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DNA-methylation in the medial prefrontal cortex regulates alcohol-induced behavior and plasticity.

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Abstract

Background: Recent studies have suggested an association between alcoholism and DNA methylation, a mechanism that can mediate long-lasting changes in gene transcription. Here, we examined the contribution of DNA methylation to the long-term behavioral and molecular changes induced by a history of alcohol dependence.

Methods: In search of mechanisms underlying persistent rather than acute dependence-induced neuroadaptations, we studied the role of DNA methylation regulating medial prefrontal cortex (mPFC) gene expression and alcohol related behaviors in rats 3 weeks into abstinence following alcohol dependence.

Results: Post-dependent (PD) rats showed escalated alcohol intake, which was associated with increased DNA methylation as well as decreased expression of genes encoding synaptic proteins involved in neurotransmitter release in the mPFC. Infusion of the DNA methyltransferase (DNMT) inhibitor RG108 prevented both escalation of alcohol consumption and dependence-induced down-regulation of 4 out of the 7 transcripts modified in PD rats. Specifically, RG108 treatment directly reversed both down-regulation of synaptotagmin 2 (*Syt2)* gene expression and hypermethylation on CpG#5 of its first exon. Lentiviral inhibition of *Syt2* expression in the mPFC increased aversion-resistant alcohol drinking, supporting a mechanistic role of *Syt2* in compulsive-like behavior.

Conclusion: Our findings identified a functional role of DNA methylation in alcohol dependence-like behavioral phenotypes and a candidate gene network that may mediate its effects. Together, these data provide novel evidence for DNMTs as potential therapeutic targets in alcoholism.

Introduction

Alcoholism is a complex disorder that results from the interplay between genetic and environmental factors. Although it has been demonstrated that alcoholism is associated with long lasting drug-induced changes in gene expression and neuronal plasticity (Tapocik et al., 2012), the molecular mechanisms modulating these changes and their maintenance are still unclear.

Substantial evidence suggests that epigenetic mechanisms are involved in the regulation of alcohol-related behaviors. For instance, the histone deacethylase (HDAC) inhibitor trichostatin A (TSA) decreases alcohol intake in the alcohol preferring P rats and prevents the development of alcohol withdrawal-related anxiety (Pandey et al., 2008; Sakharkar et al., 2012; Sakharkar et al., 2014). While several studies support a role of histone acetylation in regulation of alcohol-related behaviors (Tabakoff et al., 1986; Pandey et al., 2008; Agudelo et al., 2011; Sakharkar et al., 2012), much less is known about the possible role of DNA methylation. DNA methylation can lead to long-lasting and stable changes in gene expression, has the ability to change dynamically in response to external factors and provides a mechanism through which the environment can influence gene expression and hence behavioral phenotypes of importance for addiction. Accordingly, environmental factors including stress (Weaver et al., 2004; Murgatroyd et al., 2009) and exposure to drugs of abuse regulate methylation patterns in the brain (Wong et al., 2011; Tian et al., 2012). Several studies also indicate that DNA methylation may influence reinforcing properties of drugs of abuse (Wong et al., 2011). For example, inhibition of DNMT activity in the nucleus accumbens (NAc) increased, whereas

hypermethylation induced by NAc-specific *Dnmt3a* overexpression attenuated cocaine reward (Laplant et al., 2010). Moreover, systemic inhibition of DNMT activity decreases excessive alcohol drinking and seeking behaviors in rodents (Warnault et al., 2013).

Although previous studies suggest a role of DNA methylation in alcohol related-behaviors, mechanisms through which DNA methylation contributes to long-term neuroadaptations in alcohol dependence are presently unknown. Here, we examined the possible contribution of DNA methylation to the long-term behavioral and molecular changes induced by a history of alcohol dependence. We focused on the mPFC because of its prominent role in drug-induced neuroadaptations associated with drug seeking and alcohol dependence (Tzschentke, 2000; Kalivas, 2008; Tapocik et al., 2012; Tapocik et al., 2014). Using our model of PD rats (Rimondini et al., 2002; Tapocik et al., 2012), we first measured DNA methylation levels in the mPFC 3 weeks into protracted abstinence from alcohol vapor. Next, we functionally assessed the role of DNA hypermethylation in alcohol–related behaviors. Furthermore, we performed whole transcriptome sequencing (WT seq) and pyrosequencing analysis to identify persistent alcohol-induced changes in gene expression that are driven by DNA methylation changes. Finally, using a lentiviral approach, we confirmed the role of *Syt2*; a gene identified in the WT seq and regulated by DNA methylation, in alcohol self-administration and aversion-resistant alcohol seeking, traits thought to be hallmarks of alcohol dependence.

MATERIALS AND METHODS

Animals

Male Wistar rats (200-225g, Charles River, Wilmington MA) were housed under a reverse light cycle with food and water *ad libitum* and were habituated to the facility and handled prior to experiments. Testing took place during the dark phase. Procedures were approved by the NIAAA Animal Care and Use Committee.

Dependence induction

Dependence was induced using chronic intermittent alcohol vapor exposure as described (Rimondini et al., 2002). Briefly, rats were exposed to alcohol vapor for 14h each day (on at 7:30 pm, off at 9:30 am) for 7 weeks, resulting in blood alcohol concentrations (BACs) between 150 and 300 mg/dl. Controls were kept in identical chambers with normal air flow. Once weekly, blood was collected from the lateral tail vein. BACs were assessed using quantitative gas chromatography (Tapocik et al., 2012). Molecular and behavioral tests were performed 3 weeks after the end of the exposure to assess persistent effects of alcohol exposure (Figure 1).

Surgery

For the RG-108 experiments, rats received continuous infusion of RG108 (100 µM dissolved in 5% 2-hydroxypropyl beta-cyclodextrin (w/v)) into the lateral ventricle (coordinates relative to Bregma: anterio-posterior: -0.8; medial-lateral: +/- 1.5mm, dorsal-ventral -5.0mm) or the mPFC (coordinates relative to bregma: anterior-posterior: +2.5, medial-lateral: +/- 1.5mm, dorsal-ventral -3.5mm, 10° angle). The rats underwent surgery 3 weeks after the end of alcohol

exposure using the osmotic mini pumps 2002 (0.5 µl h^{-1}; Alzet, Cupertino, CA) and 2004 (0.25 µl h^{-1}; Alzet, Cupertino, CA) for lateral ventricle and mPFC infusion, respectively.

For the lentiviral microinjection, rats received 2 injections bilaterally (1ul per injection; rate: 0.25µl/min) directly into the mPFC (coordinates relative to bregma: anterior-posterior: +2.5 and +3mm, medial-lateral: +/- 0.7mm, dorsal-ventral -3.5mm) of a lentiviral vector containing a shRNA to *Syt2* (CTTCTCTAAGCATGACATCAT; titer: 9.7 $X10^9$ TU/ml; Sigma, St Louis, MO) and a scrambled control (titer: 2.9 $X10^9$ TU/ml). Rats were subjected to behavioral studies after a 1-week recovery period.

Two-bottle free choice

Rats had access to 2 bottles in their home cage. One bottle contained saccharin 0.2% and the second bottle contained saccharin 0.2% with increasing concentration of alcohol (3, 6 and 8%). After 10 days of stable alcohol consumption at 8%, rats were separated into 2 groups (control and PD rats). PD rats were then exposed to alcohol vapor for 7 weeks. Beginning 3 weeks after rats were removed from alcohol vapor, cannula connected to an osmotic mini pumps containing either RG108 or vehicle were implanted into lateral ventricle of PD and control rats. Rats were then tested for alcohol intake after one week recovery.

Alcohol self-administration

Training and testing for operant self-administration of 10% alcohol in water were as described (Cippitelli et al., 2010). Once self-administration was stable at a fixed ratio 1 (FR1) (baseline),

cannula connected to osmotic mini pumps containing either RG108 or vehicle were implanted into mPFC of PD and control rats. LaPlant and his collaborators have previously demonstrated that 100µM of RG108 infused at a rate of 0.25µl/min significantly decreased DNA methylation (LaPlant et al., 2014). The rats were tested for self-administration after one week recovery (Figure 1).

Behavioral test after *Syt2* inhibition:

Rats were trained to self-administer alcohol as described above. Once self-administration was stable, rats received a microinjection of *Syt2* lentiviral vector or scrambled lentiviral vector directly into the mPFC and were allowed to recover for one week. Rats were then tested for alcohol self-administration after one week recovery. Following this test, rats were assessed for compulsivity-like behavior when alcohol was mixed with increasing concentration of quinine (0.005g/L; 0.01g/L; 0.025 g/L; 0.05g/L and 0.075 g/L) during alcohol-self administration.

