SOCIAL STATUS MEDIATES ISOLATION STRESS RESPONSE AND MOOD-RELATED SYMPTOMS IN FEMALE MICE

AN HONORS THESIS

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Abstract

Social isolation (SI) is recognized as a major risk factor for physical and psychological health problems in humans and other social species. Across species, long-term SI has been shown to increase anxiety and depressive symptoms, weaken immunity, and reduce central glucocorticoid receptor sensitivity to inhibition, leading to heightened stress responses. In our study, we investigated the effect of SI on female mice since stress-related disorders such as major depression and generalized anxiety disproportionately affect women. Importantly, we want to explore how social status moderates the effect of SI stress on the manifestation of anxiety and depressive phenotypes. This study may provide valuable neurobiological insights into what makes an individual vulnerable to isolation stress, an especially timely topic given the lockdown restrictions due to the COVID-19 pandemic. In these experiments, we holistically study SI by incorporating data from behavioral paradigms, fecal corticosterone measurements, and histological assessment of c-Fos, an indirect marker of brain activity. We found that chronic SI did not induce anxiety-related behavior, but it did increase stress response and induce depression like behavior in the experimental group. Moreover, in the experimental group, subordinate animals were especially sensitive to SI in that they had significant deterioration in coat state compared to dominant mice.

Acknowledgment

First and foremost, I am extremely grateful to my principal investigator, Dr. Jonathan Fadok, and my mentor, Dr. Lydia Smith-Osborne for their invaluable advice, continuous support, and patience during the course of my honors thesis. Dr. Smith-Osborne has mentored me for the past 2 years and walked me every step of the way during this journey. Her immense knowledge and plentiful experience have encouraged me all the time in my academic research and daily life. I would also like to thank Dr. Beth Wee and Dr. David Mullin for being my second and third readers who provided technical support and valuable feedback to my research. I would like to thank all members of the Fadok lab, including Dr. Borkar, Dr. Dorofeika, and our Lab Manager Alexis Resendez for training me throughout the past 3 years and equipping me with the research skills to perform this independent research project. I am also very appreciative of all the undergrads in the Fadok Lab who have supported my research endeavor and helped create a pleasurable learning experience in the lab. My appreciation also goes out to my family and friends for their encouragement and support throughout my time at Tulane. Lastly, I would like to acknowledge the Newcomb Tulane College, the Newcomb Institute, and the Tulane University Brain Institute for supporting me financially through summer research grants.

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1. Introduction

1.1 Mental Health in Contemporary Society

Major Depressive Disorder (MDD) and Generalized Anxiety Disorder (GAD) are diseases of modernity, fueled by the interaction between genetic disposition and toxic elements of the social environment. Stressors of the contemporary environment such as social competition, economic crises, crime, inequality, and loneliness are linked to the prevalence of stress-related disorders (Hikada, 2012; Mental Health Foundation, 2014). As of 2017, MDD affects about 4.4% of the world's population, corresponding to over 322 million people; while GAD affects 3.6% of the world's population, corresponding to 264 million people (WHO, 2017). Drastic changes in lifestyle in the technological age are associated with rapid changes in the social environment, declining physical health, and the rise of psychopathology (Hikada, 2012). From 2005-2015, the number of people diagnosed with these mental health disorders increased at an alarming rate of 18.4% for MDD and 14.9% for GAD (WHO, 2017). Since the beginning of the SARS-CoV2 pandemic, the reported rates of loneliness, depressive symptoms, and anxiety symptoms have drastically increased (Cziesler et al., 2020; Robb et al., 2020). The COVID-19 lockdown period also exposed and exacerbated disparities and inequality of institutionalized racism. In this study, we investigated how factors such as social status and isolation can lead to the onset of stress-related disorders. We hope the findings from this study can contribute to determining the neurobiological roots of stress-related disorders induced by social isolation and identify contemporary risk factors for stress-related disorders and suitable prevention and treatment plans for individuals.

1.2 Major Depression Disorder and General Anxiety Disorder Comorbidity

MDD and GAD have the highest comorbid rate of any anxiety-mood comorbidity (Gorwood et al., 2004). The two disorders share overlapping symptoms such as fatigue, sleep disturbances,

difficulty concentrating, feelings of restlessness, and psychomotor agitation (Zbozinek et al., 2012). MDD and GAD have shared heritable characteristics which can be explained by common neurological mechanisms of the diseases, including a dysregulation in corticotrophin-releasing factor and vulnerable serotonin transporter gene (Gorwood et al., 2004). However, there are significant distinguishable characteristics between these classifications. GAD is significantly related to unipolar mood disorders such as MDD due to its higher load order of general distress (Zbozinek et al., 2012). Nevertheless, GAD is distinct in its strong relationship with fear, avoidance, and symptoms of worrying. Meanwhile, an individual with MDD reports symptoms such as feelings of sadness, hopelessness, and loss of interest (Gorwood et al., 2004; APA, 2013). There are also physiological symptoms that set these disorders apart. MDD is associated with flat affect, and appetite irregularities leading to weight changes of more than 5% of body weight in a month, while GAD is associated with increasing tension and fight or flight response (Zbozinek et al., 2012).

General Anxiety Disorder	**Major Depressive Disorder**
Excessive anxiety and worry (apprehensive expectation), occurring more days than not for at least 6 months, about a number of events or activities (such as work or school performance). The individual finds it difficult to control the worry. The anxiety and worry are associated with three (or more) of the following six symptoms (with at least some symptoms having been present for more days than not for the past 6 months): o Restlessness, feeling keyed up, on edge. o Being easily fatigued. o Difficulty concentrating or mind going blank. o Irritability. o Muscle tension. o Sleep disturbance	MDD is diagnosed with five or more of the symptoms listed below must be present during the same 2-week time period that represents changes in functioning. At least one symptom is either a depressed mood or loss of interest. • Depressed mood most of the day, nearly every day, as indicated in the subjective report or in observation made by others • Markedly diminished interest in pleasure in all, or almost all, activities most of the day and nearly every day • Significant weight loss when not dieting or weight gain, for example, more than 5 percent of body weight in a month or changes in appetite nearly every day • Insomnia or hypersomnia nearly every day

• The anxiety, worry, or physical symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning. • The disturbance is not attributable to the physiological effects of a substance or another medical condition. • The disturbance is not better explained by another medical disorder.	• Psychomotor agitation or retardation nearly every day • Fatigue or loss of energy nearly every day • Feelings of worthlessness or excessive or inappropriate guilt • Diminished ability to think or concentrate, or indecisiveness nearly every day • Recurrent thoughts of death

Figure 1: DSM-V Classification of GAD and MDD

In this experiment, we studied anxiety-like symptoms in mice using the Open Field Test (OFT) and Elevated Plus Maze Test (EPM). Both tests are validated pharmacologically through the treatment of anti-anxiety drugs, such as benzodiazepines, which reduce thigmotaxis and other anxiety-related behavior (Gentsch et al.,1987). In the OFT, we examined total distance traveled, time spent in the inner zone versus the outer zone, exploratory behavior such as rearing, and the number of fecal pellets left in the maze. Time spent in the outer zone represents thigmotaxis or wall-hugging behavior which is considered anxiety-related behavior (Seibenheber and Wooten, 2015). It has also been observed that the rate of defecation increases in highly stressed mice (Seibenheber and Wooten, 2015). In the EPM, we measured anxiety-related thigmotaxis behavior through time spent in the open arms. On the other hand, depression-like symptoms were measured with the sucrose preference test. The sucrose preference test assesses anhedonia behavior and maladaptation in the reward pathway, which are considered hallmark features of MDD (Liu et al., 2018).

1.3 Neurobiology of Stress

The main neuroendocrine system responsible for the stress response is the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis consists of hormone-secreting centers in the neuroendocrine system, including the hypothalamus, the pituitary gland, and the adrenal gland.

When a stressor is detected, the HPA axis is activated. First, corticotrophin-releasing hormone (CRH) is released from the hypothalamus to activate the sympathetic system and act on the anterior pituitary to cause the release of adrenocorticotropic hormone (ACTH). ACTH travels to the adrenal glands to stimulate glucocorticoid production in the adrenal cortex and epinephrine in the adrenal medulla (de Kloet et al., 2005). Glucocorticoids are adrenal steroid signaling molecules that have immunosuppressive effects; they also increase glucose levels in the bloodstream and initiate the fight-or-flight response. The predominant glucocorticoid present in humans is cortisol, while that of rodents is corticosterone.

A series of brain structures are responsible for the neural inputs that activate the HPA axis. While physiological stressors require immediate relay and response, psychological and social stressors are first processed by higher-order regulators such as limbic structures (amygdala and hippocampus) and the prefrontal cortex (Herman et al., 1997). Through GABAergic interaction with the bed nucleus of the stria terminalis, the amygdala modulates the function of the paraventricular nucleus (PVN) which initiates the HPA axis and further stress response (Herman, 1997). Meanwhile, glutaminergic projections from the limbic structures and hippocampus to the PVN are important for the inhibition of stress response (Herman, 2003). An acute stress response can be adaptive but chronic stress is pathological. In chronic stress, prolonged glucocorticoid release damages hippocampal neurons responsible for shutting off the HPA axis response, leading to a vicious cycle of dysregulation that can lead to stress-related disorders (Krugers et al., 2010).

Besides their effects on metabolism and the immune system, glucocorticoids are regulated through two distinct negative feedback cascades: a fast nongenomic and a delayed genomic feedback mechanism. The fast non-genomic feedback mechanisms take only seconds to minutes

and involve glucocorticoid binding to its membrane receptor in the respective location and inhibiting CRH secretion from the hypothalamus and ACTH secretion from the anterior pituitary. The delayed feedback takes hours to days and involves inhibition of crh gene expression in the PVN and POMC (a precursor of ACTH) in the pituitary corticotroph cells (Gjerstad et al., 2018). Glucocorticoid feedback also occurs at the level of the hippocampus and the prefrontal cortex since these structures exhibit high levels of glucocorticoid receptors. Additionally, high glucocorticoid levels increase the expression of CRH,enhance amygdala output, and increase HPA axis reactivity.

Negative feedback allows for the shutdown of the acute stress response. The acute stress response directs resources to vital body regions in response to the stressor. In contrast, chronic stress is pathological. Prolonged glucocorticoid release damages hippocampal neurons responsible for shutting off the HPA axis response. This vicious loop leads to a more elevated stress response.

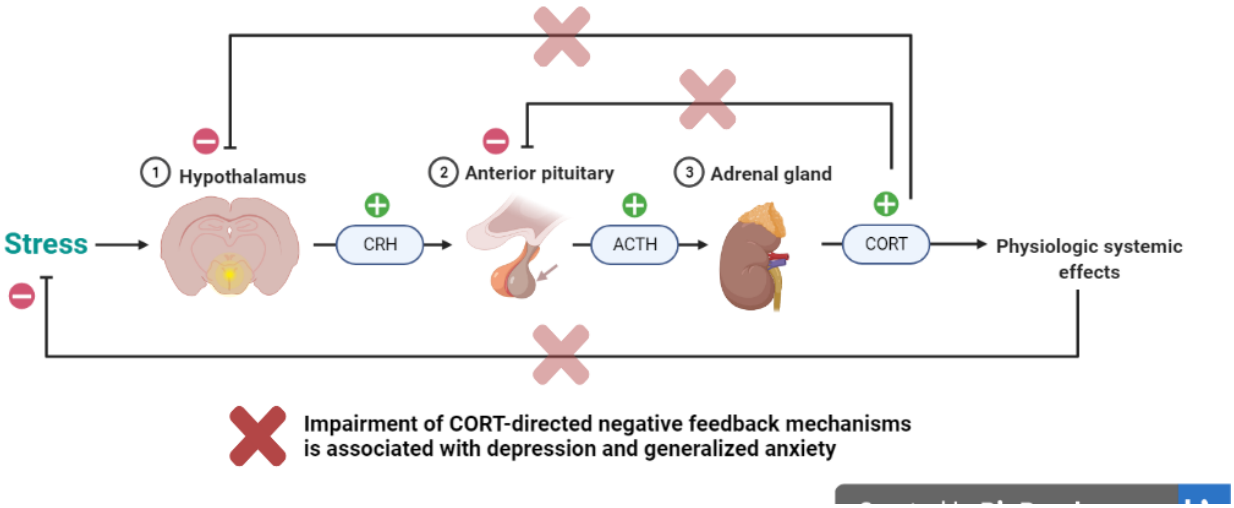

Figure 2: HPA Axis and feedback

1.4 Sex Difference in Stress Response

According to Heck& Handa, 2019, in both humans and mice, the stress response is more vigorous in females than in males because of the effect of gonadal hormones on the neuroendocrine network. Gonadal hormones such as androgens and estrogens bind to their receptors which are ligand-activated transcription factors. After ligand binding, these transcription factor units travel from the cytoplasm to the nucleus to regulate DNA transcription directly or indirectly. Gonadal hormones can regulate the HPA axis thanks to the abundance of androgen and estrogen receptors in the key neural structure controlling the HPA axis such as the PVN and the anterior pituitary. Nevertheless, estradiol, the primary female hormone, and dihydrotestosterone (DHT), a male hormone converted from testosterone, have opposing effects on the stress response and stress regulation. The sex difference in acute stress can be attributed to both activational and organizational effects of DHT and estradiol (Heck & Handa, 2019).

The level of estradiol is positively correlated with the activity of the HPA axis. Female rodents in the proestrus phase of the estrous cycle have peak estradiol levels, low progesterone, and the highest elevation in HPA output. Estradiol acts directly on the production of stress hormones as well as their feedback mechanism. At the level of the hypothalamus, estradiol increases transcription of the Crh and Avp genes. Estradiol also increases the production of the POMC gene, a vital precursor for ACTH in the pituitary. The adrenal glands also increase ACTH receptor sensitivity at the adrenal cortex, intensifying the positive feedback. Estradiol disrupts glucocorticoid feedback by reducing glucocorticoid binding in the anterior pituitary, PVN, and hippocampus. In contrast to estradiol, androgens- specifically, DHT, downregulate the activity of the HPA axis. DHT hinders the production of CRH and AVP by acting upstream on the BNST.

DHT also reduces levels of ACTH and glucocorticoid and increases CORT feedback mechanism. Moreover, neonatal and pubertal testosterone also organizationally masculinizes glucocorticoid secretion in adulthood (Heck & Handa, 2019).

Females have greater HPA axis regulation in response to chronic mild stress (CMS) and chronic variable stress (CVS). Social support in females helps buffer against the effect of long-term stress. When exposed to CMS, cellular activity in the PVN of group-housed males increased, but that of females stayed the same (Karamihalev, 2019). Like that of rodents, gonadal hormones in humans also modifies the effect of the HPA axis organizationally and activationally. This sex difference is consistent with data from studies reporting that women are more vulnerable to stress-related disorders. Stress-related disorders including MDD and general anxiety disorder disproportionately affect women (Altemus, 2014). The 2017 Global Health survey estimated that the prevalence of depressive disorder among women is 1.4 times higher than that of men, peaking in adult females aged 55-74. Similarly, GAD is 1.8 times more common in females than males (WHO, 2017). In addition to societal pressure and disparities faced by women, a neurobiological sex difference in the stress response is another major factor in the prevalence of mood disorders in women. Despite the disorder's bias in gender and age group, literature reviews reveal that most studies on the neurobiology of stress have used male animal models in the juvenile and pubescent age groups which fails to consider the sex difference in stress regulation (Planchez, 2019; Wang, 2017).

