} receptors, while the higher dose (21 mg/kg mCPP) exacerbated handling convulsions indicating that the higher dose probably activates other receptor subtypes (unpublished data). Taken together, this argues that our results with 5-HT receptor agonist and antagonist are specific to the 5-HT_{2C} receptor subtype.

Although our behavioral, sequence and expression analyses supports a mechanism whereby genetic variation in *Mpdz* affects strain differences in alcohol withdrawal severity via MPDZ interaction with 5-HT_{2C} receptors, there are quite possibility other mechanisms through associated protein networks that are important. Because *Mpdz* is known to have pleiotropic effects on convulsion severity (see Chapter III), and because *Mpdz* has the capability to interact and regulate the function of a number of neurotransmitter receptors, this suggest that multiple neurochemical systems might converge on *Mpdz* to ultimately regulate genetic differences in withdrawal severity. It should also be noted that the 5-HT_2 receptor drugs used in the behavioral studies have effects on basal handling convulsions (see Chapter III), indicating that these drugs may not be specific to alcohol withdrawal convulsions *per se*.

In summary, we identify a novel mechanism whereby genetic variation in MPDZ affects strain differences in alcohol withdrawal severity via 5-HT_{2C}

receptors. These results have direct implication for genetic studies in human alcoholics and suggest new treatment strategies for alcohol dependence. Future studies should be directed at examining how genetic variation in *Mpdz* affects receptor trafficking and examining the influence of genetic variation in *Mpdz* on other neurotransmitter receptor systems.

Chapter III

The Multiple PDZ Domain Protein regulates genetic differences in handling convulsions modulated through specific neurotransmitter receptors

Abstract

Converging evidence implicates *Mpdz*, which encodes the multiple PDZ protein (MPDZ), in regulating alcohol and pentobarbital withdrawal convulsions and other seizure phenotypes. Previous work showed that *Mpdz* genotype is genetically correlated with convulsions affecting glutamatergic and GABAergic neurotransmission. Here, we further define the role of genetic variation in *Mpdz* by comparing pharmacological modulation of handling-induced convulsions in a novel congenic strain that possesses a unique genetic variant of *Mpdz* (derived from the donor C57BL/6J or B6 strain) versus background DBA/2J (D2) strain mice. We also test this congenic for convulsions during withdrawal after chronic alcohol exposure. First, we confirm that congenic strain mice have significantly less severe acute alcohol withdrawal convulsions compared to background strain mice. Similarly, we find that congenic strain mice show a slight but significant reduction in convulsion severity compared to D2 background strain mice after chronic alcohol exposure. After i.p. administration of 1.0 mg/kg strychnine, a glycine receptor antagonist, congenic strain mice show significantly less severe handling-induced convulsions than background strain mice. Two doses (30 and 60 mg/kg) of NMDA, a glutamate-NMDA receptor agonist, produce significantly less severe convulsions in congenic than background strain mice. SB-242084

(40 mg/kg), a 5-HT_{2C} receptor antagonist, produces less severe convulsions in donor strain (B6) vs. background strain (D2) mice. However, after administration of either of two doses of PTZ (15 and 30 mg/kg), a $GABA_A$ receptor specific chloride channel blocker, congenic mice show similar convulsions compared to background strain mice. We conclude that *Mpdz* modulates alcohol withdrawal convulsion severity after acute and chronic exposure as well as convulsions modulated by some, but not all, chemiconvulsants. These results implicate *Mpdz* as a pleiotropic gene for specific handling-induced convulsion types and suggest novel molecular pathways whereby genetic variation in *Mpdz* regulates these convulsions.

INTRODUCTION

An emerging theme in neuroscience and genetic research is that genes and their protein products do not function in isolation but instead are linked in functional pathways, circuits and networks (Loomis and Sternberg, 1995). Within the last decade a field of functional proteomics has begun to evolve and at one node of this new field lies a class of proteins whose function is to link neurotransmitter receptors to signaling molecules within the neuron (Chung et al., 2002). The most common class of these proteins contain modular protein-protein interaction domains termed PDZ domains and interact in a sequence specific manner to carboxy-terminal regions of various proteins (Sheng and Sala, 2001). PDZ domain proteins attribute their name to these prototypical proteins of this class (i.e., PSD-95, Disc-Large, and ZO-1). These PDZ domain proteins are situated to efficiently funnel signals from plasma membrane receptors through

the cell and are associated with transporting receptor proteins to proper sites of action at the synapse (Sheng and Sala, 2001).

Complex neuropsychiatric diseases such as alcoholism are influenced by multiple genetic and environment factors. Teasing apart the genetic components for alcoholism has been an arduous task because of the heterogeneous nature of this disease in humans (Schuckit, 1994). However, recent advances in quantitative trait locus (QTL) mapping has proven an effective tool for dissecting genetic components for specific alcohol related behaviors in rodent models (Crabbe et al., 1999b). Using this technique we previously mapped the position of a QTL containing a gene with alleles affecting alcohol withdrawal severity on mouse chromosome 4 to a 1.5 Mb region (Fehr et al., 2002; Shirley et al., 2004). We have convergent evidence from sequence, expression and fine mapping analysis that *Mpdz* which encodes the multiple PDZ protein (MPDZ) is the actual quantitative trait gene (QTG) accounting for approximately 26% of the genetic variance in alcohol withdrawal severity in our mouse populations {Shirley, et al., 2004 }. This is the first report of a QTG that underlies a QTL and is a major step forward in the field.

In addition to *Mpdz*'s influence on acute alcohol withdrawal convulsions, it also contributes to convulsions induced during barbiturate withdrawal (Fehr et al., 2002). Subsequent to this report we also found that *Mpdz* is genetically associated with chemically induced seizures which affect glutamatergic and GABAergic neurotransmission (Fehr et al., 2004). Mid chromosome 4 in the region containing *Mpdz*, also harbors a gene for convulsions during withdrawal

from chronic alcohol exposure (Buck et al., 2002)(Bergeson et al., 2003). These data along with our findings with alcohol and barbiturate withdrawal convulsions point to potential pleiotropic effects of *Mpdz* on vulnerability to seizures. If true for *Mpdz*, rational drug design could be used to target such an important intermediate for a host of neurological disorders such as epilepsy and alcohol dependence.

Various neurotransmitter receptors have been shown to modulate convulsions. Null mutation of the 5-HT_{2C} receptor gene results in generalized susceptibility to a variety of seizures (Tecott et al., 1995; Applegate and Tecott, 1998). NMDA glutamate receptors are associated with ethanol withdrawal seizures as well as other non-drug induced seizures such as epilepsy (Hoffman and Tabakoff, 1991; Krystal et al., 2003b; Dingledine et al., 1990; Chapman, 1998). The glycine receptor is the major mediator of inhibitory neurotransmission in the brain stem and spinal chord (Laube et al., 2002). Antagonism of the glycine receptor with strychnine causes seizures in mammals (Knopman, 1975). Alterations, GABAergic neurotransmission are a well known mechanism for the generation of seizures (Gale, 1992). In particular $GABA_A$ receptor specific chloride channel blockers such as penetlyenetetrazol (PTZ) cause convulsions in a number of experimental animals (Kliouéva et al., 2001); (Ferraro et al., 1999).

The handling-induced convulsion is a sensitive measure of central nervous system (CNS) hyperexcitablity (Goldstein DB, 1973; Akers and Belknap, 1988; Crabbe et al., 1991; 1993). Handling-induced convulsions, in contrast to other convulsions (i.e., NMDA convulsions elicited by i.v. administration of the

drug) are relatively mild and non-lethal. The doses of proconvulsant drugs required to elicit the handling-induced convulsion are generally well below those needed to elicit other more severe types of convulsions. The handling-induced convulsion is also sensitive enough to detect genetic differences in CNS excitability, making it an ideal measure for determining genetic differences in sensitivity to drug responses (Crabbe et al., 1980).

In the present study, we investigate the role of *Mpdz* on handling-induced convulsions after administration of several pro-convulsant drugs and measure convulsion severity after acute and chronic alcohol exposure in a novel congenic strain possessing a unique genetic variant of *Mpdz* derived from a C57BL/6J (B6) inbred donor strain. Comparing the congenic strain to the DBA/2J (D2) inbred background strain offers a way of eliminating genetic noise from other loci in the mouse genome. Because *Mpdz* is contained in the B6 donor region, differences between the congenic and D2 background strain can be associated with genetic variation in this gene. We find that *Mpdz* mediates, in part, genetic variation in intensity of convulsions following chronic alcohol withdrawal exposure. We provide evidence that *Mpdz* also affects chemically modulated convulsions from some, but not all, convulsant drugs that have specific mechanisms of action.

MATERIALS AND METHODS

Animals. Male and female chromosome 4 congenic (CS) strain mice were bred at the Portland VA Medical center in the Portland Alcohol Research Center Animal Production core. A separate group of male and female D2 background strain mice were also bred in parallel to the CS mice. A separate cohort of B6

and D2 mice from both genders were purchased from Jackson Laboratories (Bar Harbor, ME). All experiments consisted of approximately equal numbers of mice from both genders. Food (Purina lab chow) and water were freely available at all times. Mice were housed in groups of four in wire-mesh cages with corn cobb bedding. Procedure and colony rooms were kept at a temperature of 21 ^{0}C. All mice were between 60-100 days old at time of testing. All procedures with mice were conducted in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals*, and were approved by the Portland VA Institutional Animal Care and Use Committee .

Drugs. Ethanol was purchased from Aaper Co. (Shelbyville, KY) and diluted (20% v/v) in 0.9% saline. All convulsant drugs were purchased from Sigma chemical company (St. Louis, MO). Pentylenetetrazol (PTZ), strychnine, and *N*-methyl-D-aspartate acid (NMDA) were dissolved in 0.9% saline. SB-242084 was dissolved in 8% β-cyclodextrin and 25 mM citric acid. All drugs were administered by i.p. injection. Doses for PTZ (15 and 30 mg/kg), NMDA (30 and 60 mg/kg) and strychnine (0.5 and 1.0 mg/kg) were chosen based on previous published work in mice (Crabbe et al., 1991). The dose of SB-242084 (40 mg/kg) was also chosen based on previous work (Kennett et al., 1997).

Ethanol physical dependence induction and convulsion severity assessment by handling-induced convulsion (HIC)

Chromosome 4 congenic and D2 background strain mice were made physically dependent on ethanol by chronic ethanol inhalation for 3 days as described in detail elsewhere (Terdal and Crabbe, 1994; Reilly and Buck, 2000).

One group of mice was exposed to ethanol vapor in an inhalation chamber for 3 days after an initial loading dose of ethanol (1.5 g/kg, i.p.) on the first day and injected daily with pyrazole, an alcohol dehydrogenase inhibitor, used to maintain stable blood ethanol concentration (BEC) and reduce mortality. The control group was exposed to air for 3 days and injected daily with pyrazole. On day three of chronic ethanol exposure all mice were removed from the inhalation chambers, and a 20 μl tail blood sample was collected from all ethanol dependent mice for determination of blood ethanol concentration (BEC) using a gas chromatographic assay (Terdal and Crabbe, 1994).

The handling-induced convulsion (HIC) was used to index convulsion severity following administration of drugs as previously described (Kosobud and Crabbe, 1986; Reilly et al., 2000; Goldstein and Pal, 1971). Briefly, this procedure involves lifting the mouse by the tail, gently spinning it 180 degrees if necessary and rating convulsion severity based on a 7 point scale. A score of zero indicates no convulsion after tail lift and spin. HIC scores ranging from 1to 3 require the gentle spin to elicit a tonic or clonic convulsion, whereas convulsions elicited by merely lifting the mouse by the tail are scored as 4-6. A score of 7 indicates a spontaneous convulsion prior to tail lift. Chronic alcohol withdrawal HIC was measured immediately after removal of mice from the inhalation chambers on day 3 of exposure. Then HIC was taken at hourly intervals through hour 10, and then again at hours 24 and 25. For acute drug treatments, baseline (pre-drug) HIC was measured twice at about 30 minutes prior to i.p. injection of drug. Ethanol withdrawal HIC scores were measured beginning at hr 2 post-

injection and then hourly through hr 12. NMDA, PTZ and strychnine HIC were measured at various time intervals based on previous work (Crabbe et al., 1991). Following injection of NMDA or PTZ, HIC measurements were taken at 1, 3, 5, 8, 12, 15, 20, 30, 40, 50, and 60 minutes. HIC for strychnine were taken at 1,3, 5, 8,12, 15, and 20 minutes after injection. HIC for SB-242084 were taken at hourly intervals beginning at hr 2 post-injection through hour 12 and then again at hours 24 and 25. Area under the curve (AUC) was calculated as an overall measure of convulsion severity by summing the HIC scores beginning with time points in which average drug-treated scores exceeded baseline HIC scores. There were no genotype-dependent differences in baseline HIC, so AUC is shown as raw values uncorrected for baseline HIC scores.

Statistical Analysis. For the chronic alcohol withdrawal experiment, area under the 25 hr withdrawal curve (AUC25) was computed. This is an overall measure of withdrawal severity and is calculated by summing the HIC scores beginning with those scores above hour 0. ANOVA was used to determine the effects of strain and gender on AUC25. ANOVA was also used to determine the effects of strain and gender on BEC on day 3. Because there were significant effects of gender determined by ANOVA, gender was analyzed separately. There were also significant effects of BEC and therefore a separate statistic was calculated where AUC25 scores were regressed on BEC and residual scores were used for analysis. ANOVA was used to determine the effects of strain and gender on AUC25 in air-pyrazole control animals. For the acute alcohol withdrawal experiment, AUC was calculated for the 12 hour withdrawal curve, by

summing HIC scores beginning with those that were above average baseline scores. A separate analysis using a t-test indicated no genotype-dependent difference in baseline HIC score. T-test was used to compared the effects of strain AUC scores. For experiments examining convulsion severity after acute drug treatment, ANOVA was used to determine the effects of strain, gender and dose of drug on AUC scores, which were computed as described above. Again, no baseline differences in HIC scores were observed. Post-hoc Tukey's test was used were appropriate significance level was set at alpha = 0.05.

RESULTS

Figure 3-1A shows the time course of acute ethanol withdrawal HIC after 4 g/kg ethanol in congenic and D2 background strain mice. Figure 3-1B shows area under the curves from figure 3-1A. Congenic strain mice show a significant reduction in ethanol withdrawal severity compared to D2 background strain mice ($t = 2.3$, $df = 24$, $p < 0.05$)(Figure 3-1B).

