Summary of work done

Sex difference in behavioral and molecular neural response to chronic stress in mouse models of depression and related disorders.

Introduction

Depression is one of the most prevalent mental illness worldwide with an estimated 350 million people affected by this affective disorder. It is also estimated that by 2020, depression will be the second leading cause of world disability and is expected to be the largest contributor to global disease burden by 2030. Stressful life events influence the long-term physical and mental health of an individual and have been reported to favor the development of depression and other affective disorders. Review of literature on epidemiological studies suggests a higher prevalence of depressive disorders in females than males, detected at mid-puberty through adult life. In puberty, the female brain is exposed to the cyclically fluctuating gonadal hormone levels, while men seem to be spared from these wide hormone fluctuations. More importantly, a number of reports suggest that women in menopause are not found to be at an increased risk of depression. The marked sex difference in rates of depression beginning with reproductive age implicates the role of ovarian hormones in the vulnerability of females to depressive disorders. Studies in recent past have reported a sex difference in the course of depressive illness where women have an onset of depression at a younger age and also tend to have a longer duration of episodes with a greater risk for chronicity and recurrence. In addition, the response to antidepressant drugs appears to differ depending upon the sex, and also the hormonal status of the female. There are reports showing the effect of chronic stress on the depression-like phenotypes in mouse models and the alteration of these phenotypes with the 17β-Estradiol (**E2**) administration. Moreover, **E2** has been related to the expression of Brain-Derived Neurotrophic Factor (BDNF), a downstream target for many of the antidepressants. Based on all these observations, in the present work, we hypothesized a sex difference in behavioral and molecular neural response to chronic stress in mouse models of depression, and that the ovarian hormones, especially **E2**, might have a role in these differential responses.

Summary of work done

Statement of problem

In recent times, both male and female animals are included in animal models of psychiatric disorders owing to the substantial sex differences in their prevalence, symptomatology, and treatment response. Even though ovarian hormones are considered for a higher prevalence of depressive disorders in females, a thorough understanding of their role at a behavioral and molecular level in chronic stress is required to validate ovarian hormones as a possible reason for heightened stress sensitivity. Ovarian hormone **E2** was shown to affect depression-like behavior, memory, monoamine, neurotrophic factors and neuropeptide levels. However, comparative studies of normal endogenous hormonal milieu with exogenous hormonal replacement in females and studies dissecting the roles of major ovarian hormones **E2** and Progesterone (**P4**) in chronic stress condition are very few. While studying these aspects, we have observed the differential effect of 6-days Chronic Unpredictable Stress (CUS) on mouse hippocampal memory and affective behavior. In accordance with previous studies, CUS could not affect the male behavior. Hence, we continued further studies with 21-day Chronic Variable Mild Stress (CVMS) where we observed sex differences in the effect of CVMS on mouse prefrontal cortical BDNF levels with a role of major ovarian hormones. We have carried out cDNA microarray studies in neuroendocrine hypothalamic region of male, intact female and ovariectomized (OVX) female mice to understand the differential regulation of global transcriptome profile by CVMS in the different hormonal milieu. Further, we have also performed 2D Gel Electrophoresis on the critical region of the reward circuitry i.e. Nucleus Accumbens of male, intact female and OVX female mice to understand the differential regulation of global proteome profile by CVMS.

Objectives of the study

1. To study the role of major ovarian hormones on hippocampal memory and affective behavior in CUS condition.
2. To investigate sex difference and role of major ovarian hormones on prefrontal cortical BDNF levels in CVMS condition.
3. To explore sex difference in CVMS-induced transcriptome changes in neuroendocrine hypothalamic region.
4. To uncover sex difference in CVMS-induced proteome changes in Nucleus Accumbens (NAc) region of the reward circuitry.

