Exploring Sex Difference in Epigenetic and Transcription Regulatory Mechanisms Underlying Neuroglial and Behavioral Plasticity in the Prenatal Stress-Induced Mouse Model of Depression

A thesis submitted in partial fulfilment for the award of the degree of

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By

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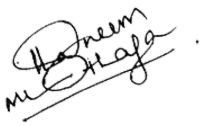
Certificate

This is to certify that this dissertation entitled "**Exploring Sex Difference in Epigenetic and Transcription Regulatory Mechanisms Underlying Neuroglial and Behavioral Plasticity in the Prenatal Stress-Induced Mouse Model of Depression**" towards the partial fulfilment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Thasneem Musthafa U K (20141107) at Centre for Cellular and Molecular Biology (CCMB), Hyderabad under the supervision of Dr Arvind Kumar, Senior Principal Scientist, during the academic year 2019-2020.

Dr Arvind Kumar

(Thesis Supervisor)



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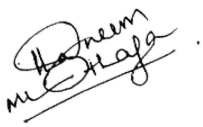
Declaration

I hereby declare that the matter embodied in the report entitled "**Exploring Sex Difference in Epigenetic and Transcription Regulatory Mechanisms Underlying Neuroglial and Behavioral Plasticity in the Prenatal Stress-Induced Mouse Model of Depression**" are the results of the work carried out by me at the Center for Cellular and Molecular Biology(CCMB), Hyderabad under the supervision of **Dr Arvind Kumar** and the same has not been submitted elsewhere for any other degree.

Dr Arvind Kumar

(Thesis Supervisor)



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Dedication

*I would like to dedicate this thesis to my parents Mrs. Fathima Rahmath K C and Mr. Musthafa U K for the continuous support and motivation to pursue my dreams. I would also like to dedicate this to Mr. Sidharth Adithyan for encouraging me to apply to CCMB, Hyderabad and work on my topic of interest for my MS thesis***.**

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LIST OF ABBREVIATIONS

%	**Percentage**
°C	**Degree Celsius**
11β HSD	**11beta-hydroxy steroid dehydrogenase type 2**
ADHD	**Attention Deficit Hyperactivity Disorder**
BDNF	**Brain Derived Neurotrophic Factor**
ChIP	**Chromatin Immunoprecipitation**
CRH	**Corticosterone Releasing Hormone**
CSDS	**Chronic Social Defeat Stress**
DNA	**Deoxyribonucleic Acid**
EPM	**Elevated Plus Maze**
EZH2	**Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit**
FST	**Force Swim Test**
GDNF	**Glial cell-line Derived Neurotrophic Factor**
Glp	**G9a related protein**
HDM	**Histone Demethylase**
HPA	**Hypothalamic-Pitutary-Adrenal Axis**
Jarid	**Jumonji/ARID Domain Containing**
Jmjd	**Jumonji C-Domian Containing**
KDM	**Lysine Demethylase**

KMT	**Lysine methyl transferase**
NFW	**Nuclease Free Water**
NORT	**Novel Object Recognition Test**
OFT	**Open Field Test**
PBS	**Phosphate Buffered Saline**
Phf	**Plant Homeodomain Finger like Domain**
pI	**Protease inhibitor**
PNS	**Prenatal Stress**
PRMT	**Protein Arginine Methyl Transferase**
SDS	**Sodium Dodecyl Sulphate**
SI	**Social Interaction**
Suv39H1	**Suppressor of variegation 3-9 homolog 1**
TST	**Tail Suspension test**
VTA	**Ventral Tegmental Area**
WHO	**World Health Organization**
µg	**Microgram**
µL	**Microliter**

Abstract

Depression is a debilitating neuropsychiatric disorder predicted to be the leading cause of disability by 2035 as per WHO, yet the underlying molecular mechanisms are unknown. Prenatal or gestational stress has a major contribution to many neuropsychiatric disorders, including depression. Here we have tried to study the effects of prenatal stress on the offspring's hippocampus in late adulthood. We used animal behavior paradigms to assess the anxiety and depression like phenotypes and indeed we were able to observe mood disorder phenotype in male offsprings and not in females. Our intense real time PCR studies to understand the epigenetic regulators involved in underlying neurobehavioural changes, specifically histone H3 and H4 lysine demethylases and lysine methyl trasferases. Western blot studies for assessing global changes in methylation specific epigenetic marks led us to uncover significant change in H3K4me3, H3K9me1 and H4K20me1, along with change in the transcript levels of demethylases/methyltransferases responsible for demethylating/methylating histone lysines. Previous lab microarray data had indicated alteration in some forkhead family transcription factors in hippocampus in sex biased manner. Here we successfully validated Foxb2 and Foxh1 in gender-specific manner. Further, our ChIP-qPCR analysis showed differential enrichment of Foxb2 on promoters of Sox2, Nestin and Synaptotagmin genes, the neurogenesis and neuroplasticity markers. Irrespective of the enrichment at the promoter region of genes that control neurogenesis, these genes were found upregulated in female while unaltered in male hippocampus. So from these novel data it is difficult to elucidate role of FoxB2 in neurogenesis; more studies are required to get better insight.

Review of Literature

1.1 Introduction

The “developmental origins of health and disease” suggests that compromises in the intrauterine environment affect the fetal development and predispose the individuals for long-range of non-communicable illnesses(O’Donnell and Meaney, 2017). Mental health issues like depression, anxiety and stress are common in pregnancy. One in three to one in five women in developing countries and one in ten women in developed countries faces significant mental health issues during their pregnancy or after childbirth (Department of Mental Health and Substance, World Health Organization, 2016). The term ‘prenatal stress’ refers to stress perceived by the fetus. It could be biological (hormones or neurochemical changes), psychological or social determinant (exposure to family violence). Children from stressed mothers tend to have increased risk of psychopathology at some point in their lifetime (Bale et al., 2010). Prenatal stress leads to neurological and psychiatric diseases such as affective disorders, autism, attention deficit hyperactivity disorder (ADHD), and schizophrenia (Bale et al., 2010). It has also been found that both male and female offspring show the difference in their psychopathology (Glover and Hill, 2012). Prenatal changes have been reported to be commonly facilitated by diverse epigenetic mechanisms. Prenatal stress alters the corticosterone releasing hormone activity in the brain regions and the incidence of depression and anxiety in rat and mouse models. An excessive amount of CRH in the human fetal brain can also lead to developmental abnormalities and depression-like phenotype. Hence it is essential to study the effect of parental stress in mice to figure out the molecular players involved in anxiety-depression like phenotypes.

1.2 Depression

All depressive disorders are characterized by the presence of feeling sad, low, empty or irritable mood, accompanied with cognitive and somatic impairments. They are categorized into disruptive mood dysregulation, major depressive disorder, dysthymia (persistent depressive disorder), permanent dysphoric disorder and medication or

unspecified depressive disorder based on the time, duration and etiology. The major depressive syndrome is marked by any five or more symptoms like consistently depressed mood in a whole day, diminished interest in activities, significant weight loss or weight gain, insomnia, psychomotor agitation, feeling of worthlessness, inability to concentrate, and suicidal ideation without a specific plan(DSM 5, 2013). The prevalence has a gender bias towards women, women experience 1.5 – 3 fold higher rates than men in early adolescence. Milder depression can be treated by psychological counselling or therapy whereas chronic depression needs medication in combination with therapy.

Etiology of depression

DSM 5 defines the cause of depression could be temperamental, environmental, genetic or physiological, and course modifiers. Neuroticism (negative affectivity) pose a high risk for development of depression. Stressful life events like adverse childhood experiences are well-established factors for developing depression. Heritability accounts for approximately 40% in depression and increases the risk of early-onset and recurrent forms. Having other personality disorders, or substance abuse could potentially increase the chances of getting depression.

FIGURE 1 AETIOLOGY OF DEPRESSION (BALDWIN AND BIRTWISTLE, 2002)

1.3 Stress and Depression

Stress is the body's reaction to any aversive stimuli from inside or outside the body. Stress is considered to be one of the most common reasons for depression. When stress is perceived body activates the neuroendocrine pathways and brain acts according to that. As mentioned earlier in depression sex, personality, history and lifestyle determine the magnitude, duration and perception of the stress (Krugers et al., 2012). Corticosteroids are released during stress for neural protection but impairs memory formation function specific to glucocorticoid receptor (De Kloet et al., 1999).

Brain derived neurotrophic factors (BDNF), Glial cell line-derived neurotrophic factor (GDNF), Neuritrophin-3 (NT-3), nerve growth factor (NGF) and Neurotrophin-4 (NT-4) are considered as stress markers. Neurotrophic hypothesis of major depression states that BDNF levels, which is involved in neuronal plasticity gets reduced in Hippocampus and specifically dentatae gyrus and antidepressant treatments restore the levels (Lee and Kim, 2010).

Stress Response

Adrenal gland plays the major role in stress response. When a stress is perceived adrenal medulla releases fight and flight hormones epinephrine and norepinephrine. In the slower second phase adrenal cortex releases glucocorticoids (GC), as an effect of Hypothalamus-Pituitary-Adrenal (HPA) axis activation. Paraventricular Nucleus (PVN) of hypothalamus releases corticotrophin releasing hormone (CRH), which stimulates anterior pituitary and it produces adrenocorticotrophic hormone (ACTH). ACTH induces adrenal cortex to produce GC. GCs exert negative feedback on HPA axis. In some cases of depression, this feedback does not work properly and leads to hypercortisolemia and causes damage in hippocampal neurons (Nestler et al., 1968). It has been found that in depressed people the hippocampal volume was reduced (Campbell et al., 2004).

1.4 Reward Pathway

The brain reward pathway mediates the reward behavior such as pleasure and motivation which reinforces approach behavior. Hippocampus exerts a negative control on HPA-axis, leading to hypercortisolemia in depressed individuals. The mesolimbic dopamine system plays crucial role in mood regulation. Nucleus accumbens (NAc) receives dopaminergic inputs from ventral tegmental area (VTA) of the midbrain. Amygdala is important in learned association between negative stimuli and environmental events. Hypothalamus has been shown to be involved in rewarding pathway of drug abuse. VTA and NAc receives strong glutamatergic inputs from frontal cortical areas, hippocampus and amygdala whereas the same areas receive projections from hypothalamic peptidergic nuclei. In context of animal models, stress affects VTA dopamine neurons and its limbic targets including NAc.

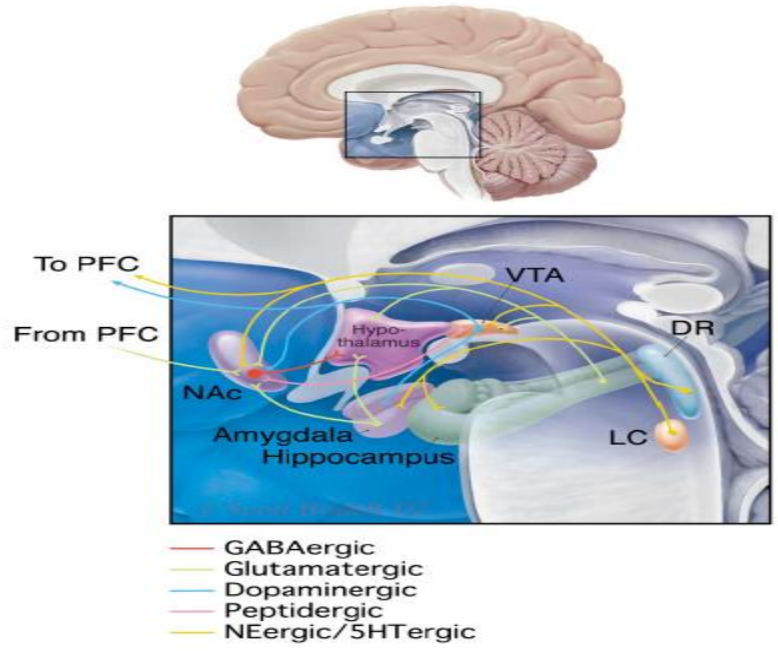


FIGURE 2 BRAIN REWARD PATHWAY (VTA-VENTRAL TEGMENTAL AREA, DR - DORSAL RAPHE, LC - LOCUS COERULEUS) (Nestler et al., 2002)

CREB (cAMP Response Element Binding protein) gets regulated in NAc by glutaminergic and dopaminergic inputs suggest an emotional gate for behavioural responsivity. Paradoxically elevations in CREB function in NAc show depressive like symptoms whereas reduced CREB function causes anxiety-like behaviour, which makes it a potential key regulator in mood disorders. The hypothalamic-NAc pathway is mediated by melanin-concentration hormone (MCH) that is important in reward (Nestler and Carlezon, 2006).

1.5 Mouse Brain Development and Adult Neurogenesis

Mouse (Mus musculus) gestational period span from 19 to 21 days. Neural stem cells (NSCs) differentiate into neurons and glial cells embryonically and postnatally. On 9th day post-conception cranial motor nuclei forms (E9). Peak of neurogenesis in amygdala happens in post-conception 12th day (E12) and in hippocampus it happens on post conceptional 15th day (E15). Nucleus accumbens forms on post-conception 16th day. The process of neural development in early stage of development ends in mouse by postnatal 30th day when the eye opening happens (Finlay and Darlington, 1995).

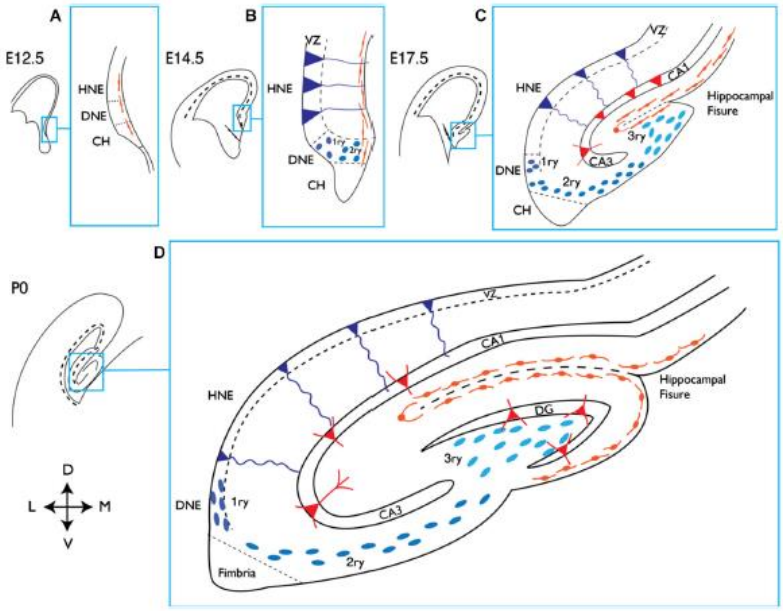


Figure 3 Development of Hippocampus (HNE - Hippocampal neuroepithelium, DNE - Dentate neuroepithelium. CH- Cortical hem) (Urbán and Guillemot, 2014)

Contradictory to earlier belief that mammalian brain doesn't produce neurons in adulthood, the process of neurogenesis happens in subventricular zone(SVZ) of olfactory pathway and Dentate gyrus(DG) of hippocampus (Zhao et al., 2008). Neural stem cells (NSCs) and/or neural progenitor cells (NPCs) residing in these regions are responsible for continuous production of new neurons. Subgranular zone of the dentate gyrus is a layer beneath the granular layer in hippocampus. The NSCs of SGZ produce granular cells, single type and get incorporated into upper granular layer (Cameron and Mckay, 2001).

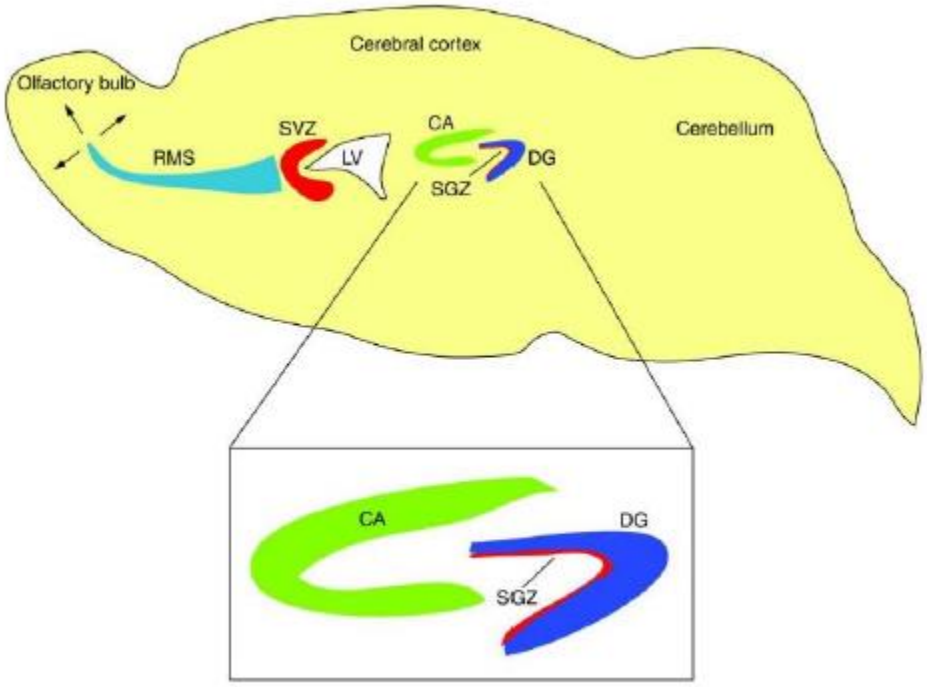


FIGURE 4 ADULT NEUROGENESIS (RMS - ROSTRAL MIGRATORY SYSTEM, SVZ-SUBVENTRIULAR ZONE, LV - LATERAL VENTRICLE, DG - DENTATE GYRUS, SGZ-SUBGRANULAR ZONE) (POZNIAK AND PLEASURE, 2006)

There has been reports on increase in corticosteroids during stress affect adult neurogenesis (Fuchs and Gould, 2000). PJ Lucassen has drawn three important observations regarding stress-depression and neurogenesis: There is reduction in hippocampal volume and neurogenesis during stress. Antidepressants work slowly to

be effective, its similar to neurogenesis. Thirdly antidepressant treatment enhances serotonin which increase the neural proliferation (Lucassen, P.J. et. al., 2016).

1.6 Animal Models of Neuropsychiatric Diseases

Depression, bipolar disorder, schizophrenia and autisms are quite prevalent and cause high burden on society (Kessler et al., 2005). Because of ethical and practical issues, despite having high burden, studying these diseases with live human brain is limited. Therefore, either the researchers should rely on post-mortem brain or with live animal models. Considering the changes happen after the death in brain, it is not easy to draw conclusions from post-mortem brain to live human brain. However, it is also challenging to mimic human scenario in animal models because of the subjective nature of symptoms and non-availability of proper diagnostic marker. Many of the neuropsychiatric disorder symptoms like guilt feeling, suicidal tendency, sadness etc it is impossible to mimic or assess them in animal models. But there are anxiety-like and depressive like features like reduction in social interaction, affected cognition or anhedonia we can assess using behavioural tests and make approximate correlation with human conditions. Genetic manipulation or stress induction using injecting drugs are other ways to mimic the symptoms of neuropsychiatric disorders. But depression has been considered multigenic and no reliable model exists so far. But despite these limitation several animal models have been developed for depression and they have enhanced the understanding of the disease. In rodent models, psychomotor and physiological symptoms can be measured using behaviour tests. Exposure to stress increases the chance of being depressed in human beings, therefore most of the models are based on environmental stressors to induce depression-like phenotype.

