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Prenatal Stress Leads to Chromatin and Synaptic Remodeling and Excessive Alcohol Intake Comorbid with Anxiety-like Behaviors in Adult Offspring

Erbo Dong[1], **Alessandro Guidotti**[1], **Huaibo Zhang**[1,2], and **Subhash C. Pandey**[1,2]

[1]Center for Alcohol Research in Epigenetics, Psychiatric Institute, Department of Psychiatry, College of Medicine, University of Illinois at Chicago IL, 60612 USA

[2]Jesse Brown VA Medical Center, Chicago IL, 60612 USA

Abstract

Epidemiologic evidence suggests that individuals during their prenatal development may be especially vulnerable to the effects of environmental factors such as stress that predisposes them to psychiatric disorders including alcohol use disorder (AUD) later in life. Currently, the epigenetic mechanisms of anxiety comorbid with AUD induced by prenatal stress (PRS) remain to be elucidated. Here, we examined anxiety-like and alcohol drinking behaviors in adult offspring of prenatally stressed dam (PRS-mice) using elevated plus maze, light/dark box and two-bottle free-choice paradigm. It was found that PRS-mice exhibit heightened anxiety-like behaviors and increased alcohol intake in adulthood and these behavioral deficits were associated with a significant decrease in dendritic spine density (DSD) in medial prefrontal cortex (mPFC) relative to their controls (NS mice). To determine the mechanisms by which PRS reduces DSD, we examined the expressions of key genes associated with synaptic plasticity, including activity regulated cytoskeleton associated protein (*Arc*), spinophilin (*Spn*), postsynaptic density 95(*Psd95*), tropomyosin receptor kinase B (*TrkB*), protein kinase B (*Akt*), mammalian target of rapamycin (*mTOR*) and Period 2 (*Per2*) in mPFC of PRS- and NS-mice. The mRNA levels of these genes were significantly decreased in PRS-mice. Methylated DNA and chromatin immunoprecipitation studies revealed that DNA methylation or reduced histone H3K14 acetylation on promoters of above genes suggesting that epigenetic dysregulation may be responsible for the deficits in their expression. Findings from this study suggest that prenatal stress induced abnormal epigenetic mechanisms and synaptic plasticity-related events may be associated with anxiety-like and alcohol drinking behaviors in adulthood.

Keywords

prenatal stress; epigenetics; DNA methylation; dendritic spine; synaptic plasticity; anxiety; alcohol intake

Corresponding author information Dr. Erbo Dong, Center for Alcohol Research in Epigenetics, Department of Psychiatry, College of Medicine, University of Illinois at Chicago IL, 60612 USA, 1601 W. Taylor St. Chicago IL. 60612, USA, erbodong@uic.edu, Tel: 312-3552438.

Conflicts of Interest

The authors declare no potential conflicts of interest in this research.

1. Introduction

The alcohol use disorder (AUD) is highly prevalent among people with mood disorders, including anxiety and depression and contribute a gross public health burden globally (Bijl and Ravelli, 2000; Burns and Teesson, 2002; Hasin et al., 2007). Recent studies show that people with anxiety/depressive disorders have an increased risk to develop alcohol use disorders (de Graaf et al., 2003; Robinson et al., 2009; Schuckit and Hesselbrock, 1994; McDolnald and Meyer, 2011). Several studies have also demonstrated strong association with anxiety-like and alcohol drinking behaviors in preclinical models (Pandey et al, 2004, Pandey et al., 2017). Also, adolescent intermittent alcohol exposure leads to development of anxiety-like and alcohol drinking behaviors in rats during adulthood (Kyzar et al., 2016; 2017). In addition, accumulated evidence suggests that molecular mechanisms of AUDs and anxiety behaviors may be associated with epigenetic dysregulation of candidate genes in specific neurocircuitry via chromatin remodeling characterized by aberrant DNA methylation and histone modifications that leads to altered gene expressions (Tsankova et al., 2007; Mehler, 2008; Day et al., 2015; Pandey et al.,2017; Starkman et al., 2012; Qiang et al., 2014; Warnault et al., 2013; Ponomarev, 2013; Manzardo et al., 2013; Barbier et al., 2015).

Epidemiologic evidence accumulated over decades suggests that individuals during their prenatal development may be especially vulnerable to the effects of environmental factors that predispose them to psychiatric disorders including alcoholism later in life (Becker et al., 2011; Gordon, 2002; Sinha, 2007, 2008; Uhart and Wand, 2009; Charil et al., 2010; Fine et al., 2014; Markham and Koenig, 2011; Mulder et al., 2002; Fumagalli et al., 2007; Winstock, 2008). Currently, precise molecular mechanisms in the specific brain regions due to prenatal stress-induced epigenetic changes in the development of the comorbidity of anxiety and alcoholism remain unclear. We recently found that adult offspring (mice) born from prenatally stressed dam (referred as PRS-mice) exhibited anxiety-like behaviors characterized by reduced social interaction and it has been established that these behavioral changes may be attributed to altered DNA methylation profiles and disrupted chromatin structures in genes associated with mental disorders such as *Bdnf*, *Gad1 and Relin* in the medial frontal cortex (mPFC) (Dong et al., 2014, 2016; Zheng et al., 2016, Matrisciano et al., 2013). However, it is not clear whether long-lasting epigenetic reprogramming induced by prenatal stress leads to alteration of dendritic spine density and genes associated with synaptic plasticity since abnormal synaptic plasticity plays an important role in the pathogenesis of neuropsychiatric disorders including anxiety, depression and alcoholism (Segal, 2005; Fagiolini et al., 2009). In addition, since anxiety often predisposes one to increase of alcohol consumption, it is necessary to explore whether prenatal stress induced anxiety-like behaviors is comorbid with altered alcohol drinking behaviors. Therefore, in this study, we first examined the effects of prenatal stress on behavioral phenotypes of anxiety and alcohol consumption in adult offspring using PRS mouse model. We also investigated dendritic spine densities and epigenetic changes in the genes associated with spine formation and plasticity in the mPFC of PRS and control adult mice. We focused our study in mPFC, as this region has been shown to play a major role in vulnerability to stress and also is associated with neuropsychiatric disorders including AUD (Duman et al., 2016;

Bludau et al., 2016; Heilig et al.,2017). Our novel results suggest prenatal stress can lead to long-lasting epigenetic modifications of synaptic plasticity-associated genes, thereby causing synaptic remodeling in mPFC and producing behavioral phenotypes of anxiety and alcoholism in adulthood offspring.

2. Methods

2.1 Animals and PRS procedure

All procedures were performed according to NIH guidelines for animal research (Guide for the Care & Use of Laboratory Animals, NRC, 1996) and were approved by the Animal Care Committee of the University of Illinois at Chicago. Pregnant mice (Swiss albino ND4, Harlan, Indianapolis, IN, USA) were individually housed with a 12-h light–dark cycle, and food and water *ad libitum*. Control dams were left undisturbed throughout gestation, whereas stressed dams were subjected to repeated episodes of restraint stress, as described previously (Dong et al., 2014, 2016; Zheng et al., 2016, Matrisciano et al., 2013). The stress procedure consisted of restraining the pregnant dam in a transparent tube (12 × 3 cm) under a bright light for 45 min three times per day from the seventh day of pregnancy until delivery. After weaning (PND 21), male mice were selected for the study and housed five per cage separately by condition. A maximum of one or two male pups was taken from each litter for each measure to remove any litter effects (Becker and Kowall, 1977; Chapman and Stern, 1979). All behavioral tests, including drinking experiments were performed in one set of mice whereas biochemical measurements in the brain were conducted in separate sets of mice that were not subjected to behavioral tests.

