**Female WMI rats with genetic stress hyper-reactivity show enhanced contextual fear memory without deficit in extinction of fear**

**Running Title:** Stress reactivity does not affect extinction.

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## **Abstract**

The prevalence of post-traumatic stress disorder is higher in females than males, but pre-clinical models are established almost exclusively in males. This study is aimed to investigate the Stress Enhanced Fear Learning model of post-traumatic stress disorder in females. The model mirrors post-traumatic stress disorder symptomology, whereby prior stress leads to extinction resistant exaggerated contextual fear memory. As stress-reactivity is highly relevant to the study and risk for post-traumatic stress disorder, females of the stress hyper-reactive Wistar Kyoto More Immobile (WMI) and its nearly isogenic control the Wistar Kyoto Less Immobile (WLI) strains were employed. Adult females of both strains were either not stressed or exposed to a two-hour restraint stress, and 48 hours later underwent contextual fear conditioning. Fear memory was measured 24 hours later, followed by extinction trials for a week. Enhanced fear memory following contextual fear conditioning was found in WMIs compared to WLI females and was neither exaggerated by prior stress nor showed extinction deficit. The novel stressor of a glucose challenge test resulted in subtle strain- and prior stress-induced differences in plasma glucose and corticosterone responses. Hippocampal expression levels of learning and memory related genes, glucocorticoid receptor, estrogen receptors, and glucose transporter 1, only changed in WLIs by prior stress. Taken together, results indicate that stress hyper-reactive WMI females do not model post-traumatic stress disorder using the stress enhanced fear learning paradigm, and control WMI females are likely in a state of chronic stress, as additional stressors produced no effects in most measures.

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## **Introduction**

Post-traumatic stress disorder (PTSD) is a debilitating condition that is characterized by severe emotional distress, intrusive thoughts, nightmares, and suicidal ideations, and is known to be precipitated by major stressful events (Mann & Marwaha, 2024). Although a large number of both men and women are affected, 7% of the US population, PTSD prevalence rates are doubled in women compared to men, even after correcting for cultural influences or quantity of traumatic events (Kessler et al., 1995; Koenen et al., 2017; Tolin & Foa, 2006). Current preclinical paradigms emphasize the effects of prior stress on fear memory, as a parallel to PTSD, however they are focused nearly exclusively on the relevant biological mechanisms in males. One commonly used PTSD model is the Stress-Enhanced Fear Learning (SEFL) paradigm, established almost exclusively with male rodents, although recently females were also employed (Gonzalez et al., 2021). Since the SEFL model depends on the effect of prior stress by contextual fear conditioning (CFC), exploring the effect of genetic stress hyperreactivity specifically in females would fill gaps in our knowledge of stress and sex related components of PTSD.

The SEFL paradigm produces an extinction-resistant fear memory response by exposing the animal to prior stress before a typical CFC test (Perusini & Fanselow, 2015; Rau et al., 2005). Exposure to this prior stressor exaggerates fear memory, which is resistant to extinction. The paradigm’s extinction resistance is crucial for the classification of the SEFL paradigm as a model of PTSD as in humans, PTSD diagnoses include the difficulty in extinguishing the intense fear memory/trauma response (Maren & Holmes, 2016). The original SEFL model uses 15 unsignaled foot-shocks as prior stressors, one day prior to the CFC paradigm. Other variations of the SEFL model have used restraint stress as prior stressor in male models and have successfully induced the same enhanced fear memory as the original paradigm (Cordero et al., 2003; Manzanares et al., 2005). Previous use of this adapted SEFL model in our lab has led to increased fear memory in both male and female rats of a strain identified as a genetic model of depression (Lim et al., 2018b; Przybyl et al., 2021).

Prior stressors often alter the responses to subsequent stressors depending on the type and strengths of both (Martí et al., 2001). Response to a novel stressor following the SEFL paradigm and extinction is relevant to our understanding of the generalizability of the stress responses in this PTSD model. For this project, an intraperitoneal glucose tolerance test (also known as glucose challenge, GTT) was used as a novel stressor following SEFL, as glucose challenge induces a stress response in rodents (Small et al., 2022), and glucose metabolism is often disrupted in PTSD patients (Michopoulos et al., 2016). This test is also performed in a novel environment to avoid the context-based fear induced in the SEFL model, again examining the generalizability of stress responses in this model. As PTSD is highly associated with type 2 diabetes, the glucose challenge also served to evaluate potential metabolic dysregulation resulting from the SEFL model (Michopoulos et al., 2016; Oroian et al., 2021).