Stress Exposure in Early Life stage

1. **Prenatal Stress**

Prenatal stress studies how traumatic environment experiences by the pregnant mother affects mental and physical health of the offspring in later life. The stress has been perceived by the mother is communicated to pups inside the womb through hormonal imbalances. This is majorly mimicked by restraint stress (immobilisation stress) for 6 times more depending upon the protocol. The effect of restraint prenatal stress varies at which point of gestational period the stress is provided and to what duration and frequency. Prenatal stress can also include immunological imbalances because that is common in human beings (Gerardin et al., 2005). It also induces impaired feedback mechanisms for HPA-axis and transformed neuroplasticity (Maccari and Morley-fletcher, 2007).

2. **Early Life Stress**

Childhood traumatic experiences lead to psychopathology in later life in humans. It can be modelled in animals in two ways: early handling or maternal separation. In early handling, for the first few weeks, pups are separated from their mother for a certain short period and returned to the home cage. Pups when they reach adulthood are subjected to physical stress, they turn to be more resilient towards it (Pryce et al., 2001). Maternal separation includes separation of pups for unpredictable duration and longer duration ranging 3h-24h. In contrast to early handling stress they show depressive phenotypes after postnatal day (PND) 2 to PND 14 maternal separation (Uchida et al., 2010). On the other hand, Wistar rats after maternal separation from PND1 TO PND21 showed higher exploratory behaviour and altered risk taking ability. Therefore, it's difficult to conclude whether early life stress induces susceptibility or resilience upon exposure.

Stress Exposure in Adult Stage

Stress experienced by an adult human being is completely different from stress perceived in early life stages. Most of these stress events are unpredictable with their occurrence, nature and duration. Mimicking human situations of chronic stress, there are several paradigms of stress for animal like restraint stress, chronic unpredictable(mild) stress, variable stress and social defeat.

Restraint Stress

Restraint stress or immobilisation stress protocols vary from acute (one-time) stress to a chronic (six to ten days or more). During the stress the animal is kept inside a restrainer, which limits the normal movement of it. It effectively mimics physico-psychological stress and induce anxiety and depression like phenotypes.

Chronic Unpredictable Mild Stress (CUMS)

It is more like day-to-day life of human where multiple mild stressors are given over period of 7 days to 8 weeks. The mild stressors include tail suspension, forced swim, wet bedding, ice bedding, restraint stress, cage tilt, water/food deprivation and light/dark cycle interruption. Animals are exposed to any of the two stressors every day.

Chronic Unpredictable Stress

It is a variation of CUMS but is more aggressive. It includes restraining for 1hr, social defeat for 10 minutes and foot shock for 1 hr in consecutive days. This stress cycle can be repeated multiple times.

Chronic Social Defeat Stress (CSDS)

In chronic social defeat model, the experimental male animal is put together with a aggressive dominant male animal for 5-10 minutes for 10-20 days. After this the animals are kept in same cage separated by a perforated plexiglass barrier in between. CSDS result in passive submissive character, anhedonia, social avoidance, anxiety and other depression like phenotypes.

1.7 Prenatal Stress

Animal studies have established prenatal stress as determinant of adulthood physiological and behavioural outcome (Weinstock, 2017). The developing and maturing brain circuitries during the critical gestational periods are sensitive to various stressors and result in severe and long lasting consequences (Pryce et al., 2005) How the programming is affected depend on stimulus type, intensity, duration and timing of the exposure.

When mother is subjected to stress HPA axis activation happens, leads to rise in circulating cortisol levels in mothers. It can cross the placental barrier and cause developmental alterations in foetus. Under normal circumstances because of the breakdown of cortisol by 11 beta- hydroxy steroid dehydrogenase type 2 (11ß-HSD-2) the fetal level of cortisol will be 5-10 times lower compared to maternal level (O'Donnell et al., 2009). Fetus does not produce its own cortisol until late gestation. Prenatal stress disrupts this cortisol rises sharply along with the reduction of 11ß-HSD-2 (Mairesse et al., 2007, Glover et al., 2009). Exposure to the higher cortisol level affect the normal development of fetal HPA -axis and its negative feedback mechanism (Weinstock et al., 1992 , Fujioka et al., 1999). Also, cortisol induces CRH release by placenta which has negative consequences like impairment in development of brain regions (Sandman et al., 2006, Raison and Miller, 2003, Kofman, 2002).

Alterations in Neural Pruning, Brain Structure and Connectivity

Microglia play a crucial role in pruning process, where surplus neurons and synapses existing during the beginning of life is cleared and hence brain development happens. This pruning process employ multiple mechanisms (Schafer et al., 2012). This include activity dependent selective elimination. MHC class I proteins recruit myocyte enhancer factor 2(MEF2) transcription factors in activity dependent manner through calcineurin pathway. MEF2 controls levels of genes involved in synapse elimination. MEF2 interact with histone deacetylase (HDAC) class IIa and act as MEF2-HDAC transcriptional repression axis. Prenatal stress has been found to influence microglial function (Ślusarczyk et al., 2015). Alterations in microglial activity during critical periods have

negative consequences for circuit formation and neuronal development. In Autism spectrum disorders (ASD) and schizophrenia it has been implicated microglial dysfunction also contribute to synaptic deficits. Abnormal MHCI-MEF2 signaling has been implicated in mouse model of maternal immune activation (MIA) associated ASD and schizophrenia (Elmer et al., 2013).

Gestational stress is associated with structural changes in white matter of amygdala, prefrontal, parietal and corticolimbic regions (Rifkin-Graboi et al., 2013, Chen et al., 2014). It causes reduced grey matter volume in cortex, medial temporal lobe, amygdala and cerebellum (Buss et al., 2010, El Marroun et al., 2016, Sandman et al., 2015). Prenatal stress affects dentate gyrus (DG) neurogenesis and hippocampal plasticity (Lemaire et al., 2000, Lemaire et al., 2006). It leads to impaired cognitive abilities. Prenatal stress has also been reported to induce reduction the CA1 area dendritic arborization in the hippocampus and CA3 area synapse number.

1.8 Prenatal Stress and Epigenetics

Epigenetic mechanisms like DNA methylation, histone modification s and non-coding RNA expression plays important role in development hence in prenatal stress induced brain reprogramming. DNA methyl transferases DNMT1 and DNMT3A are essential for synaptic plasticity, neurogenesis, and learning and memory. Mutations in methyl binding protein MeCP2 have been implicated in autism, Rett syndrome, X-linked mental retardation etc (D. et al., 2012, M.L. and J.M., 2010). Placental miRNA expression and DNA methylation patterns also have important role in brain development. Epigenetic processes in the placenta and different brain regions get influenced by the prenatal stress with genes like 11ß-HSD-2 gene, glucocorticoid receptor (Nr3c1) gene and brain derived neurotrophic factor (BDNF) (Kundakovic and Jaric, 2017, Peña et al., 2012,Oberlander et al., 2008, T.L. et al., 2009). These and other epigenetic modifications impact endocrine systems and important brain structures.

Reduction in 11ß-HSD-2 expression is associated with higher amount of DNMT3a mRNA levels and hypermethylation of CpG sites of 11ß-HSD-2 gene promoter in the

placenta. This has been shown in humans as well. After early prenatal stress adult male offspring showed depression like behaviour has found to have reduction in Nr3c1 in hippocampus and hypermethylation in CpG island (Mueller and Bale, 2008). They also found hypomethylation of CRH gene in hippocampus and amygdala hence elevated CRH expression. Sex dependent differences induced by prenatal stress, affected many placental genes including DNMT1 (Oberlander et al., 2008).

DNA methylation and histone modification machinery has been reported to show alterations as an effect of gestational stress (Dong et al., 2015, Zheng et al., 2016). Maternal exposure to methyl mercury induced depression-like behavior with reduced BDNF in DG. It is shown that it is associated with H3K27me3, decrease in histone acetylation and DNA methylation (Onishchenko et al., 2008) Benoit et al., 2015).

In response to prenatal exposure of lead, a lifelong reduction H3K4me2 and H3k27me3 observed (Cory-Slechta et al., 2017). A 10 fold reduction of H3K9me3 was found postnatal day 6 after lead exposure during gestation along with cognitive decline (Schneider et al., 2016).

1.9 Forkhead family proteins and Neuroepigenetics

Forkhead box (FOX) proteins are transcription factors important in development, organogenesis, regulation of metabolism and immune system. The first FOX gene was discovered in Drosophila, the mutation gave it a fork-headed appearance. There are 50 different FOX genes in humans and 44 in mouse divided to 19 subfamilies. The DNA binding domain is a winged-helix domain called forkhead domain and is well conserved across all the families and across species. The forkhead domain is about 100 amino acids s in length, monomeric and consists of three alpha-helices, three beta-sheets and two large loops (wing region). FOXO subfamily is the well-studied one amongst all other subfamilies because of its member’s importance in metabolic pathways. FOXA proteins can open tightly compacted chromatin without the involvement of SI-SNF (Switching-sucrose non fermenting) chromatin remodelling complex by the interaction of its c-terminal with histone H3 and H4.

FoxO are targets of phosphatidylinositol 3-kinase/protein kinase B(PI3K/Akt) signaling pathway and its involved in neuronal differentiation. FoxO transcription factors binds or interacts with molecules like Notch, wnt and miRNAs. Wnt-1 increases neuronal proliferation whereas Wnt-3a regulates neuronal differentiation (Wen et al., 2012).

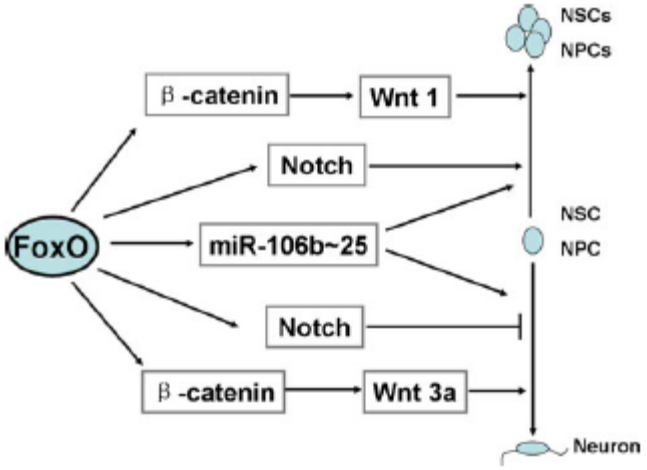


Figure 5 FoxO and Neuronal Differentiation (Wen et al., 2012)

Table 1. Fox factors involved in adult neurogenesis

Gene	Mice	Phenotype	References
Foxg1	Foxg1$^{-/-}$	Die around birth, with a severe reduction in the size of the cerebral hemispheres.	[47]
	Foxg1$^{+/-}$ and Frizzled9-Foxg1$^{f1/f1}$	Increased neuronal death and reduced postnatal DG neurogenesis. NSCs prematurely exit the cell cycle and differentiate into neurons.	[48–50]
	Overexpression of Foxgl by lentiviral infection	Enhances neurogenesis and proliferation of NSCs.	[51]
Foxj1	Foxj1$^{-/-}$	Majority die before weaning. Left/right asymmetry with loss of motile cilia and flagella. Ependymal cells fail to differentiate. Defects of proliferation and differentiation, which induced severe OB dysgenesis.	[52–55]
Foxo1	Foxo1$^{-/-}$	Die around E11.	[56]
	GFAP^{cre} Foxo1$^{f1/f1}$	No appreciable changes in apoptosis are observed both in NSCs and neurons.	[57]
Foxo3	Foxo3$^{-/-}$	Number of NSCs is reduced. NSCs have decreased self-renewal and impaired ability to generate different neural lineages.	[58, 59]
	CaMKII-tTA; Foxo3-CA	Loss of neural progenitors and increased apoptosis.	[60]
Foxo6	Foxo6$^{-/-}$	No role in adult neurogenesis	[61]
Foxo1/3/4	GFAP^{cre}; Foxo1/3/4$^{f1/f1}$	Enlarged brain. Deregulation of NSC proliferation followed by a decline in NSC pool and neurogenesis.	[57]
Foxm1	Foxm1$^{-/-}$	Die perinatally between E13.5 and E17.5 due to multiple abnormalities (mainly cardiovascular and hepatocytes defects). Impairment of self-renewal of embryonic NSCs.	[62–66]
	Math1^{Cre} or Nestin^{Cre}; Foxm1 $^{f1/f1}$	Delay in brain development.	[67]

Summary of mouse lines carrying fox gene mutations and the corresponding impact on adult neurogenesis.
Abbreviations: DG, dentate gyrus; NSC, neural stem cell; OB, olfactory bulb.

FIGURE 6 FORKHEAD FACTORS INVOLVED IN ADULT NEUROGENESIS (Genin et al., 2014)

Foxg1 the only member of forkhead G family regulates rate of neurogenesis by keeping cells in proliferative stage. Foxg1 is highly expressed in dentate gyrus. Foxg1 is also required for postmitotic neurons. Foxg1 blocks neuronal cell death. Foxg1 needs to physically interact with transducin like enhancer of split1 (TLE1) to promote cell survival. Foxj1 is important to produce motile cilia in ependymal cells. Foxm1 controls G1/S phase transition and G2/M progression. Foxm1 is ubiquitously expressed in embryonic tissue like proliferating cells of epithelial or mesenchymal cells. Foxm1 is highly expressed in neural stem cells and neural precursor cells (Genin et al., 2014).

Previous microarray and proteomics studies from the lab, we found foxb2 is differentially regulated in sex dependent manner. Foxb2 has been reported to drive neuroendocrine differentiation of prostate cancer through Wnt pathway. Foxb2 expression is restricted to developing brain (Moparthi et al., 2019).

Materials and Methods

2.1 Materials

 Acrylamide, Bis-acrylamide, Agarose, BSA fraction V, Ethylene-diamine-tetraacetic acid (EDTA), Ethidium Bromide, , Triton X-100, Trypsin, Trypsin Inhibitor, Ammonium Per Sulphate (APS), TRIzol Reagent were obtained from Sigma.

 β-Mercaptoethanol were purchased from Serva.

 Sodium Dodecyl Sulphate (SDS), Tetramethylethylenediamine (TEMED), Tris buffer, Sodium Chloride, Potassium Chloride, Disodium Hydrogen Phosphate, Sodium Dihydrogen Phosphate, Potassium Dihydrogen phosphate, Glycine, EDTA and Sodium Hydroxide were obtained from Fisher scientific.

 Glycerol and Tween-20 were purchased from MP Biomedicals.

 Protein A/G Dyna beads, Nuclease-free water, RNase A, PrimeScript first strand cDNA synthesis kit, SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) 2x buffer were obtained from Takara

 Formaldehyde, Chloroform and Isopropanol were procured from Calbiochem.

 Protease inhibitor cocktail (PI) used in experiments was from Roche.

 Syringe Filters (0.22 µm and 0.45 µm), PVDF membranes were obtained from Merck-Millipore.

 Western lighting chemiluminescence reagent (ECL) and medium molecular weight marker proteins for SDS-PAGE were obtained from Thermo Fischer.

 Plasmid isolation kit, Gel, and PCR elution kit were purchased from Macherey-Nagel.

 T4 DNA ligase used was from Kapa Biosystems.

 DNA Oligos and dNTPs were synthesized from Bioserve.

 DNA ladders, Restriction Enzymes and DNaseI were procured from New England Biolabs.

 Whatman filter papers were from Whatman International Ltd.

 RNA isolation mini kits, microRNA isolation kit, miRNA mimics, miRNA inhibitors were used from Ambion / Life Technologies.

 Primary and secondary antibodies used were from Abcam, Millipore, Cell Signaling, Life Technologies and Sigma.

 Vectastain with DAPI used in immunostaining experiments were from Vectashield.

 Other reagents were from local suppliers such as Qualigens, SRL, SD fine chemicals Ltd. and Merck India Ltd. All reagents used were of analytical grade.

Antibodies

Mouse Monoclonal anti H3K9me1 from Abcam cat#ab9045
Rabbit polyclonal anti H3K27me2 from Abcam cat#ab24684
Rabbit polyclonal anti H4k20me1 from Abcam cat#ab9053
Rabbit polyclonal anti H4K20me2 from Millipore cat#07-367
Rabbit polyclonal anti H3K4me3 from Millipore cat#07-473
Mouse anti b-actin from Sigma cat#A2228
Rabbit monoclonal FoxB2 antibody from Abcam cat#ab170897
Rabbit Control IgG from Abcam cat. # ab172730
Mouse Control IgG from Abcam cat#ab

Stock Solutions

30% Acrylamide solution: 29 g acrylamide and 1 g of bis-acrylamide were dissolved in sterile Milli-Q water, filtered and stored at 4°C in the dark colored bottle

1x Tris-Glycine running buffer: 25 mM Tris, 250 mM Glycine and 0.1% SDS were dissolved in Milli-Q water.

1x Transfer buffer: 96 mM Tris, 50 mM Glycine, 0.1% SDS and 20% Methanol were dissolved in Milli-Q water.

1x TBS or Tris-buffered saline: 137 mM NaCl, 2.7 mM KCl and 20 mM Tris Base were dissolved in Milli-Q water and pH was adjusted to 7.4 using HCl.

Ethidium bromide: 10 mg/ml solution in water

1x TAE or Tris-acetate-EDTA buffer: 40 mM Tris, 20 mM acetic acid and 1 mM EDTA were dissolved in Milli-Q water

1x PBS or Phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 2 mM KH2PO4 were dissolved in Milli-Q water and pH was adjusted to 7.4 using HCl.

2.2 **Animal Experiments**

C57BL/ NCrl female mice of age 2-3 months used for breeding. They were issued at gestational day 12. The pups both females and males obtained from the pregnant mothers at the age of 7-8 months are used for behavioral and molecular studies. All experiments were performed in accordance with protocols approved by the Institutional Animal Ethical Committee (IAEC). All mice were kept at 12/12hr light/dark cycle at constant temperature (25^0C), 60% relative humidity and water and chow *ad libitum*.

Stress Model

From the pregnant mice at gestational day 15, randomly chosen ones where subjected to 4 days restraining stress paradigm twice in a day for 30 minutes each with a gap of 5 hrs in between. Control mothers were kept undisturbed. Pups were born between gestation day 20-21, weaned after sexual

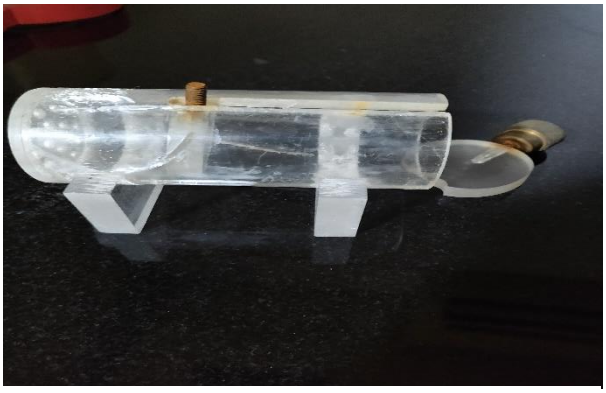


FIGURE 7 RESTRAINER

dimorphism was visible, on postnatal day 18.

Behavioral Tests

Behavioral tests are conducted after animals 7-8 months to see the depression/anxiety like behaviors and cognitive decline, if any, has been induced due to stress.