Two independent studies detected QTL on mid chromosome 4 that affect chronic ethanol withdrawal severity (Buck et al., 2002; Bergeson et al., 2003). We therefore compared chromosome 4 congenic strain and background (D2) strain mice for chronic ethanol withdrawal severity. Figure 3-2 shows results from this study where congenic and D2 background strain mice were exposed to ethanol vapor for 3 days and then scored for HIC for a period up to 25 hours post-ethanol. Results form ANOVA indicated a significant main effect of gender [$F(1, 399) = 48.5$, $p < 0.001$] and a significant interaction between gender and strain on BEC data [$F(1,399) = 4.5$, $p < 0.05$. Post-hoc analysis indicated that D2

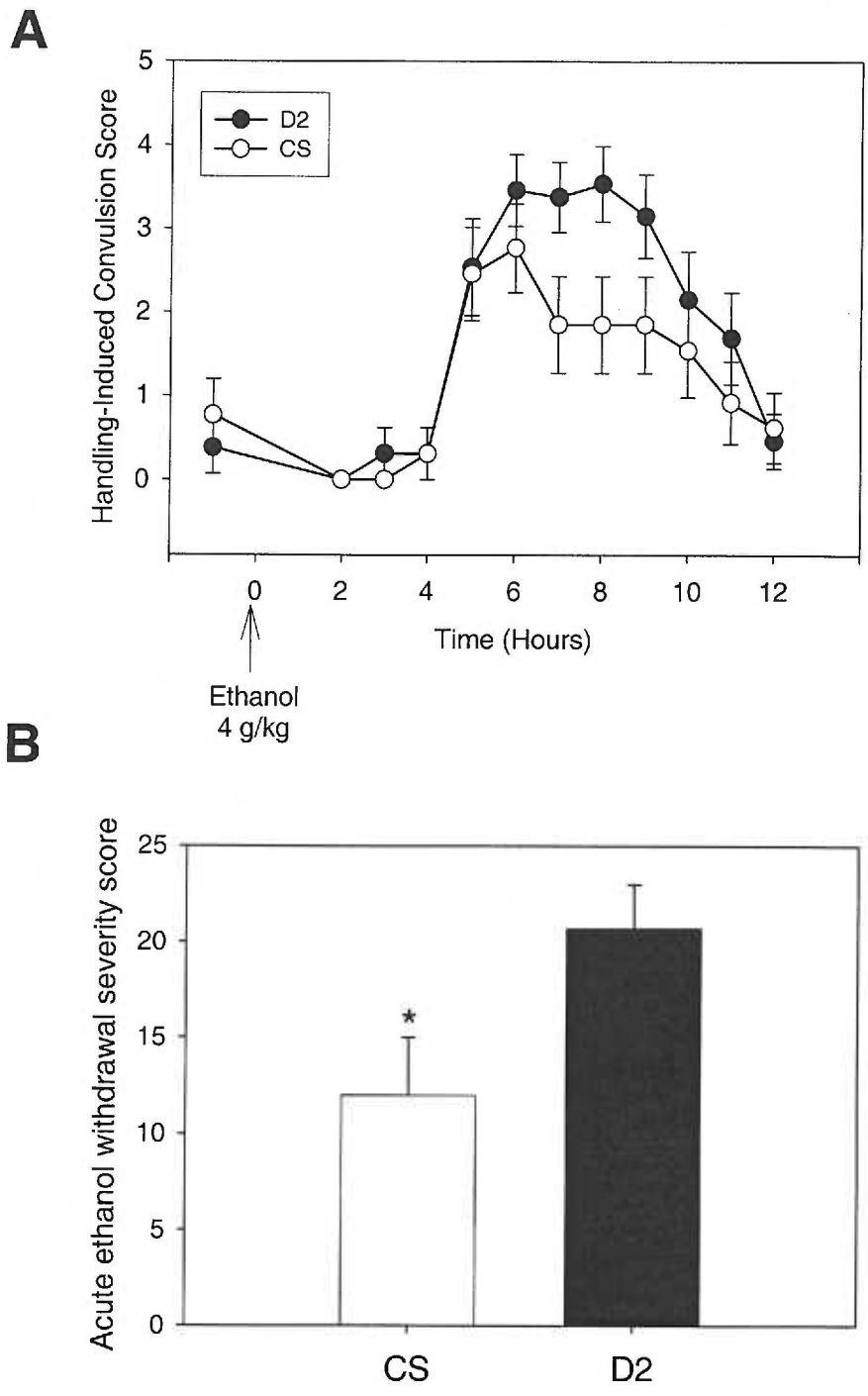

Figure 3-1: Chromosome 4 congenic mice show significantly less severe acute alcohol withdrawal severity compared to D2 background strain mice. (**A**): Time course of acute alcohol withdrawal HIC following i.p. injection of 4 g/kg ethanol. HIC was taken twice 30 minutes prior to and at hourly intervals up to 12 hours following ethanol administration. (**B**): Area under the withdrawal curves shown in Figure 1A. Congenic strain mice (CS) with the genomic interval containing the B6 allele of *Mpdz* show significantly less severe ethanol withdrawal severity compared to D2 background strain mice ($p < 0.05$). Bars represent the mean ± SEM for 13 mice per strain.

males had higher BEC compared to D2 females ($p < 0.0001$) and congenic males had higher BEC compared to congenic strain females ($p < 0.0001$). ANOVA also indicated a significant main effect of strain on AUC25 in air-pyrazole control mice [$F(1.57) = 8.3$, $p < 0.01$], and therefore AUC25 shown in figure 3-2 were corrected for differences in control data. Figures 3-2A-C show results from area under the 25 hour withdrawal curves (AUC 25) in males and females combined (Figure 6A) and separated by sex (Figures 2 B and C). No strain differences were observed in any case in ethanol withdrawn mice. Figures 3-2D-F shows regression residuals obtained from regressing BEC at 72 hours on AUC25 scores. This analysis corrects for differences in BEC and is important for distinguishing between pharmacokinetic versus pharmacodynamic factors. Figure 3-2D shows results from males and females combined, whereas Figures 3-2E and 3-2F show results separated by sex. There was a significant reduction of regBEC values in male congenic mice versus male D2 background strain mice ($t = -3.0$ $df = 180$, $p < 0.01$). (Figure 3-2F).

Knockout of the 5-HT_{2C} receptor gene results in generalized susceptibility to a variety of seizures (Tecott et al., 1995; Applegate and Tecott, 1998). We tested B6 and D2 strain mice (progenitor strains used for creation of our chromosome 4 congenic strain mice) for HIC following i.p. administration of a selective 5-HT_{2C} receptor antagonist (SB-242084) (Kennett et al., 1997). Figure 3-3A shows HIC scores in B6 and D2 mice 30 minutes before drug (baseline) and at hourly intervals up to 12 hours and then at hour 24 and 25 hours after i.p.

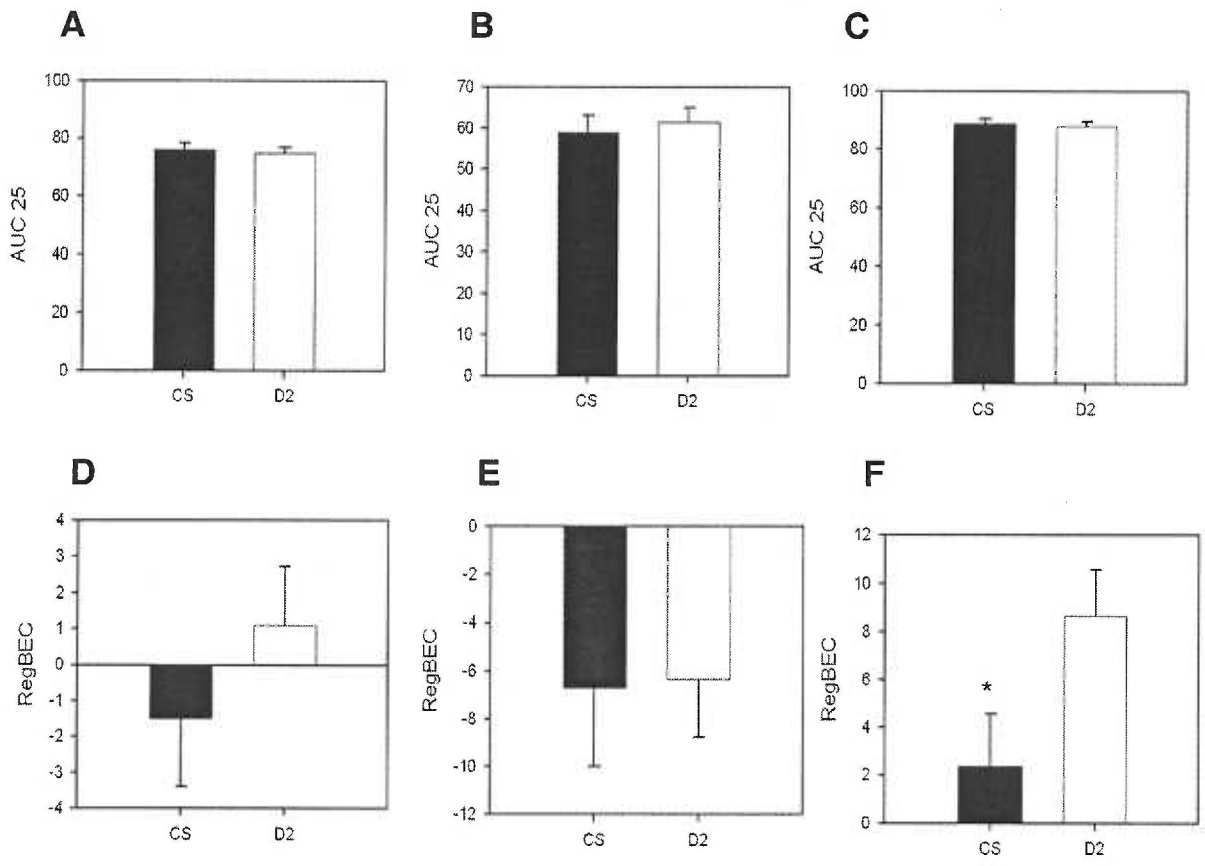

Figure 3-2: Chronic ethanol withdrawal severity in chromosome 4 congenic (CS) versus D2 background strain mice. **A**, **B**, and **C** show AUC25 results for data collapased across males and females **(A)** and seperated by gender **(B)** females, and **(C)** males. **D**, **E**, and **F** show residual scores obtained by regressing AUC25 on BEC. **(D)** shows males and females together, **(E)** shows females alone, and **(F)** shows males alone. There was a significant reduction in alcohol withdrawal severity in male congenic mice compared to male D2 background strain mice ($p < 0.05$). Bars represent the mean ± SEM for n = 146-196 mice per genotype combine among sex and n = 62-98 mice per genotype per sex. BEC for males and females combined was CS = 1.53 ± 0.04 mg/ml, D2 = 1.41 ± 0.05 mg/ml. BEC for males was CS = 1.81 ± 0.04 mg/m, D2 = 1.60 ± 0.05 mg/ml, and BEC for females was CS = 1.16 ± 0.06 mg/ml, D2 = 1.23 ± 0.08 mg/ml.

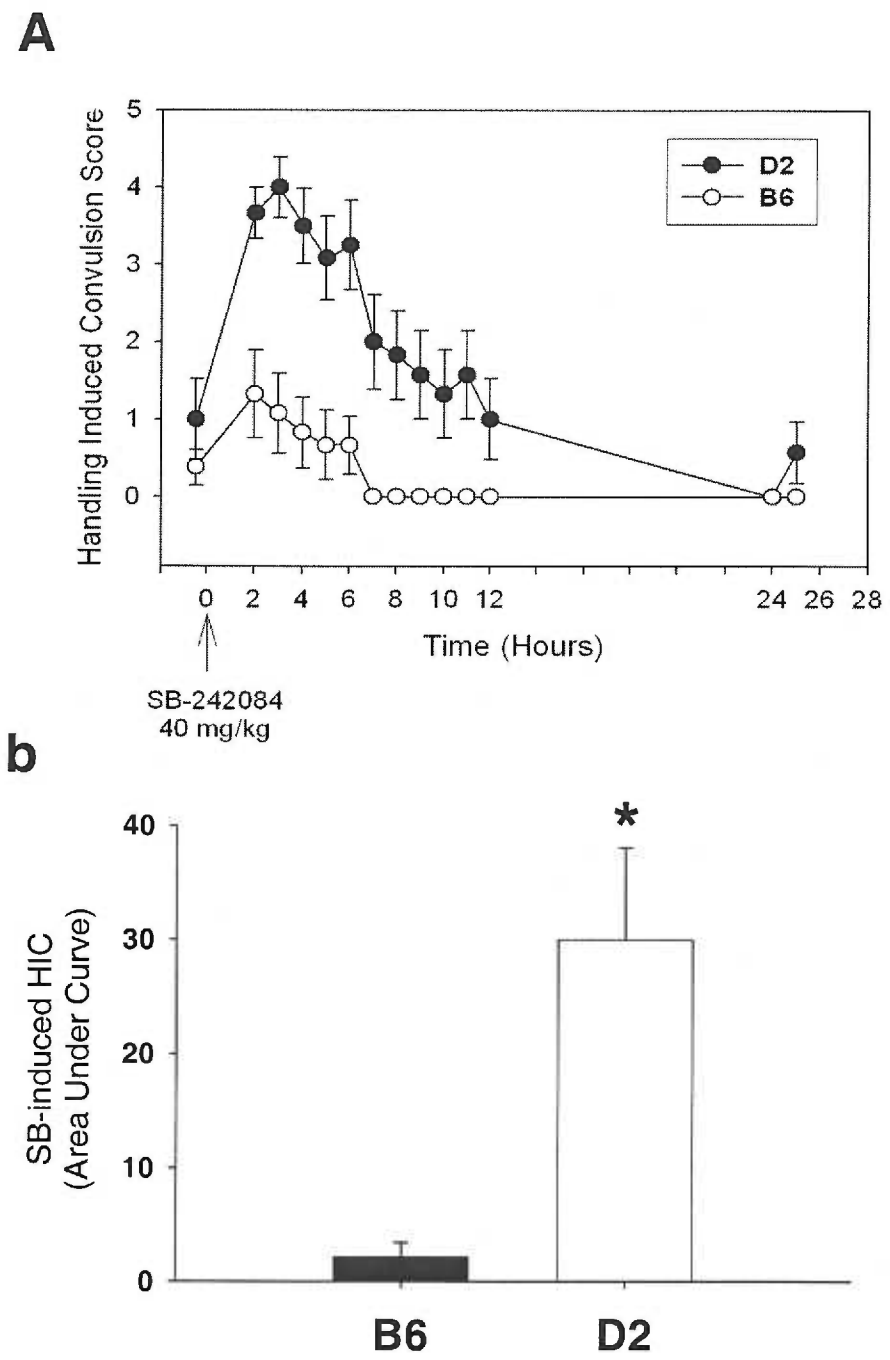

Figure 3-3: SB-242084, a 5-HT_{2C} receptor antagonist, produces significantly more severe HIC in background (D2) vs. donor (B6) strain mice. (**A**): Time course of handling-induced convulsions following i.p. adminstration of 40 mg/kg SB-242084 in donor (B6; n = 12) and D2 background strain mice (n = 12). Baseline HICs were taken 30 minutes prior to drug injection (hr 0, arrowhead). (**B**): Area under curve for data shown in panel A. Bars represent the mean ± SEM. D2 mice show a significantly more severe HIC response following SB-242084 vs. B6 strain mice (* $p < 0.01$).

injection of 40 mg/kg SB-242084, a 5-HT_{2C} receptor antagonist. Figure 3-3B shows the AUC from data shown in Figure 3-3A. D2 strain mice show significantly greater AUC compared to B6 strain mice ($t = -3.4$, $df = 22$, $p < 0.01$). These data show that B6 and D2 mice show a differential HIC response to a 5-HT_{2C} receptor antagonist.