Hippocampal expression of fear learning and memory genes are also relevant to the novel learning that leads to extinction. The glucose transporter isoform 1 (*Glut1*) is the main mediator of glucose reaching the brain, and its hippocampal expression is known to be associated positively with memory consolidation and learning (Choeiri et al., 2005). Estrogen receptors, *Esr1* and *Esr2,* are both involved in hippocampus-related memory as well (Frick et al., 2018; Fugger et al., 1998, 2000). Additionally, increased expression of the glucocorticoid receptor (*Nr3c1*)is suggested to be a risk factor for PTSD in humans (van Zuiden et al., 2011). Furthermore, *Nr3c1* methylation, which inhibits gene expression, is negatively correlated with PTSD risk (Yehuda et al., 2015). However, these *Nr3c1* associations relate purely to the risk of PTSD development and are from whole blood samples. The current literature on *Nr3c1* expression in PTSD patients is much more complex, especially that suppressed hypothalamic-pituitary-adrenal (HPA) activity may identify a risk factor and not an effect of PTSD (Eckart et al., 2009; Speer et al., 2019). Nonetheless, it is understood that *Nr3c1* through its role in the hippocampal inhibition of the HPA axis is relevant to PTSD symptomology.

In the current study, female rats from an inbred stress hyper-reactive strain were used together with females from a nearly isogenic control strain. These strains were bred bidirectionally from the Wistar Kyoto (WKY) parental strain, based on immobility measures during a forced swim test, resulting in two distinct inbred strains, the Wistar Less Immobile (WLI) and the Wistar More Immobile (WMI) (Will et al., 2003). These inbred and nearly isogenic strains (de Jong et al., 2021) display large differences in depression-like behavior, as well as behavioral stress responses (Andrus et al., 2012; Lim et al., 2018a; Przybyl et al., 2021; Redei et al., 2023). Interestingly, females of the WMI and WLI strains show no differences in anxiety-like behavior (Mehta et al., 2013). These differences are relevant to the current study as PTSD is highly correlated with anxiety symptoms, although it is no longer classified as an anxiety disorder (Breteler et al., 2021; Spinhoven et al., 2014). The uniqueness of the WMI model is the dual characteristics of enhanced depression-like behavior and stress hyper-reactivity, as PTSD is known to have high comorbidity with depression (Campbell et al., 2007; Koopowitz et al., 2021), and both disorders are known to be worsened or precipitated by prior stress (Radell et al., 2020). Therefore, the WMI strain and its nearly congenic control strain present an opportunity to study the effects of these behavioral characteristics in the SEFL paradigm. The overall goal of this study was to test whether the stress hyper-reactive WMI females will show PTSD phenotypes, enhanced fear memory with impaired extinction, and/or abnormal plasma glucose and corticosterone (CORT) responses to the novel GTT stressor compared to WLI females.

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## **Methods**

**Animals**

Animals were housed at Northwestern University Feinberg School of Medicine under the care of the Center for Comparative Medicine. Procedures were approved by the Northwestern Institutional Animal Care and Use Committee. The treatment of the animals, and their conditions, were in accordance with NIH policies. Housing was temperature and humidity controlled through a 12-hour light-dark cycle starting at 6:00 AM. Food and water were available *ad libitum*. Females were 3–5-month-old adult inbred *WLI/Eer* and *WMI/Eer* (referred to as WLI and WMI) rats from the 47th- 48th generations.

**Stress-Enhanced Fear Learning Paradigm**

The experimental design is shown in **Figure 1**. Adult 3-5 months old females were either exposed to restraint stress (RS) or left undisturbed (non-stressed, control group). 48 hours following the RS test, both groups were exposed to contextual fear conditioning (CFC), followed by an initial fear memory test 24 hours later. Subsequently, fear memory was measured every day for a week throughout the extinction period. An intraperitoneal glucose tolerance test (GTT) was performed 24 hours after the last extinction trial. Animals were sacrificed by decapitation 120 minutes after the GTT. Trunk blood and brains were collected.

**Restraint Stress**

Animals in the experimental group were placed in DecapiCone® (Braintree Scientific, Braintree, MA, USA) plastic containers with an opening to allow free breathing but restrain movement for two hours. Restraint stress was conducted between 10:00 AM to 4:00 PM. Following restraint, females were returned to their home cages.

