2023

Effect of Chronic Stress in Female PAC1 Mice

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A thesis submitted in partial fulfillment of the requirements for the degree of:

Bachelor of Science

in

Psychological Sciences

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The University of Vermont Honor's College

Spring 2023

Acknowledgments

This project would not have been possible without the help of Mahafuza Aktar, who personally guided me through every stage of the process. Her patience in working with me is greatly appreciated and I will miss my time in the lab with her.

Dr. Hammacks willingness to take me as an advisee last minute is the only reason that I was able to even begin this project, and I feel honored for the opportunity. He has been so encouraging throughout and his confidence in my abilities as a student were always made clear.

And of course, a big thanks to my best buddy Ms. Lidia M Anderson for her support in completing this project. She put hours of her time into editing and even more just listening to me talk about my work. Her ability to put up with my antics is unmatched and I am so grateful for her presence.

I would also like to thank the members of my committee for making space in their schedules during the busiest time of the semester to participate in my defense. I am so very grateful.

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Abstract

Stress related disorders such as generalized anxiety disorder and post-traumatic stress disorder (PTSD) weigh heavily on the healthcare system in the United States (DuPont et al. 1996). Pituitary adenylate cyclase-activating peptide (PACAP) system dysregulation has been implicated with stress disorders such as anxiety and Post Traumatic Stress Disorder (PTSD). This project seeks to add to existing research on the PACAP- PAC1 system's correlation with a maladaptive response to chronic variant stress (CVS) exposure, and a possible sex difference in these outcomes. The experiment involved a two-group design with female PAC1-Ires-Cre mice comparing anxiety like behavior from CVS exposure. We hypothesized that after exposure to chronic stress, there would be an observable difference in the distribution and projection of PAC1 expressing neurons in the BNST of female PAC1 Cre mice as well as increased anxiety-like behavior. Results indicated no significant difference in anxiety-like behavior, and an increase in projection fibers in the substantia nigra, lateral hypothalamus, paraventricular cortex, and the bed nucleus of the stria terminalis. Further research to investigate the role of estrogens in relation to these findings should be explored.

Keywords: Pituitary adenylate cyclase activating polypeptide, Bed nucleus of the stria terminalis, Substantia nigra, Paraventricular nucleus, Chronic variant stress, Sex differences.

Effect of Chronic Stress in Female PAC1 Mice

Minimal research investigating the effects of stress in female mice has been performed, thus, leaving a large gap in the literature. Understanding the impact chronic stress exposure has on females that leads to the development of stress disorders such as PTSD and anxiety could help explain the sex differences in the incidence of these conditions. Exploring the impact of stress on the physical anatomy of neuronal connections from the bed nucleus of the stria terminalis in female mice could provide better understanding of their functions. This project will explore the behavioral effects of chronic stress and the presence of PAC1 projecting neurons from the BNST in female mice.

Stress

The term stress refers to any situation or action that brings the body away from homeostasis. Multiple categories of stress exist and can result in different outcomes that are mediated by specific characteristics of the stressor (such as intensity, duration, and predictability) and the resources available to the individual (Richard & Bryant, 2017). Mild stressors are experienced daily as the body strays from homeostasis such as experiencing hunger or engaging in physical activity. Moderate or tolerable stressors such as presentations and tests are not within control of the individual but resources for coping are available to reduce the impacts. These low-level stressors are part of normal life and even have positive effects on health from reducing the risk of heart disease to increasing the numbers of immune cells in the innate immune system (Sanchis-Gomar et. al., 2012). Toxic stressors such as financial concerns, work, school, interpersonal relationships, as well as traumatic events can have dramatic impact on the body's system for managing stress (Sanchis-Gomar et. al., 2012). This is often referred to as an

allostatic load, a point in which the body's persistent efforts to return itself to homeostasis causes wear and tear on the systems involved in the stress response (Burrow et al. 2019).

