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# Time of Day Variation in Mechanisms Underlying Social Stress

by

Emma Shaughnessy

Under the Direction of Kim Huhman, PhD

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2024

ABSTRACT

Social stress is a salient risk factor for the etiology and persistence of multiple neuropsychiatric diseases including schizophrenia, anxiety, and mood disorders and is often studied in rodents using social defeat models. A goal of understanding the neural substrates and molecular mechanisms underlying neurophysiological responses to social stress is to discover potential targets for novel, more effective treatments for these debilitating diseases. The overarching goal of this project was to evaluate two potential mechanisms underlying social stress-induced behavioral changes: brain derived neurotrophic factor (BDNF) and perineuronal nets (PNNs). In Aim 1, we tested the hypothesis that BDNF, a neurotrophic factor implicated in the function of anti-depressants, affects responses to social stress differentially in the light and the dark. Indeed, we found that manipulation of BDNF signaling has opposing effects on the response to social stress in the light versus the dark phase of the daily cycle. In Aim 1a, we examined cellular activation in key brain regions that are responsive to social defeat stress and BDNF-signaling but none were differentially affected by lighting. In Aim 1b, we tested the hypothesis that downstream effectors of BDNF-signaling are differentially expressed in the light and the dark. We found that the BDNF receptor tropomyosin kinase receptor B (TrkB) transcripts increase in the light in ACC and that *gad1*, a genetic marker for GABAergic cells, is lower in the light than in the dark in the BLA. In Aim 2, we investigated whether perineuronal nets (PNNs), an extracellular proteoglycan matrix protein involved in synaptic plasticity, were changing across the light:dark cycle or after social defeat stress. In hamsters, PNNs did not vary across the light:dark cycle, but they did change in a sex-specific manner after social defeat stress specifically in

hippocampal area CA1. There were also sex differences in WFA expression in BLA and SSC. Collectively, these data revealed that both BDNF and PNNs are candidates in the modulations of behavioral responses to social defeat stress, and, in particular, the current data emphasize the importance of time-of-day as a critical variable when assessing potential therapeutic potential of novel interventions for stress-related neuropsychiatric disorders.

INDEX WORDS Brain-derived neurotrophic factor, social stress, Syrian hamster, perineuronal nets

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May 2024

## DEDICATION

This is dedicated to my wonderful husband, for all of his support and encouragement throughout this process. I couldn't have done it without you. I would also like to dedicate this to my dad, the original Dr. Shaughnessy (PhD). Thank you for your love of science, curiosity, and discovery.

## ACKNOWLEDGEMENTS

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# 1 INTRODUCTION

## 1.1 Overview

Roughly 400 million people suffer from mood disorders worldwide, and that number has skyrocketed in the past few years during the COVID19 pandemic (Kessler et al., 2003; Twenge & Joiner, 2020), a finding that likely is related to the important role of stress in the etiology and expression of these disorders. Depression is the number one cause of disability worldwide, but shockingly only 40% of patients respond to currently available treatments (Huh et al.; *Suicide Facts*, 2021; Trivedi). It is critical that we develop a better understanding of the molecular mechanisms underlying neuropsychiatric illness in order to create novel and more effective therapeutic options.

The overarching goal of this project was to investigate how stress leads to anxiety- and depression-like behaviors with the ultimate goal of supporting discovery of better, more targeted interventions and paying close attention to time of day as a critical variable in this behavioral shift. Two potential mediators of anxiety- and depression-like behaviors are brain-derived neurotrophic factor (BDNF) and perineuronal nets (PNNs). Interestingly, we have shown that BDNF has a differential effect on the behavioral response to stress depending on the phase of the daily light:dark cycle during which it is delivered. Aim 1 is focused on investigating potential mechanisms underlying this unique, time-dependent finding, including investigating whether the neural circuitry mediating stress-related behavioral responses is differentially responsive to social stress depending on time of day and identifying potential molecular mechanisms of BDNF signaling that might vary in the light versus the dark. Aim 2 investigated whether perineuronal nets might modulate depressive-like behavioral changes associated with

stress and to test whether they vary over the daily cycle and potentially alter how stress or pharmacological interventions alter brain and behavior during the day versus during the night.

### 1.2 Stress

Stress was defined by Hans Selye as the nonspecific response of the body to any demand for change. This encompasses mental, emotional, and physical responses to environmental changes or threats (Selye, 1956). Stress acts through the hypothalamic-pituitary-adrenal (HPA) axis, ultimately leading to the release of stress hormones such as cortisol/corticosterone and adrenaline. The HPA axis is regulated by a negative feedback loop of stress hormone receptors in the hippocampus (Sheng et al., 2021). The adaptation to stress is termed allostatic load. Acute stress is adaptive and can help an organism respond to an immediate threat. However, chronic exposure to stress (a cumulative increase in allostatic load) can alter this mechanism and lead to negative outcomes such as anxiety, heart disease, and substance abuse (Ramanathan & Desrouleaux, 2022). There are a variety of methods for inducing stress in laboratory rodents, including restraint stress, social stress, tail-suspension, and forced swim test (Atrooz et al., 2021).

### 1.3 Social stress in Syrian hamsters

One of the most commonly reported risk factors for developing a mood disorder is social stress (Bjorkqvist, 2001). Social stress is present throughout the animal kingdom, and perception of loss of or low social status has a profound impact on behavior (Bartolomucci et al., 2004; Gilbert et al., 2002). The most common laboratory method to study the effect of social stress is a resident-intruder model,

wherein a novel opponent is introduced to the home cage of a conspecific. Typically, the resident exhibits territorial aggression towards the intruder, causing the intruder to exhibit submissive behavior, thus establishing a dominance hierarchy with the resident generally establishing dominance over the intruder.

In our lab we use Syrian hamsters to study the mechanisms through which social stress, particularly social defeat, causes changes in brain and behavior. Syrian hamsters an ideal model with which to study social stress and agonistic (i.e., related to competition) behavior because hamsters are highly territorial under standard laboratory housing conditions, and they readily defend their home cage against an intruding conspecific (Huhman et al., 1990; Huhman et al., 1991; Kim L. Huhman et al., 2003). In Syrian hamsters, agonistic encounters are highly ritualized and easily quantified (H.E. Albers et al., 2002). Brief encounters in hamsters rarely result in injury, enabling us to study the effects of social stress, alone, without the confound of stress caused by tissue damage and the resulting inflammatory response that often accompany agonistic encounters in other rodent models of social stress. When hamsters lose an agonistic encounter they mount a robust hypothalamic-pituitary-adrenocortical response, which is not exhibited by their winning, or dominant, opponent (Huhman et al., 1990; Huhman et al., 1991). Losing, or subordinate, hamsters also subsequently fail to display normal territorial aggression and instead exhibit only submissive and defensive behaviors towards a novel hamster, even if this opponent is an intruder into their home cage. Defeated hamsters also display increased social avoidance of a caged opponent in a novel arena, which is the standard dependent measure used in mouse models of social defeat stress. The shift from territorial aggression to submission and avoidance in

hamsters has been termed conditioned defeat. Conditioned defeat requires sensing the aversive stimulus of the aggressor and formation of a fear memory. and molecular changes in the brain. Socially defeated animals show an increase in anxiety- and depressive-like behaviors, as well as increased social avoidance and submissive behavior (Kim L. Huhman et al., 2003). These changes in behavior can persist for at least 30 days after exposure to a repeated agonistic encounter, making this a particularly useful model for long term behavioral changes seen in humans after exposure to a stressor (Kim L. Huhman et al., 2003). An important added benefit of using hamsters as a model for social stress is that both male and female hamsters show a similar range of agonistic behaviors and territorial defense so that they can be tested under identical conditions and potential sex differences can be directly examined. This is in marked contrast to the most commonly used mouse and rat models, wherein only males show territorial aggression under standard housing conditions (Solomon, Karom, & Huhman, 2007). Given that women are more than twice as likely to develop a mood disorder, it is critical that scientists are able to study sex as a biological variable in their model organisms (Bjorkqvist, 2001).

### 1.4 Brain Regions Mediating Behavioral Responses to Social Stress

A putative circuit underlying the response to social defeat stress in rodents has been defined (For review, see (Diaz & Lin, 2020)). Because social defeat stress requires learning about an aversive stimulus (aggressor attacking) and producing a behavioral response (subject fleeing, showing submissive behaviors) it is not surprising that the basolateral amygdala (BLA) has been implicated in this circuit. The BLA is the core region of fear conditioning (Fanselow & LeDoux, 1999; Maren, 2001). Temporary

inactivation of the BLA by activating $GABA_A$ receptors with the agonist muscimol decreases submissive behaviors following defeat (Jasnow & Huhman, 2001). Similarly, inhibition of protein synthesis within BLA blocks conditioned defeat behavior while overexpression of cAMP response element binding protein (CREB), which is a mediator of fear learning, in the BLA increases submissive behaviors exhibited by defeated animals(Jasnow et al., 2005; Markham et al., 2010). CREB is a downstream effector of BDNF-signaling as well. Because of these data, we chose to focus on BLA as one of the critical nodes within the social defeat circuit.

Medial prefrontal cortex (mPFC) is also strongly involved in modulating behavior following social defeat stress and fear conditioning (Bloodgood et al., 2018; Sierra-Mercado et al., 2011). The BLA has projections to and receives inputs from the mPFC (Huang et al., 2020; Liu et al., 2020). In hamsters, temporary inactivation of the mPFC with the $GABA_A$ receptor agonist muscimol increases, while blocking $GABA_A$ receptors there reduces social avoidance following social defeat in hamsters (Markham et al., 2012). The mPFC can be subdivided into 3 subregions, anterior cingulate cortex (ACC), prelimbic cortex (PL), and infralimbic cortex (IL) (Laubach et al., 2018). ACC is thought to be involved in threat-assessment and contextual fear memory (de Lima et al., 2022). The PL is required for fear acquisition, while the IL is required for fear extinction, and both regions have been shown to project to the BLA (Bloodgood et al., 2018; Sierra-Mercado et al., 2011). Mice that are considered susceptible to acute social defeat stress show morphological changes in PL to BLA projections following defeat: susceptible mice show higher mushroom spine density on basal dendrites in neurons projecting from PL to the BLA compared to resilient mice, which do not exhibit social avoidance

after social defeat. In addition, when the PL is chemogenetically inhibited in mice, social avoidance is reduced (Grossman et al., 2022). Conversely, when the IL is chemogenetically activated, defeated hamsters show less submissive behaviors toward a conspecific compared to defeated animals given vehicle (Dulka et al., 2020). Therefore, we also focused on the subdivisions of the mPFC in this study.

The nucleus accumbens (NAc) is also necessary for the acquisition and expression of conditioned defeat in hamsters (Gray et al., 2015; Luckett et al., 2012) and is immediately downstream of the BLA (Diaz & Lin, 2020). The NAc is important for active avoidance and for the behavioral response to social stress (Ramirez et al., 2015). Interestingly, the NAc is also one of the only components of the circuit mediating behavioral responses to social defeat stress wherein manipulation of synaptic transmission can restore territorial aggression towards a non-aggressive intruder in previously defeated hamsters (Gray et al., 2015; Luckett et al., 2012).

### 1.4. BDNF and Stress

BDNF has been strongly implicated in the etiology and treatment of mood and anxiety disorders (Duman et al., 2021). There is a strong correlation between decreased BDNF in the hippocampus and increased anxiety- and depression-like behaviors in humans and rodents (Martinowich et al., 2007). A single-nucleotide polymorphism (SNP) in the *bdnf* gene in humans, Val66Met, has been linked to increased susceptibility to developing mood disorders as well as to resistance to traditional antidepressant treatment in the clinical population and in rodent models with this SNP (Chen et al., 2006; Kocabas et al., 2011). Commonly prescribed antidepressant medications increase the mature form of BDNF in multiple brain

regions including the NAc and BLA. Genetically modified mice lacking endogenous BDNF and given antidepressants do not show improvement of anxiety-like behavior while wildtype littermates do show improvement (Saarelainen et al., 2003).

There are certainly ample data that stress impacts BDNF systems. Exposure to chronic stressors (including social defeat stress) decreases BDNF protein expression in the hippocampus and PFC in (Duman & Monteggia, 2006; Krishnan et al., 2007). Human patients with depression or who have died by suicide show decreased BDNF in cortical regions (Castren & Kojima, 2017; Duman et al., 2016). Selective knockdown of BDNF expression in hippocampus is sufficient to cause depressive-like behaviors (Taliaz et al., 2010). BDNF levels are increased following social defeat stress (Berton et al., 2006; Dulka et al., 2016). Our lab has shown that infusion of BDNF protein into BLA decreases submissive behaviors towards a non-aggressive intruder in defeated hamsters (Rosenhauer et al., 2019). BDNF regulates the expression of K+/Cl- co-transporter, KCC2, which has been shown to be dephosphorylated and down-regulated in corticotropin releasing hormone (CRH) positive neurons following social defeat stress, contributing to a shift in GABAergic transmission following acute and chronic stress (Maguire, 2014).

However, other labs have found that BDNF promotes responses to social defeat stress. Partial deletion of the BDNF gene in the VTA prevents the increase in social avoidance typically seen after chronic social defeat in mice (Berton et al., 2006). In addition, BDNF protein increases in the NAc following chronic social defeat (Berton et al., 2006; Krishnan et al., 2007), and infusion of BDNF directly into VTA decreases latency to immobility in the forced swim test (Eisch et al., 2003), suggesting that BDNF

promotes anxiety- and depression-like behavior. Together, these data suggest that BDNF within the mesolimbic dopamine circuit promotes the response to social defeat stress, which infers that BDNF may have some pro-depressant-like effects under at least some conditions.

Thus, there is a critical disconnect in the literature examining the role of BDNF in mood and behavior with some studies showing that BDNF promotes behavioral responses to stress, or is “pro-depressant”, while others indicate that BDNF has anti-depressant-like effects in that the effects of antidepressants and antianxiety medications are dependent on increases in BDNF. It is imperative that this conundrum is addressed. There are a few obvious differences in these two sets of data that might help to unravel the inconsistencies in the literature. The first difference is that many of the studies showing that BDNF promotes behavioral responses to stress (e.g., appears more “pro-depressant”) use mice, while studies showing a more antidepressant-like effect have been done in hamsters. (Rosenhauer et al., 2019) showed that, at least in our hands, defeated mice also show a decrease in social avoidance when given the TrkB agonist, 7,8-dihydroxyflavone (7,8-DHF), suggesting that a simple species difference is not the cause of the inconsistent data. Another difference in the experimental designs is that the vast majority of the studies done in mice are done in the light (or inactive) phase of the light-dark cycle, while the studies in hamsters are done in the dark (or active) phase of the cycle. Therefore, the current project also focuses on time of day of the behavioral and pharmacological experiments as a major factor. We hypothesize that there are time of day changes in BDNF/TrkB transmission that underly the observed opposite actions of BDNF in the light versus in the dark. This hypothesis was investigated in **Aim 1a**.

It is also the case that the studies in this area focus on the action of BDNF in different nodes of the neural circuit mediating behavioral responses to social defeat stress. As described briefly earlier, there are many brain regions involved in the production of the behavioral response to social defeat stress. Some of the BDNF literature has focused primarily on the mesolimbic dopamine system, including the nucleus accumbens and ventral tegmental area. Our lab has focused more on the amygdala and prefrontal cortex in addition to the mesolimbic dopamine system. It is thus possible that different areas are activated by social defeat stress and/or BDNF signaling and, further, that this could occur in a time-dependent manner. These hypotheses are investigated further in **Aim 1b.**

### 1.5 Perineuronal Nets and Neuronal Plasticity

Perineuronal nets (PNNs) are extracellular matrix proteoglycans surrounding a variety of cells in brain, although primarily around GABAergic neurons, and develop postnatally (Bosiacki et al., 2019). Functionally, PNNs are thought to inhibit synaptic plasticity and to create critical periods within development. Young songbirds do not express high levels of PNNs and readily learn new songs (Cornez et al., 2018). However, as they age and the PNNs develop fully, they lose the ability to learn new songs. Thus, PNNs appear to prevent synaptic plasticity, the foundation of learning and memory—indeed, when PNNs are reversibly degraded, adult songbirds can learn new songs (Cornez et al., 2021). PNNs are primarily expressed in cortical regions and the hippocampus (Sorg et al., 2016). In addition to being critical in development, it has more recently been shown that PNNs are critical for fear learning in adults. Following auditory fear conditioning, PNN expression increases significantly in the auditory cortex

(Banerjee et al., 2017). Because PNNs change after fear conditioning, it is possible that they also play a role in the learning associated with social defeat. Further, it is possible that this role could vary over the light-dark cycle so that learning during the light varies from in the dark. These possibilities were investigated in **Aim 2**.

## 1.6 References


Berton, O., et al., *Essential Role of BDNF in the Mesolimbic Dopamine Pathway in Social Defeat Stress.* Science, 2006. **311**(5762): p. 864-868.

Bjorkqvist, K., *Social defeat as a stressor in humans.* Physiology & Behavior, 2001. **73**(3): p. 435-42.

Diaz, V. and D. Lin, *Neural Circuits for Coping with Social Defeat.* Current Opinion in Neurobiology, 2020. **60**: p. 99-107.

Dulka, B., et al., *Proteolytic cleavage of proBDNF into mature BDNF in the basolateral amygdala is necessary for defeat-induced social avoidance.* Learning and Memory, 2016. **23**(4): p. 156-160.

