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# Examining the intersection of sex and stress in modeling neuropsychiatric disorders

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## Abstract

Sex-biased neuropsychiatric disorders, including major depressive disorder and schizophrenia, are the major cause of disability in the developed world. Elevated stress sensitivity has been proposed as a key underlying factor in disease onset. Sex differences in stress sensitivity are associated with CRF and serotonin neurotransmission, important central regulators of mood and coping responses. To elucidate the underlying neurobiology of stress-related disease predisposition, it is critical to develop appropriate animal models of stress pathway dysregulation. Further, the inclusion of sex difference comparisons in stress responsive behaviors, physiology, and central stress pathway maturation in these models is essential. Recent studies by our lab and others have begun to investigate the intersection of stress and sex where the development of mouse models of stress pathway dysregulation via prenatal stress experience or early life manipulations has provided insight into points of developmental vulnerability. In addition, examination of the maturation of these pathways including the functional importance of the organizational and activational effects of gonadal hormones on stress responsivity is essential for determination of when sex differences in stress sensitivity may begin. In such studies, we have detected distinct sex differences in stress coping strategies where activational effects of testosterone produced females that displayed male-like strategies in tests of passive coping, but were similar to females in tests of active coping. In a second model of elevated stress sensitivity, male mice experiencing prenatal stress early in gestation showed feminized physiological and behavioral stress responses, and were highly sensitive to a low dose of SSRI. Analyses of expression and epigenetic patterns revealed changes in CRF and glucocorticoid receptor genes in these mice. Mechanistically, stress early in pregnancy produced a significant sex-dependent effect on placental gene expression supportive of altered fetal transport of key growth factors and nutrients. These mouse models examining alterations and hormonal effects on development of stress pathways provide necessary insight into how specific stress responses can be reprogrammed early in development resulting in sex differences in stress sensitivity and neuropsychiatric disease vulnerability.

## Introduction

In order to determine how central stress dysregulation may result in an increased vulnerability to neuropsychiatric disorders, we must first dissect the underlying neurobiology that is associated with the disease state. While females tend to show both heightened stress sensitivity and an increased presentation of affective disorders, male-biased diseases such as schizophrenia have been associated with prenatal and early life exposures to stress. Defining a true ‘sex difference’ is complex, and thus it is important in both basic and clinical research to distinguish between those features that can be attributed

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to hormonal modulation (i.e. estrogenic vs. androgenic) from that which is a true biological sex difference initiated by genes encoded on the sex chromosomes (1,2), both of which have been manipulated in rodent models examining stress responsiveness and motivational behaviors (3,4).

## Stress and Neuropsychiatric Disorders

Stress experience has been correlated with affective disorder onset as well as programming of early life predisposition toward neuropsychiatric disorders such as schizophrenia (5,6). While the stress response is essential for maintenance of homeostasis, maladaptive responses to stress can lead to disease, including an elevation in risk factors for depression and anxiety (7). A critical aspect necessary in defining pathways and mechanisms involved in potential sex differences in such diseases is the development of appropriate animal models to examine alterations in relevant physiology, behavior, gene expression and cytoarchitectural output measures. In such models, exaggerated responses or a failure to respond to stress insults may be designated as possible biomarkers of affective disorders and psychiatric disease (8). For instance, one example of a stress-sensitive model is the CRF receptor-2 deficient mouse that displays exaggerated physiological and behavioral responses to stress, and has been utilized to explore stress pathway maturation and the prenatal, pubertal and adult maladaptive stress responses likened to a ‘depressive’ phenotype (9–13). Evaluation of such models in the context of physiological and behavioral stress responses, both acute and chronic, may define periods of sex-specific plasticity where stress can be detrimental or predictive of long-term disease risk.