2.2 Behavioral Experiments

2.2.1 *Elevated Plus-Maze Test* (EPM): To examine the anxiety behavior of gestational-stress offspring, the elevated plus-maze was performed in similar way as described by us (Pandey et al., 2015). Briefly, it consisted of two open and two closed arms and was made of Plexiglas. The closed arms had transparent Plexiglas walls at the sides and end. The floor was made of black Plexiglas and elevated to a height of 50 cm above the floor. At the start of each test, mice were placed individually on the central platform and their behavior monitored by computer for 10 minutes. The number of entries for each arm and the time spent in each arm were recorded and analyzed. The percentage of open arm entries (open arm entries x100/total arm entries) and percentage of time spent in open arm (time spent in open arm x100/time spent in open and closed arms) were used as indices of anxiety. The number of closed arm entries is represented as general activity of mice.

2.2.2 *Light/Dark Box Exploration test* (LDB): The LDB consists of a dark compartment without illumination and a light compartment with illumination (0.25 Amp; light-emitting diode light). Both compartments are connected through an opening. On the day of testing, each mouse was allowed a 5-min pretest habituation period in the room before testing. Then, the mouse was gently placed in the dark compartment with its head facing away from the opening. The mouse was observed for a 5-min test period, and the time spent in each compartment was monitored and recorded by computer. The percentage of time spent in either the dark compartment or light compartment was calculated for each

animal. Total ambulation in the light and dark compartments was represented as the general activity of the mouse (Pandey et al., 2015, Sakharkar et al., 2014).

2.2.3 Alcohol Preference: Alcohol preference was measured by the two-bottle free-choice paradigm (Pandey et al., 2004). Mice were placed in individual cages and have *ad libitum* access to food and water, in two bottles, and were habituated to drink water from either bottle. Bottle positions were changed daily so that the mice would not develop a position habit. Once they started drinking water equally from either bottle, mice were provided with 3% (v/v) alcohol solution in one bottle and water in the other bottle daily for 3 days, and then concentrations of ethanol were increased to 7% for 3 days, 9% for 3 days and to 12% for another 3 days. Consumption of ethanol and water (ml) was measured daily at 6:00 PM, and fresh water and ethanol (3, 7, 9, or 12%) solution in water were provided every day at the start of dark cycle. The mean percentage of alcohol intake and the percentage of water intake were calculated from their total fluid intake for 3 days for 3, 7, 9, and 12% alcohol. We measured body weight of mice before and after each dose of alcohol. The alcohol intake was presented as g/kg/day.

2.3 Histological study

Spine Density Measurement: The Golgi-Cox staining procedure was performed to measure the dendritic spine density in the pyramidal neurons of mPFC using the FD Rapid Golgi Stain Kit (Pandey et al., 2008). Brains were rapidly immersed in impregnation solution for at least 2 week. Then 200 μm brain sections were cut, mounted and stained according to the protocol provided by Kit manufacturer. After staining, sections were dehydrated and cleared in xylene solution and then cover slipped using mounting medium. Sections were observed under a light microscope at high magnification (100x). Spines from neurons where dendrites are connected to soma and showing complete impregnation were marked and then counted using IMAGE J Program (Orlowski and Bjarkam, 2012). Spines from dendrites (a total of 9 dendrites) from three adjacent brain sections were counted and then averaged for each mouse. Total of 5 mice from each group was analyzed. The dendritic spine density was represented as mean ± SEM of the number of dendritic spines/10μm of dendritic length. All reconstructions were conducted with a ZEISS Axioskop2 microscope.

2.4 Biochemical measurements

2.4.1. Quantitative real-time PCR: The quantitative PCR measurements were carried out using the Applied Biosystems Real-Time PCR System with a SYBR green master mix (Fermentas, Glen Burnie, MD, USA). After behavioral tests, total RNA from the mPFC of PRS and NS mice, was isolated using TRIZOL reagent (Life Technologies, Grand Island, NY, USA), and was further purified using the RNeasy kit (QIAGEN, Valencia, CA, USA). The expressions of Arc, PSD95, Spn, TrkB mTor, Akt, were measured using RT-qPCR. ActB gene was chosen for normalizing mRNA expression. To confirm amplification specificity, the PCR products were subject to a melting curve analysis, in which only one peak was observed. Each sample was run in duplicate and repeated twice. Primers were designed to span at least one intron–exon boundary. PCR efficiency was carried out to confirm the specificity of the primers. The relative gene expressions were calculated using

ΔΔCt method. The primer sequences used to amplify the genes analyzed are summarized in supplementary Table 1.

2.4.2 Immunoblotting: Total protein from mPFC, extracted using RIPA lysis buffer and quantified by Enhanced BCA Protein Assay Kit (Beyotime P0010S), was separated by SDS-PAGE and transferred to PVDF membrane. After being blocked in TBS buffer containing 0.05% Tween-20 and 5% skim milk, the membranes were incubated overnight at 4°C with the following primary antibodies: anti-Arc (Abcam; ab118929; 1:1000), anti-PSD95 (Abcam, ab18258, 1:1000), anti-spinophilin (Upstate 06–852, 1:1000), anti-TrkB (Sigma-Aldrich, 07–225, 1:1000), anti-mTOR (Abcam, ab2972, 1:1000), anti-Akt (Cell Signaling, #9272, 1:1000) and anti-Per2 (Novus biologicals NB100–125; 1:1000), After incubation with the corresponding secondary antibody, the immunoreactive signals were visualized by LI-COR ODYSSEY Fc Western Blotting Detection System (and quantitated using Image Studio Ver5.2. The levels of these proteins in the stress offspring versus non-stress offspring were normalized by β-actin protein levels. In order to estimate background caused by non-specific binding of secondary antibody, a secondary control without the primary antibody was performed.

2.4.3 DNA Methylation Procedure: DNA methylation (enrichment of 5-methylcytosine, 5-mC) on the promoters of genes listed above were measured using MeDIP (Methylated DNA immunoprecipitation Diagenode, Denville, NJ, USA) as previously described by us (Dong et al., 2014, 2016). Genomic DNA isolated was sonicated to 200bp using Bioruptor (Diagenode) and subjected to immunoprecipitation using specific 5-mC antibody followed by qPCR. Primers (Table 1) were designed to be specific to the CpG enriched regions near the promoters of the genes. Input genomic DNA that was not subjected to the methylation enrichment procedure was used as a control. The percentage of methylated vs. unmodified promoter was calculated using the following equation: $\%[(\text{meDNA}-\text{IP}/\text{total input})] = 2^{[(\text{Ct}(10\%\text{ input})-3.32)-\text{Ct}(\text{meDNA}-\text{IP})]} \times 100\%$.

2.4.4 Chromatin Immunoprecipitation Assay: We performed ChIP assays based on protocols previously described (Dong et al., 2014, 2016). Briefly, about 10 mg of tissue was used for this procedure. Tissue slices were fixed with 1% formaldehyde at 37°C for 15 min to crosslink acetylated histones with the target genomic DNAs. After being washed six times with cold PBS containing protease inhibitors, slices were homogenized in 200–400 μl of SDS lysis buffer. To obtain consistent chromatin fragmentation, the lysates were sonicated by a Sonic Dismembrator, Model 500 (Fisher Scientific) at 70% of output power for 10 s on ice and repeated 4 times. The sizes of the majority of sonicated genomic DNA fragments included 250 to 500 bp. The ChIP procedure was carried out by using the ChIP assay kit and protocol (Upstate Cell Signaling Solutions). The concentration of ChIP grade anti-acetyl-histone H3 (lysine 14) (AcH3K14) antibody (Millipore, Billerica) was suggested by the manufacturer. An aliquot (1–2%) of the sonicated lysate without antibody served as an Input. At the end of the ChIP procedure, the protein/DNA cross-linked nucleosomal chromatin complex immunoprecipitated by antiboy was reverse cross-linked. Protein-free DNA then was extracted for detection and quantification of genes above. The percentages of immunoprecipitated DNA were calculated as described above for the MeDIP.