**Contextual Fear Conditioning and Extinction**

Animals were placed into a sound-attenuated fear conditioning chamber from Technical and Scientific Equipment (TSE, Bad Homburg, Germany). Rats were exposed to three minutes of habituation, followed by three shocks of 0.8mA intensity (one-second duration once every minute) over the course of three minutes. 24 hours later, the rats were returned to the same chamber for three minutes without shock. Fear memory was measured by freeze duration and distance traveled on both Day 1 and Day 2 using a computerized infrared beam system (detection rate 10Hz). Rats that did not respond to the initial shocks on Day 1 were excluded from the study. Beginning 24 hours after Day 2 of the CFC, each rat was returned to the same CFC chamber for three minutes without shock, every day for the seven days of the extinction trial. Fear memory was observed through freezing behavior and distance traveled as measured by the automated TSE system. Between animals, the chamber was cleaned with 75% ethanol to eliminate behavioral changes caused by odor.

**Glucose Tolerance Test**

Twenty-four hours after the seventh day of extinction, a GTT was conducted. Prior to the test, the animals were weighed and fasted overnight for 16 hours. The next morning, blood was collected from the tail vein to determine fasting glucose levels. Animals were then injected intraperitoneally with 2µl/g body weight of 1g/ml glucose solution. Tail blood was collected at 30- and 60-minutes post-glucose using heparinized capillary tubes. At 120 minutes, the rats were sacrificed by fast decapitation, and trunk blood and brain samples were collected. Blood samples were collected into EDTA-coated tubes (0.3µL/0.5mL whole blood, 0.5M EDTA), centrifuged at 4°C and 4000 RPM for 10 min, and the plasma was separated for storage at -80°C. Brains were collected in RNAlater™ (Invitrogen, Carlsbad, CA, USA) for dissection later and kept at -80°C.

**Hippocampal quantitative RT-PCR**

Whole hippocampi were dissected using the following coordinates: AP −2.12 to −6.0, ML 0–5.0, DV 5.4–7.6 (Wilcoxon et al., 2005), and placed into RNAlater™. For RNA extraction, tissue was homogenized in TRI Reagent (Sigma-Aldrich, Saint Louis, MO, USA), and total RNA was isolated using the Direct-zol RNA Miniprep Plus kit (Zymo Research, Irvine, CA, USA) according to the manufacturer’s protocol. RNA quality and concentration were measured using the Nanodrop 1000 Spectrophotometer (ThermoFisher, Waltham, MA, USA). Reverse transcription (RT) was carried out on 1µg RNA using Superscript VILO™ Master Mix (Invitrogen, Waltham, MA, USA) as directed by the manufacturer. 5ng of cDNA/sample/well was analyzed in qPCR using PCR 2X MasterMix Universal for SYBR Green Assay (Lamda Biotech, Saint Louis, MO, USA) in the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems) in triplicates. Primer pairs were designed by Primer-BLAST (NCBI, Bethesda, MD, USA). Primer sequences are shown in Supplemental Table 1. Relative quantification (RQ) of transcripts was determined using *Gapdh* as the housekeeping gene and a calibrator (non-stressed WLI cDNA sample) and calculated by the QuantStudio™ Software in which RQ = 2- ΔΔCT.

**Plasma Hormone and Glucose Assays**

Blood glucose levels of animals were analyzed using the Amplite® Colorimetric Glucose Quantitation Kit (AAT Bioquest, Sunnyvale, USA) from plasma samples diluted to a 1:100 ratio, according to the manufacturer’s protocol. The assay was performed in duplicates with a standard curve generated using linear regression from the concentration-absorbance data using GraphPad Prism version 10.0 (GraphPad Software, La Jolla, CA, USA).

Plasma CORT levels were measured by a commercially available competitive ELISA kit (Corticosterone Competitive ELISA kit, ThermoFisher, USA) according to the manufacturer’s protocol. The sensitivity of the assay was 18.6pg/mL and samples were diluted to a 1:1000 ratio. The ELISA was performed in duplicates with a standard curve generated using linear regression from the log-transformed concentration - absorbance data (GraphPad Software, La Jolla, CA, USA).

**Statistics**

All statistical analyses were performed using GraphPad Prism version 10.0 (GraphPad Software, La Jolla, CA, USA) to determine significant differences between the experimental groups. Two-way ANOVAs (stress and strain) were used to analyze CFC data for Days 1 and 2, for glucose and CORT levels, and gene expression. A three-way ANOVA with repeated measures (stress, strain, and days) was used to compare fear memory and distance traveled throughout extinction trials. Post-hoc analyses were employed after significant ANOVAs, using the two-stage linear set-up procedure of Benjamini, Krieger, and Yekutieli (Benjamini et al., 2006). Significance after correction for multiple comparisons was defined as q<0.05 and p<0.05 for individual p-values. All data was represented as the mean ± standard error of the mean (SEM). ANOVA results are indicated in the results sections, and post-hoc analyses are shown in the figures. Pearson’s correlations were performed to determine associations between behaviors, hormones, and genes of interest with significance defined as p<0.05.