Methodologies Used and Sample Results

In this study, initially, we used a 6-day CUS paradigm to induce depression and anxiety-like phenotype in intact female mice (mice with ovaries). CUS could induce depression-like behavior (despair behavior) as assessed in Forced Swim Test (FST), but could not significantly alter the anxiety-like behavior as evaluated by Open Field Test (OFT). Later, intact female mice were subjected to 8-day Morris Water Maze (MWM) test to assess the effect of CUS on spatial learning and memory. Intact female mice had an improved spatial memory after the CUS. There was significant increase in the mRNA levels of NMDAR subunits *GRIN2A* and *GRIN2B* in hippocampus, shown to be involved in cognition. To understand the effect of ovarian hormones OVX female mice were used where CUS could not induce despair behavior in FST but could increase anxiety-like behavior in OFT. Also, CUS could not improve the spatial memory of OVX female mice in MWM test. Even there was no significant increase in the *GRIN2A* and *GRIN2B* mRNA levels. To study the role of major ovarian hormones **E2** and **P4**, the hormones were individually administered to the OVX female mice subjected to CUS. **E2** administration alone could mitigate the despair behavior in FST where as neither **E2** nor **P4** administration could alter anxiety-like behavior. Interestingly, **E2** administration alone improved the spatial memory in OVX female stress group mice and also increased the *GRIN2A* and *GRIN2B* mRNA levels. Thus, it appears that OVX female mice are at higher risk for anxiety than intact female mice in CUS paradigm. CUS has a divided response in both the affective and cognitive behavior in intact female and OVX female mice, as shown by the despair behavior and spatial reference memory, respectively. The protective effect of **E2** administration in recovery from despair and improvement in spatial reference memory suggests the importance and multiple roles of estrogen. Altered *GRIN2A* and *GRIN2B* levels appear to mediate the effect of **E2** in hippocampus in chronic stress condition. From the above results, it can be concluded that **E2** treatment might help in coping stressful situations. Further, a slightly modified CUS paradigm that included a severe stressor i.e. social defeat, also yielded similar results as reported earlier by LaPlant et al. 2009 i.e. failed to induce depression or anxiety-like behavior in male mice assessed by using Social Interaction test and OFT, respectively, which indicates a lower stress sensitivity of males than females.

To further understand the molecular and behavioral sex differences in stress response, we used CVMS model to induce depression and anxiety-like behavior both in male and intact female mice. CVMS could induce depression-like behavior (despair

behavior) assessed in FST, but could not significantly alter the anxiety-like behavior assessed in OFT and Elevated Plus Maze (EPM) test. CVMS though failed to induce anxiety-like behavior, it did induce hyper-locomotor activity. Here, CVMS induced a sex-specific response in sucrose preference measured in Sucrose Preference Test (SPT), where intact females showed a decreased sucrose preference. The protein expression level of the neurotrophic factor, BDNF, was measured in the prefrontal cortex (PFC). The male stress group had a decrease, not significant though, in the level of BDNF protein in PFC. However, in response to the similar CVMS paradigm, intact female stress group showed a significant down-regulation in the BDNF protein level. Further, CVMS resulted in an increase in the expression levels of few stress-responsive genes *CRH*, *NR3C1*, *CART* and *NPY* in PFC of the intact female stress group, but no significant effect was observed in the male stress group. To understand the effect of ovarian hormones OVX female mice were used. CVMS could induce despair behavior in FST but failed to increase anxiety-like behavior in OFT and EPM. Similar to male and intact female stress groups of mice, there was a CVMS induced hyper-locomotor activity in OVX stress mice. Similar to male stress mice, in OVX female stress mice there was no significant change in the sucrose preference, prefrontal cortical BDNF protein levels, and the few stress-responsive genes. To study the role of major ovarian hormones, **E2** and **P4** were individually administered to the OVX female mice subjected to CVMS. **E2** administration alone could mitigate the despair behavior while either **E2** or **P4** administration failed to alter anxiety-like behavior. Interestingly, **E2** administration alone improved the sucrose preference in OVX female stress mice and increased the prefrontal cortical BDNF protein levels. Thus, it appears that OVX females resembled males in behavioral and molecular responses to CVMS. To conclude, a divided response for anhedonic behavior was observed in this paradigm where only intact females, not males and OVX females, were affected. **E2** administration, but not **P4** administration, alleviated despair behavior and improved the hedonic capacity. BDNF showed a sex-specific response to CVMS, with intact females exhibiting decreased BDNF levels, while in males and OVX females the levels were not significantly affected. This sex-specific response of PFC to chronic stressful events and modulation of BDNF by **E2** suggests a possible role of the neurotrophin in reward pathway and related sex difference in depressive condition.