Health impacts of chronic stress

Chronic unpredictable stress can cause dysregulation of the stress response system resulting in disorders such as anxiety, a condition effecting the lives of over 40,000 individuals in the United States (DuPont et. al., 1996). There is also evidence that chronic stress has an impact on the immune system and can cause long term inflammation in the body's arterial system. (Miller & Blackwell, 2006). This inflammation can lead to atherosclerosis, the precursor to coronary heart disease (CHD), the leading cause of death for adults in developed countries (Miller & Blackwell, 2006). A study performed by Miller & Blackwell (2006) found that children who experience chronic stress at a young age become 3.1 times more likely to develop CHD in adulthood. Like stress and anxiety, Post Traumatic Stress Disorder (PTSD) has also been implicated to have serious medical consequences (Richard A. Bryant, 2017). The impacts of severe stress on individuals and the healthcare system are large and efforts are being made to better understand the underlying mechanisms in attempt to relieve this weight.

PTSD sex difference

Post-traumatic stress disorder (PTSD) is a condition that effects around 6% or 13 million American adults (*Va.gov: Veterans Affairs* 2018). However, not every adult exposed to a trauma will develop PTSD the incidence rate is lower than the number of individuals who experience or witness a traumatic event. Not everyone exposed to a traumatic event will develop PTSD and the reasons for this are unclear. Women report a prevalence of generalized anxiety and PTSD twice as much as men; however, specific etiology has not yet been defined (Richard & Bryant 2017), (Veterans association 2017). One environmental factor impacting this data is the type of trauma

experienced by the individual (Kessler et. al., 2017). Women are at higher risk of experiencing sexual or relationship traumas than their male counterparts, and these experiences may be correlated with more PTSD symptoms (Kessler et. al., 2017). A possible neurobiological correlation impacting sex difference in stress disorder prevalence was demonstrated through a study of a civilian population with PTSD symptoms (Vyas et al. 2002). results indicated increased rates of the enzyme PACAP38 in female participants compared to their male counterparts (Vyas et al. 2002). Dysregulation of endocrine systems related to stress have been implicated as possible causal factors for the development of PTSD.

HPA axis

The hypothalamus-pituitary-adrenal pathway (HPA-axis) is a feedback loop in the endocrine system critically involved in activation of the stress response (Borrow et. al., 2019). It is also responsible for returning the body to homeostasis once the stressor has dissipated (Borrow et. al., 2019). When exposed to a stressor, the HPA axis functions by activating the sympathetic nervous system to prepare the body for fight or flight response, such as increased heart rate and pupillary dilation (Borrow et. al., 2019). In response to stress, the hypothalamus releases corticotropin release factor (CRH) which signals to the anterior pituitary to release adrenocorticotropic hormone (ACTH) (Borrow et. al., 2019). Next, ACTH communicates with the adrenal cortex to release anti-inflammatory glucocorticoids (Borrow et. al., 2019). One example of this is the release of cortisol, a strong anti-inflammatory hormone that helps the body recover from stress. After the threat has dissipated the HPA axis functions as a negative feedback loop, communicating with the medial prefrontal cortex to return to homeostasis (Borrow et. al., 2019). When the body is experiencing toxic stress, this pathway can become hyperactive resulting in desensitization to cortisol (Borrow et. al., 2019). This is referred to as the

glucocorticoid resistance model (Borrow et. al., 2019). When chronic toxic stress is experienced, levels of cortisol remain in a constant high level, resulting in desensitization to its anti-inflammatory effects overtime (Borrow et. al., 2019). When this occurs the negative feedback loop of the HPA axis is unable to function, and the pathway continues to fire in the absence of a threat (Borrow et. al., 2019). The HPA axis may be controlled by several limbic circuits and peptides including Pituitary adenylate cyclase activating polypeptide (PACAP).

