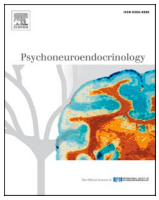
Psychoneuroendocrinology 161 (2024) 106920

Contents lists available at ScienceDirect

Psychoneuroendocrinology

journal homepage: www.elsevier.com/locate/psyneuen



Chronic stress and its effects on behavior, RNA expression of the bed nucleus of the stria terminalis, and the M-current of NPY neurons

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ARTICLE INFO

Keywords:
Chronic stress
BNST
Corticosterone
RNA-Sequencing
NPY
Avoidance behavior

ABSTRACT

Mood disorders, like major depressive disorder, can be precipitated by chronic stress and are more likely to be diagnosed in cisgender women than in cisgender men. This suggests that stress signaling in the brain is sexually dimorphic. We used a chronic variable mild stress paradigm to stress female and male mice for 6 weeks, followed by an assessment of avoidance behavior: the open field test, the elevated plus maze, the light/dark box emergence test, and the novelty suppressed feeding test. Additional cohorts were used for bulk RNA-Sequencing of the anterodorsal bed nucleus of the stria terminalis (adBNST) and whole-cell patch clamp electrophysiology in NPY-expressing neurons of the adBNST to record stress-sensitive M-currents. Our results indicate that females are more affected by chronic stress as indicated by an increase in avoidance behaviors, but that this is also dependent on the estrous stage of the animals such that diestrus females show more avoidant behaviors regardless of stress treatment. Results also indicate that NPY-expressing neurons of the adBNST are not major mediators of chronic stress as the M-current was not affected by treatment. RNA-Sequencing data suggests sex differences in estrogen signaling, serotonin signaling, and orexin signaling in the adBNST. Our results indicate that chronic stress influences behavior in a sex- and estrous stage-dependent manner but NPY-expressing neurons in the BNST are not the mediators of these effects.

1. Introduction

Chronic stress can precipitate the development of mood disorders, such as major depressive disorder (MDD) and generalized anxiety disorder (de Kloet et al., 2005). These disorders lead to a significant decrease in the quality of life and life expectancy (Felker et al., 1996). However, the neurophysiological underpinnings that contribute to these diseases remain poorly understood. To better serve individuals who present with these diagnoses, we must find better and more efficacious treatment to these nebulous diseases. Many of these mood disorders are more prevalent in cisgender women than in cisgender men, suggesting that there is a sex-related difference in their development, in the stress axis, and in the central stress response that influences the causes of these disorders. For example, major depressive disorder is diagnosed 1.6x more frequently in cisgender women, who also exhibit worse symptoms than cisgender men (Lai, 2011; Ferrari et al., 2013). Additionally, many psychiatric disorders, such as stress-related disorders like MDD and anxiety, are known to be affected by the menstrual cycle with severity of symptoms being related to sex hormone levels (Endicott, 1993). Therefore, it is also important to understand how fluctuating levels of gonadal steroids affect stress-related behaviors in mice.

When considering the stress response, most focus is on the hypothalamic-pituitary-adrenal (HPA) axis. In this system, the brain receives signals from the sensory system that the organism is in danger. This causes a cascade of signaling in which corticotropin releasing hormone (CRH) is released from the paraventricular hypothalamus (PVH) into the portal vessels of the median eminence to the pituitary inducing a release of adrenocorticotropin hormone (ACTH) from the anterior pituitary. ACTH acts on the adrenal gland to release glucocorticoids, namely cortisol in humans and corticosterone (CORT) in rodents

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https://doi.org/10.1016/j.psyneuen.2023.106920
Received 24 July 2023; Received in revised form 6 November 2023; Accepted 5 December 2023
Available online 7 December 2023

(Pariante and Lightman, 2008). While this is an essential part of the stress response, when studying chronic stress, examining the actions of CRH throughout the nervous system is necessary. CRH and its receptors are localized in many brain regions, such as the central nucleus of the amygdala, the piriform cortex, and the bed nucleus of the stria terminalis (BNST) (Peng et al., 2017). To understand chronic stress, it is important to understand CRH signaling in regions outside of the PVH. Additionally, there are sex differences in CRH signaling in the brain that may be driven, in part, by gonadal steroids. For example, 17β-estradiol increases the expression of CRH, and estradiol excites PVH CRH+ neurons (Lalmansingh and Uht, 2008; Hu et al., 2016). In addition, the BNST exhibits sex-related differences in the expression of critical signaling- and hormone-related genes, including higher aromatase expression in males, and higher expression of estrogen receptor α in the posterior BNST and more CRH-expressing neurons in the dorsolateral BNST in females (Kelly et al., 2013; Uchida et al., 2019). Thus, central stress responding functions differently depending on sex.

The BNST is often considered part of the "extended amygdala" because one function is to serve as a relay circuit between the amygdala and the prefrontal cortex (Alheid, 2003). This region is of particular interest as it seems to be solely activated by long duration stressors and not by short-context stress (Hammack et al., 2015). Previous research from our lab has shown that a chronic variable mild stress (CVMS) paradigm resulted in increased CRH signaling, increased miniature excitatory post-synaptic signaling, and suppressed M-current activity in the oval nucleus of the BNST in male mice (Hu, Liu et al., 2020). We have also recently demonstrated that chemogenetic activation of BNST CRH neurons reduced motivational behaviors as measured by effort-related choice in males and females (Maita et al., 2023). Studying the BNST neuronal populations involved in behaviors will be vital to elucidate the maladaptive effects of chronic stress.

The BNST is a heterogeneous region of the brain containing many different neuron types that may have a role in controlling stress sensitive behaviors (Beyeler and Dabrowska, 2020). One such cell type expresses neuropeptide Y (NPY), which is often seen as the counterpart to CRH. Intraventricular administration of NPY or agonists of NPY receptors facilitate reduction of anhedonic and avoidance behaviors (Koob and Heinrichs, 1999; Sajdyk et al., 2002) by dampening the effects of CRH and causing termination of the central stress response. NPY may be related to stress signaling and stress-related avoidance as overexpression of NPY leads to less avoidant animals and NPY knockout mice exhibit greater avoidance (Bannon et al., 2000; Lin et al., 2010). It is therefore essential to understand the signaling properties of NPY-expressing neurons in relation to stress and how that signaling may differ under chronic stress conditions.

Therefore, we hypothesized that chronic stress would lead to an increase in avoidance behaviors and changes in signaling in NPY-expressing neurons of the BNST which would be reflected in changes in the transcriptome of this region. We also hypothesized that there would be sex-related differences in these parameters as well. To examine this hypothesis, we used behavioral, molecular, and neurophysiological techniques. After administration of a 6-week CVMS paradigm or control conditions, three cohorts of mice were used to: 1) test of avoidance behaviors, 2) evaluate RNA expression of the BNST, and 3) investigate M-current activity of NPY-expressing neurons in the BNST. This experiment was conducted to elucidate the mechanisms underlying chronic stress, how chronic stress can lead to mood disorders, and the cellular and molecular origins of sex differences. The results of this experiment will allow others to research more effective and targeted treatments for anxiety-related disorders.

2. Materials and methods

2.1. Animals

All procedures were in accordance with National Institutes of Health standards and approved by the Rutgers Institutional Animal Care and Use Committee and agree with ARRIVE's guidelines for reporting animal research. Mice used for behavior and RNA-sequencing cohorts were adult male and female C57BL/6 J mice that were bred in house. For electrophysiology, neuropeptide Y (NPY)-GFP transgenic mice with GFP expression in NPY-expressing neurons were purchased from Jackson Laboratory (Cat# 006417) and bred in house. Mice used for the RNA-Sequencing or electrophysiology cohorts did not encounter the behavior tests and were sacrificed the day after the last stressor. Control or stress conditions were started at 7–10 weeks of age. Except for stressors, mice were housed in a temperature and humidity-controlled room (22 °C, 30–70% humidity), on a 12/12 h light/dark cycle, and provided food and water *ad libitum*. Sample sizes of approximately $n = 12$ mice for behavior per group per treatment, and $n = 8–10$ cells for electrophysiology were based on previously published studies in our lab (Hu, Liu et al., 2020; Hu, Maita et al., 2020). This allowed for detection of affects with a power $= 0.8$ at $\alpha = 0.05$. Sample sizes for female behavior according to estrous stage were more varied as we allowed the mice to cycle naturally which did not allow us to control which cycle stage the females were in prior to testing. In addition, tracking of estrous stages during behavior tests allowed us to monitor the cyclicity of the females and we found that the females were continuing to cycle normally. Sample size for RNA-Sequencing was increased from the typical 5 samples per group per treatment to $n = 9$ to account for interindividual differences and the heterogeneity of the region.

2.2. Chronic variable mild stress paradigm

A modified CVMS paradigm was performed as previously described (Sterrenburg et al., 2011). It used 1–2 daily stressors during a 6-week period. These stressors included: alteration of the light/dark cycle (lights on overnight or lights off during the day for 180 min), temperature alteration (15 min of cold stress), bedding alteration for 3 or 8 h (removing the bedding, replacing the bedding with wet bedding, replacing the bedding with one centimeter of room-temperature water, or housing them in the bedding of a novel mouse of the same sex), frequent cage changes (5 or 6 times that day), forced swim (in 21 °C water for 4 min or 4 °C water for 2 min), isolation stress for 8 h or overnight, restraint stress for 1 h, cage tilting at a 45° angle for 8 h, and predator sounds for 15 min. The stressors are randomized during the 6-week paradigm to ensure that the mice do not get accustomed to any one stressor. All food deprivation and water deprivation stressors were removed from the protocol so that the stressed mice were not accustomed to deprivation prior to behavior testing.

2.3. Corticosterone assay

Blood was collected at termination through trunk blood after decapitation. Blood was then allowed to clot after which serum was isolated, collected, and stored at − 80 °C in 0.65 ml Eppendorf tubes for later CORT concentration measurements. CORT concentration was measured by an ELISA kit (RTC002R; BioVendor). The lower limit for detection in this kit is 6.1 ng/ml with an intra-assay coefficient of variation between 5.9% and 8.9% and an inter-assay coefficient of variation between 7.2% and 7.5%. A logarithmic regression was then used to convert the absorbance to concentrations in ng/ml.