Huh, J., et al., *Treatment of generalized anxiety disorder: a comprehensive review of the literature for psychopharmacologic alternatives to newer antidepressants and benzodiazepines. LID - PCC.08r00709.* (2155-7780 (Electronic)).

Huhman, K.L., et al., *Conditioned defeat in male and female Syrian hamsters.* (0018-506X (Print)).

Jang, S.-W., et al., *N-acetylserotonin activates TrkB receptor in a circadian rhythm.*

Kessler, R.C., et al., *Lifetime prevalence and age-of-onset distributions of DSM-IV in the National Comorbidity Survey Replication.* Archives of General Psychiatry, 2003. **62**(6): p. 593-602.

Maguire, J., *Stress-induced plasticity of GABAergic inhibition.* (1662-5102 (Print)).

Sasi, M., et al., *Neurobiology of local and intercellular BDNF signaling.* (1432-2013 (Electronic)).

Solomon, M.B., M.C. Karom, and K.L. Huhman, *Sex and estrous cycle differences in the display of conditioned defeat in Syrian hamsters.* Hormones and Behavior, 2007. **52**(2): p. 211-219.

Twenge, J.M. and T.E. Joiner, *U.S Census Bureau-assessed prevalence of anxiety and depressive symptoms in 2019 and during the 2020 COVID-19 pandemic.* Depression and Anxiety, 2020. **37(10)**: p. 954-956.

Trivedi, M.H., *Major depressive disorder: remission of associated symptoms.* Journal of Clinical Psychiatry. **67**(0160-6689 (Print)): p. 27-32.

Yang, C., et al., *Antidepressant Drugs Correct the Imbalance Between proBDNF/p75NTR/Sortilin and Mature BDNF/TrkB in the Brain of Mice with Chronic Stress.* Neurotoxicity Research, 2020. **37**(1): p. 171-182.

# 2 TIME OF DAY VARIATION IN BDNF SIGNALING RESPONSE INFLUENCES RESPONSE TO SOCIAL STRESS

## 2.1 Introduction

Social stress, arguably the most common form of stress experienced by humans, is known to be a major risk factor for mood and anxiety disorders (Almeida, 2013; Bjorkqvist, 2001; Jaycox et al., 2009). While there are treatments available for these debilitating diseases, many patients fail to adequately respond to standard medications, thus there is a critical need for novel therapeutic targets for treating anxiety and mood disorders (Trivedi, 2006; Zhdanava et al., 2021).

Our laboratory studies an animal model of social stress in Syrian hamsters. Hamsters of both sexes readily produce ritualized, territorial behaviors in the lab leading to the rapid establishment of clear dominant-subordinate hierarchies (H.E. Albers et al., 2002; Huhman, 2006; Huhman et al., 1992; McCann et al., 2014). Interestingly, we have shown that hamsters that lose (i.e., become subordinate) but not those that win (i.e., become dominant) agonistic interactions exhibit a marked hormonal stress response and long-lasting behavioral changes including increases in submissive and defensive behavior and social avoidance (Huhman, 2006; Huhman et al., 1992; McCann et al., 2014; Rosenhauer et al., 2019). A benefit of using hamsters as a model of social defeat stress is that identical procedures can be used in both sexes, and defeat-induced changes in hormones and behavior can be observed after only a single exposure to social defeat in the absence of any injuries or tissue damage. These changes in behavior are similar to symptoms seen in patients with mood and anxiety disorders (Menard et al., 2017). In addition, many key brain regions that mediate social stress-

induced behavioral changes in hamsters and mice have been identified, including medial prefrontal cortex (mPFC), nucleus accumbens (NAc), and amygdala (Luckett et al., 2012; Markham et al., 2012; Markham et al., 2010). (For review, see(Diaz & Lin, 2020)).

Brain-derived Neurotrophic Factor (BDNF), which binds to tropomyosin kinase receptor B (TrkB) to activate multiple signaling cascades, promotes cell survival and learning and memory (Caviedes et al., 2017). BDNF has also been proposed as a candidate molecule for the development of novel treatments for stress-related disorders such as depression because a BDNF response is necessary for a therapeutic response to common antidepressant drugs and other interventions (Björkholm & Monteggia, 2016; De Vry et al., 2016; Duman et al., 2021; Duman & Monteggia, 2006).

There are conflicting data on the role of BDNF in stress-related behavioral change, however. Some experimental data show that BDNF promotes, or is necessary, for social stress-induced behavioral changes, which is consistent with the possibility that BDNF might instead have pro-depressant-like effects. For example, knocking down BDNF in the ventral tegmental area in mice prevents social defeat-induced social avoidance (Berton et al., 2006), and mice that exhibit social avoidance following defeat, but not those that don’t, show a marked increase in BDNF protein in the nucleus accumbens (Krishnan et al., 2007). Conversely, other data suggest that BDNF reduces behavioral responses to social defeat stress, which is consistent with the idea that increasing BDNF signaling is anti-depressant. For example, it has been shown that systemic administration of a BDNF-mimetic small molecule, 7,8-dihydroxyflavone (7,8-DHF), decreases submissive behaviors in socially defeated hamsters and decreases

avoidance of a caged opponent in mice (Rosenhauer et al., 2019), indicating that activation of TrkB signaling during or after social defeat stress prevents the typical behavioral response to both acute and chronic social stress.

If we presume that both findings are true, it is critical that we understand differences among these studies to assess the true potential therapeutic potential of manipulating BDNF signaling. One critical difference among studies is the rodent species used with many studies using C57Bl/6 mice while our lab uses hamsters. We have shown, however, that administration of 7,8-DHF in C57Bl/6 mice following an acute defeat produces similar blunting of subsequent social avoidance (Rosenhauer et al., 2019). Another important, and often overlooked, variable that differs among studies is the time of day when behavioral manipulations and pharmacological treatments are done. Most laboratory rodents are nocturnal, and therefore naturally exhibit most behaviors during the dark, or active, phase of the daily cycle. However, many, if not most, labs conduct experiments during the light phase of the cycle.

The purpose of this study was to investigate how time of day might influence responses to social stress and to manipulations of BDNF signaling. First, we tested whether BDNF-active drugs might reduce responses to social defeat in the dark phase but promote behavioral responses in the light phase. After we found that this, indeed, appears to be the case, at least in hamsters, the subsequent studies were focused on beginning to elucidate where this effect may be occurring in brain as well as identifying potential BDNF-related molecular mechanisms that might underlie this effect. We tested the hypothesis that these opposing behavioral effects are controlled by separate brain regions and that administration of BDNF into these specific regions would be sufficient

to differentially alter avoidance behavior after defeat in the light versus the dark. Additionally, we hypothesized that there are time-of-day-dependent variations in TrkB receptor and downstream signaling molecule expression and that this difference might account for the differences in behavior after social defeat stress in the light and the dark.

## 2.2 Material and Methods

### *2.2.1 Hamsters*

Syrian hamsters (*Mesocricetus* auratus) were purchased from Charles Rivers Laboratory (New York, NY or Wilmington, MA) and singly housed upon arrival and given a minimum of a week after shipment to habituate to the animal facility. Subjects were at least 90 days old and between 110 and 150 g at the beginning of experimentation. Resident aggressors used for social defeat training were older, same-sex opponents whose weight exceeded 150 g. All animals were housed in polycarbonate cages (23 x 43 x 20 cm) with corncob bedding, cotton bedding materials, tubes for environmental enrichment, and wire topped cages and were housed on a 14:10 reverse light-dark cycle to maintain gonadal patency (Ottenweller et al., 1987). All animals were given *ad libitum* access to food and water. Before testing, hamsters were handled daily for at least 7 days prior to experiments to habituate them to the experimenters. Experimental designs are as described below and are shown in each figure. All procedures and protocols were approved by the Georgia State University Institutional Animal Care and Use Committee and were in accordance with standards outlined by the National Institutes of Health Guide for Care and Use of Laboratory Animals.

### *2.2.2 Experimental Design*

*Experiment 1*: Males (n=35) were defeated within the first two hours of the onset of the light or the dark phase of the light-dark cycle, as described below. Immediately after the defeat, subjects were removed from the aggressor's cage and given an intraperitoneal injection of 1.0 mg/kg 7,8-dihydroxyflavone (7,8-DHF; TCI America, Montgomeryville, PA) in 50% DMSO/50% saline or vehicle alone. Subjects were returned to their home cage. 24 hours after their defeat session, subjects were tested for social avoidance, as described below.

*Experiment 2*: Males (n=31) were defeated within either the first two hours of the light or the dark cycle. Immediately following defeat, they were given an intraperitoneal injection1.0mg/kg ANA12 i.p. in 100% DMSO or vehicle only. 24 hours later social avoidance was tested.

*Experiment 3*: Animals (males n=15; females n=16) were defeated within the first two hours of the onset of either the light or the dark cycle. 90 minutes after the end of the defeat, they were perfused and brains were harvested. They were sectioned on a cryostat at 40 um. Immunohistochemistry (IHC) against CFos was performed using 1:500 (abcam Cat ab208942).

*Experiment 4*: Males (n=25) had cannula guides placed in mPFC (+3.3 AP +/- 1.2 ML -2.5 DV measured from dura) and a separate group of males had cannula guides placed in BLA (n=31) (-0.7 AP +/-3.9 ML -2.5 DV measured from dura) because these were the brain regions in which we observed significant effects of defeat or drug in Experiment 3. Both groups underwent surgery at P60-P67 and were given a week to recover and to habituate to handling by the experimenter. Animals were defeated as

described below and given 0.4ng/200nl sterile saline vehicle rhBDNF or 200 nl sterile saline microinjected into the brain region of interest. 24 hours later, animals were tested for social avoidance.

*Experiment 5*: Males (n=16) were sacrificed at the onset of the light or the dark cycle, and brains were harvested, and frozen on dry ice; after which all brains were stored at -80ºC until sectioned. Brains were sectioned at 14µm on a cryostat onto Frosted Plus slides (ThermoFisher). RNAscope© *in situ* hybridization was performed using gad1 and ntrk2 probes. Control probes were ppib, polr2a, and ubc. Images were taken on a Keyence microscope (Osaka, Japan) at 40X in preselected regions (ACC, PL, IL, NAc, and BLA). Number of *ntrk2* and/or *gad1* positive neurons were counted using Cell Counter in FIJI and number of puncta was calculated using IMARIS software.

*Experiment 6*: Hamsters (n=6) were sacrificed at the onset of the light or the dark cycle and brains were harvested and frozen on dry ice; brains were stored at -80ºC. 1mm circular punches from pre-selected regions (ACC, PL, IL, NAc, and BLA) were taken. To increase yield, each sample is from bilateral punches of each region in each animal. Protein was isolated from the punches.15 ng of protein were loaded into each well of a 10 well 4-12% bis-tris SDS-PAGE gel. The gel was transferred to a nitrocellulose membrane and tagged with KCC2 antibody (1:500 Millipore cat 07-432) and B-actin (1:10,000 GeneTex GTX629630) as a loading control. Images of the membranes were taken using the Licor Odyssey CLx. Bands were identified based on size compared to the protein ladder loaded in the first well on the right of each membrane. They were measured using the Gel tool in FIJI. Ratios of KCC2 to B-actin were shown to correct for any error in loading.

### *2.2.3 Pharmacological Agents*

Doses and dosing regimens were those we have used previously and have shown to be effective (Rosenhauer et al., 2019). The small molecule TrkB agonist 7,8-dihydroxyflavone (7,8-DHF) (TCI America, Montgomeryville, PA) at 0.1mg/kg dose or vehicle (50% DMSO 50% saline) was administered intraperitoneally (IP) immediately after social defeat. The selective TrkB antagonist ANA-12 ([N2–2-2-Oxoazepan-3-yl amino] carbonyl phenyl benzo (b) thiophene- 2-carboxamide) (Sigma; St. Louis, MO) at 0.1 mg/kg dose or vehicle (100% DMSO) was also administered IP immediately after social defeat stress. BDNF (rhBDNF; Invitrogen; Waltham, MA) was administered site-specifically at 0.4ng/200nl immediately after social defeat training.

### *2.2.4 Social Defeat Training*

Subjects experienced a single social defeat as described in (K.L. Huhman et al., 2003). Briefly, subjects were placed for 15 minutes in the home cage of a resident aggressor (RA) that had been screened to ensure that they rapidly attacked an intruder placed in their home cage and that they also exhibited reliable but not extreme aggression (i.e., they produced no wounding of opponents). No defeat controls were placed in an empty RA cage to control for the stress of handling and exposure to a novel cage and scent of an aggressive conspecific.

### *2.2.5 Social Avoidance Testing*

Novel RAs were placed in a Plexiglas box with perforated walls and then placed in on one side of a clean cage. Subjects were placed on the opposite side of the cage facing away from the caged opponent and were allowed to freely explore for 5 minutes. The amount of time spent interacting with the caged RA (nose touching the cage

containing the novel opponent operationally defined as “social interaction"), amount of time spent on the half of the cage containing the caged RA but not directly interacting with the cage (operationally defined as “near”), and amount of time spent on the opposite half of the cage away from the caged opponent (operationally defined as “social avoidance”) were measured using Noldus Observer by experimenters naïve to experimental condition.

#### *2.2.6 Immunohistochemistry*

Perfused brains were sectioned to 40m on a Leica Cryostat (Somewhere, Somewhere). 3 sections from each animal were blocked using 10% normal donkey serum in PBS with 0.1% Triton-X (Sigma) for one hour. They were then incubated in primary antibody (Abcam) for 18 hours at room temperature. Sections were washed in PBS with 0.1% Triton-X and then moved to secondary antibody. They were washed again and mounted onto superfrost Plus slides (Thermo) and counterstained with DAPI (Vector Labs). Images of each brain region were taken at 4X. Images were opened in FIJI and the cell counter tool used to quantify number of CFos+ cells. Brain regions were selected based on their involvement in social defeat stress: the prefrontal cortex (divided into its subregions of anterior cingulate cortex, prelimbic cortex, and infralimbic cortex (Alexander & Brown, 2011; Markham et al., 2012)), nucleus accumbens (Luckett et al., 2012), and the basolateral amygdala (Markham et al., 2010).

#### *2.2.7 Cannulation, microinjections, and histological verification*

Animals were deeply anesthetized with isoflurane (2.5-5%). Animals were fit with ear bars to prevent movement during surgery and to level the skull. All stereotaxic coordinates were measured from bregma. For mPFC cannula guide placement, the

coordinates were measured from Bregma and are -0.7 AP, +/-3.9 ML, and -2.5 DV (measured from dura). BLA cannula guides were measured from Bregma and were placed at +3.3 AP, +/-1.2 ML, and 2.1 DV (measured from dura). A dummy stylet was placed in the cannula guides. These were removed and replaced daily during habituation to ensure patency and habituate the animals to microinjections. Microinjections were performed with an infusion pump (Harvard Apparatus, Holliston, MA) and a 1 ul Hamilton syringe that was connected to the injection needle with 50-gauge polyethylene tubing. To minimize damage to the injection site a shorter 26-gauge cannula guide was used with a longer injection needle, 33-gauge projecting 1.2 mm beyond the cannula guide. Microinjections were administered over 90 seconds and the needle held in place an additional 60 seconds to ensure drug diffusion from the needle tip. Microinjections were considered successful if the solution flowed easily and a small air bubble between drug and water moved in the tubing.

At the conclusion of the experiment, all animals were given an overdose of sodium pentobarbital and 200 nl of India Ink were microinjected into the cannula guides using the same 33-gauge projection needle to verify needle placement. Brains were collected and stored in 10% phosphate buffered saline. Brains were sectioned to 40 uM using a cryostat (Leica CM 3050S) and mounted onto SuperFrost Plus Slides (Thermo). Sections were visualized at 4X on a Zeiss brightfield microscope (Zeiss Axioplan) and ink placement was identified based on the Golden Hamster Brain Atlas (Morin & Wood, 2001). Only injections within 0.3 mm of target region, as determined by an observer blind to experimental group, were included for final analysis.

### *2.2.8 RNAScope© In Situ Hybridization*

Hamsters (n=16) were sacrificed at the onset of either the light or the dark phase of the cycle. Brains were harvested in an RNAse-free environment and frozen on dry ice. Brains were sectioned at 14µm and mounted onto Superfrost Plus slides (Thermofisher). Slides were stored at -80C until used. We used the RNAscope Multiplex Fluorescent v2 Reagent Kit (Advanced Cell Diagnostics) and performed ISH according to the user manual for fresh-frozen tissue. Slides were fixed in 4% paraformaldehyde then serially dehydrated with 50, 70, then 100% ethanol for 5 minutes each. Slides were stored at -20ºC in 100% ethanol overnight. On the second day, slides were dried at room temperature (RT) for 10 minutes. A hydrophobic barrier was drawn around each section to limit the spread of solutions. Slides were first treated with hydrogen peroxide for 10 minutes at RT, washed, and then a protease solution (Protease IV) for 30 minutes at RT. Next RNAscope probes against ntrk2 and gad1 were applied and slides were incubated in the HybridEZ oven (Advanced Cell Diagnostics) at 40ºC for 2 hours. Control slides used RNAscope probes against ppib, ubc, and polr2a for the same amount of time at same temperature. Every target probe contained a mixture of 20 ZZ oligonucleotide probes bound to target RNA, as follows: gad1-C2 (GenBank accession XM_013121730.3, cat 1215311-C2), ntrk2-C3 (custom designed from GenBank NW_024429196.1), ppib-C1 (GenBank accession XM_005075522.2, cat 890851-C1), ubc-C3 (GenBank accession NW_004801705.1 cat 890861-C3), and polr2a-C2 (GenBank accession XM_013111776.1, cat 890871-C2).Next we incubated slides in preamplifier and amplifier AMP1 30 min at 40ºC, AMP2 30 min at 40ºC, and AMP3 30 min at 40ºC). Each probe was developed sequentially with horseradish peroxidase and

VIVID dye. Each slide set had a combination of probes corresponding to each channel: green (Opal520), red (Opal570), or magenta/far red (Opal690). All slides were incubated with DAPI for 20 seconds, washed, dried, and coverslipped with ProLong Diamond Antifade Mountant (cat P36961, ThermoFisher). Slides were then imaged at 40X on a Keyence microscope (Osaka, Japan).