2.5 Statistical analysis

Significant differences between two groups (PRS vs NS) were assessed by Student *t*-test for LDB, EPM, DSD, mRNA, Western blot, MeDIP and ChIP assays (two tailed), or 2 -way repeated ANOVA followed by Bonferroni *post hoc* comparisons for alcohol drinking behavior (using group and ethanol as two factors) using IBM SPSS Statistic 24 (SPSS, Chicago, IL, USA). Values are represented as mean ± S.E.M. The criterion for statistical significance was $p<0.05$. The statistical significance (PRS vs NS) was represented as: $*p < 0.05$, $** p < 0.01$ and $***p < 0.001$.

3. Results

3.1. Prenatal stress induces anxiety like behaviors in adulthood

We previously reported that (Dong et al., 2014, 2016; Zheng et al., 2016, Matrisciano et al., 2013) prenatal stress induces behavioral deficits in adult offspring, including hyperlocomotion, stereotype behavior, social interaction and fear conditioning deficits. Here, we used another test for anxiety measures. In EPM test as shown in Fig 1A, PRS mice exhibited a significant decrease in both duration of stay in and number of entries into open arms (Fig. 1A) when compared with their NS counterparts $p < 0.05$ for open arm entries, $**p <0.01$ for duration in open arms, Student *t*-test, N = 8 per group. There were no significant differences of closed arm entries between two groups, suggesting no changes in the general activity of the mice in EPM test. Similar results were found in LDB test where PRS mice spent significantly more time in dark than light compartments (Fig. 1B) in comparison with NS group $*p < 0.05$, Student *t*-test, N = 8 per group (Fig. 1B). The total ambulation in LDB of PRS mice did not significantly differ from NS mice (Fig. 1C) showing no changes in the general activity of the mice. These findings suggest that prenatal stress can lead to anxiety-like phenotypes in adult offspring.

3.2. PRS Promotes Alcohol Drinking Behaviors in Adult Offspring

Accumulated evidence suggests that anxiety may result in a risk of alcoholism (Becker et al., 2011; Uhart and Wand, 2009; Fumagalli et al., 2007; Weinstock, 2008). We therefore next tested the drinking behaviors in PRS mice using two bottle free choice paradigm. It was found that PRS mice showed higher alcohol consumption when pharmacologically relevant concentrations of 3–12% ethanol were offered (Fig. 2A). At the concentration of 7–12%, PRS-mice consumed about 3-fold more alcohol than their control counterparts [$F_{(1, 14)} = 26.547$; $***p < 0.001$, 2-way repeated ANOVA, N = 8 per group]. The drinking behavior of PRS mice is characterized by increasing intake of alcohol solution [$F_{(1, 14)} = 20.533$; $***p < 0.001$, 2-way repeated ANOVA, N = 8 per group] and decreasing water intake [$F_{(1, 14)} = 20.578$; $***p < 0.001$, 2-way repeated ANOVA, N = 8 per group] (Fig. 2B and C) but there were no significant differences between two groups (NS vs PRS) in total fluid intake (Fig. 2D). These data suggest that prenatal stress induces higher alcohol preference in adult offspring as compared with control non-stress adult offspring.

Prenatal stress disrupts synaptic plasticity related events

Studies reveal that impaired synaptic function characterized by substantial dendritic abnormalities in the pyramidal neurons of mPFC is found to be associated with psychiatric behavioral phenotypes in PRS mice (Duman et al., 2016; Christoffel et al., 2011; Korb and Finkbeiner, 2011; Segal, 2005). To determine whether prenatal stress results in impaired dendritic spine formation, we assessed dendritic spine density using Golgi-Cox staining method. The impregnated pyramidal neurons were identified by their triangular somal shape, the presence of an apical dendrite and numerous dendritic spines. Nine pyramidal neurons from each animal were selected for count spines. All reconstructions were conducted with a ZEISS Axioskop2 microscope. Figure 3A and B show the typical images of dendritic spines on apical dendrite of pyramidal neurons in mPFC of NS and PRS mice from which significant differences in morphology and density of spines between two groups were viewed. The result of quantitative analyses showed that there is about 30% less (***$p <$ 0.001, Student *t*-test, N = 5 per group) dendritic spine density in PRS neurons than in NS controls (Fig. 3E).

To explore the possible molecular mechanisms by which prenatal stress leads to reduction of dendritic spines, we focused on the expressions of key genes related to synaptic formation, stability and function, including activity regulated cytoskeleton associated protein (*Arc*), spinophilin (*Spn*), postsynaptic density 95(*Psd95*), tropomyosin receptor kinase B (*TrkB*). As shown in Fig. 4A, the mRNAs transcribed by above genes were decreased by about 30–50% in mPFC of PRS mice as compared with NS counterparts (Arc: **$p < 0.05$; Spn: **$p <$ 0.01; Psd95: *$p < 0.05$; TrkB: **$p < 0.01$; Student *t*-test, N =8 per group). To confirm RT-PCR observation, we conducted immunoblotting with specific antibodies. The results show that all proteins encoded by above synaptic genes were significantly decreased in PRS mice (ARC: ***$p < 0.001$; SPN: **$p < 0.01$; PSD95: *$p < 0.05$; TRKB: *$p < 0.05$; Student *t*-test, N = 5 per group) (Fig. 4B). These findings provide molecular evidence supporting the notion that reduced dendritic spine density may be related to reductions in synaptic plasticity associated gene expression in the cortical structures of prenatal stress mice.

3.4. Prenatal stress induces epigenetic dysregulation on genes associated with altered synaptic function.

Our previous findings indicated that in PRS brain, DNMT1 and DNMT3a are significantly overexpressed, which suggests that the reduction of candidate genes associated with prenatal stress may occur via promoter hypermethylation (Dong et al., 2014, 2016; Zheng et al., 2016, Matrisciano et al., 2013). To test the hypothesis, we examined the promoter methylation status of *Arc, Spn, Psd95* and *TrkB* using MeDIP by measuring the enrichment of 5mC with specific antibody. As shown in Fig. 5A, there was significant enrichment of 5-mC found on *Arc* and *Spn* but not *Psd95* and *TrkB* promoters (Arc: *$p < 0.05$; Spn: *$p <$ 0.05; Student *t*-test, N = 8 per group) in PRS-mice compared to their NS counterparts, indicating that decreased expression of Arc and Spn may largely due to hypermethylation on their promoters.

To further explore the possible mechanisms of Psd95 and TrkB downregulation, we analyzed histone acetylation levels around their promoters using ChIP assay. By using ChIP grade

specific antibody that recognize acetylation at the lysine-14 (K14) residue of histone H3 (AcH3K14) (Zheng et al., 2016), we found that there was significant reduction in the acH3K14 (TrkB: $^{**}p < 0.01$; Student *t*-test, N = 8 per group) occupancy associated with *TrkB* but not *Psd95* promoters (Fig. 5B). The results provide evidence that decreased acH3K14 on *TrkB* promoter may lead to its reduction in PRS mice.