Open Field Test (OFT)

The open field test (OFT) is used to assess the animal's anxiety and exploratory behaviour. Ambulation is the most studied behaviour. The equipment contains an open field as the name suggests with four opaque or clear walls of size 40 x 40 cm. Lighting should be overhead to avoid the shadows. Walls should be easily detachable to clean properly. Grid floor inserts are used to track the animal in the video recording. The grid divides the open area into small squares marking a central area, an outer area near the walls. Animal's innate response to avoid the brightly lit open area and exploratory drive contribute to the response. It's an evolutionary adaptation for animals to remain in the darker enclosed area to escape from numerous ways of threat including predation. This test does not require any training. There are many parameters which can be analysed with the test include frequency of lines crossing in the grid, frequency of central square entries, time spent in the centre of the arena, time spent close to walls, the frequency of rearing behaviour and frequency of stretch postures. More the anxiety, mice will show less mobility, and it will prefer outer areas as compared to normal or anxiolytic (anxiety reducing drugs) treated mice will prefer the central area, and it will be more active. The limitation of this test is it does not differentiate between fear and anxiety-induced behaviours. In fear induced activities the escape response or avoidance will lead to termination of fear but in an anxiety-induced response there exist a tendency of approach in addition to avoidance, which leads to an increase in anxiety. A video recording is made for 5

FIGURE 8 OPEN FIELD ARENA

minutes and parameters like latency to enter the central area, time spent in central/outer area are analysed.

Elevated Plus Maze Test (EPM)

Elevated plus maze is a behavioural assay for rodents to assess the anxiety-related behaviours. The apparatus is in a plus shape consist of two open arms, two closed arms and a central area. The size of it is 60x60cm. The animal is either placed facing one of the open arms in the central region or on one of the open arms. Arms should be at least 50cm above the ground level. The position where we kept the animal should be constant in all the trials. The test relies on animal's tendency to towards the dark, enclosed region as compared to the open region and avoidance behaviour to the height and open space. Depressed or anxious animal prefer to stay in the enclosed region whereas the normal or anxiolytic treated animals explore all the arms and central region. A video recording is made for 5 minutes using EthoVision. Latency to enter any of the closed arms, time spent in closed/open arm and frequency with which enters each arm are measured. The results vary with the exposure to a novel environment just prior to the test, gender, age, strain and the oestrous cycle. Multiple exposures to the elevated plus maze reduce the latency to enter the enclosed arm. If the animal falls off the maze it should be put back rapidly to the maze and should allow it to complete the 5mins. Care should be taken while analysing the data.

FIGURE 9 ELEVATED PLUS MAZE ARENA

Social Interaction Test (SI)

The apparatus contains an interaction box inside an open arena. Open arena is of size 60x40cm. A mouse which has not interacted with the experimental animals is kept inside the interaction box. The experimental mouse is released at one of the corners and allow it to freely move and interact

FIGURE 10 SOCIAL INTERACTION ARENA

with the mouse inside the interaction box. For every mouse two trials are done- one with no mouse inside the interaction box and followed by one with the animal inside it. The approach-avoidance behaviour is measured by the time spent by the animal around the interaction zone and by the frequency with which it approaches the zone. Time spent in corners also calculated. The new target animal could be same stain, different strain or different gender. Here we have used same gender same strain unfamiliar animals to assess the interaction. The time spent in interaction zone is calculated by Ethovision video tracking software for 5 minutes. Social avoidance is characteristics of typical depression. So this experiment assess the depression like phenotype in animal.

The interaction ratio is calculated by [**Time spent in in the interaction zone (with target) / time spent in the interaction zone (with target + without target)] x 100**

Forced Swim Test (FST)

FIGURE 11 FORCED SWIM TEST

Forced swim test is a classical experiment for assessing depression like phenotype. Mice are placed into a non-escapable transparent 10L beaker filled upto 2/3 with water of temperature 23-25 degree Celsius. 5 minutes recordings are done with camera. Four trials are done together with cardboard sheets separating each beaker. The videos are analysed with EthoVision for their mobility which is defined as their escaping tendency. Latency to first immobility is considered in analysing the despair behaviour. Stressed mice show less mobility and escaping behaviour, compared to control mice.

Tail Suspension Test

FIGURE 12 TAIL SUSPENSION TEST

The tail suspension test is for assessing depression-like phenotype in mice. Mice

are suspended by their tail with tape, in way that it can't escape to hold to nearby surfaces. Escaping behaviours in the coarse of 5 minutes test time is quantified. Social interaction cages are kept upright to get the 60cm hight and white contrast background. Four animal trials are done together, each animal is separated from other with a cardboard sheet. Autoclaved tape was used to stick the tails to the edge and stuck 3cm from the tail end. Time spent immobile is measured with Ethovision software and plotted to see the difference between groups. The advantage of tail suspension test over forced swim test is that animal won't get hypothermia because of submerging in water.

Novel Object Recognition Test (NORT)

Novel object recognition test is a test for learning and memory. It has two stages: habituation and familiarization and test. It is a 4-day protocol where in first 2 days animals are trained with two similar (called familiar object) placed 10 cm away from the walls of the social interaction open arena. Two days 5 minutes trials are conducted to familiarise the object. Nothing has been done on 3rd day. Fourth day one of the familiar object is replaced with a novel object, which varies in shape, colour and pattern. 5 minutes trial is employed and assessed how much time animal spent with the new object. Animal's innate ability is to explore the new object. But if it hasn't learned from the previous training period it will fail to recognise the new object and familiar object. From that we can assess the learning and memory of the animal. Ethovision software is used to monitor the 5-minute trials and to analyse the interaction time.

Sacrificing Animals

3rd day after last behavioural test tail suspension test, mice were sacrificed by cervical dislocation. The animals were decapitated, brain was removed immediately and rinsed in ice cold autoclaved 1x PBS. Using a brain matrix (Zivic rodent brain slice matrix) 1mm thick slices were obtained. The samples for ChIP protocol, the whole hippocampus is put in a single tube and immediately added 940µL 1% formaldehyde and kept in rotor for 12 minutes for crosslinking. To stop the reaction 62.5µL 5M glycine is added for 5minutes and mixed. 3 washes are given with protease inhibitor mixed 1x PBS to remove residual formaldehyde. After removing residual PBS samples are snap

frozen and kept in -80^{0}C. Samples for qPCR and western blotting, PFC and hippocampus from each side is separated, snap froze with liquid nitrogen and stored in -80^{0}C.

2.3 Molecular Methods

Isolation of RNA using TRIzol (Sigma) method

RNA was isolated using TRIzol reagent-a monophasic solution of phenol and guanidine isothiocyanate. TRIzol is added in a proportion for 100mg tissue 1mL TRIzol. Initially 200µL added to tissue and homogenised with handy homogeniser and autoclaved probes. After homogenisation rest of TRIzol is also added and 20minutes incubated. Chloroform, one fifth of the amount of TRIzol added to the solution mixed well and kept on ice for 15minutes before centrifuging at 16000rpm for 15minutes at 4^{0}C. The aqueous layer is collected to a different tube and equal amount of isopropanol is added. Mixed well and incubated at -30^{0}C for 2hrs. Samples are taken out and centrifuged at 12000rpm for 15minutes at 4^{0}C. Solution part is removed without disturbing the white pellet. In the washing step, 1mL 70% distilled ethanol with NFW is added and centrifuged for 10minutes at 10000rpm and 4^{0}C. This step is repeated twice. The samples are dried at 45^{0}C using dry bath. 20µL of NFW is added to dissolve the RNA. Nanodrop2000 spectrophotometer reading has been taken and 1µL ran on agarose gel to see the degradation, if any.

DNase Treatment

The RNA samples were subjected to DNase treatment with 10x DNase I buffer and 1 unit of DNase I enzyme (New England Biolabs) were added to a maximum of 20µL RNA sample. It is incubated at 37^{0}C for 40 minutes using Thermomixer. 1µL .5M EDTA is added to stop the reaction and heated to 75^{0}C for 10minutes.

cDNA Synthesis

TAKARA PrimeScript cDNA synthesis kit is used to synthesis cDNA from 1µg of RNA.

TABLE 1 RNA PRE-MIX

Component	Volume
Oligo dT Primer (50µM)	1µL
dNTP mixture (10mM each)	1µL
Template RNA	1µg
RNAase free water	x 12µL
5X PrimeScript Buffer	4 µL
RNAase inhibitor (40U/µL)	0.5 µL
PrimeScript RTase (200U/µL)	1 µL

Out of this oligo dT primer, dNTP mixture, template RNA and RNAase free dH_2O is added to PCR tube and incubate the solution at 65^0C and then cool immediately on ice. Add rest of the component, mix gently and heat it upto 42^0C for 1hr and 95^0C for 5 minutes in a PCR and cool on ice.

Real-time PCR

cDNA made from 1µg is used as a template for real-time PCR to quantify the mRNA levels of the respective genes. Real-time PCR forward and reverse primers were designed for the gene studied. Lyophilised primers were dissolved in 1X TE buffer to

make a concentration of 100µM, which was further diluted into 1µM in milliQ water. SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) 2X buffer (Takara) was used for setting up the real-time PCR as shown in table 2. All reactions were set in triplicates in the Applied Biosystem® Quant Studio in 384 -well reaction plate. B-actin and Gapdh (Glyceraldehyde 3-phosphate dehydrogenase) are used as house-keeping genes.

TABLE 2 COMPONENTS REAL-TIME PCR REACTION

Components	Volume in µL
SYBR Green(2X)	15
Forward Primer(1µM)	3
Reverse Primer(1µM)	3
cDNA	1
NFW	8
Total	30

Figure 12 Real Time PCR Temperature Conditions

Table 3 Real Time PCR Primer Sequences

Gene	Primer Sequence
BDNF	Forward: CGGCGCCCATGAAAGAAGTAA Reverse: GGTCCTCATCCAGCAGCTCTT
GDNF	Forward: TCTCTCTTCGAGGAGCGCTG Reverse: TGACCAGTGACTCCAATAT
NT4	Forward: GGCTGCTCTACCGTGCCTTAT Reverse: CCAGGGTCCTCTCTGATGTT
Phf2	Forward: ATCACCTGGATGCCTGCTTGCTTTC Reverse: TACGGAGCATCGTCTGAGCCTT
Phf8	Forward: TTGGGAAAGGAAAAGGCGAACCTG Reverse: ACTCCCATTCCCAAGCTTGCTTTC

Riken	Forward: AGGTGGGTGGACCTGGCTGT Reverse: TGCCTTGGTCTTGTCCCCCACT
Jmjd 2c	Forward: TGGAGAGTCCCCTAAATCCCA Reverse: CCTTGGCAAGACCTGCTCG
Jmjd 2d	Forward: AGCCCAAGACCTGCTCG Reverse: CTGGGTGATGCGACCAAAG
PRMT5	Forward: CAGTGCTTCATGGCTTCGCAG Reverse: GGAATTGCTGCATCGCCAGAA
G9a	Forward: TGCCTATGTGGTCAGCTCTCAG Reverse: GGTTCTTGCAGCTTCTCCAG
Glp	Forward: ATTGACGCTCGGTTCTATGG Reverse: ACACTTGGAAGACCCACACCC
Suv39H1	Forward: CTGTGCCGACTAGCCAAGC Reverse: ATACCCACGCCCACTTAACCAG
Mll3(Kmt2c)	Forward: GGCTGAAGTCGTGACCTTTGA Reverse: CGGAATCTTGTGCTGGTCATCT
Sox2	Forward: CAACGGCAGCTACAGCATGAT Reverse: TGCGAGTAGGACATGCTGTAGGT
Nestin	Forward: TTGAGTGGGGCTGCAGCTAATGTT

	Reverse: GGGGCATCTAAATGGTCAATCGCT
GFAP	Forward: AGTGGCCACCAGTAACATGCAA Reverse: GCGATAGTCGTTAGCTTCGTGCTT
Foxb2	Forward: CCTCTCGGAAAGCCGGATAC Reverse: AGGAGGACCAAGGCAAAAGG
Foxp3	Forward: GGTTTACTCGCATGTTCGCC Reverse: CTTCCCCACATGTCTAGCCC
Foxh1	Forward: CCACCTTATGGAAGCACCGA Reverse: CTCCAATCCTCACCTCACGG

Protein Isolation

3x Laemelli buffer was made using 1.88mL 1M tris pH 6.8, 600mg SDS, 3mL glycerol and 8.5mL autoclaved milliQ. 1x solution is made adding 1.7mL stock solution, 300µL β-mercaptoethanol, a pinch of bromophenol blue, 240µL of protease inhibitor cocktail, 60µL PMSF and 3.7mL autoclaved milliQ.

For half hippocampus 200µL of 1X milliQ added, 3 rounds of alternative bath sonication and boiling had been done. Centrifuged at 4^0C at 14000rpm and collected the supernatant to fresh tube.

Protein Estimation

Standard solution of 1mg/mL BSA solution made and aliquoted 5µL, 20µL, 40µL and 80 µL into fresh tubes. 100µL 1X lamelli buffer was added to each tube. 10µL samples were taken from each tube and made it upto 100µL with milliQ and added 100µL lamelli

buffer into each tube. 800 µL amido black solution added to all tubes and vortexed well, followed by centrifugation at 14000rpm for 10minutes at room temperature. The supernatant had been discarded with 1mL pipette. Added 800µL wash buffer (90% methanol and 10% ethanol) and vortexed well. Centrifuged at 14000rpm for 10minutes at room temperature. Repeated it until all the amido black solution washes away. 1mL .2N NaOH added and vortexed well. Using spectrophotometer, the OD is measured at 615nm with 0.2N NaOH as blank. Stand curve is plotted and the concentrations were extrapolated from the graph.

Western blotting

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the method devised by Ornstein and Davis and developed by Laemmli (Laemmli, 1970; Ornstein, 1964). Resolving gel (0.375 M Tris-HCl pH-8.8, 10 to 12% acrylamide, 0.1% SDS, APS and TEMED) and stacking gel (0.125 M Tris-HCl pH- 6.8, 5% acrylamide, 0.1% SDS, APS and TEMED) of 1.0 or 1.5 mm thickness were polymerized in the glass plates of Bio-Rad Gel Cast Apparatus. Running buffer was filled in the tank and samples were loaded into the wells of the gel and were run at a constant current of 20 mA per gel.

Protein transfer and Blotting

Resolved proteins from the SDS-PAGE gel were transferred onto a PVDF membrane using wet mode of transfer at a constant voltage, 30 V for 3.5 h at room temperature. Subsequently, the membrane was incubated in 5% BSA in TBST for 2 h at room temperature for blocking. The membranes were then incubated with the appropriate dilution of respective primary antibody in 2.5% BSA in TBST at 4°C overnight. This was followed by three washes with TBST of 10 min each and incubation with an appropriate dilution of respective secondary antibody in 2.5% BSA in TBST for 1 h at room temperature. After three washes (10 min each) with TBST, membranes were developed by a Bio-Rad ChemiDoc system with enhanced chemiluminescence (ECL) Western Blotting HRP substrate (Abcam). Membranes were exposed to varying time periods to

obtain signals with appropriate intensities. Band intensities were quantified using ImageJ software. β actin was used as a loading control in these experiments.

Chromatin Immunoprecipitation

Reagents required:

1X PBS (500ml)

NaCl – 4g
KCl – 100mg
Na_2HPO_4 – 720mg
KH_2PO_4 – 120mg
Add mQ water and make final volume to 500ml after adjusting pH to 7.4. ---- *Autoclave*

1% Formaldehyde (40ml)

Formaldehyde 39% w/v – 1ml
Sterile 1x PBS – 39ml

2M Glycine (5ml)

Glycine (MW: 75.07) – 0.75g
mQ water – 5ml --- *filter sterilise*

3M Na Acetate pH 5.2 (100ml)

CH_3COONa (MW: 82.03) – 24.61 g
$CH_3COONa.3H_2O$ (MW: 136.03) – 40.81 g
Add mQ water and make final volume to 100ml after adjusting pH to 5.2 ----- *Autoclave*

1M Tris HCl pH 6.5 (100ml)

Tris buffer (MW: 121.14) – 12.11g
Add mQ water and make final volume to 100ml after adjusting pH to 6.5 ----- *Autoclave*

1M Tris HCl pH 8.1 (100ml)

Tris buffer (MW: 121.14) – 12.11g

Add mQ water and make final volume to 100ml after adjusting pH to 8.1 ----- *Autoclave*

0.5M EDTA pH 8.0 (100ml)

EDTA (MW : 372.24) – 18.612g

Add mQ water and make final volume to 100ml after adjusting pH to 8.0 ----- *Autoclave*

5M NaCl

NaCl (MW : 58.45) – 29.22g

Add mQ water to make final volume to 100 ml ----- *Autoclave*

10% SDS

Dissolve 10g SDS in autoclaved mQ water and make final volume to 100 ml

1X PBS with PI (Protease Inhibitor)

500ul of 100X PI in 50ml 1X PBS

ChIP Lysis Buffer (5ml) solutions	**Final Concentration**	**Volume taken from stock**
0.5M EDTA	100mM	100ul
1M Tris HCl pH 8.1	50mM	250ul
10% SDS	1%	500ul
Protease Inhibitor (100X)	1%	50ul
mQ Water		4100ul

------- *Sterilize with 0.22 µ filter*

ChIP Dilution Buffer (5ml)

0.5M EDTA	1.2mM	12ul
1M Tris HCl pH 8.1	16.7mM	83ul
10% SDS	0.01%	5ul

Protease Inhibitor (100X)	1%	50ul
5M NaCl	167mM	167ul
10% Triton X-100	1.1%	550ul
mQ Water		4133ul

------- *Sterilize with 0.22 µ filter*

Low Salt Buffer (20ml)

0.5M EDTA	2mM	80ul
1M Tris HCl pH 8.1	20mM	400ul
10% SDS	0.1%	200ul
5M NaCl	150mM	600ul
10% Triton X-100	1%	2000ul
mQ Water		16720ul

------- *Sterilize with 0.22 µ filter*

High Salt Buffer (20ml)

0.5M EDTA	2mM	80ul
1M Tris HCl pH 8.1	20mM	400ul
10% SDS	0.1%	200ul
5M NaCl	500mM	2000ul
10% Triton X-100	1%	2000ul
mQ Water		15320ul

------- *Sterilize with 0.22 µ filter*

LiCl Buffer (20ml)

0.5M EDTA	1mM	40ul
1M Tris HCl pH 8.1	10mM	200ul

Na deoxycholate	1%	0.2g
NP 40	1%	200ul
LiCl (MW: 42.41)	0.25M	0.212g
mQ Water		

------- *Sterilize with 0.22 µ filter*

TE Buffer (20ml)

0.5M EDTA	1mM	40ul
1M Tris HCl pH 8.1	10mM	200ul
mQ Water		19760ul

------- *Sterilize with 0.22 µ filter*

Elution Buffer (10ml) Fresh

$NaHCO_3$ (MW : 84.01)	0.1M	84mg
SDS (10%)	1%	1ml
mQ (RNase/ DNase Free Water)		9ml

------- *Sterilize with 0.22 µ filter*

Tissue Collection, Cross-linking and Storage:

- Animal is sacrificed and brain is dissected out, washed with 1X PBS and placed into the brain matrix.
- Region of interest are taken as punches from the 1mm brain slices in PBS + PI.
- Transfer the punches in 1.5ml tubes and add 940ul of 1% formaldehyde, put the tube on rotator at slow speed for 10' @ RT – ***Cross linking of proteins with chromatin***
- Add 62.5ul 2M glycine in the tube and rotate for 5' @ RT – ***to stop cross linking reaction***

- Discard formaldehyde + glycine from the tissue samples and wash them 3-4 times with 1ml of cold 1X PBS + PI – ***to remove the residual formaldehyde***
- Remove PBS completely from the tube and snap freeze in liquid N2
- Keep the samples @ -80^0 C

Take samples out from -80^{o}C and add 200µl ChIP lysis buffer and keep for sonication with Bioruptor.

- **Target – to get the DNA fragments less than 500bp (Avg 300bp)**

Keep the samples for sonication for 16 cycles each of 16 minutes having 30s ON and 30s OFF cycle ----- total 96minutes

A. Reverse Cross linking:

Take 40 µl of lysate (from 200 µl homogenized tissue lysate) in a new tube and add 360 µl of ChIP dilution buffer. Further, add 41 µl of the following master mix in this mixture.