## **Results**

**Contextual Fear Conditioning and Extinction**

CFC was conducted over two days. On the first day, fear conditioning occurred via the pairing of unsignaled foot-shocks with the context where it happened. On Day 1 of the CFC, freeze duration after the foot-shocks did not differ significantly between strains and by stress (**Figure 2A**). However, the inverse of freezing, distance traveled, revealed greater activity of control WMI females than WLIs, which was decreased by stress in WMIs (strain, F[1,26]=5.06, p<0.05; **Figure 2B**).

The second day of CFC consists of re-exposing the animals to the same context without foot-shock and evaluating freezing behavior as a measure of fear memory. Fear memory of WMI females, as measured by freeze duration on Day 2 of CFC, was greater compared to WLI females, regardless of stress (strain, F[1,27]=21.70, p<0.01; **Figure 2C**). As expected, the decreased distance traveled by WMI females compared to those of WLI females showed an inverse relationship to the increased freezing behavior, also regardless of stress (strain, F[1,24]=43.98, p<0.01; **Figure 2D**). Prior stress decreased the distance traveled by the WLI females (stress x strain, F[1,24]=4.88, p<0.05).

During the week of extinction, both WMIs and WLIs showed an attenuation of freeze duration following Day 2 of CFC, which is marked as day 0 of extinction (days, F[7,181]=60.02, p<0.01; **Figure 3A**). Although WMIs had significantly greater freeze duration on extinction days 0 and 1 compared to WLI females, by day 3 of extinction, there was no significant difference in freezing behavior observed between strains. This shows a steeper extinction rate in WMIs compared to WLIs (days x strain, F[7,181]=9.97, p<0.01). Moreover, the extinction rate of freeze duration was even steeper in stressed WMI females compared to control WMIs as can be seen through the freezing behaviors of stressed versus control WMIs on day 2 of extinction (stress, F[1,30]=3.98, p=0.05; days x strain x stress, F[7,181]=2.36; p<0.05). By the last day of extinction, there were no significant differences in freeze duration between WMI and WLI females.

Distance traveled during extinction showed an inverse relationship to freeze duration, as expected (**Figure 3B**). Throughout the extinction trials, the distance traveled increased consistently (days, F[7,166]=42.75, p<0.01), but remained significantly decreased in WMI compared to WLIs until day 3 of extinction, confirming a steeper rate of fear memory extinction in WMIs (day x strain, F[7,166]=10.22, p<0.01). Additionally, stress increased distance traveled more precipitously in WMIs compared to control WMIs as is evident from the second day of extinction. Meanwhile, stressed WLI females showed increased distance traveled on days 6 and 7 of extinction (stress, F[1,29]=3.80, p=0.06; day x stress, F[7,166]=3.63, p<0.01; day x stress x strain, F[7,166]=2.55, p<0.05).

**Hormone Levels Following Extinction**

It was expected that both plasma glucose and CORT levels would increase after GTT and then return to baseline at 120 minutes post-glucose injection. The strain differences in the pattern of glucose and CORT responses would indicate whether any metabolic disturbances result from the SEFL protocol and the stress hyper-reactivity of the WMIs.

Analysis of plasma glucose levels following overnight fasting (marked as 0 minutes in the GTT) showed that prior stress increased fasting glucose levels in general, with no significant main effect of strain or significant interaction between stress x strain (stress, F[1,27]=6.12, p<0.05; **Figure 4A**).

Glucose levels significantly increased during the GTT between 0-60 minutes, and then returned toward baseline by 120 minutes (time, F[3,56]=231.6, p<0.01; **Figure 4B**). While this pattern was maintained between all groups, there were significant differences in the slope of glucose levels change between WMI and WLIs in controls, and in response to prior stress. Prior stress exposure decreased blood glucose levels in WLIs at 60 min compared to control WLIs, while it decreased glucose levels in WMIs only after 120 minutes compared to controls (time x stress, F[3,71]=4.98, p<0.01; time x stress x strain, F[3,71]=5.55, p<0.01).

WMI females, regardless of stress, showed decreased CORT levels following the overnight fasting (strain, F[1,26]=4.19, p=0.05; **Figure 4C**). This was especially pronounced between the stressed WLIs and stressed WMIs, but no other significant effects were observed.