DNA isolation and global DNA methylation analysis

Cannula connected to osmotic mini pumps containing either RG108 or vehicle were implanted in lateral ventricles. Animals were decapitated two weeks after surgery (Figure 1). Bilateral samples from the mPFC were dissected out as described (Bjork et al., 2006), and stored at -80°C. DNA was extracted from mPFC using QIAamp DNA Mini Kit (Qiagen, Valencia, CA), following manufacturer's instructions.

Global DNA methylation was measured using Epigentek's Methylamp Global DNA Methylation Quantification Ultra Kit (Farmingdale, NY) following the manufacturer's instructions. Raw values were colorimetrically quantified and total methylation level was estimated by generating a standard curve from Epigentek's methylated DNA standard. Values are represented as methylation percentage relative to vehicle control. For table 1 and 2, samples were dissected from rats that received vehicle micro-infusion.

For Pyrosequencing analysis, DNA was sent to EpigenDx (Hopkinton, MA) a DNA methylation and pyrosequencing laboratory service, which designed and analyzed our pyrosequencing assays.

Western blot

Tissue was homogenate in lysis buffer (RIPA buffer, DTT, Cell Signaling, Danvers, MA). Supernatant containing proteins was collected after 10 min centrifugation (10000g at 4° C). Protein concentration was assessed using the Pierce BCA protein assay kit (Thermo Scientific, IL). Protein samples were denaturated at 70°C for 10 min and run on a 4-12% Bis-Tris gel (NuPAGE® Novex®, Life Technologies, Grand Island, NY), then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA). The membrane was blocked with 5% nonfat dry milk and incubated overnight with the primary antibody (rabbit anti-DNMT1 (1/1500, Epigentek, Farmingdale, NY), rabbit anti-DNMT3a (1/100: SantaCruz, Dallas, TX), rabbit anti-DNMT3B (1/200; SantaCruz, Dallas, TX) or rabbit anti- βtubulin (1/10000; Abcam, Cambridge, MA). The membrane was washed with TBST and then incubated with secondary antibody anti-rabbit HRP (1:10000, Cell Signaling, Denver, MA) for 1 hour. Detection and densitometric

evaluations were performed using the ECL western blotting detection reagent (GE Healthcare, Piscataway, NJ) and ImageJ software (Bethesda, MD).

Immunohistochemistry

Three weeks after completion of alcohol exposure, animals were intracardially perfused with 4% paraformaldehyde-1X PBS. Brains were harvested, post-fixed for 2 hours, dehydrated in 30% sucrose solution; snap frozen in isopentane and stored at -80˚C. Sections were incubated in rabbit anti-DNMT1 (1µg/ml; Abcam, Cambridge, MA) or mouse anti-5Mec (1:300, Acris, antibodies, Götingen, Germany) and chicken anti-NeuN (1:500; EMD Millipore, Billerica, MA) for 48 hours at 4˚C and with the secondary antibody for 2 hours at room temperature (donkey anti-rabbit 488 for DNMT1, donkey anti-mouse 555 for 5MeC and donkey anti-chicken 633 for NeuN (1:1000; Life Technologies, Grand Island, NY). Cells stained positive for NeuN and DNMT1 or 5MeC were quantified using BioQuant Image Analyzer (Nashville, TN, USA). DNMT1 and 5MeC expression were quantified on Bioquant (Nashville, TN, USA) by measuring the average density of expression per neuron. Average density represents DNMT1 and 5MeC total density divided by total number of neurons.

RNA Isolation and Whole Transcriptome Library Preparation

Total RNA was isolated (as described above) and then quantified on a Bioanalyzer (Agilent, Santa Clara, CA). Whole transcriptome sequencing libraries (4 samples / condition, 8 libraries in total) were prepared following the manufacturer's instructions for the Whole Transcriptome

Sample Prep Kit (Illumina, San Diego, CA). Total RNA was ribosomal depleted, fragmented, hybridized and ligated to 3' and 5' adaptor primers, followed by reverse transcription. cDNA was size selected and amplified. Each library template was hybridized to one lane in the flow cell and amplified to form clusters, followed by sequencing.

Whole Transcriptome sequencing

All FASTQ files were uploaded and stored on Simbiot (Umylny, 2012) and all processing was performed using Simbiot system (Genewiz, South Plainfield, NJ). Quality check was performed using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and all sequencing reads were mapped using tophat (Langmead et al., 2009; Trapnell et al., 2009) to Ensembl (Flicek et al., 2012) version 63 of the Rat genome. The mapped results were processed using cufflinks (Trapnell et al., 2012), producing Fragments per Kilobase of transcript per Million mapped reads (FPKM) data matrix, and with htseq-count (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html), producing the raw hit counts data matrix. The raw hit counts gene matrixes was transformed using DESeq (Anders and Huber, 2010) variance stabilization algorithm and normalized using quantile normalization function built into the Bioconductor (Gentleman et al., 2004) Limma (Smyth, 2005) package. The normalized data matrix was then analyzed using Limma and SAMR (Tusher et al., 2001) algorithms.

Pathway Analyses of Whole Transcriptome Sequencing Data

Normalized values for each gene were bioinformatically analyzed using Ingenuity Pathways software (IPA, Qiagen, Valencia, CA). Methodological details on IPA can be found at http://www.ingenuity.com/science/knowledge_base.html. For IPA analysis, we selected genes from RNA-seq dataset with a 1.2 fold change cut-off and a p value < 0.05.

Reverse transcription and quantitative polymerase chain reaction (qPCR)

cDNA was reverse transcribed from total RNA and qPCRs were carried out using standard TaqMan chemistry and a laser-equipped thermal cycler to detect changes in fluorescence in real time (Applied Biosystems Inc., Foster City). cDNA concentrations were calculated according to the ΔΔCt method, corrected for differences in PCR efficiency, and normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

Statistical analysis

All data results were analyzed using ANOVA, with factors for the respective analysis indicated in conjunction with its results. When appropriate, post hoc comparisons were performed using the Newman-Keuls test. The accepted level of significance for all tests was $p \leq 0.05$.

RESULTS

A history of alcohol dependence leads to DNA hypermethylation in the mPFC

To determine whether history of alcohol dependence was associated with changes in DNA methylation, we first measured global DNA methylation in brain regions associated with addiction. We found that alcohol exposure increased DNA methylation in the mPFC and the nucleus Accumbens (Nac) but not the amygdala (Amg) or the hippocampus (Hipp) (One Way ANOVA: $F_{(1\text{-}9)}$=5.32; p=0.04; n= 6-8/group; Table 1). We chose to focus our study on the mPFC, which showed the more pronounced change in DNA methylation.

We measured protein expression levels of DNA methyltransferases DNMT1, DNMT3a and DNMT3b in the mPFC. Western blot analysis showed no changes in protein expression after history of alcohol dependence (n=8/group; Figure 2A). However, using an immunohistochemistry approach that allows us to look at specific cell types, we found that DNMT1 was increased specifically in neurons (n=6-7/group; p=0.03; Figure 2B-C). These results are consistent with previous studies suggesting that neurons are epigenetically more sensitive to environmental conditions than non-neuronal cells in the central nervous system (Feng et al., 2010; Iwamoto et al., 2011). Furthermore, we found that increased DNMT1 expression in neuronal cells of mPFC was associated with increased neuronal DNA methylation (n=12-13/group, p<0.05; Figure 3).

DNA methylation modulates alcohol intake

To understand the role of DNA methylation in alcohol-related behaviors, we pharmacologically decreased DNA methylation using the DNMT inhibitor RG108. Similar to what was found by Warnault and collaborators (Warnault et al., 2013), we showed that intracerebroventricular (ICV) infusion of RG108 prevented escalated alcohol intake, which suggests a role of DNA methylation in alcohol consumption. Two way ANOVA indicated a significant interaction between dependence history (control vs. PD) and drug treatment (vehicle vs. RG108); $F_{(1,21)}=8.28$; $p<0.01$. *Post hoc* tests showed a significant increase in alcohol consumption in PD compared to controls rats ($p<0.05$) and a significant decrease in alcohol consumption in PD-RG108 compared to PD-vehicle rats ($p<0.05$; n=5-7/group; Figure 4). In addition, RG108 treatment lowered the increased DNA methylation in the mPFC of PD rats, but did not influence it in control rats. Two way ANOVA showed a significant interaction between group and drug treatment ($F_{(1-23)}=4.2$; $p\leq0.05$). *Post hoc* tests showed a significant increase in DNA methylation in the mPFC of PD compared to control rats and a significant decrease in DNA methylation in the mPFC of PD-RG108 compared to PD-vehicle rats ($p=0.01$); n=6-8/group; Table 2).

