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Essential role of ovarian hormones in susceptibility to the consequences of witnessing social defeat in female rats

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Abstract

Background—Women are at greater risk of developing depression and comorbid disorders such as cardiovascular disease compared with men. This enhanced risk begins at puberty and ends following menopause, suggesting a role for ovarian hormones in this sensitivity. Here, we used a model of psychosocial witness stress for the first time in female rats to determine the stress-induced neurobiological adaptations that underlie stress susceptibility in an ovarian hormone dependent manner.

Methods—Female rats (intact or ovariectomized, OVX) were exposed to 5 daily 15-minute witness stress exposures. Witness stress-evoked burying, behavioral despair, and anhedonia were measured. Cardiovascular telemetry was combined with plasma measurements of inflammation, epinephrine and corticosterone as indices of cardiovascular dysfunction. Finally, interleukin-1 β (IL-1 β) and corticotropin releasing factor (CRF) were assessed in the central amygdala (CeA).

Results—Witness stress produced anxiety-like burying, depressive-like anhedonia and behavioral despair selectively in intact females, which was associated with enhanced sympathetic responses during stress including increased blood pressure, heart rate, and arrhythmias. Moreover, intact females exhibited increases in 12-hr resting systolic pressure and heart rate and reductions in heart rate variability. Notably, OVX females remained resilient. Moreover intact, but not OVX, females exposed to witness stress exhibited a sensitized cytokine and epinephrine response to stress and distinct increases in CRF and IL-1 β in the CeA.

Conclusions—Together these data suggest that ovarian hormones play a critical role in the behavioral, inflammatory and cardiovascular susceptibility to social stress in females and reveal putative systems that are sensitized to stress in an ovarian hormone-dependent manner.

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Keywords

psychosocial stress; heart rate variability; vicarious social defeat; corticotropin releasing factor; cytokine; central amygdala; hippocampus

Introduction

Social stress including abuse or witnessing violent or traumatic events can precipitate the emergence of psychosocial disorders such as depression and anxiety (1–3). Women are more than twice as likely to suffer from depression and anxiety compared with men (4, 5) and are also at a greater risk of developing inflammatory-related comorbidities such as coronary heart disease and myocardial infarction (6). This enhanced susceptibility to the psychological and physiological consequences of stress emerges at the onset of puberty and remains until menopause (4, 5), suggesting that ovarian hormones may play a crucial role in this enhanced stress sensitivity in females. Although preclinical studies suggest that ovarian hormones can confer behavioral (7) and cardiovascular (8) protection under stress-free conditions, in the context of stress ovarian hormones have proven to increase stress susceptibility (9). Therefore, identification of discrete stress-sensitive systems that are impacted by ovarian hormones under conditions of social stress will be critical to understanding the mechanisms that underlie enhanced stress susceptibility in cycling females.

Much like stress, ovarian hormones modulate a variety of physiological processes including inflammation and the expression of corticotropin releasing factor (CRF) in brain (10, 11). Both CRF and inflammation have been reported as dysregulated in depression and anxiety (12–18) and are known to regulate cardiovascular activity (19, 20). Specifically, proinflammatory cytokines and CRF increase the activation of brain regions known to regulate cardiac, autonomic, endocrine, and behavioral responses to fear including the central amygdala (CeA) (21, 22). Importantly, innate, fearful responses such as burying, avoidance of open spaces, startle responses, and anhedonia are driven in part by the CeA (23–25). Moreover, behavioral responses regulated by CeA occur in parallel with activation of the sympathetic nervous system; CeA activation increases blood pressure and heart rate in awake rats (26, 27) and CeA lesions decrease the hemodynamic response to noise stress (28). Importantly, since the CeA exhibits sensitivity to ovarian hormones (24, 29), it represents an important target to study the pathogenesis of comorbid behavioral and cardiovascular dysfunction in females. Like the CeA, the hippocampus is a stress (30–32), CRF (33) and ovarian hormone (34) sensitive region that can regulate heart rate (35) and behavioral responses to fear (36), making it ideal to assess along with the CeA to determine whether witness stress demonstrates global or region specific effects (34, 36).