At the level of stress physiology, examining sex differences in the hypothalamic-pituitary-adrenal (HPA) axis stress response may provide clues as to the possible involvement of stress sensitivity and negative feedback in the vulnerability to disease. In rodents, females display a greater physiological stress response than males as seen by higher corticosterone levels following a variety of stressors (14), regardless of estrous cycle stage (15). Further, rodent studies have demonstrated similar sex differences in behavioral responses to stress in which females utilize more passive strategies, such as increased immobile time in the forced swim and tail suspension tests compared to males (16,17). In humans, such passive responses to stress are associated with the occurrence of depressive symptoms (18). However, increases in female stress responsivity detected in animal models are not as straightforward in humans. Studies have found that while women may be more sensitive to the effects of catecholamines, the evidence is not as clear for differences in rising cortisol levels where life experiences and the extensive fluctuations in reproductive hormone levels across the life span likely influence such outcomes (19–22). As evidence of such effects, fMRI analyses in women have reported fluctuations across the menstrual cycle in response to emotional stimuli in the orbifrontal cortex, a brain region important in affect determination (23). These distinctions between rodent and human stress circuitry highlight the necessity to determine the independent roles of gonadal hormones from that of sex chromosomes in the neurobiology of stress circuitry.

Orchestration of the components of stress responsivity and recovery can influence a host of endocrine and neuroendocrine factors that may have long-term impacts on future sensitivity and ultimately lead to disease when maladaptive in nature. Therefore, the continued ability throughout life to appropriately respond to stress perturbations is a necessary aspect in homeostatic maintenance and disease prevention. Examination of the developmental timing of disease onset and window of sex disparity in disease presentation may elucidate susceptibility factors in neuropsychiatric disorders. Prior to puberty, minimal differences between sexes are detected in the hypothalamic-pituitary-adrenal (HPA) axis stress response in rodents (24–26). Similarly, males and females have an equivalent presentation of

affective disorders, both for major depression and anxiety, prior to puberty. However, during adolescence, maturation of stress neurocircuitry blunts male responsiveness that is likely related to the rise in testosterone (27). Following adolescence, there is an escalation in female presentation of affective disorders compared to males. These clinical findings suggest an involvement of gonadal hormones in shaping brain plasticity in key emotional centers, and may be important in modulating stress responsivity. Further, in humans and rodents, brain regions involved in stress regulation and emotional affect including the prefrontal cortex (PFC), amygdala and hippocampus, continue to mature well into adolescence supporting a plastic period in which steroid hormones can mold stress neurocircuitry, and a point of additional vulnerability to perturbations in long-term programming of stress responsiveness (26).

In addition to the hormonal modulation beginning at puberty, gonadal hormones are the determining factor in differential brain development (28). In rodents, it has been well established that testosterone exposure via aromatization to estradiol in males during early brain formation is critical for the development of the sexually dimorphic brain, influencing masculinization of reproductive and stress behavioral neurocircuitry (29). Some of these morphological effects can be reproduced in the female via testosterone exposure during this critical developmental period.

## Maturation of stress pathways

We currently have a much clearer understanding of the timeline of sexually dimorphic brain development than we do of how these differences relate to the dichotomy in stress pathway sensitivity. We have begun to explore the intersection of these components of stress sensitivity and sex to develop better models in which affective disorders can be studied. As such, we can take advantage of the ability to masculinize females in early life or during adolescence by administering testosterone (TP) on either postnatal day one (PN1) to examine organizational effects, or beginning at puberty to determine a more activational role for testosterone. Recent studies in rats illustrated the importance of this neonatal masculinization on the reduction of male HPA axis responsiveness to stress via enhances in androgen receptor levels (30). Masculinized neonatal female rats also show a blunted HPA axis response to stress as adults compared to intact females (31). To determine the possible contributions of activational vs. organizational testosterone on blunting adult stress reactivity, our lab has recently examined physiological and behavioral responses to stress in adult female mice exposed to testosterone either at birth or beginning at puberty (3).