#### *2.2.9 Western Blot*

At the onset of either the dark or the light phase of the daily cycle, animals were euthanized with an overdose of sodium pentobarbitol. Brains harvested and fresh frozen using dry ice. 1mm punches of each brain region were taken from both hemispheres. Protein was isolated using RIPA buffer. Protein concentration was measured using a BCA Assay (ThermoFisher). 15 ng of protein were loaded into each well of a 12 well 4-12% bis-tris gel (Thermo) along with ChameleonDuo ladder (Licor, Lincoln, NE). Gels were run with MES buffer for 35 minutes at 150 volts. Gels were transferred to a nitrocellulose membrane using the iBlot transfer system. Membranes were blocked in Odyssey Blocking buffer (Licor) for 3 hours. The membrane was then incubated for 18 hours at room temperature in antibody against KCC2 (1:500 Millipore cat 07-432) and B-actin (1:10,000 GeneTex GTX629630) in blocking buffer. Excess primary antibody was removed with PBS with 0.1% Tween. The membrane was then incubated in secondary antibody Goat anti-Mouse 800 for B-actin (Licor Biosciences, cat 926-32210) and Goat anti-Rabbit680 for KCC2 (Licor Biosciences, cat 926-68071). The membranes were imaged on the Licor Odyssey Clx machine. The images were opened in FIJI and the Gel tool was used to quantify. To control for unequal loading, all data are expressed as a ratio of KCC2 to B-actin.

#### *2.2.10 Statistical Analysis*

All statistics were calculated using GraphPad Prism (9.2.1), unless otherwise noted. Student's t tests were used when data were normal (measured by Shapiro Wilke's test) and Mann Whitney U was used when data were not normal. Two- and three-way ANOVAs were used where appropriate and Fisher's LSD was used for post-hoc analysis. Effect size was calculated using Cohen's d in Microsoft Excel. The effect size was considered large if d was greater than or equal to 0.8 and medium if d was between 0.5 and 0.8.

## 2.3 Results

### *2.3.1 Activation of TrkB has opposing effects on social avoidance in the dark and the light*

Hamsters were socially defeated either at the onset of the dark (active) phase or the light (inactive) phase, given an ip injection of 7,8-DHF, and 24 hours later social avoidance was tested (Figure 1a). Activation of TrkB during the dark phase of the daily cycle decreased social avoidance while activation of TrkB during the light phase increased social avoidance (Figure 1b). There was a significant interaction of drug x lighting $F(1,31)=9.63$ $p=0.0041$. There was not a significant main effect of drug ($F(1,31)=0.05998$; $p=0.8081$) or lighting ($F(1,31)=0.3166$; $p=0.577$). Post-hoc analysis revealed a significant difference between dark vehicle and dark 7,8-DHF ($p=0.0228$) as well as between dark vehicle and light vehicle ($p=0.0155$). Interestingly, there was also a trend towards a significant difference between light vehicle and light 7,8-DHF ($p=0.0546$). There were large effect sizes observed when comparing dark vehicle and

dark 7,8-DHF (Cohen's d=1.398515506), light vehicle and light 7,8-DHF (Cohen's d=0.813834517), and dark 7,8-DHF and light 7,8-DHF (Cohen's d=0.82421507).

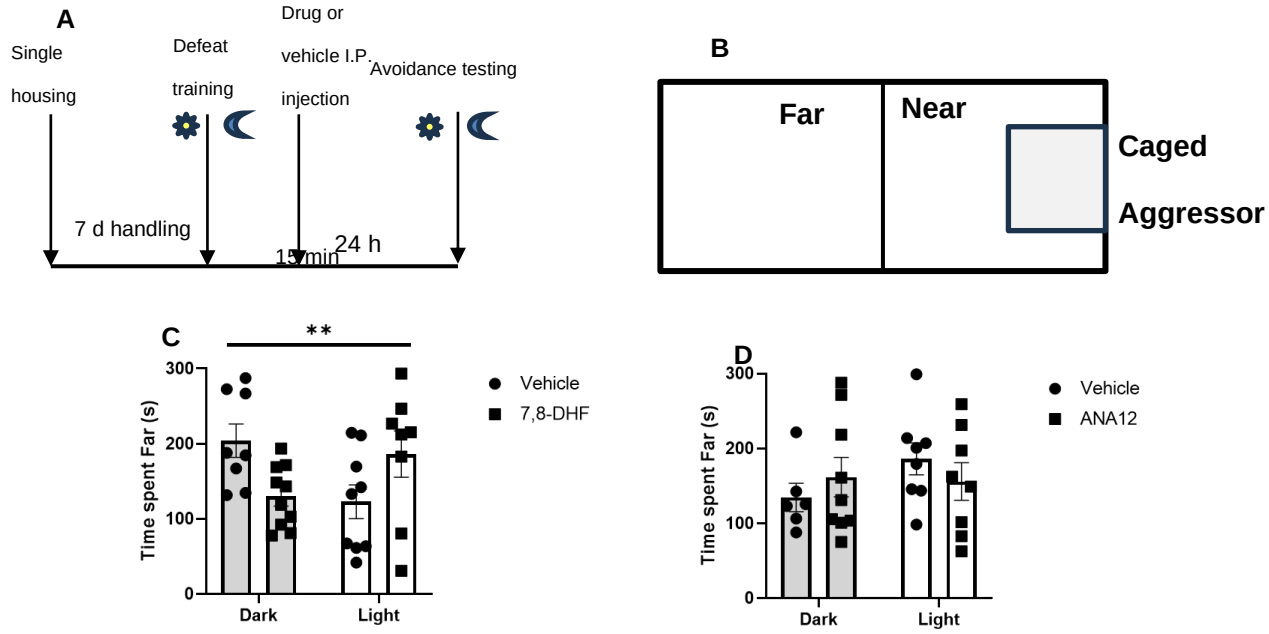



Figure 2.1 Figure 2.1 Systemic administration of tropomyosin kinase receptor B (TrkB) agonist, but not antagonist, produces opposing effects on avoidance behavior following social defeat in the light and the dark. A) Schematic of the experimental design for both pharmacological studies B) Schematic of avoidance testing arena. Seconds spent in the "Far" zone were operationally defined as "avoidance" of the caged aggressor C) There was a significant interaction of lighting and 7,8-dihydroxyflavone (7,8-DHF, TrkB agonist) on avoidance behavior following social defeat when drug was given immediately after the end of the social defeat training ($F(1,31)=9.63$ $p=0.0041$). Hamsters given 10 mg/kg 7,8-DHF I.P in the dark after defeat training showed a decrease in avoidance behavior the next day (Fisher's LSD $p=0.0228$). However, animals given the same dose IP immediately after defeat training in the light showed an increase in avoidance behavior. D) Animals given ANA12 ([N2-2-2-Oxoazepan-3-yl amino] carbonyl phenyl benzo (b) thiophene- 2-carboxamide), a TrkB antagonist, did not show any difference in avoidance behavior following social defeat in the light or the dark. ** indicates $p<0.01$

### *2.3.2 Blockade of TrkB does not alter social avoidance in the light or the dark*

Because activation of TrkB by 7,8-DHF has an interesting opposite effect on social avoidance in the light and the dark, we hypothesized that blockade of TrkB would have a similar lighting-dependent effect on social avoidance. However, ANA12 does not alter social avoidance in the light or the dark (Figure 1c). There was no significant interaction ($F(1,27)=1.391$; $p=0.2488$), main effect of lighting ($F(1,27)=0.8784$; $p=0.3569$), or main effect of drug ($F(1,27)=0.00409$; $p=0.9495$). There was a large effect size between dark vehicle and light vehicle (Cohen's $d=0.95758$), indicating there may be a baseline difference in response to social stress between the light and the dark that merits further investigation.

### *2.3.3 The BLA and PL show significant differences in neuronal activation after defeat or 7,8-DHF administration*

The next step was to begin to determine what brain regions might be differentially activated by 7,8-DHF in the light and the dark after social defeat stress. Using immunohistochemistry (IHC) for the immediate early gene product CFos as a proxy, cellular activation in each region was measured. All animals were socially defeated and given either 7,8-DHF or vehicle and sacrificed 90 minutes later (Figure 2a). An IHC against CFos protein was performed and number of CFos+ cells were counted in pre-selected regions. While both males and females were used for this experiment, no sex differences were observed ($p>0.05$) so data from both sexes are collapsed for further analysis. In BLA, there was a significant interaction between defeat and drug (Figure 2b, $F(1,38)=5.613$ $p=0.023$). In PL, there was a significant main effect of drug ($F(1,21)=5.9$

p=0.0242) and a significant main effect of defeat (F(1,21)=5.12 p=0.0344). However, there was no significant effect of lighting (Figure 2c). There was a trend in the ACC of defeat x lighting effect F(1,19)=4.246 (Figure 2d). IL showed no differences in CFos expression based on defeat, drug, or lighting and there were no significant interactions (Figure 2e, p>0.05). NAc did not show any significant effects of defeat, drug, lighting, or any interaction thereof (Figure 2f, p>0.05).

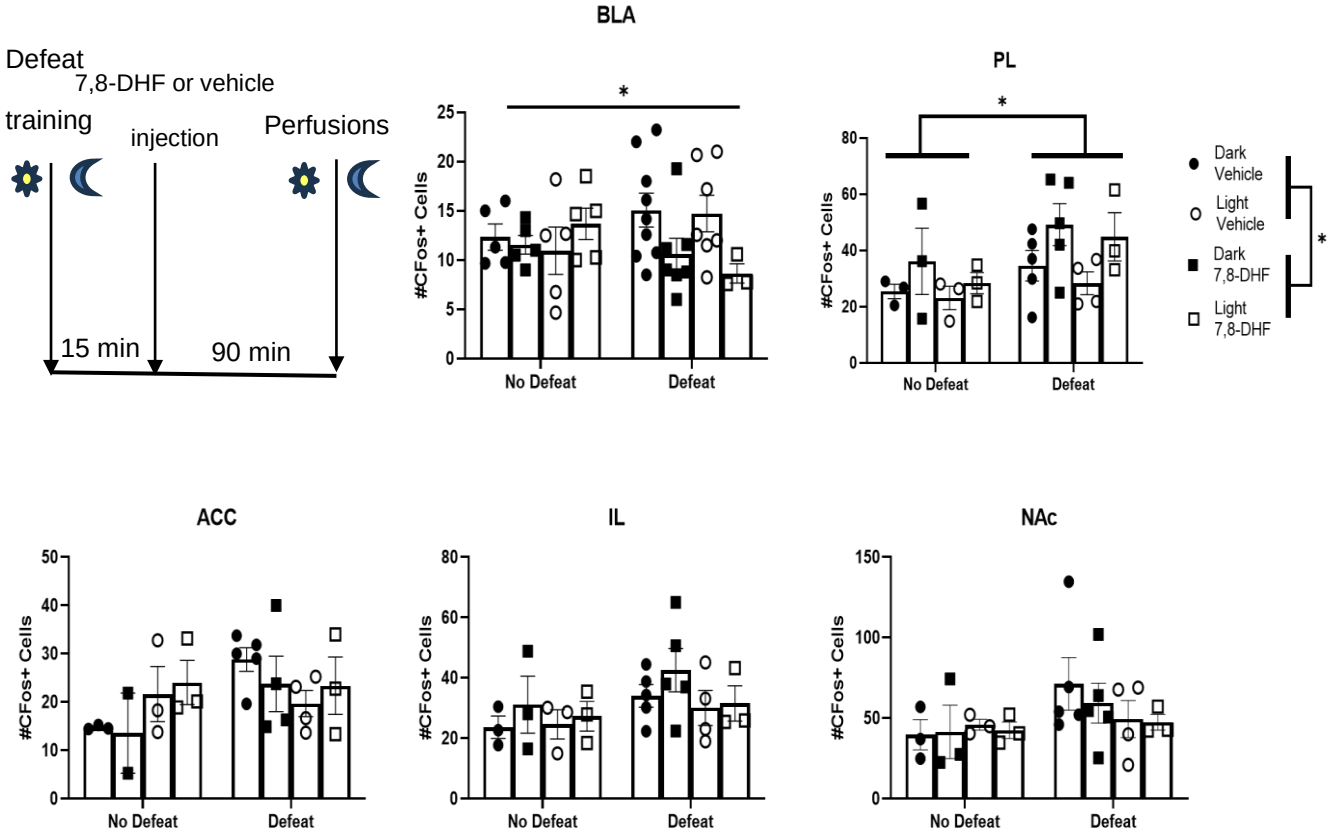



Figure 2.2 Cellular activation, as measured by CFos immunoreactivity, shows differential patterns in some areas based on defeat or drug but not lighting. A) Schematic of experimental design showing timeline of defeat to sacrifice. B) BLA displayed a significant interaction of drug x defeat (F(1,38)=5.613 p=0.023). Control, no defeat animals showed no changes in number of CFos+ cells based on lighting or defeat status, but defeated animals who received 7,8-DHF showed decreases in number of CFos+ cells regardless of lighting phase. C) PL showed a significant effect of drug (F((1,21)=5.9 p=0.0242); animals given 7,8-DHF showed a higher number of CFos+ cells compared to vehicle controls. There was also a significant effect of defeat (F(1,21)= 5.12 p=0.0344) where defeated animals showed higher number of CFos+ cells D) ACC showed a trend towards a significant defeat x lighting effect (F(1,19)=4.246 p=0.0533) where animals defeated in the dark showed higher number of CFos+ cells compared to no defeat controls. E) IL did not show any significant differences in number of CFos+ cells based on lighting, defeat, or drug F) NAc also did not show any significant differences in number of CFos+ cells based on lighting, defeat, or drug. * indicates p>0.05

### *2.3.4 Microinjection of BDNF into BLA and mPFC does not alter social avoidance behavior following social defeat*

Because there were significant differences in neuronal activation in PL and BLA, we tested the hypothesis that direct administration of BDNF into the mPFC or BLA will alter behavioral response to social defeat stress (experimental design shown in Figure 3a). Previous work has shown that administration of BDNF directly into the BLA can decrease submissive behaviors towards a non-aggressive intruder following social defeat stress (Rosenhauer et al., 2019), In mPFC, there was no significant effect of drug ($F(1,15)=0.1314$; $p=0.7220$), lighting ($F(1,15)=0.8601$; $p=0.3684$) or drug x lighting interaction ($F(1,15)=0.5292$; $p=0.4781$) on avoidance (Figure 3b). Additionally, there was only a medium effect size when comparing dark drug and dark vehicle (Cohen's $d=0.57573$). In BLA, there was no significant effect of drug ($F(1,27)=0.7913$; $p=0.3816$), lighting ($F(1,27)=0.3035$; $p=0.5862$) or interaction ($F(1,27)=0.3167$; $p=0.57582$) on avoidance (Figure 3c). There was a medium effect size between light drug and light vehicle (Cohen's $d=0.5578$), but no other noteworthy effect sizes. Injection sites are shown in Figure 3d and e.

### *2.3.5 Ntrk2 expression increases in the light in ACC*

One potential mechanism underlying the behavioral differences in the light and the dark is changing expression of TrkB or its mRNA, *ntrk2.* Number of *ntrk2* puncta increases in ACC in the light compared to the dark (Mann Whitney U, $p=0.0480$) (Figure 4a). Other brain regions examined did not show any significant changes in number of *ntrk2* puncta (PL Figure 4b $t=0.9447$, $p=0.3635$; IL Figure 4c $t=0.5822$, $p=0.5712$; NAc Figure 4d $t=0.6228$, $p=0.5451$; BLA Figure 4e $t=0.6669$, $p=0.5236$). Interestingly,

number of *ntrk2*-positive cells is not changing in any brain region (ACC Mann Whitney, p=0.3939 d=0.348261292; PL t=1.303 p=0.2170 d=0.672106045; IL t=1.008 p=0.3335 d=0.51463156; NAc t=0.5736 p=0.5778 d=0.328953958; BLA Mann Whitney p=0.5273 d=0.149626492).