Our current understanding of social stress susceptibility in females is limited, due to the difficulty associated with conducting social stress/defeat in females. Several groups have successfully utilized social defeat in females; however, this requires the use of a different species (i.e., highly territorial Syrian hamsters or California mice) or modifying the resident rat/mouse by using DREADDs to activate the ventromedial hypothalamus in males or a

lactating female as the aggressive resident (37–45). While effective in adult females, some suggest that the physical interaction of social defeat may have a greater impact on males than females (37, 41). A recent modification to the resident-intruder paradigm has emerged and consists of a rodent bearing witness to a social defeat episode between a male resident and a male intruder (46–49). This model effectively combines social isolation and visual, olfactory, and auditory exposure to social defeat. Studies utilizing this model have identified that both physical (intruder) and psychological (witness/vicarious) social stress results in the emergence of robust anxiety- and depressive-like behaviors (46–50) as well as enhanced peripheral immune (50, 51) and neuroendocrine (49) responses in males. Moreover, important studies have recently emerged documenting the effectiveness of witness stress in inducing depressive- and anxiety-like behaviors in female mice (52), yet, whether ovarian hormones mediated susceptibility was not determined. The aim of the current study was to identify if female rats display susceptibility to the behavioral and cardiovascular consequences of repeated witness stress and whether these responses were dependent on the presence of ovarian hormones. Additionally, CRF and neuroinflammation were assessed in the CeA and hippocampus to determine a putative neuronal mechanism.

Methods and Materials

Full material and methods are presented in the Supplementary Information.

Animals

Female Sprague-Dawley rats (175–200g, control/witness), male Sprague-Dawley rats (225–250g, intruders) and male Long-Evans retired breeders (650–850g, residents; Charles River, Durham, NC) were individually housed under a 12-hour light/dark cycle in standard cages with ad libitum access to food and water. Care and use of animals was approved by the University of South Carolina's IACUC and was in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Study Design

Four separate studies were conducted as outlined in Figure 1.

Surgery

To minimize inflammatory consequences of surgical manipulation and ensure washout of endogenous hormones following ovariectomy (OVX) (53), all surgical procedures were conducted 10 days prior to the start of stress/control. At approximately 9 weeks of age, all females were subject to brief ovary manipulation (Intact) or surgical removal of ovaries (OVX). Females in studies A-C (Fig 1) were implanted with HD-S11 radio-telemetric transmitters (Data Sciences Int.) in the modified lead II configuration as published (50, 54).

Cycle Tracking

The estrus cycle was monitored in Studies C and D using daily lavage (Figure S2) to determine if stress-induced behavioral or cardiovascular deficits were estrus cycle dependent. Importantly, estrous cycle length was unaffected by witness stress exposure (Table S2).

Social Stress

Witness Stress—Female witnesses were placed behind a perforated Plexiglas partition in a novel resident's cage and observed social defeat between a male resident and male intruder for 15 minutes on 5 consecutive days (Figure S1). This stress duration was chosen as we previously determined that 5 days of repeated witness stress was sufficient to induce behavioral, cardiovascular, and inflammatory consequences in males (50). Control animals were briefly handled for 15 seconds/day.

Male-paired controls—To determine whether stress effects in intact females were driven merely by exposure to a male, independent of social defeat, Study B evaluated the behavioral and cardiovascular effects of intact females placed behind the Plexiglas partition in the home cage of a Sprague Dawley male (15 min on 5 days) in the absence of the aggressive resident.

Cardiovascular telemetry

All cardiovascular data collected in studies A-C were acquired in Dataquest Art (Data Sciences Int.) and analyzed in the Ponemah Physiology platform (Data Sciences Int.) as published (50).

Chronic dark cycle systolic blood pressure (BP) and heart rate (HR)—HR and BP were collected for 9 consecutive days. Data were calculated as a change from baseline using each individual rat's 12-hour dark cycle average collected 48 hours prior to the initial stress/control (Table S1).

Chronic dark cycle heart rate variability (HRV)—Frequency domain analysis of HRV was conducted to determine the spectral power within the low frequency (LF; nonspecific index of sympathetic activity (55)) and high frequency (HF; parasympathetic (vagal) activity (56)) domains and LF:HF ratio (estimate of sympathovagal balance (57)) as published (54, 58, 59).

Cardiovascular response to stress/control—On days 1 (acute) and 5 (repeated) of witness/control, continuous cardiovascular recordings (mean arterial pressure (MAP) and HR) were obtained while resting in the home cage (30 minutes pre-stress/control) and used to calculate the change from each rat's baseline during stress/control exposure. Premature ventricular contractions (PVCs) were also quantified during baseline and stress exposure on days 1 and 5 as published (50).

