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MPDZ EXPRESSION IN THE CAUDOLATERAL SUBSTANTIA NIGRA PARS RETICULATA IS CRUCIALLY INVOLVED IN ALCOHOL WITHDRAWAL

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Abstract

Association studies implicate the multiple PDZ domain protein (MUPP1/MPDZ) gene in risk for alcoholism in humans and alcohol withdrawal in mice. Although manipulation of the *Mpdz* gene by homologous recombination and bacterial artificial chromosome transgenesis has suggested that its expression affects alcohol withdrawal risk, the potential confounding effects of linked genes and developmental compensation currently limit interpretation. Here, using RNA interference, we directly test the impact of *Mpdz* expression on alcohol withdrawal severity and provide brain regional mechanistic information. Lentiviral-mediated delivery of *Mpdz* short hairpin RNA (shRNA) to the caudolateral substantia nigra pars reticulata significantly reduces *Mpdz* expression and exacerbates alcohol withdrawal convulsions compared to control mice delivered a scrambled shRNA. Neither baseline nor pentylenetetrazol enhanced convulsions differed between *Mpdz* shRNA and control animals, indicating that *Mpdz* expression in the caudolateral substantia nigra pars reticulata does not generally affect seizure susceptibility. To our knowledge, these represent the first *in vivo Mpdz* RNA interference analyses, and provide the first direct evidence that *Mpdz* expression impacts behavior. Our results confirm that *Mpdz* is a quantitative trait gene for alcohol withdrawal and demonstrate that its expression in the caudolateral substantia nigra pars reticulata is crucially involved in risk for alcohol withdrawal.

Keywords

Mpdz; MUPP1; alcohol; substantia nigra; RNA interference; withdrawal; mouse; HICs; lentivirus; genetics

Introduction

Alcohol is widely abused for its euphoric and sedative effects, and dependence on alcohol (alcoholism) has tremendous cost to afflicted individuals and society. A multitude of twin and adoption studies demonstrate that risk for alcoholism is 50-65% genetically determined (Kendler, 2009, Palmer, 2012, Reich, 1999). Unfortunately, genetic determinants of risk remain largely unknown, hindering effective prevention and treatment of dependent individuals.

Although no animal model exactly duplicates alcoholism, models for specific factors, such as risk for physiological dependence and associated alcohol withdrawal (AWD) episodes,

which constitute a motivational force that perpetuates alcohol use/abuse and contribute to relapse (Little *et al.*, 2005), are useful to identify potential genetic determinants of liability in humans. Using a robust behavioral model of physiological dependence, positional cloning, and molecular analyses, we identified *Mpdz* as a putative quantitative trait gene (QTG) for AWD in mice, demonstrating allelic variation in sequence (structure) and expression, either one or both of which could contribute to the quantitative trait locus (QTL) phenotypic effect on predisposition to AWD (Fehr, 2002, Shirley, 2004). *Mpdz* and its human homolog (*MPDZ*) encode the multiple PDZ domain protein (MUPP1/MPDZ) (Simpson *et al.*, 1999, Ullmer *et al.*, 1998). As a member of the PDZ protein family, MUPP1 may impact the trafficking and function of the protein signaling complexes it scaffolds (Romero *et al.*, 2011).

Recent studies using conventional *Mpdz* knockout heterozygotes and bacterial artificial chromosome (BAC) transgenic mouse models further support an inverse relationship between *Mpdz* expression and AWD severity (Milner *et al.*, 2013), but cannot disentangle *Mpdz* actions from potential effects of developmental compensation and/or linked genes. Fortunately, RNA interference (RNAi) has emerged as a powerful approach to disentangle target gene effects from these potential confounds (Bahi & Dreyer, 2012). Further, RNAi spread beyond the microinjection site is minimal, thus limiting target gene knockdown to a discrete region to provide additional mechanistic information (Bahi & Dreyer, 2012). Success of the RNAi approach is therefore dependent upon targeting the appropriate brain region(s). Here, we target the caudolateral substantia nigra pars reticulata (clSNr). cFos induction analyses have implicated the SNr as involved in genetically determined differences in AWD in a manner dependent upon QTL status, and bilateral lesions of the clSNr, but not rostromedial SNr, significantly mitigate AWD using acute and repeated alcohol exposure models (Chen *et al.*, 2011b, Chen *et al.*, 2008), confirming that the clSNr itself (rather than fibers of passage) is intrinsically involved in AWD.

For these reasons, the present studies use an RNAi approach to directly and rigorously test the hypothesis that reduced *Mpdz* expression in the clSNr results in more severe AWD, without generally affecting seizure susceptibility. In both humans and mice, spontaneous convulsions are rare, but seizure threshold for some stimuli is reduced during AWD. The present studies assess AWD using the handling-induced convulsion (HIC), a quantitative index of AWD, which is highly correlated with other signs of AWD and substantially genetically determined (Crabbe *et al.*, 1991a, Crabbe *et al.*, 1983).