2.4. Behavior assays

Behavior tests started the day after the culmination of the CVMS paradigm. Males and females were tested on separate days to ensure that behavior was not influenced by the scent from other sexes. Mice were acclimated to the behavior room for at least 24 h prior to the first test. The mice were tested on avoidance behaviors and the order of the tests was the open field test (OFT), the elevated plus maze (EPM), the light/dark box emergence test (LDB), and the novelty suppressed feeding test

(NSF) as previously described (Liu et al., 2020; Wiersielis et al., 2021). These tests were conducted in this order to avoid crossover effects. OFT was conducted first as it is the least stressful, EPM was conducted second as there needed to be a gap for washout between the OFT and LDB since they are conducted in the same apparatus, and NSF was conducted last to avoid confounding effects of food deprivation on the other behavior tests. The mice used for behavior testing were euthanized the day after their last test and were not used for any other experiments. All tests were recorded and analyzed by the ANY-Maze behavior monitoring software (ANY-Maze, Version 6, Stoelting, USA). Vaginal cytology was performed on the females after every behavior test to check estrous stage.

2.4.1. Open field test

The OFT was performed in a plexiglass box with opaque sides (40 cm long x 40 cm wide x 40 cm tall), an open top, and a 1 cm × 1 cm grided floor. The mouse was dropped in the lower left corner and the ANY-Maze software tracked the mouse as it moved through different zones in the apparatus for 10 min. The software tracked the amount of time spent and well as entrances into the perimeter, the 20 cm center, and the 10 cm center.

2.4.2. Elevated plus maze

The EPM was performed in a plexiglass plus-shaped maze where two of the arms were left open and two of the arms were surrounded by walls. Arms are 30 cm long x 5 cm wide and connected at a 5 cm square center. The walls surrounding the closed arms are 15 cm tall with open tops. The mouse was dropped into the center of the maze and allowed to freely explore for 5 min. ANY-Maze tracked the amount of time spent in each arm, the number of entries into each arm, and the amount of time spent at the very ends of the open arms.

2.4.3. Light/Dark box emergence

The LDB was performed in the same apparatus as the OFT but with an opaque black insert (20 cm long x 40 cm wide x 40 cm tall) covering half of the box with a closed top. This insert had a hole at the bottom center to allow the mouse to travel between the light half and the darkened half created by the insert. The mouse was dropped in the bottom left corner and allowed to freely explore for 10 min. ANY-Maze tracked the amount of time spent and number of entries in the light zone, the dark zone, and the transition zone between the two as well as the number of stretch attend postures that occurred in the transition zone.

2.4.4. Novelty suppressed feeding

For the NSF, the mice were first fasted for 24 h prior to the test. This test was conducted in two trials. The first trial used a novel arena (30 cm long x 50 cm wide x 16 cm tall) with fresh bedding. At the center of the arena was a pedestal (10 cm diameter) with a food pellet attached. The mouse was dropped in the lower left corner of the novel arena and allowed to freely explore for up to 10 min. ANY-Maze tracked the amount of time it took for the mouse to actively feed on the food pellet. Immediately after the mouse ate from the pellet or after the 10 min elapsed, the pedestal with the food pellet was removed and placed in the mouse's home cage. For the second trial the mouse was then dropped in the lower left corner of their home cage and allowed to freely explore for 5 min. ANY-Maze again tracked the latency to eat in the home cage. Data was also collected on the amount of the pellet that was eaten and the amount of weight that was lost during the 24 h fasting period. Feed was returned to the mice immediately after the conclusion of the test.

2.5. RNA-sequencing

To study differential gene expression between males and females and stressed and control mice, RNA from the adBNST was isolated and used for RNA-sequencing. Mice were decapitated 16-18 h after the culmination of the CVMS paradigm then the brain was removed and sliced into 250 mm thin slices using the vibratome as described in the electrophysiology methods. These slices were then submerged in RNA-later solution (Invitrogen). Later the slices were microdissected isolating only the adBNST and the tissue was again placed in RNAlater and stored at − 80 °C until RNA isolation. RNA was extracted using a RNAqueous Micro Isolation kit (Invitrogen; AM1931). RNA integrity was assessed with an Agilent 2100 Bioanalyzer. Any samples with an RNA integrity number (RIN) below 8 were excluded. The samples were then sent to the JP Sulzberger Columbia Genome Center (New York, NY) for sequencing. Library preparation was performed with the TruSeq Stranded mRNA Library Prep Kit (Illumina) and pooled libraries were sequenced on the Illumina NovaSeq 6000 with 100 bp paired-end reads at a 40 million read depth. The resulting reads were quality trimmed with the FastX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) with a minimum quality score of 20.0. They were then aligned to the mm10 reference genome using STAR and the NCBI RefSeq mm10 gene annotation. Reads were counted with FeatureCounts (Dobin et al., 2013; Liao et al., 2014). Data was then imported to R and differential expression was analyzed using DESeq2 with the following design parameters: (design ~ sex + condition + sex:condition) (Love et al., 2014). Principle component analyses (PCA) were also generated by PCAExplorer to check for outliers (Marini and Binder, 2019). Sequencing data can be accessed at GEO GSE235108.

2.6. Electrophysiology

Electrophysiology was conducted as previously described (Hu, Liu et al., 2020). Mice were quickly decapitated in the morning between 8:30 am and 10:00 am. Brains were removed and fully submerged in 4 °C high-sucrose artificial cerebral spinal fluid (aCSF) made of (in mM) 208 sucrose, 2 KCl, 26 NaHCO3, 10 glucose, 1.25 NaH2PO4, 2 MgSO4 1 MgCl2, 10 HEPES with a pH of 7.35 and osmolarity of 290–310 mOsm. Once cooled, the brain was then placed into a vibratome with the reservoir containing the same cold high sucrose aCSF where 250 μm thin slices were collected, isolating only slices that contained the BNST. These slices were placed in an auxiliary chamber containing room-temperature oxygenated aCSF made of (in mM) 124 NaCl, 5 KCl, 2.6 NaH2PO4, 2 MgCl2, 2 CaCl2, 26 NaHCO3, 10 glucose with a pH of 7.35 and osmolarity of 290–310. Individual slices were then transferred to recording chamber mounted to an Olympus BX51WI fluorescent microscope. Slices were continuously perfused with 35 °C oxygenated aCSF flowing at 1.6 ml/min. Neurons were visualized and targeted using an Olympus water-immersion 40x lens.

Using a borosilicate glass pipette pulled with PC-10 micropipette puller (Narishige) and filled with internal solution containing (in mM) 10 NaCl, 128 K-gluconate, 1 MgCl2, 10 HEPES, 1 ATP, 1.1 EGTA, and 0.25 GTP (7.35 pH and 290–300 mOsm) standard whole-cell patch clamp electrophysiology recordings were collected. Targeting of neurons was restricted to the anterodorsal BNST (adBNST). NPY-expressing neurons were selected by presence of fluorescence using an Excelitas X-Cite fluorescent lamp and green fluorescent filter. Data collection and analysis was done using 1550B Axon Instruments Digidata acquisition system, 700B Axon Instruments Multiclamp amplifier, and pCLAMP software (version 11.1) from Molecular Devices. To ensure the health of the cell is not affecting results, input resistance, membrane resistance, access resistance, and membrane capacitance were monitored throughout the recordings. Input resistance was calculated from the slopes of I-V plots in the range of − 60 mV to − 80 mV that were constructed from voltage steps from − 50 to − 140 mV at 10-mV increments applied at 1-second intervals from a holding potential of − 60 mV. If any of these parameters changed by more than 20% during the recordings, the cell was excluded from the data.

2.6.1. M-current recordings

All drugs were purchased from Tocris unless otherwise specified. All voltage clamp recordings were done with a holding potential of − 60 mV unless otherwise specified (Hu, Liu et al., 2020). To record the