3.5. Prenatal stress induces downregulation of Akt - mTOR signaling cascade and circadian gene Per 2.

Akt (protein kinase B) and mTOR (mammalian target of rapamycin) are important components playing multiple roles in regulating local protein synthesis (Li et al., 2010; Akama and McEwen, 2003), including Psd95. To investigate whether decreased PSD95 in PRS mice is linked to downregulation of Akt and mTOR, we measured their mRNA and protein expressions. As shown in Fig. 6A and B, there were significant reductions of Akt and mTOR mRNA/protein in PRS mice as compared with NS group (*Akt*: $^{*}p < 0.01$; *mTOR*: $^{**}p < 0.05$; Student *t*-test, N = 8 per group) (AKT: $^{*}p < 0.05$; MTOR: $^{*}p < 0.05$; Student *t*-test, N = 5 per group). MeDIP data (Fig. 6C) revealed high levels of 5mC on their promoters, suggesting that hyper DNA methylation may be responsible for their downregulation (*Akt*: $^{***}p < 0.05$; *mTor*: $^{*}p < 0.001$; Student *t*-test, N = 8 per group). These finding suggest that Akt and mTOR signaling cascade was disrupted by prenatal stress, which may contribute to the reduction of Psd95.

Recent studies revealed that circadian genes, including *Period* (*Per*) can influence alcohol consumption behavior (Spanagel et al., 2005; Blomeyer et al., 2013; Gamsby et al., 2013). To determine whether prenatal stress also produce changes in the expression of these genes, we measured mRNA and protein levels of Per2. The results showed that Per2 levels significantly decreased in PRS mice as compared with NS group (Fig. 6A-C) (*Per2*: $^{*}p < 0.001$, N = 8; PER2:* $p < 0.05$; N = 5; Student *t*-test). MeDIP data show high levels of 5mC on its promoter, suggesting that DNA methylation may lead to downregulation of Per2 expression (Per2: $^{*}p < 0.05$; Student *t*-test, N = 8 per group). These findings provide evidence that prenatal stress can influence circadian gene that may be associated with the phenotype of excessive alcohol intake observed in PRS mice.

4. Discussion

In this study, using prenatal stress mouse model of neurodevelopmental disorders (Dong et al., 2014, 2016; Zheng et al., 2016, Matrisciano et al., 2013) we demonstrated that PRS mice exhibit not only anxiety-like but also alcohol drinking behaviors. These findings support the notion that early life stress is a risk factor in the development of AUD comorbid with anxiety disorders (Becker et al., 2011; Gordon, 2002; Markham and Koenig, 2011; Mulder et al., 2002; Fumagalli et al., 2007; Winstock, 2008). Another finding of this study is that PRS-induced deficits in histone acetylation and increased DNA methylation possibly leading to decreased expression of synaptic plasticity-associated genes in mPFC. To establish association between deficits in synaptic genes and impairs synaptic function in PRS adult offspring, we examined dendritic spine density using Golgi-Cox staining in adult offspring (PND75). Interestingly, a significant reduction of dendritic spine density of pyramidal

neurons is found in the mPFC of PRS-mice compared to NS counterparts. We believe that this morphological change induced by prenatal stress may be involved in the behavioral deficits observed in PRS mice, including excessive alcohol intake. The decreased spine density in PRS mice is supported by the findings from qPCR and immunoblotting that the genes/proteins associated with synaptic formation, stability and function such as Arc, spinophilin, PSD95 and TrkB are remarkably decreased in the same brain region. Among many proteins implicated in synaptic function, Arc is a master regulator of neuronal function (Korb and Finkbeiner, 2011) and as a critical effector molecule downstream of many signaling pathways. Arc, plays an important role in facilitating LTP consolidation, modulating dendritic spine density and regulating cognitive functions (Shepherd and Bear, 2011). Dysregulation of Arc expression can lead to synaptic dysfunction which is observed in a number of neurological disorders (Li et al., 2015). Downregulation of Arc in the central nucleus of amygdala (CeA) by its antisense oligonucleotides lead to reduced DSD and provoked anxiety-like behaviors and increased alcohol intake in rats (Pandey et al., 2008), suggesting a crucial role of Arc in the pathophysiology of AUD. Furthermore, lower Arc levels and DSD were also found in the amygdaloid structures of alcohol preferring rats as compared with non-preferring rats (Moonat et al., 2011). Here, we observed that PRS-induced deficits in Arc and other genes due to aberrant chromatin architecture might be involved in anxiety and higher alcohol consumption phenotypes in adult offspring. Spinophilin, as one of typical synaptic markers, facilitates spine growth. The deficit of this protein indicates an impairment of synaptic formation (Feng et al., 2000). PSD95 is notable in its integral role within the postsynaptic machinery mediating glutamate receptor anchoring and synaptic stability (Chen et al., 2011). TrkB, an important receptor for BDNF, modulates spine density and morphology (Kellner et al., 2014, Cao et al., 2013, Bramhan and Messaoudi 2005). To further explore the mechanisms by which above described synaptic genes are reduced by prenatal stress, we examined the status of DNA methylation on their promoter regions as we previously reported PRS-mice exhibits altered DNA methylation profiles at genes typically expressed in glutamatergic neurons and in GABAergic neurons (Dong et al., 2014, 2016). We have established that in the cortico-limbic structures of PRS-mice these promoters are hypermethylated and their transcription is downregulated (Dong et al., 2014, 2016). Thus, we hypothesized that decreased synaptic genes measured in this study may be due to promoter hyper DNA methylation. The MeDIP data demonstrate that although high levels of 5mC were found on the promoters of genes measured, the significant group effect was observed in Spn and Arc but not Psd95 and TrkB. It has been reported that Arc expression is regulated by BDNF-TrkB signaling pathway (Cao et al., 2013; Bramham et al., 2008). Thus, we can conclude that downregulation of Arc induced by prenatal stress may be result of promoter methylation and disruption of BDNF-TrkB signaling cascades. Since histone modifications, especially acetylation at lysine 14 on histone3, plays an important role for transcriptional activity, we then performed ChIP to explore the status of AcH3K14 on the promoters of Psd95 and TrkB. Data revealed a reduced binding of AcH3K14 on TrkB promoter, suggesting that low level of acetylated histone on its promoter may limit its expression.

The above results also indicate that the decreased PSD95 expression may be through different other than epigenetic mechanisms. To account for this, we examined the

expressions of Akt and mTOR because these two genes are the key components of pathways which are essential for regulation of synaptic structure, maturation and function (Akama and McEwen, 2003). Activation of mTOR signaling pathway via Akt leads to de novo synthesis of synaptic proteins, including PSD95 (Akama and McEwen, 2003; Li et al., 2010). Our qPCR and immunoblotting data indicate that Akt and mTOR are notably decreased in PRS mice. Findings from MeDIP provide evidence that aberrant promoter methylation may be responsible for their downregulation. Thus, disruption of synaptic Akt-mTOR signaling pathway in PRS mice may be responsible for the decreased expression of PSD95.

Recent advance in AUD studies suggest that disruptions in circadian functions are associated with a wide variety of disorders, including anxiety and excessive alcohol intake. For example, downregulation of circadian clock components such as *Period 2* (*Per2*) play a direct role in regulating ethanol consumption (Spanagel et al., 2005; Blomeyer et al., 2013; Gamsby et al., 2013). This prompted us to examine whether expression of Per2 is also altered in the mPFC of PRS mice. We found that Per2 was significantly reduced in PRS mice through an aberrant promoter methylation. Thus, disruption of circadian rhythms may be at least in part involved in excessive ethanol intake.

Taken together, we provide evidence that prenatal stress induces anxiety-like behaviors and excessive ethanol intake in adult offspring through epigenetic dysregulation of genes and signaling pathway associated with synaptic formation, and stability as well as function (Fig. 7). However, more studies are required to explore their causality. In this study, we specially focused on mPFC, as this brain region play a critical role in stress response and AUDs (Duman et al., 2016; Bludau et al., 2016; Heilig et al., 2017). However, the findings from this region are not enough to draw a conclusion that the causality of behavioral deficits induced by prenatal stress is only attributed by the mPFC. The role of other brain regions, such as hippocampus and amygdala cannot be ruled out since these two regions are also associated with psychiatric disorders and AUDs. Therefore, further studies are needed on epigenetic changes in these brain regions and compared them with mPFC in this model to address the region specificity of the findings.