Additional research is needed to understand the interaction of gender and the manifestation of stress-related pathologies, especially those induced by psychosocial factors. Thus, in this study,

we aimed to bridge the gap in sex differences of stress research by focusing on the onset of anxiety and depression after SI exclusively in the adult female model.

1.5 Social Hierarchy in Humans and Animals

Social status is one of the main social stressors in highly social organisms such as humans and mice. Social status is formed through socioeconomic status (SES) in humans and social confrontation in mice (Farrah et al., 2017; Karamihalev et al., 2020). Despite humans' hierarchy being significantly more complex than that of mice, the effect of perceived and objective SES in humans and social status in mice on the stress response and psychiatric symptoms are similar (Karamihalev et al., 2020). In humans, lower SES is linked to higher anxiety and an increase in depressive symptoms and psychosis (Farrah et al., 2017). In an experiment done to measure stress resilience in an interview setting, subjects with higher SES in a stable hierarchy felt more in control in a stressful situation and had better interview performance (Knight et al., 2016). The biological response was concurrent with their behavior: individuals with higher SES showed little cortisol change when facing a stressor and quick cortisol decline during the recovery period after exposure (Knight et al., 2016). Similarly, ranks in mice also lead to variability in behavior and physiology. Subordinate mice have significantly higher corticosterone levels, are less explorative, and have less locomotion than their dominant counterpart. Biological and behavioral findings suggest that lower rank is associated with higher levels of stress and more anxiety and depression-like symptoms (Schur, 1987; Horii et al., 2017).

Nevertheless, the effect of social status on stress resilience is moderated by the type of stressor and sex. Larrieu et.al (2017) exposed dominant and subordinate male mice to the chronic social defeat stress (CSDS) paradigm, where the naïve subject mouse is repeatedly exposed to an aggressor mouse. The findings reveal that dominant male mice were more susceptible to chronic

defeat stress than subordinate mice. After exposure to the CSDS paradigm, both subordinate and dominant mice showed an increase in anxiety-like behavior. However, the dominant groups had more weight fluctuation and higher social avoidance scores than the subordinate group following stress exposure. While corticosterone levels in subordinate mice were statistically similar before and after defeat, there was a significant change in corticosterone in dominant mice. The researcher proposed that mice higher in rank are particularly challenged by defeat, but mice lower in rank are already constantly subjected to defeat in the established hierarchy, and therefore develop resilience for subsequent antagonistic interaction (Larrieu et al., 2017). However, this stress resilience response does not generalize to female mice or other types of stress. Research exploring stress in a sex-specific manner found that females and males show an opposite correlation between social status and depressive-like, anxiety-like behaviors following exposure to chronic mild stress (CMS) (Karamihalev et al., 2020). After being exposed to mild stressors such as overcrowding, tilted cages, and wet bedding, dominant females had a decrease in anxiety-like behaviors while subordinate males showed an increase in locomotion compared to control mice that were not exposed to any stressors (Karamihalev et al., 2020). Karamihalev's study observed the main effect of dominance on corticosterone levels but did not detect the main effect of CMS on corticosterone levels. This contradicts findings from their behavioral experiment indicating the significant amount of stress endurance by the mice. The authors acknowledge the limitation of their plasma corticosterone measurement being performed too long after CMS after the mice have recovered from the effect of the stressors. Because male hierarchies are aggressive, subordinate males endure the most antagonistic encounters and dominant males are more territorial and protective of their rank in the hierarchy (Karamihalev et al., 2020). Conversely, female hierarchies are less rigid and the relationships between members are more communal and affiliative (Karamihalev et al., 2020).

All females in the group benefit from the social bond and the dominant female gains the most from the interactions. The contrasting nature of males and female mediates the effect of social dominance on stress. Moreover, the stress paradigms used in Larrieu's and Karamihalev's studies are notably different. Social defeat induced a stronger response in dominant male mice than other stressors since it is closely related to the loss of dominance status (Larrieu, 2017; Karamihalev et al., 2020). This raises questions on whether the stress coping mechanism is sex divergent for other types of social stressors.

1.6 Social Isolation as a Psychological Stressor

One type of social stressor that is recognized as a major risk for physical and psychological health in humans and other social species is social isolation (SI). SI in humans is normally induced by disruption of significant social relationships and expressed as the feeling of loneliness. The physiological and psychological effect of loneliness includes weakened immune response, altered sleep schedule, fatigue, hostility, increased anxiety and depressive symptoms, and increased risk of age-related cognitive decline diseases such as Alzheimer's and Dementia (Cacioppo et al., 2016). Subjects with higher self-report loneliness showed greater activation of the HPA axis and higher urinary and salivary cortisol levels (Steptoe, 2004). Animal models in research allow us to manipulate SI conditions and examine the causal link between SI and neuroendocrine responses. Exposure of rodent models to short-term and chronic isolation causes subsequent vigorous HPA axis activity, elevated glucocorticoid levels, and significant change in cardiovascular activity. Moreover, chronic SI can lead to a decrease in the sensitivity of glucocorticoid receptors in the brain, leading to an inefficient negative feedback system to inhibit the activity of the HPA axis. Therefore, subject animals that experienced social isolation have increased responses to future stressors (Cacioppo et al., 2016).

In the present day, due to lockdown guidelines following health and safety measures to prevent the spread of the COVID virus, there has been a drastic increase in rates of loneliness, as well as reported depressive and anxiety symptoms (Cziesler, 2020; Robb, 2020). In a survey conducted by the CDC, the percentage of the American population reporting depressive symptoms in 2020 was almost four times that of the percentage obtained from surveys from 2019 (Cziesler, 2020). Similarly, the report of frequent loneliness increased from 2% pre-lockdown to 20% post-lockdown (Robb, 2020). If these trends persist, long-term loneliness can lead to even more detrimental effects on psychological health.

2. Hypothesis:

Considering the recent trend of loneliness and mental health, especially in vulnerable populations, our research aimed to investigate the interaction of social status and social isolation on stress response. By using female mice models, we sought to address the current experimental bias for male models and gain further understanding of the disproportionate effect of MDD in females. Beyond the main effects of higher status mice having lower corticosterone levels and mice subjected to isolation stress having higher corticosterone levels, we hypothesized that female mice with higher social status would be more resilient when exposed to isolation stress and have a lower corticosterone level. The goal of our study was to contribute to understanding the disparities in mental health and pave directions for future research on treatment methods.

3. Methods and Material:

After 24 mice were pair-housed for a period of 24 hours, they were tested for hierarchical status using the competitive exclusion test (CE) until a stable hierarchy was confirmed. Prior to social isolation, mice were tested for anxiety levels using the Elevated Maze Plus (EPM) and Open Field Test (OFT). 16 experimental mice were single housed for the next 6 weeks, while 8 control

mice continued to be paired-housed. During weeks 2-8, mice were tested weekly for coat state and nest building for indicators of well-being. To analyze the change of corticosterone (CORT) over time, fecal samples were collected twice a week on Tuesday and Thursday and processed through enzyme-linked immune absorbent assay (ELISA). At the end of the isolation period, mice were re-tested for anxiety levels using the Elevated Plus Maze and Open Field Test and were tested for depressive symptoms using the Sucrose Preference Test. After the isolation period, mice were sacrificed, and brain slices were harvested for immunohistochemistry. The results combine data from these 24 mice with 8 mice from the pilot study. The 8 mice from the pilot study were paired-housed and subjected to the same behavioral test and fecal collection twice a week. However, OFT data, and c-Fos analysis data from the 8 mice from the pilot study were excluded in the analysis due to changes in experimental parameters (see the limitations section).

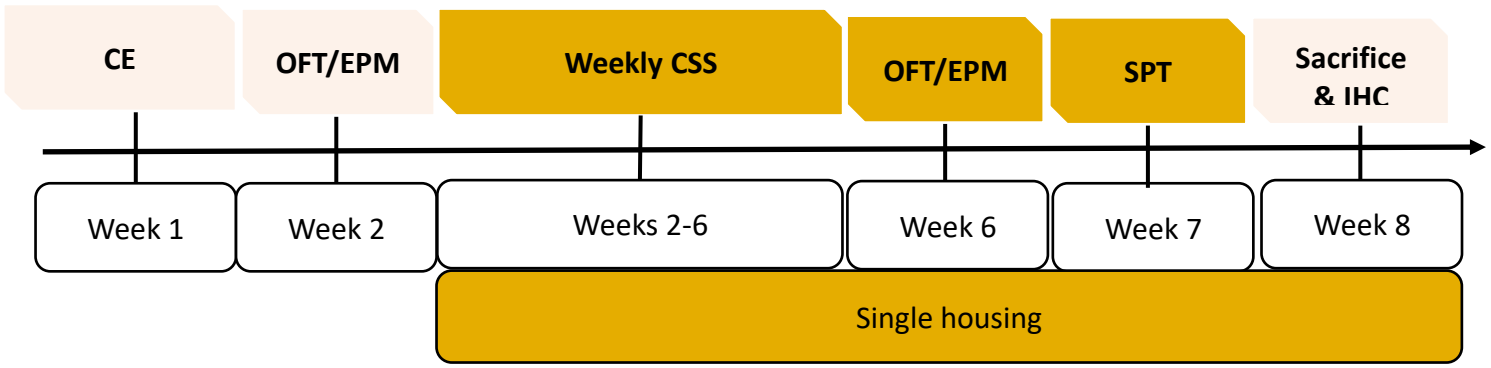

Figure 3: Experiment Timeline

Figure 3 displays the experimental timeline of the social isolation experiments. In week 1, mice were pair housed and underwent the competitive exclusion (CE) test to test for rank. In week 2, mice were tested for baseline anxiety indicators through the Open field test (OFT) and the Elevated Plus Maze test (EPM). Mice were isolated for 7 weeks from week 2 to week 8. During that time, coat state scores (CSS) were collected. In week 6, mice were retested for anxiety-like symptoms during the OFT/ EPM test. During week 7, mice were tested for anhedonia behavior through the sucrose preference test (SPT). At the end of the isolation period, mice were sacrificed. Brain tissues were collected, and tissues were stained using immunohistochemistry techniques.

2.1 Competitive Exclusion test

Mice were brought into the testing room in their home cage at least 30 minutes prior to testing for acclimation. The apparatus consisted of a clear plastic tube wide enough for the passage of one mouse (~2.6 cm in diameter) and ~30 cm in length, in which mice can pass through. During 10 trials per day for 3 consecutive days, mice were trained to run the length of the tube from one side to the other. They were trained to run the tube in both directions.

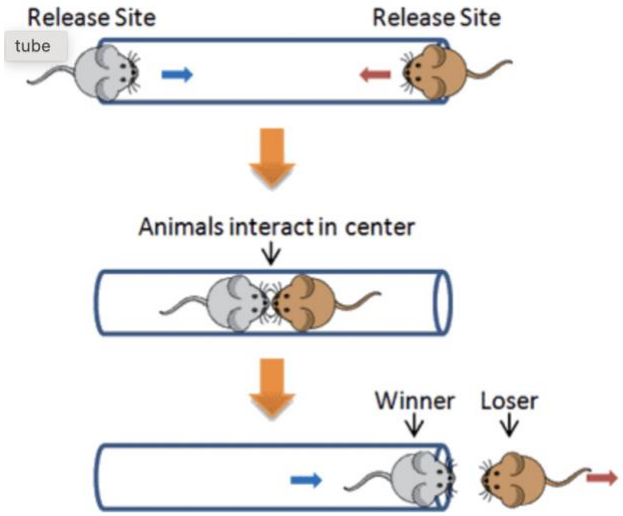

Figure 4: Competitive Exclusion Test (Stanford Medicine)

Dominance testing began after the last day of training (Day 2) and continued until pair ranks were stable for 4 consecutive days (Day 5 at the earliest). Once daily, each animal underwent two training trials before testing. Afterward, two unfamiliar mice were placed in opposite sides with the center tube divider in place. When both mice were at the center of the tube, the divider was removed, and the mice were allowed to interact. A dominance test was complete when one mouse forced the other completely back into its respective side (i.e., all four feet are in the box). This was recorded as a win for the mouse that successfully ran the length of the tube, and a loss for the mouse forced back into its starting box. The time to complete the test was recorded and

factored as a supplementary measure of relative dominance/submissiveness. Agonistic (e.g., pushing, push-back, advancing) and submissive (e.g., active retreat) behaviors dictating the win were characterized as further proof of social rank.

A total of 5 dominance trials were performed per day, and the initial side into which a mouse was placed was alternated for each trial. The dominant status for that testing session was defined as 3 or more wins. The proportion of wins to losses was used to estimate hierarchy stability (i.e., a mouse winning all 5 trials was interpreted as belonging to a more stable social hierarchy than one winning only 3 trials).

2.2 Open Field Test

Mice were brought into the testing room in their home cage 30 minutes before each session for an acclimation period. The open-field test took place under low lighting conditions (10-12 lux level) in a square, opaque plastic arena. Mice were removed from their home cage after acclimation and gently placed into the center of the arena. The testing period was 10 minutes, during which the animal was allowed to move freely around the arena while its activity is recorded to video for later analysis. After 10 minutes, the animal was returned to its home cage. Activity was recorded via an overhead camera for later analysis. After each trial the arena was cleaned with 70% ethanol.

2.3 Elevated Plus Maze Test

Mice were brought into the testing room in their home cage at least 30 minutes before each session for an acclimation period. The maze consists of two open arms and two closed arms, opposite to each other and arranged in plus shape. The entire apparatus is raised 45 cm above the floor. Mice were placed individually into the central area facing one of the open arms and allowed to freely explore the maze. The percentage of time spent in the open arms, the closed arms, and the central square is measured to evaluate the degree of anxiety of mice.

Figure 5: Elevated Plus Maze Test

2.4 Fecal Corticosterone measurement

To analyze trends in CORT production over time and assist us in assigning dominant/subordinate and susceptible/resilient status to individual mice, we took fecal samples for Fecal Corticosterone Measurement (FCM) with the EIA propriety kit from the Palme lab that measures the concentration of corticosterone metabolites. These assays are representative of glucocorticoid status approximately 8-10 hours prior to morning sample collection, a lag time which is attributed in part to the average GI transit time of 9-10 hours in mice (Touma, 2004). Because transit time is also significantly related to the time of day (Touma, 2004), we took fecal samples twice a week on Tuesday and Thursday.

The procedure for sampling was as follows (adapted from Touma et. al, 2004): prior to SI, mice were individually removed from their cage and placed in an empty cage covered with absorbent filter paper (to absorb urine) and gently handled for 2-5 minutes or until fecal pellets are

produced. Pellets for analysis were selected preferentially from areas without urine contamination when possible.