Materials and methods

Animals

A total of 80 male DBA/2J (D2) strain mice (Jackson Laboratories) were acclimated to our vivarium for 1-2 weeks. Mice were 50-57 days old at the time of surgery and behaviorally assessed starting at 70-77 days of age. Colony and procedure rooms were maintained on a 12 hr light/dark cycle with lights on from 06:00 to 18:00, and the temperature maintained at 21±1°C. All mice received water and food (LabDiet 5001Rodent Diet) *ad libitum*. All procedures were approved by the Oregon Health & Science University and VA Medical

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

Mpdz RNAi in mouse NS20Y cells

Five Sigma MISSION shRNA clones for *Mpdz*, designed and developed by the RNAi Consortium at the Broad Institute of MIT and Harvard, were additionally analyzed using BLAST to ensure specificity to reduce endogenous *Mpdz* mRNA expression in mouse neuroblastoma (NS20Y) cells compared to a control (scrambled) shRNA clone. Plasmid DNA (1 μg) was transfected into 60-70% confluent NS20Y cells using lipofectamine. Following selection for shRNA transfected cells (in 2 μg/ml puromyocin for two weeks), the cells were harvested and total RNA isolated. Relative *Mpdz* mRNA expression was assessed using a quantitative polymerase chain reaction (QPCR) assay as in previous work (Shirley, 2004). Knockdown of *Mpdz* mRNA expression was calculated relative to a scrambled control, which does not target any gene sequence in the mouse genome.

Intra-clSNr RNAi

Mice were anesthetized using isoflurane (induced at a flow rate of 1.5 liter/min oxygen, 4% isoflurane, and maintained at 0.9 liter/min oxygen, 2.5% isoflurane), injected with meloxicam (10 mg/kg ip, in 0.9% saline), and placed in a mouse stereotaxic instrument (Kopf Instruments). The skull surface was exposed and burr holes drilled at the appropriate coordinates. The clSNr coordinates were based on the Mouse Brain Atlas (Paxinos & Franklin, 2001) and empirically determined for D2 mice as follows: anterior-posterior (AP) = −3.1, medial-lateral (ML) = ±1.7, and dorsal-ventral (DV) = −4.5.

In order to use green fluorescent protein (GFP) to assess targeting and spread, the *Mpdz* shRNA and scrambled control were subcloned into the pLKO.1-hPGK-puro-CMV-TurboGFP vector, then packaged into lentivirus to a high titer (10^8 titer units [TU]/ml; Sigma). Microinjectors were created as in previous work (Lasek & Azouaou, 2010) by affixing a 33 gauge stainless steel hypodermic tube within a shorter 26 gauge stainless steel hypodermic tube, and were attached to polyethylene-20 tubing affixed to a 10 μl Hamilton syringe. Microinjectors were lowered bilaterally to the designated coordinates and 1 μl (up to 10^5 TU) per side microinjected over 5 min using an infusion pump, and left in place for an additional 10 min. During the two days post-surgery the mice received additional meloxicam and were monitored for potential weight loss, pain, and distress. Behavioral testing began 3 weeks later.

Baseline, and alcohol withdrawal enhanced HICs

Physiological dependence is characterized by the appearance of physical disturbances (withdrawal) after alcohol administration is suspended. Alcohol withdrawal induced seizures are a distinct measure of central nervous system (CNS) physiological dependence occurring in both humans, as well as rodents. McQuarrie & Fingl (1958), first reported that AWD is apparent in mice following a single hypnotic dose, and was later shown to be genetically determined (Crabbe *et al.*, 1991b). The detection and fine-mapping of *Mpdz* utilized this acute model (Buck, 1997, Fehr, 2002, Shirley, 2004), which is therefore used in the present

studies. Importantly, this acute AWD model assesses CNS sensitivity to ethanol, while chronic models can be confounded by tolerance (Crabbe *et al.*, 1991a).

AWD was assessed in two experiments (experiments 1 and 2). D2 mice were used because they demonstrate robust AWD using both acute and chronic models, allowing detection of increased *or* decreased AWD severity with experimental manipulation. Details of the AWD procedure using the 7-point HIC scale have been published (Chen *et al.*, 2011b, Metten & Crabbe, 2005). Individual mice and different genetic models can differ in baseline HIC scores. Therefore, three weeks after surgery, mice were scored twice (30 min apart) for baseline (pre-ethanol) HICs prior to AWD testing. Immediately thereafter, mice received a single hypnotic dose of ethanol (4 g/kg, i.p., 20% v/v in 0.9% physiological saline) and were scored hourly between 2 and 12 hr, and at 24 and 25 hr post-ethanol. In order to create an index of AWD that is independent of individual differences in baseline HIC scores and that reflects differences in withdrawal convulsion severity, post-ethanol HIC scores were corrected for the individual's average baseline HIC score as in our previous work (Chen *et al.*, 2008). Individual AWD severity scores were calculated as the area under the curve (AUC), which is calculated as a sum of the corrected HIC scores 2-12 hr post-ethanol as in our previous work (Chen *et al.*, 2011b).

