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Title Page:

N-Phthalyl-L-tryptophan (RG108), like clozapine (CLO), induces chromatin remodeling in brains of prenatally stressed mice

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Running Title: Antipsychotics and Epigenetic mechanisms

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Abbreviations: 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; Ab, antibody; BP, bipolar disorder; ChIP, chromatin immune precipitation; CLO, clozapine; DMSO, dimethyl sulfoxide; H3Kac, lysine acetylated histone 3; H3K27me3, trimethylated lysine 27 histone H3; HAL, haloperidol; IgG, immunoglobulin G; MeDIP, methyl DNA immuneprecipitation; meDNA, methylated DNA; mPFC, medial prefrontal cortex; NS, non-stressed; OD, optical density; PCR, polymerase chain reaction; PND, post-natal day; PRS, prenatal restraint-stressed; PVDF, polyvinylidene difluoride; RG-108, N-phthalyl-L-tryptophan, RIS, risperidone; SZ, schizophrenia; SZA, schizoaffective; TBS, tris buffered saline; VEH, vehicle; VPA, valproic acid.

ABSTRACT

Schizophenia (SZ), schizoaffective (SZA), and bipolar (BP) are neurodevelopmental psychopathological conditions related, in part, to genetic load and, in part, to environmentally-induced epigenetic dysregulation of chomatin structure and function in neocortical GABAergic, glutamatergic and monoaminergic neurons. In order to test the above hypothesis, we targeted our scientific efforts on identifying whether the molecular epigenetic signature of post-mortem brains of SZ, SZA, and BP disorder patients are also present in the brains of adult mice born from dams prenatally restraint-stressed during gestation. The brains of prenatally restraint-stressed (PRS) mice similar to the brains of SZ and BP disorder patients, show an ~ 2 fold increased binding of DNMT1 to psychiatric candidate promoters (*glutamic acid decarboxylase 67* [*Gad1],* *Reelin* [*Reln*]*,* and *Brain derived neurotrophic factor [Bdnf]*) leading to their hypermethylation, reduced expression, as well as, to the behavioral endophenotypes reminiscent of those observed in the above psychiatric disorders. To establish whether clozapine (CLO) produces its behavioral and molecular action though a causal involvement of DNA methylation/demethylation processes, we compared the epigenetic action of CLO with that of the DNMT1 competitive inhibitor, N-Phthalyl-L-tryptophan (RG108). The intracerebroventricular (icv) injection of RG108 (20 nmol/day/5 days), similar to the systemic administration of CLO, corrects the altered behavioral and molecular endophenotypes typical of PRS mice. These results are consistent with an epigenetic etiology underlying the behavioral endophenotypic profile in PRS mice. Further, it suggests that PRS mice may be useful in the preclinical screening of antipsychotic drugs acting to correct altered epigenetic mechanisms.

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INTRODUCTION

The development of new and effective drugs that also show reduced toxicity to treat the complex symptomatology of schizophenia (SZ), schizoaffective (SZA), and bipolar (BP) disorder is impeded by the lack of pertinent diagnostic tools to assess disease prodromes, progression severity, and beneficial drug responses. Additional fundamental hurdles to the identification of new drugs to effectively treat SZ, SZA, and BP disorder include an insufficient appreciation of the causal and developmental mechanisms underlying the symptoms characteristic of these diseases. Moreover, the inability to reproduce the complex nature of these disorders in laboratory animals is an additional barrier to drug discovery.

SZ and related psychiatric disorders are neurodevelopmental disorders with a significant epigenetic pathophysiology brought about by exposure to prenatal or perinatal environmental insults, including stress, drugs, maternal infection, and trauma (Khashan et al., 2008; Markham and Koenig, 2011; McGowan et al., 2011; van Os et al., 2010; Zhang et al., 2013; Cao-Lei et al., 2015; Labouesse et al., 2015; Cao-Lei et al., 2016) and a variable genetic load (Weinberger et al., 1987; Fatemi and Folsom, 2009; Sekar et al., 2016; Schizophenia Working Group of the Psychiatric Genomics Consortium, 2014). Epigenetic studies indicate that aberrant DNA methylation profiles likely play an important neurodevelopmental role in psychiatric disease pathogenesis and as a target for drug discovery (Mill et al., 2008; Ptak and Petronis, 2008; Grayson and Guidotti, 2013). We've previously established that prenatally restraint-stressed (PRS) mice exhibit SZ-like endophenotypic characteristics that can be used to identify robust and efficacious antipsychotic drugs that normalize epigenetic mechanisms (Dong et al., 2016).

The frontal cortex of PRS mice, similar to those of SZ, SZA, and BP post-mortem brains, exhibit an epigenetic profile that includes an increase in Dnmt1 binding to the *Gad1, Reln*, and *Bdnf* promoters (Matrisciano et al., 2013; Dong et al., 2015b; 2018) and an enrichment of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) at these same promoter regions

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(Dong et al., 2015a; 2016). These increases in promoter methylation are associated with reduced levels of synapse associated proteins, such as Bdnf and Reln (Dong et al., 2015a, 2016) and behavioral deficits that resemble aspects of the positive and negative symptoms (Jones et a., 2011), and the cognitive impairment, detected in SZ, SZA and BP disorder patients (Ruzicka et al., 2008, Lisman et al 2008, Fine et al., 2014, Murmu et al., 2006). Interestingly, the enrichment of 5mC at the *Bdnf, Gad1*, and *Reln* promoters in the medial prefrontal cortex (mPFC) and hippocampus of PRS mice, correlates with the behavioral endophenotypes (Dong et al., 2015a, 2016). Although there is general agreement that altered epigenetic mechanisms play an important role in the etiopathogenesis of SZ and BP disorders (Guidotti et al., 2011; Grayson and Guidotti, 2013), little is known regarding the effects of drugs, including typical and atypical antipsychotics, on the epigenetic modifications of GABAergic, glutamatergic, or monoaminergic neurons in the neocortex of SZ and BP disorder patients (Guidotti et al., 2017).