In the next series of experiments we tested handling-induced convulsions after i.p. administration of several well known chemiconvulsants drugs. Figure 3-4 shows strychnine-induced HIC expressed as AUC. ANOVA showed significant main effects for strain [$F(1.42) = 13.4$, $p < 0.001$], dose [$F(1,42) = 6.0$, $p < 0.05$], and a significant strain by dose interaction [$F(1,42) = 6.2$, $p < 0.05$]. Post-hoc analysis indicated a dose effect in D2 mice, where mice administered the 1.0 mg/kg dose of strychnine showed a greater response than D2 mice administered 0.5 mg/kg strychnine ($p < 0.01$). A genotype-dependent difference was found at 1.0 mg/kg strychnine, where D2 mice showed a greater response than congenic mice ($p < 0.001$). Congenic strain mice did not differ from D2 mice in convulsion severity after 0.5 mg/kg strychnine (Figure 3-4).

Figure 3-5 shows NMDA-induced HIC expressed as AUC. ANOVA indicated a significant main effect of strain [$F(1,48) = 25.1$, $p < 0.0001$], but no other main effects or interactions. There was a trend for a gender by treatment interaction [$F(1,48) = 3.0$, $p = 0.097$]. Examination of the data indicated that this trend was due to a dose effect in female D2 mice, where 60 mg/kg NMDA produced a greater response than 30 mg/kg NMDA. However, male and female congenic mice showed very similar response to both 30 and 60 mg/kg NMDA

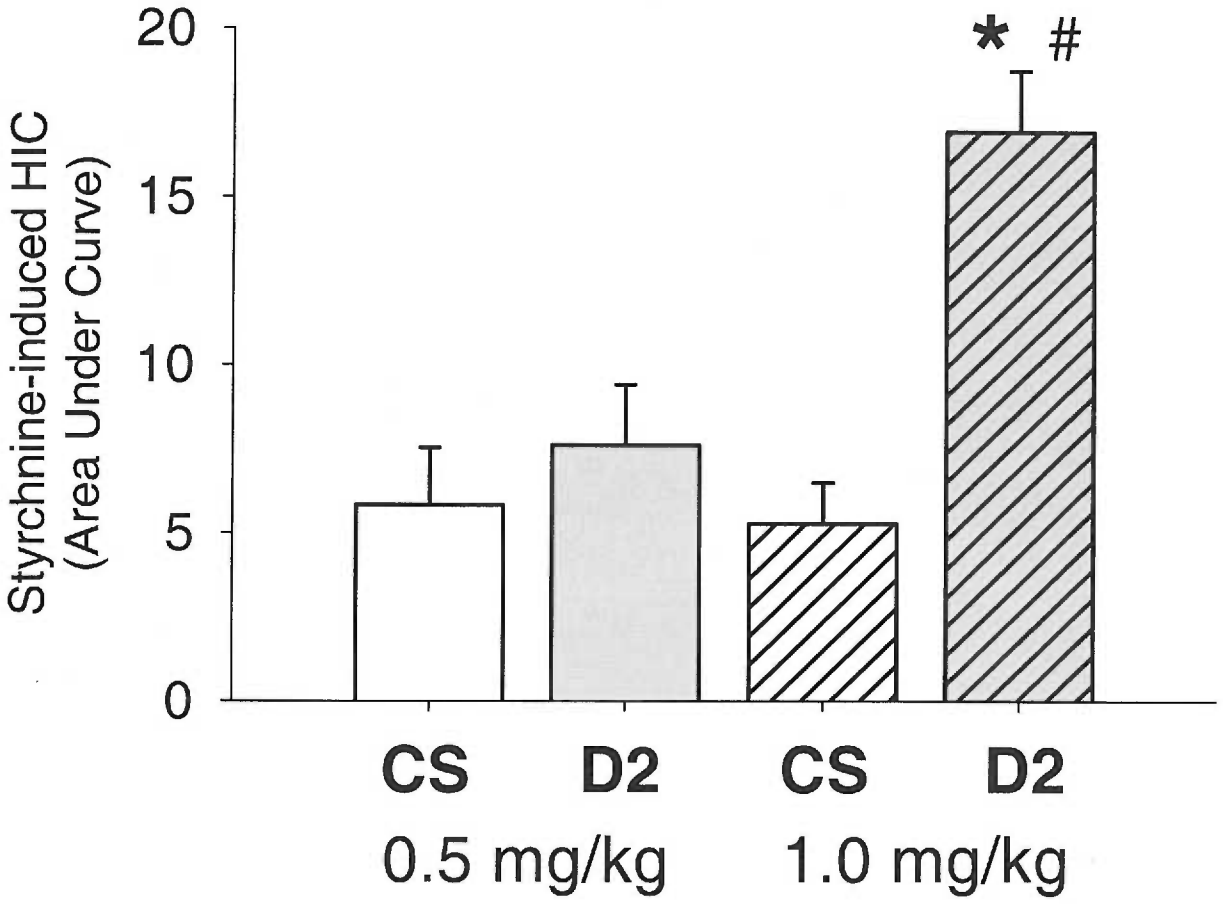

Figure 3-4: Strychnine, a glycine receptor antagonist, produces significantly more severe HIC in background (D2) vs. chromosome 4 congenic strain (CS) mice. Bars represent the mean ± SEM for 11-15 mice pre strain and dose. D2 mice show a significantly more severe HIC response following 1.0 mg/kg strychnine vs. congenic strain mice (* $p < 0.01$). D2 mice administered 1.0 mg/kg strychnine show a greater response than D2 mice administered 0.5 mg/kg strychnine (#$p < 0.05$).

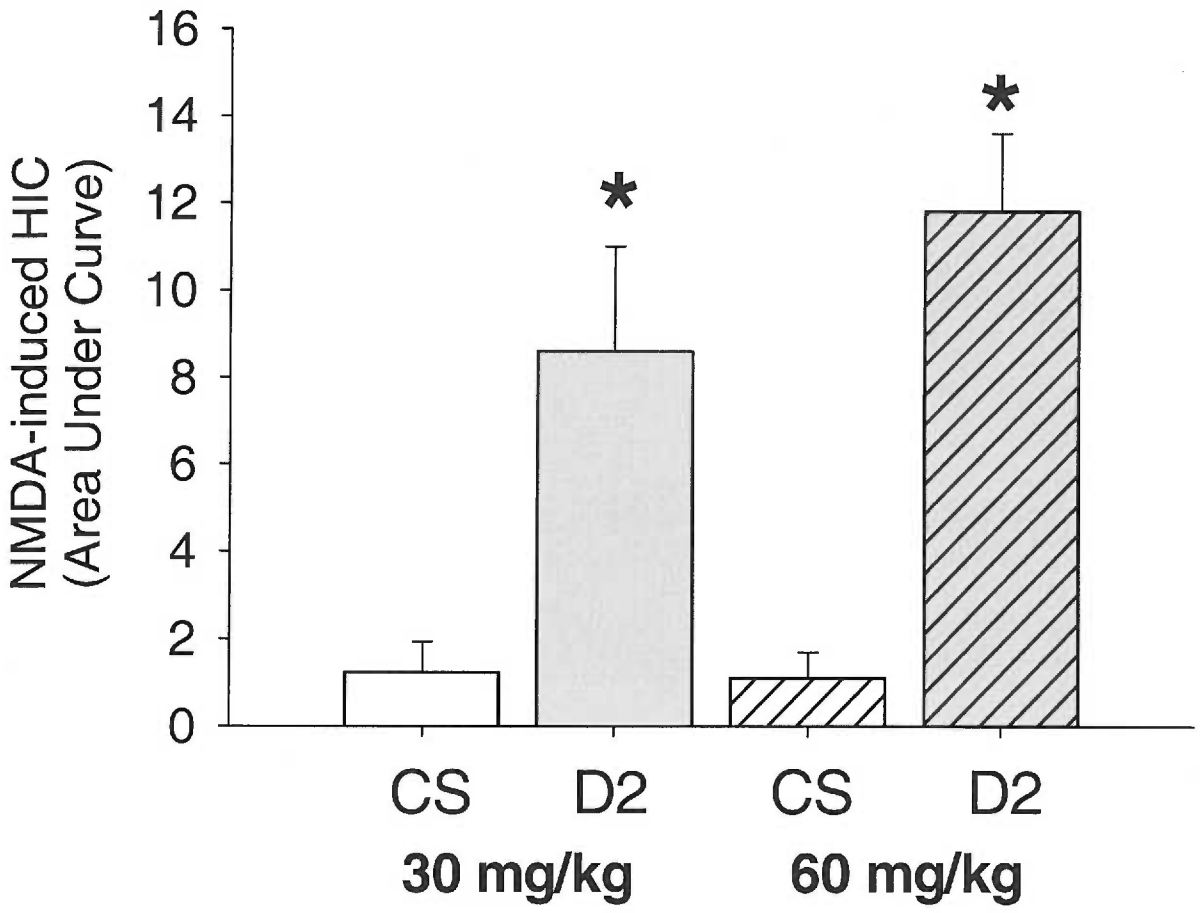

Figure 3-5: NMDA, a glutamate-NMDA receptor agonist, produces significantly more severe HIC in background (D2) vs. chromosome 4 congenic (CS) strain mice. NMDA-induced HIC expressed as Area under curve (AUC) for 30 mg/kg NMDA and 60 mg/kg NMDA. Bars represent the mean ± SEM for AUC for n = 11-16 mice per strain and dose. There was a significant main effect of strain (*$p < 0.0001$).

(Figure 3-5). These data suggest that genetic variation in *Mpdz* affects strain HIC responses to NMDA.

Figure 3-6 shows PTZ-induced HIC expressed as AUC. ANOVA indicated significant main effect of drug dose [$F(3,106) = 115.9$, $p < 0.0001$], and significant strain by gender by drug dose interaction [$F(3,106) = 3.5$. $p < 0.05$]. Post hoc analysis indicated that congenic strain female administered 15 mg/kg PTZ differed from female congenic mice given 30 mg/kg PTZ ($p < 0.0001$). Congenic male mice administered 15 mg/kg PTZ differed from congenic male mice given 30 mg/kg PTZ ($p < 0.05$). However, post-hoc analysis did not detect any strain differences (Figure 3-6). These data suggest that genetic variation in *Mpdz* does not affect strain HIC responses to PTZ.

DISCUSSION

Our findings show that *Mpdz* is a gene that pleiotropically affects handling-induced convulsion sensitivity to 5-HT_{2C}, glycine, and NMDA receptors. Because handling-induced convulsions were similar in congenic versus the D2 background strain following two doses of PTZ, it is likely that *Mpdz* does not influence handling-induced convulsions modulated through $GABA_A$ receptor specific chloride channels. Together these data show some specificity for the effects of *Mpdz* on convulsion phenotypes, which suggest that *Mpdz* does not regulate seizure severity in general. However, *Mpdz* is a gene that regulates genetic differences in both acute and chronic alcohol withdrawal severity. Overall results are summarized in Figure 3-7.

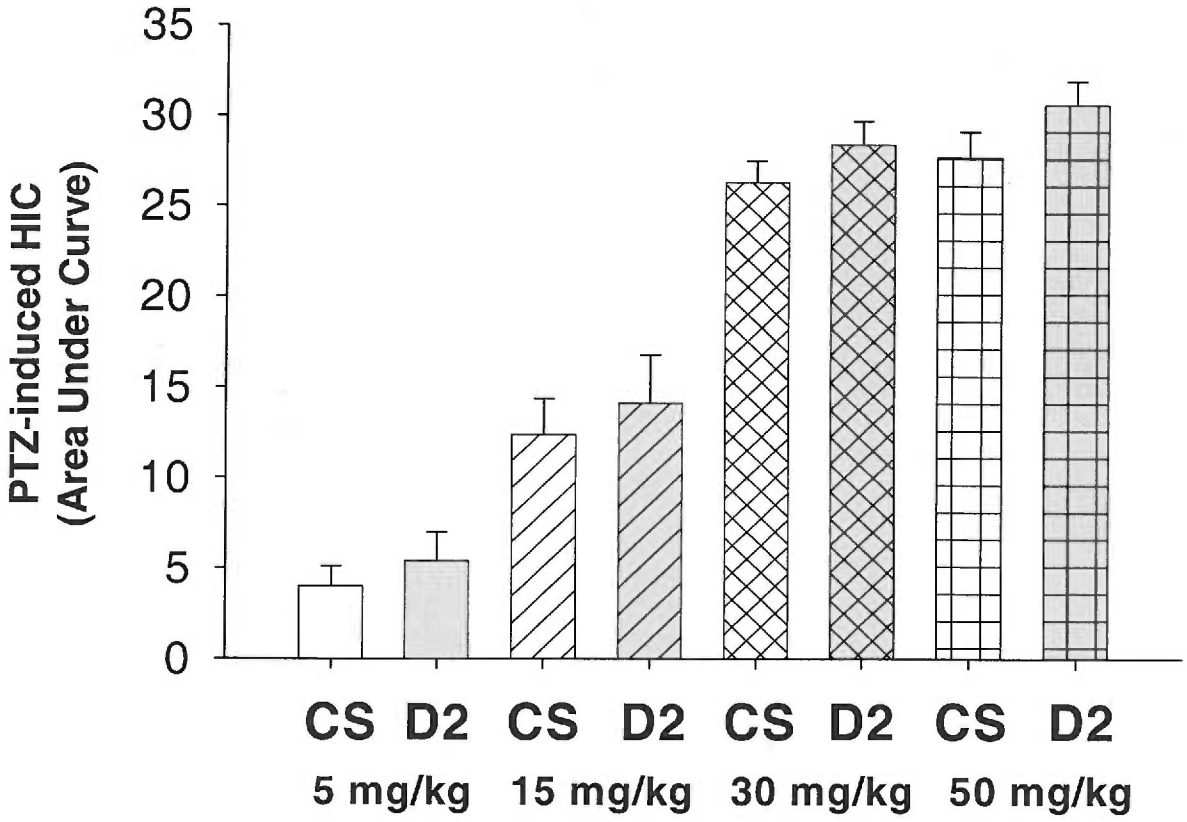

Figure 3-6: Pentylenetetrazole (PTZ), a $GABA_A$ receptor activated channel blocker, produces similar HIC in background (D2) vs. chromosome 4 congenic (CS) strain mice. PTZ-induced HIC expressed as area under curve (AUC) for 5, 15, 30 and 50 mg/kg PTZ Bars represent the mean ± SEM for AUC for n = 9-27 mice per strain and dose. D2 background strain mice show similar HIC compared to congenic strain mice.