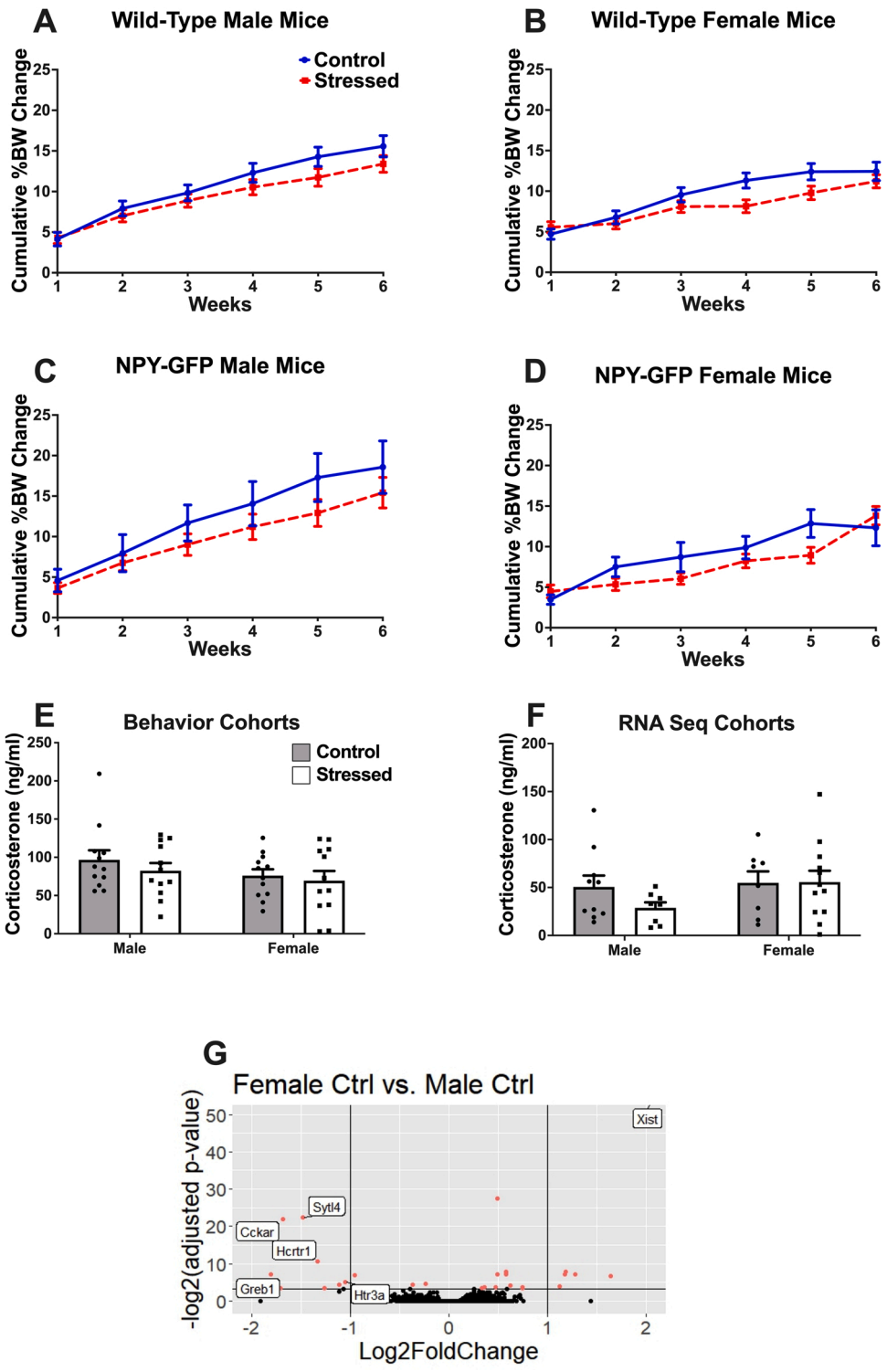
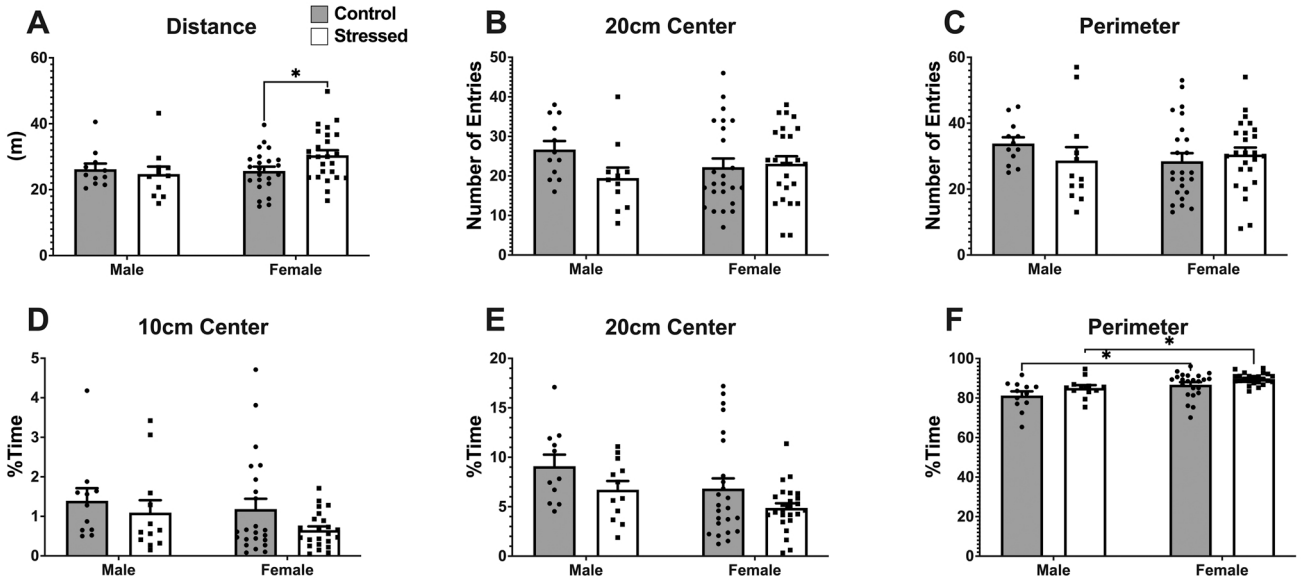
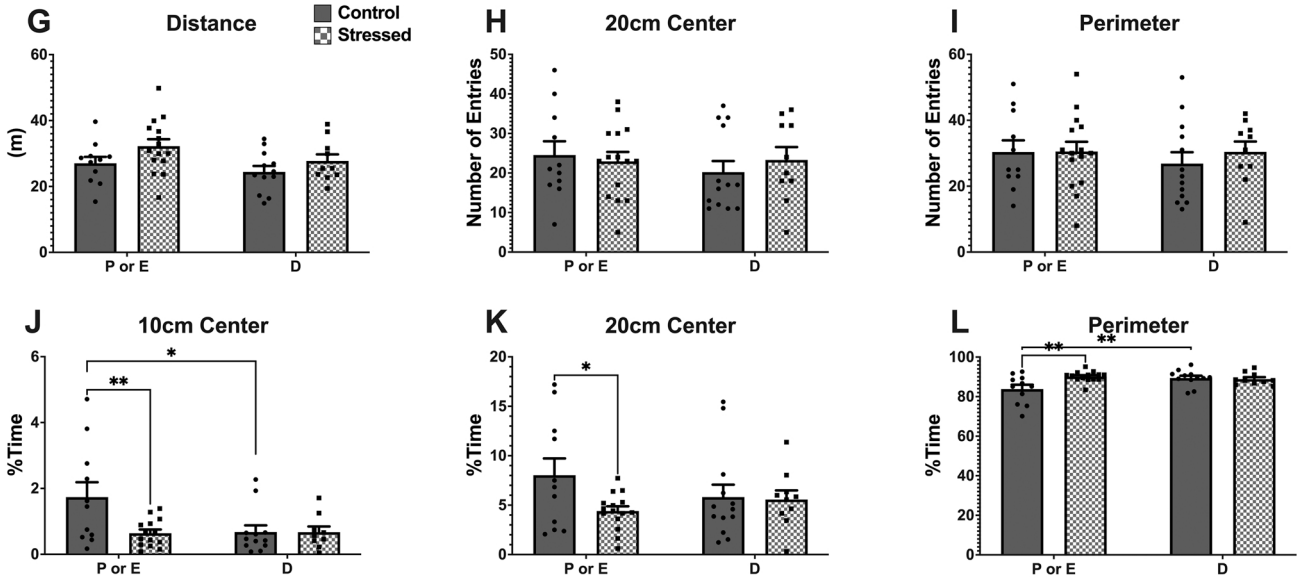


Fig. 1. Cumulative body weight (BW) change, corticosterone concentration in serum, and RNA-Sequencing of the anterodorsal bed nucleus of the stria terminalis. (A) Cumulative %BW change of male wild-type (WT) mice used for behavior tests and RNA-Sequencing. (B) Cumulative %BW change of female WT mice used for behavior tests and RNA-Sequencing. (C) Cumulative %BW change of male mice with Neuropeptide Y expressing cells tagged with GFP (NPY-GFP) used for electrophysiology. (D) Cumulative %BW change of female mice with NPY-GFP used for electrophysiology. (E) Terminal corticosterone concentrations in serum of mice used for behavior cohorts. (F) Terminal corticosterone concentrations in serum of mice used for RNA-sequencing. (G) Volcano plot showing differentially expressed genes when comparing control males and control females. Cumulative %BW and corticosterone data are presented at mean + /- SEM and analyzed by two-way repeated measures ANOVA and two-way ANOVA with Sidak post-hoc comparisons, respectively. RNA-Sequencing data analyzed by DESeq2 with significantly different genes represented as red dot. (*=0.05–0.01, **=0.01–0.001, ***=0.001–0.0001, ****=<0.0001).

Males and Females



Females separated by estrous stage



Significance Tables

M

		Sex	Treatment	Interaction
Distance	F(df)	F (1, 66) = 2.083	F (1, 66) = 0.8473	F (1, 66) = 3.031
	p-value	P=0.1537	P=0.3607	P=0.0864
20cm Center Entries	F(df)	F (1, 68) = 0.02971	F (1, 68) = 1.722	F (1, 68) = 2.799
	p-value	P=0.8637	P=0.1938	P=0.0989
Perimeter Entries	F(df)	F (1, 69) = 0.4060	F (1, 69) = 0.3165	F (1, 69) = 1.654
	p-value	P=0.5261	P=0.5755	P=0.2028
10cm Center %Time	F(df)	F (1, 65) = 1.682	F (1, 65) = 2.743	F (1, 65) = 0.2115
	p-value	P=0.1992	P=0.1025	P=0.6472
20cm Center %Time	F(df)	**F (1, 68) = 4.632**	**F (1, 68) = 5.147**	F (1, 68) = 0.04988
	p-value	**P=0.0349**	**P=0.0265**	P=0.8239
Perimeter %Time	F(df)	**F (1, 67) = 13.64**	**F (1, 67) = 6.203**	F (1, 67) = 0.1188
	p-value	**P=0.0004**	**P=0.0152**	P=0.7314

N

		Estrous Stage	Treatment	Interaction
Distance	F(df)	F (1, 44) = 3.056	**F (1, 44) = 4.371**	F (1, 44) = 0.2095
	p-value	P=0.0874	**P=0.0424**	P=0.6494
20cm Center Entries	F(df)	F (1, 45) = 0.4458	F (1, 45) = 0.0607	F (1, 45) = 0.6267
	p-value	P=0.5078	P=0.8065	P=0.4327
Perimeter Entries	F(df)	F (1, 45) = 0.3005	F (1, 45) = 0.3126	F (1, 45) = 0.2582
	p-value	P=0.5863	P=0.5788	P=0.6138
10cm Center %Time	F(df)	F (1, 42) = 3.862	**F (1, 42) = 4.435**	**F (1, 42) = 4.350**
	p-value	P=0.0560	**P=0.0412**	**P=0.0431**
20cm Center %Time	F(df)	F (1, 45) = 0.2173	F (1, 45) = 2.934	F (1, 45) = 2.245
	p-value	P=0.6434	P=0.0936	P=0.1410
Perimeter %Time	F(df)	F (1, 43) = 2.656	**F (1, 43) = 4.766**	**F (1, 43) = 6.638**
	p-value	P=0.1105	**P=0.0345**	**P=0.0135**

(caption on next page)

Fig. 2. Open Field Test. (A) Distance traveled. (B) Number of entries into the 20 cm center. (C) Number of entries into the perimeter. (D) Percent time spent in the 10 cm center. (E) Percent time spent in the 20 cm center. (F) Percent time spent in the perimeter. (G) Distance traveled with males excluded and females separated by estrous stage. (H) Number of entries into the 20 cm center with males excluded and females separated by estrous stage. (I) Number of entries into the perimeter with males excluded and females separated by estrous stage. (j) Percent time spent in the 10 cm center with males excluded and females separated by estrous stage. (K) Percent time spent in the 20 cm center with males excluded and females separated by estrous stage. (L) Percent time spent in the perimeter with males excluded and females separated by estrous stage. Data are presented at mean + /- SEM and analyzed by two-way ANOVA with Holm-Šidák post-hoc comparisons. (M) Table of significance values for omnibus statistics when males are included, and females are collapsed. (N) Table of significance values for omnibus statistics when males are excluded, and females are separated by estrous stage. Bolded cells indicate significance.