The mouse was returned to the homecage and the pellets collected in a microcentrifuge tube and stored at -20 Celsius until processing. Then the samples were homogenized in the tube, and a 0.05g aliquot was weighed out and suspended in 1 mL of 80% methanol for 1-2 minutes on a hand vortex and centrifuged at 2500g for 15 mins. The aliquot of the supernatant was diluted with assay buffer (1+9) in a 1.5 ml conical tube and stored at -20 degrees until analysis with EIA.

2.5 Perfusion

Mice were brought into the experimental room for 30 minutes of acclimation under red light before being exposed to the social stimulus. Control mice were placed in a novel environment with their partners. 6 mice in the experimental group were placed in a novel environment alone. Meanwhile, the other 8 mice in the experimental groups were placed in a novel environment and reintroduced to their original partners. The c-Fos behavioral stimulus took place for 15 minutes before mice were dark housed for another 80 minutes before being sacrificed.

A transcardial perfusion procedure was performed as follows: following intraperitoneal injection of anesthetic (tribromoethanol, 0.5 ml per mouse of 1.25% working solution), mice were assessed for the deepness of anesthesia by tail and toe pinches. The procedure continued only when mice were completely unresponsive to noxious stimuli. The mouse was secured in the supine position by pinning the paws to a pad using syringe needles or tape. Using blunt forceps to raise the sternum, sharp scissors were used to make an incision along the sternum and ribcage to expose the thoracic cavity. The diaphragm was separated from the chest wall using scissor cuts. The ribcage was then be held out of the way with hemostat clamps or completely cut away with scissors. The heart was grasped with blunt forceps and a syringe needle was inserted into the left

ventricle. The needle was held in place using a hemostat or bulldog clamp. The right atrium was then cut with scissors and the infusion pump was switched on to flush the animal with phosphate-buffered saline (PBS) (40-80 ml). Once the exiting fluid is clear, the perfusate was switched to an aldehyde-based fixative. Approximately 20-50 ml of fixative was pumped through before stopping the perfusion. The mouse was then decapitated with large surgical scissors. The cranium was subsequently dissected to allow for extraction of the brain.

2.6 Histology

60 um coronal sections were cut using a comprestome (manufacturer). Brains were stored in PBS solution with 0.025% sodium azide in a 4-degree Celsius refrigerator.

2.7 Immunohistochemistry

During day 1, brain slices were rinsed 3 times for 10 mins in 0.3% PBST. Then, slices were blocked in solution of 5% goat serum in PBST. Blocking solution was 5 ml goat serum + 95 ml PBST. Next, anti cfos rabbit primary antibody in blocking solution was added. The Primary antibody solution was diluted to the concentration: 1:2000. Slices in primary antibody solutions were incubated on a shaker in the dark for 48 hours in 10 degrees Celsius. For day 2, brain slices were rinsed 3 times at 10 mins with PBST. Slices were incubated for 2 hours in solution of 1:500 secondary antibody (Alexa fluor goat anti-rabbit 647) to blocking solution (5% goat serum) at room temperature. Slices were rinsed 3 times for 15 minutes in PBS. Finally, brain slices were mounted on slides and cover slipped with DAPI mounting media.

2.8 Imaging and Cell Counting

Slices were imaged with an Olympus confocal microscope (FV1000) and a Zeiss Axioscan slide scanning microscope. Quantification of cell counts was performed using Image J software.

4. Results

4.1 Competitive Exclusion Test

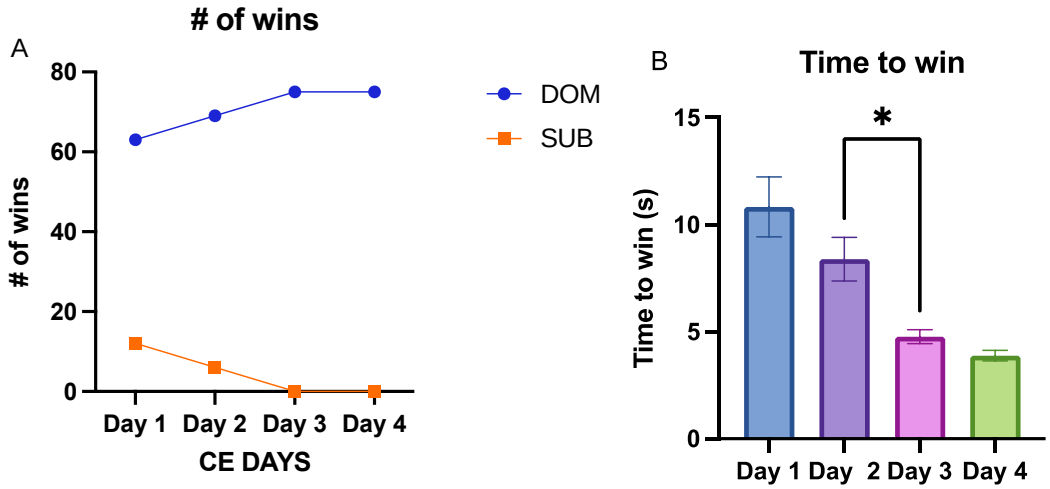

Figure 6: Competitive Exclusion Data

Figure 6A depicts the number of wins over the 4 days of competitive exclusion *(N=30*). **Figure 6B** compared the time to win from day 1 to day 4 through a 1-way ANOVA *(N=30).* The time to win from day 1 to day 2 did not significantly decrease *(p=0.1864).* Time to win from day 2 is significantly less than that of day 3 *(p=0.0107).* Time to win from day 3 was not significantly different than that of day 4 *(p=0.8625).*

Dominant females had higher overall total number of wins that that of subordinate females. All hierarchies were stable by day 3 of competitive exclusion, where the dominant won 100% of wins. The time to win significantly decreased from day 2 compared to day 3 (*p= 0.0107, p<0.05).*

4.2 Sucrose Preference Test

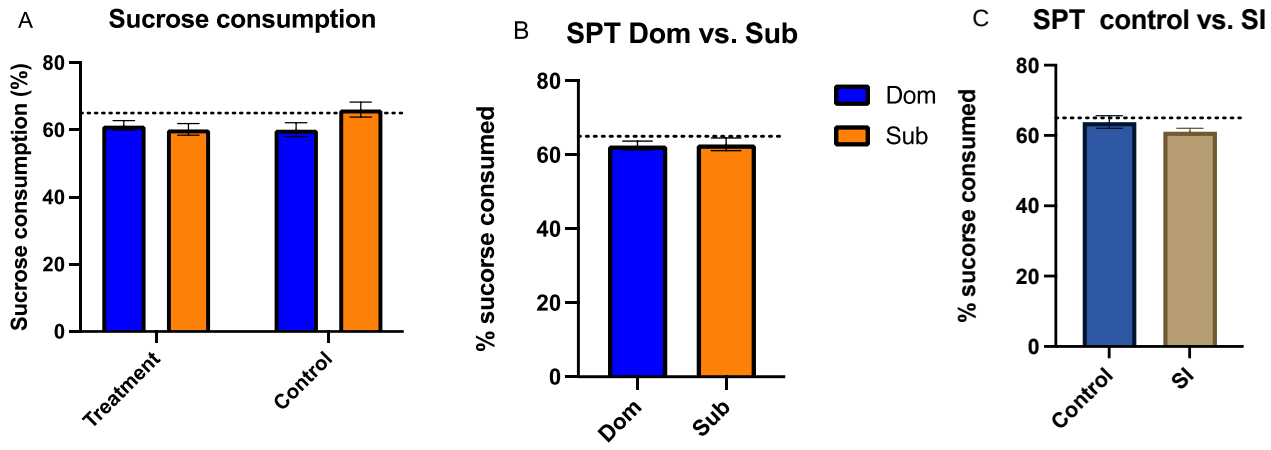

Figure 7: Sucrose Preference Data

Figure 7A compares sucrose consumption through 2 way ANOVA (N=30). There was no main effect of isolation treatment *(p=0.2252).* There was no main effect of rank either *(p=0.2402).* However, there was a trend of interaction between rank and isolation treatment *(p=0.0775).* There is a significant different between subordinate and dominant in the control group *(p=0.04092).* **Figure 7B** compares sucrose consuption of dominant vs. subordinates animals via an unpaired T-test *(p=0.8942).* **Figure 7C** compares sucrose consumption of control vs. experimental animals via an unpaired T-test *(p=0.3696).*

There was no significant different in sucrose consumption by rank or by isolation treatment. Dominant and subordinate mice subjected to isolation treatment and dominant mice in the control group had an average sucrose consumption of 62.11%, 61.62%, and 60.08% respectively. The percent sucrose consumption of these groups were less then 65%, suggesting anhedonic symptoms in dominant animals in the isolated group, subordinate animals in the isolated group, and dominant animalsl in the control group. Subordinates in the control group had an average sucrose consumption of 66.84%, suggesting that this group displayed no anhedonic symptoms. There was no significant difference between sucrose consumption of dominant and subordinates mice or experimental animals and control animals.

4.3 Coat State Score

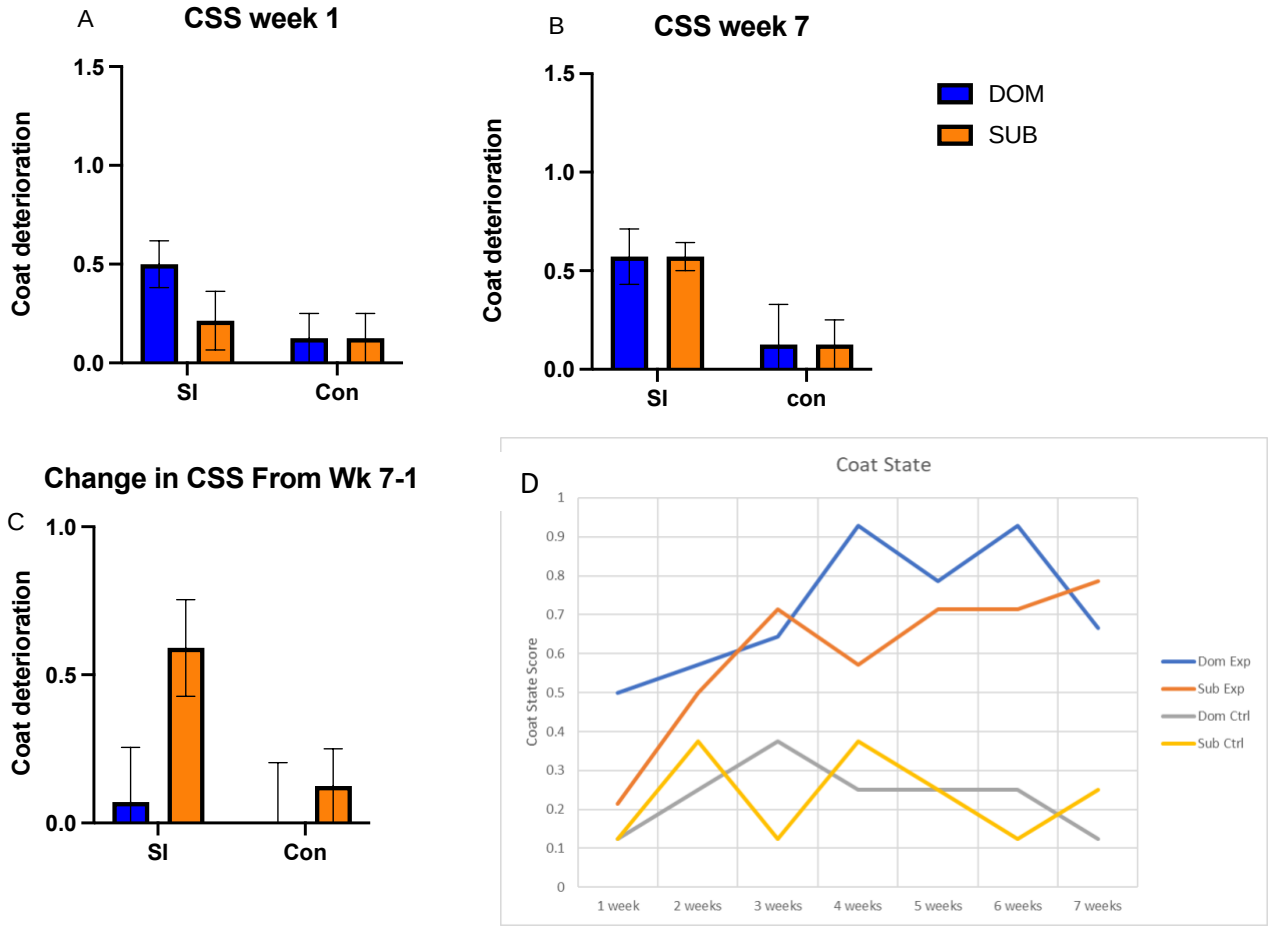

Figure 8: Coat State Score Data

Figure 8A depicts coat state comparison before social isolation (N=22). Through a 2-way ANOVA test, we observed that all groups had comparable coat state during week 1: main effect rank (*p=0.3327);* main effect SI treatment (*p=0.1231*); interaction rank x SI treatment *(p=0.3327).* **Figure 8B** depicts coat state comparison after the isolation period (N=21): main effect SI treatment *(p= 0.0038);* main effect of rank *(p>0.9999)*; interaction SI treatment x rank *(p>0.9999*). **Figure 8C** depicts change in coat state score: main effect of SI treatment *(p=0.1601);* main effect of rank *(p=0.0961);* interaction SI treatment x rank *(p=0.2959).* **Figure 8D** depicts change in coat state score over time for all groups.

Mice coat states were scored based on 8 regions of the body. Higher scores were associated with a more deteriorated coat state. At week 1, there was no significant difference in coat state by rank or SI treatment. At week 7, SI mice showed more coat state deterioration (p=0.0038; p<0.05).

There was no significant difference in change in coat state score amongst the groups. However, we saw a trend of subordinates mice in the experimental group showing most coat state deterioration.