PACAP

Pituitary adenylate cyclase activating polypeptide is a neuropeptide found throughout both the central and peripheral nervous systems (Arimura 1992). This protein plays a role in a breadth of functions including, but not limited to, vasoregulation, neuromodulation, and neurotransmission. (Arimura, 1992). It has been observed for its anxiogenic effects and is heavily concentrated in the hypothalamus and the BNST (Gargiulo et al.,2020) Chronic stress has been shown to upregulate PACAP and PAC1 receptors in the BNST (Hammack et al., 2009). Experimentation of increasing levels of PACAP inorganically through direct injection into the BNST elicited anxiety-like behaviors in mice such as light enhanced startle and anorexia (Roman et al., 2014). It has also been demonstrated that promotion of the PAC1 receptor antagonist PACAP (6-38), an enzyme that breaks down PACAP, reduced anxiety-like behaviors in mice (Roman et al. 2014). This evidence adds to the assumption that BNST PACAP is a mediator between exposure to long-duration stressors and anxiety disorders, although the exact interactions are not fully understood. Exposure to chronic stress increased dendritic length and arborization in mice, specifically in the BNST, indication that stress disorders may be related to strengthening of neuronal connections within the stress system. (Vygas, 2002).

Bed Nucleus of the Stria Terminalis

The Bed Nucleus of the Stria Terminalis (BNST) functions as an extension to the central amygdala, an area in the brain associated with emotional regulation and fear learning (Braas & May, 2010). In rodent models the BNST has been demonstrated to play a critical role in mediating emotional behaviors (Ressler et. al., 2011). The BNST has also been demonstrated to be a critical area for mediating the long-term reaction caused by a stressor exposure (Toufexis, 2007). This area has been shown to experience significant neuroplasticity through dendritic arborization in response to long duration stressors, an indication that increased neuronal signaling in the BNST may be correlated with stress disorders (Vyas et. al., 2002). This influence on stress induced behaviors is being investigated for its correlation to dysregulation of the HPA-axis.

Estrogen and PACAP on PTSD

A possible explanation for sex differences in incidence of PTSD and other stress related disorders is the hormone estrogen, as it has been demonstrated to increase BNST PACAP and PAC-1 receptors (Allen &Gorski 1990). Ressler and colleagues (2011) explored this area and found a positive correlation between estrogen and the (PAC1) *ADCYAP1R1* gene in women with PTSD. A correlation has been observed between PACAP38, an enzyme responsible for the degradation of PACAP, and the three main symptom clusters required for a diagnosis of PTSD (avoidance, intrusive re-experiencing, and hyperarousal) (Ressler et. al., 2011).

Projections

Exploring anatomical regions involved in the stress circuitry can be completed through lesion studies as well as the use of anterograde and retrograde tracers. Anterograde tracers examine afferent pathways sending information and retrograde tracers explore efferent pathways in which areas are receiving in formation. Increasing neuronal connections throughout the

efferent side of the sympathetic nervous system can result in heightened startle response and hyperactivity of the stress regulatory system, a characteristic associated with anxiety. Using fluorescent microscopy axonal fibers can be visualized. Quantifying this data can be completed using image analysis software to measure the brightness and area covered by fibers.

Behavioral measures of emotion

To examine these emotional aspects in animal models, extensive research has been conducted to classify certain rodents' behaviors as corresponding with stress (Walker and Davis 2002). Stressor exposure is correlated with reduced weight gain and anorexia in mouse models and thus are good measures to ensure validity of research. Some behaviors that indicate distress include light enhanced startle, time spent in the open arms of a plus maze, anorexia, and lack of weight gain (Carola et al. 2002). Exposure to chronic variant stress (CVS) showed both behavioral changes as well as physiological changes throughout HPA axis (Handa, 2019). Thus, CVS is recognized as valid method for inducing and observing the effects of long duration stress in animal models.

Genetically modified mice

This project utilized genetically modified animals developed to show a fluorescent marker when in the presence of the tissue-specific promotor (Kim et. al., 2018). These are called transgenic knockout mice and have been engineered to observe and measure the number of PAC1 expressing neuronal projections in samples of brain tissue (Kim et. al., 2018). This relies on fLEX switches, a method used to turn genes on or off when exposed to the specific promotor they are engineered for (Kim et. al., 2018). This is completed in one generation by breeding a cre mouse with a homozygous loxp mouse (Kim et. al., 2018). This occurs through cre recombinase which recognizes specific DNA sequences and makes a SNP flanking both sides of the codon

section, resulting in turning the gene off (Kim et. al., 2018). In this project PAC1-Ires-Cre mice injected with a fluorescent marker Adeno-associated-virus-2-EF1a-DIOmCherry will show fluorescents in the presence of PAC1 receptors.