In these studies, as a measure of HPA axis stress physiology we examined a time course of corticosterone levels following an acute restraint stress, and as expected intact females showed a substantially higher maximal rise following the restraint than males. We found that TP administered to females during puberty produced activational effects by decreasing stress-induced corticosterone levels similar to that of intact males. However, females treated with postnatal TP did not show this masculinization, but instead displayed a slower rate of stress recovery (3). This may be suggestive of an altered negative feedback related to changes in hippocampal glucocorticoid receptors (GR), pointing to a window of developmental vulnerability in females where programming of future stress sensitivity can occur.

In addition to the physiological HPA axis stress response, we also have examined the role of masculinization on known sex differences in behavioral stress responsivity in tests where females typically show more passive responses than males (17). In the tail suspension test, intact females showed a predictive increased immobile time compared to males. Activational TP administered during puberty masculinized female coping behaviors by

producing a more active response in this test (3). Similar to its lack of effect on the HPA axis stress response, postnatal organizational TP produced no change in female immobile time in the tail suspension test. In a marble burying test, intact females displayed the lowest burying behavior, indicative of predicted passive stress behavioral responses. However, females that were treated with either organizational or activational TP showed a masculinized response and buried a greater number of marbles (3). Such effects of TP to increase active burying have also been demonstrated in gonadectomized male rats (32). The differences in results between anxiety- and depression-like tasks support a distinction in the organizational and activational roles of TP having unique profiles of behavioral effects. While brain masculinization at critical developmental periods appears to be a major contributor to sex differences in physiological and behavioral stress responsivity, the mechanisms by which these effects may occur are still not known. Our studies suggest that testosterone may act on different neurobiological targets depending on the timing of exposure being during postnatal brain development or the highly plastic period of adolescence. For instance, early postnatal life is a sensitive period during which hormone exposure can have organizational effects that alter serotonin system maturation (33,34), potentially leading to long-term changes in stress sensitivity related to active coping strategies. Further, the presence of testosterone beginning in puberty may exert additional modulatory actions on serotonergic and GABAergic systems (35), further affecting active coping behaviors and stress physiology. Therefore, the coordinated impact of masculinization at both time periods may interact with components of sex chromosomes to orchestrate a complete ‘normal’ male phenotype. Disease predisposition is likely a result of perturbations that occur during these developmental windows, disrupting normal stress pathway programming and resulting in an adult with a phenotype that is at either end of the bell curve, being either too sensitive to stress or not sensitive enough. It is clear from both human and rodent studies that not only is increased stress responsiveness linked to neuropsychiatric disorders including depression, anxiety, and schizophrenia, but the inability to appropriately respond to stress to maintain homeostasis in the face of perturbations has also been shown to have detrimental effects in the brain, including cell death in regions where neurotransmission of serotonin is regulated (8).

## Early life programming

One such example of an animal model in which normal fetal programming has been disrupted resulting in a phenotype of adult heightened stress sensitivity is prenatal stress. Stress experience during gestation is associated with an increased incidence of numerous neuropsychiatric disorders, including depression, anxiety, schizophrenia, and autism (6,17,36). The mechanism through which fetal antecedents contribute to disease development is not known, though likely involves a complex interaction between maternal environment and genetics. As many of the diseases associated with prenatal stress exhibit a sex bias in presentation, elucidation of the mechanisms by which sex-specific susceptibility arises may provide critical insight into disease etiology.

While prenatal stress has been broadly associated with offspring disease, the developing nervous system is unlikely to show uniform vulnerability to perturbations over the course of gestation. We hypothesize that the impact on offspring would be dependent on the timing of stress insult, and therefore have examined outcomes specific to early, mid or late pregnancy stress. In confirmation of our hypothesis, we demonstrated that the influence of prenatal stress on hippocampal dependent learning and memory was specific to the timing of stress exposure and sex of the offspring (13,37). However, we are just beginning to appreciate the importance of the timing of stress insult during pregnancy for its impact on stress pathway development that may underlie disease predisposition. In support of such a timing specificity to the effects of stress on long-term outcome in neurodevelopmental disorders, a recent