Escalation of alcohol self-administration following a history of alcohol dependence is in part mediated by DNA hypermethylation in the mPFC

Since DNA methylation was found to be specifically increased in the mPFC and the Nac, we next wanted to determine whether hypermethylation in the mPFC alone is causally related to escalation of alcohol consumption. To address this question, rats received infusion of RG108

directly into the mPFC 3 weeks into protracted abstinence. The dose used for this experiment has previously been shown to significantly decrease DNA methylation (Laplant et al., 2010). For this study, we assessed alcohol intake using operant self-administration, because this methodology is thought to more directly gauge reinforcing properties of alcohol, and allows examination of alcohol-taking as well as alcohol-seeking behavior that is in part driven by the mPFC (Koros et al., 1999; Dayas et al., 2007). A history of alcohol dependence resulted in a significant escalation of operant alcohol self-administration ($F_{(1\text{-}17)}$ =0.003; n=10/group; Figure 5A) as determined by rewards received during a 30-minute session. Baseline was calculated as the last 3 days of self-administration before surgery. Infusion of RG108 directly into mPFC prevented the escalation of alcohol self-administration observed in PD rats but did not influence self-administration rates in control rats. ANOVA indicated a main effect of group (control vs. PD): $F_{(1, 15)}$ =5.9; p=0.02; and an interaction between time (baseline vs. test) X group X drug (RG108 vs. vehicle): $F_{(1, 15)}$ =5.9; p=0.03; n=5/group). Post hoc tests showed a significant decrease in alcohol self-administration in PD-RG108 treated rats compared to PD-Vehicle rats but no effect of RG108 on control rats (p<0.001; Figure 5A). RG108 treatment did not modify locomotor activity, making it unlikely that non-specific motor impairment or sedation would account for the findings, and supporting a specific role of RG108 in alcohol consumption (Figure 5B).

Chronic intermittent alcohol exposure regulates expression of genes involved in neurotransmitter release

Whole transcriptome sequencing analysis identified 784 genes with nominally significant changes in expression within the mPFC after a history of alcohol dependence ($p < 0.05$, no FDR correction; n=4/group). Within this list, bioinformatics analysis identified two over-represented categories of genes, related to gene expression and neurotransmission (Table 3). Based on a bioinformatics network analysis and the crucial role of neurotransmission for addiction, we selected a subset of 7 genes coding for synaptic proteins for confirmation by qPCR (n=8/group; Figure 6A-B). qPCR confirmed that these genes were significantly down-regulated in the mPFC of PD rats compared to control rats (t test; $p<0.05$). Decreased expression of these genes was specific to the mPFC, as their expression did not change or were increased in the Amg, NAc or Hipp 3 weeks after chronic alcohol exposure (n=10/group; Table 4).

Synaptic transmission genes repressed by chronic intermittent alcohol exposure are rescued by DNMT inhibition using RG108

Next, to assess whether DNA hypermethylation accounts for the expression changes of the qPCR validated differentially expressed genes, we investigated whether RG108 treatment into mPFC reversed the persistent gene expression changes (Figure 6C-F). RG108 treatment prevented the down-regulation of 4 out of the 7 genes (*Syt1, Syt2, Wnk2* and *Cacna1a*; n=7-8/group) that were confirmed by qPCR. Two way ANOVA showed a main effect of drug (vehicle vs. RG-108) on *Syt2* ($F_{(1-25)} =14.16$; $p<0.001$), *Syt1* ($F_{(1-28)} =9.72$; $p<0.005$) and *Wnk2* ($F_{(1-28)} =4.01$; $p=0.05$) transcripts. A significant interaction (treatment X drug) was found for *Cacna1a* ($F_{(1-28)} =3.988$; $p=0.05$), *Wnk2* ($F_{(1-28)} =10.30$; $p=0.003$) and *Syt1* ($F_{(1-28)} =25.65$; $p<0.0001$). Post hoc

analysis showed a significant difference in *Wnk2, Syt1* and *Cacna1* mRNA levels between vehicle- and RG108 treated PD rats. In contrast, RG108 had no effect in controls.

ICV infusion of RG108 did not influence the expression of these transcripts in the Amg, NAc, and or Hipp (Table 4), suggesting a specific effect of RG108 in the mPFC. Together, these findings suggest that chronic intermittent exposure to alcohol persistently decreases the expression of these synaptic genes through increased DNA methylation.

RG108 treatment prevented hypermethylation on exon1 of *Syt2* induced by chronic intermittent alcohol exposure

To determine whether alcohol exposure directly decreases the expression of these synaptic transmission genes through increased DNA methylation, we measured DNA methylation levels on the promoter region and exon 1 of *Syt2* and on the promoter region of *Cacna1a* in the mPFC by pyrosequencing (n=6-8). We found that CpG#5 on exon1 of *Syt2* was significantly hypermethylated in PD rats compared to control rats and that this hypermethylation was prevented by RG108 treatment (Figure 7 A-B). Two way ANOVA showed a significant interaction of group X drug ($F(1\text{-}23)=4.31$; $p<0.05$). Post hoc tests showed a significant increase in DNA methylation in the mPFC of PD rats compared to control rats ($p=0.02$) and a significant decrease of DNA methylation in PD-RG108 rats compared to PD-vehicle rats ($p=0.007$). Interestingly, we found similar results for CpG site #6 which is next to CpG#5 (Figure 7C). Two way ANOVA showed a significant interaction group X drug ($F(1\text{-}23)=4.31$; $p=0.03$. *Post hoc* tests showed an increase in DNA methylation in mPFC of PD compared to control rats that is close to significance

(p=0.06). Like promoter-silencing hypermethylation, increased DNA methylation on exon1 is associated with gene silencing (Brenet et al., 2011). Therefore, these results suggest that alcohol consumption may decrease the expression of *Syt2* through increased DNA methylation on its exon1. In contrast, DNA methylation levels on the promoter region of *Cacna1a* were similar between control and PD rats suggesting that RG108 regulates *Cacna1a* expression through indirect mechanisms (data not shown).

SYT 2 knockdown contributes to compulsive-like drinking

To determine whether decreased expression of *Syt2* plays a functional role in alcohol-related behaviors induced by a history of alcohol dependence, we inhibited *Syt2* expression specifically in the mPFC of naïve rats using a lentiviral vector. Cells infected by our "shRNA *Syt2* lentiviral vector" show no expression of *Syt2*, confirming that it inhibited its expression (Figure 8A). *Syt2* inhibition did not modify alcohol self-administration rates (n=7-9; Figure 8B). However, cortical-*Syt2* inhibition resulted in tolerance to quinine adulteration, suggesting a compulsive-like behavior similar to that observed in alcohol dependence (Vendruscolo et al., 2012). Repeated measures ANOVA showed a main effect of treatment ($F_{(1, 56)} = 4.57$; $p \leq 0.05$; Figure 8C). Cortical *Syt2* inhibition did not affect taste perception as rats with *Syt2* inhibition drank similar amounts of quinine solution as rats injected with the scrambled virus (Figure 8D-E).

DISCUSSION

In this study we sought to determine the epigenetic events that occur during protracted abstinence from alcohol dependence a stage that in human alcoholics is associated with the emergence of alcohol craving and high relapse risk (Heilig et al., 2010). We showed that a history of alcohol dependence is associated with increased DNA methylation specifically in the mPFC. Importantly, we demonstrated that DNA methylation is causally related to alcohol intake and seeking behaviors. Furthermore, our gene expression analysis suggests that DNA methylation regulates alcohol-induced neurotransmission-related gene expression changes. For instance, *Syt2*, one of the neurotransmission-related genes found to be altered, had increased DNA methylation at 2 CpG sites in exon1. RG108 treatment was able to restore both *Syt2* expression and DNA methylation levels, suggesting a direct role of DNA methylation for *Syt2* silencing. Finally, we identified a causal role of *Syt2* in compulsivity-like behavior, a hallmark of alcohol dependence.