Pentylenetetrazol (PTZ) enhanced HICs

One week later, the animals in experiment 2 were assessed for HICs in response to PTZ (30 mg/kg i.p.), which blocks $GABA_A$ receptor-mediated transmission. Previous studies show that the severity of PTZ-enhanced HICs is not influenced by prior testing for AWD or surgery (Chen *et al.*, 2008). This dose was used because it enhances HIC intensity without inducing other convulsions (e.g., tonic hindlimb extensor) associated with higher doses. Mice were scored for HICs at 10 time points from 1 to 65 min post-PTZ injection. PTZ enhanced HIC scores were corrected for individual differences in baseline HIC scores to create an index of response to PTZ independent of variability in individual baseline (pre-PTZ) scores. The PTZ enhanced HIC severity scores were calculated as AUC scores and in our previous work (Chen *et al.*, 2011b).

Histology and laser capture microdissection (LCMD)

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

Mpdz RNA expression

Total RNA was isolated from NS20Y cells using Qiagen RNeasy mini kit and from LCMD clSNr samples using the Ambion RNAqueous-Micro Kit. cDNA synthesis used a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), with preamplification (*in vivo* experiment only) using a TaqMan PreAmp Master Mix kit and probes for *Mpdz* and a control gene (*Reep5)*. *Reep5* was used because it is a highly stable reference gene expressed throughout the mouse brain (www.brain-map.org) (Kozell *et al*., 2009). Quantitative polymerase chain reaction (QPCR) utilized *Mpdz* and *Reep5* TaqMan probes (Mm00447849_m1 and Mm00492230_m1, respectively), and was performed on an ABI Prism7500 thermal cycler using TaqMan Universal PCR Master Mix and standard reaction conditions. A standard relative quantification method ($\Delta\Delta C_t$) was used which corrects for run to run variability by normalization of target and reference gene expression to calibrator sample expression (preamplified cDNA pooled from all samples) (Livak & Schmittgen, 2001, Shen & Johnson, 1997). The calibrator was set to "1" and gene expression in clSNr samples was evaluated in reference to the calibrator.

Statistical analyses

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

Results

Mpdz RNAi efficacy in mouse neuroblastoma cells

Table 1 summarizes the results for five *Mpdz* shRNAs tested for their efficacies to affect endogenous *Mpdz* expression in mouse NS20Y cells compared to a scrambled control. This validated negative control engages the RNAi pathway but does not target any sequence in the mouse genome. The TRCN0000103482 plasmid demonstrated the highest *in vitro* knockdown of *Mpdz* expression (i.e., a 77% reduction relative to the scrambled control), and was therefore utilized for the subsequent *in vivo* RNAi experiments.

Intra-clSNr Mpdz RNAi

Mpdz shRNA (TRCN0000103482) and a scrambled control shRNA were subcloned into pLKO.1-hPGK-puro-CMV and packaged in lentivirus to a high titer (10^8 TU/ml). Figures 1a and 1b show representative photomicrographs of the clSNr microinjection site at 4x and 10x magnification, respectively, evident by GFP expression. Figure 1c shows a representative LCMD tissue section of the clSNr, which were used for *Mpdz* mRNA quantification by QPCR (Fig 1d). Figure 1e is a schematic illustration of the extent of the bilateral clSNr microinjection sites and GFP expression spread, which extended from −3.1 to −3.8 mm (anterior-posterior) from Bregma, with occasional spread into the SN lateralis (Paxinos & Franklin, 2001).

Baseline and AWD enhanced HICs in Mpdz shRNA and control animals

Baseline (pre-ethanol) and AWD enhanced HICs were assessed in two experiments which were combined for increased statistical power. For those animals with confirmed bilateral targeting of the clSNr, *Mpdz* shRNA and control animals did not differ in baseline (pre-ethanol) HIC severity (mean score ± SEM = 0.5±1.1 and 0.2±0.6, n=14 and 16, respectively, $t_{1,28}$=0.99, p=0.33, NS). However, *Mpdz* shRNA mice demonstrated more severe AWD than controls ($t_{1,28}$=3.0, p=0.006, Fig 2). To our knowledge, these represent the first *in vivo Mpdz* RNAi analyses, and provide the first direct evidence that *Mpdz* expression impacts behavior. Taken together, our results conclusively prove that *Mpdz* is a QTG for AWD risk.