In the current study, we first replicated previous results on the hypermethylation of Bdnf Reln1, Gad1 promoters in PRS mice. To validate the PRS mouse as a reasonable epigenetic animal model for studying the actions of specific groups of neuroleptic drugs acting on altered DNA methylation, we compared the effects of a typical antipsychotic haloperidol (HAL) (acting at DRD2 receptors) and two atypical antipsychotics: clozapine (CLO) (active at various receptors including HTR1A, HTR2A, HTR2C and ADORA1 receptors) and risperidone (RIS) (active at DRD2, HTR2A, HTR2C, and ADORA1 receptors) (Table 1, Jarskog et al., 2007; Meltzer, 2012) with the effects of RG108, a recently identified compound that selectively target the catalytic domain of DNMT1 (for structure, see Asgatay et al., 2014).

Materials and Methods

Animals and PRS Procedure

All procedures were performed according to guidelines of the National Institutes of Health for animal research and were approved by the Institutional Animal Care Committee of the University

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of Illinois at Chicago. Timed pregnant mice (Swiss albino ND4) were obtained from Envigo. Pregnant dams were individually housed with a 12-h light-dark cycle, and food and water *ad libitum*. The pregnant mice were left to habituate to the new housing conditions for 1-2 days and were then randomly assigned to non-stressed (NS) or stressed groups. NS dams were left undisturbed thoughout gestation, whereas stressed dams were subjected to repeated episodes of restraint stress, as described previously (Dong et al., 2016). Experiments were performed on mice between PND 60 to 85. Male mice were chosen for the experiments because they exhibit more robust and consistent stereotyped behavior, reduced social interactions, prepulse inhibition and fear conditioning deficits than female mice. However, in a recent study we also established that female PRS mice show more anxiety than male mice (Dong et al. 2018).

Drug Treatment

CLO (Sandoz Pharmaceuticals, Princeton, NJ), HAL (Millipore-Sigma, St. Louis, MO), and RIS (Tocris Bioscience, Minneapolis, MN) were dissolved as previously described (Dong et al., 2008). RG108 was dissolved in 10% DMSO.

Drugs (CLO 5 mg/kg, HAL 1mg/kg, and RIS 0.5 and 5 mg/kg) were administered to PRS and NS mice at PND 60 to 75 subcutaneously (sc) twice daily for five consecutive days. It is noteworthy that drugs used in this study elicited catalepsy (HAL), and sedation (CLO, RIS) which are maximal after approximately 1 h but disappear following 18 h of washout when the animals were resubmitted to the behavioral tests.

It should be noted that drug doses and duration of treatment were chosen on the basis of preliminary studies showing that CLO (5 mg/kg) requires multiple days of treatment to improve behavioral deficits and elicits demethylation of hypermethylated Reln promoters in mice treated protractedly with large doses of methionine (Guidotti et al., 2009, 2011; Alachkar et al, 2018; Dong et al. 2008).

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RG108 is a non-competitive antagonist of DNMT1 activity (Asgatay et al., 2014). Because RG108 does not enter the brain at sufficient concentrations due to the blood brain barrier, it was administered by icv infusion using an osmotic mini pump connected with a brain infusion kit (Alzet, Duret Corporation, Cupertino, CA). The pump delivered 0.5 μl/h of a solution containing 100 nmol of RG108 in 10% dimethyl sulfoxide (DMSO) in 72 μl for 5 days. Vehicle (VEH, 10% DMSO alone) was administered to corresponding control groups using a similar pump delivery regimen. Behavioral tests were conducted 18 h after the last administration of RG108, antipsychotic, or VEH.

Behavioral Tests

Stereotyped rearing activity and social interactions were examined in succession on the same day between 10 am and 3 pm. We selected PNDs 60 to 75 for behavioral testing because at this post-natal time, the performance of the offspring was more reproducible and stable than the performance measured at earlier developmental time points (Matrisciano et al., 2013).

Stereotype rearing activity

A computerized Animal Activity Monitoring System with VersaMax software (AccuScan Instruments, Columbus, OH) was used for the quantification and tracking of vertical activity in mice as described previously (Matrisciano et al., 2013). The total number of interruptions of the vertical sensors was taken as a measure of stereotyped behavior. Activities were recorded for 15 min.

Social interaction

Social interaction was measured using the “Thee-Chambers Apparatus” described in detail elsewhere (Dong et al., 2016).

Social approaches were defined as the ratio of the sniffing time for the wire cup enclosing the stranger mouse *vs* the empty cup. The reliability of the measurements was assessed by correlating the scores of different raters.

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Experimental Design

For these experiments we used 3 independent cohorts of mice. In the first cohort, we compared CLO to HAL in 6 groups of mice: Group 1, NS mice receiving VEH; Group 2, NS mice receiving CLO; Group 3, NS mice receiving HAL; Group 4, PRS mice receiving VEH; Group 5, PRS mice receiving CLO; Group 6 PRS receiving HAL. In the second cohort, we have: Group 1, NS mice receiving VEH; Group 2, NS mice receiving RIS (0.5 mg/kg); Group 3, NS mice receiving RIS (5 mg/kg); Group 4, PRS mice receiving VEH; Group 5, PRS mice receiving RIS (0.5 mg/kg); Group 6, PRS receiving RIS (5 mg/kg). In the third cohort we have: Group 1, NS mice receiving VEH; Group 2, NS mice receiving RG108; Group 3, PRS mice receiving VEH; Group 4, PRS mice receiving RG108 (icv, 100 nmol/day/5 days). Because these experiments are labor intensive and time consuming, we used a different cohort for each drug tested.

For the statistical analyses, to reduce the effect of variability among cohorts, results of CLO, HAL, RIS, and RG108 treatments were compared to the values of the respective controls and were expressed as % of VEH-treated control mice.

Prefrontal cortex (PFC) dissection

A Jacobowitz mouse brain slicer matrix was used for the dissection of the cortex. Starting from the frontal lobe (AP=4.28 mm) thee slices of 1 mm were mounted on a cold coverslip and disks of 1.5 mm in diameter were punched from the frontoparietal motor and sensory cortices. Using this procedure, we obtained ~20 mg of tissue for DNA/RNA studies (Impagnatiello et al., 1996).