4.4 Fecal Corticosterone Measurement

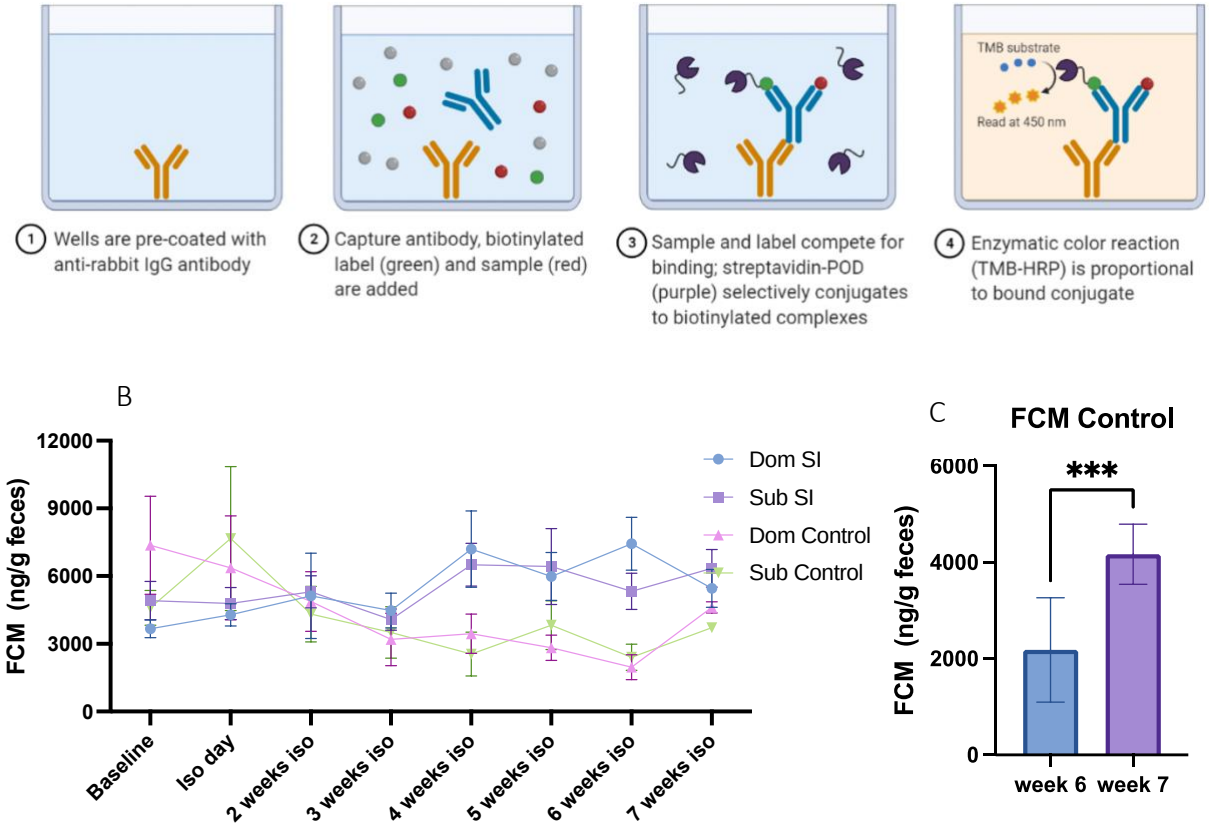

Figure 8: Fecal Corticosterone Analysis

Figure 8A shows the cellular mechanism for the Enzyme Linked Immunosorbent Assay (ELISA). **Figure 8B** depicts CORT levels over time. **Figure 8C** depicts change in FCM levels of control mice from week 6 to week 7 (N=8). There was a significant increase in CORT levels for control mice from week 6 to week 7 (p=0.0005)

There was no significant difference in fecal corticosterone measurement by rank or stress treatment from baseline to week 3 of isolation. At baseline, all groups had the same corticosterone levels. After 2 weeks and 3 weeks of isolation, SI mice had the same CORT levels as control mice

(p = 0.997, p>0.05) (for week 2), *(p = 0.9496, p>0.05)* (for week 3). However, corticosterone levels became significantly higher for experimental mice compared to control mice at week 4 of isolation (*p =0.0231, p< 0.05).* At 5 weeks of isolation, there was an unsignificant trend of SI animals having higher fecal corticosterone levels (*p =0.1298, p>0.05).* This higher corticosterone level in SI mice became more prominent in week 6 (*p= 0.002, p< 0.05)*. During week 7, there was still a trend of SI mice showing more stress (*p= 0.0784, p>0.05).* However, it should be noted that during week 7, mice in the control group are single housed to test for sucrose preference. Thus, there was a significant higher corticosterone levels for control mice in week 7 compared to week 7 *(p = 0.0005 p<0.05*. Data suggests that isolation treatment induced significant stress starting at week 4. There was no significant difference in corticosterone levels by rank throughout the experiment.

4.5 Elevated Plus Maze

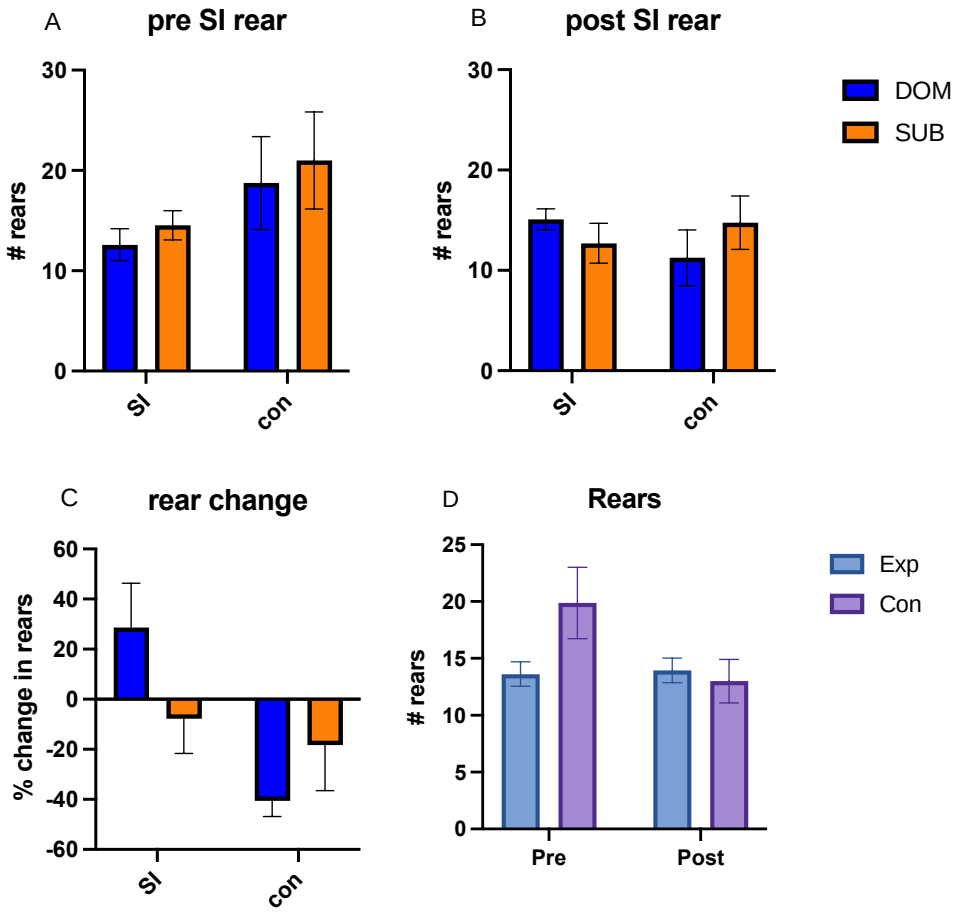

Figure 10: Elevated Plus Maze-Rears

Figure 10A depicts number of rears pre isolation during the EPM test (N=29): main effect of rank (p=0.4323); main effect of SI treatment (p = 0.0243); interaction treatment x rank (p=0.9543). **Figure 10B** depicts number of rears post isolation during the EPM test (N=29): main effect of rank (p=0.2503); main effect of SI treatment (p=0.6766); interaction treatment x rank (p=7.601). **Figure 10C** depicts % change in rears (N=28): main effect of SI treatment (p=0.0473); main effect of rank (p=0.7124); interaction SI treatment x rank (p=0.1369). **Figure 10D** depicts number of rears of pre and post isolation for the experimental and control groups (N=29): main effect of time (p=0.0540); main effect of SI treatment x time (p=0.1661); interaction SI treatment x time (p=0.0344).

Rearing behavior is considered an exploratory behavior and is used as a measure of anxiety in both EPM and OFT. Rearing consists of unsupported rears (UR) and supported rears (SR). Supported rearing is neither considered anxiolytic nor anxiogenic, while unsupported rears are stressed sensitive and more representative of anxiety symptoms (Seibenherner et al., 2015). In the

Elevated Plus Maze Test, there were no occurrences of unsupported rears due to the narrow width of the maze, thus, in our EPM analysis, rears refer only to supported rears. Pre-isolation, there was a main effect of control mice rearing more than mice subjected to SI (*p=0.0243, p< 0.05)*. However, there was no difference by rank or stress treatment post isolation. There was a trend of rears decreasing for all animals (*p= 0.0540, p>0.05)*, but there was no significant change in rears by stress treatment or rank. There was an interaction effect between time and SI treatment in that the number of rears in control grouped decreased more than that of the experimental groups after the isolation period.

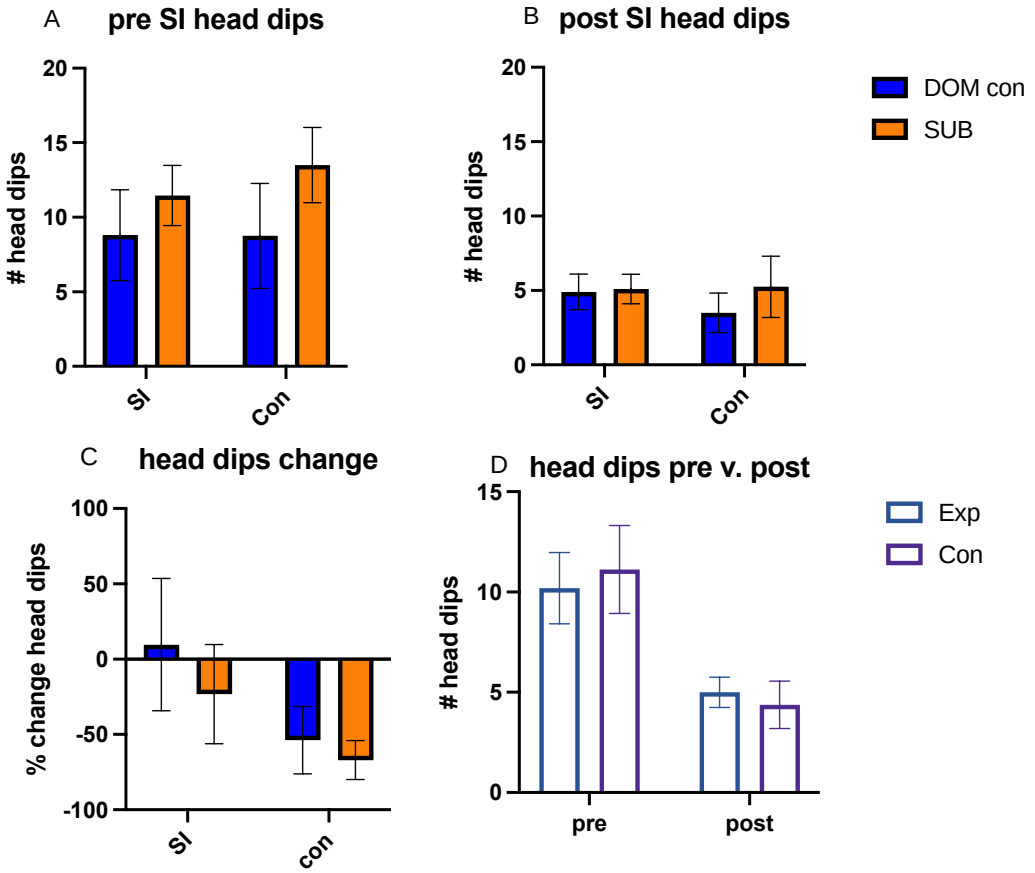

Figure 11: Elevated Plus Maze- Head dips

Figure 11A depicts the number of head dips pre isolation during EPM test (N=29): main effect of rank (p=0.2623); main effect of SI treatment (p=0.2623); interaction rank x SI treatment (p=0.7482. **Figure 11B** depicts the number of head dips post isolation during the EPM test (N= 29): main effect of rank (p=0.5195); main effect of SI treatment (0.6753); interaction rank x SI

treatment (p=0.6043). **Figure 11C** depicts the percent change of head dips from between pre and post isolation in the EPM test (N=28): main effect of rank (p=0.6147); main effect of SI treatment (p=0.2464); interaction between rank and SI treatment (p=0.8302). **Figure 11D** depicts the number of head dips pre and post isolation for the experimental and control groups (N=29): main effect of time (p=0.0061); main effect of SI treatment (p=0.9411); interaction SI treatment x time (p = 0.7098)

Head dips are also indicative of exploratory behavioral and anxiolytic behavior. During head dips, the animal is taking a risk by dipping its body below the arm to explore underneath the maze. Pre-isolation, there was no significant difference in the number of head dips by rank or stress treatment. Similarly for post-isolation, we did not find a significant difference amongst the ranks and stress treatment groups. However, when analyzing the average number of head dips before isolation and post isolation for all animals, the number of head dips significantly decreased ($p= 0.0061, p<0.05$). Nevertheless, there was no significant different in the reduction in head dips by rank or SI treatments.

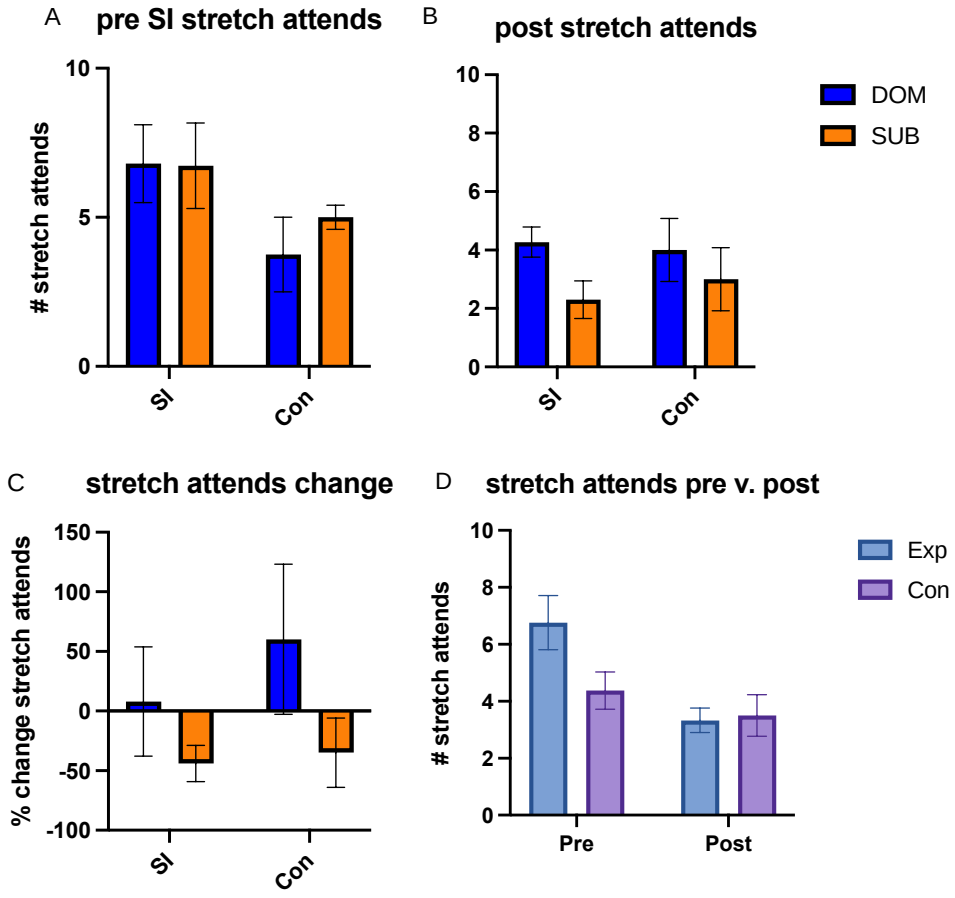

Figure 12: Elevated Plus Maze- Stretch Attends

Figure 12A depicts the number of stretch attends pre isolation during EPM test (N=29): main effect of rank *(p=0.7264*); main effect of SI treatment *(p=0.1634);* interaction rank x SI treatment *(p=0.6943).* **Figure 12B** depicts the number of stretch attends post isolation during the EPM test (N= 29): main effect of rank *(p=0.0832);* main effect of SI treatment *(p=0.7975);* interaction rank x SI treatment *(p=0.5601).* **Figure 12C** depicts the percent change in stretch attends from between pre and post isolation in the EPM test (N=28): main effect of rank *(p=0.1088)*; main effect of SI treatment *(p=0.4964);* interaction between rank and SI treatment *(p=0.6304).* **Figure 12D** depicts the number of stretch attends pre and post isolation for the experimental and control groups (N=29): main effect of time (*p=0.0210);* main effect of SI treatment *(p=0.2252);* interaction SI treatment x time *(p = 0.1640).*

Similar to head dips, stretch attends is both an exploratory and anxiolytic behavior. During a stretch attend behavior, the animal lowers its body close to the surface and stretch forward to explore. Pre-isolation and post isolation, we found that there was no significant difference in number of stretch attends by rank or stress treatment. However, the average stretch attends of all

animals after the 7 weeks treatment period were significantly lower than at the beginning (*p=0.0210, p<0.05).* The reduction in stretch attend was more prominent in the SI groups than the control groups.