This project explores the effects on chronic stress on PAC1 transcriptions within the stress circuitry of female PAC1 Cre mice. Data was collected through observational behavioral testing and fluorescent microscopy analysis of anatomical areas. Results indicated projection fibers from the bed nucleus of the stria terminalis.

Methodology

Animals

Results from multiple power analysis indicated that 8 animals per experimental condition should allow for adequate statistical power behavioral and anatomical experiments. An example from previous work within this lab pertaining to pERK staining in rats (control 43.1 cells vs stimulated 74.5 cells) with an average standard deviation of 19.01, expected power of 0.8 and 4 pairwise comparisons, results from a power analysis indicated a sample size of 8 per condition. This is consistent with past numbers we have used for behavioral and anatomical studies with mice.

16 (Female PAC1 Cre mice between the ages of 12 and 15 weeks old were obtained from Dr. Bradford Lowell's laboratories at Deaconess Hospital, Harvard University). Mice were given a one-week period after delivery to allow for recuperation before experimentation. Animals were housed in groups and followed a 12-hour light/ darkness schedule with food and Institutional Animal Care and Use Committee of the University of Vermont.

Procedures

Adeno-associated-virus-2-EF1a-DIOmCherry (~1 × 1012 viral genomes/ml) was used to identify PAC1 cells. The virus was obtained from the University of North Carolina at Chapel Hill Vector core and was be injected stereotaxically into the BNST. This procedure was performed while anesthetized by 1.5-2% isoflorine administered in an induction chamber. After animals were sedated, their heads were shaved, and they were secured in position with ear bars. Scalp was sterilized with povidone-iodine and an incision was made to expose the skull. Two burr holes were then made into the skull. Next, 0.5 µl of the AAV2-EF1a-DIO-mCherry reporter vector was injected using a Hamilton 7001 KH syringe, 1ul, needle size- 25s gauge and length - 70 mm into the BNST at the following coordinates: AP = +0. 1 mm, ML= -1/+1 mm, DV = - 4.6 mm. This was injected using a pump over a 5-minute duration. The syringe was kept in place for another period of 5 minutes before being removed slowly over 3 minutes, both practices functioned to minimize any efflux of the virus. After the procedure, mice were sutured using Vicryl and administered ketoprofen (5mg/kg) as an analgesic to reduce pain. Mice were then placed on a heating pad until they awoke, where they were replaced back into their home cages. The day after surgery the mice were weighed and administered a final dose of analgesic. The mice were then given 14 days of recovery time before experimentation began.

Chronic Stress Paradigm

After recovery from surgery mice were then randomly assigned to either control or experimental groups. Control groups were handled daily while the experimental groups underwent various types of stressors including foot shock, forced swim, pedestal standing, oscillation, and restraint for 14 days. Stressors were administered at the same time daily in random order to elicit anxiety-like behavior (Hammack et. al, 2009)**.** Foot shocks were administered by placing mice in a plexiglass conditioning chamber that is 30cm x 25cm x 35cm

(L x W x H). After five minutes of habituation animals were administered two 1.0 mA, 2 second foot shocks at intervals of 5 minutes through the grid floor. Three minutes after the final foot shock animals were then returned to their home cages. Forced swim involved placing mice in a cylindrical container 29cm x 37 cm (D x H) that was 2/3 full of room temperature water. Animals were forced to swim for 5 minutes before being removed and placed into a fresh cage to dry for 30 minutes. Once dried, mice were then returned to their home cages. Pedestal standing included placing mice on a pedestal for 30 minutes and then directly returning them to their home cages. The oscillation exercise was completed by securing mice on a clinical rotator where they were oscillated for 30 minutes at medium speed. Restraint exercise was performed by placing mice in a 200ml glass beaker for one hour.