CORT levels throughout the GTT showed a pattern very similar to the glucose GTT results, with an increase from 0-60 minutes and a decrease between 60-120 minutes (time, F[3,72]=202.9, p<0.01; **Figure 4D**). Plasma CORT levels differed by strain at 30 min post-glucose administration (strain, F[1,28]=9.12, p<0.01; time x strain, F[3,72]=6.08, p<0.01). Specifically, CORT production quickly increased by 30 minutes in WLIs, with only a small increase between 30-60 minutes, while CORT levels in WMI females peaked at 60 minutes, suggesting a somewhat sluggish CORT stress response in WMI females. Elevated plasma CORT levels in response to GTT returned to baseline in WLI control females, but not in control WMIs and stressed counterparts (stress x strain, F[1,27]=5.90, p<0.05). Baseline and 120 min CORT levels differed between control and stress conditions.

**Hippocampal Expression of Genes**

Transcript levels of both *Glut1* and *Esr1* were significantly greater in the hippocampi of control WMIs compared to WLI controls, although this difference reached a significant strain effect for *Glut1* only (strain, F[1,23]=4.56, p=0.04; **Figure 5A and B,** respectively). Prior stress increased their expression in WLIs, but decreased their expression in WMIs (*Glut1*, stress x strain, F[1,23]=11.88, p<0.01; *Esr1*, stress x strain, F[1,25]=11.79, p<0.01).

*Nr3c1* expression was increased in the hippocampi of control WMIs compared to control WLIs (**Figure 5C**). Additionally, prior stress exposure decreased expression in the WMI hippocampi only, indicating a strain dependent stress response on *Nr3c1* expression (strain x stress, F[1,26]=7.86, p<0.01).

Expression of *Esr2* in WLI trended towards a general increase compared to WMI females, although this effect did not quite reach significance (strain, F[1,26]=3.69, p=0.06; **Figure 5D**). Stress was also shown to increase *Esr2* expression in both strains to a degree, although most prominently in WLI animals, (stress, F[1,26]=4.94, p=0.04).

**Correlation of CFC, Hormonal Measures, and Hippocampal Gene Expressions**

Pearson correlation analysis of all animals revealed a significant positive correlation between fasting CORT and fasting glucose (r=0.52; p<0.01) as well as positive correlations between hippocampal expression of *Glut1* and *Esr1*(r=0.76; p<0.01; **Figure 6A**).

However, when correlations were carried out by strain, additional associations were found within WLI females (**Figure 6B**). As seen in the overall correlation analysis, fasting glucose and fasting CORT were still positively correlated to each other (r=0.63, p<0.01). The positive correlation between *Glut1* and *Esr1* was also still observed (r=0.83, p<0.01). However, *Esr1* was further correlated with fasting glucose in WLIs (r=0.51, p<0.05), and fear learning and fear memory, as measured by freezing duration on days 1 and 2 of the CFC, were positively correlated with each other in WLI females (r=0.53, p=0.05). In analysis of exclusively the WMI females, the only significant correlation that remained was between *Glut1* and *Esr1* (r=0.69, p<0.01; **Figure 6C**).

## **Discussion**

The main findings of the study indicate that, as expected, genetic stress hyper-reactivity and depression resulted in exaggerated contextual fear memory. Prior stress did not enhance fear memory further, in contrast to the findings in male SEFL models. Furthermore, both WMI and WLI females, regardless of stress, showed diminished fear memory by the end of extinction, suggesting no deficits in novel learning ability in either strain. The subtle strain- and prior stress-induced differences in the plasma glucose and CORT responses to the GTT suggest that WMIs do not show exaggerated responses to this novel stressor after being exposed to restraint stress prior to CFC, fear conditioning, and fear memory extinction. Hippocampal expressions of the learning- and memory-related genes, *Esr1, Esr2, Nr3c1,* and *Glut1*, showed opposite patterns in response to the restraint stress in WLI and WMI females. Generally, while exposure to stress prior to CFC in WLIs resulted in increased expression of these genes, no stress effect was observed in WMIs. These results suggest that the stress hyper-reactive WMI females at baseline are already at the ceiling of their ability to respond to additional stressful stimuli.