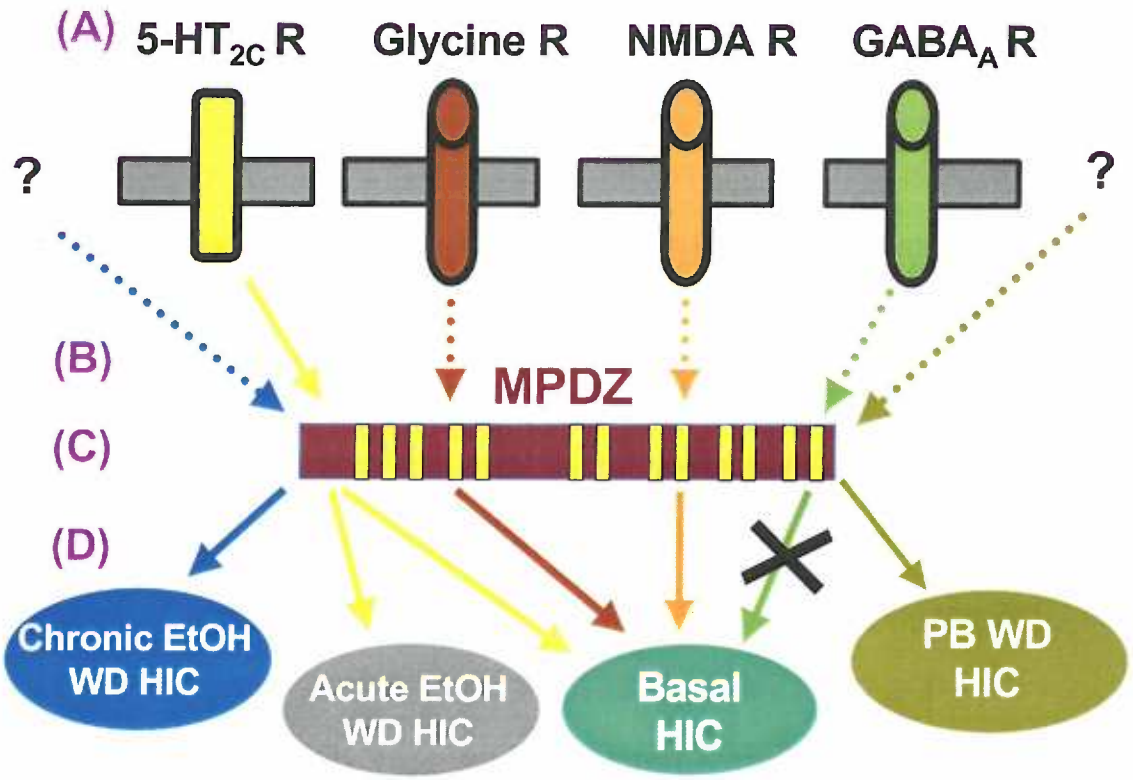

Figure 3-7: Potential pleiotropic effects of *Mpdz* on handling-induced convulsion severity. (**A**): Neurotransmitter receptors examined through pharmacological modulation with chemiconvulsant drugs. Question marks indicate no receptor mechanism examined. (**B**): The solid arrow from the 5-HT_{2C} receptor to MPDZ indicates that a direct interaction between these two proteins has been identified (Becamel et al., 2001). Broken arrows indicate that a direct protein-protein interaction has not been identified. (**C**): The Multiple PDZ Domain Protein (MPDZ). (**D**): Handling-induced Convulsion (HIC) phenotype measured. Pentobarbital (PB) convulsions were tested by Fehr et al., 2002. Unbroken arrows projecting from MPDZ to ellipses with HIC phenotypes indicate regulation of this convulsion phenotype through MPDZ. The (**x**) through the arrow originating from the $GABA_A$ receptor indicates that MPDZ does not regulate basal HIC via blockade of $GABA_A$ receptor operate chloride channels. Abbreviations: EtOH (ethanol); WD (withdrawal); Handling-induced convulsion (HIC); PB (pentobarbital)

(Becamel et al., 2001; Parker et al., 2003). Our finding that the B6 donor strain significantly differs from the D2 background strain in severity of handling induced convulsion sensitivity to 5-HT_{2C} receptor antagonist suggests a functional relevance of the 5-HT_{2C} receptor and MPDZ interaction. We speculate that the large difference in response to the 5-HT_{2C} receptor antagonist is due to genetic variation in *Mpdz*, but the specific mechanism is not yet known. Some possibilities are that genetic variation in *Mpdz,* affecting MPDZ expression and /or structure, differentially regulates 5-HT_{2C} receptor trafficking and/or 5-HT_{2C} receptor mediated signal transduction. Indeed, we have found possible differences in 5-HT_{2C} receptor subcellular localization between congenic and D2 background strain mice, suggesting differences in receptor trafficking (see Chapter II). Overall MPDZ protein abundance in brain is higher in congenic versus D2 background strain mice (see Chapter II), which could potentially differentially affect the dynamics of the interaction between MPDZ and 5-HT_{2C} receptor proteins in congenic versus D2 background strain mice.

Our data support a role of the 5-HT_{2C} receptor in seizure susceptibility. 5-HT_{2C} receptor null mutant mice have a genetic susceptibility to variety of convulsions induced by chemical, electrical and sound stimulation (Brennan et al., 1997; Applegate and Tecott, 1998). Other studies using pharmacological manipulations of 5-HT_2 receptor neurotransmission also support a role for this receptor in seizure susceptibility. mCPP, a 5-$HT2_{C/B}$ receptor agonist, micro-injected into the substantia nigra protects against electrical seizures evoked from area temptas (Pasini et al., 1996). Genetic variation in *Mpdz* in our mouse

populations is very likely to affect strain differences in convulsion severity between B6 and D2 mice because 5-HT_{2C} receptor sequence is identical between B6 and D2 mouse strains (see Chapter II).

A novel and as of yet undefined pathway by which genetic variation in *Mpdz* affects handling convulsions modulated by glutamate NMDA receptors is another key finding of this investigation. Thus far there is no report of a direct interaction of MPDZ with NMDA receptors. However, PSD-95 as well as other PDZ domain proteins have a direct protein-protein interaction with NMDA receptors through a carboxy terminus PDZ recognition motif which is contained in both NR1 and NR subunits (Wenthold et al., 2003). Therefore, a MPDZ and NMDA receptor interaction is possible. Further studies are needed to confirm a direct MPDZ and NMDA receptor interaction.

Glutamate receptors have been implicated in genetic susceptibility to seizure disorders such as epilepsy as well as genetic differences in alcohol withdrawal severity (Crabbe et al., 1990a; Dingledine et al., 1990). Increased protein for AMPA and NMDA glutamate receptor subunits are found in patients with temporal lobe epilepsy (Mathern et al., 1998). It is interesting to hypothesize that regulation of glutamate receptor subunit expression in patients with epilepsy is influenced by effects of *Mpdz*. Our results support this hypothesis along with a previous report demonstrating that levels of the PDZ domain binding protein, Mint1, are altered during seizures in a rat model of epilepsy (Scorza et al., 2003). Mint1's function is to regulate the transport of NR2B subunits of NMDA receptor to proper post-synaptic sites. This is a

plausible mechanism affecting seizure susceptibility by controlling synaptic availability of NMDA receptors. We speculate that there is a similar mechanism involved with the differences we observe in handling-induced convulsion sensitivity to NMDA which involves genetic variation in *Mpdz*. In addition, Mint1 protein abundance is altered with seizures and this is consistent with our finding that MPDZ protein abundance is greater in the congenic versus the D2 background strain (see Chapter II). Enhanced sensitivity to NMDA has been observed in Withdrawal Seizure-Prone (WSP) compared to Withdrawal Seizure-Resistant (WSR), which have been selectively bred to have severe or mild handling convulsions during withdrawal from chronic alcohol exposure, respectively (Crabbe et al., 1991; Finn and Crabbe, 1999). Crabbe et al. (1991) found that ethanol-naïve WSP mice were more sensitive to handling-convulsions after NMDA compared to WSR mice, while Finn and Crabbe (1999) found that ethanol withdrawing WSP mice were more sensitive than ethanol-withdrawing WSR mice to NMDA convulsions produced through i.v. administration of the drug. However, ethanol-naïve WSR mice are more sensitive than WSP mice to NMDA convulsions induced through i.v. administration of the drug (Kosobud and Crabbe, 1993). The differences in these studies are likely due to whether the mice are naïve to ethanol exposure and the specific seizure endpoint (i.e., handling-induced convulsion versus NMDA convulsions). Nonetheless, it is interesting to speculate that *Mpdz* may be associated with differences in sensitivity to excitatory amino acids in WSP and WSR mice. Preliminary data support this hypothesis because both WSR replicate lines posses the B6 allele of

Mpdz. However, WSP replicate line 1 also has the B6 allele, while WSP replicate line 2 possess an *Mpdz* allele which is similar to strains with protein variant two of MPDZ (Buck et al., unpublished data). In addition, QTL mapping using crosses between inbred WSP x WSR strains, identified a QTL for chronic alcohol withdrawal severity on mid chromosome 4 within a the region containing *Mpdz* (Bergeson et al., 2003). Overall, our results offer a new mechanism in which handling-induced convulsions modulated through NMDA receptors are potentially regulated by genetic variation in *Mpdz*, and suggests an interaction between these two proteins that has not yet been defined.

Our results also indicate a novel mechanism in which handling-induced convulsions are modulated by glycine receptors through MPDZ. In contrast to NMDA receptors, glycine receptors do not contain PDZ recognition sites, which makes the interpretation of the strychnine results a little more complex. However, glycine receptors interact with gephyrin, which is a tubulin binding protein, and this interaction is responsible for accumulating glycine receptors at the plasma membrane (Hanus et al., 2004). Therefore, one interpretation of our results with strychnine is that MPDZ might regulate convulsions through glycine receptors in an indirect way, possibility through an interaction with proteins similar to gephyrin. However, there is no reported evidence of such an interaction. But this idea is support by the finding that MPDZ interacts with stargazin, a transmembrane AMPA receptor regulatory protein, and not the AMPA receptor itself, providing an indirect mechanism by which MPDZ could affect AMPA receptor function (Dakoji et al., 2003). The proteome of glycine

receptor subunits has not been defined completely, but our data suggest that MPDZ will be included in the list of proteins associated with this receptor.

Like the glycine receptor, $GABA_A$ receptor subunits do not contain PDZ recognition domains, but have been shown to interact with gephyrin and GABARAP, a $GABA_A$ receptor associated protein that does contain a Class II PDZ domain (Fehr et al., 2004). GABARAP binds to the γ2 subunit of $GABA_A$ receptors, which is required for proper targeting and assembly of the receptor at post-synaptic sites (Essrich et al., 1998). Our results with PTZ do not support an interaction of $GABA_A$ receptors and MPDZ. One possible interpretation for this finding is that because PTZ is a non-competitive chloride channel blocker of $GABA_A$ receptors, blocking this chloride channel may have no direct effect on a potential subunit specific interaction with MPDZ.

The pattern of results we obtained for each convulsant drug in congenic versus D2 background strain mice is strikingly similar to the pattern of results obtained when WSP and WSR mice were tested for handling convulsions after administration of some of the same drugs (Crabbe et al., 1991). PTZ is one of the only convulsants that produced comparable handling convulsions in WSP and WSR mice; while NMDA and strychnine exacerbated handling convulsions only in WSP mice. Crabbe et al. (1991) postulated that the greater HIC response seen in WSP mice following administration of most convulsants had more to do with handling convulsions in general and not to mechanisms specific to ethanol withdrawal. Furthermore the authors also speculated that there may be a final common pathway for handling-induced convulsions which is associated with

several neurotransmitter systems (Crabbe et al., 1991). Because our results with congenic and D2 background strain mice are so similar to the results of Crabbe et al. (1991), we speculate that *Mpdz* might be one of the final common molecular components of the pathway connecting various neurotransmitter receptors, which would then have an overall influence on convulsion intensity. This indeed is a very intriguing possibility because of the potential ability of MPDZ to interact with a host of intracellular proteins via its thirteen PDZ domains. As more information becomes available about interaction partners for MPDZ, more light will be shed on the specific neurochemical pathways and behaviors that MPDZ regulates. Our study is the first attempt at defining this pathway for handling-induced convulsion severity.

In a previous study from our laboratory we found that MPDZ protein status was genetically correlated with seizures induced by specific chemiconvulsant drugs in a panel of standard inbred mouse strains (Fehr et al., 2004). Our initial sequence analysis of *Mpdz* in a panel of nine standard inbred strains, identified three protein variants of MPDZ (Fehr et al., 2002). Fehr et al. (2004) took the sequence information for MPDZ obtained in the previous study by Fehr et al., 2002 and correlating it with behavioral data obtained from the same panel of inbred strains on timed tail vein i.v. administration of several convulsant agents. The most significant correlations were found for kainic acid and PTZ, suggesting that there is an association with genetic variation in *Mpdz* and seizures modulated through glutamatergic and GABAergic neurotransmission. Results with PTZ in the present study do not support this finding. Another discrepancy is

that in the present study we find a large difference in strychnine's effects on handling convulsions between the congenic and D2 background strain, while Fehr et al. (2004) reported no evidence of a genetic correlation between MPDZ status and strychnine convulsions induced by i.v. administration of the drug through the lateral tail vein. There are several plausible explanations of these differences. First, the study by Fehr et al. (2004) was a correlational study, and thus only provides indirect evidence that *Mpdz* actually has a role in seizure modulation induced by chemiconvulsants. Secondly, two different types of seizure endpoints were studied. In the present study chemical modulation of convulsions induced by handling were studied, while in the study by Fehr et al. (2004) correlations were obtained for seizures induced by i.v. administration of drugs through the lateral tail vein. Finally, because of the almost complete elimination of genetic noise from other loci in the mouse genome through comparisons of the congenic versus the D2 background strain, differences in the present study and the one by Fehr et al. (2004) that used a panel of inbred mouse strains could result from the influence of other genes on convulsions. However, taken together these results support the notion of a previous finding from our group that independent mechanisms underlie different convulsant signs elicited by a single drug (Kosobud and Crabbe, 1990). Handling-induced convulsions are a mild type of seizure. On the other hand, chemical seizures induced by time tail vein i.v. administration of drug (as used by Fehr et al., 2004) are more severe (i.e., they are usually lethal). Therefore, independent mechanisms are likely to underlie each distinct type of convulsion.