After 24 hours from the final stressor exposure, mice were placed on an elevated plus maze where they were tested and observed for anxiety-like behavior **(Figure 1)**. The plus maze apparatus consists of two sets of arms in which two are open and measured to be 25cm x 5 cm x 0.5cm (L x W x H) and two enclosed arms of the same length and width but the walls are taller (16cm) in height. The room in which the plus maze exercises are completed in was illuminated with a red bulb 6 Lux (lx). Animals were placed individually in the center of the maze facing towards a closed arm. The door to the room was then shut to avoid disruptive noise, and the mouse was allowed to explore the maze for 5 minutes. All activity was recorded through a webcam on the ceiling and was observed using Quicktime player installed on a Mac laptop computer. Scoring was then completed by an observer who is blind to the treatment condition to eliminate bias. Mice were score by time spent in closed arms, open arms, and number of times they cross from

the closed to open arm. Mice who exhibit anxiety- like behavior spend more time in the closed arm than the open arm, and cross between the two less frequently as well (Carola et al, 2002).

Analysis

Directly after mice were removed from the plus maze, they were perfused using PBS and 4% paraformaldehyde (PFA). After removal from the skull the brains were left in a 4% PFA as a fixative for 24 hours. After one day the brains were then treated with a 30% solution of sucrose, a cryoprotectant. Next, samples were sectioned into 40 µm thick slices with the cryostat machine before being mounted on Superfrost Plus (Fisher Scientific) slides. They were then covered with citifluor antifadent solution to preserve them. After mounting, images were collected through an epifluorescence microscope in which PAC1 receptor presenting neuron fibers show fluorescence and images were collected with Stereo Investigator software. Quantification and analyzation of results was completed using the software Image J. Significance Data was calculated using SPSS to measure the significance of the data between experimental and control conditions.

Results

Attrition

One animal from the control condition did not survive the surgery phase of the experiment resulting in the control group including 7 animals and the stress group containing 8 animals. All other animals recovered from procedures and were included in the results of the project. One animal from the control condition was not recorded in the plus maze data due to technological error.

During microscopy 3 samples were removed from analysis as the initial injection of the virus Adeno-associated-virus-2-EF1a-DIOmCherry was not injected into the correct stereotaxic coordinates to align with the BNST. Thus, the final data pool of included 5 control mice in the control condition and 7 in the stress condition.

Behavioral Testing

No significant difference was observed in total number of crossings with independent sample t test result of t (12) = 1.078, p =.151 **(Figure 3b)**. Time spent in open arms of the plus maze was not measured to be significant t (12) = .717, p = .224 **(Figure 3c)**. Number of entries to open arms of the plus maze were not significant with t (12) = 1.136, p = .139 **(Figure 3a)**. Weight change between stress and control conditions did not show a significant difference t (12) = 1.649, p = .063 **(Figure 2)**.

Projection Fibers

Images collected from fluorescent microscopy in the stress condition showed projections from the bed nucleus of the stria terminalis to the paraventricular nucleus, substantia Nigra, and lateral hypothalamus **(Figure 4)**. This is possible evidence for these regions as part of the stress response circuitry as result of chronic variant stress exposure. Results of independent samples t test comparing the sample means from a pixel analysis were non-significant t (47.8), P = .959 **(Figures 5,6)**

Discussion

Results from behavioral testing were measured to be non-significant for anxiety-like behavior across conditions. Evidence for sex differences in stress behaviors is emerging in the literature but this major gap in data is significant to this project. As previous research has focused only on male mice, behavioral measures of stress and anxiety may be irrelevant for measuring female stress induced behaviors (Palanza & Parmigiani, 2017). Indicators of stress in female mice may be related to movement levels of increased grooming behaviors, instead of increased light enhanced startle and time spent in closed arms of the plus maze (Toufexis, 2007). As behavioral data was recorded with video footage, future work could be done with existing data to score for these specific behaviors. Total weight change across cohorts was not statistically significant although, a trend towards significance was observed. Results from image analysis of pixel fluorescence were not statistically significant across cohorts. Therefore, the null hypothesis was retained for this experiment.