In animals in which clSNr targeting was unilateral only, neither baseline HICs (mean score ± SEM = 0.4±0.4 and 0.4±0.7, n=14 and 20, respectively; $t_{1,32}$=0.01, p=0.9) nor AWD severity (mean score ± SEM = 22.3±7.4 and 24.3±10.0, n=14 and 20, respectively, $t_{1,32}$=0.7, p=0.5, NS) differed between *Mpdz* shRNA and control animals. These results are consistent with our previous data showing that bilateral but not unilateral clSNr lesions mitigate AWD (Chen *et al.*, 2011b, Chen *et al.*, 2008)

PTZ enhanced HICs in Mpdz shRNA and control animals

In order to assess whether *Mpdz* RNAi might have more general effects on enhanced HICs beyond AWD, the animals in experiment 2 were assessed for PTZ enhanced HICs. *Mpdz* shRNA and control animals with confirmed bilateral targeting of the clSNr did not differ in baseline (pre-PTZ) HIC scores (mean score ± SEM = 1.1±1.5 and 1.4±1.7, n=8 and 7, respectively, $t_{1,13}$=0.36, p=0.7, NS; Fig 3a) or PTZ enhanced HIC severity ($t_{1,13}$=0.14, p=0.9, NS, Fig 3).

RNAi significantly reduces intra-clSNr Mpdz expression

In order to assess the impact of RNAi on target gene expression it is important to specifically examine the ROI using a robust, quantitative method. We therefore used LCMD followed by QPCR to determine expression of *Mpdz* in the clSNr. As indicated in Figure 2d,*Mpdz* shRNA significantly reduced *Mpdz* mRNA expression in LCMD-clSNr compared to control animals. *Mpdz* mRNA was reduced by 33% in experiment 1 (mean score ± SEM = 0.88±0.05 and 0.59±0.04, respectively, $t_{1,28}$ = 4.2, p = 0.0002). A significant reduction in *Mpdz* mRNA was also apparent in experiment 2 (mean score ± SEM = 1.5±0.07 and 1.2±0.08, respectively, $t_{1,28}$= 3.6, p = 0.001), and, not surprisingly, was somewhat smaller in magnitude (25% decrease) than in experiment 1 owing to the expected reduction in titer over time.

Discussion

Naturally-occurring genetic (allelic) variation affects *Mpdz/MPDZ* sequence and expression in mice and humans, but the functional and behavioral consequences remain largely unknown. Association studies implicate the mouse and human homologs in risk for alcohol physiological dependence and alcoholism (Fehr, 2002, Karpyak *et al.*, 2009, Shirley, 2004, Tabakoff, 2009). However, despite the recent creation of *Mpdz* transgenic and knockout heterozygote models (Milner *et al.*, 2013), direct evidence that *Mpdz* impacts these (or any)

behavior has been lacking. Here, we report the creation of the first *in vivo Mpdz* RNAi animal model, and provide the first direct evidence that *Mpdz* expression impacts behavior. Specifically, we demonstrate that *Mpdz* shRNA reduces *Mpdz* expression in the clSNr, resulting in more severe AWD severity compared to control animals, but no change in baseline and PTZ enhanced convulsions. These data provide conclusive proof that *Mpdz* is a QTG affecting AWD risk.

The SNr is a main output of the basal ganglia circuit and has been implicated in CNS hyperexcitability including AWD (Chen *et al.*, 2008) and limbic seizures (Shehab *et al.*, 1996, Veliskova *et al.*, 2005). A growing body of literature indicates that the caudal and rostral subregions are functionally distinct in their regulation of AWD as well as related limbic seizure phenotypes (Shehab *et al.*, 1996, Veliskova *et al.*, 2005). The caudal SNr, active during pre-clonic seizures, is thought to be an early gateway for seizure initiation/propagation, and is a sensitive target for the suppression of tonic seizures (Shehab *et al.*, 1996, Veliskova *et al.*, 2005). In contrast, the rostral subregion becomes activated after a motor seizures occurs (Collins *et al.*, 1986, Shehab *et al.*, 1996, Veliskova *et al.*, 2005). Interestingly, the present studies find that intra-clSNr *Mpdz* RNAi impacts AWD, but not baseline or PTZ enhanced HICs, demonstrating that the clSNr, and particularly *Mpdz* expression within the clSNr, has an important role in AWD but does not generally affect seizure susceptibility. This is consistent with and builds upon our data showing that clSNr (but not rostral SNr) lesions mitigate AWD but do not affect baseline or PTZ enhanced HICs (Chen *et al.*, 2011b, Chen *et al.*, 2008). Taken together, these studies consistently demonstrate that the clSNr subregion plays a crucial role in mediating AWD convulsions, and that *Mpdz* expression is inversely related to AWD severity.