Cocktail:

5M NaCl ---------- 80 µl
1M Tris pH 6.5 ----------80 µl
0.5M EDTA ----------40 µl
Proteinase K (20mg/ml) ------- 5 µl
Total ----------205 µl

Keep the samples @ 65^{o}C for 4-5 hrs

B. DNA isolation:

- Use Phase lock gel (PLG) tubes for DNA isolation
- Spin PLG tubes @ 14000 rpm for 30s @ RT
- Add 450 µl Phenol Chloroform Isoamyl Alcohol (PCI) and 440 µl chromatin lysate
- Mix and shake
- Spin PLG tubes @ 14000 rpm for 8’ @ RT

- Transfer upper 400 µl layer to a fresh tube and add 40 µl of 3M Na Acetate (pH5.2), mix well
- Add 1ml of ice cold absolute alcohol and mix well in a 2ml tube
- Keep @ -80^{o}C for O/N

Morning -

- Spin @ 14000 rpm @ 4^{o}C for 30'
- Discard supernatant and add 1ml 70% Alcohol
- Spin @ 14000 rpm @ 4^{o}C for 15'-20'
- Discard supernatant and dry the pellet @ 37^{o}C dry bath
- Add 20 µl DNA elution buffer (M/N), mix and vortex to dissolve the DNA pellet
- Quantify the eluted DNA using nanodrop and load on 1% Agarose gel

(If size of the fragmented DNA is less than 500bp in length, we can proceed for antibody binding)

A. Sonicated/ Fragmented chromatin is quantified using nanodrop, lysis buffer is used as blank.
B. Based on nanodrop readings, 50 µg of chromatin is taken from every sample for each antibody.
C. For every sample, final volume is made to 1100 µl using ChIP dilution buffer in a 1.5ml tube.
D. Mix well and take 100 µl out as **input** and keep at 4^{o}C and rest 1000 µl is used for further antibody binding.

➢ **<u>Target - pre-clearing of the samples to reduce the nonspecific background</u>**

a. Add 30 µl of protein A agarose beads --- based on the antibody used for pull down (in the animal which it is raised) in 1ml volume of chromatin and incubate on a rotor for 30' @ 4^{o}C at high speed
b. (Magnetic beads can also be used in place of protein A agarose beads)

c. Separate the beads either by centrifugation @ 4000 rpm for 1 min (protein A agarose beads) or by using magnetic separator stand (magnetic beads) and transfer the 1ml of supernatant to a fresh tube.

Immunoprecipitation

a) Add 10µL of the Foxb2 antibody for 50 µg chromatin (@ 1 µg antibody for 10 µg chromatin) and incubate @ 4^{o}C on a rotor at slow speed for 12-13 hrs.
b) For control, incubate 50 µg chromatin either with antibodies against IgG or without any IgG.

   (Follow all steps for Antibody of interest, IgG and no antibody control as well)
c) Add 30 µl of beads (either Protein A agarose or magnetic ones) to bind with the chromatin bound antibodies and incubate for 2 hrs @ 4^{o}C on a rotor at slow speed.
d) Separate the beads and discard the supernatant.

Washing the Beads – Antibody – Protein – DNA complex

All steps @ RT

A. Add 1ml ChIP dilution buffer to each tube.
B. Rotate for 5 min in the rotor.
C. Spin @ 4000rpm for 1 min or use magnetic separation stand (for magnetic beads) to separate beads from the solution.
D. Aspirate and discard the liquid from the tubes without disturbing the beads.

Repeat the above steps (A-D) with **cold**

1. 1ml Low salt Buffer – one wash
2. 1ml High Salt Buffer – one wash
3. 1ml Lithium Chloride Buffer – one wash
4. 1ml TE Buffer – two washes

Elution of Antibody – Protein – DNA Complex from the beads

A. Remove the TE buffer completely and carefully from the tubes without disturbing the beads.
B. Add 200 µl of freshly prepared elution buffer to the beads and mix well (or vortex for few seconds)
C. Rotate for 25-30 minutes @ medium speed
D. Separate the beads by either spin @ 9500 rpm for a minute or using magnetic separator stand and transfer 190 µl to freshly labeled tubes.
E. Again add 200 µl elution buffer, vortex, rotate, spin and transfer to the same tubes to a total volume of 390 µl.

Reverse Cross linking:

- ✓ Add 41 µl of master mix (having Tris HCl pH6.5, NaCl and EDTA) to 390 µl elution product, mix well and keep @ 65^{o}C for overnight. (Keep the input samples also for reverse cross linking)
- ✓ Add 1 µl of proteinase K to the mix in the morning and further keep for 1-2 hrs @ 65^{o}C to digest some remaining proteins

DNA purification by PCI method:

a. Use Phase lock gel tubes for DNA isolation
b. Spin PLG tubes @ 14000 rpm for 30s @ RT
c. Add 450 µl Phenol Chloroform Isoamyl Alcohol and 440 µl chromatin lysate
d. Mix and shake
e. Spin PLG tubes @ 14000 rpm for 8' @ RT
f. Transfer upper 400 µl layer to a fresh tube and add 40 µl 3M Na Acetate (pH5.2)
g. Add 1ml ice cold absolute alcohol and mix well in a 2ml tube
h. Keep @ -80^{o}C for O/N

Morning -

a) Spin @ 14000 rpm @ 4^{o}C for 30'
b) Discard the supernatant

c) Add 1ml 70% Alcohol and mix well

d) Spin @ 14000 rpm @ 4^{o}C for 15'-20'

e) Discard supernatant and dry the pellet @ 37^{o}C dry bath

f) Add 20 µl DNA elution buffer (M/N), mix and vortex to dissolve the DNA pellet

NOTE::: This dissolved DNA can be diluted further and can be used as a template for real-time PCR to quantify the changes in the binding of the protein of interest to its targets (promoter of several genes)or for ChIP-Sequencing to identify all the targets (new or old) of the protein of interest. Input serves as a positive control while IgG or no antibody serves as negative

Results

3.1 Behaviour analysis

Pregnant mouse was subjected to stress and pups were assessed for anxiety-like, depression-like and cognitive impairment phenotypes at adult stage (7-8 months) and compared with control animals who were born from non-stressed dams. Open field test and elevated plus maze test were employed to assess anxiety-like phenotype. Forced swim test, tail suspension test and social interaction test were done to analyse depression-like phenotype. Novel object recognition test was done to assess cognitive impairment.

3.1.1 Anxiety Like Phenotype

In male prenatally stressed group, anxiety was seen at significant level in both elevated plus maze test and open field test in male, whereas in females there was a trend only (Figure 11,12,13,14). Data were analysed by Students' T-test. Bar graphs represent average and error bars represent standard mean error (SEM); * represents significant difference between groups.

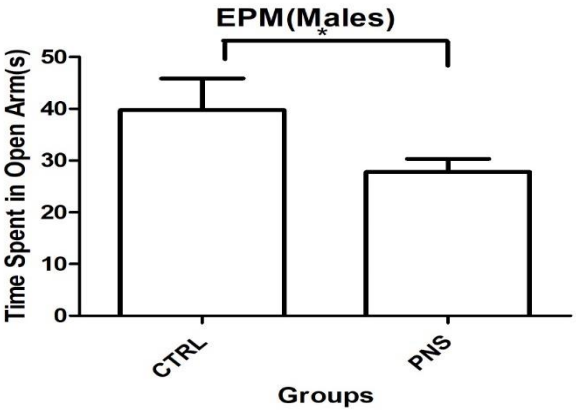


FIGURE 14.1 ELEVATED PLUS MAZE MALE (P VALUE=0.0451)

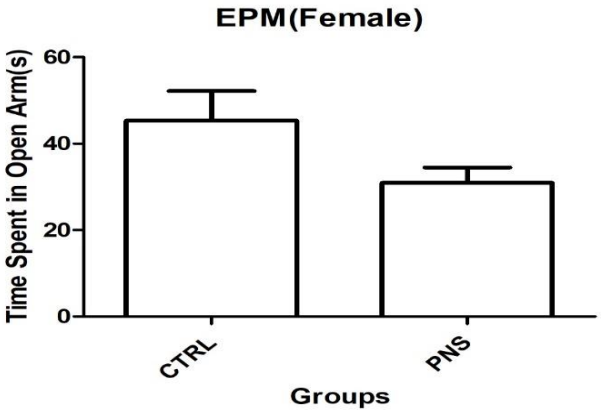
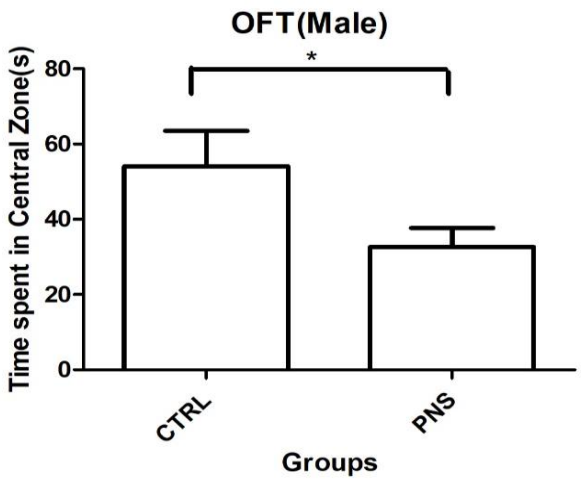


FIGURE 14.2 ELEVATED PLUS MAZE FEMALE (P VALUE=.154)



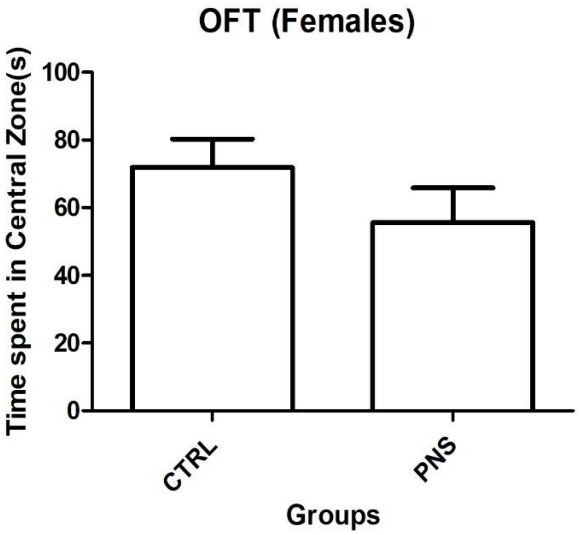
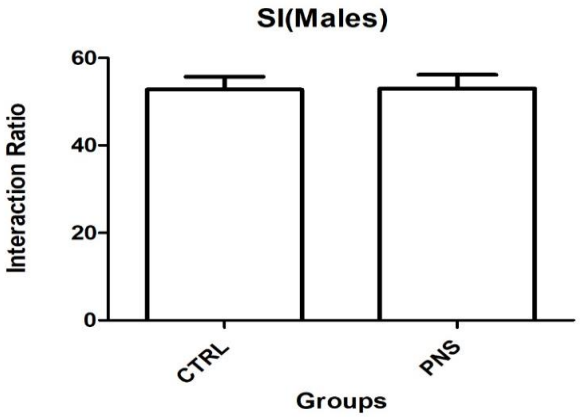
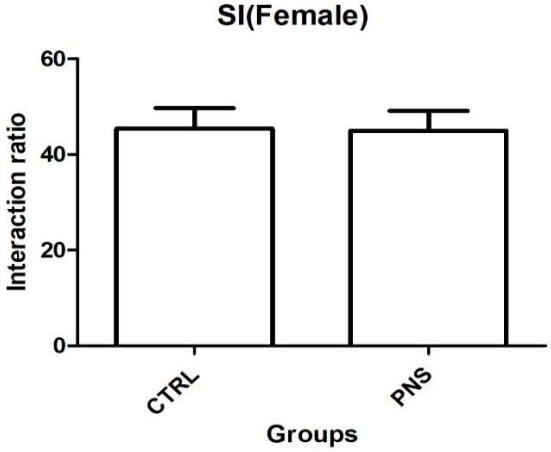


Figure 15.1 Open field test male (p value=0.036) Figure 15.2 Open field test female (p value=.238)

3.1.2 Depression Like Phenotype





Depression-like phenotype was not observed in males or females from PNS group in forced swim test or social interaction test (Figure 13, 14), but in tail suspension test male PNS group was found to show significant depressive phenotype (Figure 15). The male animals in PNS group were less mobile during the tail suspension, indicating

despair like condition, hallmark of depression. Locomotory disability was not found in any test or control animal.

FIGURE 16.1 SOCIAL INTERACTION TEST MALE (P VALUE=0.9579) FIGURE 16.2 SOCIAL INTERACTION FEMALE (P VALUE=0.9431)

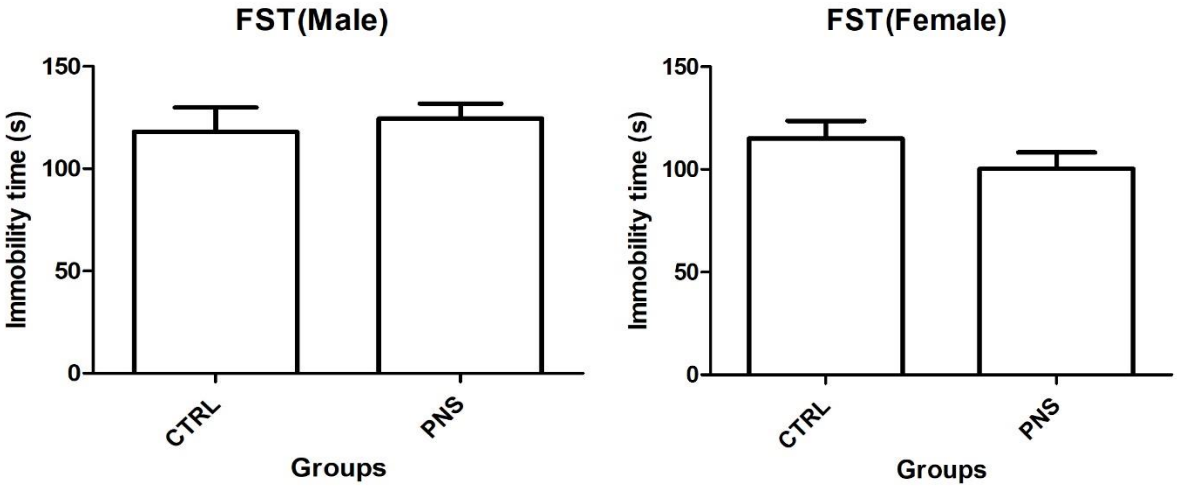


Figure 17.1 Forced swim test male (p value=0.6330) Figure 17.2 Forced swim test female (p value=0.2493)

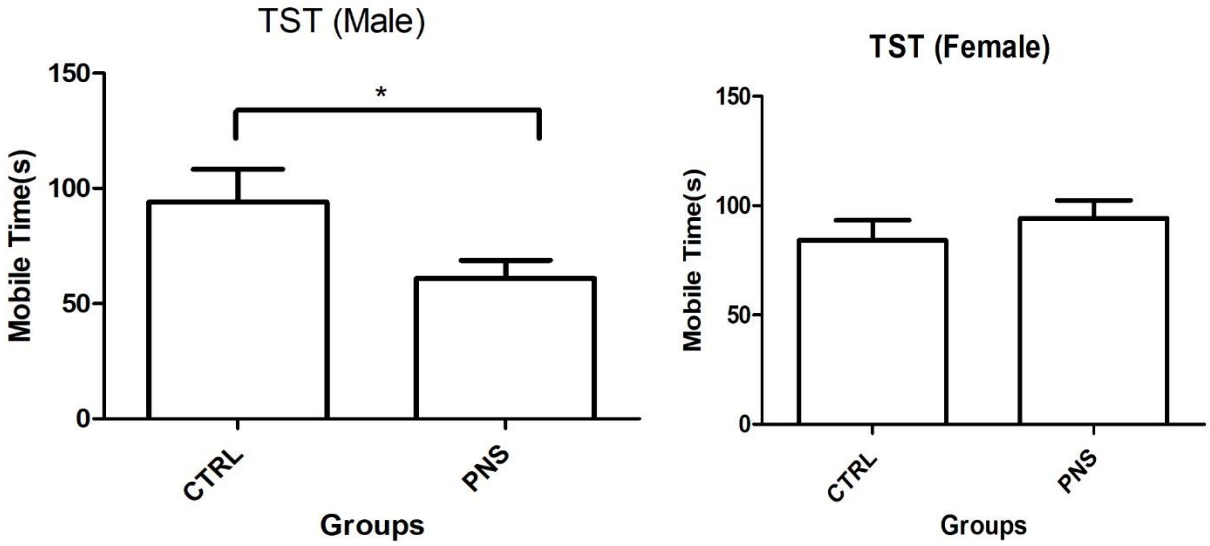
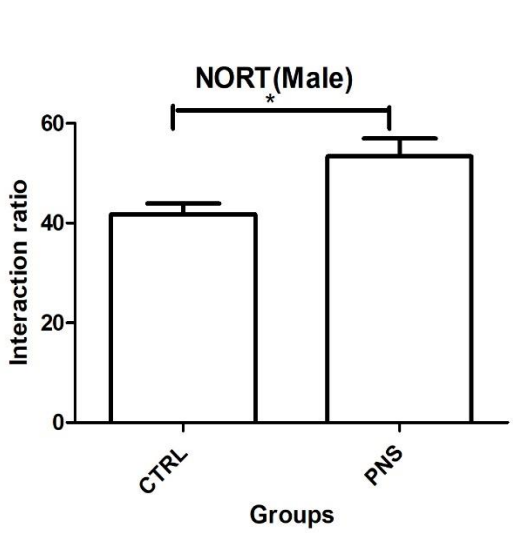


Figure 18.1Tail suspension test male (p value=0.0346) Figure 18.2 Tail suspension test female (p value=0.4636)

3.1.3 Learning and/or Memory test



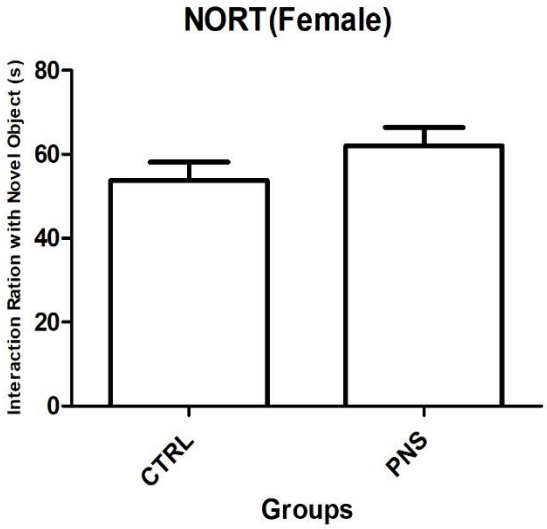
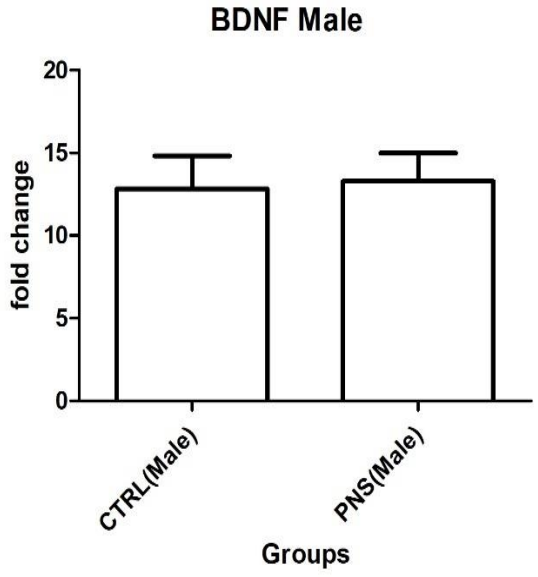


Figure 19.1 NORT male (p value=0.0302) Figure 19.2 NORT female (p value=0.2493)

In novel object recognition test (NORT) a significant improvement in reference memory was found in the male PNS group, whereas no such change was observed in female PNS group

3.2 Assessing neurotrophic markers known to be affected in anxiety and depression

Unlike reported attenuation in the levels of some neurotrophic factors in earlier studies involving depression and anxiety like phenotype, our data failed to show the change in BDNF levels in both male and female hippocampus in our PNS model. In males there is slight trend in upregulation of GDNF and NT4 levels in prenatally stressed group.