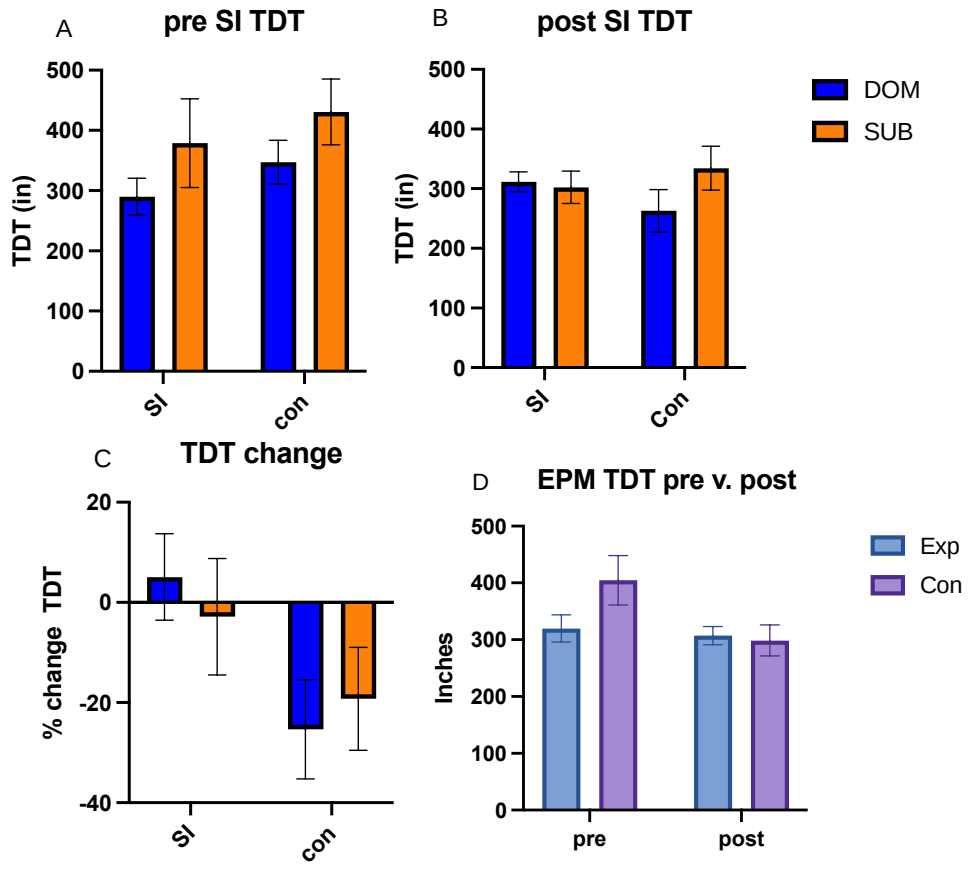

Figure 13: Elevated Plus Maze- Total Distance Travel (TDT)

Figure 13A depicts total distance travel (TDT) in inches pre isolation during EPM test (N=29): main effect of rank *(p=0.0934*); main effect of SI treatment *(p=0.2765);* interaction rank x SI treatment *(p=0.9556).* **Figure 13B** depicts the TDT post isolation in inches during the EPM test (N= 29): main effect of rank *(p=0.3028);* main effect of SI treatment *(p=0.7864);* interaction rank x SI treatment *(p=0.1877).* **Figure 13C** depicts the percent change in TDT from between pre and post isolation in the EPM test (N=28): main effect of rank *(p=0.9414)*; main effect of SI treatment *(p=0.0726);* interaction between rank and SI treatment *(p=0.5790).* **Figure 13D** depicts total distance travel pre and post isolation for the experimental and control groups (N=29): main effect of time (*p=0.0398);* main effect of SI treatment *(p=0.1816);* interaction SI treatment x time *(p = 0.1048).*

Total Distance Travel (TDT) was a measure of locomotor activity in both EPM and OFT. There was no significant difference by rank or stress treatment in TDT both pre- and post-isolation. TDT decreased across all animals ($p=0.0398$; $p<0.05$) from pre to post 7-week isolation period. When looking at the change in TDT, we saw a trend that control animals showed a greater decrease in TDT than SI animals after 7 weeks ($p=0.0726$; $p>0.05$).

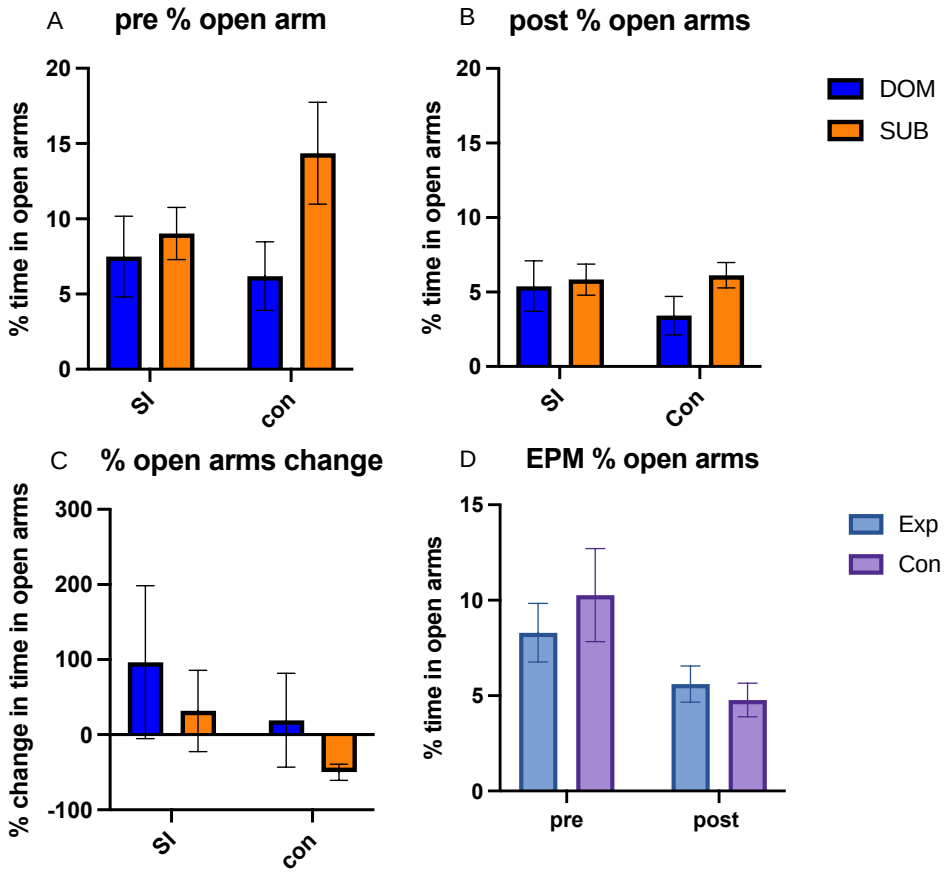

Figure 14: Elevated Plus Maze- % Time Spent in Open Arms

Figure 14A depicts % time spent in open arms pre isolation during EPM test (N=29): main effect of rank *(p=0.1006*); main effect of SI treatment *(p=0.4855);* interaction rank x SI treatment *(p=0.2549)*. **Figure 14B** depicts the % time spent in open arms post isolation during the EPM test (N= 29): main effect of rank *(p=0.3748);* main effect of SI treatment *(p=0.6336);* interaction rank x SI treatment *(p=05222)*. **Figure 14C** depicts change in % time spent in open arms between pre and post isolation in the EPM test (N=28): main effect of rank *(p=0.4892)*; main effect of SI treatment *(p=0.4127);* interaction between rank and SI treatment *(p=0.9819)*. **Figure 14D** depicts % time spent in open arms pre and post isolation for the experimental and control groups (N=29):

main effect of time (*p=0.0173);* main effect of SI treatment *(p=0.7323);* interaction SI treatment x time *(p = 0.3035).*

Percent time spent in open arms and closed arms are critical measures of anxiety in EPM test. Time spent in open arms demonstrates anxiolytic characteristics while time spent in closed arms demonstrates anxiogenic characteristic. There was no significant difference in time spent in the open arms by rank or by SI treatment both pre- and post-isolation time point. On average for all animals, there was a reduction in time spent in the open arms *(p=0.0173, p<0.05).*

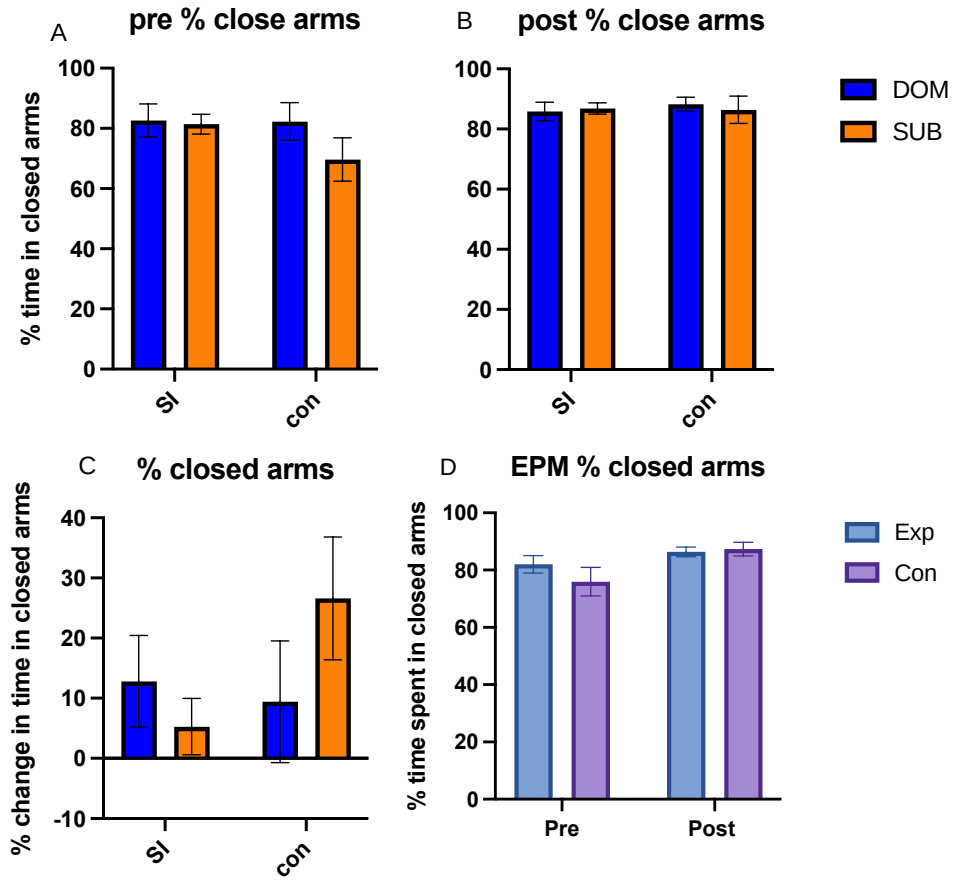

Figure 15: Elevated Plus Maze- % Time Spent in Closed Arms

Figure 15A depicts % time spent in closed arms pre isolation during EPM test (N=29): main effect of rank *(p=0.2490*); main effect of SI treatment *(p=0.3099);* interaction rank x SI treatment *(p=0.3385).* **Figure 15B** depicts the % time spent in closed arms post isolation during the EPM test (N= 29): main effect of rank *(p=0.8899);* main effect of SI treatment *(p=0.7747);* interaction rank x SI treatment *(p=0.6807).* **Figure 15C** depicts change in % time spent in closed arms

between pre and post isolation in the EPM test (N=28): main effect of rank *(p=0.5708)*; main effect of SI treatment *(p=0.2967);* interaction between rank and SI treatment *(p=0.1548).* **Figure 15D** depicts % time spent in closed arms pre and post isolation for the experimental and control groups (N=29): main effect of time (*p=0.0213);* main effect of SI treatment *(p=0.4499);* interaction SI treatment x time *(p = 0.2917).*

As expected, percent time spent in close arms had an inverse relationship with percent time spent in open arms. There was an increase in time spent in closed arms across all animals *(p=0.0213; p < 0.05).*