Convergent evidence suggests that up-regulated DNMT1 contributes to the maintenance of alcohol-induced DNA hypermethylation. First, RG108 has been designed to selectively target the catalytic domain of DNMT1 (Brueckner et al., 2005). Second, DNMT1 was upregulated in the mPFC after repeated cycles of alcohol intoxication. Third, increased DNMT1 expression was associated with increased DNA methylation in neuronal cell. Finally, DNMT1 is the main enzyme that maintains DNA methylation, the mechanisms that we inhibited with RG108. Rather than inducing *de novo* methylation, the main role of DNMT1 is to maintain DNA methylation already in place. We therefore do not believe that elevated activity of DNMT1 caused DNA

hypermethylation as such in our study. Instead, it is more likely that it contributed to maintaining alcohol-induced DNA hypermethylation, which was already in place 3 weeks after alcohol exposure, before RG108 was administered. Our hypothesis is that repeated cycles of alcohol intoxication followed by protracted abstinence induced DNA hypermethylation, which was then maintained by DNMTs and specifically DNMT1. However, we cannot rule out a possible interaction between DNMT1 and the other DNMT enzymes, specifically DNMT3a which can interact with DNMT1 to maintain DNA methylation (Feng et al., 2010; Iwamoto et al., 2011). Furthermore, although RG108 was designed to inhibit DNMT, it is unclear whether it may also affect DNMT3a and DNMT3b activity.

To identify the molecular mechanisms through which DNA methylation might affect alcohol consumption, we investigated transcriptome changes induced by a history of alcohol dependence and assessed whether some of these changes may be driven by DNA hypermethylation. Because the magnitude of long-term neuronal gene expression changes is frequently small and easily lost in attempts to apply transcriptome-wide false discovery rate corrections, we relied on nominal expression values and bioinformatics pathway analysis for our discovery effort, and validated the functionally relevant candidate hits by qPCR.

Using this strategy, we found that a history of alcohol dependence had a global effect in the mPFC on a gene network that includes proteins regulating synaptic vesicle formation and function, such as *Syt1, Syt2, Wnk2* and *Cacna1a*. These data suggest a persistent dysregulation of synaptic transmission in PD mPFC neurons that may be responsible for the escalated alcohol

consumption we observed following a history of alcohol dependence. These genes regulate Ca^{2+}-evoked neurotransmitter release (Chapman, 2008) and may therefore be involved in changes in neurotransmitter release observed after alcohol exposure. Consistent with our data, Worst and colleagues observed a down-regulation of *Syt1* expression in the prefrontal cortex of Alko alcohol accepting rats (AA) compared to their alcohol non-accepting counterparts (ANA) (Worst et al., 2005). Interestingly, a recent study from Varodayan and colleagues showed that the expression of *Syt1* is upregulated in mouse cortical neurons following a single acute alcohol exposure (Varodayan et al., 2011). If these tissue culture findings reflect processes that occur *in vivo*, these observations collectively suggest the possibility of an allostatic process, in which acute upregulation of *Syt1* expression and function by alcohol is followed by a persistent downregulation to a new set point.

Importantly, we demonstrated that RG108 can reverse more than 50% of the persistent gene expression changes, supporting that they are mediated by DNA hypermethylation. The observation that RG108 did not uniformly rescue all gene expression changes is unsurprising, and suggests that DNA methylation does not account for all alcohol-induced neuroadaptations. However, DNA methylation can have a significant impact on genes involved in important neuronal functions such as neurotransmitter release. Because of the complexity of how DNA methylation is regulated, we chose to first focus on the role of DNA methylation on select genes to begin elucidating the possible functional contribution of at least some of them.

We show two observations likely pointing to two different mechanisms through which DNA methylation can regulate gene expression in the postdependent state. For instance, we found

that repression of *cacna1a* is likely to be indirectly mediated by increased DNA methylation. Indeed, although RG108 treatment restored *cacna1a* expression levels, DNA methylation profile of *cacna1a* was not altered by alcohol exposure. In contrast, *Syt2* seems to be directly regulated by DNA methylation. Our pyrosequencing data showed that repeated alcohol intoxication induced hypermethylation on exon 1 of *Syt2*. Similar to promoter hypermethylation, DNA methylation on exon1 is associated with gene silencing (Brenet et al., 2011). Furthermore, RG108 infusions restored both *Syt2* and DNA methylation levels suggesting that repeated cycles of alcohol intoxication followed by protracted abstinence down-regulated *Syt2* through increased DNA methylation.

Recent publications suggest that DNA can be actively demethylated (Wu and Zhang, 2014). It is possible that when DNMT enzymes are inhibited, active demethylation reverses alcohol-induced hypermethylation and therefore restores gene expression levels. Additional research will be required to examine this hypothesis.

Furthermore, we found that *Syt2* has a mechanistic role in alcohol dependence-induced behaviors. Inhibition of *Syt2* directly in the mPFC induced compulsivity-like behavior, thought to be one of the hallmarks of alcohol dependence. In contrast, *Syt2* inhibition did not modify alcohol self-administration, suggesting that *Syt2* alone may not be sufficient to cause an escalation in alcohol consumption after alcohol dependence. Principal component analysis of behaviors assessed PD animals from a prior study (Vendruscolo et al., 2012) indicates that compulsivity-like, quinine-resistant alcohol consumption and escalation of self-administration rates load on separate factors (data not shown). Overall, interaction between *Syt2* and other synaptic transmission genes may be necessary to alter alcohol consumption in PD rats. This

points to the possibility that the complex behavioral phenotype induced by a history of alcohol dependence is driven by multiple factors and that therapeutics may have better prospect of being effective if they target pathways rather than individual molecular targets. In that context, DNMT inhibition may offer an attractive therapeutic mechanism as it can simultaneously control the expression of multiple genes. Gene expression regulation by DNA methylation is very complex, and future experiments investigating genome-wide changes in alcohol-induced DNA methylation will be needed to better understand the mechanisms through which DNA methylation regulates alcohol-induced neuroadaptations.

In conclusion, we found that DNMT inhibition prevented both escalated alcohol intake and gene expression changes induced by a history of alcohol dependence. Thus, DNMT inhibitors may have a potential to be pharmacotherapies for alcohol dependence. DNMT inhibitors such as 5-azacytidine and 5-aza-2'-deoxycytidine are currently FDA approved for the treatment of myelodysplastic syndrome (Fandy, 2009). Our findings provide an initial rationale for exploring the potential of these or other DNMT inhibitors as a treatment for alcohol dependence.

Conflict of interest,

The authors declare that, except for income received from our primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Figure 1: Experimental timeline: Rats are exposed to alcohol vapor for 7 weeks (14 hrs. per day). A. AMG, HIPP, Nac and mPFC were collected 3 weeks after the end of alcohol exposure. B. Alcohol self-administration (SA) was measured 3 weeks after alcohol exposure. Once SA was stable (baseline), Cannula connected to an osmotic mini pumps containing either RG108 or vehicle were implanted into mPFC of PD and control rats. The rats were tested for SA after one-week recovery.

Figure 2: History of alcohol dependence increases DNMT1 expression in neuronal cells in mPFC. A. Western blot analysis shows no difference in DNMT1, DNMT3a and DNMT3b protein expression. The mean values (+/- SEM) of control rats are shown in black bars. The mean values (+/- SEM) of PD rats are shown in grey bars. Protein expression is represented as DNMT/βtubulin relative to control. B. and C. Immunohistochemical detection of DNMT1 (in green) and neuronal cells (in red) in the mPFC of control (upper panels) and post-dependent rats (lower panels). Right upper and lower panels show merge DNMT1/NeuN. C. Average density of DNMT1 in neuronal cells. #$p<0.05$ control vs. post-dependent rats

Figure 3: A. Immunohistochemical detection of 5MeC (in red) and neuronal cells (in green) in the mPFC of control (upper panels) and PD (lower panels). Right upper and lower panels show merge 5MeC/NeuN. B. Average density of 5MeC in neuronal cells. #$p<0.05$ control vs. post-dependent rats

Figure 4: Intracerebroventricular infusion of RG108 prevents escalation in alcohol intake in PD rats. The mean values (+/- SEM) of control rats are shown in black bars. The mean values (+/- SEM) of PD rats are shown in grey bars. PD rats show escalated alcohol consumption that is

prevented by i.c.v. infusion of RG108. #p<0.05 control vs. post-dependent rats; *p<0.05 vehicle vs. RG108.