We recently demonstrated that *Mpdz* knockout heterozygotes voluntarily consume less alcohol than wildtype littermates, providing the first evidence that *Mpdz* may also influence alcohol consumption and reward (Milner *et al.*, 2013). Recent association studies using human clinical populations also implicate *MPDZ* in alcohol consumption and dependence (Gizer, 2011, Karpyak *et al.*, 2009, Tabakoff, 2009) in a manner dependent upon AWD risk (Karpyak *et al.*, 2009), apparently similar to our results in mouse *Mpdz* genetic models (Milner *et al.*, 2013). Although, to our knowledge, the clSNr has not been implicated in alcohol consumption phenotypes, it receives robust projections from the rostroventral striatum (Chen & Buck, 2010), which in turn has reciprocal connections to the dorsal striatum, a region known to have an important role in alcohol seeking behavior, its habitual use, and alcoholism risk (Chen *et al.*, 2011a, Jeanblanc *et al.*, 2009). The lateral SNr also receives projections from the central nucleus of the amygdala and the intercalated cells of the amygdala (Shinonaga *et al.*, 1992), another region with a proven role in alcohol consumption and preference in mice (Dhaher *et al.*, 2008, Funk *et al.*, 2006). Future studies will be needed to rigorously test the role of MUPP1 in one or more of these brain regions, as well as the role of its expression in the clSNr beyond AWD to alcohol seeking, consumption, and preference behaviors.

MUPP1 and other PDZ domain proteins impact signaling by regulating supramolecular complex assembly and synaptic protein localization, as well as the rate and fidelity of the signal transduction pathways of the receptors and proteins which they scaffold (Romero *et*

al., 2011, Sheng & Sala, 2001). MUPP1 is not thought to have an intrinsic catalytic domain, suggesting that its impact on AWD and alcohol consumption is mediated by the protein complexes that it scaffolds. MUPP1 contains thirteen PDZ domains allowing for a large diversity of interactions, so multiple MUPP1 partners may be involved in MUPP1 actions on AWD, as well as alcohol consumption/preference. Yeast two hybrid analyses have begun to identify MUPP1 association partners, including G-protein coupled $GABA_B$ and 5-HT_2 receptors (Balasubramanian *et al.*, 2007, Ullmer *et al.*, 1998) and the $NMDA_{2B}$-SynGAP (synaptic GTPase-activating protein)-CaMKII (Ca^{2+}/calmodulin-dependent kinase) complex (Krapivinsky *et al.*, 2004), all three of which have been implicated in AWD and/or alcohol consumption/reward (Chen *et al.*, 2011a, Humeniuk *et al.*, 1994, Lal *et al.*, 1993, Panocka & Massi, 1992, Vlachou & Markou, 2010). In addition, we have previously demonstrated that the degree to which a selective $GABA_B$ receptor agonist and a selective 5-HT_{2C} receptor antagonist enhance HICs may be dependent upon QTL/*Mpdz* status (Reilly *et al.*, 2008). Furthermore, ongoing neurophysiological in clSNr neurons and behavioral studies indicate that $GABA_B$ and 5-HT_2 receptor function are altered in MUPP1 knockdown mice (K Buck, L Kruse, and D Rossi, unpublished data) which also show enhanced AWD severity compared to wildtype (Milner *et al.*, 2013). In addition, the role of $GABA_B$ receptors in alcohol dependence can be extended beyond AWD to alcohol consumption, since baclofen, a selective $GABA_B$ receptor agonist, is currently being evaluated in clinical trials as a therapeutic treatment for alcoholism (Addolorato *et al.*, 2011, Leggio *et al.*, 2013). Taken together with the knowledge that MUPP1 plays a role in mediating $GABA_B$ and 5-HT_2 receptor function, both of which have been implicated in AWD and alcohol consumption, the MUPP1 interaction with one or both of these receptors could be a potential mechanism contributing to *Mpdz*'s role in alcohol physiological dependence and associated AWD. Future studies will be important to begin to dissociate the functional importance of MUPP1 interactions with 5-HT_2 receptors (at PDZ10) from its interactions with $GABA_B$ and SynGAP/$NMDA_{2B}$ receptors (at PDZ13) on AWD, as well as on alcohol consumption (e.g., using the CRISPR/Cas9 system) to further elucidate the role of *Mpdz* in alcohol dependence.

To our knowledge, the present studies are the first to employ state-of-the-art RNAi techniques for targeted gene expression to advance the understanding of the important interplay between brain region specific gene expression and its influence on the severity of AWD. These studies present clear evidence that *Mpdz* is a QTG for physiological dependence and associated AWD, and demonstrate that its expression within the clSNr is crucially involved. Further, taken together with previous work from our lab (Chen *et al.*, 2011b, Chen *et al.*, 2008), these studies suggest that the clSNr plays an important role as a critical modulator of AWD severity. Though our work contributes significantly to the understanding of the genetic determinants of alcohol physiological dependence and associated withdrawal, there are some limitations. First, in order to confidently assess *Mpdz* expression in the clSNr we employed LCMD followed by QPCR to quantify *Mpdz* mRNA. Although this technique did not allow us to quantify MUPP1 protein in the clSNr, our previous work demonstrates that reduced *Mpdz* mRNA results in a comparable reduction in MUPP1 protein in the brain (Shirley, 2004). Importantly, while the present RNAi studies were performed in D2 mice, evidence supporting the role of *Mpdz* expression in AWD is not limited to this strain. Recently, we have demonstrated that *Mpdz* knockout heterozygote