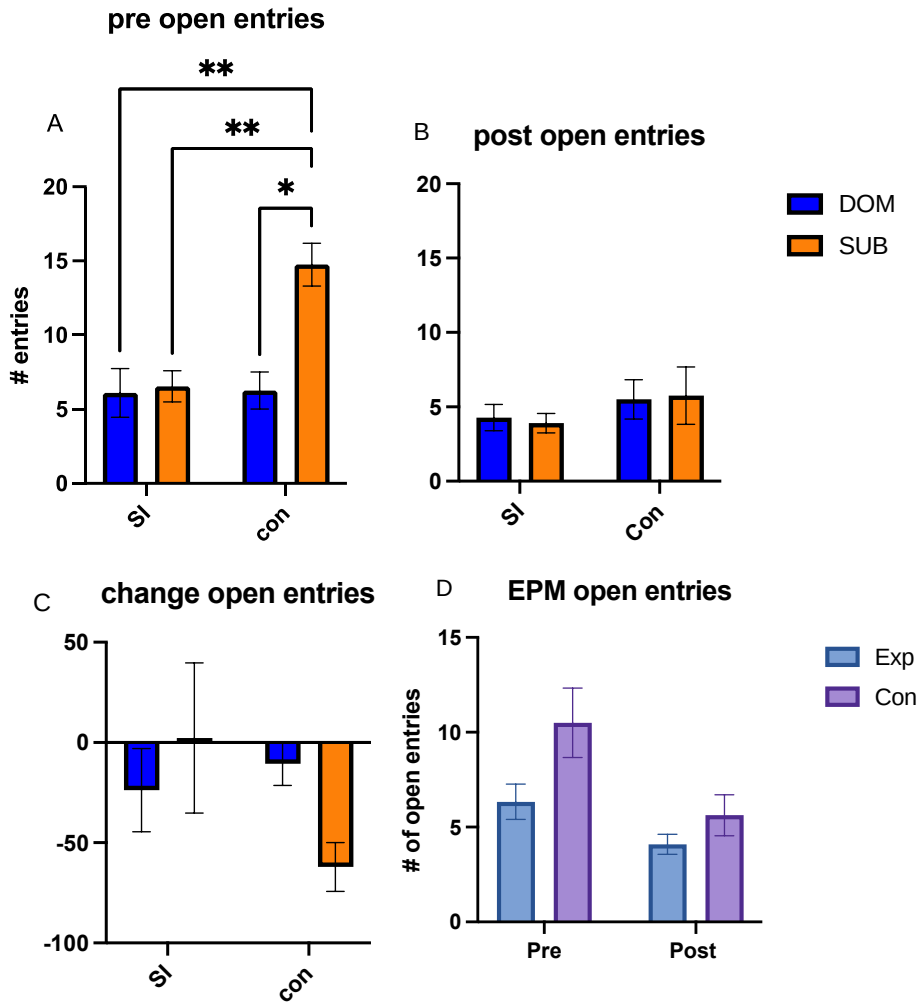

Figure 16: Elevated Plus Maze- Open Entries

Figure 16A depicts number of open entries pre isolation during EPM test (N=29): main effect of rank *(p=0.0132*); main effect of SI treatment *(p=0.0198);* interaction rank x SI treatment *(p=0.0241).* **Figure 16B** depicts the number of open entries spent in closed arms post isolation

during the EPM test (N= 29): main effect of rank *(p=0.9570);* main effect of SI treatment *(p=0.1838);* interaction rank x SI treatment *(p=0.7844).* **Figure 16C** depicts percent change in open entries between pre and post isolation in the EPM test (N=28): main effect of rank *(p=0.7207)*; main effect of SI treatment *(p=0.4713);* interaction between rank and SI treatment *(p=0.2796).* **Figure 16D** depicts number of open pre and post isolation for the experimental and control groups (N=29): main effect of time (*p=0.0018);* main effect of SI treatment *(p=0.0112);* interaction SI treatment x time *(p = 0.2295).*

Before isolation, subordinate animals had a significantly higher number of open entries than all other groups. After isolation, the number of open entries among all groups were the same. The number of open entries significantly reduced for all groups from pre to post isolation *(p=0.0018, p<0.05).* There was also the main effect of control groups having more open entries both pre and post isolation *(p=0.0112, p<0.05).* However, there was no interaction between time and SI treatment.

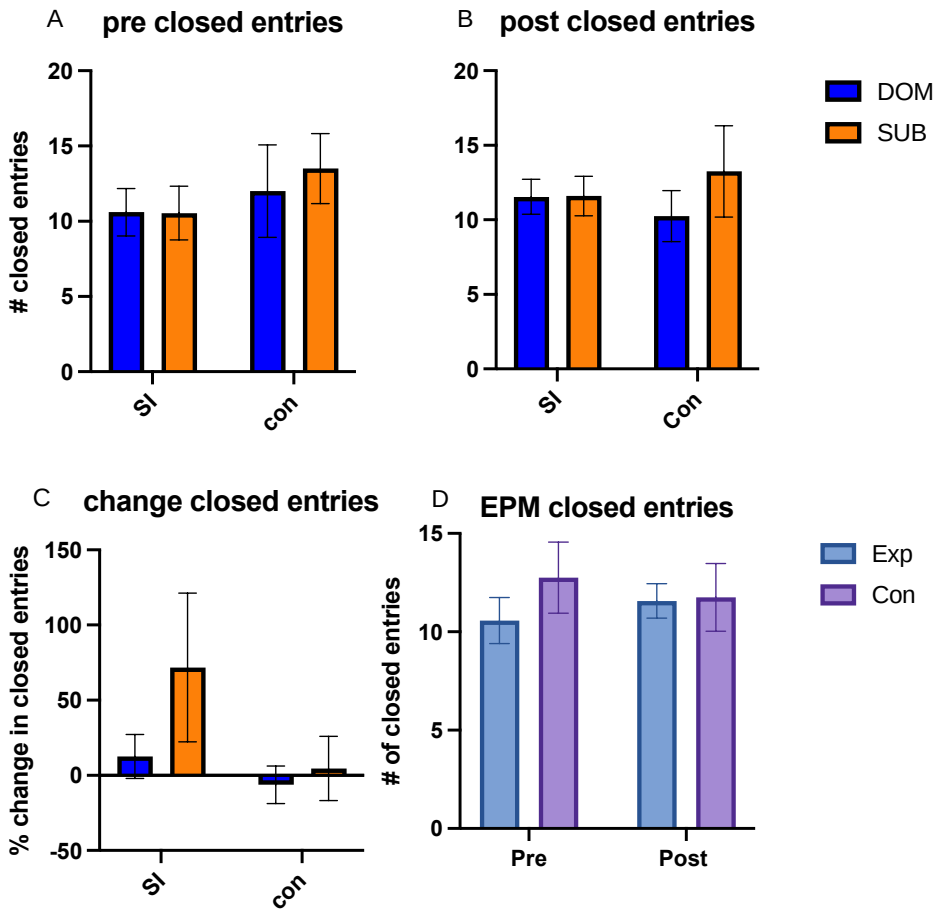

Figure 17: Elevated Plus Maze- Closed Entries

Figure 17A depicts number of closed entries pre isolation during EPM test (N=29): main effect of rank *(p=0.7538)*; main effect of SI treatment *(p=0.3487);* interaction rank x SI treatment *(p=0.7360).* **Figure 17B** depicts the number of closed entries spent in closed arms post isolation during the EPM test (N= 29): main effect of rank *(p=0.3984);* main effect of SI treatment *(p=0.9213);* interaction rank x SI treatment *(p=0.4152).* **Figure 17C** depicts percent change in closed entries between pre and post isolation in the EPM test (N=28): main effect of rank *(p=0.4178)*; main effect of SI treatment *(p=0.3208);* interaction between rank and SI treatment *(p=0.5730).* **Figure 17D** depicts number of open pre and post isolation for the experimental and control groups (N=29): main effect of time (*p=0.9999);* main effect of SI treatment *(p=0.4068);* interaction SI treatment x time *(p = 0.4811).*

There was no difference by rank or SI treatment in closed entries both before and after isolation. There was also no significant change in closed entries for all animals between pre and post SI period.

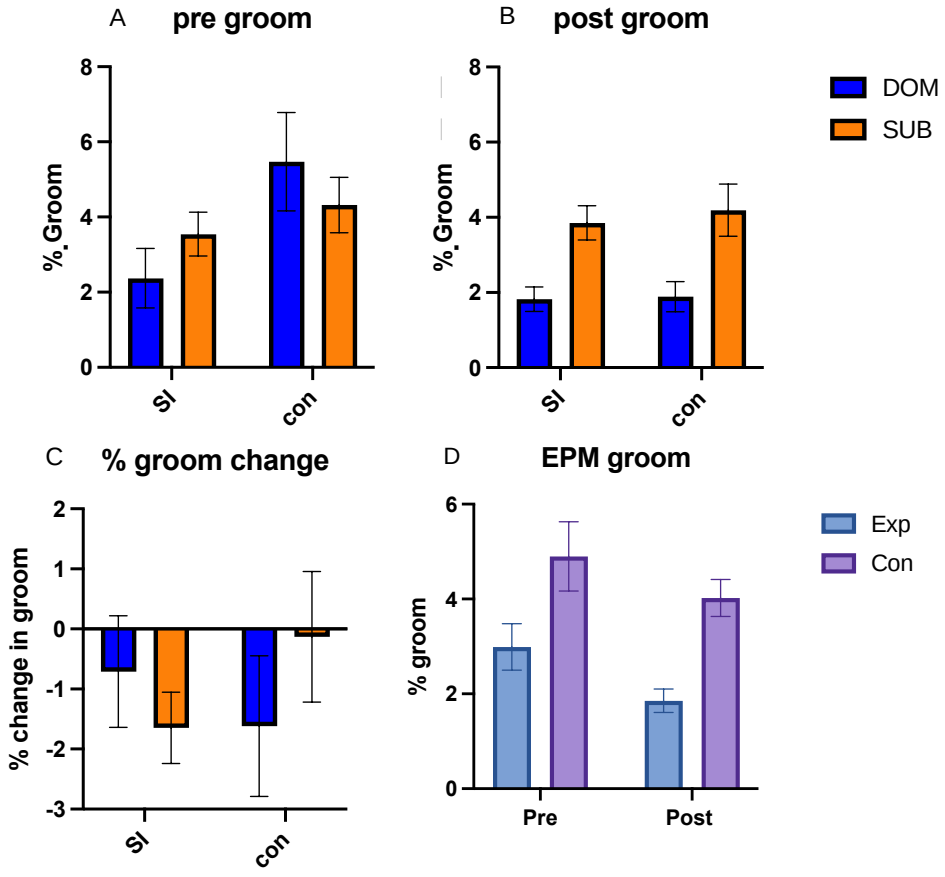

Figure 18: Elevated Plus Maze- Groom

Figure 18A depicts % time grooming pre isolation during EPM test (N=29): main effect of rank *(p=0.9908)*; main effect of SI treatment *(p=0.0440);* interaction rank x SI treatment *(p=0.2146).* **Figure 18B** depicts % time grooming post isolation during the EPM test (N= 29): main effect of rank *(p=0.0003);* main effect of SI treatment *(p=0.6973);* interaction rank x SI treatment *(p=0.7945).* **Figure 18C** depicts percent change in closed entries between pre and post isolation in the EPM test (N=28): main effect of rank *(p=0.7876)*; main effect of SI treatment *(p=0.7664);* interaction between rank and SI treatment *(p=0.2419).* **Figure 18D** depicts number of open pre and post isolation for the experimental and control groups (N=29): main effect of time (*p=0.0553);* main effect of SI treatment *(p=0.0002);* interaction SI treatment x time *(p = 0.8025).*

Grooming behavior has been observed in rodents when level of alertness is decreased, suggesting that groom has anxiolytic effects (Nunes, 2012). Grooming is also a self-care behavior and is linked to coat state maintenance (analyzed in section 4.3). Pre-isolation, control mice groomed more than experimental mice *(p=0.0440; p<0.05)*. Post isolation, there was no difference in % time grooming in the experimental and the control group. However, post isolation, there was a difference by rank. Subordinate mice groomed more than dominant mice *(p=0.002, p<0.05).* However, when comparing change in % grooming, there was no significant difference by rank or SI treatment. Across groups, we saw that there was a trend of decrease time grooming *(p=0.0553, p>0.05).*

4.6 Open Field Test

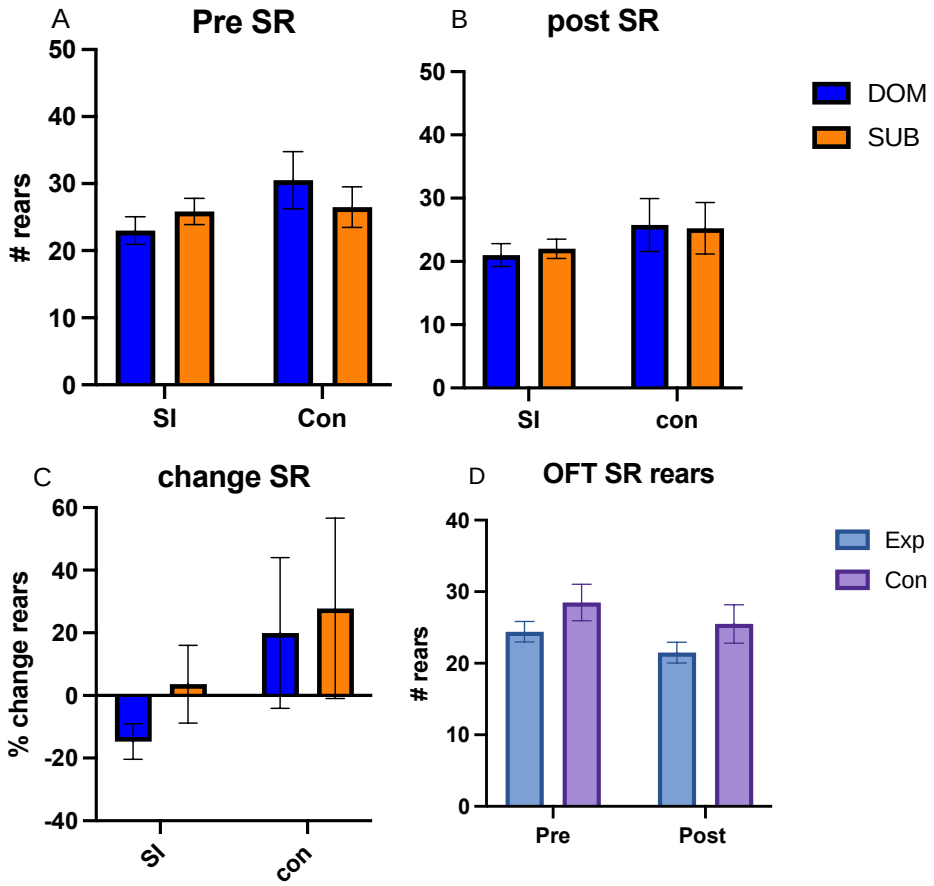

Figure 19: Open Field Test- Supported Rears

Figure 19A depicts number of supported rears pre isolation during OFT (N=22): main effect of rank *(p=0.8356)*; main effect of SI treatment *(p=0.1509);* interaction rank x SI treatment *(p=0.2226).* **Figure 19B** depicts number of unsupported rears post isolation during the OFT test (N= 22): main effect of rank *(p=0.9241);* main effect of SI treatment *(p=0.1360);* interaction rank x SI treatment *(p=0.7752).* **Figure 19C** depicts percent change in supported rears between pre and post isolation in the OFT test (N=22): main effect of rank *(p=0.4356)*; main effect of SI treatment *(p=0.0897);* interaction between rank and SI treatment *(p=0.7538).* **Figure 19D** depicts number supported rears pre and post isolation for the experimental and control groups (N=22): main effect of time (*p=0.13293);* main effect of SI treatment *(p=0.0432);* interaction SI treatment x time *(p = 0.9853).*

There was no significant difference in rears by rank or stress treatment both at the pre- and post-isolation time point. There was no significant change in supported rears before and after

isolation. There was a main effect of control mice rearing more than experimental mice both pre and post isolation *(p=0.0432, p>0.05).*

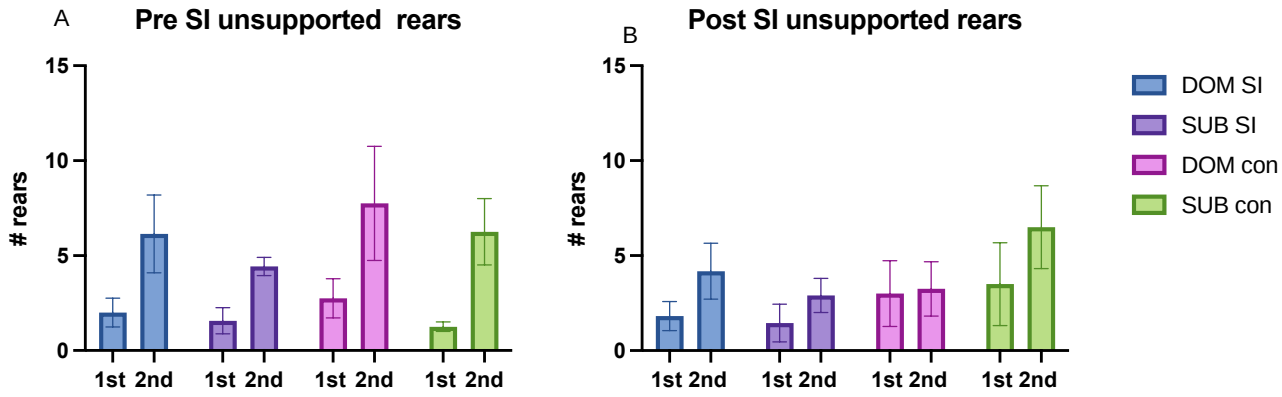

Figure 20: Open Field Test- Unsupported Rears (1)

Figure 20A depicts the number of unsupported rears in the first vs. the second half of the Open Field Test pre isolation (N=22): main effect of time (p=0.0002). **Figure 20AB** depicts the number of unsupported rears in the first vs. the second half of the Open Field Test post isolation (N=22): main effect of time (p=0.0852).