5. Conclusions

The findings from the present study suggest that prenatal stress can induce alterations of multiple genes and networks in the mPFC of adult offspring, leading to anxiety-like behaviors and excessive alcohol intake. The PRS mice may serve as a useful model for exploring the development of AUD comorbid with anxiety and may be useful in preclinically screening for the potential efficacy of drugs acting on altered epigenetic mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Prenatal stress induces excessive alcohol intake and anxiety-like behaviors in adult offspring.
- Prenatal stress downregulates genes associated with spine formation and plasticity via epigenetic mechanisms.
- Prenatal stress leads to decreased dendritic spine density in the medial frontal cortex of adult offspring.

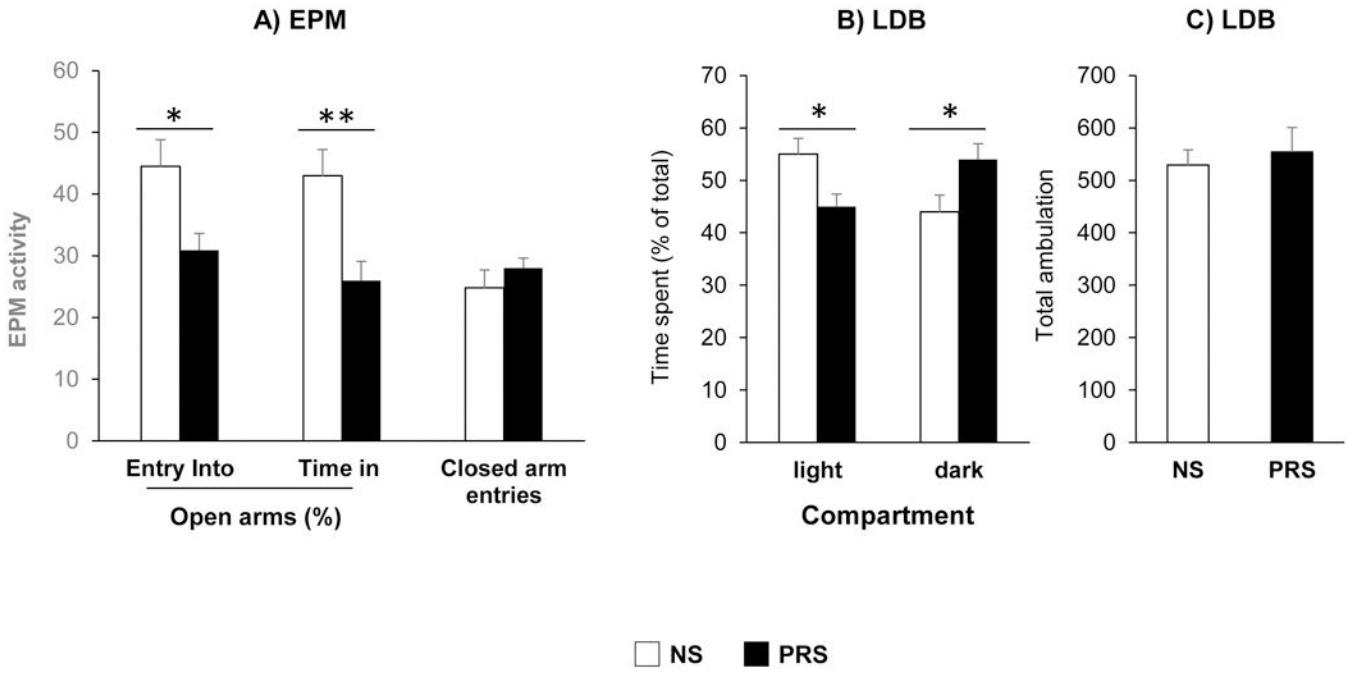

Figure 1:

Prenatal stress induces anxiety-like behavior in adult offspring at age of postnatal day 75 (PND75). Anxiety-like behaviors were measured using the elevated plus maze (EPM) and Light/Dark Box (LDB) tests. Compared with NS mice, PRS mice exhibited low percentage of entering into, and time spent in open arms in EPM test (**A**), high preference to dark compartment in LDB test (**B**) and similar ambulation of LDB exploration (**C**) Data are presented as mean ± SEM. For EPM, $*p < 0.05$ (open arm entries), $**p < 0.01$ (duration in open arms) vs. NS mice, respectively, N= 8 for each group. For LDB, $*p < 0.05$, NS vs PRS mice, N= 8 for each group.

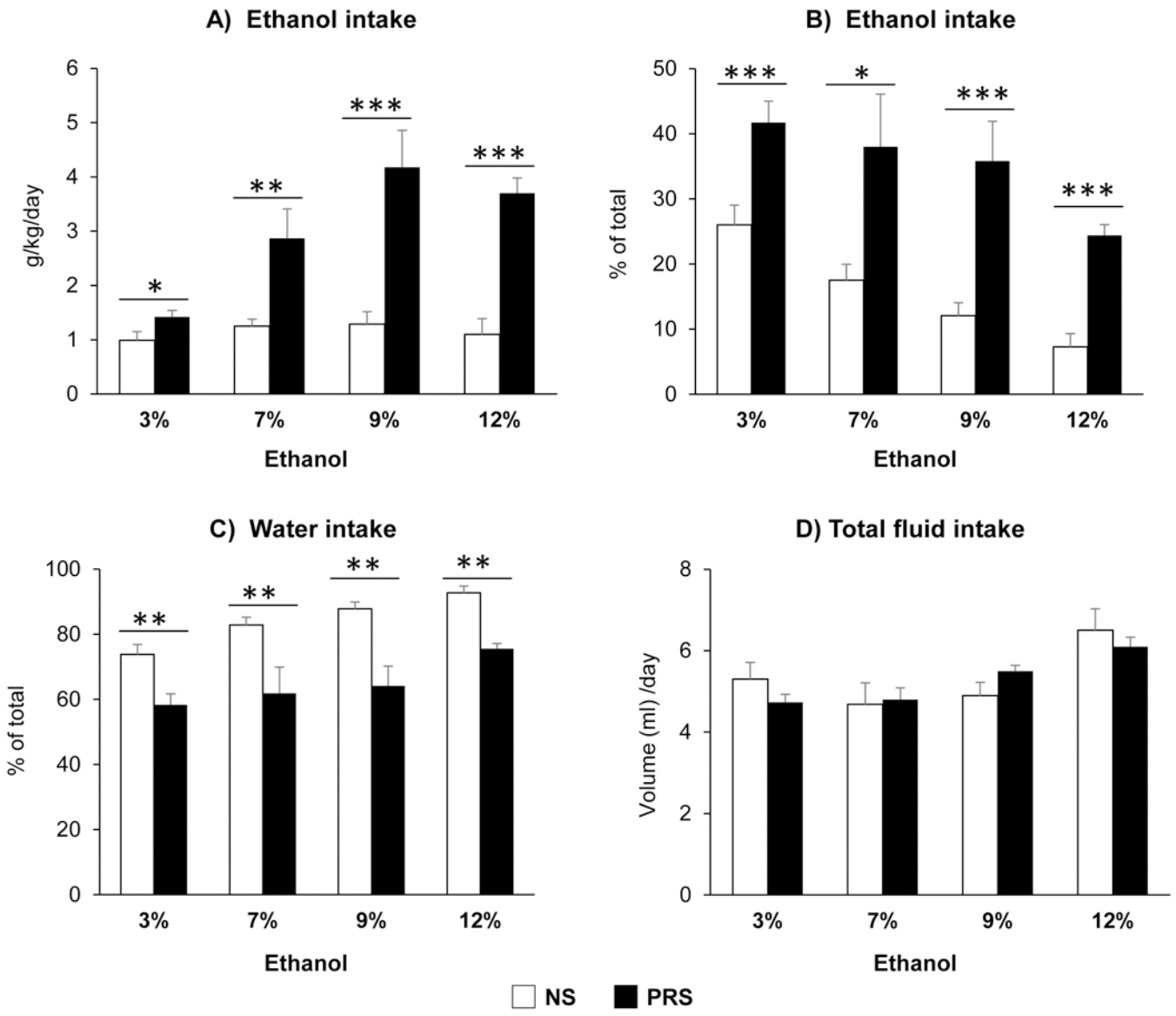

Figure 2:

Prenatal stress induces alcohol intake in adult offspring (PND75). Compared with NS mice, PRS mice consumed more alcohol (g/kg/d) (**A**) [NS vs PRS mice, *$p < 0.05$ (3%); **$p <$ 0.01(7%); ***$p < 0.001$(9%) and ***$p < 0.001$ (12%), N = 8 for each group] and exhibited high preferences to alcohol (**B)** [NS vs PRS mice, ***$p < 0.001$ (3%); *$p < 0.05$(7%); *$p <$ 0.001(9%) and **$p < 0.001$(12%), N=8 for each group] and less to water intake (**C**) [NS vs PRS mice, **$p < 0.01$ (3%); **$p < 0.01$(7%); **$p < 0.01$ (9%) and **$p < 0.01$ (12%), N=8 for each group] in the two-bottle free-choice test. There was no significant difference in total fluid intake between two groups (**D**). Values are the mean ± SEM.

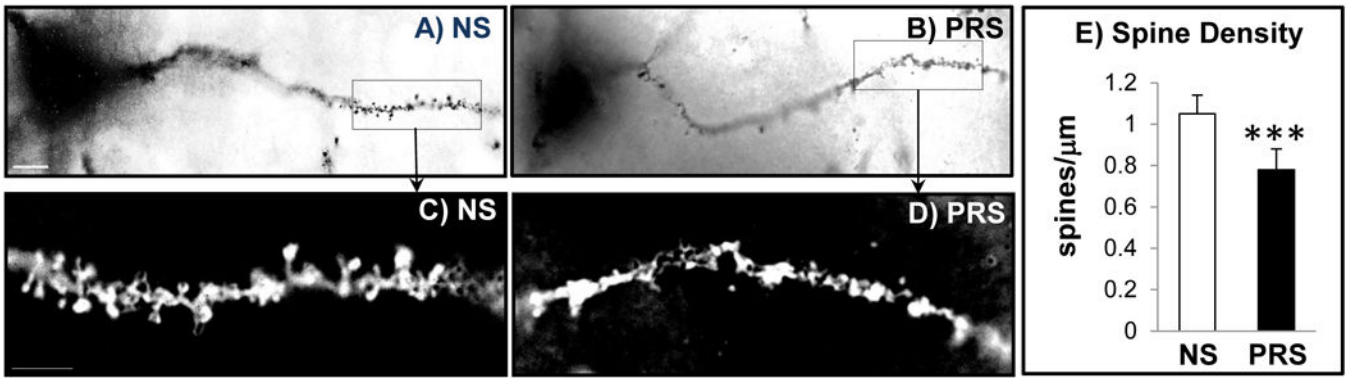

Figure 3:

Prenatal stress (PRS) induces reduction of dendritic spines on mPFC neurons. **A** and **B** are representative photomicrographs of dendritic spines of pyramidal neurons from mPFC of NS and PRS mice (PND75). **C** and **D** are the magnified views of boxed areas in A and B. **E** is the bar diagram showing quantification of dendritic spine density in mPFC of adult NS and PRS mice. Values are the mean ± SEM. ***$p < 0.001$, N=5 mice for each group. Scale bars in **A** and **B**, 10µm; in **C** and **D**, 5µm).

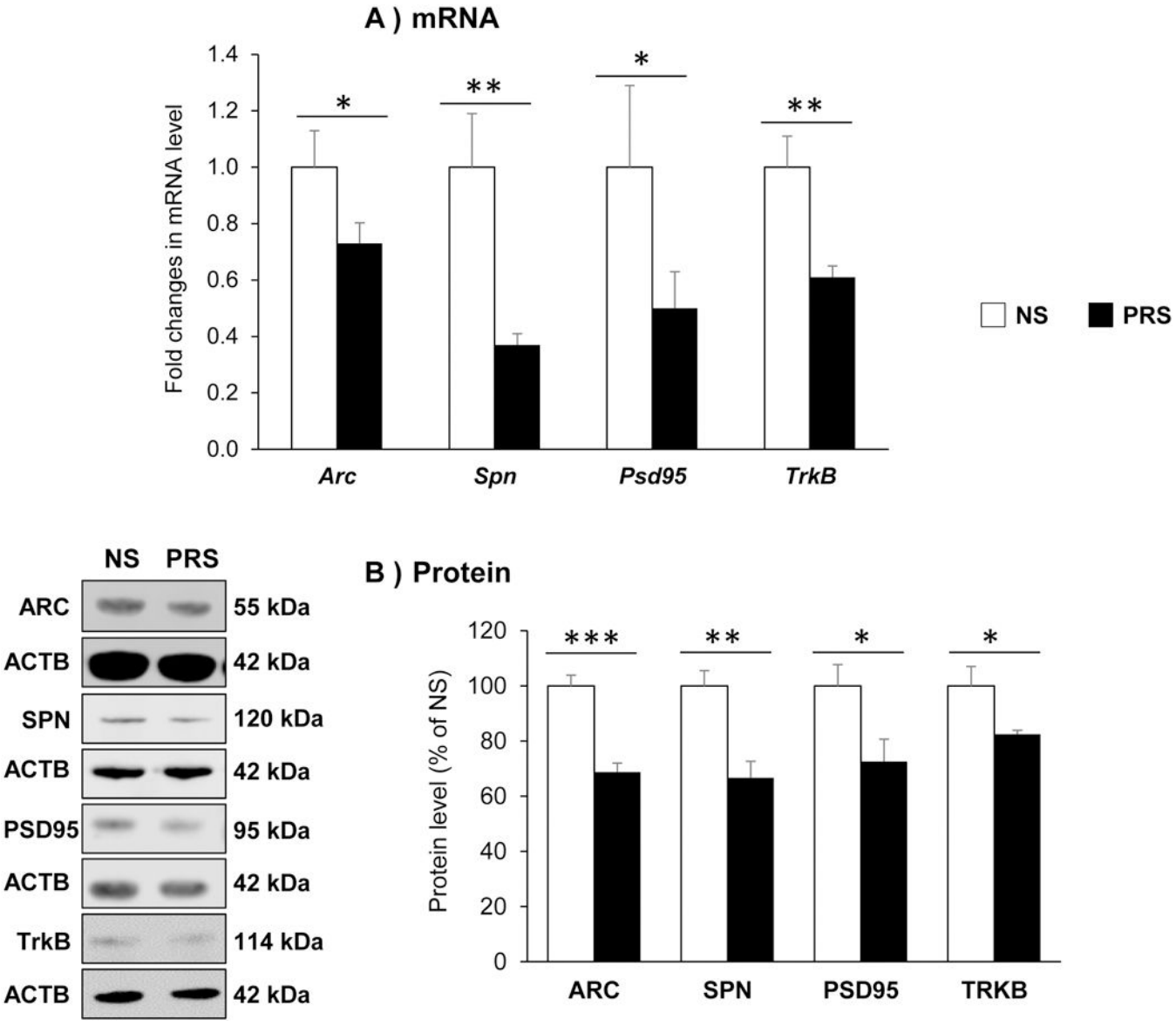

Figure 4:

Prenatal stress induces a decrease in the expressions of specific synaptic genes (**A**) and their encoded proteins (**B**) in the mPFC offspring of PND75. The data are expressed as mean ± SEM. For genes: Arc (*$p < 0.05$), Spn (**$p < 0.01$), Psd95 (*$p < 0.05$) and TrkB (**$p < 0.01$); for proteins: ARC (***$p < 0.001$), SPN (**$p < 0.01$), PSD95 (*$p < 0.05$) and TRKB (*$p < 0.05$) NS vs PRS mice, N=8 in each group for gene expression and N = 5 in each group for Western immunoblotting.