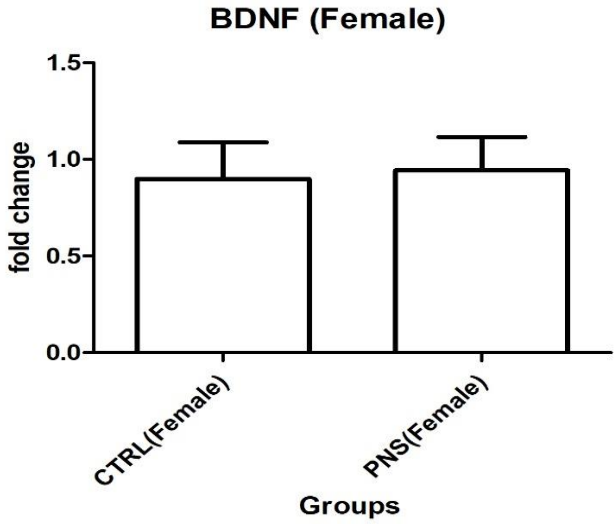
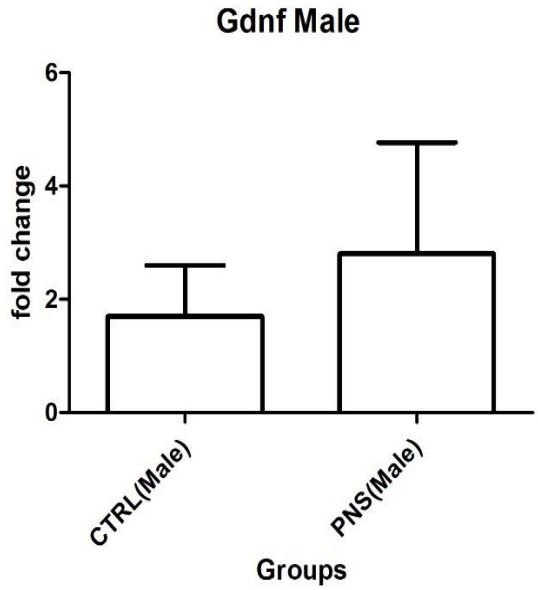


FIGURE 20.1 BDNF MALE (P VALUE= 0.8659) **FIGURE 20.2 BDNF FEMALE (P VALUE=0.8645)**



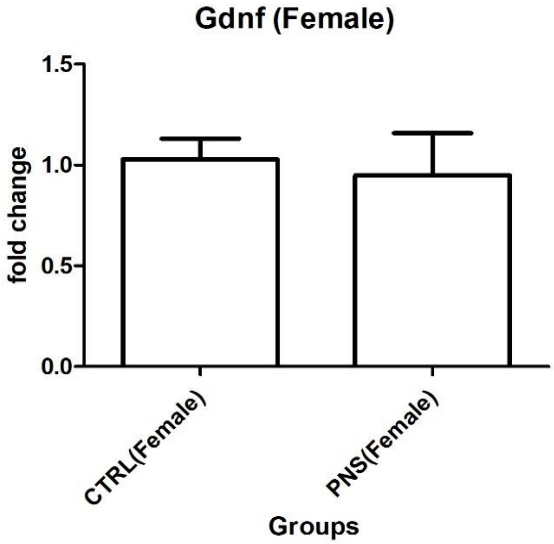
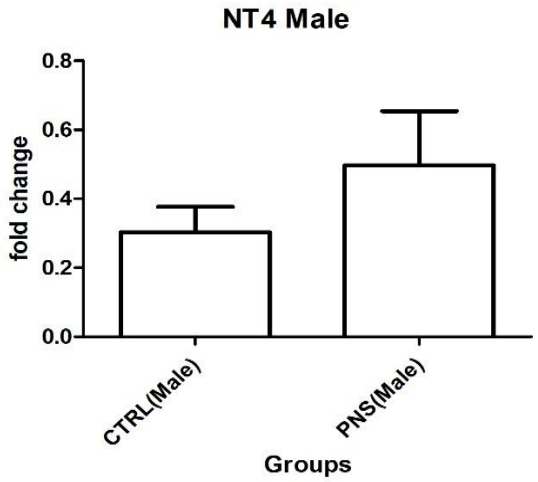


Figure 21.1 GDNF male (p value = 0.6028) Figure 21.2 GDNF Female (p value= 0.7503)



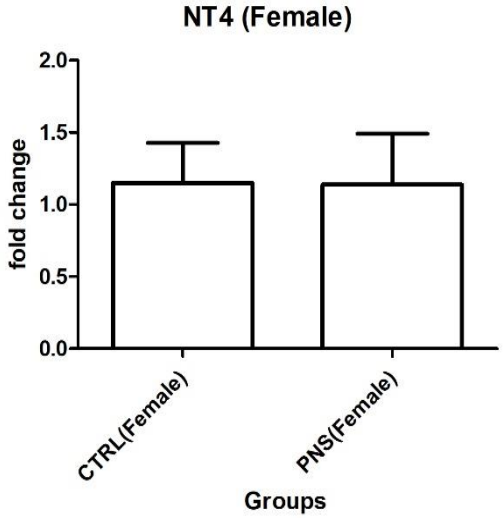
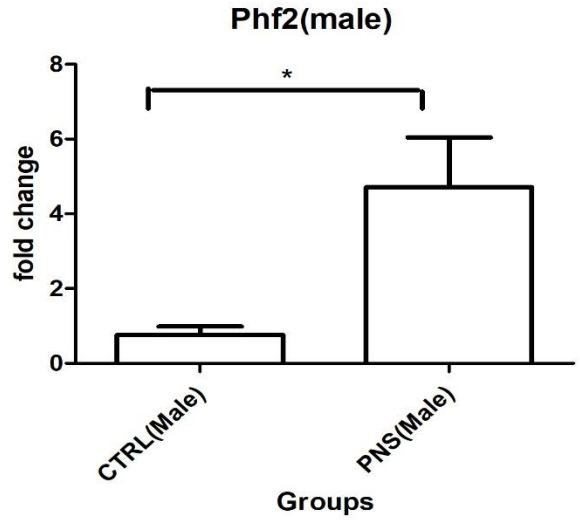


FIGURE 22.1 NT4 MALE (P VALUE= 0.0886) **FIGURE 22.2 NT4 FEMALE (P VALUE= 0.9820)**

3.3 Assessing the role of some epigenetic regulators that control histone methylation and demethylation

3.3.1 Histone Demethylases

Phf2, Phf8 and Rik, the lysine specific histone demethylases that contain a plant homoedomain (hence the name Phf2) and jumonji C-domain containing histone demethylase domain. These epigenetic erasers (named because they are involved in removing or erasing methyltion from lysine residues) belong to KDM7 family and demethylate both histone and non-histone proteins. Phf2 demethylates H3K9me2. Phf8 demethylates H3K9me1, H3K9me2, H3k27me2 and H4K20me1. When mapped using RT-qPCR, we found dysregulation in few of these demethylases in hipocampus.



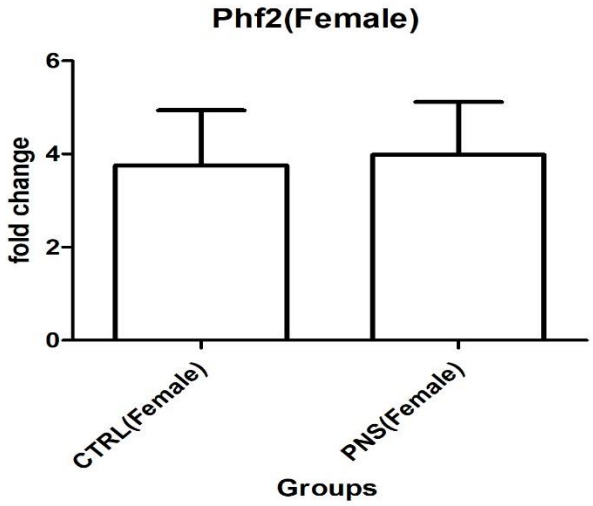
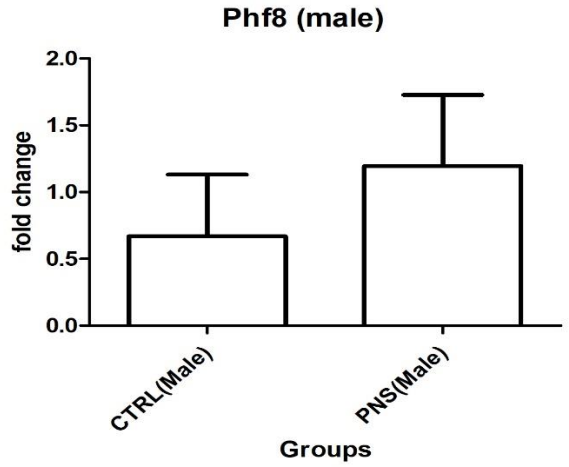


FIGURE 23.1 PHF2 MALE (P VALUE= 0.0205) **FIGURE 23.2 PHF2 FEMALE (P VALUE= 0.8935)**



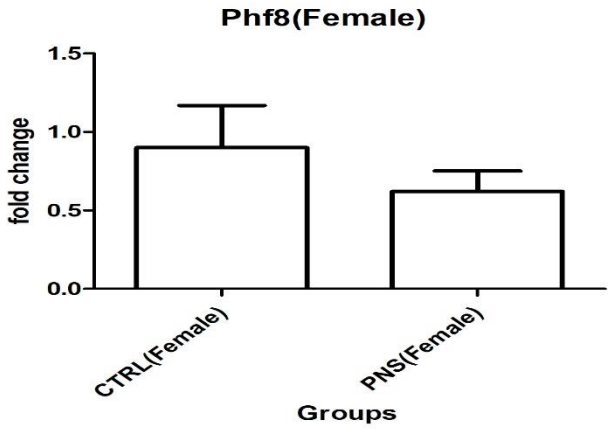
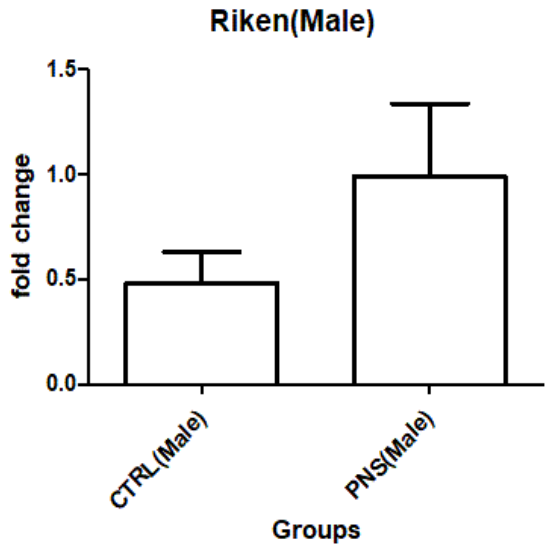


FIGURE 24.1 PHF8 MALE(P VALUE= 0.4975) **FIGURE 24.2 PHF8 FEMALE(P VALUE= 0.3286)**



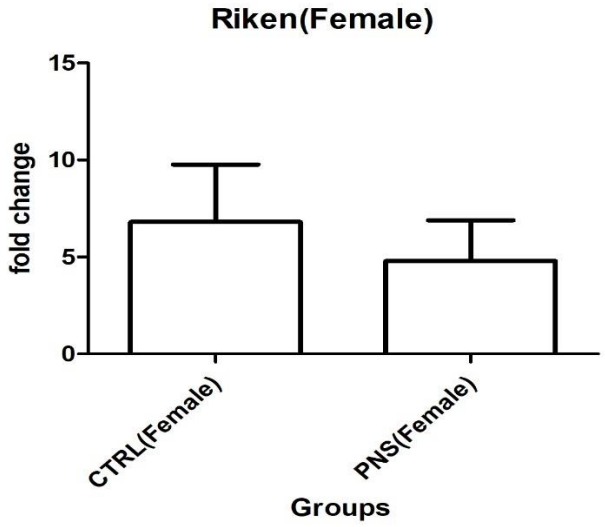
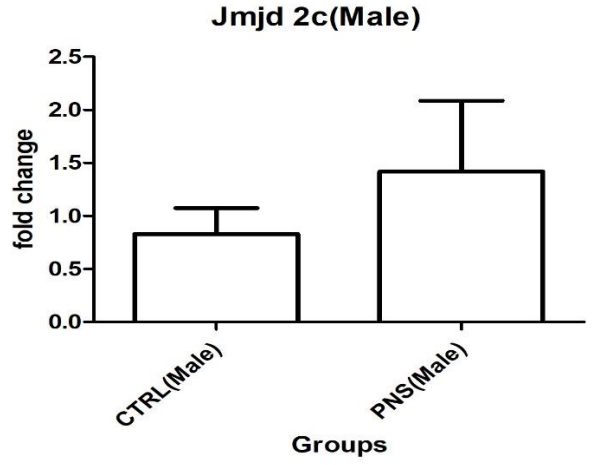


FIGURE 25.1 RIKEN MALE (P VALUE=0.2690) **FIGURE 25.2 RIKEN FEMALE (P VALUE=0.5834)**

In our study, we also checked two members of KDM4 family of demethylases. Jmjd2c and Jmjd2d, both of which contain a Jumonji domain Jmjd2c demethylates H3K9me3 and H3K36me3. Jmjd2d demethylates H3K9me2 and H3K9me3.



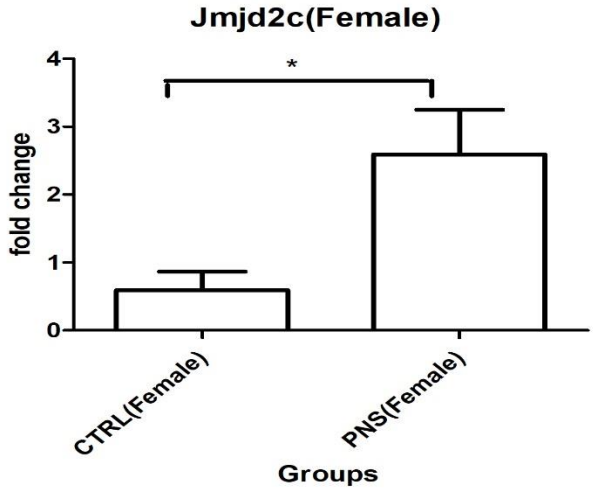
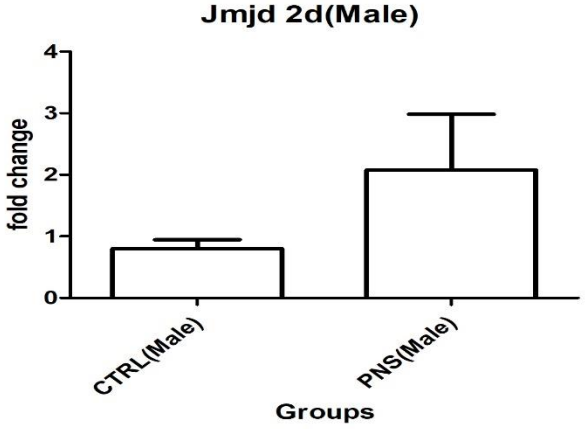


FIGURE 26.1JMJD2C MALE (P VALUE = 0.3980) FIGURE 26.2 JMJD2C FEMALE (P VALUE =0.0375)



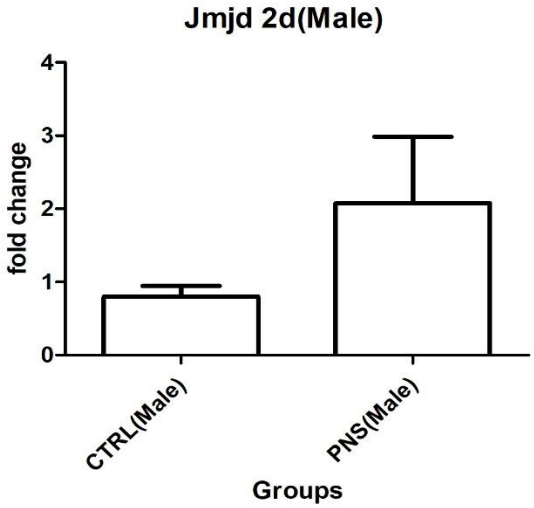
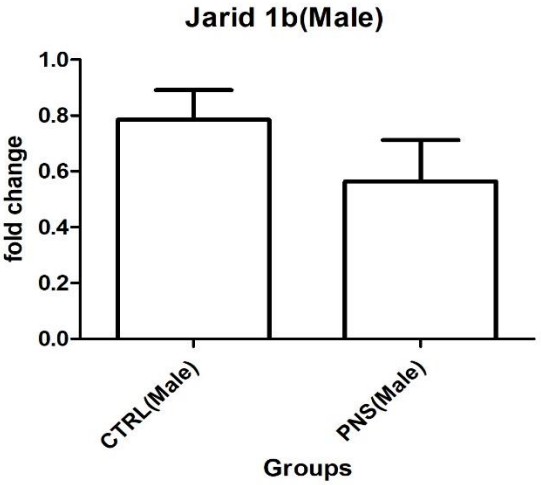


FIGURE 24.1JMJD 2D MALE (P VALUE= 0.1299) FIGURE 24.2 JMJD 2D FEMALE (P VALUE= 0.2638)

Jarid1b and Jarid1c from lysine demethylase family 5 (KDM5) which contain a jumonji /ARID domain and demethylate H3K4me1, H3K4me2 and H3K4me3, act as corepressor along with FOXG1B and PAX9. The qPCR analysis shows no change.