clinical study revealed an association between maternal stress experience only during the first trimester of pregnancy with an increased risk of schizophrenia in males (38). Studies in guinea pigs have demonstrated that the timing of prenatal stress insult as well as offspring estrous cycle stage during testing as adults was critical in behavioral outcome (39). Further, these studies found that the timing of pregnancy stress on behavioral and physiological stress responsivity was dependent on offspring sex, supporting the need for mechanistic examination of parameters altering programming of stress circuitry during critical periods of sexually dimorphic brain development (40,41). While rodent models provide a controlled environment in which pregnancy manipulations can be conducted, it is important to note the disparity in the timing of rodent and human brain development when considering effects of fetal antecedents. However, influences of early gestational perturbations affecting developmental programming in humans and rodents may contribute to mechanisms whereby changes in maternal hormones or placental gene expression patterns may impact the developing embryo throughout gestation. Therefore, recent studies from our lab have examined the temporal specific outcomes of stress during pregnancy on long-term programming of offspring stress physiological and behavioral sensitivity.

In our studies, male offspring that had been exposed to early prenatal stress (days 1–7; E-PS) exhibited maladaptive behaviors in both the tail suspension and forced swim tests with elevated levels of immobility, likened to stress-induced learned helplessness. No effect of E-PS was detected in females in these tests (17). As the increased immobility in the tail suspension and forced swim tests by E-PS males was suggestive of a depression-like phenotype, we further examined anhedonia-like behaviors using a sucrose preference test. E-PS males exhibited a diminished preference for a 1% sucrose solution compared to controls. Presentation of increasing sucrose concentrations of 5% or 10% ameliorated this effect and revealed a diminished basal sensitivity to hedonic rewards in E-PS males. Further, following an acute stress, E-PS males consumed significantly more of the 10% sucrose than controls if access had been restricted prior to the stress, suggesting that stress-induced binge-like behaviors in E-PS males are another hallmark of stress pathway dysregulation found in neuropsychiatric disorders (17).

Given the phenotype of these E-PS males, we examined behavioral responses to an acute administration of a selective serotonin reuptake inhibitor (SSRI), citalopram to determine if alterations in serotonergic signaling might be present. E-PS males exhibited a greater sensitivity to a low dose of citalopram with decreased immobile time and an increased latency to first immobile bout (17). As this test measures active coping behaviors that are responsive to acute changes in synaptic serotonin, the greater sensitivity in E-PS males suggested a possible alteration in serotonin neurocircuitry. We detected reduced serotonin transporter levels in the CA1 region of the hippocampus and a trend for increased tryptophan hydroxylase-2, the rate-limiting enzyme in 5-HT production, in the dorsal raphe (17). Thus, increased serotonin output and decreased re-uptake may underlie the increased sensitivity to a lower dose of SSRI.

Increased physiological stress responsivity is a hallmark of affective disorders and is also present in many other neuropsychiatric diseases (36). In examination of the HPA stress axis in our studies, we found that E-PS males also showed a significant increase in stress-induced corticosterone levels. We analyzed expression of genes important in stress pathway modulation in these mice and detected a significant increase in CRF expression in the central nucleus of the amygdala and a decrease in hippocampal glucocorticoid receptor expression in E-PS males (17). Glucocorticoid receptors in the hippocampus provide negative feedback for the HPA stress axis. While previous studies examining effects of prenatal stress have reported similar biochemical and physiological changes in offspring

exposed to stress late in gestation, our results determined a wider developmental window during which stress neurocircuitry is vulnerable (42).