Another important outcome of our study is that we have demonstrated some specificity of the effects of MPDZ on pharmacologically modulated convulsions by showing that PTZ, a $GABA_A$ receptor activated chloride channel blocker, has similar effects on handling convulsions in congenic versus D2 background strain mice. This indicates that the effects of MPDZ on handling convulsions do not affect all types of pharmacologically modulated convulsions. This result was rather surprising because of the previous finding of a suggestive PTZ-induced seizure locus on mid chromosome 4 in a region containing *Mpdz* (Ferraro et al., 1999). However, Ferraro et al., 1999 used much different seizure parameters which included latencies to focal clonus, generalized clonus, and maximal seizure. These are different types of convulsions compared to the milder handling convulsions studied in our present study. Given this apparent discrepancy with the literature, our results nonetheless suggest that MPDZ is not an intermediate in PTZ modulated handling convulsions. However, it remains to be determined if MPDZ determines responsiveness to more severe PTZ induced convulsions such as those studied by Ferraro et al. (1999).

These studies also show that acute and chronic ethanol withdrawal are not identical. This is supported by comparisons of previous studies examining acute and chronic alcohol withdrawal in B6 and D2 mice and mice derived from these strains (Crabbe, 1998; Buck et al., 2002; Buck et al., 1997). Buck et al., 2002 reported only a very weak QTL on mid-chromosome 4 for chronic ethanol withdrawal in B6D2 F2 mice. Furthermore, this QTL was not detected in BXD recombinant inbred mouse strains (Crabbe, 1998). A very strong QTL for acute

ethanol withdrawal, however, was detected on mid-chromosome 4 (Buck et al., 1997). Therefore, the differences in our results between acute and chronic ethanol withdrawal and the role of *Mpdz* in these phenotypes is not surprising. It is likely that during the 3 day ethanol exposure for the chronic alcohol withdrawal model, additional genetic factors come into play.

In conclusion, we have identified potential neurochemical pathways for handling-induced convulsion severity in mice that involves genetic variation in *Mpdz*. Serotonin type 2C, glycine, and NMDA receptors all modulate handling convulsion severity through MPDZ, while MPDZ appears to not play a role in regulation of convulsions by $GABA_A$ receptor specific chloride channels. Convulsions after chronic alcohol exposure are also mediated, in part, through *Mpdz*. Our results provide a framework to study in more detail the pathways and the proteins that interact with genetic variants of *Mpdz* to influence complex behaviors such as convulsion severity. Furthermore, our results suggest that MPDZ is an important target for development of pharmacotherapies for addiction as well as seizure disorders such as epilepsy.

Chapter IV

Genetic differences in alcohol withdrawal severity are associated with differential activation of an extended limbic circuit in DBA/2J compared to C57BL/6J mouse strains

Abstract

The C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains are used extensively to study ethanol-related phenotypes. The D2 strain shows severe withdrawal following chronic ethanol exposure, while the B6 stain displays less severe withdrawal severity. Previous studies have used the immediate early gene, *cfos,* as a sensitive neuronal marker to identify brain regions associated with ethanol withdrawal in rodents. However, the specific brain regions that mediate differential ethanol withdrawal in the B6 and D2 inbred strains have not been determined. Here we used FOS immunohistochemistry to identify brain regions associated with differential ethanol withdrawal in the B6 and D2 strains. Male B6 and D2 mice were exposed to ethanol vapor in an inhalation chamber for 3 days and injected daily with pyrazole. The control group was exposed to air for 3 days and injected daily with pyrazole. Mice were killed seven hours after removal from the inhalation chambers, which is a time point where mice exhibit peak withdrawal convulsions, and brains removed and processed for immunohistochemistry.

Overall, ethanol withdrawn D2 mice show a greater FOS activation in limbic regions (including the hypothalamus and several subregions of the

hippocampus) compared to ethanol withdrawn B6 mice. Several regions were similarly activated by ethanol in both strains including the ecthorhinal and posterior parietal cortex, specific subregions of the amygdala and the caudate putamen. Ethanol-naïve control B6 and D2 mice did not differ in FOS activation of any brain region examined. These results suggest that severe ethanol withdrawal in D2 mice involves activation of an extended limbic circuit.

INTRODUCTION

The C57BL/6J (B6) and DBA/2J (D2) inbred mouse strains represent the most widely used animal models for the study of ethanol-related phenotypes. B6 and D2 mice differ on a number of responses to ethanol including voluntary consumption (Phillips et al., 1994) tolerance to the ataxic effects (Gallaher et al., 1996) sensitization to the stimulant effects (Phillips, 1997) and acute and chronic withdrawal severity (Metten and Crabbe, 1994; Buck et al., 1997; Crabbe et al., 1983; Crabbe, 1998). These strain differences in sensitivity to ethanol responses have made B6 and D2 mice and crosses between these strains useful for examining the genetics underlying ethanol related traits.

Ethanol dependence, in part, is thought to represent a neuroadaptation resulting from perturbations caused by excessive exposure to ethanol. Heightened central nervous system (CNS) excitability is one of the hallmark signs of ethanol dependence, which typically manifests as convulsions or seizures. Ethanol withdrawal convulsions are an important aspect of the withdrawal syndrome because they can be life threatening if left untreated and they are a powerful motivational force perpetuating continued drinking (i.e.,

negative reinforcement) (Koob and Le Moal, 2001). Dora Goldstein in 1973 was the first to demonstrate a genetic component that influences the severity of alcohol withdrawal in mice (Goldstein, 1973). Subsequently, B6 and D2 mice were found to show a substantial difference in severity of withdrawal following chronic alcohol exposure; D2 mice showed severe withdrawal, while B6 mice had more moderate withdrawal (Crabbe et al., 1983)

Changes in gene expression are thought to be responsible for at least some of the neuroadaptations to chronic ethanol (Reilly et al., 2001; Daniels and Buck, 2002). The expression of the immediately early gene, *cfos*, is known to be sensitive to a number of stimuli challenges such as seizures (Zhang et al., 2002) and stress (Kovacs, 1998). cFOS forms a transcription factor with members of the Jun family (c-jun, JunB, and JunD) which is referred to as the activator protein-1 (AP-1) complex (Hughes and Dragunow, 1995). However, the expression of cFos in the central nervous system has been used most extensively as a functional anatomical mapping tool to identify cells and extended circuits that become activated under various conditions (Herdegen and Leah, 1998). Furthermore, because *cfos* expression is induced quite rapidly after certain challenges (mRNA peaks 30-60 minutes and protein peaks around 1-3 hrs), it is a useful marker of neuronal activation (Kovacs, 1998). In addition, because of its low level of basal induction, *cfos*, is a useful marker to study the effects of drug-specific brain activation. However, major limitations of the use of *cfos* as a mapping tool are that it does not mark cells with a net inhibitory drive

and the target genes (and cell phenotypes) that are activated by *cfos* are not known without additional work (Kovacs, 1998).

A number of studies have examined the brain regions that underlie ethanol dependence using *cfos* expression as a sensitive neuronal marker of brain activation (Olive et al., 2001; Knapp et al, 1998, 2000, Moy et al., 2000; Ryabinin et al., 2000). However, there is little information on how genetic variation in alcohol withdrawal severity affects withdrawal-specific brain circuits (Olive et al., 2001). In particular, there is no information on the brain regions that may underlie differential ethanol withdrawal in the B6 and D2 inbred mouse strains. Here, we use FOS immunohistochemistry to identify brain regions associated with severe ethanol withdrawal in the D2 strain compared to brain regions associated with more moderate withdrawal severity in the B6 strain. Our results indicate that activation of an extended limbic circuit is associated with severe alcohol withdrawal in the D2 mouse strain.

MATERIALS AND METHODS

Animals. Male B6 and D2 mice were purchased from the Jackson laboratories (Bar Harbor, ME). Upon arrival at the AALAC accredited Portland VA Medical Center, mice were housed in groups of 4 per cage with corn cobb bedding and allowed to acclimate to this housing condition for at least one week. All mice were 70-100 days old at time of testing. Food (Purina lab chow) and water were freely available at all times. Colony and procedure rooms were maintained at an ambient temperature of 21 ^{0}C. All procedures were approved

by the institutional IACUC committee and followed guidelines for the care and use of laboratory animals from the National Institute of Health.

Ethanol Physical Dependence Induction

Male B6 and D2 strain mice were made physically dependent on ethanol by chronic ethanol inhalation for 3 days as described in detail elsewhere (Terdal and Crabbe, 1994; Reilly and Buck, 2000). One group of mice was exposed to ethanol vapor in an inhalation chamber for 3 days. On day one ethanol groups were injected with a 1.5 g/kg loading dose of ethanol to elevate blood ethanol concentrations (BEC). All mice were injected daily with pyrazole, an alcohol dehydrogenase inhibitor that is necessary to reduce mortality and maintain stable BEC. The control group was exposed to air for 3 days and injected daily with pyrazole. On day 3 of chronic ethanol exposure all mice were removed from the inhalation chambers, and a 20 μl tail blood sample was collected from all ethanol dependent mice for determination BEC using a gas chromatographic assay (Terdal and Crabbe, 1994). All mice were killed seven hours after removal from the inhalation chambers (when ethanol withdrawal severity is at its peak), brains removed and submerged in 4% Para formaldehyde for 48 hours. Brains were then submerged in 30% sucrose for 24 hours.

cFOS Immunohistochemistry

cFOS immunohistochemistry procedures were performed according to Hitzemann and Hitzemann (1999). Brains were frozen on dry ice and coronally sectioned on a freezing microtome at 30 μm. Slices were collected in 10 mM PBS. Brain sections were rinsed in 0.3 % hydrogen peroxide in PBS for 15

minutes to block endogenous peroxidase activity and then washed six more times in PBS to remove residual hydrogen peroxide. Sections were blocked in the immunoreaction buffer (10 mM PBS containing 0.25 % Triton-X 100 and 3% normal goat serum) for 2 hours. Sections were incubated in primary antibody recognizing the cFOS peptide (Oncogene Science Inc., Cambridge, MA) for 72 hours at 4 ^{0}C. This antibody recognizes amino acid residues 4-17 of human cFOS and was raised in a goat. Chan and Sawchenko (1995) have shown that specific staining with this FOS antisera is blocked following preadsorption overnight with 100 μM of synthetic immunogen.

Sections were rinsed three times in 10 mM PBS and incubated with biotinylated goat anti-rabbit IgG (1:200) in 10 mM PBS containing 0.3 % Triton-X 100 and 3% goat serum for 2 hours at room temperature. Sections were incubated with horseradish peroxidase avidin-biotin complex in 10 mM PBS for 2 hours at room temperature. Sections were rinsed in PBS and placed in 0.1 M Tris (pH 7.4) for 5 min. Sections were incubated in a solution containing diaminobenzidine (50 mg/100ml), 0.01 % nickel ammonium sulfate, and 0.035% hydrogen peroxide to complete the chromatic reaction. Sections were mounted onto slides, dehydrated, and cover-slipped in Permount.

FOS positive cells were quantified using an Olympus BX60 light microscope. Images were captured at 10 X magnification through a Spot Insight digital color camera. The mouse atlas by Paxinos and Franklin (1997) was used to identify brain regions. Common landmarks (e.g., anterior commissure) were identified on the tissue sections to ensure comparable brain regions between

mice for analysis (Paxinos, 1997). Image analysis was performed using ImagePro Plus software. Immunoreactivity was measured using the number of Fos positive cells for a defined area of interest. FOS positive cells were generated by manually outlining the immunoreactivity present in brain regions showing positive staining.

Data analysis . ANOVA was used to determine the effects of strain and treatment condition on the number of FOS positive cells in each brain region. Post-hoc tukey's test were conducted where appropriate. Significance level was set at $\alpha = 0.05$. Two D2 ethanol withdrawn mice were removed from the analysis because of the occurrence of overt convulsions to avoid cause versus effect issues in cFOS expression. In addition, because animals were killed in proximity to other ethanol withdrawing animals, the order in which each mouse was killed was examined to rule out potential confounds associated with environmental stress on cFOS expression; no consistent effects of this type of stress were observed.

RESULTS

The following results are divided into major regions of the brain, cortical, basal ganglia, limbic, thalamus, hypothalamus, and septum. In all the brain regions examined, there were no significant differences between control (air-pyrazole) B6 and D2 mice in FOS expression. A list of abbreviations used for brain regions is shown in Table 4-1.