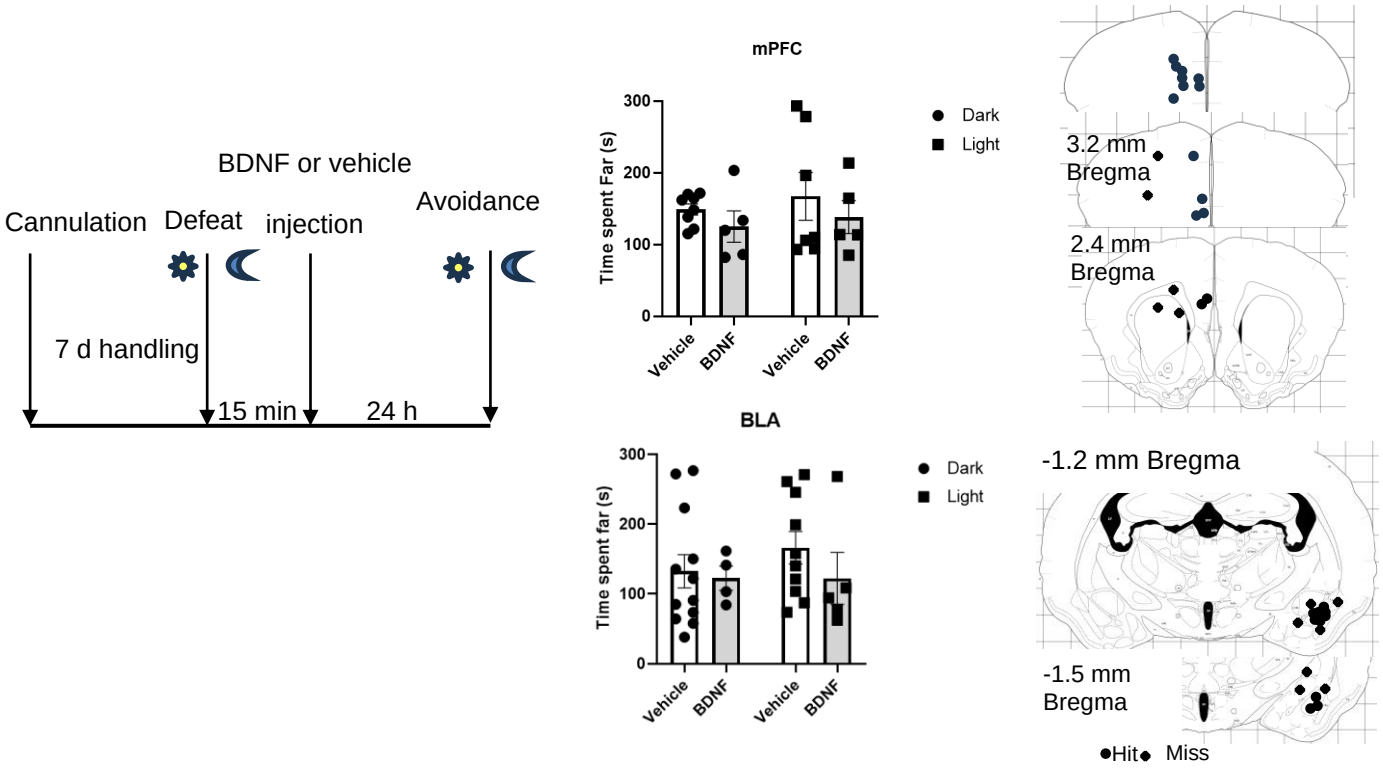



Figure 2.3 Administration of BDNF directly into either mPFC or BLA was not sufficient to alter avoidance behavior after social defeat stress in the light or the dark. A) Schematic of experimental design of surgical implantation of cannula guides and subsequent behavior B) Infusion of BDNF protein into mPFC did not affect avoidance behavior in defeated animals in the dark (circles) or the light (squares). C) Schematic for injection sites for data showed in panel B. Each mark represents one or more animals. Ink injections were used to verify needle placement at the completion of the experiment. Animals with ink injections within 0.3 mm of the targeted site were considered hits (shown by black circles); misses were recorded and shown by black x's. Illustrations were modified from (Morin & Wood, 2001). D) Infusion of BDNF protein directly into BLA did not significantly impact avoidance behavior in the dark (circles) or light (squares). E) Schematic for injection sites for data shown in panel D. Each mark represents one or more animals. Ink injections were used to verify needle placement at the completion of the experiment. Animals with ink injections within 0.3 mm of the target region were considered hits (shown in black circles); misses were recorded and shown by black x's. Illustrations were modified from (Morin & Wood, 2001).

In addition to evaluating *ntrk2* expression, we hypothesized that there may be differences in signals downstream of TrkB. We hypothesized that KCC2 and *gad1* expression may be changing between the light and the dark. We assessed number of

cells co-expressing *gad1* and *ntrk2* as well as number of puncta of *gad1* and number of cells expressing *gad1.* In BLA, there was number of cells expressing both *gad1* and ntrk2 in the light compared to the dark was significantly lower (Figure 5a t=2.347, p=0.0409; d=1.498588). Significant effects of lighting were not observed in other brain regions (ACC Figure 5b Mann-Whitney U, p=0.2739 d=0.295842608; PL Figure 5c t=0.1662 p=0.8710 d=0.091512607; IL Figure 5d t=1.625 p=0.13 d=0.858400972; NAc Figure 5e Mann-Whitney U p=0.637 d=0.278721067).There was no significant difference in number of *gad1* puncta in any of the brain regions investigated (ACC t=0.01997 p=0.9844 d=0.011386165; PL t=1.075 p=0.3054 d=0.58652; IL t=0.5843 p= 0.5734 d=0.36932; NAc t=0.4022 p=0.6952 d=0.22077667; BLA t=0.7519 p=0.4713 d=0.312597252). However, in the BLA there were much more *gad1*-expressing cells in the dark compared to the light (Figure 5f t=2.347 p=0.0409 d=1.498588008). This could explain the observed differences in number of cells expressing both *ntrk2* and *gad1* between the light and the dark. In the other brain regions, there were no statistically significant differences in number of *gad1* expressing cells in the dark versus the light

*Figure 2.4 In situ hybridization showing differences in expression of ntrk2 (gene that encodes tropomyosin kinase receptor B, TrkB) in the light and the dark A) Representative photomicrograph taken from NAc blue shows DAPI (counterstain for nuclei), magenta shows ntrk2 and red shows gad1 (gene encoding glutamate decarboxylase, an enzyme highly expressed in GABAergic cells). The scale bar represents 50 um. B) ACC shows a significant increase in number of ntrk2 puncta in the light (squares) compared to the dark (circles; p=0.0480). Other regions do not show differences in number of ntrk2 puncta based on lighting C) PL D) IL E) NAc. F) Number of cells containing at least 3 ntrk2 puncta in any brain region studied did not differ in the light and the dark G) ACC H) PL I) IL J) NAc K) BLA * indicates p>0.05*

(ACC: Figure 5g; Mann Whitney p=0.8357, d=0.295842608; PL: Figure 5h; t=0.1662, p=0.8710, d=0.091512607; IL: Figure 5i; t=1.625, p=0.13, d=0.858400972; NAc: Figure 5j; Mann Whitney p=0.637, d=0.278721067).

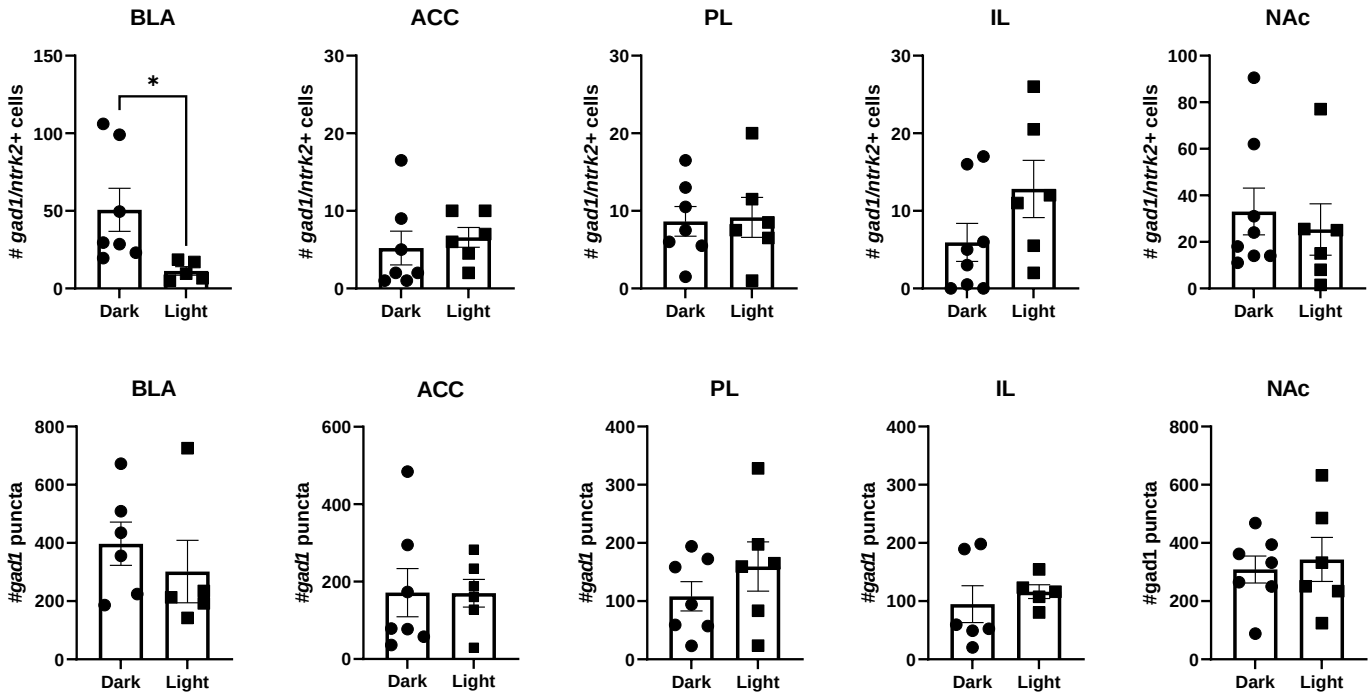
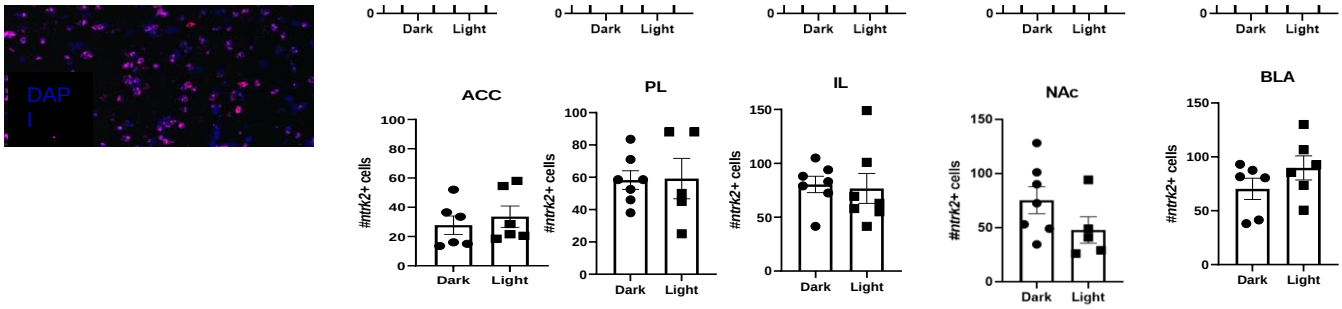


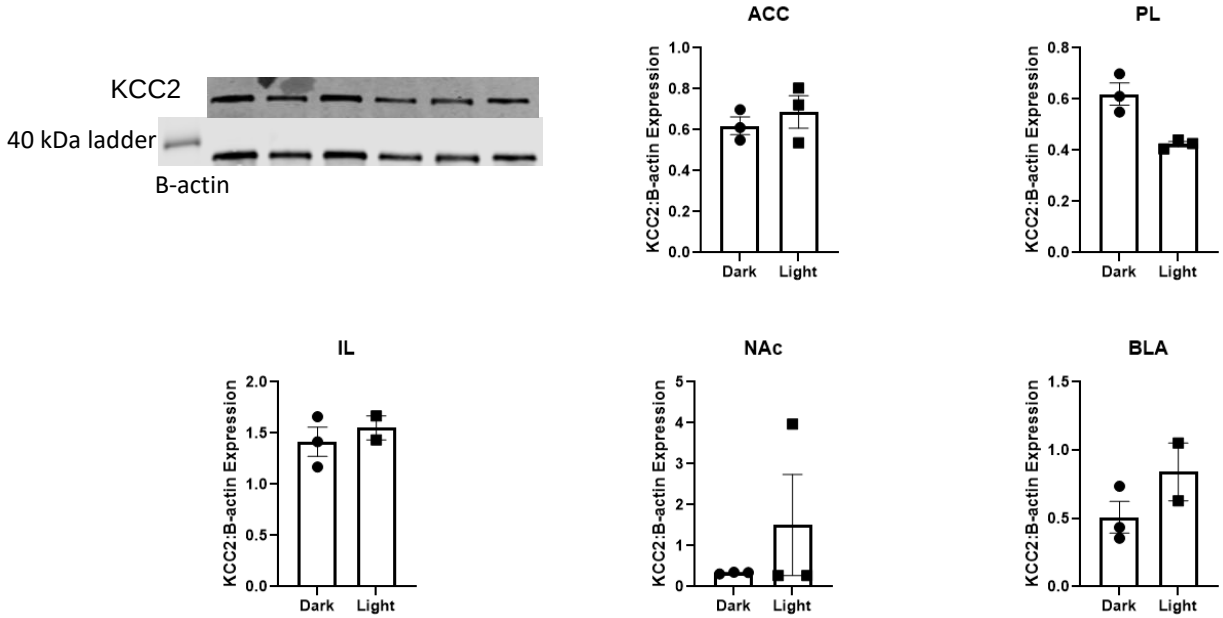

Figure 2.5 Number of cells expressing both *gad1* and *ntrk2* in the light and the dark A) BLA showed significantly higher number of cells expressing both *gad1* and *ntrk2* in the dark (circles) compared to the light (squares). Other regions did not show significant differences in number of cells expressing both *gad1* and *ntrk2* B) ACC C) PL D) IL and E) NAc. To understand if this difference is driven by lighting-dependent changes in *gad1* expression, number of *gad1* puncta in each region are shown. There is no difference in number of *gad1* puncta in any region examined F) BLA G) ACC H) PL I) IL J) NAc. * indicates p>0.05




### *2.3.6 KCC2 does not vary across the light-dark cycle*

In mice, KCC2 expression varies across the light-dark cycle. I hypothesized that because there is a difference in behavioral response to social stress following TrkB

activation, there is an underlying difference in KCC2 expression. Western blotting data indicate that there are no significant changes in KCC2 expression across the daily light-




dark cycle in any brain region examined (ACC Figure 6a t=0.6616 p=0.5232 d=0.5557; PL Figure 6b Mann Whitney U p=0.4000 d=0.61062; IL Figure 6c t=0.6699 p=0.5509 d=0.64877; NAc Figure 6d t=0.9492 p=0.2963 d=0.775; BLA Figure 6e t=1.532 p=0.2231 d=1.30785). A sample blot from IL is shown in Figure 6f.

## 2.4 Discussion

To our knowledge, we are the first to demonstrate that the BDNF mimetic drug 7,8-DHF can have opposing effects on behavioral responses to social defeat stress depending on time of day that the drug is administered. That is, when given in the dark,

*Figure 2.6 Western blot data showing KCC2 expression relative to B-actin. None of the brain regions examined showed significant differences in KCC2 expression based on lighting. A) Representative western blot from IL showing KCC2 band located at 120 kDa (protein ladder not shown) and B-actin located at 40 kDa (protein ladder on left). B) ACC C) PL D) IL E) NAc and F) BLA do not show significant differences in expression of KCC2 (relative to B-actin) between the light and the dark.*

or active, phase of the daily cycle for a nocturnal rodent, 7,8-DHF decreases social stress-induced avoidance, but when given in the light, or inactive, phase, 7,8-DHF increases social stress-induced social avoidance, leading to a significant lighting by drug interaction.

To determine if this interaction is also observed if TrkB receptors are blocked, we next administered a TrkB antagonist to determine whether it would have similarly opposing effects on the behavioral response to social defeat stress in the dark versus the light. Interestingly, the TrkB antagonist ANA12 did not significantly affect social avoidance in either the light or the dark. This was somewhat surprising given that we have previously demonstrated that ANA12 administered following defeat during the early dark phase of the light-dark cycle via the same dosing regimen used here, increases the behavioral response to social defeat (Rosenhauer et al., 2019). The dependent variable measured in earlier study was submission exhibited in response to a smaller conspecific introduced in the home cage, while the current study measured social avoidance of a caged opponent in a novel cage. It is possible that the latter behavioral endpoint was less sensitive to the effects of ANA12. In addition, it is also possible that this experiment was under-powered. It may be worth noting that the ANA12 data did appear to follow a similar, opposing pattern as did the behavioral data in Experiment 1. That is, there appeared to be a modest increase in avoidance in the dark but a slight decrease in the light. While this apparent interaction did not reach statistical significance, we did observe medium effect sizes of vehicle versus drug in both the dark and light. Overall, the ANA-12 data, albeit non-significant still suggest that

time of day may be an important factor when considering behavioral and pharmacological assays.