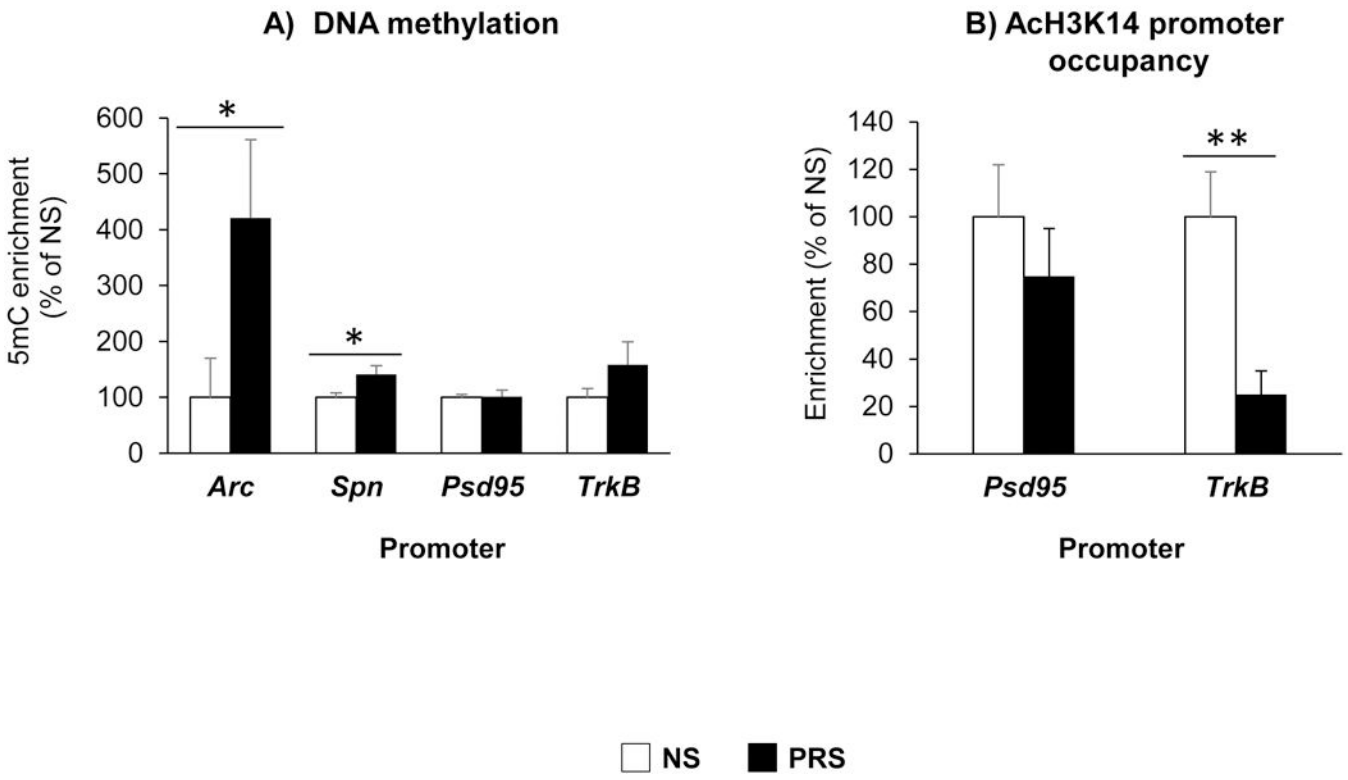

Figure 5:

In mPFC of PND 75 old offspring, prenatal stress induces, (**A**) altered DNA methylation (defined as levels of 5mC) on *Arc* (*$p < 0.05$), *Spn* (*$p < 0.05$) promoters, NS vs PRS mice, N = 8 per group; (**B**) decreased histone H3K14 acetylation (AcH3K14) on *TrkB* (**$p < 0.01$) promotors. The data are expressed as mean ± SEM. NS vs PRS mice, N = 8 for each group.

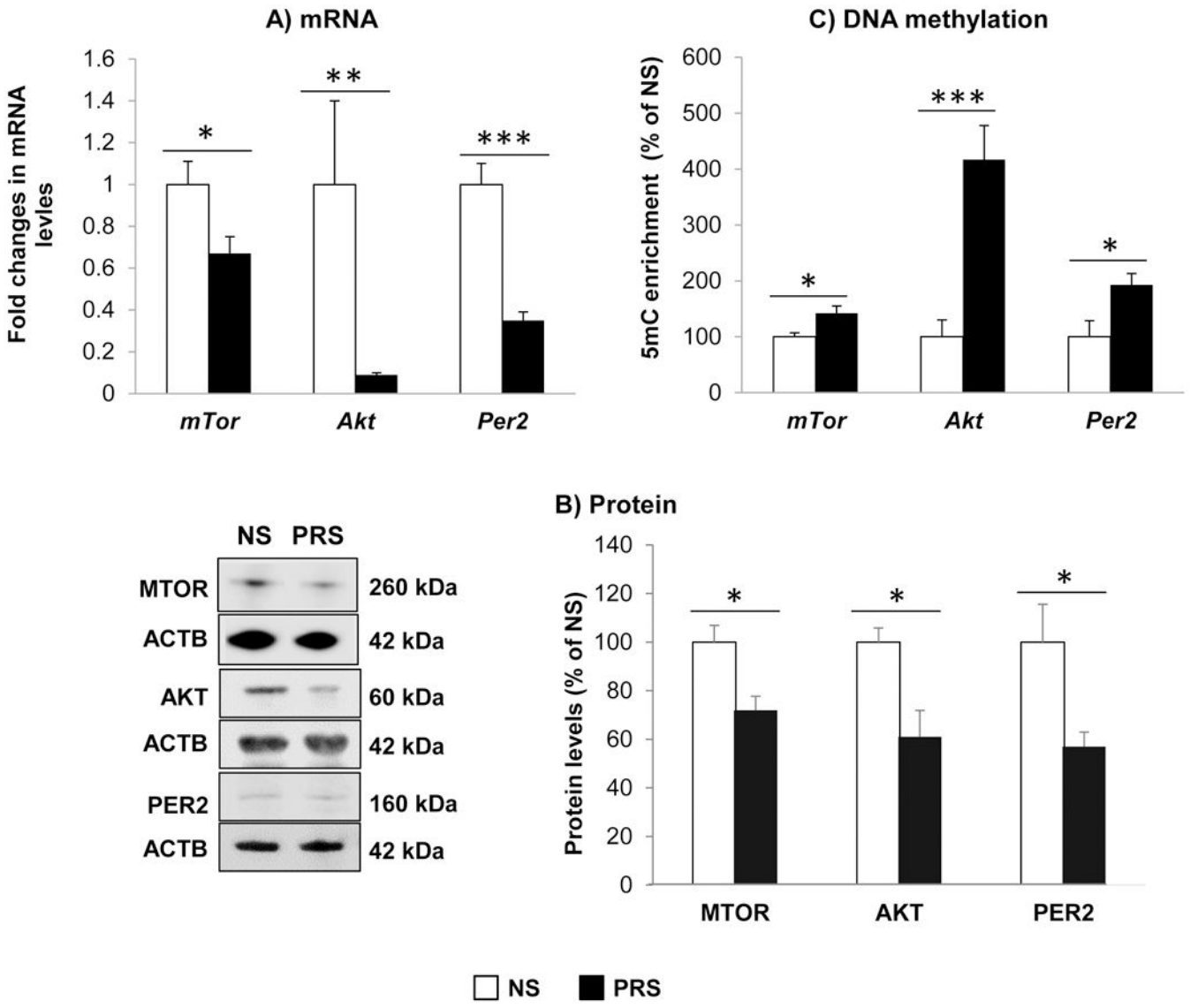

Figure 6:

Prenatal stress induces: (**A**) decrease in the expressions of *mTor* (*$p < 0.05$), *Akt* (**$p < 0.01$), and *Per 2* (***$p < 0.001$); (**B**) their encoded proteins [MTOR (*$p < 0.05$), AKT (*$p < 0.05$), and PER 2 (*$p < 0.05$); and (**C**) DNA hyermethylation (defined as levels of 5mC) on their promoters: *mTor* (*$p < 0.05$), *Akt* (***$p < 0.001$), and *Per 2* (*$p < 0.05$) in mPFC of PND 75 old offspring. The data are expressed as mean ± SEM. NS vs PRS mice, N = 8 in each group for RT-PCR and MeDIP, N = 5 in each group for Western immunoblotting.

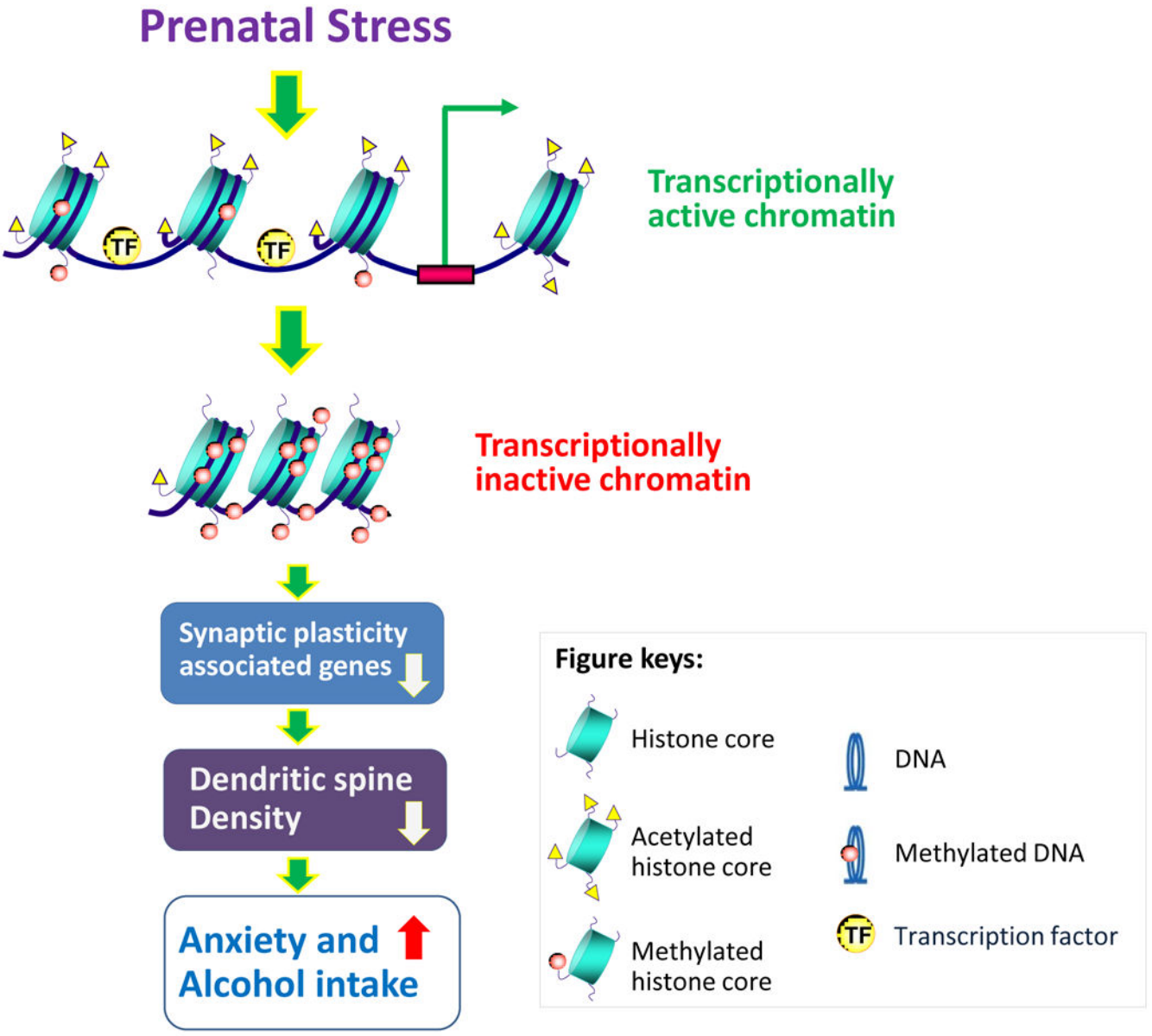

Figure 7: Schematic diagram of putative mechanisms by which prenatal stress induces excessive alcohol intake comorbid with anxiety behaviors in adult offspring.

Epigenetic regulation of gene expression is controlled by remodeling of chromatin structure. Specifically, chromatin condensation and relaxation block or allow the accessibility of gene promoters to the transcriptional machinery. Prenatal stress induces increased DNMTs and HDACs (Dong et al., 2014, 2016, Zheng et al., 2016), leading to closed chromatin and to transcriptional silencing of genes associated with synaptic formation and plasticity, which may be responsible for the dendritic spine density, possibly operative in regulating anxiety-like and excessive alcohol intake phenotype in adult offspring.

Table 1

Primer sequences for mRNA expression		
Genes	**Forward primer (5’−3’)**	**Reward Primer (5’−3’)**
Akt	CCTCTGCTTTGTCATGGAGTAT	CACAATCTCCGCACCATAGAA
Arc	CCTGAGCCACCTGGAAGAGTA	GGCCCATTCATGTGGTTCTG
Mtor	GGTGTGGCATGTGGTTCTGT	CCATCCAATCTGATGCTGGA
Per2	TTGGTGTGTGGGTTGTTGTG	CTACCTGGTCAAGGTGCAAGAG
PSD95	TGACGACCCATCCATCTTTATC	CCCGGACATCCACTTCATT
Spn	GATCCAAGTATTCAGCACCTACTC	CACTCGCTTCTCTAGCTCATATTC
TrkB	ACTAAGATCCACGTCACCAATC	CAGGGTGTAGTCTCCGTTATTC
b-Actin	TAAGGCCAACCGTGAAAAGATGAC	ACCGCTCGTTGCCAATAGTGATG
Primer sequences for MeDIP and ChIP assays		
Genes	**Forward primer (5’−3’)**	**Reward Primer (5’−3’)**
Akt	CTACTGGAGGGAGGTCTTCTAT	GTGATCTACCCATCTCTGGTTC
Arc	AATAACCTGCCTTAGCCTCATC	CCGAGTGACTAATGTGCTCTG
Mtor	CTCACGACTGATTGGCTCTT	GGGAGGAACATCCACCAATAA
Per2	GCTGGTCAGTTTAGGAAGTAGG	GGATGTCTCACACCGTCAATAA
PSD95	CATTGCCCTGAAGAACGC	ATGGATCTTGGCCTCGAA
Spn	ATGAATCCAAGAAGGAGGACTTC	GATGAAGAAGACGAAGAGGACG
TrkB	CAAAGACCCGTTACCGACTT	GTGATCTACCCATCTCTGGTTC