Figure 4-1 shows cortical brain regions examined for expression of FOS positive cells in ethanol withdrawn B6 and D2 mice and corresponding control B6

Table 4-1

Abbreviation	Region
Aud cx	Auditory cortex
BLA	Basolateral amydgala
BMA	Basomedial amygdala
BNST	Bed nucleus of the stria terminalus
CA1	CA1 field of hippocampus
CA2	CA2 field of hippocampus
CA3	CA3 field of hippocampus
DG	Dentate gyrus
CeC	Central nucleus of amygdala (capsular)
CeL	Central nucleus of amygdala (Lateral)
CeM	Central nucleus of amygdala (Medial)
Cing cx	Cingulate cortex
CPu, dm	Caudate putamen, dorsal medial
Entorh cx	Entorhinal cortex
Hyp PVN	Hypothalamus paraventricular nucleus
Insular cx	Insular cortex
Lat sep	Lateral septum
Med sep	Medial septum
Motor cx	Motor cortex
NAc core	Nucleus accumbens core
NAc shell	Nucleus accumbens shell
Pir cx	Piriform cortex
PPta cx	Posterior parietal cortex
Prelimb cx	Prelimbic cortex
Sens cx	Sensory cortex
SN	Substantia nigra
Thal APVN	Thalamus anterior paraventricular nucleu
Thal MD	Thalamus mediodorsal nucleus

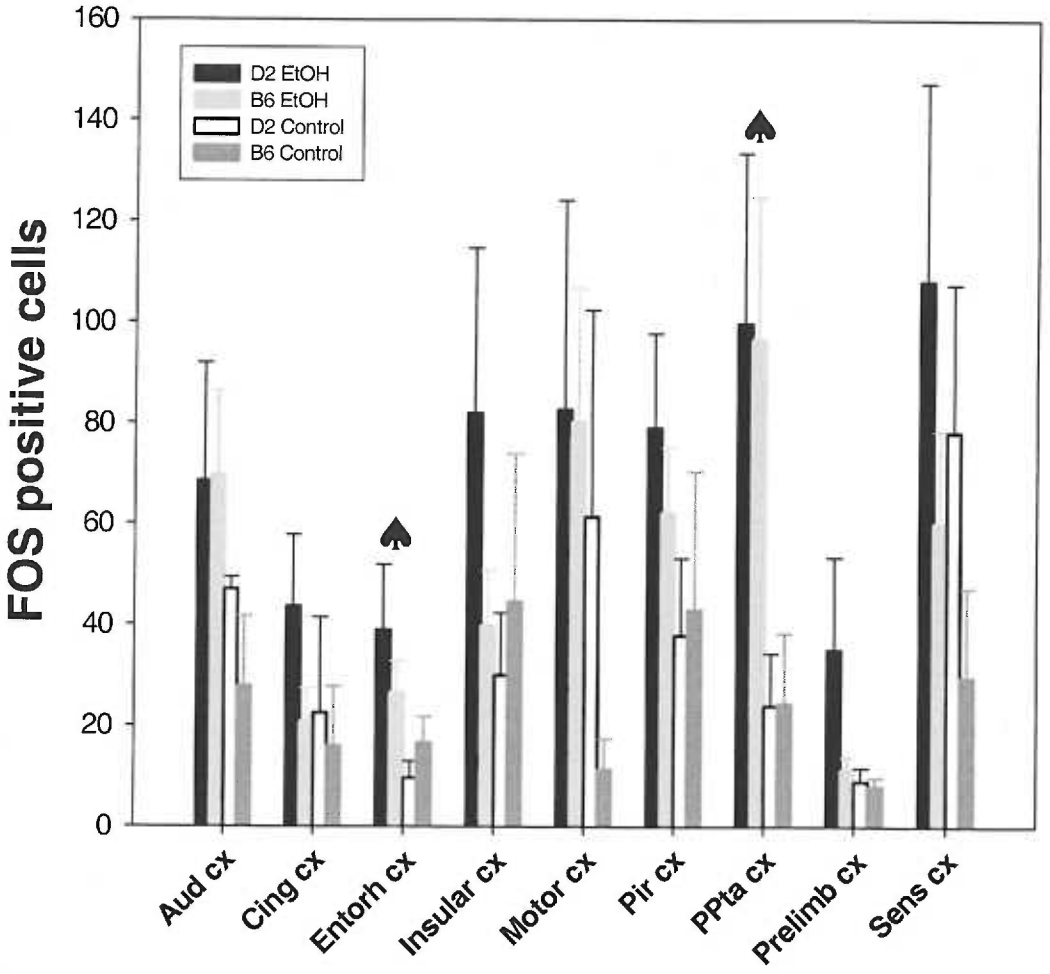

Figure 4-1: FOS positive cells in cortical regions. Figure shows ethanol withdrawn B6 (B6 EtOH n = 8-12) and ethanol withdrawn D2 (D2 EtOH n = 7-10) mice, and control B6 (B6 control n = 6) and D2 mice (D2 control n = 3-6). BEC in ethanol withdrawn B6 mice (1.64 ± 0.12). BEC in ethanol withdrawn D2 mice (1.64 ± 0.09). Enthorhinal and posterior parietal cortex show main effect of treatment (♠$p < 0.05$). Bars represent the mean ± S.E.M.

and D2. The ecthorhinal and posterior parietal cortex showed significant main effects of treatment $F(1, 28) = 5.0$, $p < 0.05$ and $F(1, 28) = 6.4$, $p < 0.05$, respectively. There were no main effects of strain or interactions in these regions. In the remaining cortical regions there were no significant effects of strain, treatment or strain by treatment interactions.

Figure 4-2 represents regions of the basal ganglia including the caudate putamen (dorsal medial division) and substantia nigra. A significant main effect of treatment was observed in the CPu, dm region $F(1, 28) = 6.2$, $p < 0.05$), but no strain or strain by treatment interaction. There was a significant strain by treatment interaction in the substantia nigra $F(1, 28) = 5.8$, $p < 0.05$, but no main effect of strain or treatment. However, post-hoc Tukey's test indicate no significant group differences.

Figure 4-3 shows limbic brain regions. Figure 4-3A shows regions of the hippocampus including the CA1, CA2, and CA3 fields along with the dentate gyrus. In the CA2 there was a significant main effect of strain $F(1, 27) = 4.4$, $p < 0.05$, but no main effect of treatment or strain by treatment interaction. In CA3 there was a significant main effect of treatment $F(1,29) = 4.8$, $p < 0.05$, an almost significant main effect of strain $F(1, 29) = 3.9$, $p = 0.057$, but no treatment by strain interaction ($p = 0.11$). In the dentate gyrus there was a significant main effect of strain $F(1, 27) = 5.3$, $p < 0.05$, and a strain by treatment interaction $F(1, 27) = 5.3$, $p < 0.05$. Post hoc Tukey's test showed that D2 ethanol withdrawn mice have significantly greater number of FOS positive cells than B6 ethanol withdrawn mice ($p < 0.01$); Tukey post-hoc analysis also showed that D2 ethanol

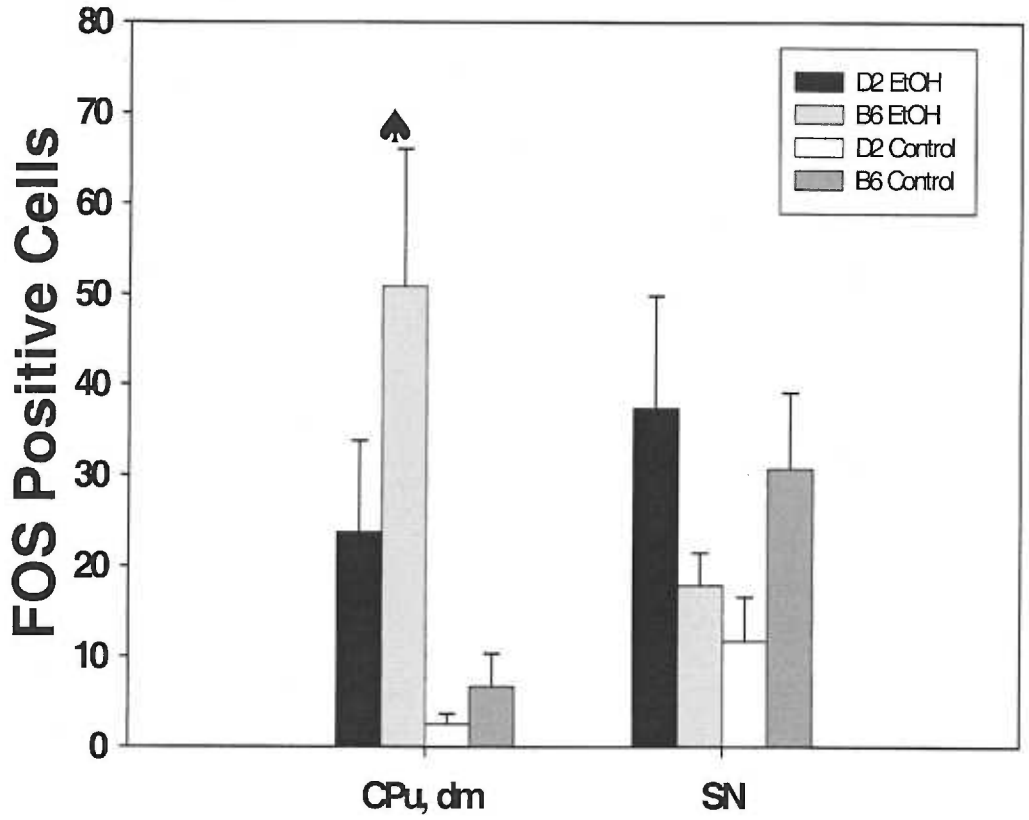

Figure 4-2: FOS positive cells in the basal ganglia. Figures show ethanol withdrawn B6 (B6 EtOH n = 8-12) and ethanol withdrawn D2 (D2 EtOH n = 7-10) mice, and control B6 (B6 control n = 6) and D2 mice (D2 control n = 3-6). BEC in ethanol withdrawn B6 mice (1.64 ± 0.12). BEC in ethanol withdrawn D2 mice (1.64 ± 0.09). The dorsal medial divison of the caudate putamen showe a significant main effect of treatment (*$p < 0.05$). Bars represent the mean ± S.E.M.

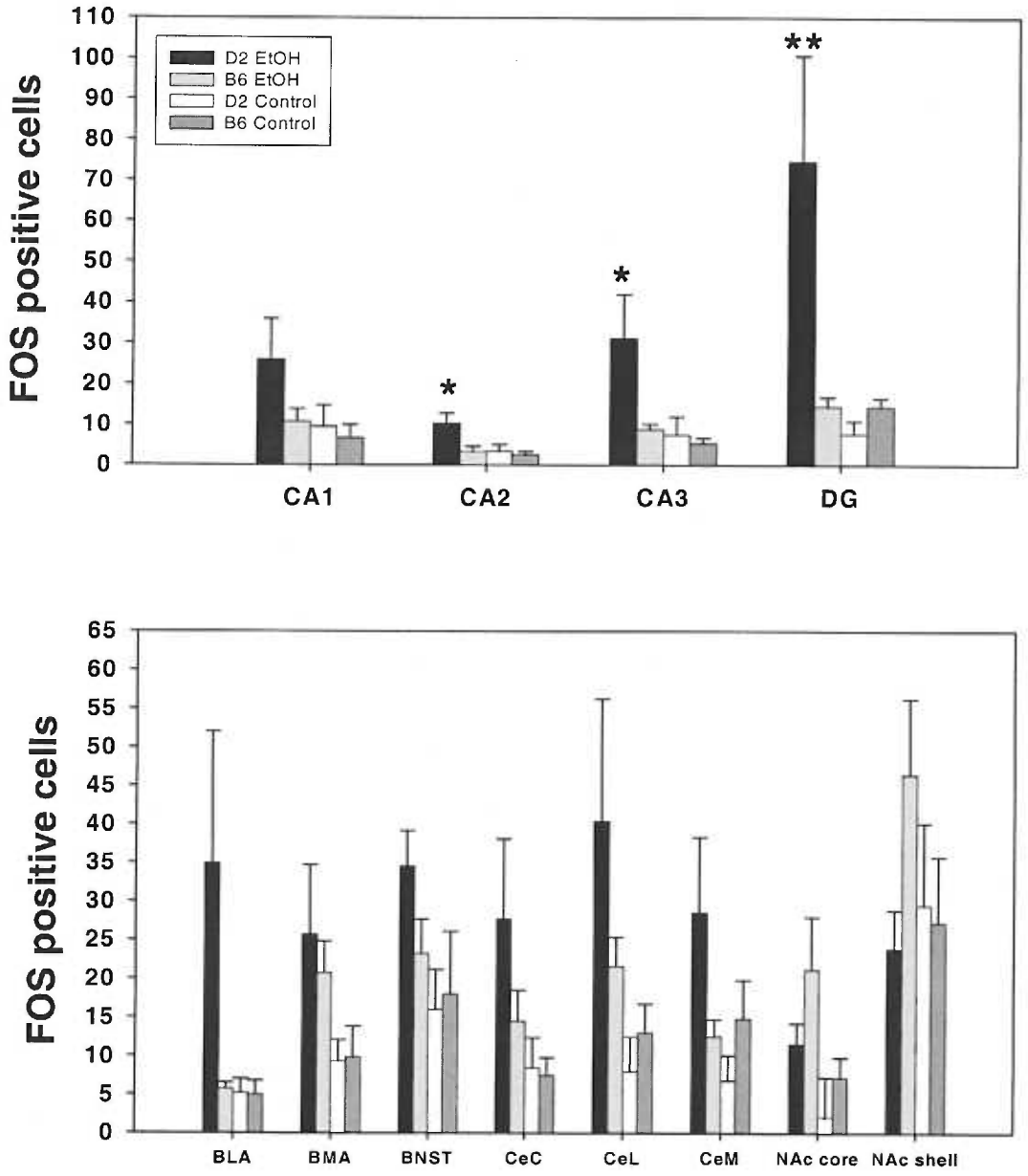

Figure 4-3: FOS positive cells in limbic system. Figures show ethanol withdrawn B6 (B6 EtOH n = 8-12) and ethanol withdrawn D2 (D2 EtOH n = 7-10) mice, and control B6 (B6 control n = 6) and D2 mice (D2 control n = 3-6). BEC in ethanol withdrawn B6 mice (1.64 ± 0.12). BEC in ethanol withdrawn D2 mice (1.64 ± 0.09). **(A):** Figure represents regions of the hippocampus including CA1, CA2, and CA3 fields and the dentate gyrus. D2 ethanol withdrawn mice show a greater number of FOS positive cells in the dentate gyrus compared to B6 ethanol withdrawn mice (**$p < 0.01$). There were significant main effects of strain in CA2 and CA3 ($p < 0.05$). **(B):** Figure represents regions within the extended amygdala including the basolateral and central amygdala as well as the nucleus accumbens. There was a significant main effect of ethanol treatment in the capsular and lateral divisions of the central nucleus of the amygdala (*$p < 0.05$). Bars represent the mean ± S.E.M.

withdrawn mice have a significantly greater number of FOS cells compared D2 control mice ($p < 0.05$).

Figure 4-3B shows regions within the extended amygdala. There was a significant main effect of treatment in the basomedial amygdala $F(1, 28) = 4.2$, $p < 0.05$, but no effects of strain or strain by treatment interaction. There was a significant main effect of treatment in the lateral division of the central nucleus of the amygdala $F(1, 28) = 5.1$, $p < 0.05$, but no effects of strain or strain by treatment interaction. There was a significant strain by treatment interaction in the medial division of the central nucleus of the amygdala $F(1, 28) = 4.2$, $p < 0.05$, but no main effects of strain or treatment. However, post-hoc Tukey's test indicate no significant group differences.

Figure 4-4 shows results from the thalamus and hypothalamus. The paraventricular nucleus of the hypothalamus showed a significant main effect of strain $F(1, 28) = 6.2$, $p < 0.05$, but no effect of treatment or strain by treatment interaction. There were no significant main effects or interactions observed in the thalamus.