Behavioral measurements

Video recordings of witness stress on day 1 and 5 were scored for the spontaneous expression of witness stress-evoked burying by an experimenter blinded to the animal's treatment (Studies A-C). Anhedonia was assessed using the 2-bottle choice sucrose preference test ($[\text{volume } 1\% \text{ sucrose}/\text{total volume consumed}] \times 100\%$; Studies A, B) as published (50, 60, 61). Behavioral despair was assessed using the Porsolt forced swim test (FST; Study D) as previously published (62, 63) and the water depth was modified to 35cm.

Video recordings of test sessions were scored using a time sampling technique by two experimenters blinded to animal conditions (62–64).

Euthanasia and tissue collection

Tissues collected from rats in study A were used for physiological and neuroendocrine analyses. To screen for systems that may be sensitized following witness stress exposure, females in study A were subjected to a 15-minute context re-exposure immediately before euthanasia (day 10): Witnesses were placed behind a Plexiglas partition in a soiled resident cage in the presence of the intruder but in absence of the resident; controls were handled and returned to their home cage. Brains were flash frozen with ice-cold 2-Methylbutane, hearts were dissected to obtain ventricular weights, plasma was collected following centrifugation (50, 61), and uteri were weighed for verification of OVX (Figure S5).

Peripheral substrates activated by contextual re-exposure

Plasma corticosterone and cytokine concentrations in response to contextual re-exposure were assessed per manufacturer protocol with ELISA (Enzo Life Science) and a Bio-Plex Multiplex Assay (Bio-Rad Laboratories), respectively. Plasma epinephrine was determined using high performance liquid chromatography as published (50, 61).

CeA and hippocampus tissue collection and analysis

Frozen brains were sliced coronally to the level of the CeA (65) and hippocampus (65). Bilateral 1×1mm punches were obtained and histologically verified (Figure S3, S4). Tissue was homogenized and assayed for protein concentration using a Pierce BCA assay (Thermo Scientific) as published (50, 61). Western blot was used to quantify CRF expression in the CeA and hippocampus using rabbit anti-CRF (1:5000; Abcam), mouse anti-GAPDH (1:1000; Abcam), goat anti-rabbit IRDye 680RD (1:15000; LiCor Biosciences), and goat anti mouse IRDye 800CW (1:15000; LiCor Biosciences) as published (50). Neither stress ($F_{(1,33)}=0.7$, $p=0.4$) nor hormonal condition ($F_{(1,33)}=0.3$, $p=0.6$) altered the normalizing protein GAPDH. CeA neuroinflammation was determined using a Bio-Plex assay (Bio-Rad) as published (61).

Statistical Analysis

The Modified Thompson Tau outlier test identified statistical outliers as published (50, 61). Statistical analysis was conducted using GraphPad Prism 6 and SAS JMP 10 software. Multivariate analysis of variance ($\alpha=0.05$) with repeated measures was conducted for the FST and cardiovascular data to determine effects of stress, hormone, and time. Witness stress-induced burying and hormone-induced differences in resting HRV between intact and OVX were analyzed using standard two-tailed unpaired t-tests ($\alpha=0.05$). All other data sets were analyzed using standard Two-Way ANOVAs ($\alpha=0.05$) to determine effects of stress and hormone. All ANOVAs were followed by Fisher LSD post-hoc analysis. Statistical statements from data represented in the figures are included in each figure legend.

Results

Acute witness stress exposure produces anxiety-like behaviors in intact females

Intact females exhibited significantly greater anxiety-like behaviors as evidenced by shorter latencies to begin witness stress-evoked burying (Average $\pm$ SEM (s) Intact: 25.7 $\pm$ 7.4s; OVX: 65.3 $\pm$ 5.7s; $t_{(9)}=4.8$, $p<0.001$) and greater bury durations (Figure 2A) compared to OVX witnesses. Although control handling sessions for study A were not video recorded, exposure to the male-paired control manipulation was evaluated (Figure S11A) and did not significantly induce burying behaviors. Interestingly, intact witnesses exhibited habituation to the anxiogenic effects of witness stress exhibiting similar burying behaviors to that of OVX witnesses by the 5th stress exposure (Latency: $t_{(12)}=0.0$, $p=1.0$; Duration: $t_{(11)}=0.9$, $p=0.4$, data not shown). While burying behavior exhibited by intact females was independent of the estrus cycle stage (Figure S6), preliminary data indicate that estradiol replacement using silastic implants reinstated burying behavior in OVX females (Figure S9A). These distinct group differences in witness-stress evoked burying (as well as the cardiovascular and inflammatory findings below) were not driven by differences in the quantity or intensity of observed attacks, as the average number of attacks received by the intruder was the same regardless of the hormonal status of the witness behind the partition (Table S3).