The current finding of enhanced fear memory on day 2 of CFC in female WMI controls confirms previous results. Specifically, early adolescent WMI females, and adult WMI females of the same age as in the current study exhibit subtly or significantly enhanced fear memory compared to same age WLIs, respectively (Kim et al., 2021; Przybyl et al., 2021). However, aging has abolished and even reversed this difference between WLI and WMI females. WMI females of 6-7 months of age show no difference in contextual fear memory, but by 12-13 month of age their fear memory is attenuated compared to same age WLIs (Lim et al., 2018a, 2018b). This age dependence in fear memory of naive WMI females suggest that aging itself, or accumulated minor stressors affect fear memory.

However, the lack of further enhancement of fear memories by stress in the SEFL paradigm conflict with previous results (Lim et al., 2018a; Przybyl et al., 2021). These studies have found strain-dependent effects of stress; exaggerated fear memory in WLI females, while unaltered or attenuated fear memory in stressed WMI females (Lim et al., 2018a; Przybyl et al., 2021). Fear memory is enhanced by prior restraint stress in WLI females and is attenuated in WMIs in these studies. A potential explanation for the lack of effect of prior stress on WLI females in the current study may be that the experimentally set 0.8mA foot shocks were not consistently delivered to the animals via the computerized system. As the lower 0.6mA shock-induced fear memory in the study from Przybyl and colleagues (2021) shows the same pattern as that in the current study, this possibility cannot be excluded. However, this explanation does not hold for the lack of effect of prior stress in the current study compared to the previous observations of enhanced fear memory of WMIs in response to the lower intensity foot-shock during fear conditioning in Przybyl’s study. Additional similarities are present in fear learning and memory in WLI, but not WMI, females between the two studies. Freeze duration correlates between day 1 and day 2 in the Przybyl study (2021), while this correlation could be observed only in WLI females of the current study. Thus, no convincing, simple explanation can be provided for these discrepancies between the studies.

Throughout extinction, WMI and WLI freezing duration and distance traveled decreased and increased, respectively. These patterns are both indicative of diminishing fear memory, despite the initial enhanced fear memory response in WMI females. The rapid extinction of fear memory observed in the WMI females indicates that genetic stress hyper-reactivity does not dampen learning processes. Moreover, the steepness of the WMI female’s fear memory extinction suggests improved learning, particularly after stress. These findings show that WMI females are not modeling elevated risk for PTSD. The original SEFL model of PTSD generates a form of “extinction resistant” fear memory (Long & Fanselow, 2012; Rau et al., 2005). While the restraint stress version of the SEFL model is believed to enhance fear learning to the same degree as the original paradigm, extinction was not studied with these modified SEFL (Cordero et al., 2003; Manzanares et al., 2005; Przybyl et al., 2021). It is possible that prior restraint stress is not sufficient to engrave extinction-resistant fear, as the magnitude of fear memory might depend on the strength of the initial stressor, as suggested previously (Sandi & Pinelo-Nava, 2007). The observed attenuation of fear memory in both WLI and WMI strains within only a few days of extinction may also indicate that females require a stronger or different stressor than males to induce lasting extinction-resistant fear memory. Alternatively, while sex-specific factors may indeed play a role in learning and memory, genetic stress hyper-reactivity could affect fear learning and extinction via divergent pathways. It’s important to note that the stress hyper-reactive WMI females are known to show increased depression-like behaviors but the same anxiety-like behaviors as female WLIs (Mehta et al., 2013). Thus, the enhanced fear memory of the WMI females compared to WLIs in the SEFL model of PTSD seems to be independent of their anxiety-like behavior.

Response to a novel stressor following extinction, as measured by plasma glucose and CORT response to GTT, suggests a subtle strain- and stress-dependence. While prior stress resulted in a lower glucose peak after GTT in WLI females, no stress effect was seen in WMI females in glucose response. The lack of metabolic dysregulation in WMI females further suggests that they are not models of PTSD, as glucose metabolism is known to be disrupted in PTSD patients (Michopoulos et al., 2016; Oroian et al., 2021). The plasma CORT response of WMI females was slower to reach the peak and did not return to baseline when CORT levels of control WLI females did. Fasting plasma glucose and CORT levels were correlated in WLIs but not in WMIs. Interestingly, the low fasting plasma CORT levels in the WMI females were concurrent with plasma glucose levels similar to those of WLIs, showing a greater CORT sensitivity of the WMIs to release glucose by gluconeogenesis in the liver. In contrast, the sluggish increase and prolonged CORT response to GTT in WMIs indicates a slow negative feedback regulation of HPA, which agrees with the attenuated hippocampal *Nr3c1* expression in stressed WMIs. Decreased *Nr3c1* expression, as found in the stressed WMI hippocampus, is known to dampen this feedback loop (Palma-Gudiel et al., 2015).