Unsupported rears significantly increased from 1st half of the Open Field Test compared to 2nd half of the test in Pre-isolation (*p= 0.0002, p< 0.05)*. Concurrent with past literature pre-isolation data suggests that unsupported rearing is stress sensitive (Seibenheber and Wooten, 2015). The mice were less likely to explore the maze when first placed in the maze than when they are more familiar with the surrounding. After the 7 weeks isolation period, there was still a trend of more unsupported rears in the second half of the OFT compared to the first half (p=0.0852). However, it was not a significant difference since the number of unsupported rears in the second half of the video did not increase as much.

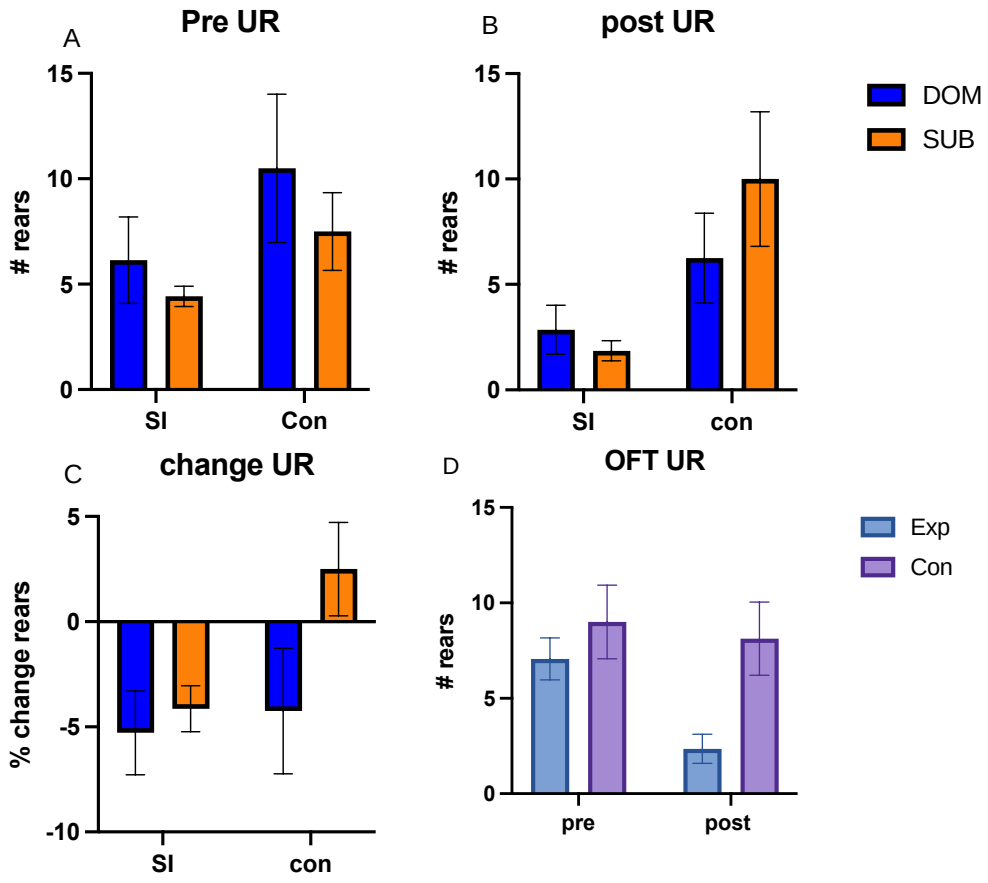

Figure 21: Open Field Test- Unsupported Rears (2)

Figure 21A depicts number of unsupported rears pre isolation during OFT (N=22): main effect of rank *(p=0.2601)*; main effect of SI treatment *(p=0.0835);* interaction rank x SI treatment *(p=0.7548).* **Figure 21B** depicts number of unsupported rears post isolation during the OFT test (N= 22): main effect of rank *(p=0.3721);* main effect of SI treatment *(p=0.0008);* interaction rank x SI treatment *(p=0.1288).* **Figure 21C** depicts percent change in unsupported rears between pre and post isolation in the OFT test (N=22): main effect of rank *(p=0.0697)*; main effect of SI treatment *(p=0.0769);* interaction between rank and SI treatment *(p=0.1874).* **Figure 21D** depicts number supported rears pre and post isolation for the experimental and control groups (N=22): main effect of time (*p=0.0467);* main effect of SI treatment *(p=0.0070);* interaction SI treatment x time *(p = 0.1679).*

Pre-isolation, there was a trend that control mice had a higher number of unsupported rears than mice in the experimental group (*p= 0.0835, p>0.05).* Post-isolation, we saw that the

difference in unsupported rears in the control group and the SI group exacerbated (*p= 0.0008, p<0.05).* There were no significant trends in rank in both pre- and post-isolation. However, when looking at changes in unsupported rears, we saw that there is a trend of subordinates displaying more unsupported rears (*p=0.0697, p> 0.05)* than dominant animals, and the control groups displaying more unsupported rears *p= 0.0697, p>0.05)* than control animals, but no interaction between rank and stress treatment (*p= 0.1874, p>05).*

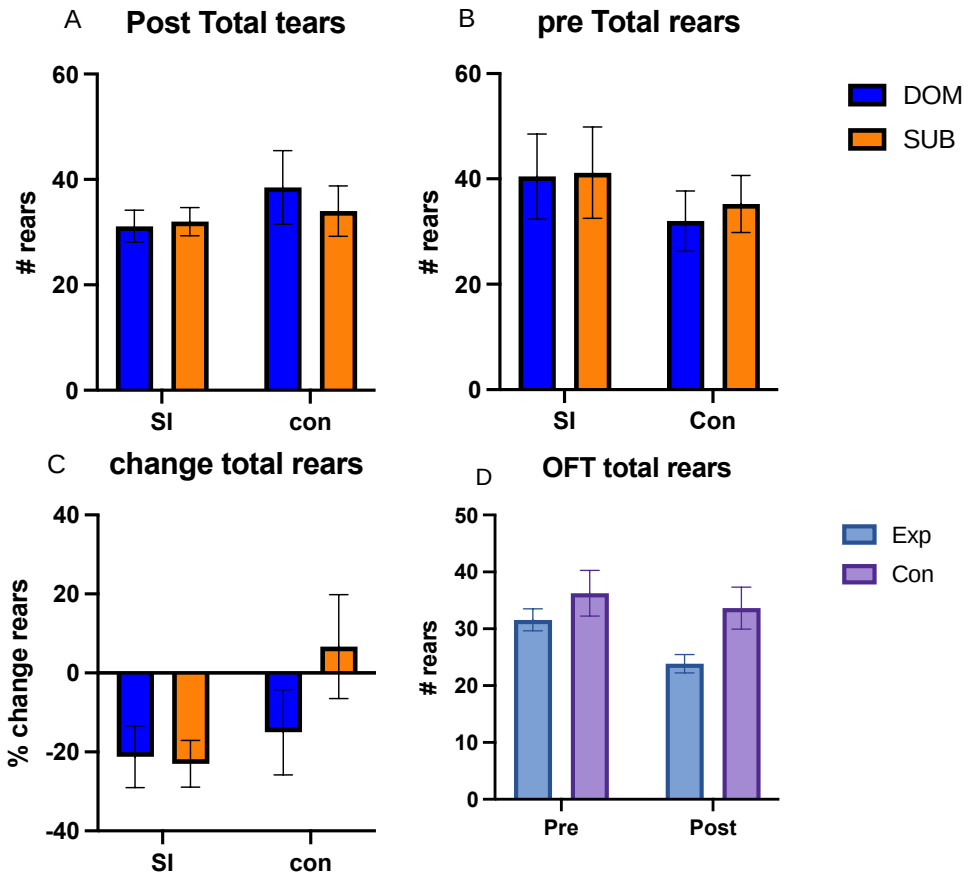

Figure 22: Open Field Test- Total Rears

Figure 22A depicts total number of rears pre isolation during OFT (N=22): main effect of rank *(p=0.6621)*; main effect of SI treatment *(p=0.2687);* interaction rank x SI treatment *(p=0.5217).* **Figure 22B** depicts number of total rears post isolation during the OFT test (N= 22): main effect of rank *(p=0.9035);* main effect of SI treatment *(p=0.4912);* interaction rank x SI treatment *(p=0.9035).* **Figure 22C** depicts percent change in total rears between pre and post isolation in the OFT test (N=22): main effect of rank *(p=0.2850)*; main effect of SI treatment *(p=0.0628);*

interaction between rank and SI treatment *(p=0.2103)*. **Figure 21D** depicts number supported rears pre and post isolation for the experimental and control groups (N=22): main effect of time (*p=0.0571);* main effect of SI treatment *(p=0.0092);* interaction SI treatment x time *(p = 0.3407).*

There was no significant difference in total rears by rank or stress treatment both at the pre- and post-isolation time point. There is no significant change in supported rears before and after isolation. When looking at the number of rears pre and post isolation, we do see a main effect of SI treatment (p=0.0092, p<0.05), showing that control mice reared more than experimental mice.

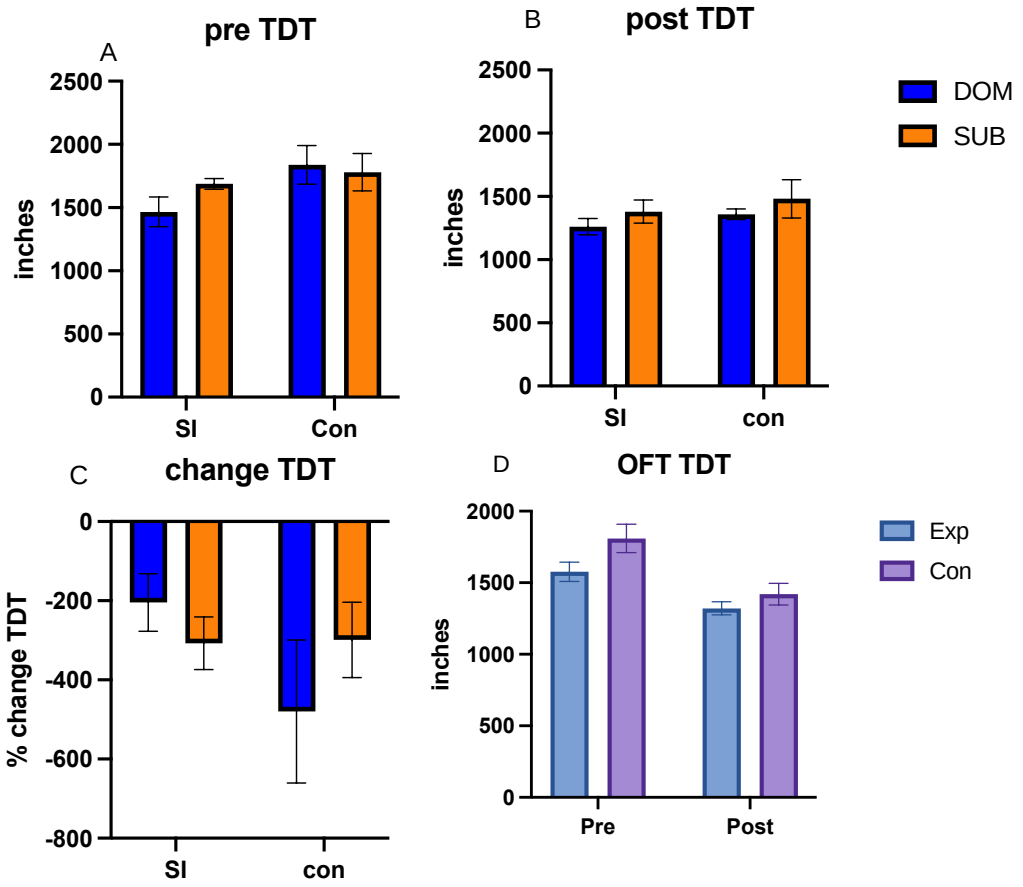

Figure 23: Open Field Test- Total Distance Travel

Figure 23A depicts total distance traveled pre isolation during OFT (N=22): main effect of rank *(p=0.4824)*; main effect of SI treatment *(p=0.0566);* interaction rank x SI treatment *(p=2364).* **Figure 23B** depicts total distance traveled post isolation during the OFT test (N= 22): main effect of rank *(p=0.2189);* main effect of SI treatment *(p=0.3089);* interaction rank x SI treatment *(p=0.9823).* **Figure 23C** depicts percent change in total distance traveled between pre and post isolation in the OFT test (N=22): main effect of rank *(p=0.7016)*; main effect of SI treatment

(p=0.1963); interaction between rank and SI treatment *(p=1706).* **Figure 23D** depicts total distance travel pre and post isolation for the experimental and control groups (N=22): main effect of time (*p<0.0001);* main effect of SI treatment *(p=0.0345);* interaction SI treatment x time *(p = 0.3867).*

There was a significant reduction TDT across all animals ($p < 0.0001$, $p < 0.05$) from before compared to after the 7-week experimental period. There was a main effect of control animals rearing more than experimental animals cross pre and post isolation. Moreover, the change in TDT was the same across all groups.