M-current, slices were first perfused with 1 μM tetrodotoxin (TTX) in the aCSF to prevent Na+ -spike-dependent synaptic inputs. Under voltage clamp, a standard deactivation protocol was used to measure K^+ currents elicited during 500-millisecond voltage steps from − 30 to − 75 mV in 5-mV increments after a 300-msec prepulse to − 20 mV. The amplitude of the M-current relaxation or deactivation was measured as the difference between initial (<10 msec) and sustained current (>475 msec) of the current trace in the control conditions (1 μM TTX; 5 min perfusion). After baseline recording, XE-991 (40 μM with 1 μM TTX) was perfused for 10 min, and the protocol was repeated. Deactivation protocol was repeated twice for each perfusion condition and averaged for analysis. Rundown tests of the NPY neurons exposed to TTX indicated that current of the neurons significantly decreased after 20 min (Fig. 7 B) so all recordings were completed within 20 min.

2.7. Statistical analysis

All parameters unless otherwise specified were analyzed using Prism (GraphPad Prism, Version 10, Dotmatics) and used a p-value or adjusted p-value of < 0.05 for significance. The CORT assay, body weight tracking, and behavior tests were first checked for outliers by the Grubbs' test using a critical value of 0.05 as the cut off. Data were then analyzed by a two-way ANOVA followed by a Šidák post-hoc comparison. All behavior data was first analyzed with the variables of treatment and sex. It was then analyzed a second time to exclude males and separate the females by estrous stage, separating proestrus and estrus (P/E) females (representing a state of flux in ovarian hormones) from metestrus and diestrus (D) females (representing a stable ovarian hormone state with low estrogen) so that the resulting variables were treatment and estrous stage. Proestrus and Estrus mice were first separated but once no significance between the two stages was found, they were combine. Probability to eat in the novelty suppressed feeding test was also analyzed by a Kaplan-Meier survival curve followed by a log-rank test. A table of sample sizes and number of excluded data points, including outliers and censored data points, is available in the supplementary figures as Supplementary Table 1. RNA-Sequencing data was analyzed by DESeq2 on R Studio. The IV plots of the M-current recordings were analyzed by a repeated-measures two-way ANOVA followed by a Šidák post-hoc comparison. Max peak of the current at − 35 mV, resting membrane potential, and change in input resistance were analyzed by a two-way ANOVA followed by a Šidák post-hoc comparison.

3. Results

3.1. Body weight and corticosterone levels

Body weight was measured weekly for the 6-week CVMS protocol to determine physiological effects of stress. Cumulative %body weight change was calculated to measure how the stress was affecting the weight gain or weight loss. As seen in Fig. 1A, B, C, and D, the mice exposed to CVMS did not have significantly different weight in either genotype. Serum was collected from the mice at the culmination of the stress paradigm through trunk blood after euthanization. The data in Fig. 1E and F show no significant difference between the stressed mice and their control counterparts.

3.2. adBNST transcriptome

BNST from control and stressed male and female mice was collected the day after the last stressor for RNA Sequencing. No differentially expressed genes (DEGs) were found when comparing control males to stressed males or control females to stressed females. This could possibly be due to the gap in time between the last stressors and euthanasia in which any regulation of genes would have ceased. Future studies will ensure that the BNST is collected at most 1–2 h after the last stressor. However, DEGs were found between males and females though some were already known to be differentially expressed, such as X-linked or Y-linked genes. Some genes of interest that were differentially expressed were: *Hcrtr1* (the orexin receptor) (log2FoldChange = −1.335, adjusted p = 0.0006), *Greb1* (a protein involved in the estrogen signaling pathway) (log2FoldChange = −1.808, adjusted p = 0.0066), and *Htr3a* (a subunit of the type 3 serotonin receptor) (log2FoldChange = −1.058, adjusted p = 0.0279) (Fig. 1G) (See Supplementary Table 2 for all DEGs). These genes could be important areas for future research.

3.3. Behavior tests

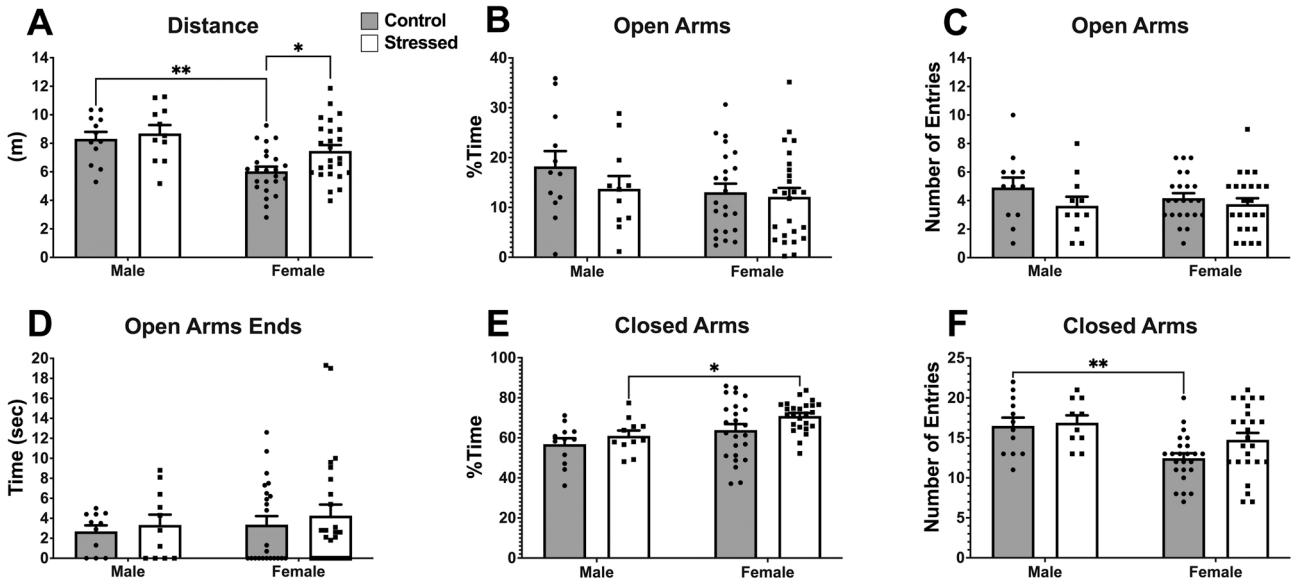
3.3.1. Open field test

The OFT was the first behavior test conducted on the mice. This test can serve as both a measure of overall locomotion and avoidance behavior. In this test, we saw effects of sex, estrous stage, and treatment in various factors. In terms of distance (m), stressed females moved more than their control counterparts [F (1, 44) = 4.37,1 p = 0.04080] (Fig. 2A). We also observed that overall, female mice [F (1.68) = 4.632, p = 0.0349] and stressed mice [F (1, 68) = 5.147, p = 0.0265] spent less time (% of total time) in the 20 cm center (Fig. 2M). Specifically, control females in P/E spent more time in the 20 cm center than their stressed counterparts (p = 0.0471) (Fig. 2K). When observing the more restricted 10 cm center, we also see effect of treatment within the females regardless of estrous stage [F (1, 42) = 4.435, p = 0.0412] and an interaction effect between treatment and estrous stage [F(1, 42) = 4.350, p = 0.0431] (Fig. 2N). Specifically, control P/E females spent more time (sec) in the 10 cm center than both their stressed and D counterparts (p = 0.0068, p = 0.0118 respectively) (Fig. 2J). In the perimeter of the OFT, there were effects of sex and treatment where females [F (1, 67) = 13.64, p = 0.0004] and stressed mice [F(1,67) = 6.203, p = 0.0152 respectively] spent more time (% of total time) in the perimeter (Fig. 2M). Additionally, when excluding males and separating out by estrous stage, we found a treatment effect [F(1, 43) = 4.766, p = 0.0345] and an interaction effect of treatment and estrous stage [F(1, 43) = 6.638, p = 0.0135] in the females (Fig. 2N). Specifically, control P/E females spent less time in the perimeter than their stressed counterparts (p = 0.0024) and D counterparts (p = 0.0099) (Fig. 2L).

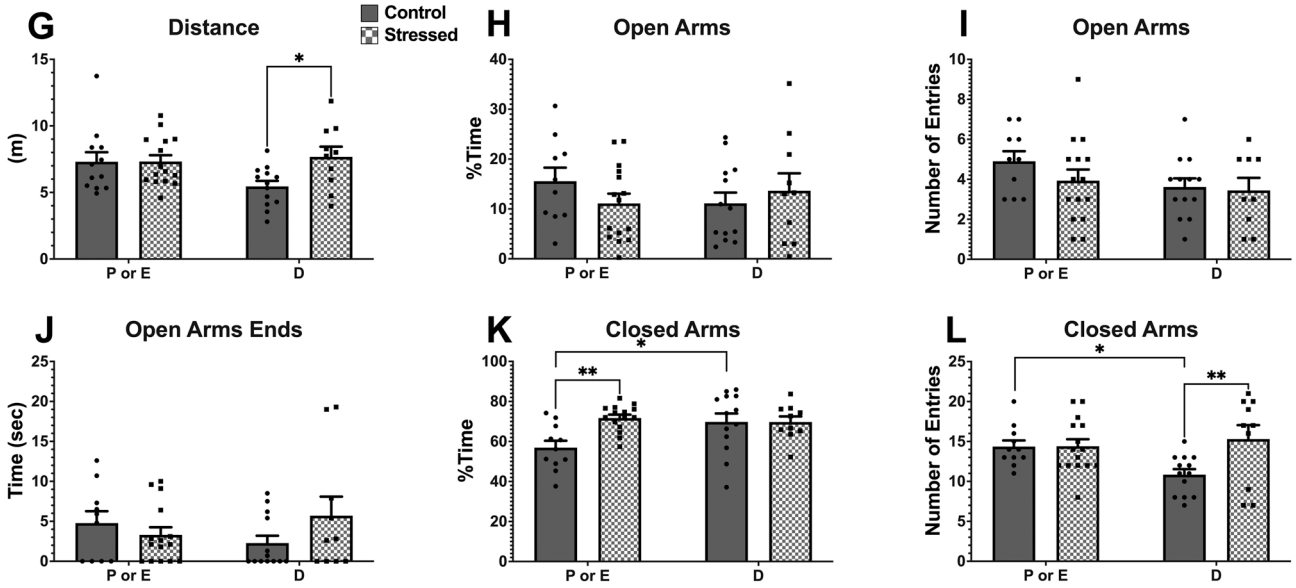
3.3.2. Elevated plus maze

The second test in the behavior paradigm was the elevated plus maze, which evaluates avoidance behaviors. In general, females exhibited less distance (m) traveled than males [F (1, 69) = 9.752, p = 0.0026] (Fig. 3M) with specifically control males being significantly different than control females (p = 0.0147) (Fig. 3A). An effect of sex was also observed in the time (% of total time) spent in the closed arm [F (1, 68) = 9.152, p = 0.0035] and the number of entries into the closed