As previously stated, some implications for the role of estrogen to influence PACAPergic systems in rodents has been demonstrated, a possible causal factor for the sex difference in PTSD incidence. Evidence for the impact of sex hormones estrogen and testosterone providing different evolutionary advantages is a focus for current research. Female animals provide a caregiving role and when exposed to a threat must expend energy to protect their young, thus it is evolutionary advantageous to move more in response to a stressor. Males with higher levels of testosterone exhibit heightened light enhanced startle in multiple studies (Toufexis, 2007). However, there is conflicting results published in the literature regarding estrous effects on stress behaviors (Toufexis, 2007). Difference in gonadal hormone levels could account for the lack of anxiety-like behavior observed in the stress condition in this experiment, as some stages of the

cycle may serve as a protective factor for fear learning (King et al., 2017). A factor likely contributing to the contradictory literature surrounding the effect of estrogens on fear learning are differences in life stages such as pregnancy or lactation (King et al., 2017), (Toufexis, 2007). Such as evidence that lactating mice are more resistant to fear learning and show higher aggression than during pregnancy (Toufexis, 2007). Without proper background information about behavioral indicators of stress in female mice, and as estrous cycles were not recorded, no major implications can be made regarding anxiogenic effects from this project.

PAC1 transcriptions projecting from the BNST were observed in the lateral hypothalamus, paraventricular nucleus, and substantia nigra. Variables in the statistical analysis of this data could be due to inconsistency in the injection sites in the surgery phase of the trial. PAC1 transcriptions in these areas of the hypothalamus implicates dendritic branching and arborization, evidence of cross talk between regions, a possible indication for involvement in the stress circuitry.

Paraventricular Nucleus (PVN)

The paraventricular nucleus is a section of the hypothalamus in the supraoptic region and is understood to be a major control center for regulation of the autonomic nervous system including controlling functions such as stress, metabolism, growth, reproduction, as well as digestion and other basic autonomic and neuroendocrine functions (Boucher et al., 2022). The PVN has afferent projections to many regions throughout the nervous system to respond to and control such functions (Boucher et al., 2022). Relevance to this project includes as the PVN has connections that help to control the HPA axis and corticotropin release hormone (Boucher et al., 2022). Connections between the BNST and PVN have been previously established and align

with existing data implicating stress as a predictive factor for psychopathologies such as anxiety and PTSD.

Lateral Hypothalamus

The lateral hypothalamus is associated with endocrine functions involved with feeding and eating behaviors (Berthoud et al., 2019). This has been demonstrated through lesion studies in which damage to this region resulted in anorexia in rats (Fakhoury et. al., 2020) The lateral hypothalamus is also associated with fear responding (Berthoud et al., 2019).

Substantia Nigra

This area of the midbrain is involved in controlling motor movements as well as governing of the reward system (Das et. al., 2007). This connection to the reward system has led to understanding the substantia nigra plays role in habit formation (Hassan & Benarroch, 2015). As there is evidence for stress increasing habit formation, our result indicating PAC1 projections from the BNST to the SN may provide further insight into this phenomenon (Hassan & Benarroch, 2015). Increased PACAP in this area has also been implicated as a possible treatment for Parkinson's disease due to its ability to serve as a protective factor for dopaminergic neurons. Our observation of PAC1 projection fibers from the BNST to the SN is a new finding that provides many routes for future research to better understand the implications.

Limitations

Although camera settings were unanimous for image capture, control for photo bleaching from length of fluorescent exposure was not, leading to a possible reduction of expression in some samples. Although this information effected the ability to quantify data between control and experimental conditions, it does not minimize data collected regarding anatomical locations of interest as that is observed in a qualitative manner. The lack of existing data for behavioral

models of stress and anxiety in female mice posed a major limitation to this work. Without recording estrous cycles in the subjects, or having a male control group, it is not possible to draw conclusions about a sex difference from this data.