Real-time polymerase chain reaction (PCR)

The PCR was carried out using an Applied Biosystems Real-Time PCR System using SYBR green master mix (Fermentas, Glen Burnie, MD). For additional details see Dong et al., 2016.

Methylated DNA immunoprecipitation

Methylated DNA at the *Gad1*, *Reln*, and *Bdnf* IX promoters were measured using immunoprecipitation (MagMeDIP), as described by the manufacturer (Diagenode, Denville, NJ).

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We used immunoprecipitation with specific antibodies to distinguish 5mC from the approximately 20% of methylcytosines in the brain that are 5hmC (Wen et al., 2014). The percentage of methylated *vs* unmethylated promoter was calculated using the following equation: % [(meDNA−IP/total/input)] = $2^{[(Ct(10\%input)-3.32)-Ct(meDNA-IP)]}\times 100\%$. The efficiency of MeDIP was determined by qRT-PCR using internal positive and negative DNA controls (methylated/hydroxymethylated and unmethylated DNA), as well as, control primers for testis-specific H2B histone gene (TSH2B, which is methylated in all somatic cells but not in testis), and GAPDH promoter, which is poorly methylated. Data obtained with internal positive and negative DNA controls indicate a specificity of ~96% (Specificity % = 1-(enrichment unmethylated/enrichment methylated) x 100). No PCR signal was obtained when using an IgG control (Gatta et al. 2017).

Chomatin immunoprecipitation assays

We performed chomatin immunoprecipitation (ChIP) assays for DNMT1 binding based on protocols previously described (Dong et al., 2016). The percentages of immunoprecipitated DNA were calculated as described for MeDIP assay. ChIP grade DNMT1 specific antibodies (Imgenex, San Diego, CA), were used to co-precipitate cross-linked chomatin as previously described (Dong et al., 2016). No PCR signal was detected using an IgG control sample.

Western Blotting

All extraction procedures were carried out with an EpiQuik Total Histone Extraction Kit at 4°C, accordingly to manufacturer's (Epigentek, Farmingdale, NY) instructions. To ensure similar protein loading, the total protein concentration of these extracts was determined by using the bicinchoninic acid assay method (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA) using bovine serum albumin as a standard.

Samples were diluted in Laemmli buffer, denatured at 75°C for 10 minutes, run on 4-12% Bis-Tris gel (Thermo Fisher Scientific), and blotted onto PVDF membranes (Millipore). Membrane

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preparations were incubated overnight at 4°C with anti-histone antibodies diluted in 5% dry milk, 0.1% Tween 20 in TBS, washed, and further processed with horseradish peroxidase-conjugated secondary antibodies (GE HealthCare, Chicago, IL). Immunocomplexes were revealed by enhanced chemiluminescence with an Immobilon Western Chemiluminescent HP Substrate Kit (Merck, Burliongton, MA) by using the LI-COR ODYSSEY System and analyzed with Image Studio 5.2 (iS5.2) software.

To profile the pattern of histone tail modifications, we used the following antibodies: polyclonal anti-histone3 (H3) (Millipore, 1:100); polyclonal anti-H3Kacetylated (H3Kac) (Millipore Sigma, 1:1000); monoclonal anti-H3 dimethyl K9 (H3K9me^2) (1:1000; Abcam, Cambridge, MA); monoclonal anti-H3 trimethyl lysine-4 (H3K4me^3) (1:1000, Cell Signaling Technology, Danvers, MA) and monoclonal anti-histone H3 trimethyl lysine-27 (H3K27me^3) (1:500, Cell Signaling Technology, Danvers, MA) The antibodies were highly specific. After immunoblotting, only a single reactive band was detected, and the corresponding molecular weight was that of expected modified histone. In addition, preadsorbing Ab with the corresponding synthetic peptides resulted in a complete loss of signal. In all blots, the optical density (OD) of the H3Kac, H3K4me^{3,} and H3K27me^3 bands were quantified and normalized relative to the OD of the correspondent H3 band. The anti-H3 antibody detects both unmodified and modified H3 variants (total H3). Samples from VEH- and drug-treated animals were analyzed in the same immunoblot, and values were calculated relative to VEH-treated mice.

Statistical analysis

Results are expressed as mean ± SD and are presented as % of respective NS mice receiving VEH. Experimental differences were assessed by two-way ANOVA followed by Tukey's *post-hoc* comparisons, using Predictive Analytics SoftWare (PASW) v.18 (SPSS, Chicago, IL). The criterion for significance was $p<0.05$, two tailed. Correlation analyses were performed using Person's correlation test. Western blotting data were analyzed using Student's t test.

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Results

CLO, but neither HAL nor RIS, corrects the behavioral endophenotype in PRS mice. Table 1 reports the high affinity neurotransmitter receptor profile of thee antipsychotics: CLO, HAL, RIS, each of which were tested for their ability to correct the behavioral endophenotypes and the molecular abnormalities in PRS mice. We previously established that PRS mice exhibit several behavioral alterations, including increased stereotypy, deficits in social interaction time, hyperlocomotor activity in an open field, and deficits in prepulse inhibition of acoustic startle (Matrisciano et al., 2013). The behavioral studies with PRS mice include increased vertical activity (stereotyped behavior) and reduced social interaction. These tests were selected because they produce limited distress to the animals that are then used for subsequent biochemical studies (Dong et al., 2016). For the behavioral experiments, young adult (PND 60-75), NS and PRS male mice were treated for five days with either VEH or the drug under study and then withdrawn for 18 h to eliminate any acute effects of antipsychotics on behavior. As shown in **Fig. 1**, we replicated previous reports (Dong et al., 2016) that CLO corrects the behavioral endophenotypes associated with PRS mice, while HAL fails to do so. In addition, we observed that RIS (0.5 or 5 mg/kg) was ineffective at reducing the increased stereotyped behavior (**Fig. 1A**) or relieving the social interaction deficit of PRS mice (**Fig. 1B**). To establish whether CLO produces its behavioral effects via a causal control of DNMT-mediated promoter methylation, we compared the epigenetic action of CLO with that of the DNMT1 competitive inhibitor, RG108, in PRS mice. The i.c.v. infusion of RG108 (100 nmol/day for 5 days), similar to CLO (5mg/kg twice a day for 5 days), corrects the behavioral endophenotypes (**Fig. 1**).