Males and Females



Females separated by estrous stage



Significance Tables

M

		Sex	Treatment	Interaction
Distance	F(df)	**F (1, 68) = 14.34**	F (1, 68) = 3.809	F (1, 68) = 1.282
	p-value	**P=0.0003**	P=0.0551	P=0.2615
Open Arms %Time	F(df)	F (1, 67) = 2.225	F (1, 67) = 1.417	F (1, 67) = 0.6112
	p-value	P=0.1405	P=0.2380	P=0.4371
Open Arms Entries	F(df)	F (1, 66) = 0.3796	F (1, 66) = 2.786	F (1, 66) = 0.7035
	p-value	P=0.5399	P=0.0998	P=0.4046
Open Arms Ends Time	F(df)	F (1, 66) = 0.5080	F (1, 66) = 0.4721	F (1, 66) = 0.01270
	p-value	P=0.4785	P=0.4944	P=0.9106
Closed Arms %Time	F(df)	**F (1, 68) = 9.152**	**F (1, 68) = 4.027**	F (1, 68) = 0.2633
	p-value	**P=0.0035**	**P=0.0487**	P=0.6096
Closed Arms Entries	F(df)	**F (1, 67) = 11.10**	F (1, 67) = 2.120	F (1, 67) = 1.050
	p-value	**P=0.0014**	P=0.1500	P=0.3091

N

		Estrous Stage	Treatment	Interaction
Distance	F(df)	F (1, 46) = 1.645	F (1, 46) = 3.628	F (1, 46) = 3.531
	p-value	P=0.2060	P=0.0631	P=0.0666
Open Arms %Time	F(df)	F (1, 44) = 0.1371	F (1, 44) = 0.1454	F (1, 44) = 1.902
	p-value	P=0.7130	P=0.7048	P=0.1748
Open Arms Entries	F(df)	F (1, 43) = 2.627	F (1, 43) = 1.081	F (1, 43) = 0.5289
	p-value	P=0.1124	P=0.3043	P=0.4710
Open Arms Ends Time	F(df)	F (1, 44) = 0.001495	F (1, 44) = 0.4717	F (1, 44) = 2.975
	p-value	P=0.9693	P=0.4958	P=0.0916
Closed Arms %Time	F(df)	F (1, 45) = 2.959	**F (1, 45) = 5.445**	**F (1, 45) = 5.508**
	p-value	P=0.0923	**P=0.0242**	**P=0.0234**
Closed Arms Entries	F(df)	F (1, 45) = 1.606	**F (1, 45) = 4.726**	**F (1, 45) = 4.574**
	p-value	P=0.2116	**P=0.0350**	**P=0.0379**

(caption on next page)

Fig. 3. Elevated Plus Maze. (A) Distance traveled. (B) Percent time spent in open arms. (C) Number of entries into open arms. (D) Amount of time spent at the ends of the open arms. (E) Percent time spent in the closed arms. (F) Number of entries into the closed arms. (G) Distance traveled with males excluded and females separated by estrous stage. (H) Percent time spent in open arms with males excluded and females separated by estrous stage. (I) Number of entries into open arms with males excluded and females separated by estrous stage. (J) Amount of time spent at the ends of the open arms with males excluded and females separated by estrous stage. (K) Percent time spent in the closed arms with males excluded and females separated by estrous stage. (L) Number of entries into the closed arms with males excluded and females separated by estrous stage. Data are presented as mean + /- SEM and analyzed by two-way ANOVA with Holm-Šidák post-hoc comparisons. (*=0.05–0.01, **=0.01–0.001). (M) Table of significance values for omnibus statistics when males are included, and females are collapsed. (N) Table of significance values for omnibus statistics when males are excluded, and females are separated by estrous stage. Bolded cells indicate significance.

arm [F (1, 67) = 11.10, p = 0.0014] (Fig. 3M). Specifically, stressed males spent less time in the closed arm in comparison to stressed females (p = 0.0315) (Fig. 3E). However, control males had more entries into the closed arm than control females (p = 0.0046) (Fig. 3F). When excluding males and separating females by estrous stage, we find an effect of treatment where stressed females spent more time in the closed arms [F (1, 45) = 5.445, p = 0.0242] and an interaction effect of treatment and estrous stage [F (1, 45) = 5.508, p = 0.0234] (Fig. 3N). Specifically, control P/E females spent less time in the closed arms than their D (p = 0.0124) and stressed (p = 0.0028) counterparts (Fig., 3 K). We also observe that control females had fewer entries into the closed arms [F (1, 45) = 4.726, p = 0.0350] and an interaction effect of treatment and estrous stage in the number of entries into the closed arms [F (1, 45) = 4.572, p = 0.0379] (Fig. 3N). Control D females had fewer entries into the closed arms than both P/E (p = 0.0404) and stressed (p = 0.0096) counterparts (Fig. 3L).

3.3.3. *Light/Dark box*

The third behavior test conducted was the LDB which involved tracking mice between light and dark areas of a chamber. In this test, we observed an effect of sex on the distance (m) traveled [F (1, 66) = 4.988, p = 0.0289], the amount of time (% of total time) spent in the light zone [F (1, 68) = 4.161, p = 0.0453], the number of entries into the light zone [F (1, 68) = 8.356, p = 0.0052], and the amount of time spent in the transition zone [F (1, 68) = 5.739, p = 0.0193] with the females showing the more avoidant behaviors in all of these parameters (Fig. 4M). There was also an effect of sex on the number of stretch attend postures in the transition zone [F (1, 65) = 19.30, p < 0.0001] though this may be due to the females spending less time in the transition zone in general (Fig. 4M).

3.3.4. *Novelty suppressed feeding*

The final test was the novelty suppressed feeding test which tracks the mouse's latency to eat in different arenas as well as their probability to eat. In general, stressed animals had a shorter latency to eat (sec) in their home cage in comparison to their control counterparts [F (1, 61) = 7.483, p = 0.0081] (Fig. 5M) and specifically stressed males had a shorter latency than their control counterparts (p = 0.0403) (Fig. 5E). In terms of the probability to eat as shown by Kaplan-Meier curves, there was an effect of sex in the novel arena where males where more likely to eat (p = 0.0388) (Fig. 5M). In the home cage, there was an effect of treatment where stressed animals were more likely to eat in the home cage (p = 0.0440) (Fig. 5M) and specifically stressed males were more likely to eat in the home cage than control males (p = 0.0418) (Fig. 5F).

3.3.5. *NPY M-current activity*

Whole-cell patch clamp electrophysiology was conducted in NPY-expressing neurons of the BNST in slice preparation after CVMS or control conditions looking at the activity of the M-current. There were no differences between groups in the cell's resting membrane potential as all neurons were relatively hyperpolarized. RMP of control males: − 74.43 mV + /− 0.086, control females: − 74.34 mV + /− 0.071, stressed males: − 74.75 mV + /− 0.318, stressed females: − 74.39 + / − 0.069 (Fig. 6C). There was no difference in the change of input resistance during the recordings (Fig. 6D). We also observed no differences in the activity of the M-current between the groups regardless of sex or treatment (Fig. 6E-G and I-K). However, we did find the M-current is active in these cells as XE-991 was able to significantly reduce the current recording and max peak current of the cell regardless of sex or treatment [Males: F(1, 18) = 146.7, p < 0.0001; Females: F(1, 18) = 34.70, p < 0.0001] (Fig. 6H and L).

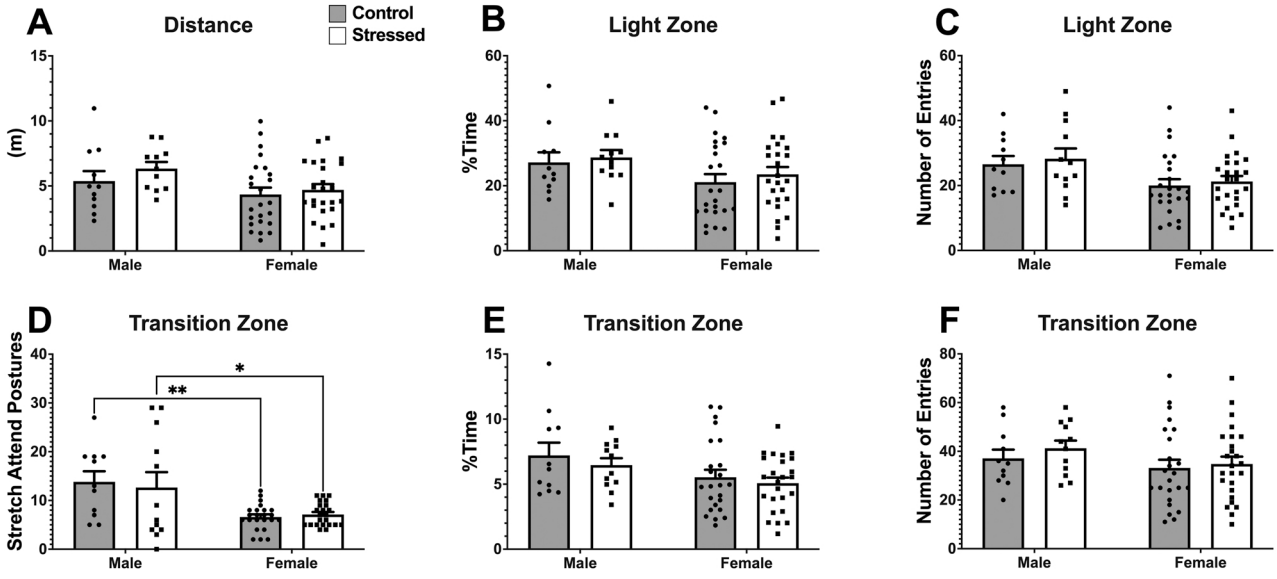
4. Discussion

This research has shown that the CVMS model results in behavioral differences in mice relating to sex and to estrous stages in females. Females, regardless of estrous stage were more sensitive behaviorally to CVMS, and females in P/E displayed more avoidance behaviors than diestrous females after chronic stress. However, the NPY neurons in this region appear to not be the main mediators of chronic stress as the M-current was not affected. Similarly, we found no significant differences in the transcriptome between stressed and non-stressed mice, though there were notable differences across the sexes in the control groups.