Intact females selectively exhibit stress-induced depressive-like behaviors

Repeated witness stress selectively induced anhedonia in intact females as measured by the sucrose preference test (Figure 2B). These anhedonic effects were not driven by differences in total fluid intake (Effect of Hormone $F_{(1,33)}=0.9$, $p=0.4$; Effect of Stress $F_{(1,33)}=0.4$, $p=0.5$) or pre-existing differences in sucrose preference (Effect of Hormone: $F_{(1,39)}=2.1$, $p=0.2$; Effect of Stress $F_{(1,39)}=0.2$, $p=0.7$), and did not occur following exposure to a male in the absence of social defeat (Figure S11B). Moreover, estradiol replacement did not reinstate anhedonic behaviors in OVX females (Figure S9B). In intact females, witness stress also induced behavioral despair as measured by increased immobility (Figure 2C) and shorter climbing durations in the FST (Figure 2D, indicative of shifts in noradrenergic neurotransmission (66)) compared with intact controls and OVX witnesses. Changes in immobility and climbing behaviors were independent of the estrus cycle stage (Figure S7).

Cardiovascular response to witness stress is enhanced selectively in intact females

The presence of ovarian hormones enhanced the magnitude of pressor and tachycardic responses to the acute (day 1) witness stress such that intact females demonstrated the greatest stress-induced MAP (Figure 3A) and HR (Figure 3C) compared with OVX females. This sensitized cardiovascular stress response in intact females was maintained throughout the 5-day stress period (Figure 3B and D), whereby the magnitude of stress-induced MAP and HR was greatest in intact females on day 5. Importantly, witness stress-induced cardiovascular responses returned to baseline (i.e., resting levels immediately preceding stress) within two hours following the termination of witness stress in intact females (Figure S10A–D). Since social stress can precipitate arrhythmic events (59, 67), it was not surprising that witness stress evoked PVCs in intact females, but not OVX females (Figure 3E). Despite the persistence of stress-induced tachycardia during the 5th stress exposure, intact witnesses no longer exhibited arrhythmic responses by day 5 (Figure 3F). It should also be

noted that the magnitude of stress-induced cardiovascular measures on days 1 and 5 were independent of the estrus cycle stage (Figure S8) and were not merely a function of exposure to a male in the absence of social defeat (Figure S11C–D), suggesting that it is the observation of social stress under conditions of intact ovarian hormones that contributes to the enhanced cardiovascular sensitivity exhibited by intact females.

Consequences of prior stress exposure on resting cardiovascular function are dependent on ovarian hormones

To assess whether the presence of ovarian hormones impacts the long-lasting cardiovascular repercussions observed following stress exposure, differences in systolic pressure, HR, and HRV were assessed during the 12-hr dark cycle (while all rats were unstressed, resting in their home cage). Surprisingly, prior to the onset of stress or control manipulations, the HF component of HRV (indicative of vagal tone) was blunted in intact females compared with OVX, resulting in elevated LF/HF ratio (Figure 4A). Moreover, once witness stress was initiated, the HF component remained unchanged (Effect of Stress $F_{(1,28)}=0.5$, $p=0.5$; data not shown), while the LF component of HRV (reflecting in part, sympathetic tone) was increased (Figure 4B), resulting in elevated LF/HF ratio (Figure 4C) selectively in intact females suggesting enhanced sympathetic cardiac outflow in rats predisposed to blunted vagal tone. In support of these HRV findings, intact witnesses also demonstrated enhanced systolic pressure (Figure 4D) and HR (Figure 4E) as early as the first dark cycle following the initial stress exposure. Importantly, these cardiovascular effects were absent in OVX females and male-paired controls (Figure S11E–F). Importantly, there were no differences in general locomotor activity between stress and hormone groups across the duration of the study (Effect of Stress $F_{(1,20)}=0.4$, $p=0.5$; Effect of Hormone $F_{(1,20)}=3.7$, $p=0.07$), suggesting that these shifts in resting cardiovascular measures were not induced by differences in activity levels. Furthermore, intact females exhibited greater ventricular weights, a factor known to be associated with enhanced sympathetic outflow (68), compared with OVX females (Figure 4F).