CLO, but neither HAL nor RIS, corrects the increased DNMT1 binding and 5mC promoter hypermethylation of SZ-associated genes in PRS mice. To establish the concept that Bdnf promoter methylation modulates the expression of its mRNA, we tested whether HAL or RIS could correct the local DNA methylation enrichment at cytosines proximal to the

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transcriptional start site (TSS) of the Bdnf-ix promoter in the dorsal hippocampus of PRS mice. We observed that the enrichment of cytosine methylation at the Bdnf-ix promoter (**Fig. 2**) was not reduced in either PRS or NS mice treated with HAL- or RIS. In contrast, in a replication of our previous study (Dong et al., 2016) with new cohorts of PRS and NS mice, we showed that CLO corrects both the increased Dnmt1-binding (**Fig. 3A**) and 5mC enrichment (**Fig. 3B**) to selected target promoters (Bdnf-ix, Gad1, and Reln). Collectively, these data suggest that CLO, unlike HAL or RIS, may limit DNA methylation, either by reducing the expression of Dnmts, or more likely by interfering with Dnmt1-DNA binding capability (Grayson and Guidotti, 2013; 2016) or Dnmt1 enzymatic activity.

RG108, like CLO, corrects the behavioral phenotype and alterations in target promoters in the PFC of PRS mice. We also established that RG108, administered icv, corrects the methylation enrichment, as well as, the increased binding of DNMT1 to the *Gad1, Reln, and Bdnf-ix* promoters in the cortex of PRS mice (compare **Fig. 3** and **Fig. 4**). An important observation of this study is that CLO, like valproic acid (VPA, Dong et al., 2016), has no effect on either the behavior or promoter methylation or the expression of mRNA and protein that we studied when administered to control mice or when injected only 1 time, 18 h before testing. A possible explanation for the failure of CLO to demethylate target promoters in NS mice is that the dynamic state of DNA methylation may be accelerated in PRS mice when compared with the NS mice. Hence the steady-state methylation process may be very sensitive to short term inhibition in PRS mice.

The restorative action of CLO on altered epigenetic mechanisms is not mediated by histone- post-translational covalent modifications? The restorative effects of CLO on the molecular and behavioral endophenotypes of PRS mice can be duplicated by the histone deacetylase (HDAC) inhibitors, VPA (Tremolizzo et al. 2005) directly acting on histone acetylation levels. Of note, VPA increases histone acetylation levels in both NS and PRS mice (Tremolizzo

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et al., 2005). In addition, there is one report (Huang et al., 2007) that demonstrates an increase in the levels of H3K27me^3 in the cortex of mice treated chonically with (CLO-5 mg/kg, daily for 21 days). Therefore, we tested whether single (1 h before) or repeated injections of CLO (5 mg/kg twice a day for five days and followed by 18 h withdrawal) alter the levels of H3Kac or H3K4me^3, or H3K27me^3 in NS mice. In **Fig. 5**, we show representative images of the western blot for H3K27m^3. The values were normalized to the levels of non-methylated or non-acetylated H3. No significant differences in the levels of these histone variants were detected in the mPFC of mice receiving CLO. For example the levels of H3Kac in the PFC are 110 +/-10 and 101+/-16 % of control (n=5) in single and repeated CLO treated mice respectively. The levels of H3K27me^3 were 127+/-7.6 % of controls and the values of H3K4me^3 were 80+/-18 % of controls (n=5, non-significant) in PFC of mice treated for 5 days with CLO.

Discussion

In this study, we describe a neurodevelopmental, epigenetic mouse model for psychiatric disease (the *in utero* stressed mouse or PRS mouse) that in the cerebral cortex expresses an increased DNMT1 binding and 5-cytosine hypermethylation of promoters (Reln, Gad1, and Bdnf) that represent the core epigenetic pathophysiological signature underlying SZ, SZA and BP disorders. This model responds to the administration of CLO with striking behavioral and neuroepigenetic improvements, while HAL and RIS fail to do so. The behavioral and molecular effects of CLO are mimicked by the icv injection of the DNMT inhibitor, RG108 (Asgatay et al., 2014), suggesting that PRS mice may help to identify novel families of neuroleptic drugs capable of reducing DNA-hypermethylation and correcting the behavioral endophenotypes associated with these disorders.

Mice born from mothers stressed during gestation show that, like in humans, environmental insults occurring at critical periods of central nervous system development can trigger epigenetic modifications at psychiatric disorder candidate genes including *Reln, Gad1, and Bdnf*, leading to

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their downregulation (Khashan et al., 2008; Markham and Koenig 2011; Mcgowan et al., 2011; van Os et al., 2010; Zhang et al., 2013; Cao-Lei et al., 2015; 2016; Scheinost et al., 2016). These epigenetic changes are paralleled by behavioral deficits that are phenotypically reminiscent of those observed in psychiatric diseases (Dong et al., 2016). Hence, the PRS mouse model has value as an experimental epigenetic model for SZ, SZA, and BP, disorders and can be useful for screening potential new anti-SZ/SZA/BP disorder drugs for pre-clinical efficacy and perhaps prophylactic intervention.

Despite the large numbers of preclinical and clinical efforts aimed at delineating the role of major neurotransmitter receptors in the anti-SZ profile of different classes of antipsychotic drugs, little is known regarding the role that these receptors play in the regulation of the neurodevelomental epigenetic pathology underlying SZ and related psychiatric disorders. Here, we show that by investigating the behavioral and molecular neuroepigenetic alterations induced by typical and atypical neuroleptic drugs (Jarskog et al., 2007), it should be possible to identify or exclude the contribution of each receptor or combination of receptors and their corresponding signal transduction mechanisms to the regulation of altered chomatin remodeling events in PRS mice.