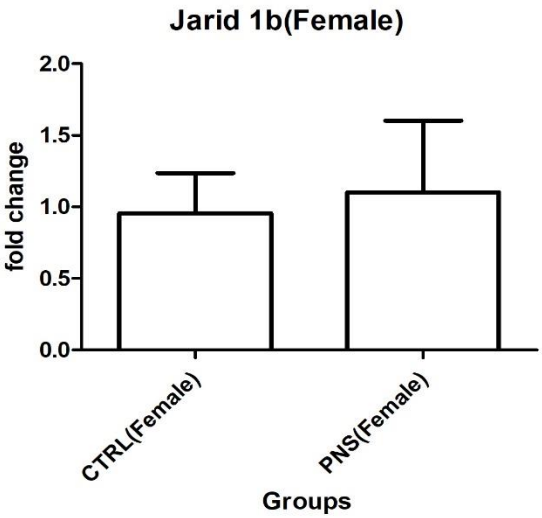
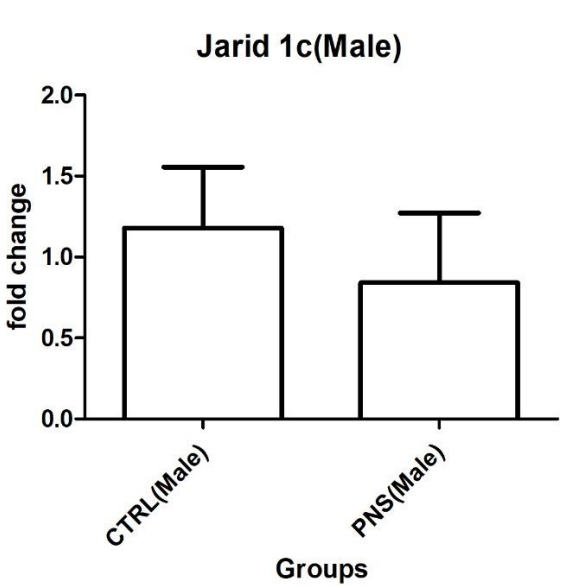


FIGURE 28.1 JARID 1B MALE (P VALUE=0.2407) FIGURE 28.2 JARID 1B FEMALE (P VALUE= 0.8251)



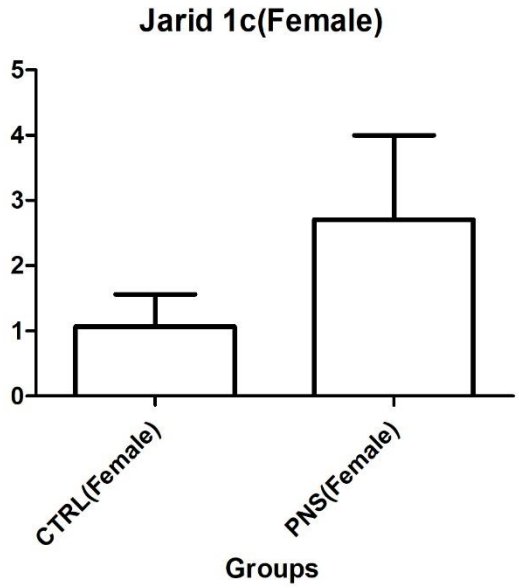
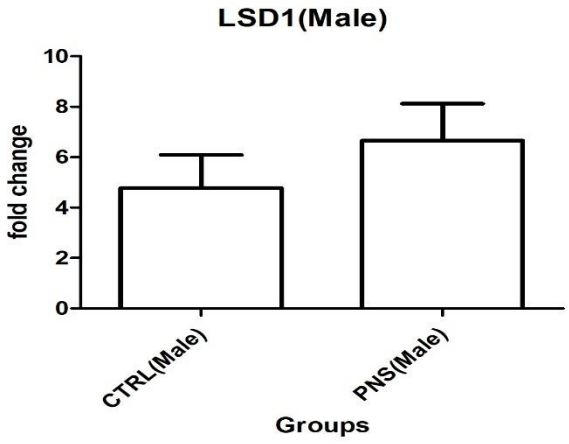


Figure 29.1 Jarid 1c male (p value= 0.5647) Figure 29.2 Jarid 1c female (p value=0.3300)

Lysine specific demethylase 1 (LSD-1), the first histone demethylase discovered, demethylates H3K4me1, H3K4me2 and H3K9me2 in redox process. However, in this study LSD1 was not dysregulated in hippocampus.



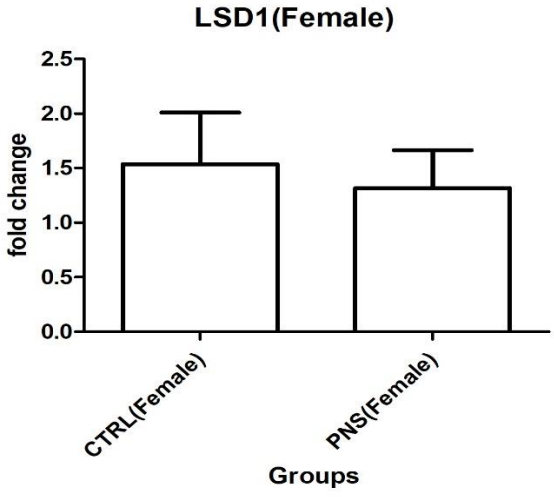
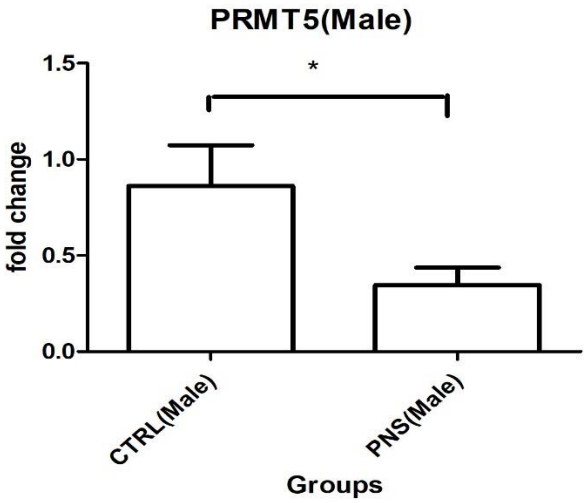


FIGURE 30.1 LSD1 MALE (P VALUE= 0.2900) **FIGURE 30.2 LSD1 FEMALE (P VALUE=0.7121)**

In males all three members of KDM7 family have shown increasing trend where Phf2 upregulation is significant. In females Phf8 and Riken are showing downregulated trend whereas Phf2 is unaltered. In KDM4 family both Jmjd2d and Jmjd2c have upregulated trend in both males and females. In KDM5 family members assessed Jarid 1b is unaltered in females whereas in males its showing slight downregulation trend. In case of Jarid 1c its showing opposite trends in males and females. LSD-1 is also showing slight increase in case of males which is absent in females. The only arginine methyltransferase checked, in males its downregulated whereas in females in shows upregulating trend.

Protein arginine methyl transferase 5(PRMT5) methylates H3R8me.



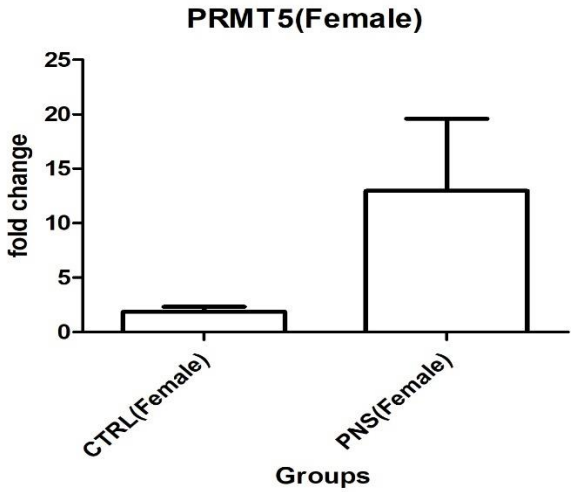
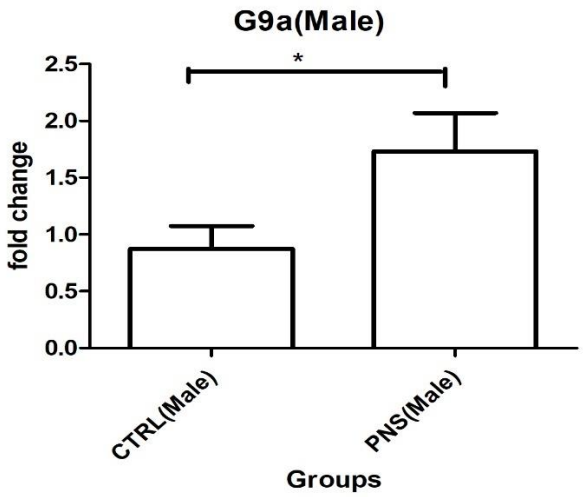


FIGURE 31.1 PRMT5 MALE (P VALUE= 0.0275) **FIGURE 31.2 PRMT5 FEMALE (P VALUE=0.1258)**

3.3.2 Histone Lysine Methyltransferases

G9a and G9a related protein Glp are important for H3K9 methylation. In both males and females G9a has been increased. Whereas in case of Glp the trend is opposite. In males, like G9a it is showing an increasing trend, but in females it is a decreasing trend.



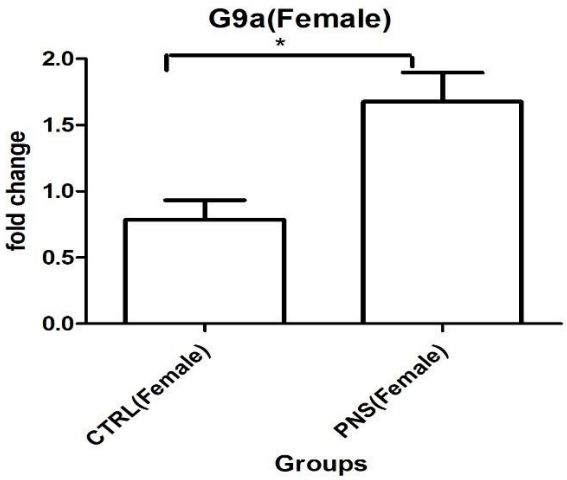
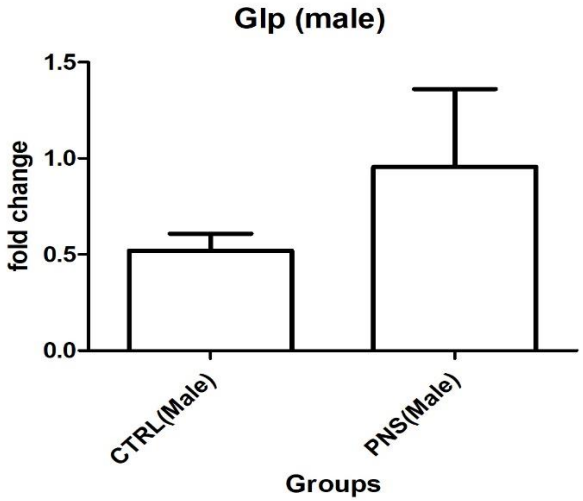


FIGURE 32.1 G9A MALE (P VALUE= 0.0492)

FIGURE 32.2 G9A FEMALE (P VALUE=0.0157



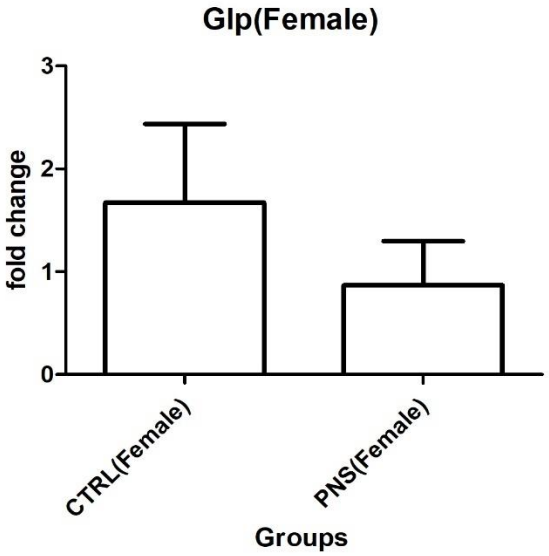
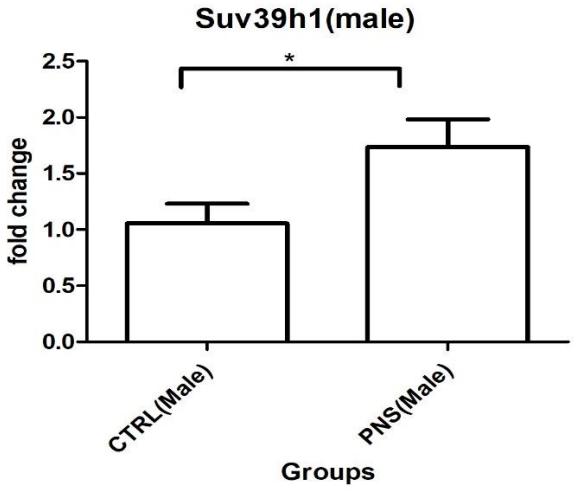


FIGURE 33.1 GLP MALE (P VALUE=0.3639)

FIGURE 33.2 GLP FEMALE (P VALUE=0.3624)

Suppressor of variegation 3-9 homolog 1(Suv39H1) is also a H3K9 methyltransferase. KMT2C, lysine methyl transferase 2c is a H3K4 methyltransferase. Enhancer of Zeste 2 (EZH2), a Polycomb Repressive Complex 2 Subunit, methylates H3K9me and H3k27me.In males Suv39h1 and kmt2c are fond to be significantly upregulated. Kmt2c is upregulated in males and downregulated in females. Ezh2 is showing decreasing trend in both sexes.



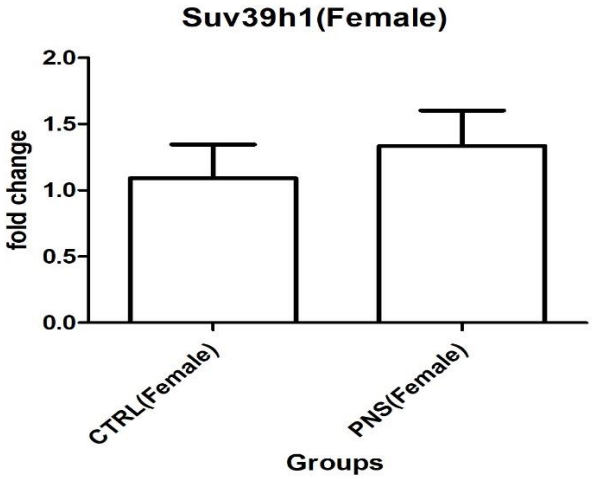
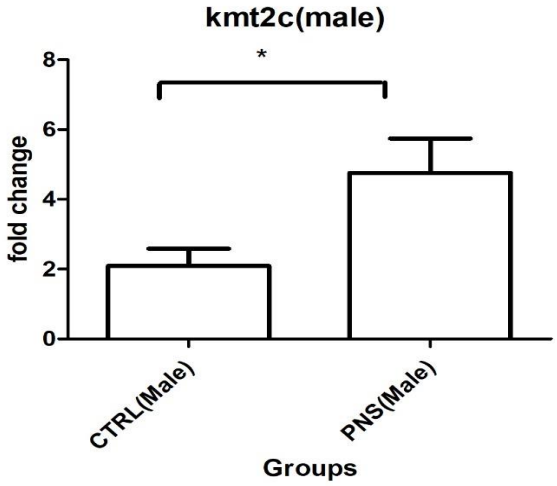


FIGURE 34.1 SUV39H1 MALE (P VALUE= 0.0473) **FIGURE 34.2 SUV39H1 FEMALE(P VALUE= 0.5471)**



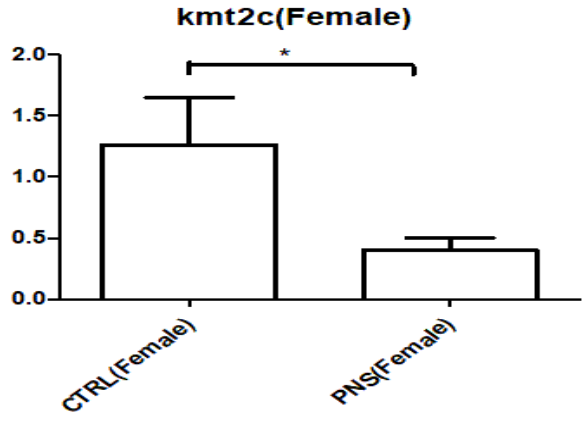
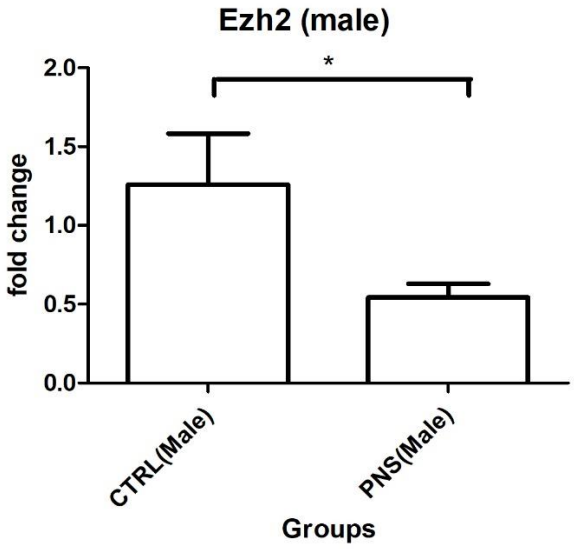


FIGURE 35.1 KMT2C /MLL3 MALE(P VALUE=0.0280) **FIGURE 35.2 KMT2C/MLL3 FEMALE(P VALUE=0.0412)**



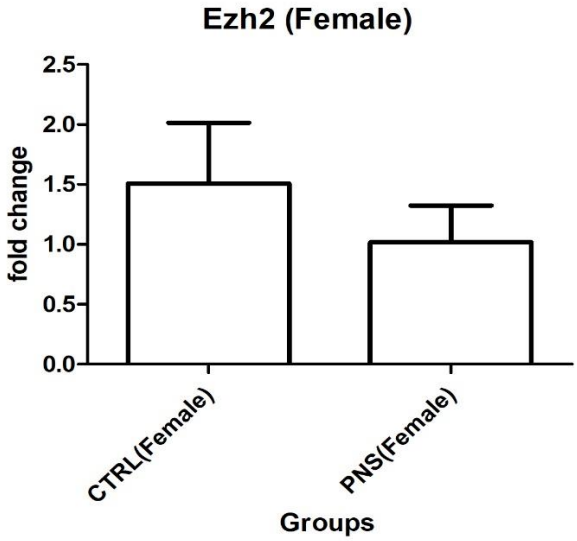


FIGURE 36.1 EZH2 MALE (P VALUE=0.0321)

FIGURE 36.2 EZH2 FEMALE (P VALUE= 0.3990)

The dysregulation in demethylases and methyl transferases in our study is summarized in the table below:

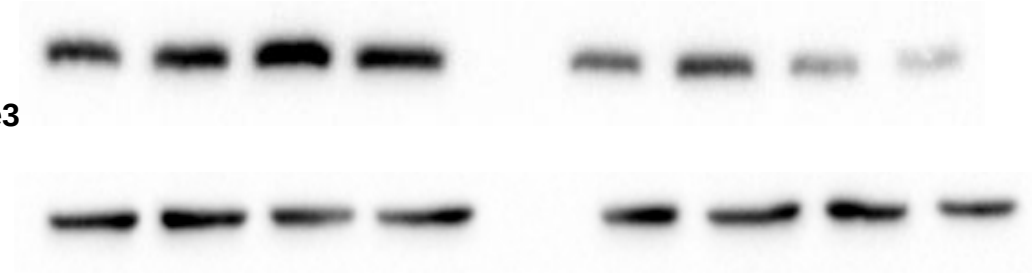
Table 4 Dysregulation of epigenetic regulators controlling histone methylation/ demethylation in mouse hippocampus (* indicates significant)

Gene	Male (PNS w.r.t. CTRL)	Female (PNS w.r.t. CTRL)
Demethylases		
Phf2	↑ (*)	—
Phf8	↑	↓
Riken	↑	↓
Jmjd 2C	↑	↑ (*)
Jmjd 2D	↑	↑

Jarid 1B	↑	—
Jarid 1C	↓	↑
LSD1	↑	↓
Arginine Methyltransferase, PRMT5	↓ (*)	↑
Histone Lysine Methyltransferases		
G9a	↑ (*)	↓ (*)
Glp	↑	↓
Suv39H1	↑ (*)	—
Mll3	↑ (*)	↓ (*)
Ezh2	↓ (*)	↓

3.3.3 Changes in global histone lysine methylation level in hippocampus

Immunoblotting study was performed to ascertain the change in global histone H3 and H4 lysine methylation level in hippocampus and the results are shown in Figure 37,1 and 37.2.