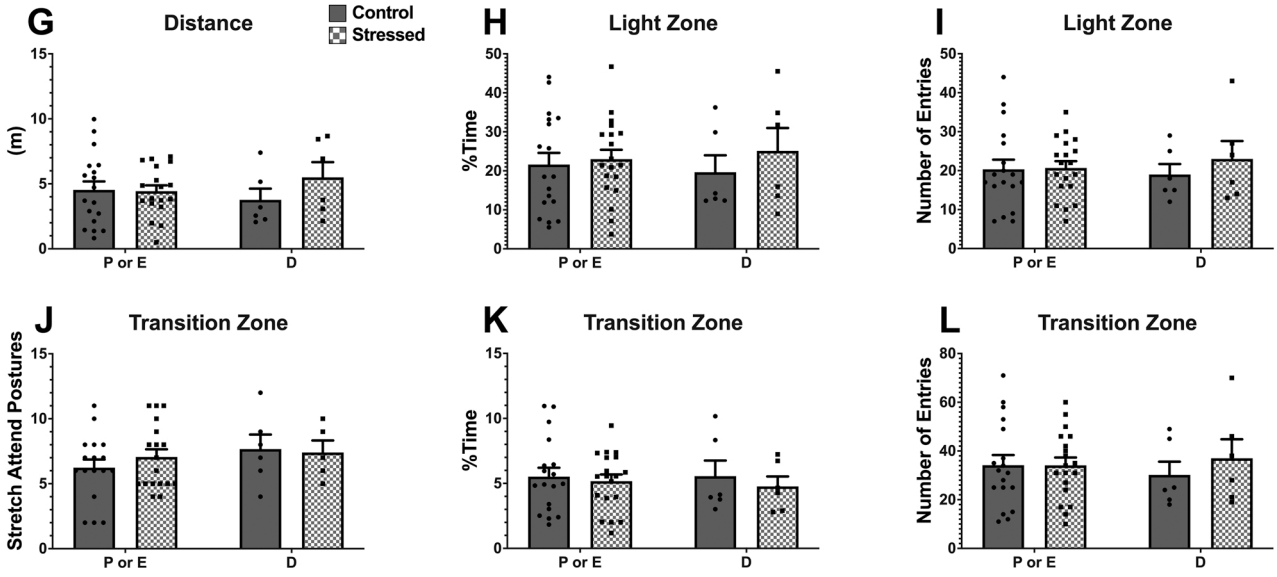
Much like with neurological consequences of chronic stress, the metabolic consequences of chronic stress are poorly understood and seem to result in conflicting data depending on type of stress, previously established metabolic phenotype of the mice, or any other number of confounding factors (Lin et al., 2015; Pałucha-Poniewiera et al., 2020). Some research has shown that mild stress paradigms results in hypophagia and decreased body weight gain (Martí et al., 1994; Michel et al., 2005) while other studies show hyperphagia and increased body weight gain (Teegarden and Bale, 2008; Sanghez et al., 2013). Reviews have suggested that it can be difficult to predict the body weight changes of mice exposed to chronic stress (Razzoli and Bartolomucci, 2016). Our mice did not show any differences in weight gain between the conditions. However, to ensure that the NSF test results would not be affected by previous acclimation to food restriction our stress paradigm significantly reduces the number of metabolic stressors, such as water restriction or food restriction, that are often used in unpredictable stress models like CVMS (Willner, 2005). The lessening of metabolic stressors may be another reason why the mice did not show any changes in their body weight.

Plasma CORT levels naturally raise as a response to stress as a part of a functional HPA axis. Therefore, during chronic stress, CORT levels may be elevated. Our data contradicts this hypothesis as there was no difference in CORT levels between stressed and control groups. One study found that changes in plasma CORT concentrations was affected by the mouse's susceptibility to stress (Kim et al., 2013) while another found that the duration of the chronic stress model can be a determinant of CORT levels (Pałucha-Poniewiera et al., 2020). The Pałucha-Poniewiera et al. publication found that after longer periods of stress (36 days), CORT levels were not as elevated as during the shorter-term stressors. This suggests that there is an adaptation to the chronic stressor or there is a compensatory mechanism that returns CORT levels back to basal levels if the stress period is sufficiently long. Our stress period of 6 weeks is longer than the stress period employed in this study (Pałucha-Poniewiera et al., 2020), and may be the reason CORT levels are not elevated. This is supported by other rodent studies that have similarly found CORT levels returning to basal after a sufficiently long period of chronic stress(Kant et al., 1987; Retana-Márquez et al., 2003; Marin et al., 2007). Future studies will bleed the mice at the beginning and throughout the paradigm to measure the changes in CORT levels as the mice are continually stressed to see where this shift in CORT levels occurs. While we do not see changes in conventional measures of stress, we

Males and Females



Females separated by estrous stage



Significance Tables

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		Sex	Treatment	Interaction
Distance	F(df)	**F (1, 66) = 4.988**	F (1, 66) = 1.221	F (1, 66) = 0.2565
	p-value	**P=0.0289**	P=0.2732	P=0.6142
Light Zone %Time	F(df)	**F (1, 68) = 4.161**	F (1, 68) = 0.5024	F (1, 68) = 0.02483
	p-value	**P=0.0453**	P=0.4809	P=0.8753
Light Zone Entries	F(df)	**F (1, 68) = 8.356**	F (1, 68) = 0.3943	F (1, 68) = 0.00981
	p-value	**P=0.0052**	P=0.5322	P=0.9214
Stretch Attend Post.	F(df)	**F (1, 65) = 19.30**	F (1, 65) = 0.04713	F (1, 65) = 0.3327
	p-value	**P<0.0001**	P=0.8288	P=0.5661
Transition Zone %Time	F(df)	**F (1, 68) = 5.739**	F (1, 68) = 0.8771	F (1, 68) = 0.05123
	p-value	**P=0.0193**	P=0.3523	P=0.8216
Transition Zone Entries	F(df)	F (1, 68) = 1.996	F (1, 68) = 0.6224	F (1, 68) = 0.1183
	p-value	P=0.1622	P=0.4329	P=0.7319

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		Estrous Stage	Treatment	Interaction
Distance	F(df)	F (1, 44) = 0.03534	F (1, 44) = 1.030	F (1, 44) = 1.295
	p-value	P=0.8518	P=0.3157	P=0.2613
Light Zone %Time	F(df)	F (1, 45) = 0.0004133	F (1, 45) = 0.7609	F (1, 45) = 0.2686
	p-value	P=0.9839	P=0.3877	P=0.6068
Light Zone Entries	F(df)	F (1, 45) = 0.02601	F (1, 45) = 0.5101	F (1, 45) = 0.3588
	p-value	P=0.8726	P=0.4788	P=0.5522
Stretch Attend Post.	F(df)	F (1, 42) = 1.025	F (1, 42) = 0.09964	F (1, 42) = 0.3841
	p-value	P=0.3171	P=0.7538	P=0.5388
Transition Zone %Time	F(df)	F (1, 45) = 0.04785	F (1, 45) = 0.4522	F (1, 45) = 0.0683
	p-value	P=0.8278	P=0.5047	P=0.7950
Transition Zone Entries	F(df)	F (1, 45) = 0.01081	F (1, 45) = 0.4060	F (1, 45) = 0.4208
	p-value	P=0.9176	P=0.5273	P=0.5198

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Fig. 4. Light/Dark Box Emergence Test. (A) Distance traveled in the light and transition zones. (B) Percent time spent in the light zone. (C) Number of entries into the light zone. (D) Number of stretch attend postures while in the transition zone. (E) Percent time spent in the transition zone. (F) Number of entries into the transition zone. (G) Distance traveled in the light and transition zones with males excluded and females separated by estrous stage. (H) Percent time spent in the light zone with males excluded and females separated by estrous stage. (I) Number of entries into the light zone with males excluded and females separated by estrous stage. (J) Number of stretch attend postures while in the transition zone with males excluded and females separated by estrous stage. (K) Percent time spent in the transition zone. (L) Number of entries into the transition zone with males excluded and females separated by estrous stage. Data are presented as mean + /- SEM and analyzed by two-way ANOVA with Holm-Šidák post-hoc comparisons. (*=0.05–0.01, **=0.01–0.001). (M) Table of significance values for omnibus statistics when males are included, and females are collapsed. (N) Table of significance values for omnibus statistics when males are excluded, and females are separated by estrous stage. Bolded cells indicate significance.

believe that the previous literature establishing this protocol (Antoniuk et al., 2019) and our observed changes in behavior in this experiment supports this paradigm as sufficiently stressful.

The transcriptome of various brain regions are known to be affected by stress (Flati et al., 2020). However, the transcriptomes from different brain regions expressing CRH can be differentially affected by the stress, which can be sex dependent (Brivio et al., 2020; Flati et al., 2020). In the BNST, research has been inconsistent with how stress affects gene expression. Previous research has found changes in expression of retinoic acid signaling and metabolism, cytoskeleton proteins, phospholipase activity, adrenergic acid signaling, and myelination (Laine et al., 2018; Gururajan et al., 2022). However, these studies often contradict each other. For example, genes involved in G-protein coupled receptor activity can be downregulated after chronic stress (Gururajan et al., 2022), or upregulated (Laine et al., 2018), dependent on the chronic stress paradigm used and tissue collection timepoints. This makes it difficult to compare the research and make any conclusions about the effect of stress on the transcriptome of the BNST. Our research found no significant DEGs regarding chronic stress. It is possible that any differences were washed out due to the heterogeneity of the region or due to a loss from the transient nature of gene expression as our tissue was collected 18 h after the last stressors. In future studies, we can employ two strategies to more accurately assess the adBNST transcriptome: 1) single-cell RNA-Sequencing to target specific subpopulations and 2) collect tissue only 1 h after the last stressors. While there were no differences in the stress, there were sex-related differences. Many of these genes could be used to direct studies in the future.