For example, we compared two atypical (CLO and RIS) and one typical (HAL) antipsychotic on the behavioral and molecular (chomatin remodeling) alterations observed in PRS mice. We confirmed previous results that doses of HAL that are capable of blocking DRD2 function and inducing extrapyramidal side effects but do not have serotonin receptor affinity, fail to normalize the altered behavioral endophenotypes or reduce promoter hypermethylation in PRS mice (**Table 1**). In contrast, CLO, that binds with high affinity to serotonin receptors but with low affinity to DRD2 receptors (Table 1), normalizes the altered endophenotypes (**Figs. 1A 1B**) and aberrant chomatin remodeling (**Figs. 2 and 3**). These data suggest that the effects of CLO are not mediated solely by the blockade of DRD2 function.

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To further clarify this issue, we studied RIS (an antipsychotic with high affinity for both DRD2 and HTR1A, HTR2A, HTR2C, and ADORA1 receptors, see Table 1). This atypical antipsychotic failed to induce the restorative behavioral and molecular effects reported with CLO (Figs. 1A, 1B and 2). The lack of effects of HAL or RIS on the behavioral and molecular neuroepigenetic endophenotypes of PRS mice support the concept that DRD2 receptor, as well the HTR1A, HTR2A, HTR2C, and ADORA1 receptors are not necessary to explain the corrective action of CLO in our PRS mouse model. However, we cannot rule out a role for CHM1 receptors at this point. The question of whether CLO modulates DNA-methylation dynamics bypassing specific neurotransmitter receptors by directly interfering with the binding of DNMT1 to candidate SZ gene promoters or by activating DNA demethylation processes, also remains unresolved.

To establish whether the restorative effects of CLO on altered Dnmt1 binding and DNA-methylation processes are independent from their action on neurotransmitter receptors and instead dependent on a direct action on DNA methylating and demethylating enzymes and cofactors, we treated mice with RG108, a competitive Dnmt1 antagonist with no known neurotransmitter receptor agonist or antagonist affinity (Asgatay et al., 2014). This drug reduces the increased DNMT1 binding to the *Bdnf, Reln*, and *Gad1* promoters, and also reduces the enrichment of 5mC to these promoters, and restores the behavioral deficits in PRS mice. To explain the behavioral and biochemical action of CLO we studied whether CLO, like VPA, could alter histone post transcriptional modifications. In addition, there has been at least one report showing increased H3K4me^3 in the mouse cerebral cortex following CLO administration (5 mg/kg) for 21 days (Huang et al., 2007). However, no CLO-mediated action on histones was detected with the drugs and doses tested in our study. We also studied the effects of CLO on the expression of DNMTs and TETs (Ten eleven translocation family of proteins) and observed that these enzymes are slightly increased in the cortex of PRS mice. CLO normalizes the increased levels in the PRS mice but fails to change the basal level of the enzymes (Dong et al 2016).

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In preliminary studies, we tested whether CLO has Dnmt1 inhibitory activity using purified recombinant protein *in vitro.* CLO was ineffective at inhibiting Dnmt1 activity up to concentrations of 10^{-3} M (Valentina Locci, unpublished data). Hence, the mechanisms underlying the marked effects of CLO on DNA-methylation are not yet adequately explained.

Overall, the data suggest that drugs or conditions that reduce DNA methylation by reducing DNMT levels or by blocking DNMT binding or activity to SZ-target genes, may be desirable or even required, either alone or in association with antipsychotics, to restore normal behavioral activity in PRS mice and like-wise to provide beneficial effects to BP, SZA, and SZ patients with an underlying epigenetic etiopathogenesis.

The PRS mouse model is insensitive to neuroleptics (i.e. HAL, RIS) that do not have an epigenetic action but is sensitive to drugs like CLO, VPA, (Matrisciano et al., 2013; Dong et al., 2016), and RG108 (present results) because these drugs modulate chomatin function. In this context, PRS mice behave like “antipsychotic-resistant” SZ patients, predicting treatment responses to CLO and as yet unexplored neuroleptic agents. The above data support our scientific premise that the PRS mouse represents a relevant model for studying interactions between antipsychotic drugs and aberrant epigenetic mechanisms that underlie the behavioral pathology associated with SZ, SZA and BP disorder.

Collectively, the behavioral and biochemical data with neuroleptic drugs support the hypothesis that the PRS mouse has value to screen for novel antipsychotic drugs with improved clinical efficacy and lower side effect liability. Furthermore, the PRS model theoretically has the potential for predicting treatment responses at specific initial stages of the illness with particular attention to early disease detection and possible prophylactic intervention.

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Author's Contributions:

Participated in research design: Dong, Locci, Grayson, Guidotti.

Conducted experiments: Dong, Locci.

Contributed new reagents or analytic tools:

Performed data analysis: Dong, Locci, Gatta

Wrote or contributed to the writing of the manuscript: Dong, Locci, Gatta, Grayson, Guidotti

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Footnotes:

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Legends for Figures

Figure Legends

Fig. 1. Increased stereotype behavior **(A)** and social interaction deficits **(B)** in PRS mice is reversed by treatment with CLO but not HAL or RIS. Sixty days old PRS or NS male mice were treated subcutaneously twice daily, for five days with vehicle, 5 mg/kg CLO, 1 mg/kg HAL, and 0.5 or 5 mg/kg RIS. Stereotypic behavior and social interaction were measured 18 h after the last antipsychotic treatment. The data are expressed as mean ± S.D. of 5-6 mice per group. Because we used different vehicle groups for each drug used, to compare the effect of the drugs, the values are expressed as % of the corresponding non-stressed (NS) groups receiving vehicle. **A,** Two-Way ANOVA, group x treatment effect $F_{5,76}$ = 4.23, *P*=0.002 followed by Tukey's post-hoc analysis. **P*<0.05 when PRS mice are compared with the respective NS vehicle treated mice. #*P*<0.05 when CLO and RG-108 treated PRS mice are compared to PRS mice receiving VEH. Average values of vertical activity in NS mice were 280 ± 60. **B**, The social interaction activity (ratio of sniffing time to the wire cup with a stranger mouse vs the wire cup without the stranger mouse) was measured in the same mice used in 1A about 18 h after the last antipsychotic treatment. The data are expressed as mean ± S.D. of 5-6 mice per group. Two-Way ANOVA, group x treatment effect $F_{5,88}$ = 3.45, *P*=0.007 followed by Tukey's post-hoc analysis. **P*<0.05 when PRS mice are compared with the respective NS vehicle treated mice. #P<0.05 when CLO and RG-108 treated PRS mice are compared to PRS mice receiving vehicle (VEH). Average values of social interaction ratio in 5 groups of NS mice 1.57 ± 0.43.