Figure 5: Infusion of RG108 directly into mPFC prevents escalation in alcohol intake in PD rats. A. The mean values (+/- SEM) of rats before drug injection are shown in black bars. The mean values (+/- SEM) of rats after drug injection are shown in grey bars. Infusion of RG108 into mPFC prevents the increased alcohol consumption observed in the post-dependent compared to control rats. B. RG108 does not modify locomotor activity. The Mean values (+/- SEM) of rats treated with vehicle are shown in white bars. The mean values (+/- SEM) of rats treated with RG108 are shown in black bars. #p<0.05 control vs. post-dependent rats; *p<0.05 vehicle vs. RG108.

Figure 6: RG108 prevents gene expression changes induced by a history of alcohol dependence. A. Figure from Ingenuity Pathway Analysis showing one of the top gene networks that is down-regulated by chronic alcohol exposure. This network includes genes involved in calcium release and exocytosis, two processes critical for synaptic transmission. B. Table shows the fold change in gene expression that is obtained by RNA sequencing and qPCR validation. RG108 prevents alcohol-induced decrease expression of *syt1* (C), *syt2* (D), *cacna1a* (E) and *wnk2* (F). The mean values (+/- SEM) of rats treated with vehicle are shown in white bars. The mean values (+/- SEM) of rats treated with RG108 are shown in black bars. #p<0.05 control vs. post-dependent rats; *p<0.05 vehicle vs. RG108.

Figure 7: History of alcohol dependence increases DNA methylation on exon 1 of *Syt2*. A. Figure shows sequence of exon 1 of *Syt2* and the location of the 7 CpG sites. Bar graph shows

DNA methylation level (%) at CpG#5 (B) and at CpG# 6 (C) on exon1. The mean values (+/-SEM) of control rats are shown in black bars. The mean values (+/-) of PD rats are shown in grey bars. #p<0.05 control vs. post-dependent rats; *p<0.05 vehicle vs. RG108.

Figure 8: *Syt2* inhibition increases tolerance to quinine adulteration. A. Immunohistochemical detection of *Syt2* (in red) and cell infected by shRNA lentiviral vector specific to *Syt2*. The figure shows that cells infected by lentivirus do not express SYT2 (in green). B. Number of reward for alcohol in rats that received injection of shRNA lentiviral vector specific to *Syt2* (square) and rats that received the scrambled lentiviral vector. C. Compulsive-like drinking (i.e., persistent alcohol drinking despite the aversive bitter taste of quinine added to the alcohol solution). The data represent the percent change from baseline (i.e., lever presses for alcohol alone before adulteration with quinine). D. *Syt2* inhibition does not modify quinine consumption. Bar graph shows quinine consumption as measured by two bottle free choice. E. Graph shows mapping of viral injection sites within the mPFC denoted by a ● .

Table legends:

Table 1: Measure of global DNA methylation relative to control (%) in mPFC, Nac, Amg and Hipp of control and PD rats.

Table 2: Measure of DNA methylation levels in the mPFC after RG108 treatment in control and PD rats. Data show % methylation relative to control/vehicle group (%).

Table 3: Top 6 GO functional enrichment analysis for the list of 784 significantly altered mRNAs (P<0.05, B-H, FDR<0.2). Abbreviations: B-H, Benjamini-Hochberg method; FDR, false discovery rate; GO, gene ontology; mRNA, messenger RNA. Cells shaded in gray represent a GO category that reached significance with P<0.05.

Table 4: **RG108 treatment does not regulate the expression *Cacna1a*, *Syt1*, *Syt2*, *Cacna1l*, *Slc8a2* and *Wnk2* in the Amg, NAc and Hipp.** Table listing the mRNA levels (fold change) of *Cacna1a*, *Syt1*, *Syt2*, *Cacna1l*, *Slc8a2* and *Wnk2* in the Amg, NAc and Hipp of control and post-dependent rats treated with vehicle or RG108. *p<0.05 control vs. post-dependent rats**.**

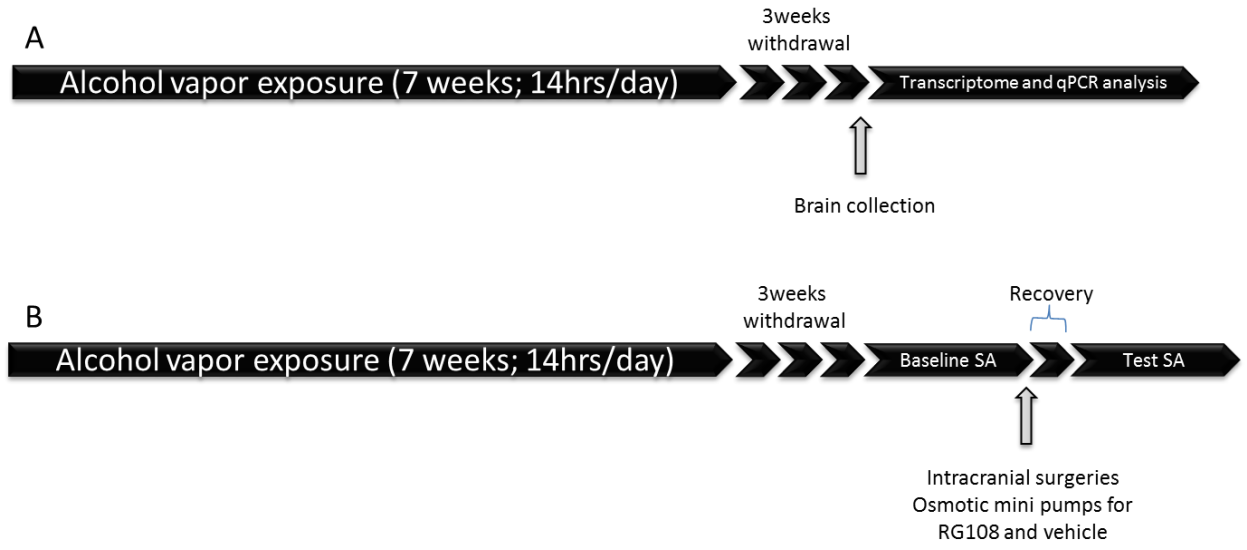

A
3weeks
withdrawal
Alcohol vapor exposure (7 weeks; 14hrs/day)
Transcriptome and qPCR analysis
Brain collection
B
3weeks
withdrawal
Recovery
Alcohol vapor exposure (7 weeks; 14hrs/day)
Baseline SA
Test SA
Intracranial surgeries
Osmotic mini pumps for
RG108 and vehicle