Although recent literature suggests that hormones play significant role in the prevalence, symptomatology, and treatment response of depressive disorders, so far there is no study to the best of our knowledge on the global transcriptome alterations induced by CVMS in the neuroendocrine hypothalamic region, well known for the sexual diamorphism. To this end, we carried out the cDNA microarray analysis on hypothalamic samples from male, intact female ad OVX female mice subjected to 21-day CVMS paradigm. Before processing for the identification of differential gene expression, all the replicate samples in their respective groups were subjected to principal component analysis and correlation coefficient analysis to inspect replicate similarity and also to identify if there is any outlier in the samples. Pair-wise correlations within the sample groups were found to be higher than the pair-wise correlations across the groups, indicating the replicate similarity and differential group expressions between the samples. To identify the pattern of differentially regulated genes, unsupervised hierarchical clustering has been performed and different clusters were identified. During the analysis of the data, we have focused on those genes whose expression was altered more than 1.2 fold ($p<0.05$). A total of 114, 226 and 121 genes were differentially expressed between stress and control mice in male, intact female, and OVX females respectively. Of these, 90, 205 and 106 genes were uniquely expressed in male, intact female, and OVX female stress group of mice, respectively. Pathway analysis using GeneGo MetaCore software showed that the most significantly enriched pathway was the Posttranslational processing of neuroendocrine peptides, and disease (by biomarker) was Depressive Disorder, Major. Neuropeptides Arginine vasopressin (AVP) and Cholecystokinin (CCK), and AVP were upregulated in male and OVX stress mice, respectively, while in intact female stress mice Oxytocin (OXT) was upregulated. qPCR was performed to validate the regulated genes in the enriched pathway and the associated genes enriched in the DAVID and STRING software. These results suggest a hormonal mechanism in the differentially regulated transcriptome profile of hypothalamus in male, intact female, and OVX female stress mice. This might signify the much-needed sex difference for specifically diagnosing and treating depressive disorders.

Behavioral differences observed in the SPT in chronic stress condition intuited us to investigate global alterations in proteome of the Nucleus Accumbens (NAc), a critical region in reward circuitry. To our knowledge, this study for the first time examined the global proteomic changes in male, female, and OVX mice, which underwent a 21-day

CVMS paradigm, using 2-dimensional gel electrophoresis. For the identification of differentially expressed proteins, Image analysis was carried out with Image Master 2D platinum analysis software. In a match set for the comparative image analysis, gels consisting of triplicate treatments were grouped accordingly and analyzed for qualitative and quantitative differences. To ensure that the differences in spot volume and density between gels are due to differential expression, all the gels were normalized using total spots density normalization tool. The minimum fold change between groups was restricted to greater than 1.2 fold and ANOVA test was performed for the statistical significance ($p \leq 0.05$) of differentially altered spots across the gel members in a matching set. A total of 46 spots were selected, among which 7 from the male, 19 from intact female and 20 from OVX female mice, we processed for protein identification by LC-MS/MS. Among them, Dihydropyrimidinase-related protein 2 [Collapsin response mediator protein-2 (CRMP2)] was selected for further validation in the Western blotting based on its differential expression across the groups (down-regulated in male stress mice and up-regulated in intact female and OVX female stress mice). It is a central nervous system protein involved in neuronal development, axonal and neuronal growth and has been linked to neurodegenerative disorders such as Alzheimer's disease and neuropathic pain, to psychiatric disorders such as schizophrenia**.** These results imply the need to understand the protein level changes in chronic stress condition, which depend on the sex and hormonal status of the female, where these criteria/conditions affect the prevalence and symptomology of depression.