We next investigated if some of the brain regions that we have previously shown to be critical for the acquisition or expression of social stress-induced behavioral responses were differentially activated by 7,8-DHF or social defeat stress in the light or the dark phase of the daily cycle to try to identify where in the circuit our lighting effect might be mediated. Using the immediate early gene product CFos as a marker of cellular activation, we assessed if any of the preselected regions showed differences in neuronal activation following stress or administration of 7,8-DHF in the light versus in the dark. Disappointingly, we did not find any statistically significantly differences in CFos activation based on lighting, although there were significant effects of both defeat and drug in the PL as well as a significant defeat x drug interaction in the BLA. While we did not find a brain region statistically significant for the opposing lighting effect on behavior, these regions are obviously sensitive to defeat and BDNF-signaling and should therefore be considered in future studies. These regions were selected based on their necessity in social defeat stress response (Luckett et al., 2012; Markham et al., 2012; Markham et al., 2010). There are, of course, other brain regions that modulate behavioral responses to social defeat stress response that were not included, such as bed nucleus of the stria terminalis and ventral hippocampus (Bagot et al., 2015; Markham et al., 2009). Future studies should evaluate if these regions are differentially activated by social stress or drugs in the light and the dark. Additionally, it may be worthwhile to assess how the suprachiasmatic nucleus of the hypothalamus responds to social defeat stress and BDNF-signaling manipulation, as this region is critical to

circadian rhythm and time of day perception (Rosenwasser & Turek, 2015). It is of note that there are rhythmic lighting-dependent changes in CFos expression in SCN (Earnest et al., 1990), thus CFos may not be the best marker for cellular activation following behavioral or pharmacological manipulation in this region, specifically, and potentially other brain regions, as well. CFos is only one immediate early gene that can be used to assess cellular activation in brain; other immediate early genes such as *arc* and *egr-1* (Hoffman et al., 1993) could be studied instead. Late arc protein in the basolateral amygdala are associated with fear memories and expression peaks 2 hours after fear conditioning (Nakayama et al., 2016). Future studies could include these other immediate early genes in the same brain regions to see if there is a lighting effect.

Next, we tested whether BDNF administered directly into the PL or the BLA would alter avoidance behavior after social defeat stress and whether these responses would be different in the light and the dark. These regions were selected because this is where we saw changes in CFos in the previous experiment. Unfortunately, we did not observe a significant effect of site-specific BDNF administration on social avoidance in either the light or the dark. We have previously demonstrated that microinjecting BDNF in BLA of Syrian hamsters significantly reduces defeat-induced submissive behavior exhibited towards a nonaggressive intruder (NAI) (Rosenhauer et al., 2019). We have previously assumed that NAI testing and social avoidance assess a similar underlying behavioral response to social stress, but the current data suggest that what is being assessed is somewhat different. Certainly, conditioned defeat, or NAI, testing measures an active response to a novel, non-threatening (smaller) conspecific introduced into the experimental animal's home cage. The common response of non-defeated hamsters to

this stimulus is aggression/attack. Defeated hamsters, conversely, exhibit no aggression and instead avoid (flee from) the conspecific. In the case of social avoidance testing, avoidance is operationally defined as time spent on the far half of a novel cage from a caged opponent that is a novel, aggressive conspecific. Together, our data emphasize that the choice of the dependent measure may dramatically impact how, or even if, we observe an effect of manipulating BDNF signaling.

We also hypothesized that there might be time-of-day variation in BDNF-signaling pathways that could underlie the opposing effects of 7,8-DHF in the light and dark. We began to test this hypothesis by examining *ntrk2* (the gene encoding TrkB receptors) mRNA expression. Additionally, we examined the downstream signaling molecules KCC2 and *gad1* to see if their expression changed over the light:dark cycle. Interestingly, we found differential expression of *gad1* and *ntrk2* between the light and the dark in the BLA but not in the mPFC or NAc. This suggests that the BLA might at least partially mediate the lighting-dependent response to peripheral TrkB agonists. Despite failing to see differential expression of CFos in the BLA based on lighting, this region may still be critical for the lighting-dependent changes in behavior following social defeat stress and BDNF-signaling activation.

One important consideration is that BDNF binding to TrkB can activate many different pathways including phospholipase C-$\gamma$, Ras/ERK, and phosphatidylinositol 3-kinase/Akt, all of which have their own signaling cascades that alter cell function in unique ways (for review see (Kowianski et al., 2018)). It is therefore important to consider which pathway is activated by TrkB agonists and how it changes cellular function. There were no differences in KCC2 protein expression, suggesting that KCC2

may not be involved in the differential behavioral response to lighting and social defeat stress in Syrian hamsters. Interestingly, KCC2 expression can increase or decrease depending on which TrkB tyrosine residue is phosphorylated (reference). A future study could look at protein expression of TrkB and its active form phosphorylated TrkB (pTrkB) to see if TrkB or pTrkB is different in the light and the dark.

Chronopharmacology is the study of the influence of biological rhythms on drug response (Dobrek, 2021). Most biological organisms have rhythmicity at multiple levels (organ, cellular, and molecular, to name a few) that could easily influence how drugs are metabolized, distributed, and bound and how they ultimately alter brain and behavior. Despite the importance of circadian and time-of-day effects, however, many published studies fail to even report the time of day or lighting conditions in which behavioral testing occurred (Nelson et al., 2021). Many other labs run behavioral and pharmacological manipulations during the light phase of the cycle when nocturnal rodents would normally be asleep. To improve the translatability of research done with rodent models, we maintain that these time-of-day variables should be reported and tested explicitly so that we can determine whether timing of drug administration might alter the response to the drug.

Overall, we maintain that the most important take-away of this study is that it is critical to consider time of day as a variable when performing behavioral or pharmacological studies. In fact, these data demonstrate that a single pharmacological manipulation may have opposite effects depending on time of day that it is administered. Thus, time of day of administration must be carefully considered when

assessing the therapeutic potential of interventions aimed at altering responses to stress.

## 2.5 References


Albers, H. E. (2012). The regulation of social recognition, social communication and aggression: vasopressin in the social behavior neural network. *Hormones and Behavior*, *61*(3), 283-292. https://doi.org/10.1016/j.yhbeh.2011.10.007

Albers, H. E., Dean, A., Karom, M. C., Smith, D., & Huhman, K. L. (2006). Role of V1a vasopressin receptors in the control of aggression in Syrian hamsters. *Brain Research*, *16*, 425-430. https://doi.org/https://doi.org/10.1016/j.brainres.2005.12.081

Albers, H. E., & Ferris, C. F. (1984). Neuropeptide Y: role in light-dark cycle entrainment of hamster circadian rhythms. *Neuroscience Letters*, *50*(1-3), 163-168. https://doi.org/10.1016/0304-3940(84)90480-4

Albers, H. E., Huhman, K. L., & Meisel, R. L. (2002). 6 - Hormonal Basis of Social Conflict and Communication. In *Hormones, Brain and Behavior* (Vol. 1). https://doi.org/https://doi.org/10.1016/B978-012532104-4/50008-1

Alexander, W. H., & Brown, J. W. (2011). Medial prefrontal cortex as an action-outcome predictor. *Nature Neuroscience*, *14*(10), 1338-1344. https://doi.org/10.1038/nn.2921

Almeida, D. M. (2013). Resilience and vulnerability to daily stressors assessed via diary methods. *Current Directions in Psychological Science*, *14*, 64-68.

Atrooz, F., Alkadhi, K. A., & Salim, S. (2021). Understanding stress: Insights from rodent models. *Current Research in Neurobiology*, *2*. https://doi.org/10.1016/j.crneur.2021.100013

Bagot, R. C., Parise, E. M., Pena, C. J., Zhang, H., Maze, I., Chaudhury, D., Persaud, B., Cachope, R., Bolanos, C. A., Cheer, J. F., Deisseroth, K., Han, M., & Nestler, E. J. (2015). Ventral hippocampal afferents to the nucleus accumbens regulate susceptibility to depression. *Nature Communications*, *6*. https://doi.org/10.1038/ncomms8062

Bartolomucci, A., Pederzani, T., Sacerdote, P., Panerai, A. E., Parmigiani, S., & Palanza, P. (2004). Behavioral and physiological characterization of male mice under chronic psychosocial stress. *Psychoneuroendocrinology*, *29*(7), 899-910.

Bath, K. G., & Johnston, R. E. (2007). Dominant-Subordinate relationships in Hamsters: Sex Differences in Reactions to Familiar Opponents. *Hormones and Behavior*, *51*(2), 258-264. https://doi.org/10.1016/j.yhbeh.2006.10.009

Berton, O., McClung, C. A., DiLeone, R. J., Krishnan, V., Renthal, W., Russo, S. J., Graham, D., Tsankova, N. M., Bolanos, C. A., Rios, M., Monteggia, L. M., Self, D. W., & Nestler, E. J. (2006). Essential Role of BDNF in the Mesolimbic Dopamine Pathway in Social Defeat Stress. *Science*, *311*(5762), 864-868. https://doi.org/10.1126/science.1120972

Björkholm, C., & Monteggia, L. M. (2016). BDNF - a key transducer of antidepressant effects. *Neuropharmacology*, *102*(1873-7064 (Electronic)). https://doi.org/10.1016/j.neuropharm.2015.10.034

Bjorkqvist, K. (2001). Social defeat as a stressor in humans. *Physiology & Behavior*, *73*(3), 435-442. https://doi.org/10.1016/s0031-9384(01)00490-5.

Bloodgood, D. W., Sugam, J. A., Holmes, A., & Kash, T. L. (2018). Fear extinction requires infralimbic cortex projections to the basolateral amygdala. *Transl Psychiatry*, *8*(1). https://doi.org/10.1038/s41398-018-0106-x

Burgoon, P. W., Lindberg, P. T., & Gillette, M. U. (2004). Different patterns of circadian oscillation in the suprachiasmatic nucleus of hamster, mouse, and rat. *Journal of Comparative Physiology: a Neuroethology, sensory, neural, and behavioral physiology*, *190*(2), 167-171. https://doi.org/https://doi.org/10.1007/s00359-003-0486-z

Castren, E., & Kojima, M. (2017). Brain-derived neurotrophic factor in mood disorders and antidepressant treatments. *Neurobiology of Disease*, *97*, 119-126. https://doi.org/https://doi.org/10.1016/j.nbd.2016.07.010

Caviedes, A., Lafourcade, C., Soto, C., & Wyneken, U. (2017). BDNF/NF-κB Signaling in the Neurobiology of Depression. *Curr Pharm Des*, *23*(21), 3154-3163. https://doi.org/10.2174/1381612823666170111141915

Chen, Z. Y., Jing, D., Bath, K. G., Ieraci, A., Khan, T., Siao, C.-J., Herrara, D. G., Toth, M., Yang, C., McEwen, B. S., Hempstead, B. L., & Lee, F. S. (2006). Genetic Variant BDNF (Val66Met) Polymorphism Alters Anxiety-Related Behavior. *Science*, *314*(5796), 140-143.

Dallman, R., Brown, S. A., & Gachon, F. (2014). Chronopharmacology: New Insights and Implications. *Annual Review of Pharmacology and Toxicology*, *54*, 339-361. https://doi.org/https://doi.org/10.1146/annurev-pharmtox-011613-135923

Day, D. E., Cooper, M., Markham, C. M., & Huhman, K. L. (2011). NR2B subunit of the NMDA receptor in the basolateral amygdala is necessary for the acquisition of conditioned defeat in Syrian hamsters. *Behavioral Brain Research*, *217*, 55-59.

de Lima, M. A. X., Baldo, M. V. C., Oliveira, F. A., & Canteras, N. S. (2022). The anterior cingulate cortex and its role in controlling contextual fear memory to predatory threats. *eLife*, *11*. https://doi.org/10.7554/eLife.67007

De Vry, J., Vanmierlo, T., Martínez-Martínez, P., Losen, M., Temel, Y., Boere, J., Kenis, G., Steckler, T., Steinbusch, H. W. M., Baets, M. D., & Prickaerts, J. (2016). TrkB in the hippocampus and nucleus accumbens differentially modulates depression-like behavior in mice. *Behavioural Brain Research*, *296*, 15-25. https://doi.org/10.1016/j.bbr.2015.08.027

Diaz, V., & Lin, D. (2020). Neural circuits for coping with social defeat. *Current Opinions in Neurobiology*, *60*, 99-107. https://doi.org/10.1016/j.conb.2019.11.016

Dobrek, L. (2021). Chronopharmacology in Therapeutic Drug Monitoring—Dependencies between the Rhythmics of Pharmacokinetic Processes and Drug Concentration in Blood. *Pharmaceutics*, *13*(11). https://doi.org/10.3390/pharmaceutics13111915

Dulka, B., Ford, E., Lee, M., Donnell, N., Goode, T., Prosser, R., & Cooper, M. (2016). Proteolytic cleavage of proBDNF into mature BDNF in the basolateral amygdala is necessary for defeat-induced social avoidance. *Learning and Memory*, *23*(4), 156-160. https://doi.org/10.1101/lm.040253.115

Dulka, B. N., Bagatelas, E. D., Bress, K. S., Grizzell, J. A., Cannon, M. K., Whitten, C. J., & Cooper, M. A. (2020). Chemogenetic activation of infralimbic cortex to basolateral amygdala projection promotes resistance to acute social defeat stress. *Scientific Reports*, *10*.

Duman, R. S., Aghajanian, G. K., Sanacora, G., & Krystal, J. H. (2016). Synaptic plasticity and depression: new insights from stress and rapid-acting antidepressants. *Nature Medicine*, *22*(3), 238-249. https://doi.org/https://doi.org/10.1038/nm.4050

Duman, R. S., Deyama, S., & Fogaca, M. V. (2021). Role of BDNF in the pathophysiology and treatment of depression: Activity-dependent effects distinguish rapid-acting antidepressants. *European Journal of Neuroscience*, *53*(1), 126-139.

Duman, R. S., & Monteggia, L. M. (2006). A Neurotrophic Model for Stress-Related Mood Disorders. In (Vol. 59, pp. 1116-1127): Elsevier.

Earnest, D. J., Iadarola, M., Yeh, H. H., & Olschowka, J. A. (1990). Photic regulation of c-fos expression in neural components governing the entrainment of circadian rhythms. *Experimental neurology*, *109*(3), 353-361. https://doi.org/10.1016/s0014-4886(05)80027-5

Eisch, A. J., Bolanos, C. A., de Wit, J., Simonak, R. D., Pudiak, C. M., Barrot, M., Verhaagen, J., & Nestler, E. J. (2003). Brain-derived neurotrophic factor in the ventral midbrain-nucleus accumbens pathway: a role in depression. *Biological Psychiatry*, *54*(10), 994-1005. https://doi.org/10.1016/j.biopsych.2003.08.003

Fan, Z., Li, W., Lee, S. R., Meng, Q., Shi, B., Bunch, T. D., White, K. L., Kong, I., & Wang, Z. (2014). Efficient Gene Targeting in Golden Syrian Hamsters by the CRISPR/Cas9 System. *PLoS One*, *9*(10). https://doi.org/10.1371/journal.pone.0109755

Fanselow, M. S., & LeDoux, J. E. (1999). Why we think plasticity underlying Pavlovian fear conditioning occurs in the basolateral amygdala. *Neuron*, *23*(2), 229-232. https://doi.org/10.1016/s0896-6273(00)80775-8

Faruzzi, A. N., Solomon, M. B., Demas, G. E., & Huhman, K. L. (2005). Gonadal hormones modulate the display of submissive behavior in socially defeated female Syrian hamsters. *Hormones and Behavior*, *47*(5), 569-575. https://doi.org/https://doi.org/10.1016/j.yhbeh.2004.11.023

Gilbert, P., Allan, S., Brough, S., Melley, S., & Miles, J. N. V. (2002). Relationship of anhedonia and anxiety to social rank, defeat and entrapment. *Journal of Affective Disorders*, *71*(1-3), 141-151.

Gray, C. L., Norvelle, A., Larkin, T., & Huhman, K. L. (2015). Dopamine in the nucleus accumbens modulates the memory of social defeat in Syrian hamsters (Mesocricetus auratus). *Behavioral Brain Research*, *286*, 22-28. https://doi.org/10.1016/j.bbr.2015.02.030

Grossman, Y. S., Fillinger, C., Manganaro, A., Voren, G., Waldman, R., Zou, T., Janssen, W. G., Kenny, P. J., & Dumitriu, D. (2022). Structure and function differences in the prelimbic cortex to basolateral amygdala circuit mediate trait vulnerability in a novel model of acute social defeat stress in male mice. *Neuropsychopharmacology*, *47*(3), 788-799. https://doi.org/10.1038/s41386-021-01229-6.

Hoffman, G. E., Smith, M. S., & Verbalis, J. G. (1993). c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. *Frontiers in Neuroendocrinology*, *14*(3), 173-213. https://doi.org/10.1006/frne.1993.1006

Huang, W., Zucca, A., Levy, J., & Page, D. T. (2020). Social Behavior Is Modulated by Valence-Encoding mPFC-Amygdala Sub-circuitry. *Cell Reports*, *32*(2). https://doi.org/10.1016/j.celrep.2020.107899

Huh, J., D., G., J, T., BY, L., & Kang, M. Treatment of generalized anxiety disorder: a comprehensive review of the literature for psychopharmacologic alternatives to newer antidepressants and benzodiazepines. LID - PCC.08r00709. (2155-7780 (Electronic)).

Huhman, K. L. (2006). Social conflict models: Can they inform us about human psychopathology? *Hormones and Behavior*, *50*(4), 640-646.

Huhman, K. L., Bunnell, B. N., Mougey, E. H., & Meyerhoff, J. L. (1990). Effects of social conflict on POMC-derived peptides and glucocorticoids in male golden hamsters. *Physiology & Behavior*, *47*(5), 949-956. https://doi.org/https://doi.org/10.1016/0031-9384(90)90023-W

Huhman, K. L., Moore, T. O., Ferris, C. F., Mougey, E. H., & Meyerhoff, J. L. (1991). Acute and repeated exposure to social conflict in male golden hamsters: Increases in plasma POMC-peptides and cortisol and decreases in plasma testosterone. *Hormones and Behavior*, *25*(2). https://doi.org/https://doi.org/10.1016/0018-506X(91)90051-I

Huhman, K. L., Moore, T. O., Mougey, E. H., & Meyerhoff, J. L. (1992). Hormonal responses to fighting in hamsters: separation of physical and psychological causes. *Physiology & Behavior*, *51*(5), 1083-1086.