Figure 1

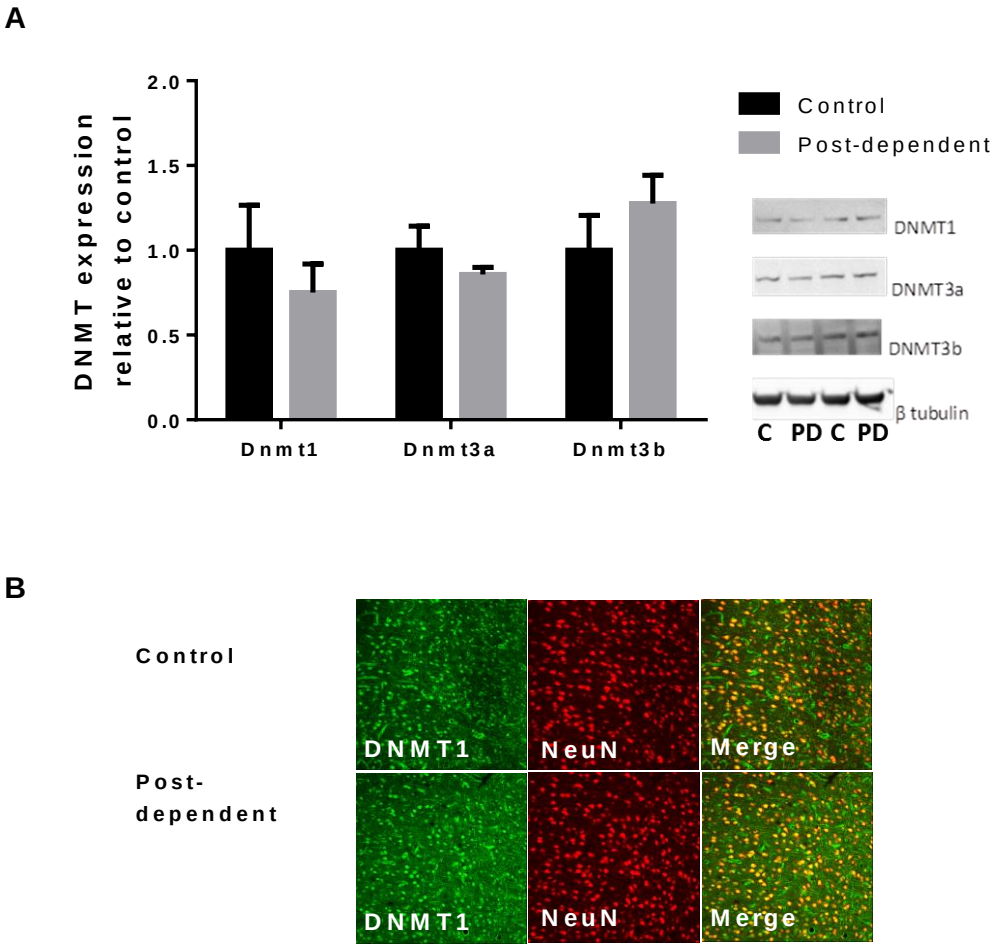

A
DNMT expression relative to control
2.0
1.5
1.0
0.5
0.0
Dnmt1
Dnmt3a
Dnmt3b
Control
Post-dependent
DNMT1
DNMT3a
DNMT3b
β tubulin
C PD C PD
B
Control
Post-dependent
DNMT1
NeuN
Merge
DNMT1
NeuN
Merge
C
Neuronal DNMT1 immunoreactivity
200
150
100
50
0
#
Control
PD

Figure 2

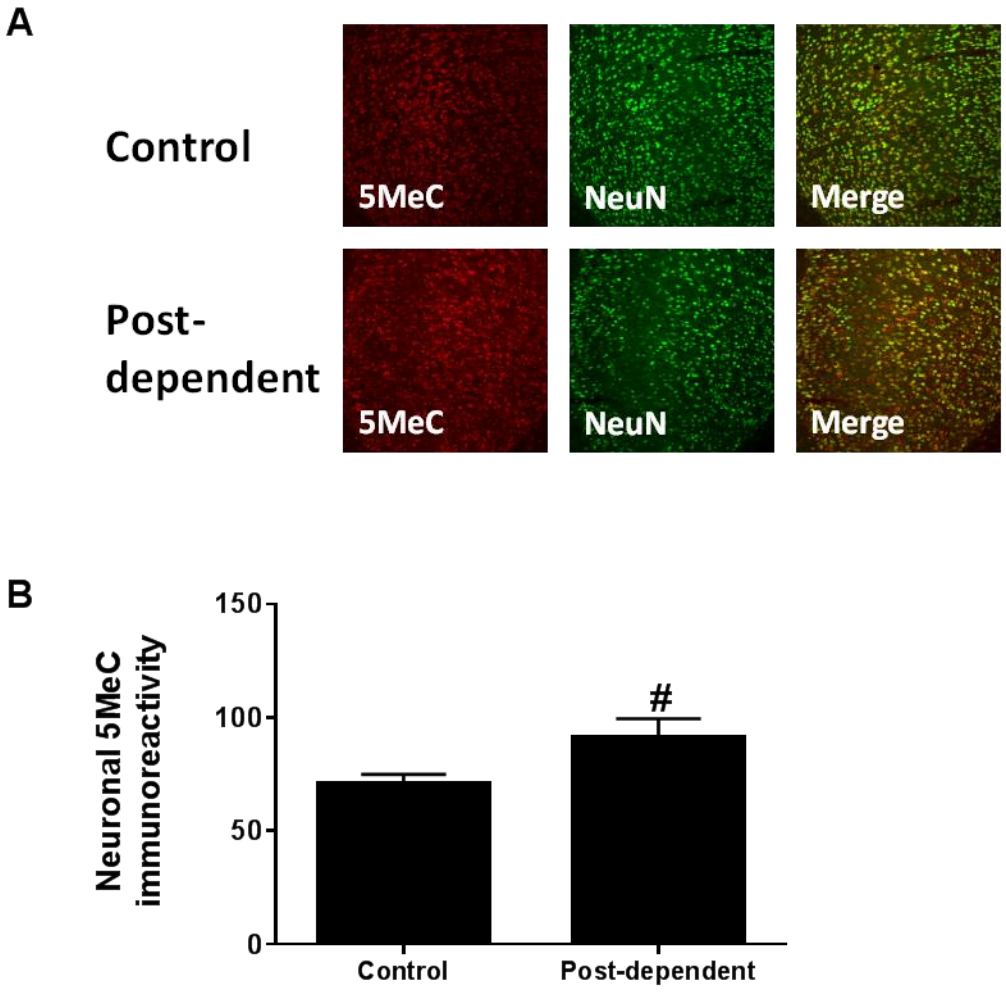

A
Control
5MeC
NeuN
Merge
Post-
dependent
5MeC
NeuN
Merge
B
Neuronal 5MeC
immunoreactivity
150
100
50
0
#
Control
Post-dependent

Figure 3

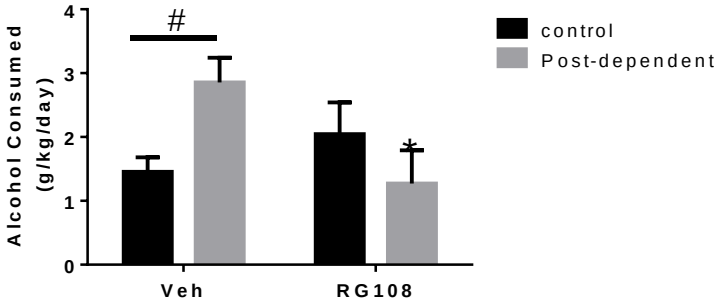

Alcohol Consumed
(g/kg/day)
4
3
2
1
0
#
*
Veh
RG108
control
Post-dependent

Figure 4

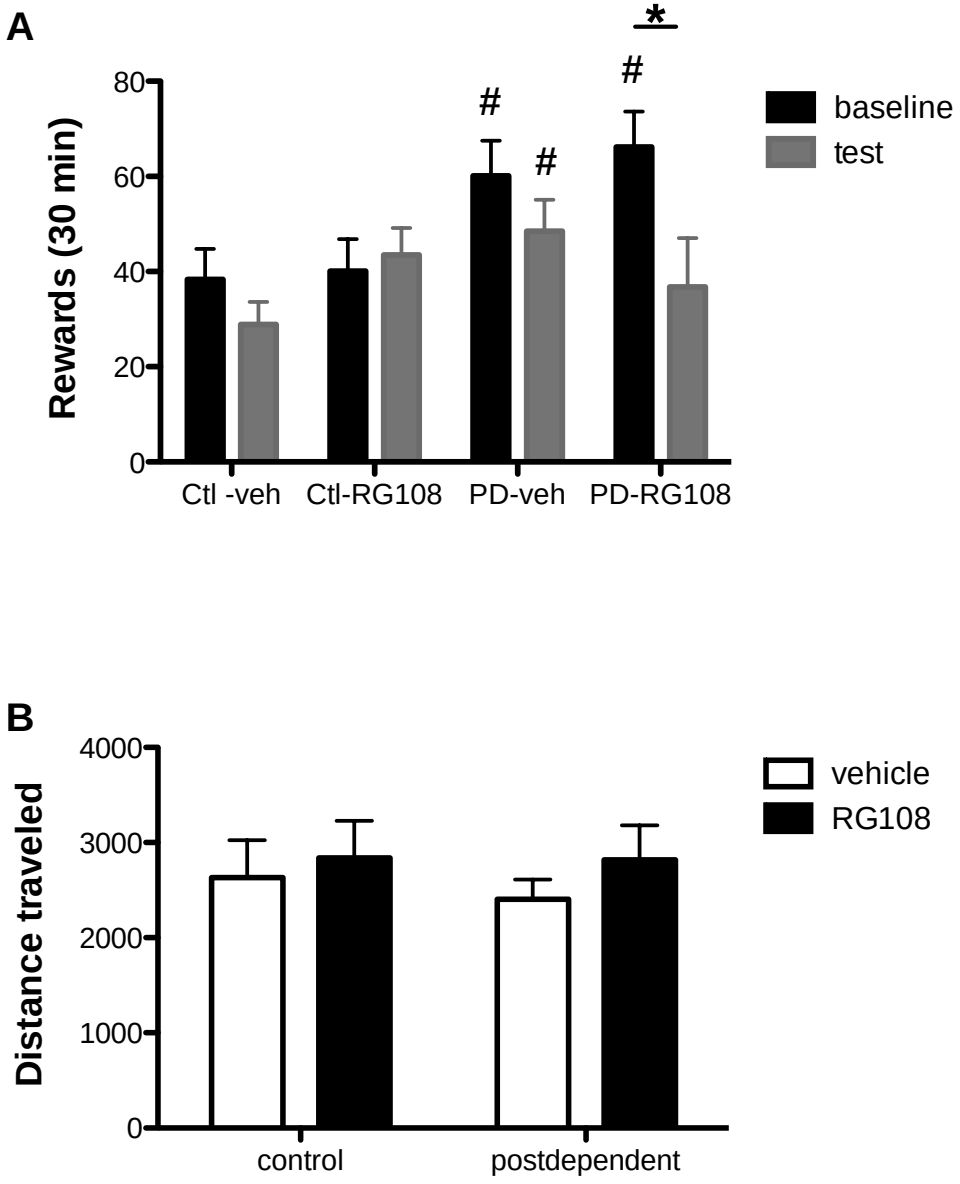
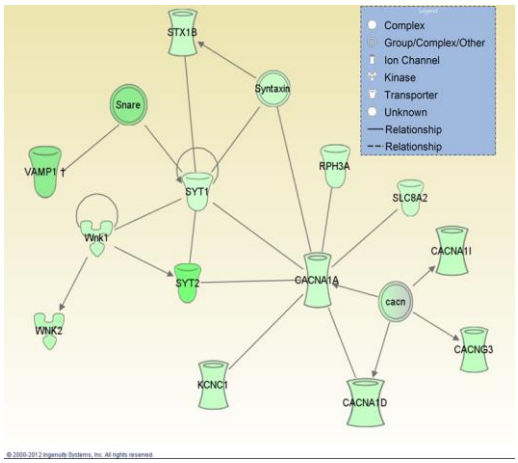