Prior witness stress exposure sensitized the plasma stress responses to re-exposure to the resident's cage in intact, but not OVX females

To determine stress-sensitive systems that may be sensitized by prior stress exposure and dependent upon ovarian hormones, plasma cytokines, epinephrine, and corticosterone concentrations were measured. Strikingly, witness stress produced opposing effects on plasma inflammatory cytokine levels depending on hormonal status; intact females with a history of witness stress exhibited enhanced inflammation upon re-exposure to the resident's cage while OVX witnesses exhibited reduced cytokine expression (Figure 5 A–C, Table S4). Intact witnesses also exhibited greater plasma epinephrine compared with intact controls (Figure S12A). Interestingly, plasma corticosterone was elevated by context re-exposure, but was not hormone dependent (Figure S12B), suggesting that despite their resilient phenotype, OVX females exhibit hypothalamic-pituitary-adrenal axis activation in response to the witness stress context.

Witness stress selectively enhances CRF and inflammation in the CeA in a hormone dependent manner

Since CRF is known to play an integral role in stress-induced behavioral and cardiovascular dysfunction (54, 62, 69, 70), CRF in the CeA was measured in intact and OVX females. History of witness stress exposure increased CRF protein in the CeA exclusively in intact females (Figure 5D). Interestingly, CRF in the hippocampus, another stress and ovarian hormone sensitive brain region [24–26], was not impacted by witness stress in either intact or OVX females (Figure 5E) suggesting that there is regional specificity in the ability of witness stress to elicit persistent increases in CRF. Subsequent Bio-Plex analysis revealed that witness stress selectively enhanced IL-1 β in the CeA of intact females (Figure 5F) while the remaining 9 cytokines were unchanged (data not shown; $p>0.05$). These findings indicate that ovarian hormone-induced mechanisms are responsible for the adaptations that occur within CRF and neuroinflammatory systems in the CeA during witness stress. Future studies are needed to determine whether IL-1 β contributes to the stress by ovarian hormone interaction that resulted in elevated CRF in the CeA.

Discussion

Clinical data supporting enhanced incidence of depression (4, 5) and co-morbid cardiovascular disease (6) in females have been well established. However, animal studies designed to determine the effects of ovarian hormones on social stress-induced behavioral and physiological consequences have produced equivocal results. To our knowledge, the current study is the first to provide insight into the combined behavioral, cardiovascular, and inflammatory consequences of witness stress in females and is the first report that witness stress is effective in female rats. Moreover, these studies determine that stress susceptibility is mediated by the presence of ovarian hormones, but not by a particular stage of estrus. Specifically, witness stress-induced anxiety-like and depressive-like behaviors, sustained cardiovascular sensitivity, and shifts in resting cardiac autonomic balance towards sympathetic dominance were dependent upon the presence of ovarian hormones, yet independent of the specific estrus cycle stage. These studies also point to adaptations in CRF and neuroinflammation within the CeA as a putative ovarian hormone-dependent mechanism that increases stress susceptibility in intact females.

Recent clinical studies have reported that ruminations (71) and arousal to subsequent negative stimuli (72) are elicited to a greater degree in normal cycling women compared to men and postmenopausal women. Several preclinical studies using non-social stress models have suggested that specific stages of the estrous cycle may drive stress sensitivity (73–76). However, findings utilizing modified female social defeat models have been largely incongruent (43, 77, 78). The current study determined that intact cycling females were more susceptible to witness stress exposure than OVX females, yet these effects were independent of estrus cycle stage. It is possible that certain consequences of witness stress develop over time and do not require a specific stage of estrous cycle. Moreover, studies utilizing unavoidable stressors such as inescapable foot shock (79, 80), thermal pain (80) and fear potentiated startle (81) have also reported cycle-independent effects (80). Therefore, the confined nature of the witness stress paradigm could be contributing to the cycle-

independent effects identified herein. Future studies comparing exogenous administration of a constant level of hormone replacement versus cyclic release will be critical in further understanding the contribution of estrogen and progesterone to witness stress susceptibility.