Figure 4-5 shows results from the septum, both the lateral and medial divisions. There were no significant main effects or interactions observed in either of these regions.

Figure 4-6 show representative photomicrographs of FOS expression in ethanol withdrawn B6 and D2 mice. D2 ethanol withdrawn mice show a greater number of FOS positive cells in the dentate gyrus (Figure 6B) as well as a greater number of FOS positive cells in the paraventricular nucleus of the

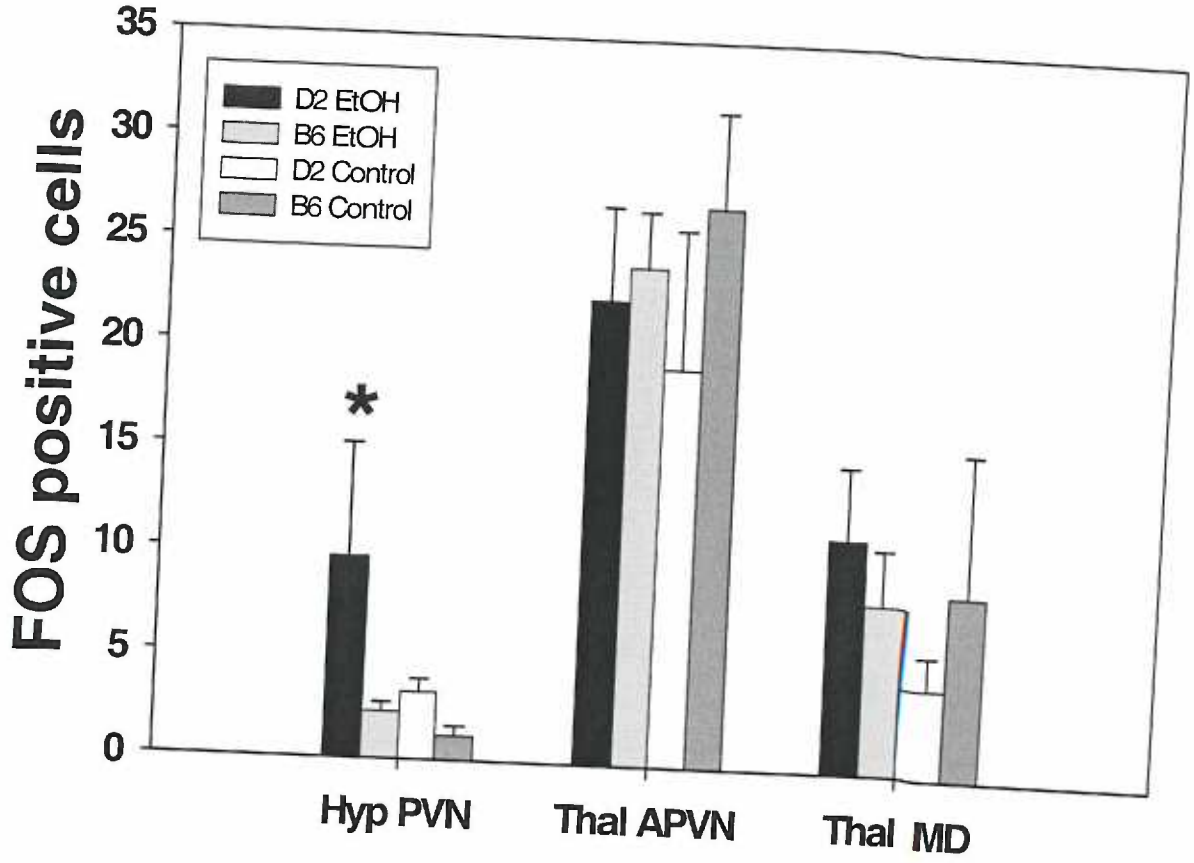

Figure 4-4: FOS positive cells in the thalamus and hypothalamus. Figures show ethanol withdrawn B6 (B6 EtOH n = 8-12) and ethanol withdrawn D2 (D2 EtOH n = 7-10) mice, and control B6 (B6 control n = 6) and D2 mice (D2 control n = 3-6). BEC in ethanol withdrawn B6 mice (1.64 ± 0.12). BEC in ethanol withdrawn D2 mice (1.64 ± 0.09). There was a significant strain effect detected in the paraventricular nucleus of the hypothalamus (*$p < 0.05$). Bars represent the mean ± S.E.M.

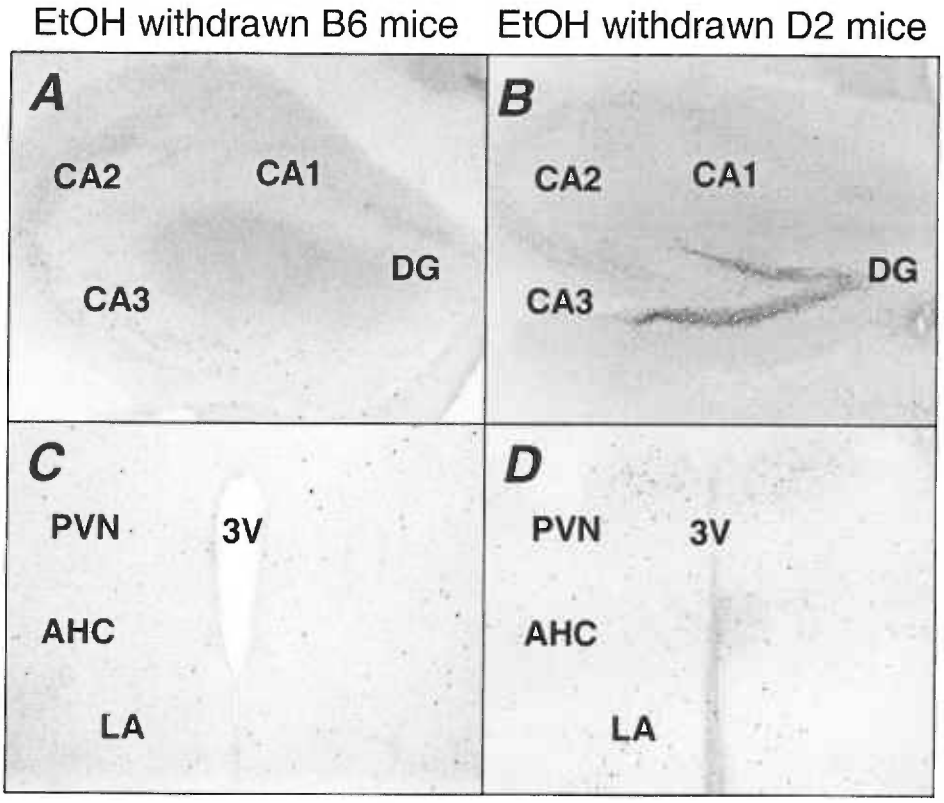

Figure 4-6: Photomicrographs (10X) showing FOS positive cells in ethanol withdrawn B6 and D2 mice. **(A):** Hippocampus in B6 ethanol withdrawn mice, **(B):** Hippocampus in D2 ethanol withdrawn mice, **(C):** Paraventricular nucleus of hypothalamus in B6 ethanol withdrawn mice, **(D):** Paraventricular nucleus of hypothalamus in D2 ethanol withdrawn mice. Abbreviations: 3V (3rd ventricle); AHC (anterior hypothalamus) CA1, CA2, CA3 (fields of hippocampus); DG (dentate gyrus); LA (lateral hypothalamus); PVN (paraventricular nucleus of hypothalamus).

hypothalamus (Figure 6D) compared to B6 ethanol withdrawn mice.

DISCUSSION

The brain is elaborately organized in functional systems and circuits. To understand brain function it is essential to understand this organization and connectedness. In the present study we have attempted to uncover brain circuits associated with genetic variation in alcohol withdrawal severity using the immediate early gene, *cfos*, as a marker of neuronal activation. The major finding is that severe ethanol withdrawal in D2 mice is associated with activation of an extended limbic circuit which includes the hippocampus and hypothalamus.

One of the key advantages of using FOS expression as a means to identify brain regions activated during alcohol withdrawal is the identification of functional circuits underlying the patterns of activation observed. Here we have found activation of an extended limbic circuit which contains regions previously grouped into the extended amygdala (Koob and Le Moal, 2001). These regions of the extended amygdala have cytoarchitectual, circuit and functional similarities that have been hypothesized to form a component of the brain reward circuit. Brain regions that modulate and feed into the extended amygdala include the hippocampus, hypothalamus, and ventral tegmental area. While regions within the extended amygdala project to orbito-frontal, dorsolateral prefrontal, and cingulate cortex. G.F. Koob has put forth allostasis as a model of drug dependence and addiction (Koob and Le Moal, 2001). Allostasis, in contrast to homeostasis, is the maintenance of stability outside of the normal homeostatic range (i.e., around a new setpoint) (Koob and Le Moal, 2001). The extended

amygdala along with its afferent and efferent connections have been identified as potential neural substrates for the positive and negative reinforcing effects of drugs of abuse and this circuit appears to be necessary for allostatic changes in reward function (Koob and Le Moal, 2001; Koob, 1999). Because we find activation of some regions in this circuit, one interpretation of our results fits with an allostatic model and suggest that genetic variation differentially affects activation of this critical 'addiction' circuit in the brain.

Despite the numerous procedural, animal model, and method of ethanol administration differences between studies of FOS expression in ethanol withdrawn animals, there is considerable consistency in the brain regions activated during withdrawal. A common theme is that regions of the limbic system including central amygdala, hippocampus, and hypothalamus show activation during ethanol withdrawal (Knapp et al., 1998, 2001; Moy et al., 2000; Ryabinin et al., 1997; Bouchenafa and Littleton, 1998; Morgan et al., 1992). The present study also identifies similar regions, but adds that activation of this extended limbic circuit is dependent on genotype. In contrast to studies examining chronic ethanol administration, preliminary studies from our laboratory have identified activation of an extended basal ganglia circuit as being associated with severe alcohol withdrawal in D2 compared to B6 mice after acute alcohol withdrawal (Buck et al., in preparation). Thus, acute and chronic alcohol withdrawal are not monolithic phenomena; this suggests that additional neuroadaptations occur during chronic exposure to alcohol which recruits different brain circuits compared to acute withdrawal challenges. This in fact is

consistent with the finding that there is not a complete overlap between QTL (either in effect size or location) for acute and chronic alcohol withdrawal severity in crosses between B6 and D2 mouse strains (Buck et al., 1997; Crabbe, 1998, Buck et al., 2002).

The region that showed by far the greatest activation in ethanol withdrawn D2 mice was the dentate gyrus. The dentate gyrus has been identified as a key structure associated with the spread of seizure discharges in temporal lobe epilepsy (Heinemann et al., 1992). Furthermore, the dentate gyrus is thought to have frequency filtering properties which are altered in several epilepsy models (Behr et al., 1998). Our results identify the dentate gyrus as a critical structure underlying genetic differences in alcohol withdrawal convulsions and suggest that some of the same electrical frequency properties of the dentate gyrus are similarly altered with alcohol withdrawal. We have also identified brain regions that show no genotype dependent affect but are nonetheless activated by alcohol withdrawal. These regions include the posterior parietal and ecthorhinal cortex, caudate putamen, central nucleus of the amygdala (lateral division) and basomedial division of the amygdala.

An alternative interpretation of the present findings is that activation of the extended limbic circuit may be due to non-specific effects of stress rather than alcohol withdrawal *per se*. This is not a likely possibility for a number of important reasons. Ryabinin et al., 1999 have reported significant differences between B6 and D2 mice after repeated handling and injection stress, where B6 mice show virtually complete habituation to handling stress after 2 weeks while

D2 mice do not (Ryabinin et al., 1999). In the present study both ethanol exposed and control B6 and D2 mice were subjected to repeated handling and injection stress over the 3 day duration of the experiment. However, control B6 and D2 mice show no difference in the number of FOS positive cells in some of the same brain regions tested by Ryabinin et al. (1999), which argues that B6 and D2 mice in the present study habituated to handling and injection stress to a similar degree. Therefore, the significant strain differences in ethanol withdrawal mice cannot be accounted for by differences in stress induced by handling or injection. However, the differences in FOS habituation between the present study (control B6 and D2 mice) and the study by Ryabinin et al. (1999) could be due to differences in duration of exposure to stress or differences in the type of stress (i.e., animals were administered pyrazole each day versus normal saline). In addition, we examined if the order in which mice were killed had an effect on FOS expression. We found that any stress associated with visual, auditory or olfactory stimuli present during killing of the mice had no consistent effect on FOS expression in either strain. Given these important factors our results argue for specific effects of ethanol withdrawal on FOS expression in B6 and D2 inbred mouse brain.

As noted previously, many studies have examined the effect of ethanol exposure and withdrawal on the induction of FOS in a number of animal models (Knapp et al., 1998, 2001; Moy et al., 2000; Ryabinin et al., 1997). However, the effects of genetic variation in alcohol withdrawal severity and the specific brain regions activated as a result of this genetic variation have only just begun to be

appreciated. Until now, only one study has examined the effects of genetic differences in alcohol withdrawal severity and induction of FOS. Olive et al. (2001) examined FOS expression in the brains of ethanol withdrawn PKCε null mutant mice compared to wild-type C57BL/6J x 129SvJae mice. Overall, their results suggested that less severe ethanol withdrawal severity in PKCε KO mice is associated with activation of the dentate gyrus, PVN of the hypothalamus, substantia nigra, and regions of the thalamus, whereas a reduction in FOS activation of the lateral septum was associated with less severe withdrawal severity in these mice (Olive et al., 2001). We find almost opposite results; the dentate gyrus and hypothalamus were associated with severe ethanol withdrawal in our study versus being associated with less severe ethanol withdrawal in the study by Olive et al., 2001. This may be explained by a number of possibilities. First, as with all null mutant studies caution in interpretation needs to be taken with regard to developmental compensations that may occur as a result of gene deletion and complications associated with genetic background (Phillips et al., 1999). Due to the null mutant targeting constructs used in generation of traditional KO mice, a small but significant amount of extra genetic material from the strain used in the ES cells surrounding the knocked out gene is usually carried along with the null mutation. This extra genetic material in the knock out region could thus have unexpected effects on the phenotype (Gerlai, 1996). In addition to these limitations of knockout studies, Olive et al., 2001 used a liquid diet procedure to induce physical dependence which is different from the inhalation procedure we used. Also, Olive et al. (2001) measured FOS protein

expression following handling-induced convulsions in their mice, while the mice used in our analysis had no overt convulsions. Finally, differences in FOS expression between the two studies could be due to differences in genotype and the intensity of withdrawal. Therefore, given these differences in our study and the study by Olive et al. (2001) it is not surprising that such strikingly opposite results were obtained.