Fig. 2. The Increased levels of 5mC at Bdnf-IX promoter region in the frontal cortex of PRS mice are not corrected by HAL or RIS. The enrichment of 5mC was measured on the sixth day, ~18 h after the last day treatment. The data are expressed as mean ± S.D. of 5-6 mice per group. Two-Way ANOVA, group effect for 5mC: *Bdnf* - IX ($F_{1,72}$= 318, * P<0.001). Average value of 5mC levels in 3 groups of NS mice is about 1% of the input.

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Fig. 3. The increased DNMT1 binding **(A)** and levels of 5mC **(B)** at *Bdnf-ix*, *Reln, and Gad1* promoter regions in the frontal cortex of PRS mice are reversed by treatment with CLO (5 mg/kg sc twice a day for 5 days). Note there was no change of 5mC in the NS mice receiving CLO. The data are expressed as mean ± S.D. of 5 animals per group and analyzed with two-way ANOVA followed by Tukey's multiple comparisons. **A,** DNMT1 binding to: *Bdnf-ix;* ($F_{1, 16}$ = 10.63 p= 0.005), *Reln* ($F_{1,16}$ = 19.2,p= 0.0005), *Gad1* ($F_{1, 16}$ = 25.26, *P*= 0.0001). **B**, 5mC enrichment at Bdnf-ix; ($F_{1,16}$ = 8.36 p= 0.01), Reln ($F_{1,16}$ = 7.32, p= 0.02, Gad1 ($F_{1,16}$ = 18.61, p= 0.0005). * P<0.05 when PRS mice are compared with the respective NS vehicle treated mice. # P<0.05 when treated PRS mice are compared to PRS mice receiving vehicle (VEH).

Fig. 4. The increased DNMT1 binding **(A)** and levels of 5mC **(B)** at Bdnf-ix, Reln, and Gad1 promoter regions in the frontal cortex of PRS mice are reversed by treatment with RG108. Note there is no change of 5mC in the NS mice receiving RG108. The data are expressed as mean ± S.D. of 5 animals per group and analyzed with two-way ANOVA followed by Tukey's multiple comparisons. **A,**.DNMT1 binding at Bdnf-ix; ($F_{1,16}$ = 11.39 p= 0.004), Reln ($F_{1,16}$ = 6.15, p= 0.02, Gad1 ($F_{1,16}$ = 9.26, p= 0.008).* P<0.05 when PRS mice are compared with the respective NS vehicle treated mice. # P<0.001 when treated PRS mice are compared to PRS mice receiving vehicle (VEH). **B**, 5mc enrichment at Bdnf-ix; ($F_{1,16}$ = 9.75 p= 0.007), Reln ($F_{1,16}$ = 6.75, p= 0.02, Gad1 ($F_{1,16}$ = 15.94, p= 0.001). * P<0.05 when PRS mice are compared with the respective NS vehicle treated mice. # P<0.05 when treated PRS mice are compared to PRS mice receiving vehicle (VEH).

Fig 5. Effect of CLO treatment on H3K27me^3 protein levels in the frontal cortex of adult male mice. CLO was administered at the dose of 5 mg/kg twice a day for 5 days Protein levels of H3K27me^3 are shown as a ratio of H3K27me3/total H3. Optical densities are expressed as a % of VEH. Data are means ± S.D. of 5 mice per experimental group. Representative Western

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blot image is shown on top. Data indicate that there are no significant differences (unpaired t-test).

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Tables

Table 1. Relative PFC DNA methylation, Neurotransmitters Receptor Affinities, and Therapeutic Profiles of Selected Antipsychotics in PRS mice

Drugs*	Antipsychotic class	Affinity for neurotransmitter receptors**						Treatment efficacy in PRS-mice (correction of SZ-like behavior)	Promoter hypermethylation
		DRD2	HTR2A	HTR1A	HTR2C	ADORA1	CHM1		
Clozapine 5 mg/kg	Atypical	+	+++	+	++	+++	++++	+++[#]	Decreased[&,#]
Haloperidol 1 mg/kg	Typical	++++	+	-	-	+++	-	-[#]	No effect[&]
Risperidone 0.5 mg/kg 5.0 mg/kg	Atypical	++++	++++	-	++	+++	-	-[&]	No effect[&]
RG108 100 nmol/72 μl (i.c.v.)	-	-	-	-	-	-	-	+++[&]	Decreased[&]

*Antipsychotics were injected s.c. twice a day for 5 days. On the sixth day, 18 h after the last injection, mice were tested for behavioral activity (vertical activity, social interaction). RG108 was administered i.c.v. at a rate of 0.5 μl/h for 5 days.

**Affinity for neurotransmitter receptors (--) minimal to none; (+) low; (++) moderate; (+++) high; (++++) marked. Data are from Jarskog et al., 2007.

[#]Data are from Dong et al., 2016.

[&]Data are from this paper.