mice (on a C57BL/6J genetic background) with an approximate 53% reduction of *Mpdz* expression, also demonstrate increased AWD severity (Milner *et al.*, 2013). Finally, our results are based on a single measure of AWD, and future studies will be needed to assess the broader role of *Mpdz*/MUPP1 in additional measures of AWD (e.g., anxiety-like and depression-like behaviors), alcohol drinking and preference, and beyond to further understand *Mpdz*'s contributing role in alcohol use and abuse. *Mpdz*'s influence on risk for alcohol dependence is of particular interest since drugs with known MUPP1 partners as an initial target are currently being used in clinical trials evaluating their efficacies to reduce craving and relapse (Addolorato *et al.*, 2011, Leggio *et al.*, 2013). As more information becomes available on precise MUPP1 partner interactions and the relevant neural circuit, the mechanism by which MUPP1 regulates AWD and its contribution to alcohol consumption, preference, and reward will become apparent and could identify “drugable” targets for new pharmacotherapies for alcohol dependence.

Acknowledgments

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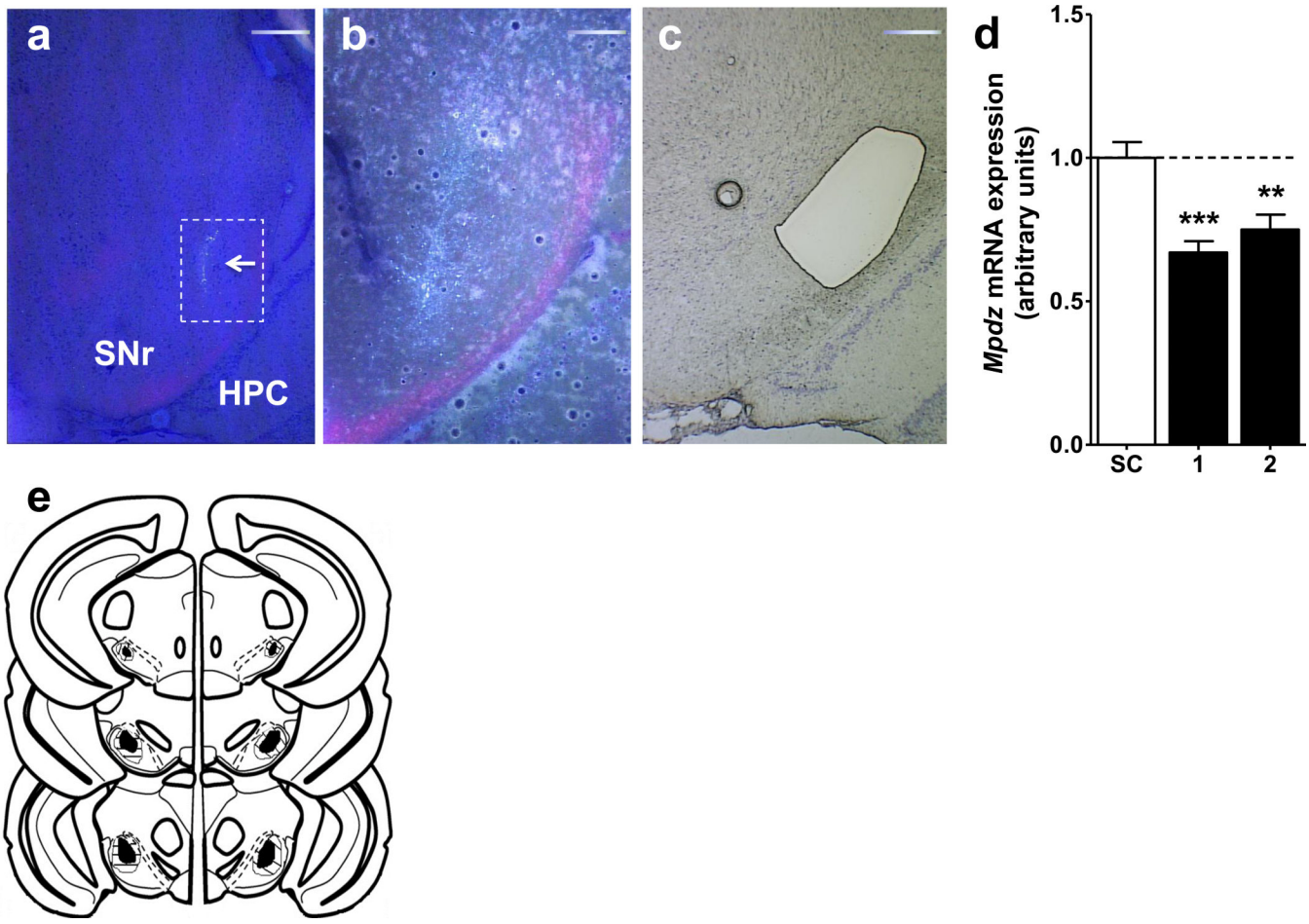