Hippocampal *Esr1* and *Glut1* expressions were generally higher in WMI females compared to WLIs. *Esr1* activity and expression have been linked to synaptogenesis (Foster, 2012) and increased hippocampal activity, resulting in enhanced learning and memory consolidation (Hojo et al., 2011). *Esr1* knockout mice display diminished performance in hippocampal-dependent spatial memory tasks (Fugger et al., 1998, 2000). Inversely, infusion of *Esr1* agonistenhances memory consolidation in the hippocampus (Frick et al., 2018). Glucose transport across the blood brain barrier participates in the facilitation of memory-related mechanisms (Cruz et al., 2022; Sajadi et al., 2023).

Highly significant correlation has been seen between *Esr1* and *Glut1* expression in the hippocampus of females of both strains, similarly to those seen in other tissues (Laudański et al., 2004). The glucose transporter isoform 1 provides a critical component of neuron health and activity (Mergenthaler et al., 2013; Takata et al., 1997; Uldry & Thorens, 2004). Increased hippocampal transcript levels of *Glut1* have also been associated with improved memory consolidation and learning (Choeiri et al., 2005). Inversely, inhibition of glucose transport in the hippocampus decreases fear learning and memory, further supporting the role of glucose transport in memory facilitation (Kong et al., 2017). Thus, increased expressions of *Esr1* and *Glut1* in the WMI hippocampus may contribute to an increased ability for novel learning and memory consolidation during extinction, compared to the WLIs. Although *Glut1* expression was positively correlated with *Esr1* expression in the hippocampus, expression of neither gene is correlated with freezing behavior on either day of CFC. One possible explanation for this may be that these genes are not involved in fear-based learning and memory, but rather mediate the non-fear induced novel learning during extinction.

It is worth noting the limitations of the current study. All hormone and gene analyses were performed eight days after the completion of day 2 CFC and following repeated exposure to the fear-associated context. As such, the significance of these findings is related to the strain and stress effects on the novel learning of extinction. Further questions, regarding differences in immediate stress responses following the SEFL procedure, can be answered in the Przybyl, et al. study (2021), in which some animals were also sacrificed immediately after day 2 CFC. Furthermore, while the control animals may be used as a comparison against the stress-exposed females, they were exposed to CFC and extinction too. Thus, they cannot be considered complete controls for interpreting the hormonal and gene expression results. Nevertheless, the current findings provide clear indication that genetic stress hyper-reactivity is not sufficient to induce the PTSD phenotype in females, using the modified SEFL paradigm.

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**Conflict of Interest Statement**

The authors declare no conflict of interest.

**Author Contributions**

Conceptualization, A.H. and E.E.R.; Methodology and Validation, A.H., M.N., M.J., L.L., A.Y., and C.K.; Analysis, A.H. and E.E.R.; Writing—Original Draft Preparation, E.E.R. and A.H.; Writing—Review & Editing, A.H., M.N., M.J., L.L., A.Y., C.K., and E.E.R.; Funding Acquisition, E.E.R and A.H. All authors have read and agreed to the published version of the manuscript.

**Data Accessibility Statement**

Raw data supporting the findings described in this work will be made available upon request.

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**Table 1: Primer Sequences for Quantitative PCR.** *F*, forward; *R*, reverse.

|  |  |  |
| --- | --- | --- |
| **Gene** | **Sequence 5’ - 3’** | |
| *Esr1* | *F* | GAA AGG CGG GAT ACG AAA AGA |
| *R* | TCT GAC GCT TGT GCT TCA ACA |
| *Esr2* | *F* | CAT CAG TAA CAA GGG CAT GGA A |
| *R* | CAC CGG GAC CAC ATT TTT G |
| *Nr3c1* | *F* | AAC AGA CTT TCG GCT TCT GGA A |
| *R* | TGG AAC GCT GGT CGA CCT AT |
| *Glut1* | *F* | TTA ATC GCT TTG GCA GGC GG |
| *R* | GTC AGG CCA CAG TAC ACT CC |
| *Gapdh* | *F* | CAA CTC CCT CAA GAT TGT CAG CAA |
| *R* | GGC ATG GAC TGT GGT CAT GA |

**Figure Legends**

**Figure 1: Overview of experimental procedure.** Rats wererandomly assigned to a control, non-stressed group or an experimental group exposed to two hours of restraint stress (RS). 48 hours later, both groups were exposed to Day 1 of contextual fear conditioning (CFC). CFC Day 1 consisted of three minutes of chamber habituation followed by one foot-shock per minute (0.8mA). CFC Day 2 reintroduced animals to the same context without foot-shocks. Starting 24 hours after Day 2 of CFC, animals were exposed to an extinction protocol lasting seven days. Extinction protocol was the same as the second day of CFC. Freezing behavior and distance traveled measures were collected for CFC and extinction as a method of evaluating fear memory. 24 hours after the last day of extinction animals were exposed to an intraperitoneal glucose challenge test (GTT), and then 120 min later sacrificed.