Genes that express neuropeptide and neurotransmitter receptors known to be involved in stress signaling, such as *Htr3a* and *Hcrtr1*, were expressed at higher levels in the males. *Htr3a* is the gene that encodes for subunit A of the type 3 receptor for serotonin. Serotonin modulates mood, reward, and learning and serotonin neurons from the dorsal raphe project to the dorsal BNST and are involved in stress signaling (Garcia-Garcia et al., 2018). An increase in serotonin sensitizes the brain to further stress (Adell et al., 1988) and changes in serotonin levels in response to stress is believed to the related to resilience or susceptibility to stress and dysfunction, a key factor in the development of MDD (Robertson et al., 2005). Serotonin is deeply correlated with the progression of MDD as selective serotonin reuptake inhibitors have become the first-line pharmacotherapy for MDD. The link to MDD and chronic stress as well as the differential expression of one of its receptors in the adBNST suggests that it may underlie some of the sex differences in the response to chronic stress.

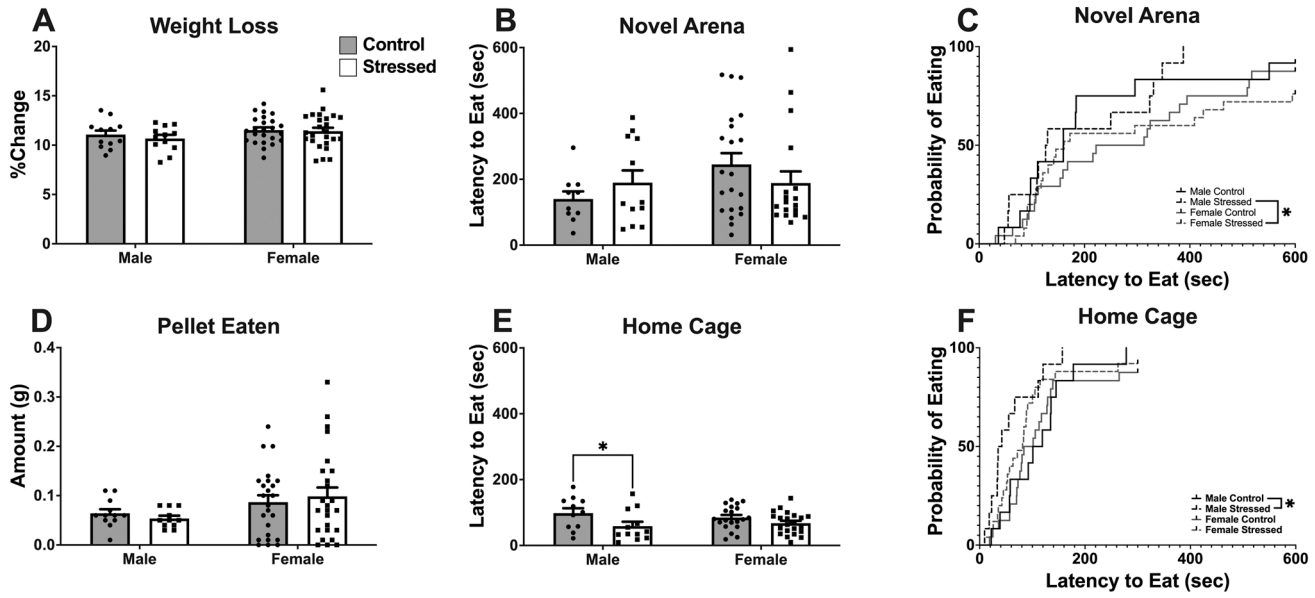
Hcrtr1 is the gene that encodes for the orexin receptor subtype 1. Orexin is a neuropeptide that is synthesized exclusively in the hypothalamus, but projects to various regions throughout the brain, including the BNST (Peyron et al., 1998). Whereas previous research has established orexin's modulation of appetite and nutrition, more recent studies have implicated its roles in chronic stress as well. This research has shown that an unpredictable chronic stress model, similar to CVMS, lead to an increase in *c*-fos expression in orexin neurons and that the orexin antagonist, almorexant, reduced stress-related depressive behaviors (Nollet et al., 2011; Nollet et al., 2012). Additionally, direct injection of orexin-A into the BNST has been shown to lead to an increase in anxiety-like behaviors (Lungwitz et al., 2012). This, along with the sex-related differential expression, indicates that orexin signaling in the BNST should be investigated for its involvement in the sex differences found in response to chronic stress.

Our behavior data indicate that there is a sex-related difference in how chronic stress is processed, particularly around the estrous cycle and the gonadal steroid milieu of the mouse. In the OFT, we see that stressed mice were generally more avoidant spending less time in the center. In females, the effects of CVMS were dependent on the estrous stage – stressed females in proestrus/estrus exhibited more avoidance behaviors than non-stressed females and females in diestrus regardless of stress condition. We also observed in the EPM where proestrus/estrus stressed females responded to CVMS by spending more time in the closed arms, which was similar to the behavior of the diestrus females regardless of treatment. Overall, these data indicate that CVMS does not alter avoidance behaviors in diestrus females despite these females exhibiting more avoidance behaviors than proestrus/estrus females.

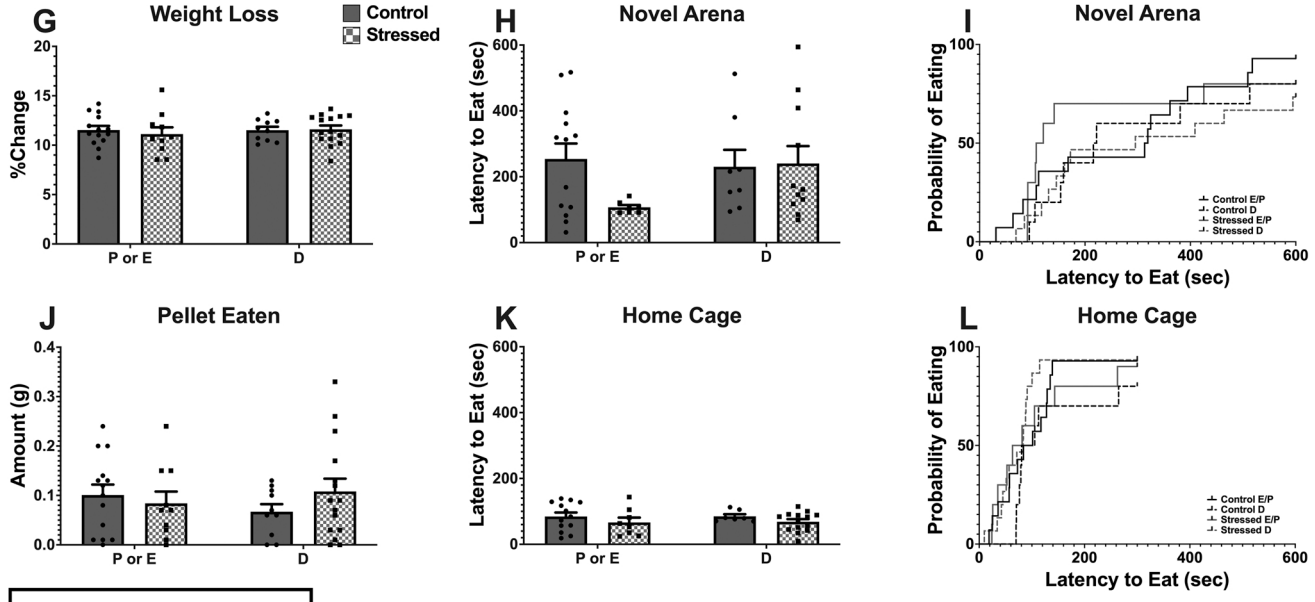
Our results agree with previously published research that found female mice in a high gonadal steroidal state or ovariectomized mice with estrogen replacement performed worse in cognitive tasks following stress than low-estrogenic counterparts, demonstrating that estrogen is a significant factor in stress signaling (Shansky et al., 2004). Thus, female mice in a high estrogen state, and more specifically proestrus, may be predisposed to anxiety-related conditions. However, we have also shown that diestrous females, regardless of treatment, were more avoidant than their proestrous counterparts, suggesting that a low-estrogenic state reduces sensitivity to chronic stress by producing more avoidance behaviors. Furthermore, many studies have also exhibited that estrogen has anxiolytic effects. One found that estrogen administration resulted in less avoidant behaviors in the LDB, supporting our results (Walf and Frye, 2009). In clinical research, postmenopausal women were more stressed according to a stress a test than premenopausal women, which was ameliorated by estrogen administration (Lindheim et al., 1992). Another one found that low estrogen levels were a risk factor for the development of PTSD in women (Glover et al., 2012). Collectively, this suggests that estrogens can have both anxiolytic and anxiogenic effects. Research has found that selective activation of ERα lead to an increase in activity of the HPA axis, while selective activation of ERβ decreased the activity (Handa et al., 2012), showing a model for which estrogens may have differing results on stress signaling, with some pathways priming for stress and others decreasing stress.