Figure 1. Bilateral *Mpdz* RNAi targeting of the clSNr

Representative photomicrograph showing fluorescence at the microinjection site (*arrow*) overlain a light photomicrograph to visualize structure and GFP expression at **(a)** 4X and **(b)** 10X (of the area outlined in white within panel a). **(c)** 4X Thionin stained light photomicrograph image with the clSNr dissected by LCMD. **(d)** Data presented are for animals with confirmed bilateral targeting of the clSNr. *Mpdz* expression in LCMD clSNr from *Mpdz* shRNA animals in two experiments conducted 4 months apart (black bars) compared to scrambled control (SC) animals (open bar). **(e)** Schematic diagram of a coronal brain section depicting microinjection sites and extent of GFP expression (indicating viral infection) in the clSNr. The darkened regions indicate the sites of clSNr microinjections from all confirmed bilateral hits, identified by GFP. The striped region indicates the extent of GFP spread (including injection sites). Based on coordinates from the mouse brain atlas (Paxinos & Franklin, 2001), the confirmed microinjection sites and lentiviral spread ranged from −3.1 to −3.8 mm AP from Bregma in the clSNr. *Additional abbreviations*: HPC, hippocampus. Scale bars are located in the upper right hand corner of each panel. **p<0.01, ***p<0.001.

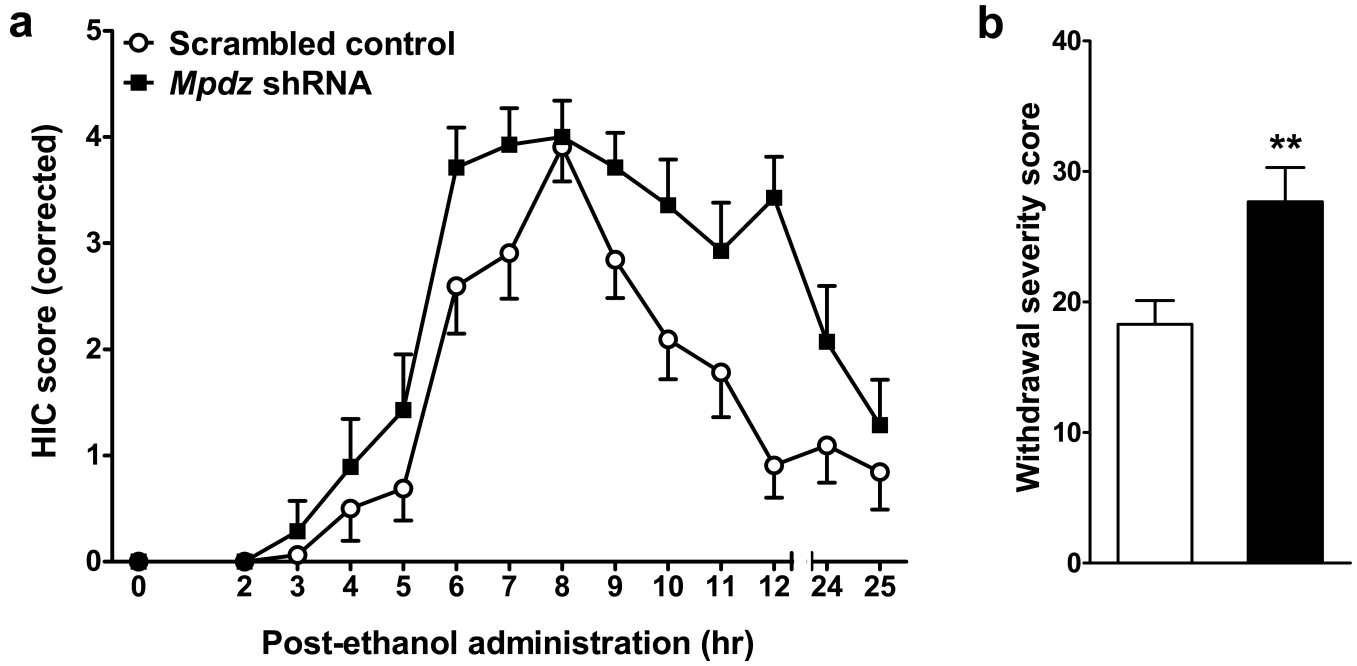

Figure 2. AWD severity is increased by intra-clSNr *Mpdz* RNAi

(a) Data represent the mean HIC score values ± SEM (corrected for individual baseline scores) for mice with confirmed bilateral targeting of the clSNr. Around 4 hr post-ethanol, HIC scores increase above baseline, indicating a state of withdrawal hyperexcitability. **(b)** Individual withdrawal severity scores were calculated as the sum of the corrected HIC scores 2-12 hr post-ethanol as in previous work (Chen *et al*., 2011b). AWD is significantly more severe in *Mpdz* shRNA (N=14) compared to control animals (N=16). **p<0.01, ***p<0.001.