H3K4me3

b-actin

Control Female **PNS Female**

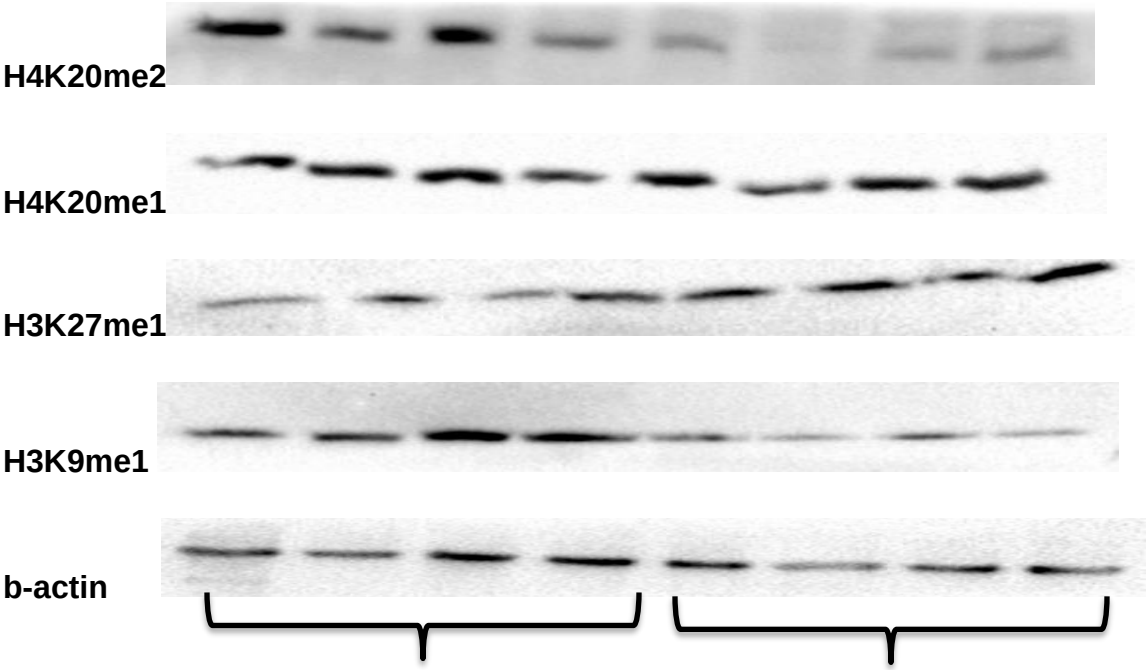


Figure 37.1 Histone H3 and H4 lysine methylation marks in female hippocampus

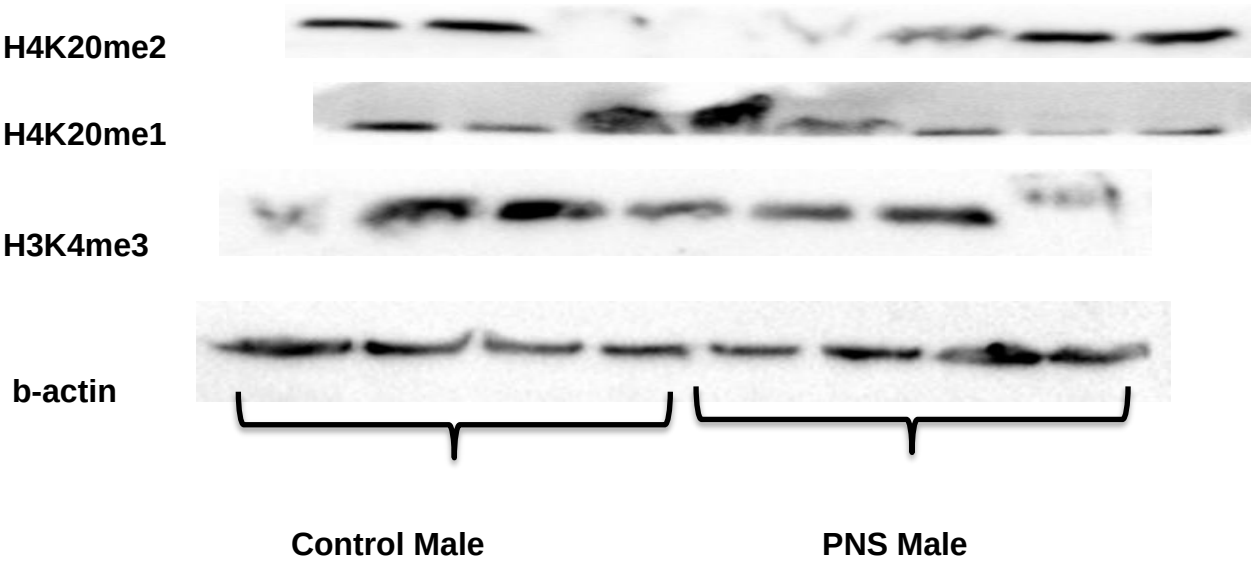
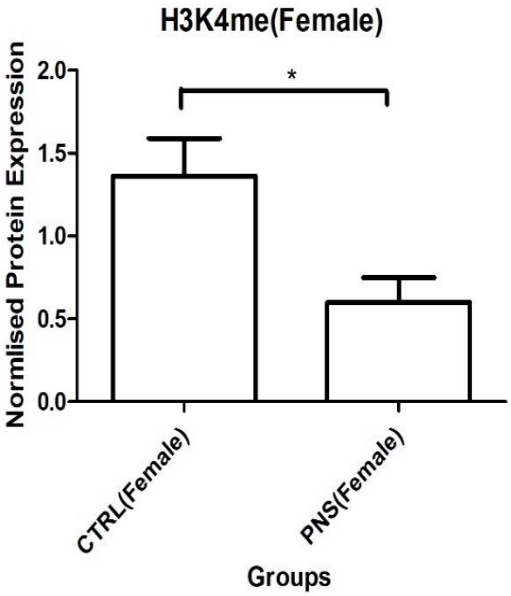


Figure 37.2 Histone H3 and H4 lysine methylation marks in male hippocampus



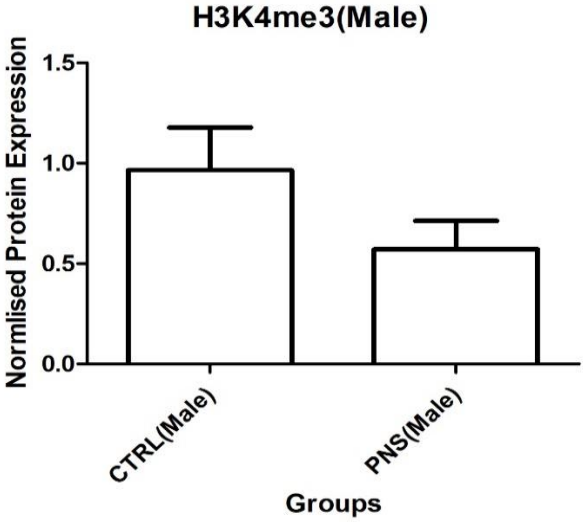
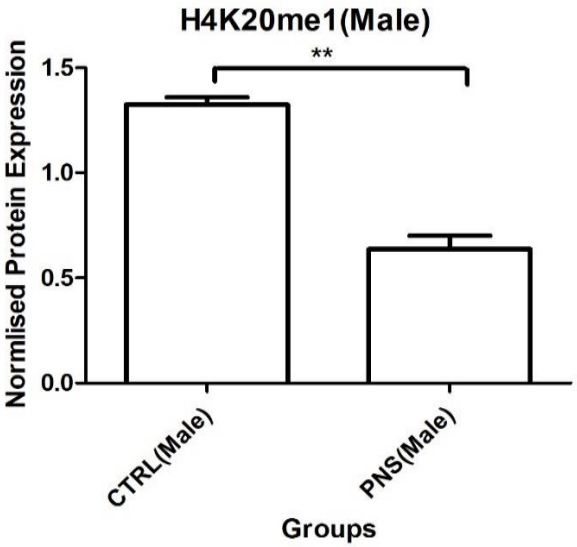


FIGURE 38.1 H3K4ME3 MALE(P VALUE=0.2148) FIGURE 38.2 H3K4ME3(P VALUE=0.0305)



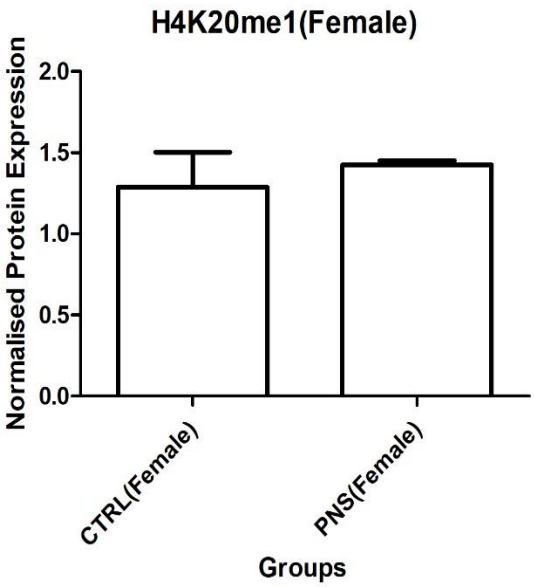
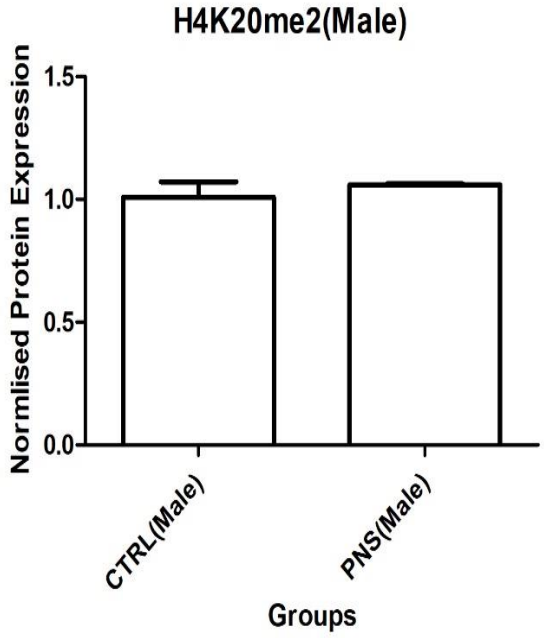


FIGURE 38.3 H4K20ME1 MALE(P VALUE=0.0039) FIGURE 38.4 H4K20ME1 FEMALE (P VALUE= 0.5508)



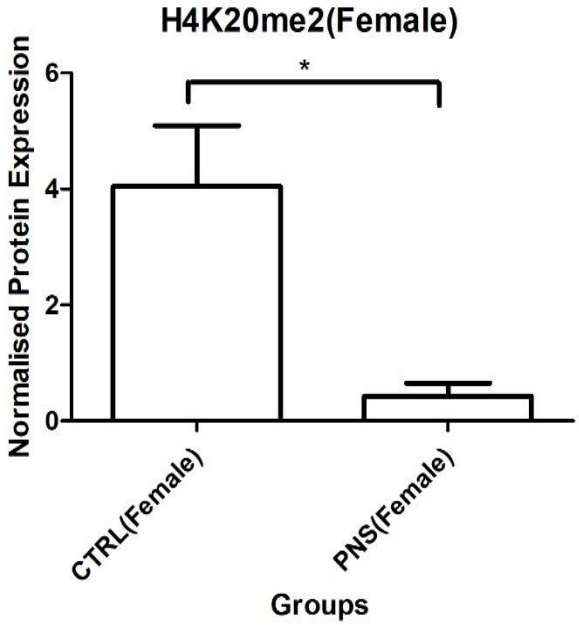
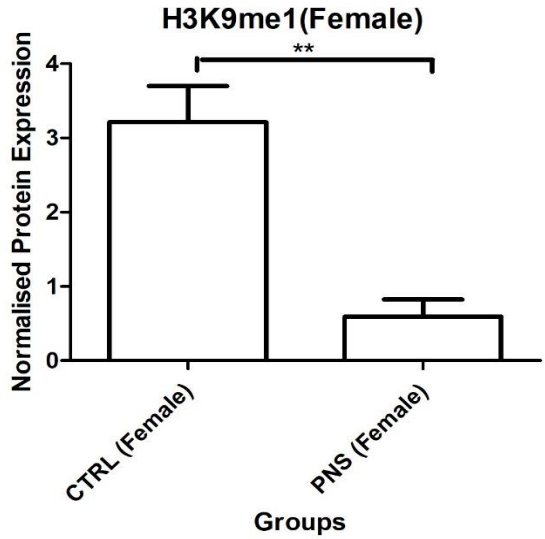


FIGURE 38.5 H4K20ME2 MALE(P VALUE=0.5113) FIGURE 38.6 H4K20ME2 FEMALE(P VALUE =0.0151)



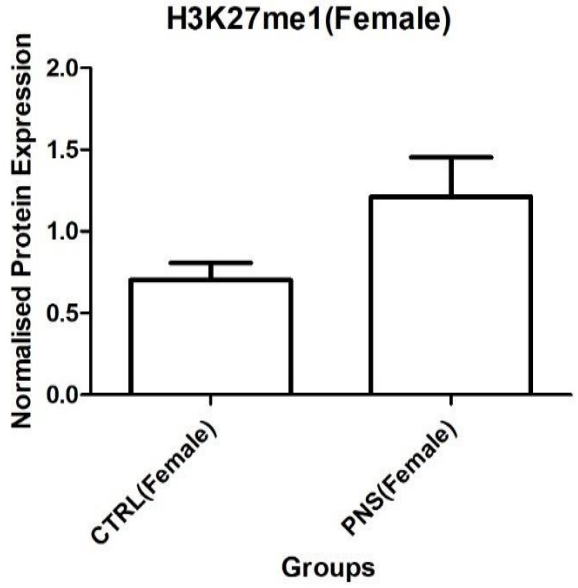


Figure 38.7 H3K9me1(p value=0.0028) Figure 38.8 H3K27me1 Female (p value= 0.102)

The results obtained in immunoblotting experiments has been summarized here in Table 5.

Table 5 Alterations in global levels of histone methylation/ demethylation in mouse hippocampus (* indicates significant)

Methylation Marks	Male (PNS w.r.t. CTRL)	Female (PNS w.r.t. CTRL)
H3K4me3	↓	↓ (*)
H3K9me1	Haven't checked	↓ (**)
H3K27me1	Haven't checked	↑
H4K20me1	↓ (**)	———
H4K20me2	———	↓ (*)

3.4 Assessing changes in Neurogenesis Markers

Previous studies have shown alteration in neurogenesis markers in chronic stress group. So, we mapped some of the markers in our model using qPCR.

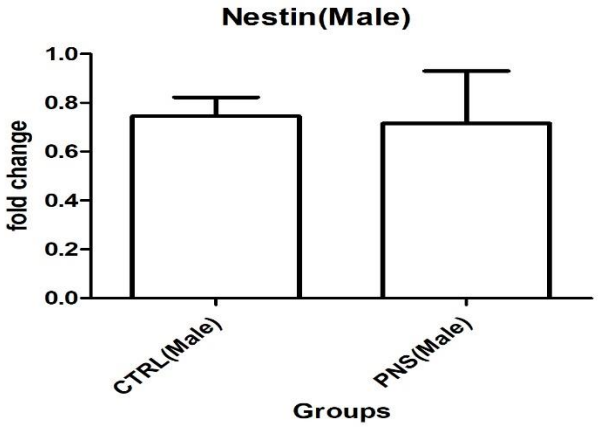


Figure 39.1 Nestin male (P value = 0.8922)

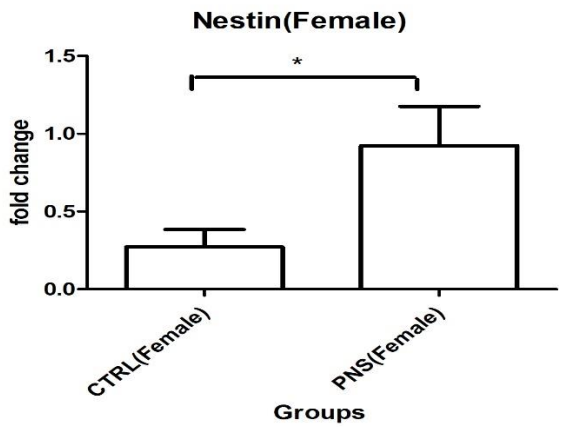


Figure 39.2 Nestin Female (P value=0.0285)

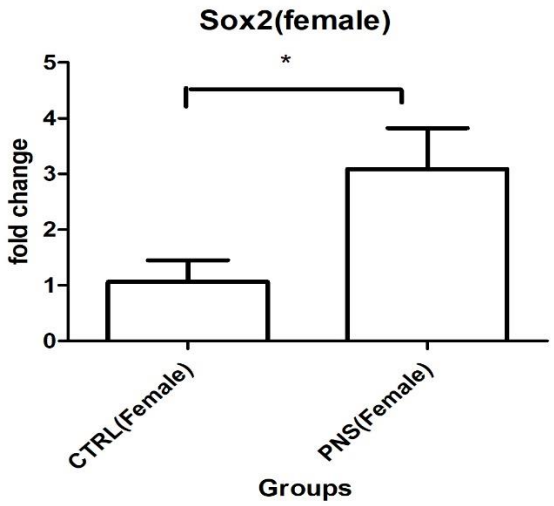
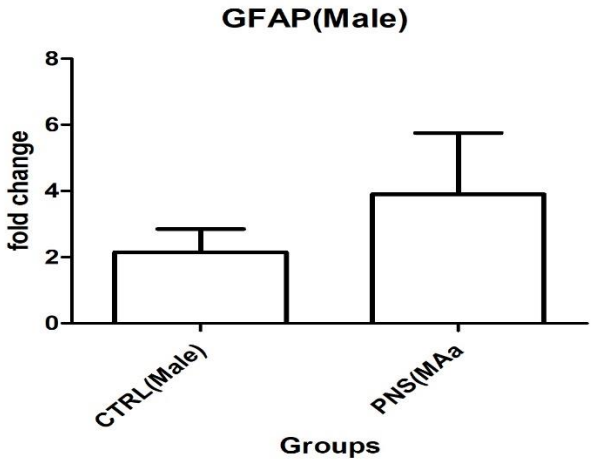


FIGURE 40.1 SOX2 MALE (P VALUE=0.9925)

FIGURE 49.2 SOX2 FEMALE (P VALUE = 0.0351)

Interestingly none of the neurogenesis markers showed difference in male PNS group. In female PNS group they (Nestin and Sox2) were found upregulated. This could be a reason why females appear resilient, not showing anxiety and depression-like behavioural phenotype as shown by male animals.

GFAP was unchanged in PNS groups, irrespective of sex.



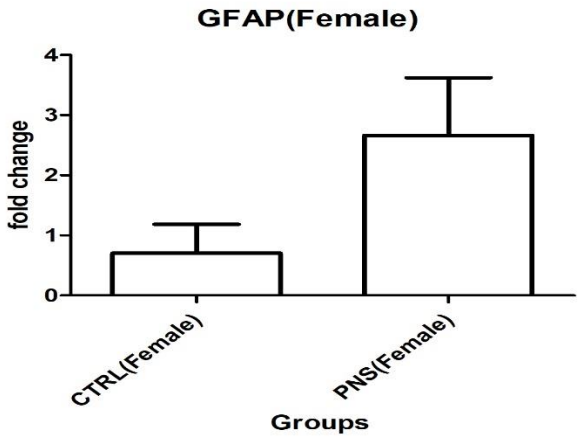


FIGURE 37.1 GFAP MALE (P VALUE=0.3424)

FIGURE 37.2 GFAP FEMALE (P VALUE=0.1361)

3.5 PNS-induced alteration in hippocampal circuitry might be mediated by few Forkhead family transcription factors and worked in gender-based fashion

As mentioned in the literature review section, earlier microarray data from the lab using PNS mouse model indicated sex dependent differential regulation of several forkhead family transcription factors in hippocampus. Foxb2 was indicated to alter in both microarray and iTRAQ proteomics data (unpublished findings). My dissertation project was intended to validate some of these findings and take it further to the next level, i.e. molecular mechanisms that mediate alteration in hippocampal circuitry following PNS by Foxb2. The analysis of qPCR results successfully led me to validate the array data; Foxb2 was significantly downregulated in female PNS group. However, it didn't change significantly in male PNS group, as found in array data. My study could also validate the changes shown in array data for the levels of Foxh1 but it altered only in female PNS group unlike array data. Foxp3, though showing a trend in alteration in male and female in opposite direction, but not to significant level as uncovered in array data in the lab. Thus, some of the Forkhead family transcription factors appear to play role in transcription regulation in sex-dependent manner.