Our results suggest several future directions. First, because the phenotype of the cells which show FOS expression are not known, it will be important to perform co-localization studies with FOS and makers of dopaminergic, glutmaterigic, GABAergic and serotonergic neurotransmission. For example, Thiele et al., 2000 used double-labeling immunohistochemistry and found that ethanol induced FOS expression was restricted to catecholamine and neuropeptide Y containing neurons in the rat brainstem (Thiele et al., 2000). Others have performed similar experiments and found that ethanol-induced FOS expression is present in pre-pro-enkephalin and GABAergic expressing neurons of the central nucleus of the amygdala (Criado and Morales, 2000). Another important and similar future direction would be to examine the pharmacological specificity of FOS induction by ethanol withdrawal. For example, drugs could be administered to mice during ethanol exposure or during withdrawal to try to block the induction or expression of physical dependence. Morgan et al., 1992 found that the dentate gyrus and piriform cortex are selectively active during ethanol withdrawal and this activation is blocked by administration of a NMDA receptor antagonist (Morgan et al., 1992). Finally, because the choice of immediate early

gene used to map brain activation (e.g., cFOS versus FosB) has been shown to affect results (Ryabinin and Wang, 1998), it will be important to examine expression of other immediate early genes. For example, in contrast to cFOS, FosB and Δ FosB are generally activated under chronic challenges (Kovacs, 1998), and persist long after drug exposure is discontinued (Nestler et al., 2001). Therefore, FosB and ΔFosB could be examined during extended ethanol withdrawal to identify brain regions that may be activated during periods when individuals are susceptible to relapse.

In conclusion, the present study identifies marked strain differences in activation of an extended limbic circuit after chronic alcohol exposure and withdrawal. These results have specific implications for understanding genetic differences in neurocircuitry activated during drug withdrawal and drugs that modulate these circuits could be used to treat drug addiction.

Chapter V

Summary and Overall Discussion

A key outcome of this work is the identification of potential mechanisms in which a quantitative trait gene affects a complex genetic trait. This is the first attempt at linking a gene underlying an addiction QTL to its function. As other quantitative trait genes are identified, this work will be useful as a model for understanding other quantitative trait genes. This work has *translational* importance; *Mpdz* could be used as a marker of susceptibility for the development of alcohol-related problems in humans or drugs could be designed that disrupted *Mpdz*'s interaction capabilities altering its function on alcohol withdrawal. Given the pleiotropic nature of this gene, investigation into *Mpdz*'s role in other addictions, other aspects of alcoholism and seizure disorders is warranted. Finally, *Mpdz* is an ideal candidate to study epistasis because of its ability to interact with numerous intracellular proteins.

In a recent review article by Phillips et al., 2002 the authors highlighted the need to move from studying single genes in a piecemeal fashion to more integrative approaches to complex trait genetics such as the influence of pleiotropism and epistasis (Phillips et al., 2002). A major aim of the present work was to examine the influence of *Mpdz*, a quantitative trait gene for acute alcohol withdrawal severity (Fehr et al., 2002) on other convulsion phenotypes. One traditional method for investigating pleiotropism is by selective breeding and examining correlated responses to selection. However, this classical genetic approach only allows one to make broad statements about the overlap of

particular genes that underlie multiple phenotypes because the genes themselves are not known (Crabbe et al., 1990b). In the present study we used a different approach. In a congenic strain, at least some information is known about specific genes within the introgressed genomic interval. Although the introgressed interval contains more than one gene, candidate genes can be eliminated based on genotype dependent sequence and expression differences. After testing our chromosome 4 congenic strain for handling-convulsions modulated by several convulsant drugs, we find marked differences compared to the D2 background strain in convulsions altering glutamatergic and glycinergic neurotransmission (but not GABAergic neurotransmission). Because we have very convincing evidence that *Mpdz* is the actual quantitative trait gene underlying the QTL for acute alcohol withdrawal (Fehr et al., 2002, Shirley et al., 2004) the difference in the congenic versus the D2 background strain in convulsion severity by glutamatergic and glycinergic drugs suggests that genetic variation in *Mpdz* also regulates these phenotypes. *Mpdz* is also likely to regulate handling-convulsion severity through serotonin type 2C receptor because of the large difference between B6 and D2 mice after administration of a serotonin type 2C receptor antagonist.

Other complex traits map to mid chromosome 4 where *Mpdz* is contained. What is particularly interesting is that a QTL for Morris water maze learning maps almost directly on top of *Mpdz* (Steinberger et al., 2003). Other PDZ domain proteins such as PSD-95 that are present in high abundance at glutamatergic synapses and couple to NMDA receptor, are known to regulate learning and

memory behaviors (Sanes and Lichtman, 1999). Because neuroadaptations to drugs of abuse represent a form of synaptic plasticity similar in some ways to plasticity associated with learning (Nestler, 2001), it is very attractive at this point to speculate that *Mpdz* may have an influence on this form of learning and memory. If this is true, then this would open up a whole new area to examine the effects of *Mpdz* in relation to alcoholism in the form of regulation of cognition (Larimer ,1999). *Mpdz* could then be implicated in things such as cognitive related effects on susceptibility to relapse and the strength of associative cues which trigger relapse episodes (Larimer, 1999).

This work demonstrates the utility of the QTL approach for identifying genes underlying complex genetic traits. Some have argued that the ultimate proof of a quantitative trait gene is demonstrating that replacement of the variant nucleotide results in the predicted phenotypic effect. Although technically difficult, this can be demonstrated using 'knock-in' technology or creation of a bacterial artificial chromosome (BAC) transgenic (Glazier et al., 2002). We are currently developing a BAC transgenic to test the influence of *Mpdz* on alcohol withdrawal convulsions and other related behaviors (Buck et al., unpublished). However, this thesis used a composite approach to provide additionally strong evidence that *Mpdz* is the quantitative trait gene, which is also accepted as proof of a quantitative trait gene (Glazier et al., 2002; Abiola et al., 2003). For example, the protein product of the quantitative trait gene must show expression in relevant cell types and tissues, the quantitative trait gene must show genotype dependent sequence and expression differences, and demonstration of

mechanism supporting the causal relationship between the quantitative trait gene and its function on the phenotype. We show here that (1): MPDZ is expressed in critical brain regions associated with genetic differences in alcohol withdrawal severity and is co-localized with 5-HT_{2C} receptors in these same brain regions, (2): MPDZ protein is differentially expressed in congenic versus D2 background strain mice, providing evidence that *Mpdz* is *cis*-regulated, and (3): genetic variation in *Mpdz* modulates alcohol withdrawal severity, in part, through 5-HT_{2C} receptors.

As more and more quantitative trait genes are discovered it is becoming increasingly evident that coding variants (i.e., allozymes) are responsible for only a fraction of the quantitative variation in a complex trait (Farrall, 2004). Rather, *Cis*-regulation, whereby genetic variation in non-coding, regulatory regions (i.e., gene promoters, *cis*-elements) affecting gene expression levels is responsible for most of the quantitative variation in a complex trait (Farrall, 2004). Papers regarding this issue of *cis*-regulation are sprouting up in the literature and it is evident that this type of regulation is not only common to the mouse (Cowles et al., 2002) but it is also present in the human genome (Lo et al., 2003; Pastinen et al, 2004; Bray et al., 2003). This type of phenomenon was predicted over 30 years ago by King and Wilson (1975). Humans and chimpanzees share about 99% identity in protein sequences, which does not explain the vast differences in behavior and anatomy that has evolved since these two species diverged over 5 million years ago (King and Wilson, 1975). Therefore, regulation of gene

expression by *cis*-regulation for example must account for at least some of these species differences.

We find that *Mpdz* undergoes *cis*-regulation. In addition to the 10 single nucleotide polymorphisms in the coding region (Fehr et al., 2002), *Mpdz* contains sequence variation in regulatory regions that affects its expression (Fehr et al., 2002). Because of the importance and common occurrence of this type of regulation in causing variation in susceptibly to human disease, this provides additional evidence that *Mpdz* is the actual quantitative trait gene. This is not to say, however, that *trans*-acting factors will not be important for influencing susceptibility to human disease. The combination of expression profiling experiments and QTL mapping studies has shown that *cis*- and *trans*-acting factors do not act in isolation but are organized in complex genetic networks which is a molecular correlate of pleiotropy (Hitzemann et al., 2003; Tabakoff et al., 2003; Schadt et al., 2003). It can be predicted from the data generated in this thesis that *Mpdz* will likely participate in *trans*-regulation of various other genes. Candidate *trans*-regulated genes evident from these studies include NMDA receptors and glycine receptors. However, the serotonin type 2C receptor is not *trans*-regulated by *Mpdz* which is surprising based on the defined direct protein interaction between these two proteins (Becamel et al., 2001; Parker et al., 2003). A plausible alternative is that *Mpdz* might *trans*-regulate some other protein associated with 5-HT_{2C} receptors (Becamel et al., 2002).

Other genes that could potentially interact with *Mpdz* to influence alcohol dependence comes from examination of candidate genes contained within

previously mapped QTL for acute alcohol withdrawal (Buck et al., 1997). Of particular note are two very compelling candidate genes on distal chromosome 1, *kcnj9* and *kcnj10*, which encode G-protein coupled inwardly rectifying potassium channels (GIRKs) (Buck et al., 2002). Both of these genes encode proteins with PDZ recognition motifs at their carboxy-terminus and thus have the capability to directly interact with *Mpdz*. Members of this protein family of channels are known to be targets for the actions of alcohol (Lewohl et al., 1999) and are differentially expressed in frontal cortex of alcoholics versus controls (Lewohl et al., 2000). Data from our group also shows that *kcnj9* is differentially expressed in our mouse mapping populations and contains a regulatory sequence polymorphism which affects gene expression (Hitzemann, RR unpublished data). Another interesting link in this potential network is that the differences in behavioral effects (abuse liability versus anti-craving effects) of $GABA_B$ receptor agonists (γ-hydroxy-butyric acid versus baclofen) on the mesolimbic dopamine reward system are due to variation in the combination of GIRK channel proteins that are expressed (e.g., *kcnj3/kcnj6* versus *kcnj3/kcnj9* combinations) (Cruz et al., 2004). Because preliminary evidence suggests that *Mpdz* might interact directly with $GABA_B$ receptor R2 subunits (Milligan and White, 2001) and because of the potential interactions between *Mpdz* and GIRK channels, this suggests a protein network that might regulate dependence. A plausible hypothesis is that genetic variation in *Mpdz* may differentially couple $GABA_B$ and GIRK channels to regulate differences in susceptibility to addiction. This could be tested in a cellular model system where cell lines or primary neuronal cultures expressing $GABA_B$ receptors

and GIRK channels are transfected with the different variants of *Mpdz*. Then electrophysiological responses from GIRK channels or K channels coupled to $GABA_B$ receptors could be measured. In addition, preliminary evidence suggests that that expression of *Mpdz* is highly correlated with expression of aquaporin subtype 4 (*aqp4*) in BXD recombinant inbred mouse strains (http://webqtl.org/cgi-bin/WebQTL.py). AQP4 has a PDZ recognition motif at its carboxy-terminus and members within this family are differentially regulated in alcoholic versus control brains (Lewohl et al., 2000).

We have identified brain regions and circuits differentially activated during withdrawal from chronic alcohol exposure in B6 compared to D2 mouse brain. Severe alcohol withdrawal in D2 mice is associated with activation of an extended limbic circuit. Others have argued that this circuit may be crucial for allostatic adaptations associated with dependence on drugs of abuse (Koob and Le Moal, 2001), and therefore our findings show that genetic variation in activation of this circuit may be critical for these allostatic adaptations to alcohol. Our results also suggest that genetic variation in *Mpdz* influences, in part, withdrawal from chronic alcohol exposure. It therefore will be important to determine if *Mpdz* is expressed in this circuit, which preliminary data suggests (Eshelman et al., unpublished data; Reilly et al., unpublished data). There is some overlap in the genomic locations associated with acute and chronic alcohol withdrawal, and *Mpdz* appears to be one of the common genes. Distal chromosome 1 is a particularity interesting genomic 'hot-spot' because QTL for both acute and chronic alcohol withdrawal (and other behaviors associated with

drugs of abuse) map to this same region (Buck et al., 1997; 2002). Very compelling candidate genes, as mentioned above, for the chromosome 1 QTL are *kcnj9* and *kcnj10* (Buck et al., 2002). Therefore, it will also be important to determine if *Mpdz* and *kcnj9* and *kcnj10* co-localize in the extended limbic circuit.

The Future

Complex traits are controlled by many genes and environmental factors. The inheritance of complex traits does not follow the simple rules of Mendelian genetics (Falconer and MacKay, 1996). The principal reasons are because the relationship between genetic variation and phenotypic variation is *non-linear*, and the relevant genes do not interact additively. Prime examples of the inherent non-linearity of complex traits are phenomenon like pleiotropism (i.e, a gene can contribute to the emergence of more than just one phenotype), epistasis (gene-gene interaction), and gene-environment interactions. Moving forward in a field such as complex trait genetics requires the adoption of a systems based approach, with the realization that the whole (the phenotype) does not equal the sum of its parts (the genes underlying a complex trait). Reductionist approaches to molecular biology and genetics have dominated scientific thought for half a century, with the central dogma of molecular biology (DNA $\rightarrow$ RNA $\rightarrow$ Protein) at the corner stone of this debate. However, once the specific genes underlying a particular complex trait are known, the next challenge will be a new synthesis with specific emphasis on the emergent properties that constitute the phenotype but are not derivable from the components themselves. These emergent properties will be evident in the relationships, patterns and *interactions* between

the components of the system (McClearn, 1993). This new synthesis is already being realized with the advent of the 'omics' revolution in many fields of biology. The mining of the vast amount of information in the human and mouse genome will undoubtedly uncover new and exciting insights to the genetics of alcoholism. But the post-genomic era in which we are now entering will surely see the utilization of strategies designed to understand biology on more global scales (Huang, 2000).

The field of alcoholism research has moved from attributing the effects of alcohol on disruption of membrane fluidity (Buck et al., 1989) to alterations in specific neuronal proteins (Mihic et al., 1997). The effects of genetic variation on key targets of alcohol are just starting to be realized. *Mpdz* will no doubt be an important mediator of the effects of alcohol on the brain, and this work indeed has shown how genetic variation in this gene can affect responses to alcohol at multiple levels. This work has set the stage to investigate hypotheses of alcohol dependence with emphasis on pleiotropism and epistasis, which are two phenomena that *Mpdz* is sure to play a crucial role.

Chapter VI

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