The role of cortisol in measuring chronic stress could be examined in relation to estrogen. Observation of other stress emotion behavior measures such as light induced startle could be investigated to examine effects of multiple types of stress in females. Other studies have shown a correlation between estrous cycles and PACAP, and some effects of PACAP on fear learning, but the combination of the two to look at estrous effects on PACAP regarding chronic variant stress have yet to be assessed.

The PACAP/PAC1 system has been recognized for its extensive capabilities including increasing neuronal growth. This can be a protective factor in regions such as the substantia nigra in individuals with Parkinson's disease, a condition characterized by neuronal death, by serving as a protective factor for dopaminergic neurons in the substantia nigra. Further research in this area is being completed to explore the use of PACAP as a therapeutic intervention for disorders such as Parkinson's disease. PACAPs ability to heighten neuronal connections can also be correlated with disorders such as PTSD and anxiety when high levels of the peptide are present within the stress circuitry. The ability for PACAP ergic systems to be either a protective or detrimental factor for disorders and disease appears to be specific to exact anatomical locations within the brain. If these differences can be understood therapies could be developed to target specific regions and the disorders correlated with them. The historical disregard for investigating sex differences in psychopathology has directly impeded the ability to develop efficacious treatments in female born individuals. When investigating these systems and their functions it is essential to include female models in the research.

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Tables

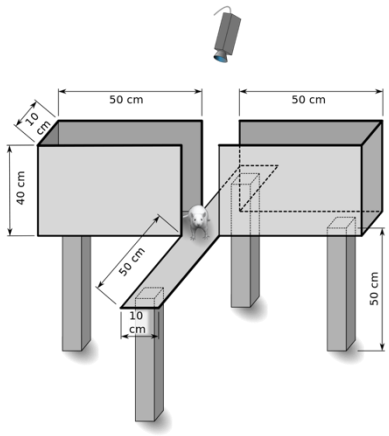
Table 1:

Day	Stressor	Duration
1	Foot shock	2 seconds (X 2)
2	Swim	5 minutes
3	Oscillation	30 minutes
4	Pedestal	30 minutes
5	Restraint	60 minutes
6	Foot shock	2 seconds (X 2)
7	Swim	5 minutes
8	Oscillation	30 minutes
9	Pedestal	30 minutes
10	Restraint	60 minutes
11	Foot shock	2 seconds (X 2)
12	Swim	5 minutes
13	Pedestal	30 minutes
14	Oscillation	30 minutes

Chronic Stress Paradigm: Table 1 displays the schedule of stressors used in the chronic variant stress paradigm over the fourteen days of the experiment, and the duration the stressor lasts for.

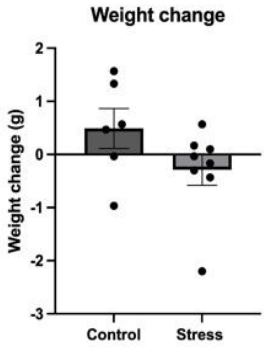
Figures

Figure 1:



Plus Maze: Figure 1 displays a sketch example of a plus maze designed to measure anxiety-like behavior in mice. It involves four arms in a x formation with two sides covered and two sides exposed. Mouse is placed in the center of the maze facing on one of the closed arms and observed for five minutes.

Figure 2:



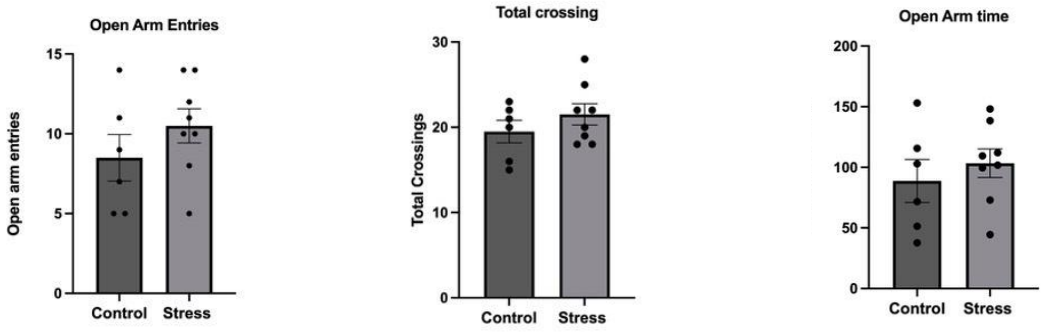
Weight Change

p = .063

Results of overall change in weights between control and stress conditions was not measured to be statistically significant. Possible skew in results from one outlier in the control condition that lost weight. P value of .063 is displayed implicating a non-significant result.

Figure 3:

(a) (b) (c)



Plus Maze

Figure 2 includes graphs displaying data from an independent samples t-test for the anxiety-like behavior as measured from plus maze recordings. (1) no significant difference was measured between the control and stress cohorts for open arm entries made in the plus maze. (2) No significant distance was measured between total number of crossings across both groups. (3) total time spent in open arms was not significantly different between groups.

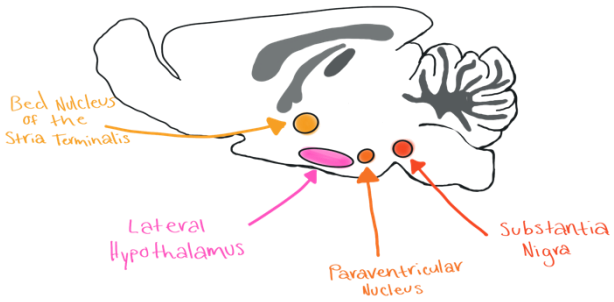
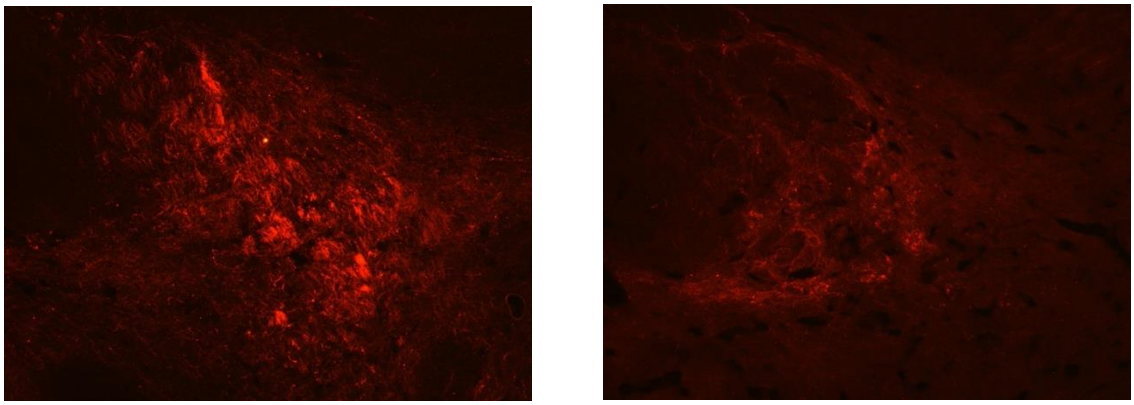


Figure 4:

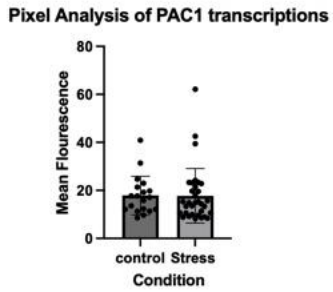
Cross section: figure 4 displays a simplified diagram of a mouse brain highlighting the regions of interest including the BNST, LH, PVN, and SN.

Figure 5:



PAC1 projections: Images display projection fibers with PAC1 receptors. No significant difference was measured between experimental and control conditions.

Figure 6:



Pixel Analysis: Figure 5 shows insignificant difference between PAC1 projection measured through pixel analysis software.