The autonomic nervous system plays a critical role in modulating the neurocardiac axis and determines how one responds to stress; for example, generating arrhythmias in a heart that is structurally normal. Vagal tone is protective against cardiac arrhythmias (59, 67) and as a result, the reduced HF power in intact females prior to stress can explain the increased susceptibility to ventricular arrhythmias. Moreover, daily exposure to witness stress further increased resting sympathetic activity, resulting in even greater increases in the LF/HF ratio and increases in blood pressure and HR both during stress and under resting home cage conditions as early as ten hours after the first stress and persisting for at least three days following the final stress. Importantly, comparable shifts in HRV are an independent risk factor for cardiovascular morbidity and mortality (82), and this enhanced sympathetic outflow could be responsible for the ventricular hypertrophy evident in intact females (68). Beyond the cardiovascular implications of HRV, a recent study in humans has linked vagal tone with susceptibility to stress. This study determined that marines with reduced HF prior to deployment were more susceptible to developing post-traumatic stress disorder (PTSD) in response to the stress of deployment (83). Taken together, these findings suggest that reduced HF may not only enhance cardiovascular vulnerability, but also highlights a strong relationship between vagal tone and behavioral stress susceptibility. Several peripheral factors, including circulating inflammation and epinephrine, have also been associated with the emergence of cardiovascular disease (84, 85) and these factors were also sensitized in intact, but not OVX females when re-exposed to the resident's cage. Importantly, mouse studies of social defeat have highlighted peripheral, circulating inflammation as being a critical driver of stress susceptibility (51).

Several stress sensitive brain regions such as the hippocampus (34, 36) and CeA (24) are regulated by ovarian hormones. Both of these regions regulate fear memory and the expression of generalized fear responses (24, 36) through modulation of a variety of peptides, including CRF (24). Interestingly, CRF was increased in the CeA of intact females five days following stress, yet CRF was unaffected in OVX witnesses. These stress effects were not ubiquitous, as hippocampal CRF was unchanged in either group exposed to witness stress, indicating that stress-induced effects on CRF are regionally specific. These data not only support previous findings regarding the role of stress-induced CRF in the CeA and behavioral dysfunction (86) and underscore the involvement of ovarian hormones in this process (87, 88), they also suggest that witness stress may selectively alter the activity and function of select neural circuits. Although the current study did not assess alteration in the function or activity of related brain regions, it is possible that repeated exposure to witness stress may have resulted in the attenuation of specific neuronal circuits leading to the habituation of behavioral anxiety-like responses (i.e., CeA-prefrontal cortex (89)) while others, namely those regulating cardiovascular function (i.e., CeA-locus coeruleus and CeA-hypothalamus (90)), remained unaffected. Stress induced IL-1 β may be a critical mediator in these region-specific effects as the IL-1 cytokine family is known to enhance transcription of the CRF gene (91). Interestingly, the IL-1 family is regulated by ovarian hormones; *in vivo* exposure to ovarian hormones potentiates LPS-induced IL-1 β mRNA in microglia (92). In

the present study, CeA concentrations of IL-1 β closely paralleled that of CRF, such that witness stress significantly enhanced CeA IL-1 β in intact females while producing little to no change in OVX females. While stress-induced adaptations in IL-1 β and CRF in the CeA are clearly dependent on ovarian hormones, future studies are needed to determine whether IL-1 β , ovarian hormones, or a combination of the two is responsible for elevated CRF expression. Nonetheless, these data provide a putative neuronal mechanism by which ovarian hormones sensitize behavioral and cardiovascular responses to stress as both inflammation and CRF are known to activate several brain regions including the CeA (21, 22).

Conclusions

By identifying neural substrates that are stress and hormone dependent these studies highlight systems capable of mediating the enhanced susceptibility to stress-induced behavioral and cardiovascular dysfunction in females. Critically, the current studies identified the utility of witness stress in female rats and further determined specific stress-related neuromodulators that are recruited during stress, but only in the presence of ovarian hormones, extending our understanding of the specific role ovarian hormones play in stress-induced adaptations in the brain. Considerable evidence points towards sex differences in CRF signaling as driving increased stress susceptibility to non-social stressors in females (93–95), as well as a role of neuroinflammation in social stress (50, 60, 61, 96–100), and these studies provide support that mechanisms engaged to increase CRF and cytokines in the context of psychosocial stress are dependent upon ovarian hormones.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Study design and timeline