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Figure 1

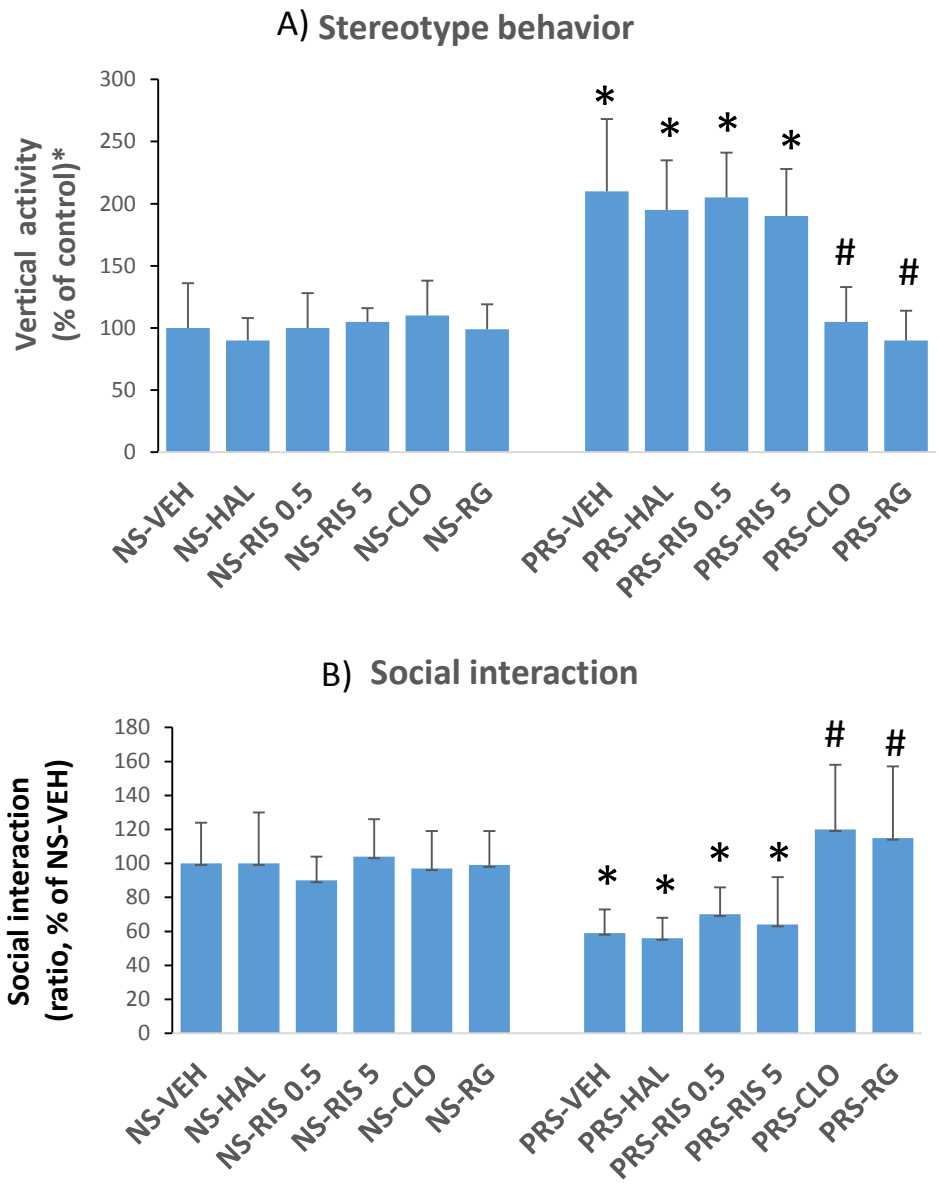

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Figure 2

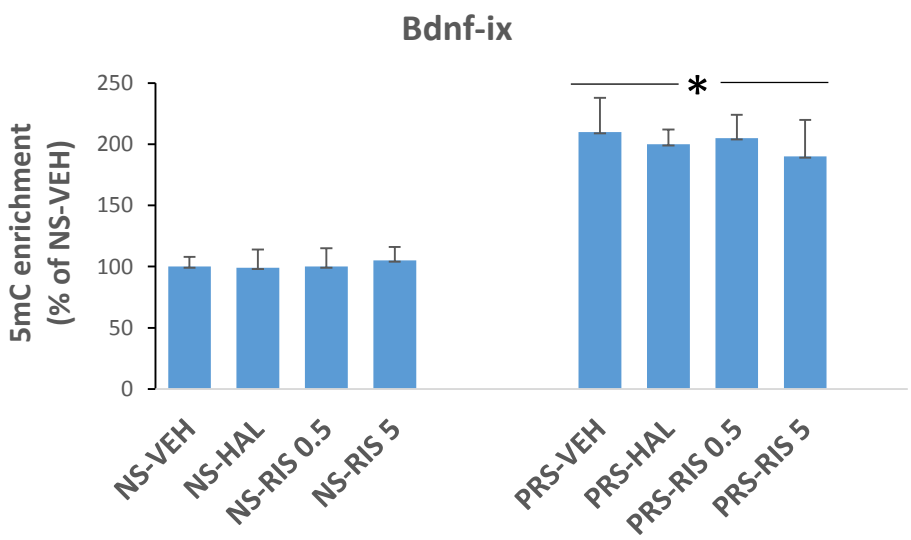

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Figure 3