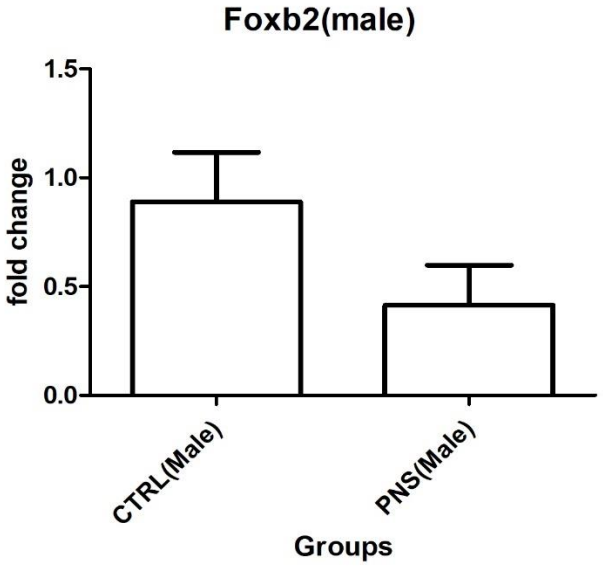


FIGURE 42.1 FOXB2 MALE (P VALUE=0.1486)

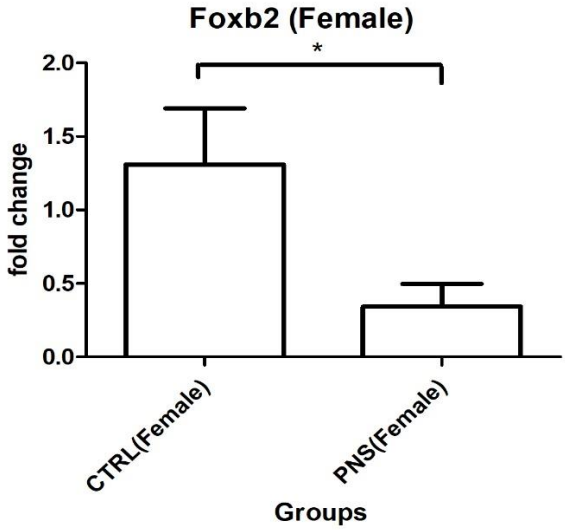
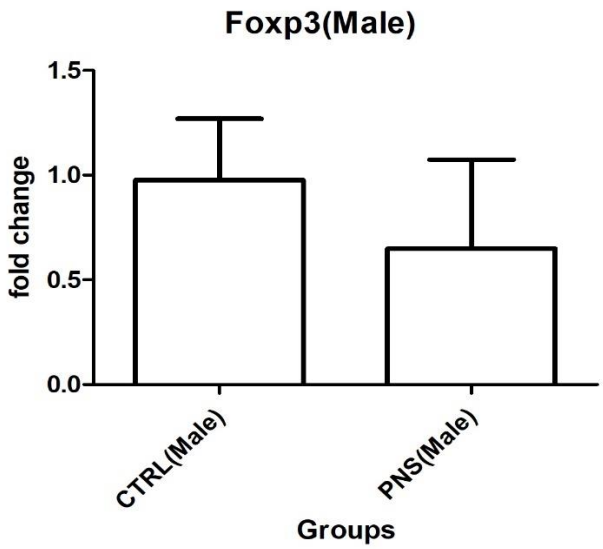


FIGURE 42.2 FOXB2 FEMALE (P VALUE= 0.0272)



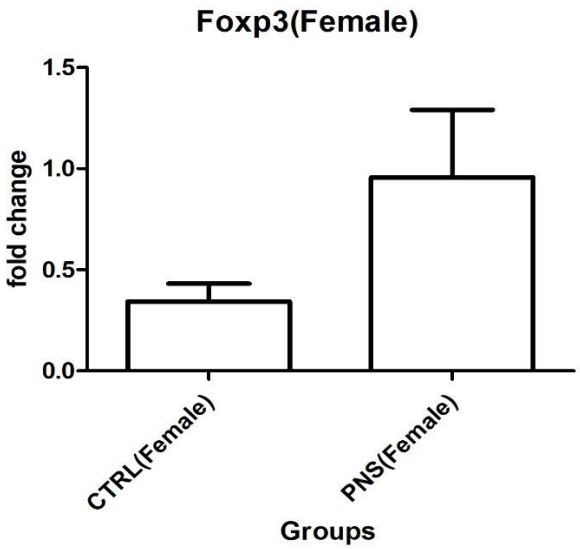
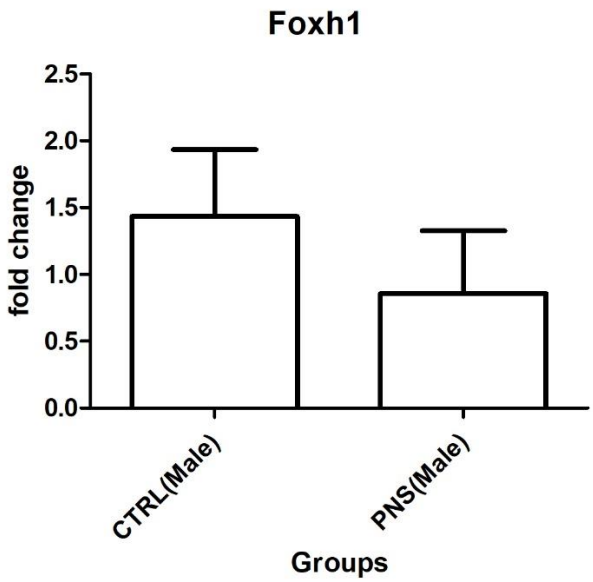


FIGURE 43.1 FOXP3 MALE (P VALUE= 0.5313)

FIGURE 43.2 FOXP3 FEMALE(P VALUE= 0.1843)



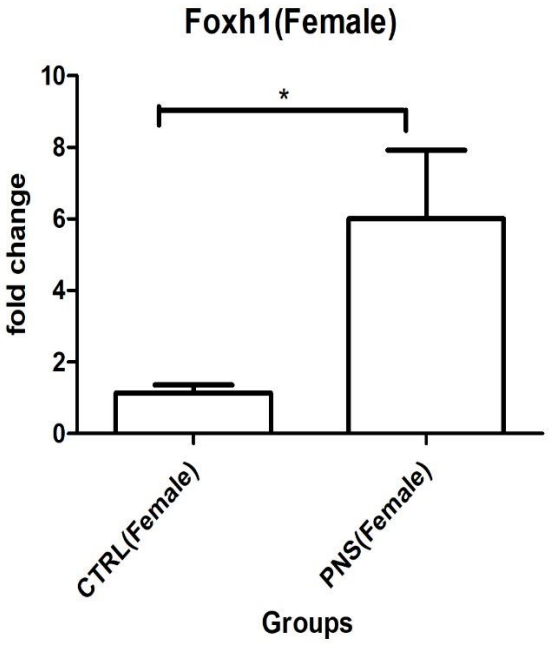


FIGURE 44.1 FOXH1 MALE(P VALUE= 0.4487)

FIGURE 44.2 FOXH1 FEMALE(P VALUE=0.0211)

3.5.1 Role of Foxb2 TF in regulation of genes involved in neurogenesis and neuroplasticity

Chromatin ImmunoPrecipitation (ChIP) was performed utilizing the antibody against Foxb2 and its enrichment or binding at the promoter regions of Sox2, NeuroD1, Nestin and Synaptotagmin genes was analysed by qPCR using the genomic DNA, eluted after the immunoprecipitation, to see the downstream effects of Foxb2 gene. The Foxb2 enrichment was significantly altered on the promoters of Sox2, Nestin, and Synaptotagmin in male PNS group compared to male control group (see Figure 38.1). The contrasting result was observed in Foxb2 enrichment on Sox2 and Nestin promoters in female PNS group compared to female control group (see Figure 38.2). Foxb2 enrichment was up on Sox2 promoter in male PNS animals while it was down in female PNS animals. On the other hand, Foxb2 enrichment was down on Nestin promoter in male PNS animals while it was up in female PNS animals. Thus, our ChIP-qPCR results indicate that Foxb2 has some role in altered neurogenesis related to prenatal stress. Interestingly, Foxb2 binding has got attenuated on Synaptotagmin promoter in male PNS group, but unchanged in female PNS group (Figure 45.1 & 45.2). This indicates that Foxb2 plays a role in altered hippocampal plasticity in male animals borne out of stressed dams and the TF acts on gene regulation in gender-specific way.

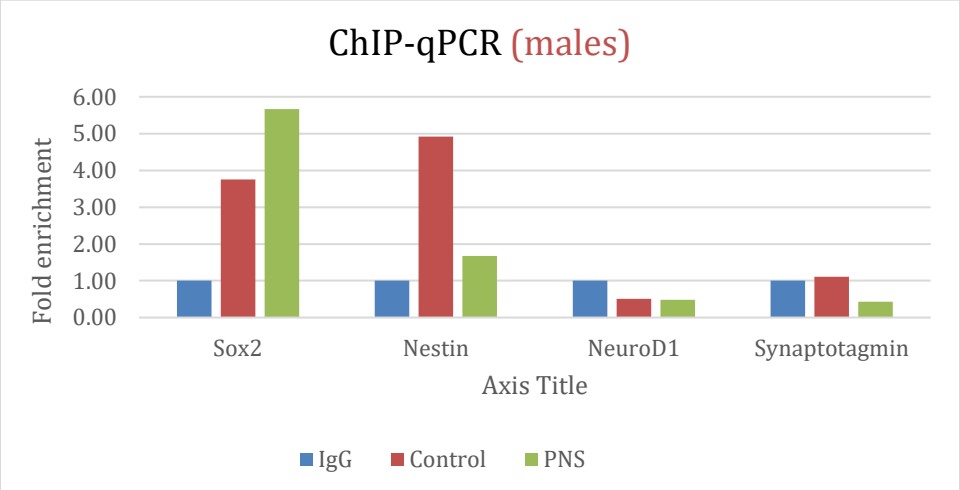


Figure 45.1 Foxb2 binding or enrichment on gene promoters in male hippocampus

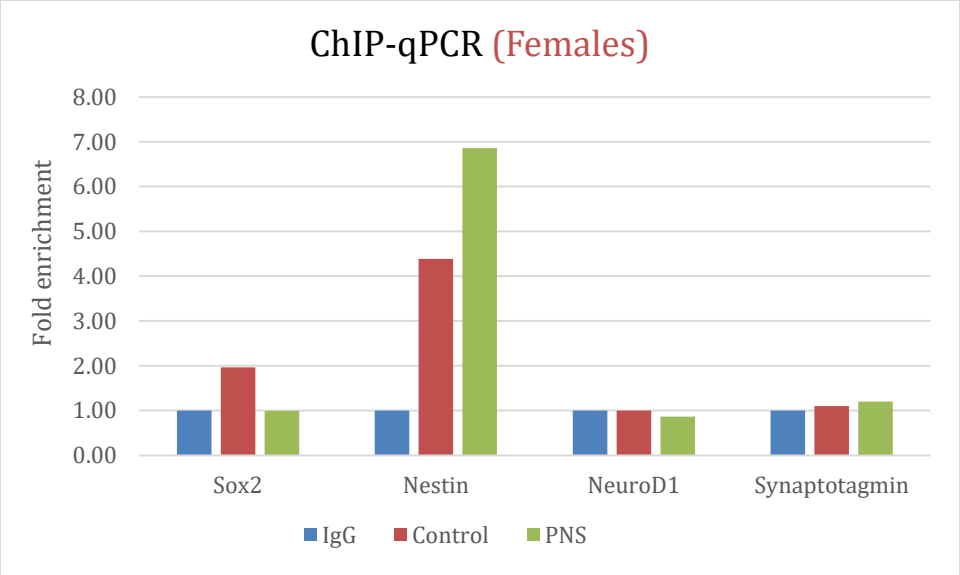


FIGURE 45.2 FOXB2 BINDING OR ENRICHMENT ON GENE PROMOTERS IN FEMALE HIPPOCAMPUS

Discussion

Here in this study we could successfully develop the prenatal or *in utero* stress-induced mouse model for late life neuropsychiatric disorders, where the male offsprings of the stressed mothers (dams) developed anxiety, depression like mood disorder phenotypes, but not even mild cognitive disorder to our surprise. Cognitively, male animals from PNS group were rather better performing than that from Control group. The female offsprings, however, failed to show similar changes in affect/mood, even in cognition. This novel finding of sex difference in the effects of PNS on offspring behavioural phenotype is interesting. Our molecular data showed that the level of neurogenic markers Sox2 and Nestin were significantly elevated in the hippocampus of females, not males, of PNS groups. It is possible that increase in adult neurogenesis in female hippocampus is somehow helping the females to be resilient and not succumbing to the effect of *in utero* stress they were exposed to.

To uncover the molecular mechanisms in sex-bias manner, underlying early life stress induced changes in the affected circuitry, hippocampus, which is involved in controlling both mood and cognition, RT-qPCR was extensively utilized. The level of neurotrophic factors Bdnf and Gdnf was found unaltered, unlike reports in few other prenatal perturbations-induced animal models of adult neurological disorders (Zheng et al., 2016 , T.L. et al., 2009). However, we could see some change in the neurotrophic factors NT4 in male borne of stressed dams, although not to the statistical significance level. Interestingly, it was not at all changed in females borne from stressed dams as shown in figure 20-22.

Recent studies have implicated diverse epigenetic mechanisms in stress-induced nerual and behavioural changes using animal models of neuropsychiatric disorders. Transcriptionally activating as well as repressing histone lysine methylation appear to play role in the underlying aetiology. The analyses of our qPCR data on the genes that code for the epigenetic regulators involved in histone methylation and demethylation led us to some interesting novel findings: we have identified a number of *epigenetic regulators which are dysregulated in hippocampus of PNS group of animals,* such as

the ones involved in histone lysine methylation G9a, Suv39h1, Mll3/KMT2C, Ezh2, and histone lysine demethylation Phf2 and Jmjd2c (figures 23-36). In female hippocampus only one repressive methylase G9a was upregulated, while in male hippocampus in addition to G9a another repressive methylase Suv39h1 was upregulated. This might be part of the molecular mechanism affecting negatively the hippocampal circuitry in male offspring but not in female and inducing depression, anxiety and related mood disorder phenotype in gender specific way. It should be noted that both G9a and Suv39h1 are responsible for repressive H3K9me2 mark, which has been shown to be up in nucelus accumbens, the critical region in reward pathway of mice in social defeat induced depression model (Pathak et al 2017). Interestingly, there is a trend in upregulation of a gene that codes for another H3K9 methyltransferase Glp, in male hippocampus in PNS group. Pathak et al also showed high level of Glp mRNA in accumbens following chronic social defeat stress.

Mll3 (Kmt2c), a methyltransferase for H3K4 mark, which is transcriptionally activating in nature was found dysregulated in PNS groups in opposite direction, in female hippocampus it was attenuated while in male brain it was upregulated. Part of our data was in agreement with a recent study where the level of Mll3 (Kmt2c) was found reduced in reward region following social defeat (Martin et al., 2017). Upregulation in the level of MLL3 in male hippocampus is suggestive of altered neurodevelopment, for Mll3 has been shown to regulate critical genes involved in early neural development. Differential regulation of Mll3 in sex specific manner might also explain differential behavioural outcome (phenotype) in male and female mice from exposed to prenatal or *in utero* stress. The immunoblot results showing attenuation in activating mark H3K4me3 in hippocampus of PNS female (figure 37.1, 38.1), which nicely correlated with downregulated Mll3 transcription. Interestingly, H3K9me1 was significantly reduced in hippocampus of female PNS group, which also correlated well with the upregulation of Phf8 and downregulation of Mll3 in females.

Ezh2, the methyltransferase regulating H3K27 methylation mark, plays important role in biology of stem cells, including neural stem cells, and neural development. Gender specific regulation of Ezh2, down in male and unchanged in female hippocampus, might also be partly responsible for differential outcome in offsprings subjected to *in utero* stress.

Our investigation also led us to uncover significant level of dysregulation in Prmt5, an arginine methyl transferase, in hippocampus of male PNS group only (figure 31.1). PRMT5 has been shown to be involved in neural stem or progenitor cell proliferation, differentiation and neurodevelopment. *This novel finding* is not reported in this type of study earlier and altered level of Prmt5 might have implication for the hippocampal function and plasticity.

As far as demethylase data are concerned, even though the change is not significant, Phf8 and Riken the KDM7 family members are showing trend of upregulation in male PNS group. Interestingly, in chronic social defeat model it has been shown to be upregulated in male depressed animals (Chen et al., 2018). Another member Phf2 was highly upregulated in hippocampus in male PNS group only, unchanged in female from PNS. Phf2, which removes methyl group from K3K9 and acts as transcriptional activator, might have affected hippocampal function and plasticity by derepressing some of the genes supposed to be repressed. In KDM4 family Jmjd2c was found significantly upregulated in females from PNS group, which is in agreement with upregulation shown in male nucleus accumbens after brief 5 days of social defeat stress (Pathak et al., 2017). This demethylase removes methyl moiety from H3K9 and H3K36 and it is possible that it is helping females in resilience phenotype, as Pathak et al has shown its level going down in accumbens in depression-like phenotype following defeat stress.

The transcription factor family Forkhead box has been implicated in neural differentiation and development; in particular some members like Foxp2 and p3 are implicated in neurodevelopmental disorders like Autism Spectrum Disorders (ASDs). Previous lab microarray data had indicated alteration in some forkhead family transcription factors in hippocampus in sex-biased manner. Here we successfully validated Foxb2 and Foxh1 in gender-specific manner. Our novel findings of

dysregulation in Foxb2 and Foxh1 in hippocampus of the offsprings of PNS mothers is the highlight of the study (figure 42-44). There was significant attenuation in the level of expression of Foxb2 in the hippocampus of female offsprings. In male offspring there was trend of attenuation in Foxb2. There was a trend of increase in Foxp3 in female offsprings brain but not at all in male. Interestingly, Foxh1 level was significantly high in female offspring's hippocampus, while there was sex difference once again In its regulation as shown by other Fox family members.

In the end to uncover the clear cut sex difference in the role of this family of TFs in prenatal or gestation stress-induced alteration in neural circuitry of offsprings, ChIP-qPCR was performed and the analysis of data suggested differential targeting of some of the gene promoters involved in neurogenesis and neuroplasticity i.e. Sox2, Nestin and Synaptotagmin, as evident by the altered level of enrichment of Foxb2 on promoter regions (figure 45.1, 45.2). Irrespective of the enrichment at the promoter region of genes that control neurogenesis, these genes were found upregulated in female while unaltered in male hippocampus. So from these novel data it is difficult to elucidate the role of FoxB2 in neurogenesis and neuroplasticity. More study is required to get better insight into the Foxb2 mediated changes in circuitry at the molecular level. Future work utilizing ChIP-Seq following Foxb2 pulldown might give us better insight into molecular mechanisms underlying the neural, behavioral and neuroplastic changes in the affected hippocampal circuitry.

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