Huhman, K. L., Solomon, M. B., Janicki, M., Harmon, A. C., Lin, S. M., Israel, J. E., & Jasnow, A. M. (2003). Conditioned defeat in male and female Syrian hamsters. *Hormones and Behavior*, *44*(3), 293-299. https://doi.org/https://doi.org/10.1016/j.yhbeh.2003.05.001

Jasnow, A. M., & Huhman, K. L. (2001). Activation of GABA(A) receptors in the amygdala blocks the acquisition and expression of conditioned defeat in Syrian hamsters. *Brain Research*, *920*(1-2), 142-150. https://doi.org/https://doi.org/10.1016/S0006-8993(01)03054-2

Jasnow, A. M., Shi, C., Israel, J. E., Davis, M., & Huhman, K. L. (2005). Memory of social defeat is facilitated by cAMP response element-binding protein overexpression in the amygdala. *Behavioral Neuroscience*, *119*(4), 1125-1130. https://doi.org/10.1037/0735-7044.119.4.1125

Jaycox, L. H., Stein, B. D., Paddock, S., Miles, J. N., Chandra, A., Meredith, L. S., Tanielian, T., Hickey, S., & Burnam, M. A. (2009). Impact of Teen Depression on Academic, Social, and Physical Functioning. *Pediatrics*, *124*(4), e596-e605. https://doi.org/10.1542/peds.2008-3348

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R., & Walters, E. E. (2003). Lifetime prevalence and age-of-onset distributions of DSM-IV in the National Comorbidity Survey Replication. *Archives of General Psychiatry*, *62*(6), 593-602. https://doi.org/10.1001/archpsyc.62.6.593.

Kocabas, N. A., Antonijevic, I., Faghel, C., Forray, C., Kasper, S., Lecrubier, Y., Linotte, S., Massat, I., J., M., Noro, M., Montgomery, S., Oswald, P., Snyder, L., Zohar, J., & Souery, D. (2011). Brain-derived neurotrophic factor gene polymorphisms: Influence on treatment response phenotypes of major depressive disorder. *International Clinical Psychopharmacology*, *26*(1), 1-10.

Kollack-Walker, S., Watson, S. J., & Akil, H. (1997). Social stress in hamsters: defeat activates specific neurocircuits within the brain. *Journal of Neuroscience*, *17*(22), 8842-8855. https://doi.org/10.1523/JNEUROSCI.17-22-08842.1997

Kowianski, P., Lietzau, G., Czuba, E., Waskow, M., Steliga, A., & Morys, J. (2018). BDNF: A Key Factor with Multipotent Impact on Brain Signaling and Synaptic Plasticity. *Cellular and Molecular Neurobiology*, *28*(3), 579-593. https://doi.org/10.1007/s10571-017-0510-4

Krishnan, K., Han, M. H., Graham, D. L., Berton, O., Renthal, W., Russo, S. J., Laplant, Q., Graham, A., Lutter, M., Lagace, D. C., Ghose, S., Reister, R., Tannous, P., Green, T. A., Neve, R. L., Chakravarty, S., Kumar, A., Eisch, A. J., Self, D. W., . . . Nestler, E. J. (2007). Molecular adaptations underlying susceptibility and resistance to social defeat in rbain reward regions. *Cell*, *131*(2), 391-404. https://doi.org/10.1016/j.cell.2007.09.018

Lai, W., Ramiro, L. R., Yu, H. A., & Johnston, R. E. (2005). Recognition of the familiar individuals in golden hamsters: a new method and functional neuroanatomy. *Journal of Neuroscience*, *25*(49), 11239-11247. https://doi.org/10.1523/JNEUROSCI.2124-05.2005

Laubach, M., Amarante, L. M., Swanson, K., & White, S. R. (2018). What, If Anything, Is Rodent Prefrontal Cortex. *eNeuro*, *5*(5). https://doi.org/10.1523/ENEURO.0315-18.2018

Liu, W., Zhang, W., Zheng, Z., Zou, J., Liu, X., Huang, S., You, W., He, Y., Zhang, J., Wang, X., & Pan, B. (2020). Identification of a prefrontal cortex-to-amygdala pathway for chronic stress-induced anxiety. *Nature Communication*, *11*(1). https://doi.org/10.1038/s41467-020-15920-7

Luckett, C., Norvelle, A., & Huhman, K. L. (2012). The role of the nucleus accumbens in the acquisition and expression of conditioned defeat. *Behavioural Brain Research*, *227*(1), 208-214.

Maguire, J. (2014). Stress-induced plasticity of GABAergic inhibition. *Frontiers in Cellular Neuroscience*, *8*. https://doi.org/10.3389/fncel.2014.00157

Maren, S. (2001). Neurobiology of Pavlovian fear conditioning. *Annu Rev Neurosci*, *24*, 897-931. https://doi.org/10.1146/annurev.neuro.24.1.897

Markham, C. M., Luckett, C. A., & Huhman, K. L. (2012). The medial prefrontal cortex is both necessary and sufficient for the acquisition of conditioned defeat. *Neuropharmacology*, *62*(2), 933-999.

Markham, C. M., Norvelle, A., & Huhman, K. L. (2009). Role of the bed nucleus of the stria terminalis in the acquisition and expression of conditioned defeat in Syrian hamsters. *Behavioral Brain Research*, *198*(1), 69-73. https://doi.org/10.1016/j.bbr.2008.10.022

Markham, C. M., Taylor, S. L., & Huhman, K. L. (2010). Role of amygdala and hippocampus in the neural circuit subserving conditioned defeat in Syrian hamsters. *Learning and Memory*, *17*(2), 109-116.

Martinowich, K., Manji, H., & Lu, B. (2007). New insights into BDNF function in depression and anxiety. *Nature Neuroscience*, *10*, 1089-1093.

McCann, K., Bicknese, C., Norvelle, A., & Huhman, K. L. (2014). Effects of inescapable versus escapable social stress in Syrian hamsters: the importance of stressor duration versus escapability. *Physiology & Behavior*, *22*(129), 25-29. https://doi.org/10.1016/j.physbeh.2014.02.039

Menard, C., Pfau, M. L., Hodes, G. E., Kana, V., Wang, V. X., Bouchard, S., Takahashi, A., Flanigan, M. E., Aleyasin, H., LeClair, K. B., Janssen, W. G., Labonté, B., Parise, E. M., Lorsch, Z. S., Golden, S. A., Heshmati, M., Tamminga, C., Turecki, G., Campbell, M., . . . Russo, S. J. (2017). Social stress induces neurovascular pathology promoting depression. *Nature Neuroscience*, *20*, 1752-1760.

Morin, L. P., & Wood, R. I. (2001). *A Stereotaxic Atlas of the Golden Hamster Brain*. Academic Press.

Nakayama, D., Hashikawa-Yamasaki, Y., Ikegaya, Y., Matsuki, N., & Nomura, H. (2016). Late Arc/Arg3.1 expression in the basolateral amygdala is essential for persistence of newly-acquired and reactivated contextual fear memories. *Scientific Reports*, *6*. https://doi.org/10.1038/srep21007

Nelson, R. J., Bumgarner, J. R., Walker, W. H., & DeVries, A. C. (2021). Time-of-day as a critical biological variable. *Neuroscience and biobehavioral reviews*, *127*, 740-746. https://doi.org/10.1016/j.neubiorev.2021.05.017

Newman, S. W. (1999). The Medial Extended Amygdala in Male Reproductive Behavior A Node in the Mammalian Social Behavior Network. *Annals of the New York Academy of Sciences*, *877*(1), 242-257.

Ottenweller, J. E., Tapp, W. N., Pitman, D. L., & Natelson, B. H. (1987). Adrenal, thyroid, and testicular hormone rhythms in male golden hamsters on long and short days. *American Jornal of Physiology*, *253*, R321-328.

Panda, S., Antoch, M. P., Miller, B. H., Su, A. I., Schook, A. B., Straume, M., Schultz, P. G., Kay, S. A., Takahashi, J. S., & Hogenesch, J. B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell*, *109*(3), 307-320. https://doi.org/10.1016/s0092-8674(02)00722-5

Ramanathan, R., & Desrouleaux, R. (2022). Introduction: The Science of Stress. *Yale Journal of Biology and Medicine*, *95*(1), 1-2.

Ramirez, F., Moscarello, J. M., LeDoux, J. E., & Sears, R. M. (2015). Active Avoidance Requires a Serial Basal Amygdala to Nucleus Accumbens Shell Circuit. *Journal of Neuroscience*, *35*(8), 3470-3477. https://doi.org/https://doi.org/10.1523/JNEUROSCI.1331-14.2015

Rosenhauer, A. M., Beach, L. Q., Jeffress, E. C., Thompson, B. M., McCann, K. E., Partrick, K. A., Diaz, B., Norvelle, A., Choi, D. C., & Huhman, K. L. (2019). Brain-derived neurotrophic factor signaling mitigates the impact of acute social stress. *Neuropharmacology*, *148*, 40-49.

Rosenwasser, A. M., & Turek, F. W. (2015). Neurobiology of Circadian Rhythm Regulation. *Sleep Medicine Clinics*, *10*(4), 403-412. https://doi.org/10.1016/j.jsmc.2015.08.003v

Ross, A. P., Norvelle, A., Choi, D. C., Walton, J. C., Albers, H. E., & Huhman, K. L. (2017). Social housing and social isolation: Impact on stress indices and energy balance in male and female Syrian hamsters (Mesocricetus auratus). *Physiology & Behavior*, *177*, 264-269. https://doi.org/10.1016/j.physbeh.2017.05.015

Saarelainen, T., Hendolin, P., Lucas, G., Koponen, E., Sairanen, M., MacDonald, E., Agerman, K., Haapasalo, A., Nawa, H., Aloyz, R., Ernfors, P., & Castren, E. (2003). Activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects. *Journal of Neuroscience*, *23*(1), 349-357.

Selye, H. (1956). Endocrine Reactions During Stress. *Anesthesia and Analgesia*, *35*(3), 182-193.

Sheng, J. A., Bales, N. J., Myers, S. A., Bautista, A. I., Roueinfar, M., Hale, T. M., & Handa, R. J. (2021). The Hypothalamic-Pituitary-Adrenal Axis: Development, Programming Actions of Hormones, and Maternal-Fetal Interactions. *Frontiers in Behavioral Neuroscience*, *14*. https://doi.org/10.3389/fnbeh.2020.601939

Shin, L. M., & Liberzon, I. (2009). The Neurocircuitry of Fear, Stress, and Anxiety Disorders. *Neuropsychopharmacology*, *35*, 169-191.

Sierra-Mercado, D., Padilla-Coreano, N., & Quirk, G. J. (2011). Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. *Neuropsychopharmacology*, *36*(2), 529-538. https://doi.org/10.1038/npp.2010.184

Solomon, M. B., Foster, M. T., Bartness, T. J., & Huhman, K. L. (2007). Social defeat and footshock increase body mass and adiposity in male Syrian hamsters. *American Journal of Physiology: regulatory, integrative, and comparative physiology*, *292*(1), R283-R290. https://doi.org/https://doi.org/10.1152/ajpregu.00330.2006

Solomon, M. B., Karom, M. C., & Huhman, K. L. (2007). Sex and estrous cycle differences in the display of conditioned defeat in Syrian hamsters. *Hormones and Behavior*, *52*(2), 211-219. https://doi.org/https://doi.org/10.1016/j.yhbeh.2007.04.007

Storch, K., Lipan, O., Leykin, I., Viswanatha, N., Davis, F. C., Wong, W. H., & Weitz, C. J. (2002). Extensive and divergent circadian gene expression in liver and heart. *Nature*, *417*(6884), 78-83. https://doi.org/10.1038/nature744

Taliaz, D., Stall, N., Dar, D. E., & Zangen, A. (2010). Knockdown of brain-derived neurotrophic factor in specific brain sites precipitates behaviors associated with depression and reduces neurogenesis. *Molecular Psychiatry*, *15*(1), 80-92. https://doi.org/10.1038/mp.2009.67

Toyoda, A. (2017). Social defeat models in animal science: What we have learned from rodent models. *Animal Science Journal*, *88*(7), 944-952.

Trivedi, M. H. Major depressive disorder: remission of associated symptoms. *Journal of Clinical Psychiatry*, *67*(0160-6689 (Print)), 27-32.

Twenge, J. M., & Joiner, T. E. (2020). U.S Census Bureau-assessed prevalence of anxiety and depressive symptoms in 2019 and during the 2020 COVID-19 pandemic. *Depression and Anxiety*, *37(10)*, 954-956. https://doi.org/10.1002/da.23077

Walker, W. H., Sprowls, S. A., Bumgarner, J. R., Liu, J. A., Melendez-Fernandez, O. H., Walton, J. C., Lockman, P. R., DeVries, A. C., & Nelson, R. J. (2021). Circadian Influences on Chemotherapy Efficacy in a Mouse Model of Brain Metastases of Breast Cancer. *Frontiers in Oncology*, *11*. https://doi.org/https://doi.org/10.3389/fonc.2021.752331

Wood, S. K., & Bhatnagar, S. (2015). Resilience to the effects of social stress: Evidence from clinical and preclinical studies on the role of coping strategies. *Neurobiology of Stress*, 164-173. https://doi.org/10.1016/j.ynstr.2014.11.002

Zhdanava, M., Pilon, D., Ghelerter, I., Chow, W., Joshi, K., Lefebvre, P., & Sheehan, J. J. (2021). The Prevalence and National Burden of Treatment-Resistant Depression and Major Depressive Disorder in the United States. *Journal of Clinical Psychiatry*, *16*(82). https://doi.org/10.4088/JCP.20m13699

# 3 PERINEURONAL NETS IN SYRIAN HAMSTERS: ANATOMICAL LOCALIZATION, SEX DIFFERENCES, DIURNAL VARIATION, AND RESPONSE TO SOCIAL STRESS

## 3.1 Introduction

Perineuronal nets (PNNs), which are extracellular matrix proteoglycans that surround neurons and glia, were traditionally thought to act only as structural support in the nervous system. Although their anatomical distribution throughout the central nervous system (CNS) is not well characterized across species, the existing data suggest that PNNs primarily surround inhibitory neurons (Seeger et al., 1994). In Rhesus macaques, perineuronal nets are mainly seen surrounding cells in the cortex and are largely absent from subcortical regions such as the hypothalamus and caudate/putamen (Mueller et al., 2016). However, in mice, PNN expression occurs throughout subcortical structures including multiple thalamic nuclei, lateral and medial globus pallidus, and the anterior hypothalamus (Ciccarelli et al., 2021). Human studies have largely focused on expression in the cortex, hippocampus, or amygdala (Pantazopoulos et al., 2010; Rogers et al., 2019) with little additional information available regarding PNN distribution in other brain regions.

As it began to be recognized that these molecules were more labile than previously believed, PNNs were studied during postnatal development and were shown to play a role in controlling neuronal plasticity (Sorg et al., 2016) and in regulating the opening and closing of so-called critical periods of development. For example, the ontogeny of PNNs is correlated with crystallization of song learning in zebra finches (Cornez et al., 2018). More recently, Banerjee et al. showed that PNNs are dynamically

regulated in adults following fear conditioning, suggesting that they may play an important role in learning and memory (Banerjee et al., 2017). In addition, PNNs appear to vary across the light-dark cycle in mice and rats (Harkness et al., 2021; Pantazopoulos et al., 2020) and may be sexually dimorphic in mice in brain regions known to modulate reproductive behavior. Recent studies have also implicated PNNs in etiology of multiple neuropsychiatric illnesses including mood and anxiety disorders (Browne et al., 2022; Sorg et al., 2016), and the current conception of PNNs as being more plastic than originally thought is consistent with recent evidence that PNNs change in response to stress and inflammation (Bosiacki et al., 2019)(for review see (Sorg et al., 2016)). Thus, PNNs are emerging as a new mechanism in the modulation of complex behavior and possibly in the development of stress-related neuropsychiatric disease, underscoring the importance of examining PNNs in a range of models so that we can better understand their distribution and potential role in gating behavior. The initial purpose of the present study was to characterize for the first time the distribution of PNNs in Syrian hamsters, which display a rich array of social and agonistic behaviors (Albers et al., 2006; H.E. Albers et al., 2002).

Exposure to stress in humans is known to be an important risk factor for developing mood and anxiety disorders and to induce or exacerbate symptoms of these mental illnesses (Kessler et al., 2003). Millions of Americans are affected by these debilitating diseases, and roughly 1/3 of patients are resistant or respond sub-optimally to standard antidepressant medications (Huh et al.; Kessler et al., 2003). Thus, there is a critical need to identify novel targets for the development of alternative treatments. Social stress, and social defeat in particular, is arguably the most common and salient

type of stress experienced by humans and nonhuman animals (Almeida, 2013; Bjorkqvist, 2001; Huhman, 2006). Both humans and nonhumans exposed to social defeat stress subsequently display increased anxiety- and depression-like signs and symptoms including social avoidance (Bjorkqvist, 2001; Huhman, 2006) (Toyoda, 2017; Wood & Bhatnagar, 2015). Much like fear conditioning, behavioral responses to social defeat stress require learning and adaptation to environmental challenges, processes that thus might involve the PNNs (Day et al., 2011; Huhman, 2006; Jasnow et al., 2005).