A
Rewards (30 min)
80
60
40
20
0
#
#
#
*
baseline
test
Ctl -veh
Ctl-RG108
PD-veh
PD-RG108
B
Distance traveled
4000
3000
2000
1000
0
vehicle
RG108
control
postdependent

Figure 5

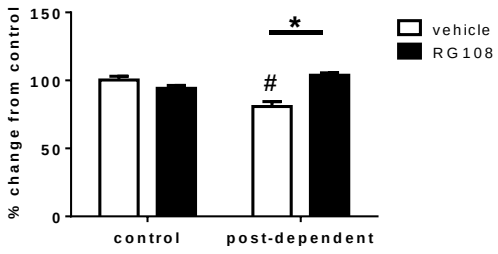
A.



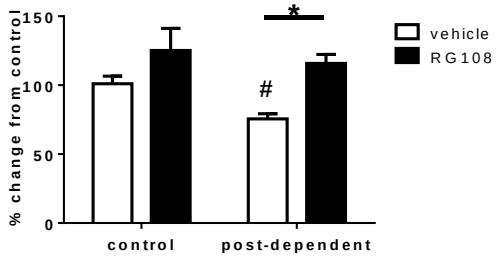
B.

	RNA sequencing		real-time quantitative PCR	
	Fold change	p values	Fold change	p values
syt1	-1.318	0.033	-1.276	0.001
syt2	-3.862	0.029	-1.335	0.002
cacna1a	-1.447	0.044	-1.306	0.035
cacna1i	-1.717	0.012	-1.111	0.105
wnk1	-1.661	0.010	-1.177	0.079
wnk2	-1.999	0.009	-1.228	0.045
kcnc1	-1.678	0.028	-1.143	0.037

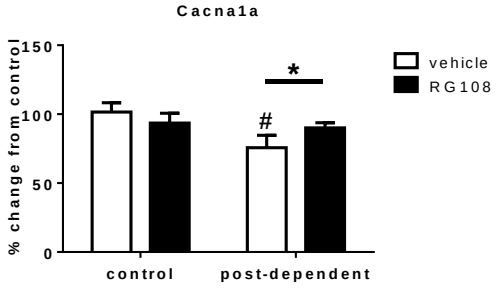
C.



D.



E.



F.

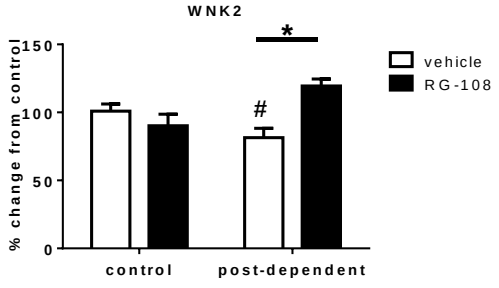


Figure 6

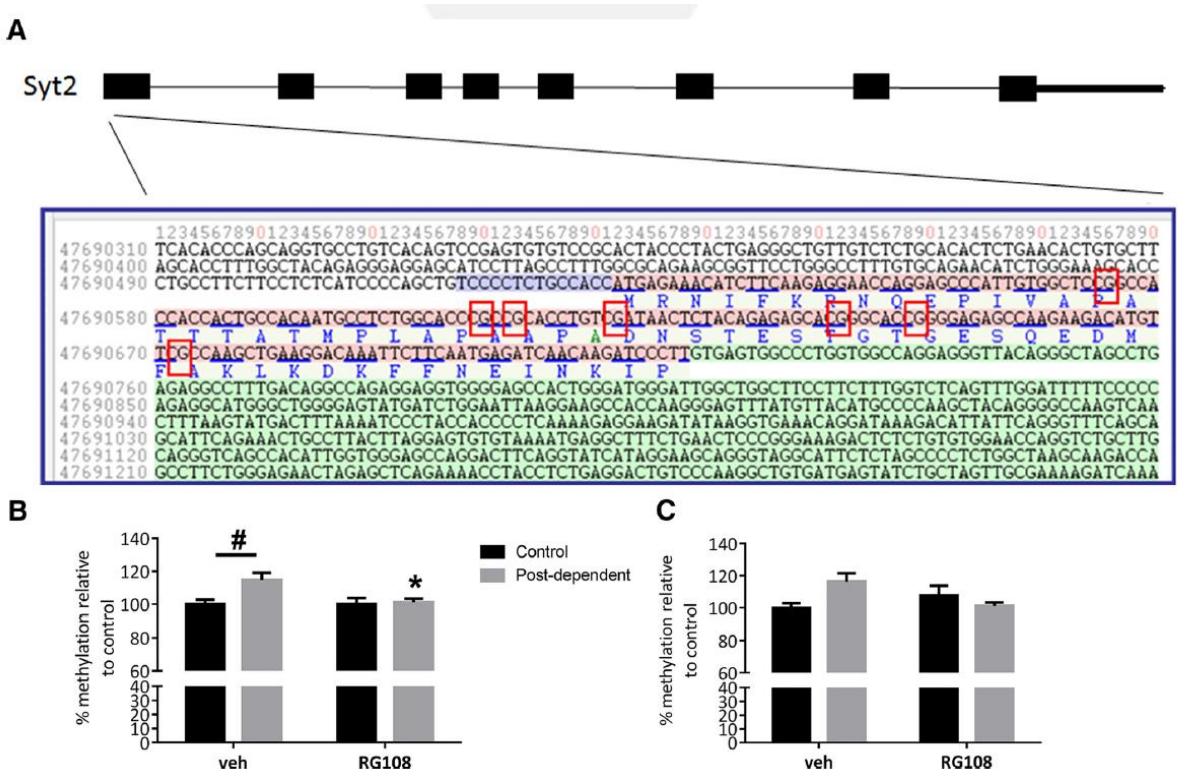

A
Syt2
B
% methylation relative to control
Control
Post-dependent
#
*
veh
RG108
C
% methylation relative to control
veh
RG108