Interestingly, we do not see many effects of chronic stress on males suggesting that CVMS is more impactful on female behavior than male behavior. Male avoidance behaviors were only altered in the probability to eat in the NSF. Previous work from our labs using a similar CVMS paradigm, but in a different building and behavior rooms, found consistent effects of CVMS on avoidance behaviors in male mice (Hu, Liu et al., 2020). Other publications have found that chronic mild stress paradigms, like our CVMS, results in increased avoidance behaviors in male rodents (D'Aquila et al., 1994; Mineur et al., 2006). However, CVMS results are historically conflicting, and some studies show that females are more susceptible to the paradigm than males (Franceschelli et al., 2014). Additionally, circulating testosterone levels in male rodents has been found to be extremely variable (Machida et al., 1981). Testosterone may also be affecting these behaviors, which we are not seeing since all males were treated as a homogenous group. It might be relevant to test testosterone levels and separate males into high and low

Males and Females



Females separated by estrous stage



Significance Tables

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		Sex	Treatment	Interaction
Weight Loss	F(df)	F (1, 69) = 2.556	F (1, 69) = 0.4667	F (1, 69) = 0.1426
	p-value	P=0.1145	P=0.4968	P=0.7069
Latency to Eat (Novel)	F(df)	F (1, 57) = 1.930	F (1, 57) = 0.00854	F (1, 57) = 2.023
	p-value	P=0.1702	P=0.9267	P=0.1604
Survival Curve (Novel)	χ square	**χ=4.269**	χ=0.02690	
	p-value	**P=0.0388**	P=0.8697	
Pellet Eaten	F(df)	F (1, 68) = 3.788	F (1, 68) = 0.00121	F (1, 68) = 0.4149
	p-value	P=0.0558	P=0.9723	P=0.5216
Latency to Eat (Home)	F(df)	F (1, 61) = 0.0464	**F (1, 61) = 7.483**	F (1, 61) = 1.197
	p-value	P=0.8302	**P=0.0081**	P=0.2783
Survival Curve (Home)	χ square	χ=0.7747	**χ=4.058**	
	p-value	P=0.3788	**P=0.0440**	

N

		Estrous Stage	Treatment	Interaction
Weight Loss	F(df)	F (1, 45) = 0.2461	F (1, 45) = 0.1204	F (1, 45) = 0.2793
	p-value	P=0.6223	P=0.7302	P=0.5997
Latency to Eat (Novel)	F(df)	F (1, 35) = 1.207	F (1, 35) = 1.886	F (1, 35) = 2.475
	p-value	P=0.2795	P=0.1784	P=0.1246
Survival Curve (Novel)	χ square	χ=1.590	χ=0.2499	
	p-value	P=0.2073	P=0.6171	
Pellet Eaten	F(df)	F (1, 45) = 0.04261	F (1, 45) = 0.2663	F (1, 45) = 1.504
	p-value	P=0.8374	P=0.6084	P=0.2264
Latency to Eat (Home)	F(df)	F (1, 38) = 0.01288	F (1, 38) = 2.278	F (1, 38) = 0.00672
	p-value	P=0.9102	P=0.1395	P=0.9351
Survival Curve (Home)	χ square	χ=0.003585	χ=1.463	
	p-value	P=0.9523	P=0.2265	

(caption on next page)

Fig. 5. Novelty Suppressed Feeding test. (A) Amount of weight loss during the 24-h fasting period as a percent of total body weight. (B) Amount of pellet eaten during the test. (C) Latency to eat in the novel arena. (D) Latency to eat in the home cage. (E) Kaplan-Meier survival curve for the probability to eat in the novel arena. (F) Kaplan-Meier survival curve for the probability to eat in the home cage. (G) Amount of weight loss during the 24-h fasting period as a percent of total body weight with males excluded and females separated by estrous stage. (H) Amount of pellet eaten during the test with males excluded and females separated by estrous stage. (I) Latency to eat in the novel arena with males excluded and females separated by estrous stage. (J) Latency to eat in the home cage with males excluded and females separated by estrous stage. (K) Kaplan-Meier survival curve for the probability to eat in the novel arena with males excluded and females separated by estrous stage. (L) Kaplan-Meier survival curve for the probability to eat in the home cage with males excluded and females separated by estrous stage. Data are presented as mean + /- SEM. A, B, C, D, G, H, I, and J analyzed by two-way ANOVA with Holm-Šidák post-hoc comparisons. E, F, K, and L analyzed by Log-rank test. (*=0.05–0.01). (M) Table of significance values for omnibus statistics when males are included, and females are collapsed. (N) Table of significance values for omnibus statistics when males are excluded, and females are separated by estrous stage. Bolded cells indicate significance.

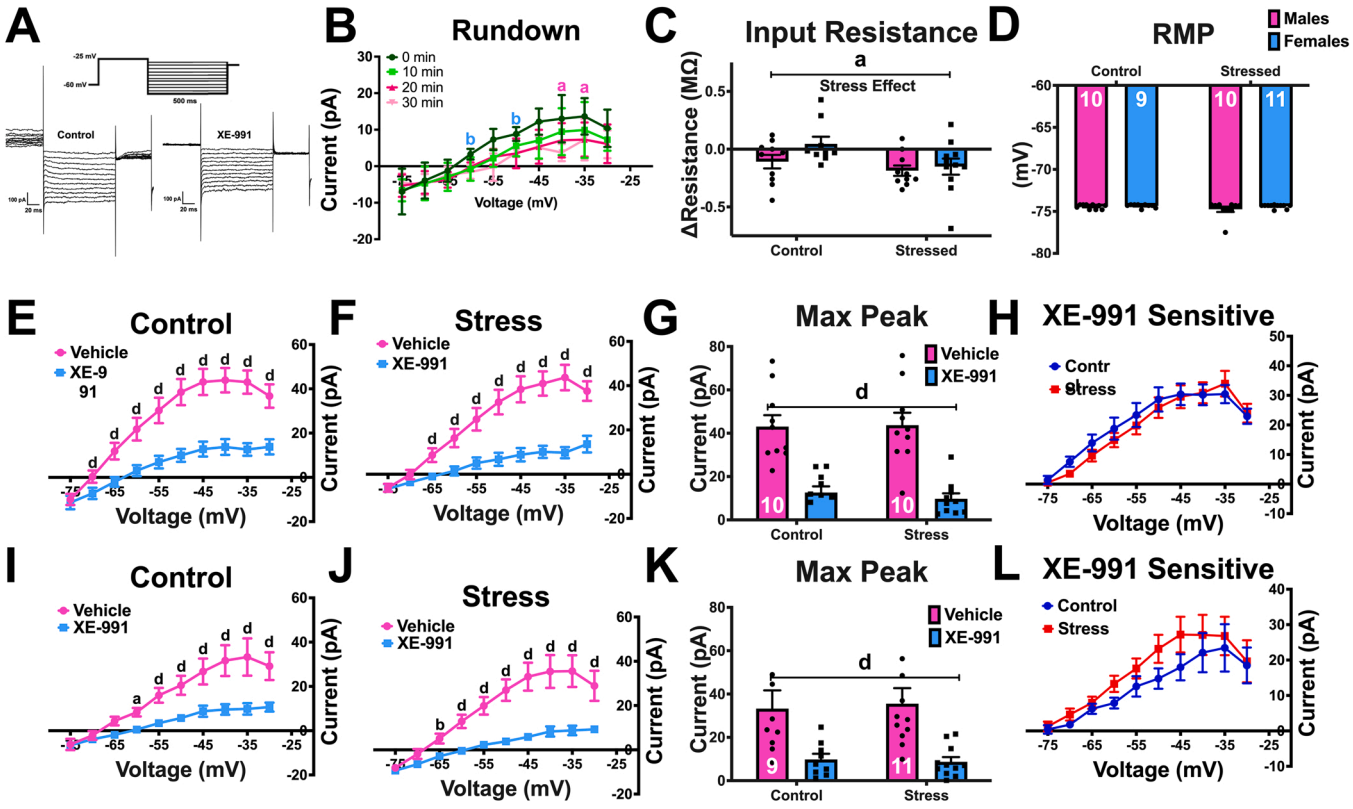

Fig. 6. M-current activity in bed nucleus of the stria terminalis Neuropeptide Y neurons from stressed and non-stressed mice. (A) M-current protocol and representative traces. (B) Rundown of M-current. (C) Resting membrane potential and (D) change in input resistance after perfusion with XE-991 from male and female stressed and non-stressed mice. I-V plots from − 75–30 mV after XE-991 perfusion in (E) control males and (F) CVMS-exposed males. (G) I-V plot of XE-991 sensitive current in males. (H) Current at max peak in males. (I-L) Control and CVMS-exposed females. Data are presented as mean + /-SEM and analyzed with two-way ANOVA with Holm-Šidák post-hoc comparisons (a=0.5–0.1, b=0.1–0.01, c=0.01–0.001, d=<0.001). Blue letters represent comparison between 0 min and 30-min recordings. Pink letters denote comparison between 0 min and 20-min recordings.

testosterone groups in future projects.

Previous research in our own lab has shown that chronic stress alters the excitability and activity of the M-current in the CRH-expressing oval BNST neurons, which was associated with an increase in anxiety-related behaviors (Hu, Liu et al., 2020). We have previously described M-current activity in hypothalamic NPY-expressing and CRH-expressing neurons (Roepke et al., 2011; Hu et al., 2016; Yasrebi et al., 2016). This is the first characterization of M-current activity in adBNST NPY-expressing neurons. As BNST NPY neurons are implicated in behavior and stress signaling, we were interested in the potential effects of chronic stress on adBNST NPY-expressing neurons. The lack of effects on the M-current of these neurons suggests that BNST NPY-expressing neurons are not responsive to chronic stress, at least in terms of M-current activity, nor are they major mediator of chronic stress. The implication that NPY neurons are not a mediator of chronic stress in the BNST is supported by previous findings that inhibitory post-synaptic currents in these neurons were not altered by a chronic stress paradigm (Pleil et al., 2012). However, the adBNST is heterogeneous region; thus, other adBNST neurons should be investigated. We are seeking to continue this research by examining the response to chronic stress in CRH-expressing neurons from male and female mice and assessing excitatory inputs, inhibitory inputs, the M-current, and firing properties in these neurons.

Our study demonstrates that chronic stress signaling in the brain is dependent on sex and hormone status of the individual and is cell-type specific. It additionally shows that the NPY neurons of the BNST are not likely candidates for the formation of anxiogenic responses following chronic stress. Further research is needed in how estrogen plays a role, which neuronal subpopulations are important for the formation of mood disorders, and how chronic stress alter the neurophysiology and transcriptome of neurons in the BNST.

CRediT authorship contribution statement

Thomas Degroat: Formal Analysis, Investigation, Data Curation, Writing - Original Draft, Project Administration. **Kimberly Wiersielis:**

Formal Analysis, Investigation, Data Curation, Writing - Review & Editing, Project Administration. **Katherine Denney:** Validation, Formal Analysis, Writing - Review & Editing. **Sowmya Kodali:** Formal Analysis, Investigation, Data Curation. **Sierra Daisey:** Formal Analysis, Investigation, Data Curation. **Jessica Tollkuhn:** Validation, Writing - Review & Editing. **Benjamin A. Samuels:** Conceptualization, Methodology, Writing - Review & Editing, Funding Acquisition. **Troy A. Roepke:** Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Project Administration, Funding Acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Aknowledgments

This research is funded by NIH/NIMH R01MH123544 (T.A.R. and B. S), USDA-NIFA NJ06195 (TAR), and P30ES05022 (T.A.R.).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2023.106920.

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