Four separate experiments were used for the completion of this study. (A) Depicts the main stress study from which most of the behavioral, cardiovascular, and physiological data were collected (Intact control $n=5$; Intact Witness $n=7$; ovariectomized [OVX] control $n=15$; OVX witness $n=11$). (B) To ensure that the robust consequences to witness stress in intact females were not driven solely by the presence of a male (in the absence of defeat), a separate set of intact females were placed into the cage of a male intruder (Intact females, $n=6$). (C) To determine whether estrus cycle was affected by witness stress and whether the stage of estrus cycle contributed to the cardiovascular effects of witness stress, a telemetry study was conducted in which females were lavaged daily for 20 consecutive days (Intact control $n=12$; Intact witness $n=7$). (D) As an additional measure of depressive-like behavior induced by witness stress, rats were subjected to the Porsolt forced swim test (FST). Rats were also lavaged to determine if estrus stage biases behavior during the FST (OVX control $n=8$; OVX

witness n=8; Intact control n=10; Intact witness n=8). X-ray transmitter figure with permission from Data Sciences Intl.

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Figure 2. The presence of ovarian hormones during witness stress produces a distinct anxiety-like and depressive-like phenotype

During witness stress exposure, intact females exhibited greater anxiety-like behaviors as demonstrated by enhanced bury durations (A; $t_{(13)}=2.9$, $p<0.05$) compared with ovariectomized (OVX) females. Importantly, controls and females placed into a male's cage in the absence of defeat did not exhibit burying behaviors (Ave $\pm$ SEM (s), Latency: 79.7 ± 19.3 s, Duration: 0.9 ± 0.3 s; Fig S11A). Intact females selectively exhibit an anhedonic phenotype 5 days following repeated witness stress exposure (B; Effect of Stress $\times$ Hormone $F_{(1,35)}=4.0$, $p<0.05$), while OVX females exhibit resilience. Similar depressive-like behaviors were exhibited in the forced swim test such that intact witnesses exhibited greater immobility (C; Time $\times$ Stress $\times$ Hormone Interaction: $F_{(4,23)}=3.5$, $p<0.05$) and reduced climbing (D; Time $\times$ Hormone Interaction: $F_{(4,23)}=3.0$, $p<0.05$) compared with OVX females. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Intact witness vs. Intact control; $^{\alpha}p<0.05$, Intact witness vs. OVX witness; $^{\#}p<0.05$, OVX witness vs. OVX control. All data are presented as the mean $\pm$ SEM, $n=7-15$ per group (see Figure 1).

Figure 3. Ovarian hormone-induced sensitivity to the cardiovascular effects of witness stress
 Witness stress evoked a robust hemodynamic response as evidenced by significantly elevated mean arterial pressure (MAP) on both day 1 (A; Effect of Stress: $F_{(1,19)}=28.1$, $p<0.0001$; Effect of Hormone: $F_{(1,19)}=10.9$, $p<0.01$; Effect of Time $F_{(14,6)}=9.03$, $p<0.01$) and day 5 (B; Effect of Stress $F_{(1,21)}=16.6$, $p<0.001$; Effect of Hormone $F_{(1,21)}=17.8$, $p<0.001$; Effect of Time $F_{(14,8)}=14.8$, $p<0.001$). Importantly, the pressor response to witness stress in intact females was blunted in ovariectomized (OVX) females. Sensitivity to the tachycardic effect of witness stress was also dependent on ovarian hormones. Stress-induced heart rate (HR) was significantly greater in intact vs. OVX witnesses on day 1 (C; Effect of Stress

$F_{(1,22)}=9.1$, $p<0.01$; Effect of Hormone $F_{(1,22)}=32.3$, $p<0.0001$; Effect of Time $F_{(14,9)}=4.1$, $p<0.05$) and day 5 (D; Effect of Stress $F_{(1,16)}=12.6$, $p<0.01$; Effect of Hormone $F_{(1,16)}=15.1$, $p<0.01$; Effect of Time $F_{(14,3)}=53.7$, $p<0.01$). Witness stress-induced tachycardia in intact females was associated with enhanced PVCs on day 1 (E; Effect of Hormone $F_{(1,33)}=9.4$, $p<0.01$) that were diminished by day 5 (F; Effect of Hormone $F_{(1,33)}=1.0$, $p=0.3$; Effect of Stress $F_{(1,33)}=5.7$, $p<0.05$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Intact witness vs. Intact control; ^a $p<0.05$, ^b $p<0.01$, Intact witness vs. OVX witness; ^δ $p<0.05$, OVX control vs. Intact control. All data are presented as the mean $\pm$ SEM, $n=7-15$ per group (see Figure 1).