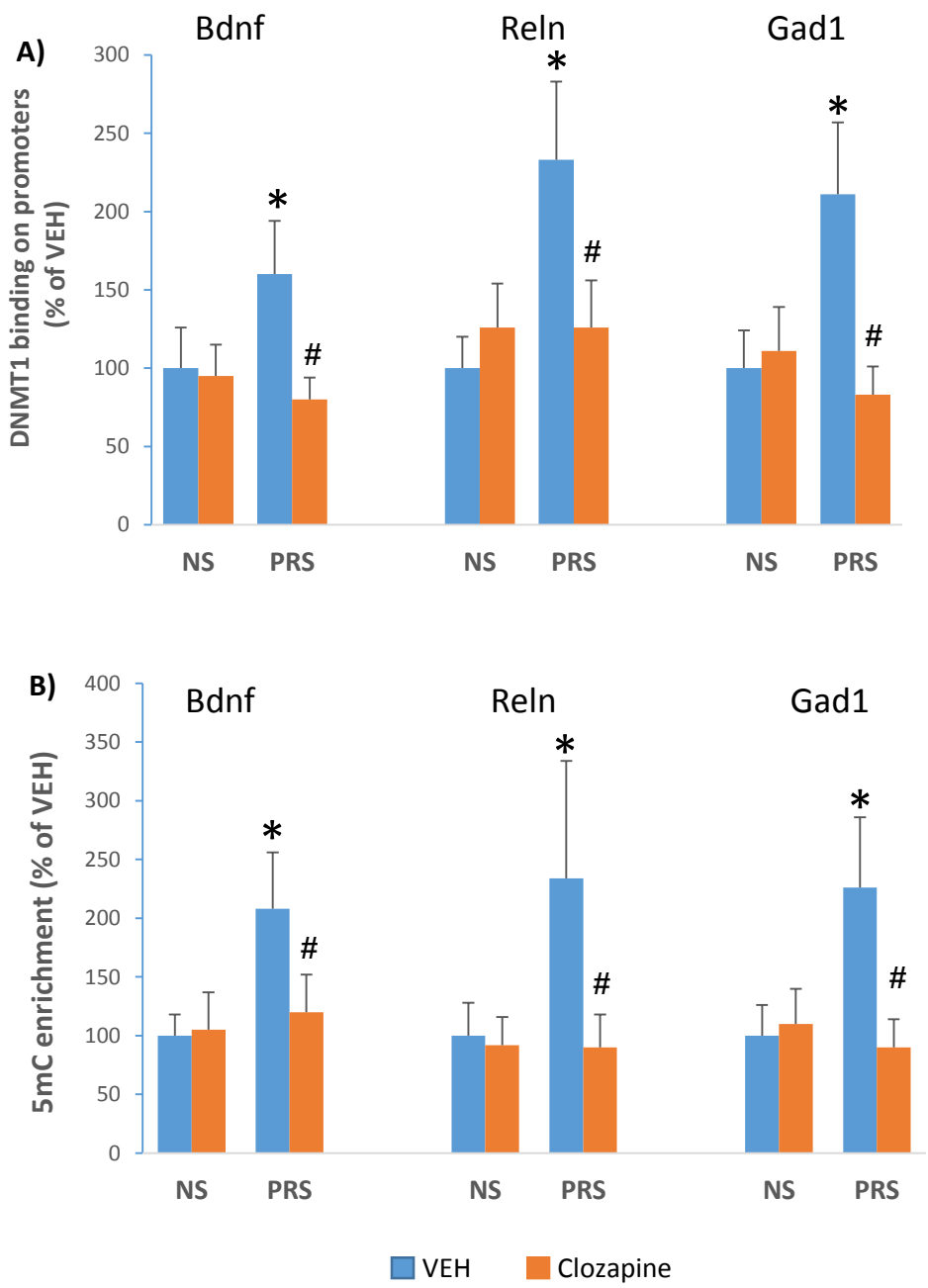

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Figure 4

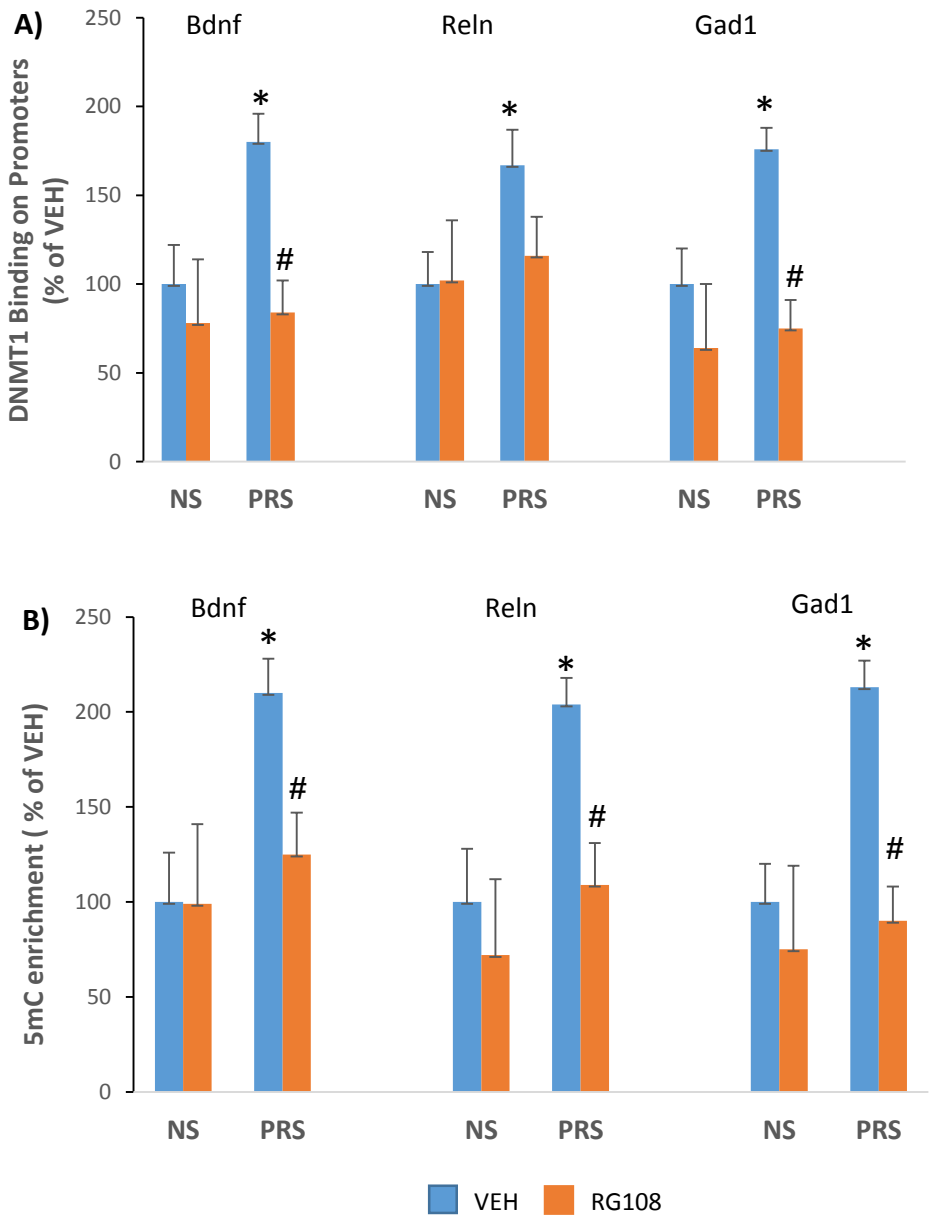

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Figure 5 Mol #113415