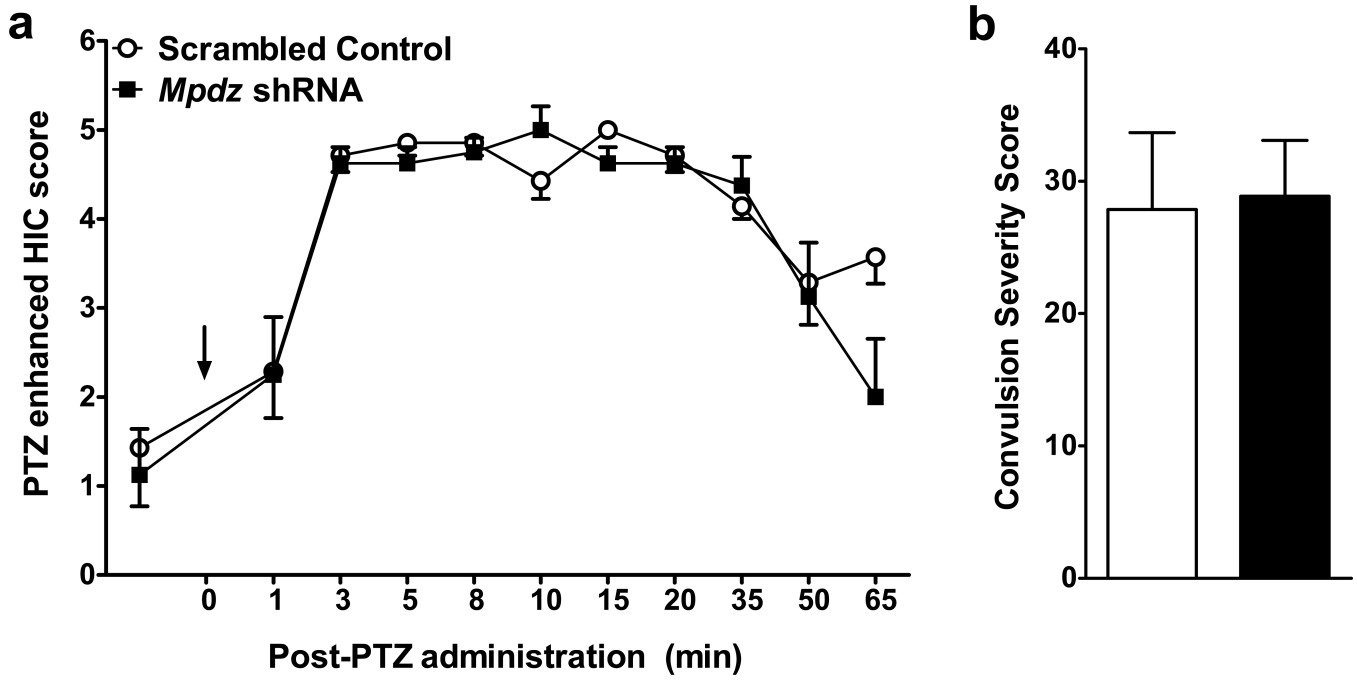

Figure 3. PTZ enhanced convulsion severity is not altered by intra-clSNr *Mpdz* RNAi

(a) Data represent the mean HIC score values ± SEM for mice with confirmed bilateral targeting of the clSNr. HICs were assessed at 10 time points from 1 to 65 min post-PTZ administration. **(b)** Individual HIC severity scores were calculated as the sum of the corrected HIC scores 2-12 hr post-ethanol as in previous work (Chen *et al.*, 2011b). PTZ enhanced convulsions did not differ between *Mpdz* shRNA (N=8) compared to control (N=7) animals.

Table 1

Mpdz knockdown in NS20Y cells. Five *Mpdz* shRNA plasmids were assessed for relative knockdown of *Mpdz* expression in NS20Y cells compared to scrambled control using QPCR. Each hairpin sequence is comprised of a 21 base stem (with the 21 underlined bases indicating the bases that hybridize to the *Mpdz* sequence in Exon 10) and a 6 base loop.

Plasmid	Targeted Sequence	*Mpdz* expression
TRCN0000103480	CCGGCGCACCAGAATGAGTGTGTTTCTCGAGAAACACACTCATTCTGGTGCGTTTTTG	**1.05**
TRCN0000103481	CCGGGCTCTGATAGATACACCTGATCTCGAGATCAGGTGTATCTATCAGAGCTTTTTG	**0.24**
TRCN0000103482	CCGGGCCTTCAGGAATCTTTGTAAACTCGAGTTTACAAAGATTCCTGAAGGCTTTTTG	**0.23**
TRCN0000103483	CCGGGCATCTGAAATTCAGGGACTACTCGAGTAGTCCCTGAATTTCAGATGCTTTTTG	**0.62**
TRCN0000103484	CCGGCATCTGAAATTCAGGGACTAACTCGAGTTAGTCCCTGAATTTCAGATGTTTTTG	**0.50**
SCH002	CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT	**1.00**