Syrian hamsters readily exhibit aggression and territorial behaviors in the laboratory and rapidly establish dominant-subordinate relationships between dyads. Because their aggression is highly ritualized, it rarely results in physical injuries, making it possible to study the effects of social defeat stress without the confound of injury and the accompanying inflammation. In addition, both sexes display similar agonistic behavior, so it is possible to evaluate sex differences in the response to social defeat stress. Therefore, we tested the hypothesis that PNNs vary following social defeat stress and, further, that they might do so in a sex-dependent manner (Huhman, 2006; Kim L. Huhman et al., 2003; Solomon, Foster, et al., 2007). Finally, we have recently observed a marked variation in the response to a pharmacological treatment aimed at reducing the behavioral response to defeat when the treatment was given at the beginning of the dark (active) phase versus at the beginning of the light (inactive) phase of the daily cycle (unpublished data). Hamsters exhibit more dramatic daily variation in behavior than do many laboratory rat and mouse species (Albers & Ferris, 1984; Burgoon et al., 2004). Therefore, we also tested the hypothesis that there is a daily

variation in PNNs in hamsters that might underlie, at least in part, the observed diurnal variation in drug response.

## 3.2 Materials and Methods

### *3.2.1 Animals*

Male and female hamsters were bred in-house using animals obtained from Charles Rivers Laboratories (New York, NY or Wilmington, MA). Hamsters were group-housed in same sex groups of 3-5 at weaning in static polycarbonate cages (23x42x20 cm) with corn cob bedding, paper nesting material, plastic tubes for environmental enrichment, and wire tops in a colony room on a 14:10 light/dark cycle as is common in hamsters in order to maintain gonadal patency (Ottenweller et al., 1987). Food and water were available ad libitum. All behavioral testing occurred between postnatal day (PND) 60 and 75, whereupon animals weighed between 110 and 160 grams. Resident aggressors (RAs; >170 g) were singly housed, older male and ovariectomized female hamsters that were known to reliably attack a same-sex intruder introduced in their home cage. Subjects were singly housed (23x42x20 cm) a minimum of 8 days before behavioral testing and gently handled each day leading up to behavioral testing to habituate them to experimenters. Syrian hamsters are thought to be territorial in the wild, and singly housing them does not have deleterious effects on their behavior or overall health (Ross et al., 2017). Estrous cycles for females were monitored by vaginal swabs for at least 8 days before testing. All females experienced their final or only defeat on Diestrus Day 1. All procedures and protocols were approved by the Georgia State University Institutional Animal Care and Use Committee and were in accordance

with the standards outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals.

### *3.2.2 Social Defeat Training*

Acute social defeat was performed as described previously in (K.L. Huhman et al., 2003). Briefly, subjects were placed in the home cage of a same-sex resident aggressor for 15 minutes and allowed to interact freely with the RA, which generally attacks an intruder within 30 sec of the beginning of the trial. No defeat controls were placed in an empty RA cage for 15 minutes to control for handling and novel cage exposure. For repeated defeat, subjects were placed in the home cage of a novel RA for 10 sessions (5 min/trial) across 9 days, to ensure that the females started and ended on Diestrus Day 1. On the first day, subjects experienced 2 defeat sessions with an intertrial interval of at least 45 minutes. For the light/dark experiments, the same protocol was used for acute defeat, but the defeats took place either within 2 hours of the onset of the dark (or active) phase of the daily cycle or within 2 hours of the onset of the light phase.

### *3.2.3 Immunohistochemistry*

Animals were anesthetized with an overdose of sodium pentobarbital. Once deeply anesthetized, animals were transcardially perfused with cold phosphate-buffered saline (PBS) and then 4% paraformaldehyde in phosphate buffer (pH=7.4). Brains were collected and stored in paraformaldehyde for 24 hours, then moved to 30% sucrose for cryoprotection for a minimum of 3 days at 4°C. Brains were sectioned on a Leica CM3050 S cryostat (Deer Park, IL) into 40 µm sections and stored in cryoprotectant (30% sucrose 30% ethylene glycol in PBS) at -20°C. For immunostaining, sections were

washed in PBS 5 times and then blocked in 5% Normal Donkey Serum in PBS with 0.1% Triton-X for 1 hour. They were then incubated with an antibody for *Wisteria Florabundin* agglutinin (WFA) with a conjugated fluorescein tag (Vector Labs, Burlingame CA) overnight at 4°C. Sections were then washed 3 times in PBS, mounted to slides, and coverslipped with VectaShield Hardset with DAPI (Vector Labs, Burlingame CA). WFA binds with specificity to the N-acetylgalactosamine epitope and is frequently used to stain perineuronal nets throughout the brain. PNNs are heterogenous but most appear to express this epitope within brain (Bruckner et al., 1993).

#### *3.2.4 Antibody Characterization*

The Wisteria floribunda agglutinin antibody (Vector Labs, FL-1351-2, used at 1:1,000) is a fluorescein conjugated antibody that binds to N-acetylgalactosamine epitopes present on multiple components of chondroitin proteoglycans that make up PNNs. While the binding specificity is not well understood, it is known to bind carbohydrates located on both terminal and internal N-acetylgalactosamine linked at the α or β position to the 3 or 6 position of the galactose (Bruckner et al., 1993).

### *3.2.3 Image Analysis*

All images were taken with a Keyence microscope (Osaka, Japan) at 4x. Original image files were opened with FIJI and the Cell Counter tool was used to count cells in each region, as determined by the Golden Hamster Brain Atlas (Morin & Wood, 2001). Due to the size and variability of the somatosensory cortex (SSC), a 1x1mm square was superimposed over the image and only cells within that block were counted. The experimenter was blinded to the experimental condition during analysis. For anatomical distribution of PNNs, entire sections were imaged at 4X and stitched using Keyence BZ-

X software (Osaka, Japan). The criteria for scoring were as follows: - (negative or no) indicates 0-5 immunopositive cells or stain in the neuropil, + (low) shows some somatic staining (>5 but <10 cells/mm$^2$) and diffuse staining of the neuropil, ++ (moderate) indicates more intense somatic staining with a cell count between 10 and 20 cells/mm$^2$, +++ (high) showed higher somatic staining with cell counts 20 or more cells/mm$^2$.

### *3.2.4 Statistical Analysis*

Data were first analyzed for normality using Shapiro-Wilke's test and homogeneity of variance using Levene's test. One-way ANOVAs with Fisher's LSD post-hoc tests and Student's t-test were performed where appropriate. Effect sizes were calculated using Cohen's d in Excel. A large effect size was ascribed at d greater than or equal to 0.8, a medium effect size was d between 0.2 and 0.8, and a small effect size was d less than 0.2. Statistical analysis was performed using GraphPad Prism (9.1.2). All graphs were created in GraphPad Prism (9.1.2).

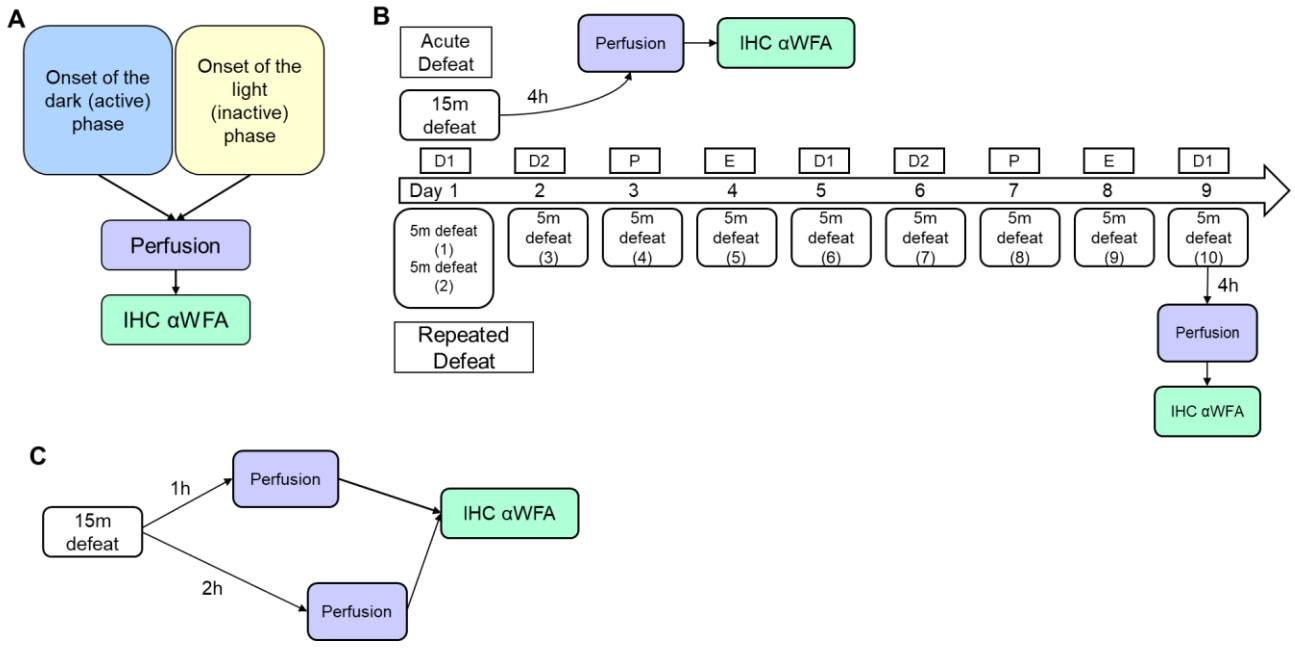



Figure 3.1 Experimental design for Experiments 2-4 Note that in Experiment 1, there were no experimental manipulations other than collection of brains, so the design is not shown here. A)

Experiment 2 tested whether there are differences in PNN expression at the beginning of the dark (active) phase versus the beginning of the light (inactive) phase of the daily light-dark cycle. B) Experiment 3 tested whether PNN expression is altered 4 hr after acute or repeated defeat versus no defeat controls. All females were defeated for the first time on Diestrus Day 1 (D1) and were sacrificed on D1, as well. (D2 is Diestrus Day 2, P is Proestrus, and E is Estrus). Animals receiving repeated defeat were defeated a total of 10 times over 9 days (number of the defeat session is shown in parentheses). A similar number of males were tested each day with the females. C) Experiment 4 was a small pilot to determine whether PNNs expression might be altered 1 or 2 hours after an acute defeat stressor.

## 3.3 Results

### *3.3.1 Anatomical localization of PNNs in male hamsters*

To date, no one has characterized where perineuronal nets are expressed in hamster brain. We observed the highest expression of WFA immunostaining in cortical regions, particularly the somatosensory and motor cortices (high expression) (Figure 2a, Table 1). The medial septum also showed high WFA immunostaining, while the lateral septum showed low staining (Figure 2b). The basal ganglia, thalamus, and most of the hypothalamus showed little no expression of PNNs. The hippocampus showed moderate expression in all subregions. The subregions of the amygdala showed no apparent expression of WFA (Figure 2c). The staining pattern of WFA in Syrian hamster showed the same mesh-like pattern surrounding soma and proximal dendrites that has been seen in other rodents and in humans (Figure 2d).

Table 3.1 Perineuronal net expression in Syrian hamster brain nuclei as labeled by WFA immunohistochemistry. - indicates 0-5 immunopositive cells and/or no staining in the neuropil, + indicates <10 immunopositive cells/mm$^2$ and diffuse staining in the neuropil, ++ indicates more intense somatic staining with a cell count between 10 and 19 immunopositive cells/mm$^2$, and +++ indicates higher somatic staining with cell counts of 20 or more immunopositive cells/mm$^2$.

| Brain Region | Relative Expression |
|---|---|
| **Cortex** | |
| *Primary somatosensory* | +++ |
| *Agranular Insular cortex* | ++ |
| *Granular Insular Cortex* | ++ |

| | |
|---|---|
| *Motor Cortex* | +++ |
| *Retrosplenial Granular* | ++ |
| *Cortex* | + |
| *Retrosplenial Agranular* | +++ |
| *Cortex* | |
| *Endopiriform Nucleus* | |
| **Hippocampus** | |
| *Dentate Gyrus* | ++ |
| *CA1* | ++ |
| *CA2* | + |
| *CA3* | + |
| **Hypothalamus** | |
| *Medial Preoptic nucleus* | + |
| *Zona Incerta* | ++ |
| *Paraventricular nucleus* | - |
| *Anterior hypothalamus* | - |
| *Posterior hypothalamus* | - |
| *Arcuate* | - |
| **Thalamus** | |
| *Mediodorsal* | - |
| *Central medial* | - |
| *Centrolateral* | - |
| **Septum** | |
| *Medial Septum* | +++ |
| *Lateral Septum* | + |
| **Striatum** | |
| *Caudate* | - |
| *Putamen* | - |
| *Globus pallidus* | - |
| *Nucleus accumbens* | - |

| | |
|---|---|
| **Amygdala** | |
| *Medial* | - |
| *Central* | + |
| *Basolateral* | - |

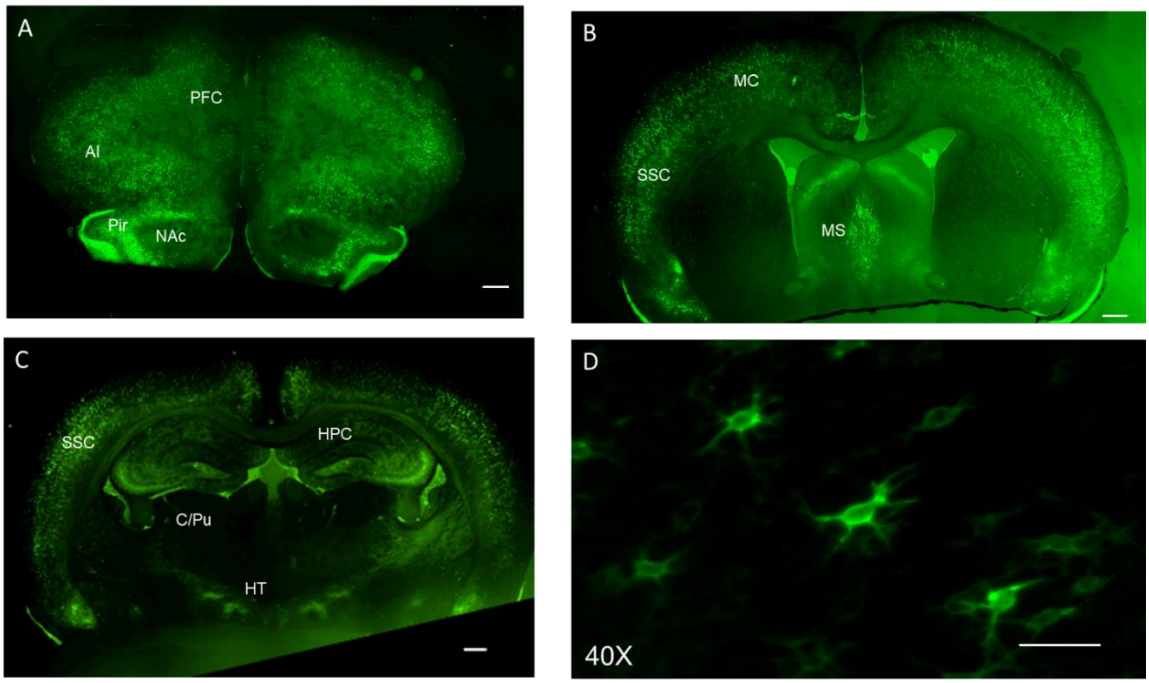



Figure 3.2 Representative micrographs showing WFA staining (green) in Syrian hamster brain. A-C are taken at 4X and scale bar represents 500 um. A) The agranular insular cortex (AI) and piriform cortex (Pir) show high expression of WFA+ cells with moderate expression in the prefrontal cortex (PFC) and little to no apparent expression in the nucleus accumbens (NAc) (+3.22mm AP from Bregma as shown in the stereotaxic atlas of hamster brain [31]. B) Both the motor cortex (MC) and somatosensory cortex (SSC) as well as the medial septum (MS) show high expression of WFA+ cells (+1.8mm from Bregma) C) The somatosensory cortex (SSC) and hippocampus (HPC) show high expression of WFA+ cells while the hypothalamus (HT) and caudate/putamen (C/Pu) show little to no apparent WFA+ cells (-0.9mm from Bregma). D) Higher magnification of perineuronal nets surrounding soma and proximal dendrites of cells in the mPFC (scale bar represents 50 um).