**Figure 2: Contextual Fear Conditioning elicits heightened fear memory in WMI females regardless of stress.** **(A)** Fear learning on Day 1 of CFC as measured by freeze duration shows no significant differences between groups. **(B)** Activity measure of distance traveled on Day 1 is higher in control WMIs compared to WLIs but decreased by stress. **(C)**. Fear memory is greater in WMI females than in WLIs regardless of prior stress. **(D)** In agreement with fear memory, distance traveled is decreased in WMIs compared to WLIs. However, within the WLI strain, exposure to prior acute stress shows a reduction in distance traveled compared to the control group. Values are shown as mean **±** SEM; *post-hoc* group comparisons were carried out by two-stage linear set-up procedure of Benjamini, Krieger, and Yekutieli following ANOVA, \*q<0.05, \*\*q<0.01. WLI control *n* = 8; stress *n* = 8; WMI control *n* = 8; stress *n* = 7.

**Figure 3: WMI females show no deficit in extinction of fear memories with or without prior stress. (A)** Day 0 of extinction is the Day 2 CFC data. Freeze duration showed significant differences between strains and/or stress groups through Day 2 of extinction. **(B)** Distance traveled showed a similar, but inverse pattern of separation between strains through Day 2 of extinction. However, Day 6 and 7 of extinction show a significant decrease in distance traveled in the WLI controls when compared to all other groups. Values are shown as mean **±** SEM; *post-hoc* were carried out by two-stage linear set-up procedure of Benjamini, Krieger, and Yekutieli following ANOVA. \*q<0.05, \*\*q<0.01 and **^**q<0.05, **^^**q<0.01 control and stressed WLI vs WMI corrected for multiple comparisons, respectively. **+**q<0.05, **++**q<0.01 control vs. stressed, corrected for multiple comparisons. Number of animals as in Figure 2.

**Figure 4: Glucose challenge test reveals strain and prior stress-induced differences in plasma glucose and CORT levels of WLI and WMI females. (A)** Baseline glucose levels following overnight fasting are heightened in stressed WLI animals. **(B)** Glucose levels throughout the GTT differ by strain and stress after an hour, specifically of WLIs. **(C)** Plasma CORT levels following overnight fasting are generally lower in WMIs compared to WLIs. **(D)** CORT responses to the stress of GTT differ between strains: both in the slope of increase after glucose injection, and in the magnitude of the response. Values are shown as mean **±** SEM; *post-hoc* comparisonswere carried out by two-stage linear set-up procedure of Benjamini, Krieger, and Yekutieli following ANOVA. \*q<0.05, \*\*q<0.01 and **^**q<0.05, **^^**q<0.01 control and stressed WLI vs WMI corrected for multiple comparisons, respectively. **+**q<0.05, **++**q<0.01 control vs. stressed, corrected for multiple comparisons. Individual p value, #p<0.05 is not corrected for multiple comparison. Number of animals as in Figure 2.

**Figure 5: Hippocampal gene expression pattern differs between control and stress groups in a strain-dependent manner. (A and B)** Hippocampal expression of *Esr1* and *Glut1* were significantly lower in control WLI females compared to both stressed WLIs and control WMIs. **(C)** *Nr3c1* transcript levels were higher in control WMIs compared to control WLIs and stressed WMI females. **(D)** Transcript levels of *Esr2* were higher in stressed WLIs compared control WLIs and to stressed WMIs. Values are shown as mean **±** SEM; *post-hoc* were carried out as described in the previous figure legend. Number of animals as in Figure 2.

**Figure 6: Strain dependent correlations between behaviors, hormone levels and gene expression. (A)** Pearson’s correlations for all groups regardless of stress status. **(B)** Correlations for WLI females, both controls and stressed. **(C)** Correlations for WMI females both controls and stressed. An increasing gradient of color is used to express increasing strength of correlations; blue represents positive correlations while red represents negative correlations. Values represent Pearson’s *r* values; significance is marked with bolded *r* values and \*p<0.05, \*\*p<0.01, ˆp<0.1. Number of animals as in Figure 2.