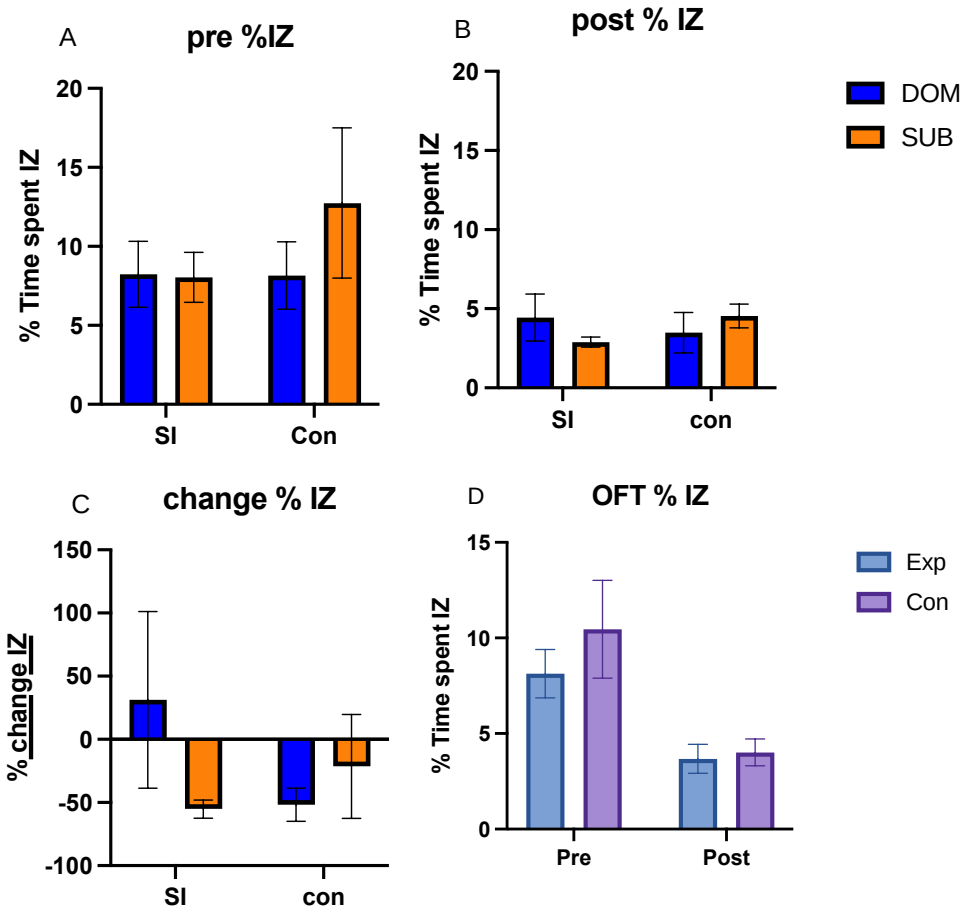

Figure 24: Open Field Test- % Inner Zone

Figure 24A depicts % time spent in the inner zone pre isolation during OFT (N=22): main effect of rank *(p=0.4077)*; main effect of SI treatment *(p=0.3825);* interaction rank x SI treatment *(p=0.3677).* **Figure 24B** depicts % time spent in the inner zone post isolation during the OFT test (N= 22): main effect of rank *(p=0.8333);* main effect of SI treatment *(p=0.7728);* interaction rank x SI treatment *(p=0.9823).* **Figure 24C** depicts percent change in time spent in the inner zone

between pre and post isolation in the OFT test (N=22): main effect of rank *(p=0.5822)*; main effect of SI treatment *(p=0.6280);* interaction between rank and SI treatment *(p=0.2585).* **Figure 24D** depicts total distance travel pre and post isolation for the experimental and control groups (N=22): main effect of time (*p=0.0003);* main effect of SI treatment *(p=0.3458);* interaction SI treatment x time *(p = 0.4815).*

Percent time spent in the inner zone (% IZ) is a crucial measure of anxiolytic behavior in OFT. There was no significant difference by rank or stress treatment both at the pre isolation and post isolation time point. However, the average percent time spent in the inner zone decreased significantly for all animals (*p= 0.0003, p < 0.05).* Nevertheless, the reduction in percent time spent in the inner zone was the same across all groups.

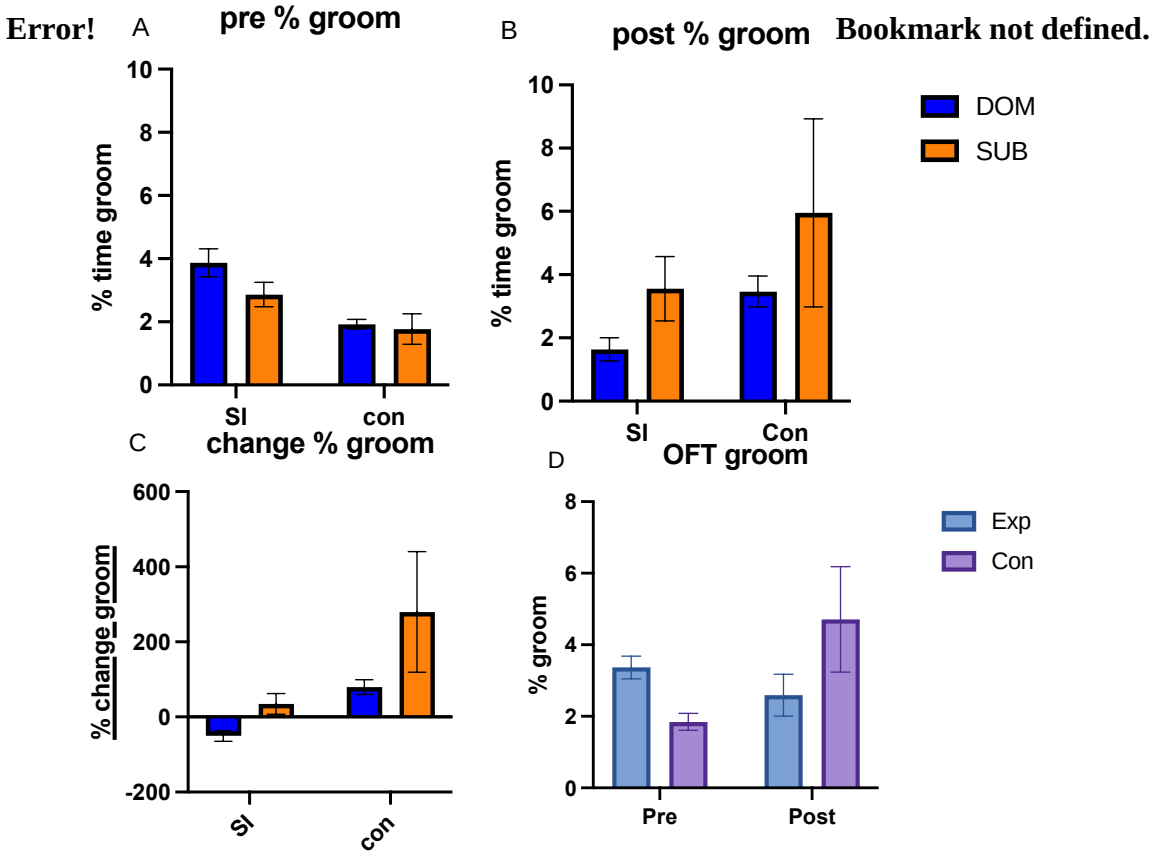

Figure 25: Open Field Test- % Groom

Figure 25A depicts % time spent grooming pre isolation during OFT (N=22): main effect of rank *(p=0.2030)*; main effect of SI treatment *(p=0.0027);* interaction rank x SI treatment *(p=0.3433).* **Figure 24B** depicts % time grooming post isolation during the OFT test (N= 22): main effect of rank *(p=0.1106);* main effect of SI treatment *(p=0.1246);* interaction rank x SI treatment

(p=0.8301). **Figure 24C** depicts percent change in time spent in the inner zone between pre and post isolation in the OFT test (N=22): main effect of rank *(p=0.0339)*; main effect of SI treatment *(p=0.0075);* interaction between rank and SI treatment *(p=0.3688).* **Figure 24D** depicts total distance travel pre and post isolation for the experimental and control groups (N=22): main effect of time (*p=0.6778);* main effect of SI treatment *(p=0.1477);* interaction SI treatment x time *(p = 0.0141).*

Pre isolation, percent time grooming was significantly higher in the experimental group compared to the control group (*p= 0.0027, p < 0.05).* After the 7-week experimental period, grooming behavior for control group increased, while that for the SI experimental mice decreased *(p= 0.0441, p<0.05).* Moreover, there was a main effect of subordinates having a greater increase in grooming behavior (*p= 0.0111, p< 0.05),* but no interaction between rank and stress treatment regarding change in percent groom. There was an interaction between SI treatment and time, in that control mice had an increasing time in grooming after the 7 weeks isolation period while experimental mice witnessed a decrease in grooming time.

4.7 Histology Data

For Histology, we stained brain slices of 30 mice with c-Fos rabbit primary antibody and goat-anti rabbit 647nm secondary antibody. We focused on five primary areas of the brain, the prelimbic regions, the dentate gyrus of the hippocampus, the amygdala (basolateral and medial amygdala), and the nucleus acumbens (both core and shell). The prelimbic cortex is responsible for attention processing and adaptation to shifting environments while the nucleus accumbens is responsible for modulating reward and pleasure (Sharpe and Killcross, 2015; Sailer, 2008). Altered responses in these regions are highly implicated in mood and anxiety disorders such as MDD, GAD, and PTSD (pleasure Sharpe and Killcross, 2015; Sailer, 2008). The amygdala, especially areas such as the central amygdala plays a key role in fear response and acts as an integrative hub for anxiety disorders (Glipin, 2015). Meanwhile, other regions of the amygdala

such as the medial amygdala and the basolateral amygdala are highly involved in the regulation of the HPA axis (Herman,1997). Through GABAergic interaction with the bed nucleus of the stria terminalis, the amygdala modulates the function of the paraventricular nucleus (PVN) which initiates the HPA axis and further stress response (Herman, 1997). Meanwhile, glutaminergic projections from limbic structures such as the hippocampus to the PVN are important for the inhibition of stress response (Herman 1997). The hippocampus is also involved in memory formation and plays a key role in the pathophysiology of major depression (Campbell, 2004). Histological analysis is ongoing and is not included in the thesis write-up.

5. Discussion

We investigated how SI differentially induces stress, depressive symptoms, and anxiety symptoms in mice of different ranks. Over the 7 weeks of isolation, we measured changes in corticosterone levels of the mice, changes in behavior during the OFT and EPM test, and coat state deterioration over time, and assessed sucrose consumption at the end of the isolation period.

After pair housing for 24 hours, mice quickly establish hierarchies consisting of one subordinate and one dominant mouse. We observed that the hierarchies become stable after 3 days of the competitive exclusion task. Dominant mice win 100% of the time and take significantly less time to win after 3 days. After rank is determined, mice were tested for anxiety symptoms, locomotor activity, and exploratory behavior in OFT and EPM before starting isolation treatment.

We looked at the effect of SI on stress by collecting fecal corticosterone samples. Based on the FCM data, we observed that isolation treatment induces a significant elevation of fecal CORT after 4 weeks. This suggests a critical period where acute isolation stress becomes chronic isolation stress. We hypothesize that after 4 weeks of isolation, there is a dysregulation in the HPA axis, leading to a faulty negative feedback loop and excess corticosterone secretion. There was no

significant difference in corticosterone levels by rank in either the control group or the socially isolated group. Thus, we cannot conclude whether isolation differentially induces stress by rank using the fecal corticosterone measurement.

Mice subjected to SI also showed significant coat deterioration compared to control mice. Coat state score is used as a measure of self-care and well-being for mice. While mice in the control groups showed no change in coat state, mice subjected to SI showed significant deterioration in coat state. Subordinate mice subjected to isolation showed the greatest coat state deterioration, suggesting that isolation affects the self-care and well-being of subordinate mice more than other that of dominant animals. We did not see a correlation between grooming behavior in EPM test with coat state score. However, in OFT, experimental animals showed a more significant change in percent grooming compared to control animals, indicating a reduction in self-maintenance behavior after isolation treatment.

When looking at Sucrose Preference Test, both dominant and subordinate animals subjected to isolation consumed less than 65% of 1% sucrose solution. However, dominant animals in the control group also had a percent sucrose consumption of less than 65%, indicating anhedonia behavior. Only subordinate animals in the control group exhibited no anhedonia symptoms. When comparing the sucrose consumption of the experimental and the control group, we did observe a trend that SI animals consumed less sucrose than control animals, suggesting that isolation may induce anhedonia behavior, but more experiments are needed.

For the Elevated Plus Maze Test, we looked at anxiety-related behaviors through time spent in the open arms, locomotor activity, and exploratory behavior such as rearing, head dips, and stretch attends. Head dips and stretch attends are risk-taking behaviors that can also be classified as anxiolytic behaviors. Pre-isolation, control mice, and experimental mice are comparable in all

measures except for the number of rears and number of open arms entries. Subordinate mice in the control group had the most open entries pre-isolation. However, all groups had a comparable number of open entries after the 7-week experimental period. It is also notable at the pre-isolation time point, subordinate mice in the control had the highest level of activity, seen through the highest level of rears (non-significant), head dips (non-significant), total distance travel (non-significant), open entries (significant), and percent time spent in the open arms (non-significant). After the 7-week period, we saw a significant decrease in the average head dips, stretch attends, and open entries of all animals pre- and post-isolation. This is due to the test decay effect. When mice are reintroduced to the EPM maze, the environment becomes less novel. Thus, the animal is less likely to explore the surrounding environment. Nevertheless, through the EPM test, we did not see that isolation treatment induces anxiety symptoms or differences in anxiety symptoms by rank.

As the findings of the EPM, we also saw a test decay for the OFT. There is a significant decrease in percent time spent in the inner zone, locomotor activity, and exploratory behavior for all groups. Regarding unsupported rears, we observed that unsupported rears reduced significantly more for SI groups than control groups, suggesting that the experimental groups are more sensitive to stress than the control group after experiencing 7 weeks of isolation. Based on the OFT, we cannot conclude how isolation treatment induces anxiety symptoms differentially by rank.

Overall, we observed isolation-induced stress, and deterioration in well-being and maintenance, especially after 4 weeks of chronic isolation. The effect of isolation on self-maintenance is especially prominent in subordinate animals. Chronic isolation induced anhedonia behavior in the experimental groups, while acute isolation induced anhedonia in the dominant

group. We also saw that acute isolation induced stress in the control groups during separation in the SPT. However, we did not see an effect of SI on anxiety-related behavior.

6. Limitations

The biggest limitation of our experiment is the small control samples. There are only 8 control animals, 4 subordinates, and 4 dominants. The small sample size introduces higher variability and many statistically insignificant comparisons between the subordinate and dominant in the control groups, and between the experimental and control group.

We were not able to include the OFT and c-Fos pilot data in our analysis due to time constraints and differences in experimental parameters. The OFT performed for the pilot group was under red light, while that of the main experiment group was performed under dim light. Because mice were more active during the dark phase, there was a significant difference between the behaviors and activity levels of the pilot group and the main experimental group. While we cannot include the pilot OFT group in the analysis, this proposes an interesting comparison of rank differences that might only be present in the active phase. C-Fos results of the pilot mice were excluded from analysis since the pilot mice were sacrificed at the end of 8 weeks, while mice from the follow-up study were sacrificed at the end of 7 weeks.

Another limitation is that running OFT and EPM twice can introduce re-test bias. We saw a reduction in locomotor activity and exploratory behavior for all mice after the 7-week experimental period. When animals are re-tested for OFT and EPM, the maze used becomes less novel during the post-test. Thus, we can expect a reduction in exploratory behavior and activity.

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