One potential mechanism whereby stress can influence fetal development and programming is through epigenetics, including histone modification and DNA methylation (43–45). Further, epigenetic analyses have been applied to the examination of the long-term outcomes attributed to disparate levels of maternal care that impact DNA methylation of specific genes (44,46). While recent emphasis has been placed on the involvement of epigenetic machinery in the programming of early development, the influence of fetal antecedents on the epigenome remains unknown. In our studies, DNA isolated from the specific brain regions where gene expression changes had been detected in adult E-PS males showed reductions in methylation at specific points within the cytosine and guanine rich region of the CRF promoter in close proximity to the cAMP response element (CRE) and glucocorticoid response element (GRE) (17). This reduction in promoter methylation corresponds with the increased CRF expression detected in this brain region. Similarly, we also detected a site-specific increase in methylation of the GR promoter in E-PS males that correlated with the decreased GR expression in the hippocampus from these mice (17). These changes in gene methylation patterns detected in adult brains may contribute to the long-term alterations detected in expression of these genes important in regulation of stress responsivity.

Although the specific mechanism whereby maternal stress contributes to disease risk via effects on epigenetic programming remains unclear, several key contributors have been suggested involving changes in the maternal hormonal milieu. Elevated glucocorticoids *in utero* can alter early life 'programming' (43). However, the placenta inactivates a significant percentage of maternal glucocorticoids via 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), protecting the fetus through the late stages of pregnancy (43). Despite the placenta's critical role in regulating the exchange of hormones, nutrients, and waste products between the maternal and fetal circulatory systems, very little is known regarding either the effects of maternal stress or the influence of fetal sex on placental function. Further, the placenta is a candidate tissue for mechanistic investigation as it is derived from the blastocyst and undergoes critical development during this period of early prenatal stress used in our studies (47). We examined effects of stress early in pregnancy on male and female placental gene expression patterns through analysis of a focused PCR array. E-PS male placentas exhibited significant *increases* in peroxisome proliferator-activated receptor alpha (PPARα), insulin-like growth factor binding protein-1 (IGFBP-1), glucose transporter 4 (GLUT4), and hypoxia inducible factor-3a (HIF3a) compared to control male levels (17). Surprisingly, E-PS female placentas showed a *reduction* in PPARα and IGFBP-1 as compared to control female levels. Expression of PPARα, a transcription factor that regulates cellular metabolism and differentiation, is increased by glucocorticoids (48). Further, PPARα directly increases expression of IGFBP-1 (49). Reductions in growth factors are linked to depression and neurodevelopmental disorders (50). Thus, an elevation in placental IGFBP-1 and consequent decrease in available growth factors during critical developmental periods may play a role in male fetal programming.

These studies reveal a sex-specific effect of stress during early pregnancy on the programming of long-term dysregulated stress neurocircuitry resulting in an adult organism with heightened stress sensitivity and maladaptive behavioral stress responsivity. Examination of sex differences and temporal specificity in these studies provided critical mechanistic information regarding the underlying developmental origin of male-biased neurodevelopmental disorders such as schizophrenia. Interestingly, these results support current findings in clinical evaluation of sex differences in schizophrenic patients. Imaging studies have revealed dysregulation of the human neuroendocrine system with fetal antecedents coinciding with brain sexual differentiation (51). Further, fMRI analyses in male

and female schizophrenics confirmed a disruption of the normal sexually dimorphic ratio of orbitofrontal cortex to amygdala where male schizophrenia patients showed a feminization of these brain regions (52). Future investigation into the underlying mechanisms whereby disruptions in normal sexual differentiation of the brain occurs may provide insight as to the timing of pregnancy stress and the specific genes targeted during this period of re-programming (5,53).

## Summary

At the intersection of stress and sex lies an increased vulnerability toward neuropsychiatric disorders. Throughout development and on into adolescence, the male and female brain continue to develop and change in response to gonadal hormones resulting in the sexually dimorphic adult brain and consequent sex differences in physiological and behavioral stress responsivity. Along this road to maturation, there are points at which perturbations may reprogram neurocircuitry important for adaptive stress coping and disease prevention (40). We have but just scratched the surface into defining mechanisms of how sex differences in disease occur. However, through exploration of the possible involvement of exciting new areas such as the intrauterine environment, epigenetics, and proteomics in long-term disease risk and determination of how these regulatory mechanisms could be built into sex-specific outcomes, we may elucidate new disease preventions and possible novel targets for therapeutic treatment.

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