Figure 7

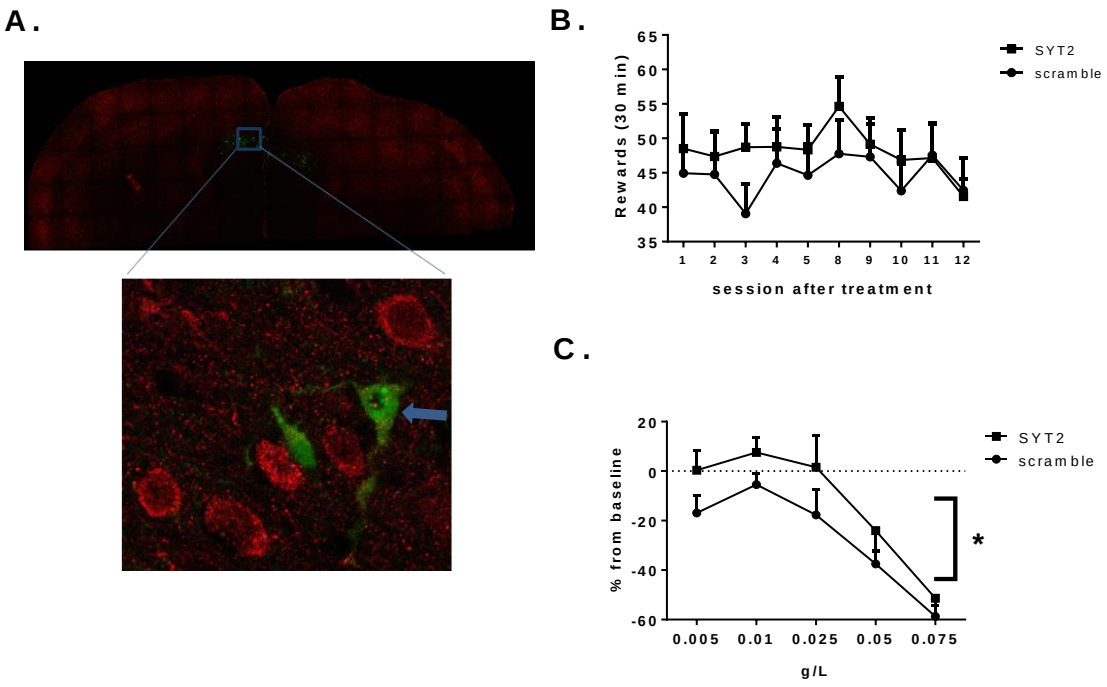

A.
B.
C.
SYT2
scramble
Rewards (30 min)
session after treatment
% from baseline
g/L
*
D.
E.
Quinine consumption (mg/kg/day)
Quinine
Scramble
Syt2
+3.7
+3.2
+2.7
+2.5

Figure 8

Table 1: Global DNA methylation levels

Brain region	Control	Post-dependent
mPFC	100 ± 17.65	200.34 ± 38.45 *
Amg	100 ± 28.7	105.79 ± 29.2
Hipp	100 ± 13.08	92.51 ± 15.13
Nac	100 ± 17.0	144 ± 6.8 *

Table 2: Effect of RG108 on global DNA methylation levels in the mPFC

Drug treatment	Control	Post-dependent
vehicle	100 ± 17.65442	200.34 ± 38.45 #
RG108	109.35 ± 11.36	119.62 ± 19.11*

Table 3: Top 6 GO functional enrichment analysis for the list of 784 significantly altered mRNAs (P<0.05, B-H, FDR<0.2).

Category	Term	Function	Term Description	Genes	Count	List Total	Pop Hits	Pop Total	Fold Enrichment	PValue	Bonferroni	Benjamini	FDR
GOTERM_BP_FAT	GO:0006836	**Neurotransmission**	neurotransmitter transport	SYT1, RAB3C, NOS1AP, SLC6A1, SLC6A11, SYT2, STX1B, RIMS1, PARK7, LIN7A, SLC1A3, SYN1, TRIM9, CACNA1A, NSF, LPHN1	16	395	100	12092	4.898025316	7.68E-07	0.002	0.002	0.001
GOTERM_BP_FAT	GO:0007269		neurotransmitter secretion	SYT1, RAB3C, NOS1AP, SYN1, TRIM9, SYT2, NSF, CACNA1A, LPHN1, LIN7A	10	395	52	12092	5.887049659	4.01E-05	0.097	0.050	0.071
GOTERM_CC_FAT	GO:0044451	**Regulation of gene expression**	nucleoplasm part	NR6A1, HR, WBP11, TBP, DMAP1, MEIS1, POLR2B, PNN, ARNT, ERCC5, BRPF1, GTF3C6, GATAD2A, EWSR1, CHD4, MYST3, ATPAF2, RARG, SMAD9, SMAD7, YWHAB, ECSIT, GPS2, MED4, EP300, SMARCE1, RBPSUH, PIAS3, HIPK2, PAF1, HDAC7, NCOR2, MED1	33	354	430	10776	2.336145053	1.20E-05	0.005	0.005	0.017
GOTERM_CC_FAT	GO:0070013		intracellular organelle lumen	NR6A1, HR, TBP, DMAP1, PNN, RBM4B, FAR2, BRPF1, IDH3G, P4HA3, MRPL36, MYST3, ATPAF2, RARG, POLR1E, ERP29, CDK9, FGF22, ECSIT, MED4, EP300, SMARCE1, PIAS3, HIPK2, ATP5C1, RANGRF, PAF1, TGFB1I1, MED1, TXN2, NFKBIE, GLUD1, WBP11, MEIS1, POLR2B, ARNT, LOC684557, ERCC5, TAP1, GTF3C6, GATAD2A, ETV6, NSUN2, CHD4, DNAJA3, EWSR1, LYZ2, SHMT2, SMAD9, SMAD7, PNO1, FDXR, YWHAB, IDH3B, ILF3, GPS2, PEO1, MRPL23, DDX56, PHF2, RBPSUH, PES1, HDAC7, NCOR2, DAP3	65	354	1178	10776	1.679663895	2.79E-05	0.011	0.006	0.039
GOTERM_MF_FAT	GO:0003712		transcription cofactor activity	DCC, HR, YWHAB, DMAP1, PPARGC1A, ARNT, GPS2, MED4, NCOA2, EP300, ATN1, ZMIZ2, WDR77, BCL11A, HIPK2, TGFB1I1, MKL1, HDAC7, NCOR2, MED1	20	387	164	11963	3.769773744	1.38E-06	0.001	0.001	0.002
GOTERM_MF_FAT	GO:0008134		transcription factor binding	DCC, HR, TBP, HSPA1A, DMAP1, ARNT, ATN1, WDR77, BCL11A, MKL1, CHD4, MYST3, RARG, YWHAB, PPARGC1A, GPS2, MED4, NCOA2, EP300, RBPSUH, ZMIZ2, HIPK2, TGFB1I1, NCOR2, HDAC7, MED1	26	387	309	11963	2.601021884	2.28E-05	0.016	0.008	0.034

Table 4: RG108 treatment does not regulate the expression *Cacna1a*, *Syt1*, *Syt2*, *Cacna1l*, *Slc8a2* and *Wnk2* in the Amg, NAc and Hipp.

	CTL/VEH		CTL/RG108		PD/VEH		PD/RG108	
Amg	Fold change	sem	Fold change	sem	Fold change	sem	Fold change	sem
Cacna1a	1.01	0.05	-1.02	0.07	-1.13	0.04	-1.12	0.05
Syt1	1.01	0.04	1.01	0.04	-1.05	0.05	-1.05	0.05
Syt2	1.02	0.07	-1.09	0.09	-1.24	0.11	-1.12	0.05
Cacna1l	1.01	0.06	1.10	0.06	1.10	0.12	1.05	0.06
Slc8a2	1.02	0.08	1.19	0.10	1.56	0.23	1.26	0.11
wnk2	1.01	0.05	1.08	0.06	1.19*	0.05	1.32*	0.14
Hipp								
Cacna1a	1.01	0.06	1.03	0.05	1.07	0.05	1.24	0.11
Syt1	1.01	0.04	1.15	0.03	1.25	0.05	1.09	0.08
Syt2	1.02	0.08	1.32	0.15	1.52*	0.13	1.50*	0.14
Cacna1l	1.01	0.07	1.00	0.06	1.11	0.11	1.18	0.11
Slc8a2	1.02	0.08	1.05	0.06	1.20*	0.06	1.17*	0.05
Wnk2	1.01	0.06	0.81	0.04	1.29*	0.16	1.01	0.05
NAc								
Cacna1a	1.12	0.19	1.29	0.12	1.30	0.15	1.19	0.13
Syt1	1.09	0.17	1.19	0.09	1.12	0.09	1.02	0.07
Syt2	1.24	0.38	1.53	0.23	1.85	0.49	1.42	0.26
Cacna1l	1.02	0.08	-1.06	0.08	1.00	0.10	0.88	0.09
Slc8a2	1.02	0.07	-1.07	0.07	-1.03	0.08	-1.22	0.07
Wnk2	1.01	0.06	-1.02	0.06	1.02	0.06	-1.19	0.05