Figure 4. Ovarian hormones and witness stress produce shifts in resting 12-hour dark cycle sympathovagal balance

Prior to stress/control exposure there are pre-existing hormone-induced differences in sympathovagal balance (A). Intact females exhibit increased LF:HF ratio (A: LF:HF: $t_{(30)}=6.9$, $p<0.0001$). Further examination of the spectral power within the LF and HF revealed that this is a result of reduced high frequency (HF, vagally-driven) domain while low frequency (LF) is not significantly different (A; HF: $t_{(32)}=3.9$, $p<0.001$; LF: $t_{(33)}=1.8$, $p=0.08$). Exposure to witness stress further induces a trend to increase the LF power as early as the first dark cycle after the 1st exposure (B; day 1, Effect of Stress: $F_{(1,27)}=3.4$, $p=0.07$) and persists for at least 24 hours following the 5th exposure (day 6, Effect of Hormone:

$F_{(1,21)}=14.0$, $p<0.01$). The HF component was unchanged by stress (day 1 $F_{(1,28)}=0.5$, $p=0.5$; day 6 $F_{(1,20)}=0.0$, $p=0.8$, data not shown), and as a result, the LF:HF ratio was increased in intact females after day 1 (C; Stress $\times$ Hormone $F_{(1,28)}=6.7$, $p<0.05$) and day 6 (Stress $\times$ Hormone $F_{(1,21)}=4.5$, $p<0.05$). As evidence of the physiologically relevant shifts in sympathovagal balance, resting 12-hour dark cycle systolic pressure was also elevated selectively in intact females (D; Stress $\times$ Hormone Interaction: $F_{(1,15)}=11.7$, $p<0.01$). Moreover, robust and persistent increases in resting 12-hour HR were also evident (E; Stress $\times$ Hormone interaction $F_{(1,22)}=8.7$, $p<0.01$). This enhanced sympathetic outflow in intact females was also associated with greater ventricular hypertrophy compared to OVX females (F; Effect of Hormone: $F_{(1,33)}=20.6$, $p<0.0001$). $^{\alpha}p<0.001$ intact vs. OVX; $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ Intact witness vs. Intact control; $^ap<0.05$, $^bp<0.01$, $^cp<0.001$ Intact witness vs. OVX witness. All data are presented as the mean $\pm$ SEM, $n=7-15$ per group (see Figure 1).

Figure 5. The presence of intact ovarian hormones selectively enhances stress-induced peripheral cytokines, in addition to corticotropin releasing factor (CRF) and interleukin (IL)-1β in the central amygdala (CeA)

Plasma and tissue samples collected in study A were subjected to Bio-Plex cytokine analysis (A–C, F) and western blot for CRF expression (D, E). In rats with a history of repeated witness stress, subsequent re-exposure to the resident's cage in the absence of the resident resulted in enhanced circulating levels of IL-1β (A; Stress*Hormone Interaction: $F_{(1,30)}=30.8$, $p<0.0001$), IL-6 (B; Stress*Hormone Interaction: $F_{(1,29)}=33.7$, $p<0.0001$), and TNF-α (C; Stress*Hormone Interaction: $F_{(1,28)}=7.0$, $p<0.05$). Interestingly, lack of ovarian hormones (OVX) resulted in inhibition of peripheral cytokines induced by re-exposure to the stress context. Similar to findings in the periphery, re-exposure to the stress context increased CRF in the CeA in intact, but not OVX, females (D; Effect of Stress: $F_{(1,33)}=12.1$, $p<0.01$). This enhancement of CRF was not generalized to other stress and ovarian hormone sensitive regions, as CRF in the hippocampus was unaffected by stress or hormone (E; Effect of Hormone: $F_{(1,32)}=0.1$, $p=0.7$; Effect of Stress: $F_{(1,32)}=2.7$, $p=0.1$). Importantly, further evaluation of the CeA identified that IL-1β was also selectively increased in intact female witnesses (F; Effect of Stress: $F_{(1,31)}=10.6$, $p<0.01$). CeA and hippocampal post punch

placement as well as representative western blots are found above and below CRF results in figures D and E respectively. ^fp=0.06, *p<0.05, **p<0.01; ***p<0.001 Intact witness vs. Intact control; #p<0.05, ^ap<0.01 OVX witness vs. OVX control. All data are presented as the mean ± SEM, n=7–15 per group (see Figure 1).

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