### *3.3.2 Diurnal variation in PNNs was not observed in male hamsters within selected brain regions that are known to mediate behavioral responses to social stress*

In C57BL/6 mice, PNNs appear to be dynamically regulated across the light-dark cycle in medial prefrontal cortex (mPFC), habenula, amygdala and all subregions of the

hippocampus (Pantazopoulos et al., 2020). Sprague-Dawley rats also show variation in PNN expression across the light-dark cycle in the mPFC (Harkness et al., 2021). As noted above, we have observed different behavioral outcomes when drugs were given at the onset of the light or dark phases of the daily cycle. A result that we hypothesized could be based, at least in part, on variation in PNNs at these time points. Therefore, we explored whether PNNs in Syrian hamsters differ between the onset of the light and the onset of the dark phases. Males were sacrificed at the onset of the light phase or the dark phase of the light-dark daily cycle, and the number of PNNs in selected brain regions were counted. The brain regions selected were those that are part of the putative Social Behavior Neural Network (Albers, 2012; Newman, 1999) and that we have previously determined are necessary for the acquisition or expression of behavioral responses to social defeat (Luckett et al., 2012; Markham et al., 2012; Markham et al., 2010) — the basolateral amygdala (BLA), nucleus accumbens (NAc), and subregions mPFC (anterior cingulate cortex, prelimbic cortex, and infralimbic cortex; ACC, PL, and IL, respectively) as well as areas showing high expression of PNNs in naïve animals—somatosensory cortex (SSC) and CA1 region of the hippocampus (CA1). BLA was selected before the study was conducted; when Experiment 1 showed no staining in the BLA (less than 5 cells), we still included BLA in Experiments 2 and 3 because previous studies have shown behavioral changes can occur even when only a few BLA cells have been manipulated (Jasnow et al., 2005). None of the areas examined showed differential expression based on lighting conditions (Figure 3) as determined by Student's t-tests: ACC $t=1.575$, $p=0.1464$ $d=0.9693$ (Figure 3a); PL $t=0.5347$ $p=0.6058$ $d=0.3222$ (Figure 3b); IL $t=-1.3755$, $p=0.2022$ $d=0.8193$

(Figure 3c); NAc t=0.02905 p=0.9775 d=0.0172 (Figure 3d); BLA t=0.5562, p=0.5933 d=0.3248 (Figure 3e); CA1 t=-0.2941, p=0.7762 (Figure 3f); SSC t=-0.1127, p=0.9130 (Figure 3g). Despite not reaching significant p-values, large effect sizes were observed in ACC and IL, and medium effect sizes were observed in PL and BLA.

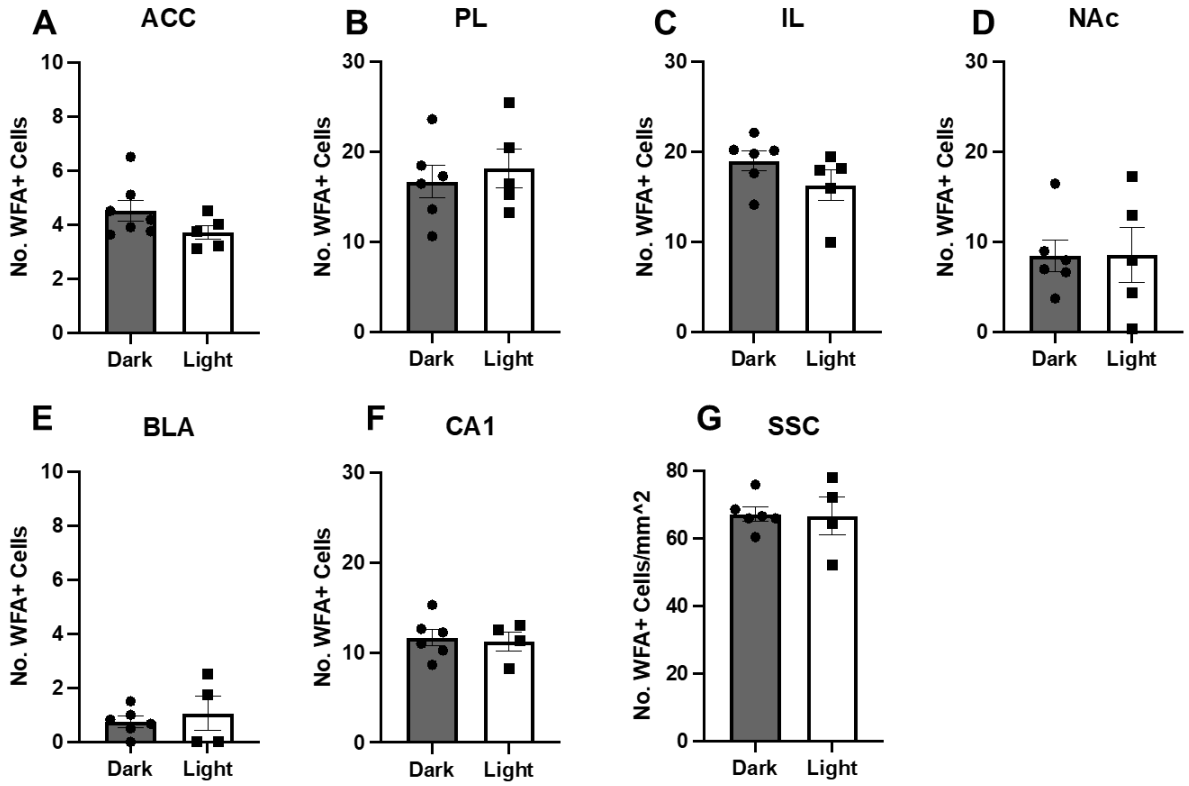



*Figure 3.3 Number of WFA+ cells does not appear to differ between the beginning of the dark (active) versus the light phase of the daily light-dark cycle in any of the brain regions studied. Similar expression of WFA+ cells was observed in the dark (gray bars with circle dots) and light (white bars with square dots) in the anterior cingulate cortex (A), prelimbic (B) and infralimbic cortices (C), nucleus accumbens (D), basolateral amygdala (E), hippocampus (F), and somatosensory cortex (G). Note the scale differences between regions. See Table 1 for quantification of PNNs in specific brain regions.*

### *3.3.3 WFA+ staining was largely stable following acute and repeated social defeat stress, except in the hippocampus, but staining did reveal some marked sexual dimorphism*

Because PNNs are dynamically regulated 4 hours after fear conditioning, it was hypothesized that PNNs would be similarly altered following social defeat stress. In

CA1, we observed a significant interaction of sex and defeat status (Figure 4a: Interaction $F(2,31)=4.629$ $p=0.0174$, Sex $F(1,31)=0.8960$ $p=0.3512$, Defeat timing $F(2,31)=0.6469$ $p=0.5306$), with acute defeat slightly increasing PNNs in males (not statistically significant) but decreasing them in females (Fisher's LSD $p=0.0064$). In the BLA (Interaction $F(2,27)=0.1284$ $p=0.88$, Sex $F(1,27)=4.596$ $p=0.0412$, Defeat timing $F(2,27)=1.147$ $p=0.3326$) and the SSC (Interaction $F(2,31)=0.4015$ $p=0.6727$ Sex $F(1,31)=5.319$ $p=0.0279$ Defeat timing $F(2,31)=2.702$ $p=0.0829$), there was no change in number of WFA+ cells after acute or repeated defeat, but we did observe a significant sex difference (Figure 4b-c) in these regions. As shown in Figure 4d-g, there were no significant of defeat or sex on number of immunostained cells, so data were collapsed across sex on the graphs. (ACC (Interaction $F(2,25)=0.4271$ $p=0.6571$, Sex $F(1,25)=1.813$, $p=0.1902$, Defeat timing $F(2,25)=0.2529$ $p=0.7785$); PL (Interaction $F(2,25)=0.2652$ $p=0.7692$, Sex $F(1,25)=0.8557$ $p=0.3638$, Defeat timing $F(2,25)=0.8882$ $p=0.4240$); IL (Interaction $F(2,25)=0.1654$ $p=0.8485$, Sex $F(1,25)=0.001399$ $p=0.9705$, Defeat timing $F(2,25)=0.6757$ $p=0.5178$); NAc (Interaction $F(2,32)=0.5564$ $p=0.5787$; Sex $F(1,32)=0.01099$ $p=0.9172$; Defeat timing $F(2,32)=0.02839$ $p=0.9720$)). Of note, although there were no significant differences among groups in the SSC, there was a large effect size of acute defeat versus no defeat in females (Fig. 4c; $d=0.9125$) and a medium effect size of acute defeat in males ($d=0.6626$).

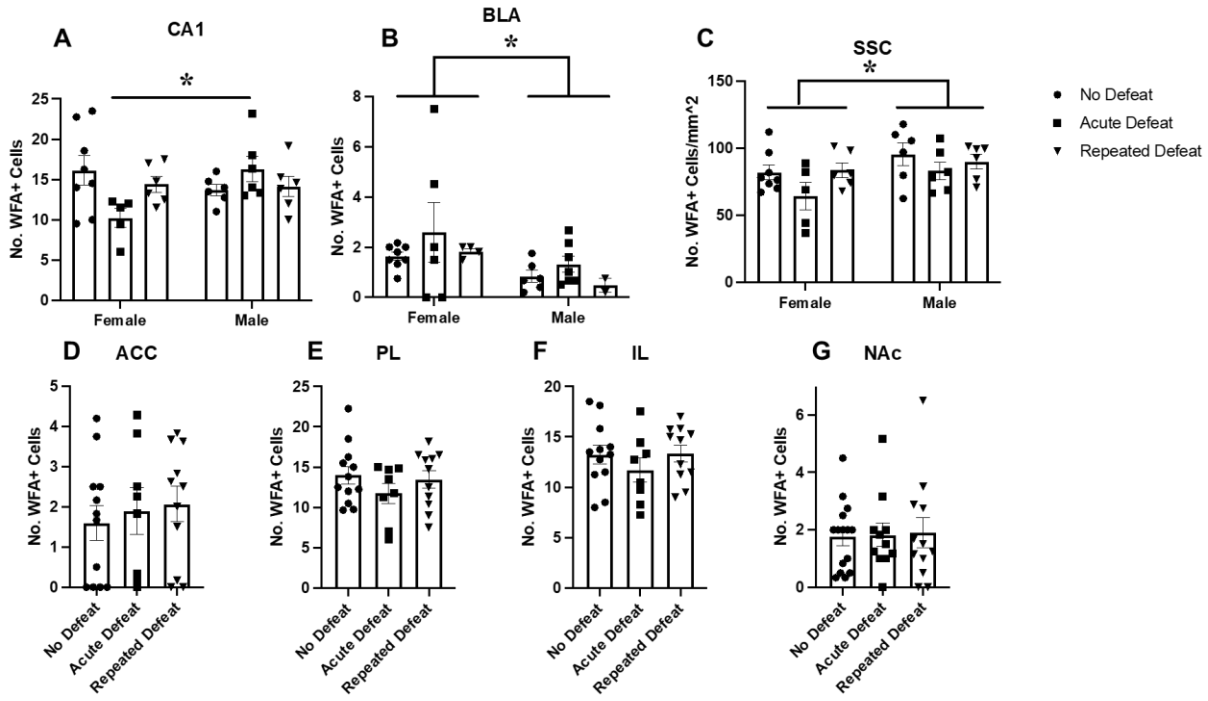



*Figure 3.4 Number of WFA+ cells in select brain regions after no defeat (white bars with circles), acute defeat (light gray bars with squares), or repeated defeat (dark bars gray with triangles). A) Hippocampal area CA1 (CA1) shows an interaction between sex and defeat status ($F(2,31)=4.629$ $p=0.0174$) with defeat appearing to reduce number of WFA+ cells in females but increase them in males, particularly after acute defeat. B) In basolateral amygdala (BLA), females display higher expression of PNN cells compared to males regardless of defeat status (significant main effect of sex $F(1,27)=4.596$ $p=0.0412$). C) In somatosensory cortex (SSC), conversely, males have higher expression of PNNs compared to females regardless of defeat status (significant main effect of sex $F(1,31)=5.319$ $p=0.0279$). D-G) There was no significant effect of sex on WFA+ cells in anterior cingulate cortex (ACC), prelimbic cortex (PL), infralimbic cortex (IL), and nucleus accumbens (NAc), thus the data were collapsed over sex in these brain regions. There was also no significant effect of defeat in these brain regions. *significant interaction of defeat status by sex; **acute defeat is significantly less than no defeat (Fisher's LSD, $p<0.05$); ***significant effect of sex regardless of defeat status; "d" indicates a medium effect size and "dd" indicates a large effect size (Cohen's d; as defined in Statistical Analysis).*

### *3.3.4 PNN expression does not vary 1-2 hours after an acute social defeat stressor*

Because there were no changes in PNN expression 4 hours after social defeat in a majority of the brain regions evaluated in *Experiment 3*, we examined the possibility that the 4-hour time point was too late to capture transient changes in PNN expression. A small pilot study was run, and animals were subjected to a single social defeat within the first 2 hours of the onset of the dark phase of the light-dark cycle and sacrificed

either 1 or 2 hours after the defeat stressor. The number of PNNs in preselected brain regions were counted. None of the regions evaluated showed significant changes in number of WFA+ cells one or two hours after defeat compared to no defeat controls (Figure 5). However, there was a trend towards an interaction between defeat and time of sampling in CA1 (Interaction $F(1,7)=5.507$ $p=0.0591$, Time $F(1,7)=0.05927$ $p=0.8146$, Defeat status $F(1,7)=0.6607$ $p=0.4431$). A two-way ANOVA was run for all brain regions examined. ACC: Interaction $F(1,6)=0.01434$ $p=0.9086$, Time $F(1,6)=0.01617$ $p=0.9030$, Defeat status $F(1,6)=0.2402$ $p=0.6414$; PL Interaction $F(1,7)=0.4370$ $p=0.5297$, Time $F(1,7)=0.4270$ $p=0.5343$, Defeat status $F(1,7)=1.505$ $p=0.2596$; NAc Interaction $F(1,7)=0.1582$ $p=0.7027$, Time $F(1,7)=0.6783$ $p=0.4373$, Defeat status $F(1,7)=0.08067$ $p=0.7846$; BLA Interaction $F(1,7)=1.550$ $p=0.2532$; Time $F(1,7)=0.010057$ $p=0.9210$, Defeat status $F(1,7)=0.1098$ $p=0.7501$; CA1 Interaction $F(1,7)=1.136$ $p=0.3218$, Time $F(1,7)=0.3315$ $p=0.5828$, Defeat status $F(1,7)=4.074e\text{-}5$ $p=0.9951$; SSC Interaction $F(1,8)=0.4776$ $p=0.5090$, Time $F(1,8)=0.6895$ $p=0.4304$, Defeat status $F(1,8)=0.05453$ $p=0.8212$. IL showed a large effect size between 1 hour no defeat and 1 hour defeat (Cohen’s $d=3.1983$), as did PL (Cohen’s $d=1.626$) and CA1 (Cohen’s $d=0.8055$).

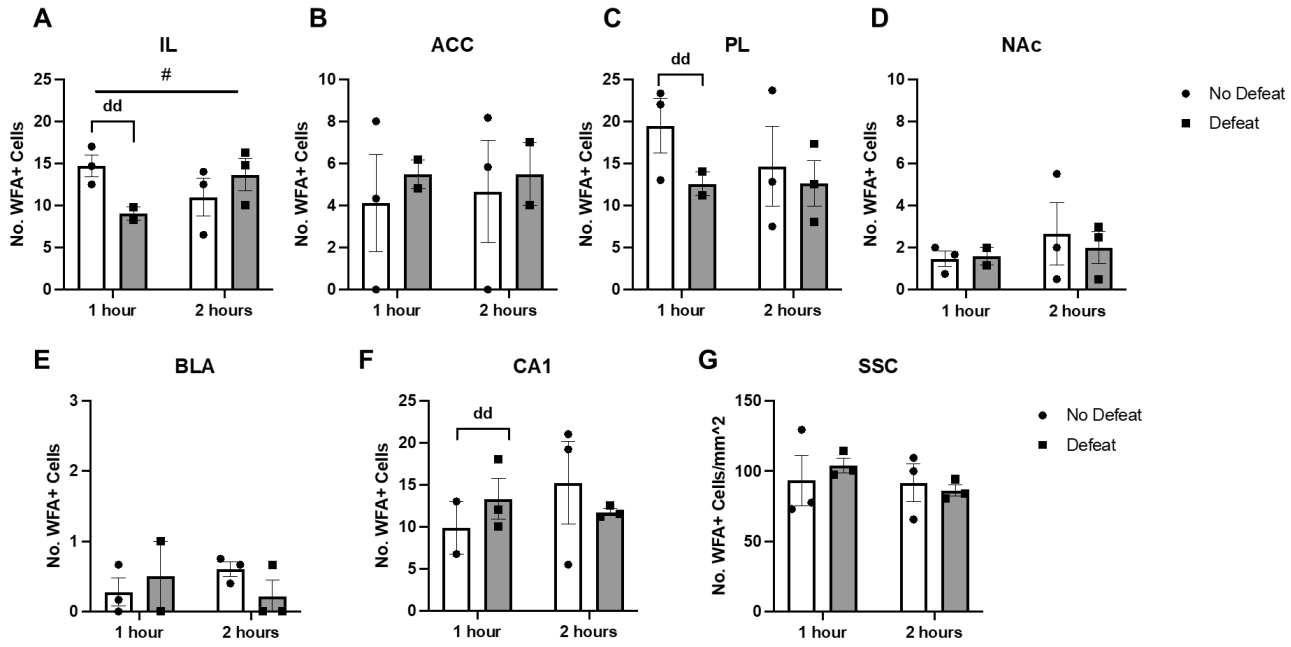



*Figure 3.5 Number of WFA+ cells one and two hours after a single social defeat (gray bars with squares) compared to no defeat controls (white bars with circles) A) Infralimbic cortex (IL) shows a trend that 1 hour after an acute defeat, the number of WFA+ cells is reduced (F(1,7)=5.067 p=0.0591, Cohen's d=3.1983) but rebounds two hours after defeat. B-G) Anterior cingulate cortex (ACC), prelimbic cortex (PL), nucleus accumbens (NAc), basolateral amygdala (BLA), CA1 region of the hippocampus, and the somatosensory cortex (SSC) do not show significant differences in WFA+ cells one or two hours after defeat compared to no defeat controls. # indicates p=0.0591; "dd" denotes large effect size (Cohen's d; as defined in Statistical Analysis).*

## 3.4 Discussion

The present study is the first to characterize the distribution of PNNs in Syrian hamsters, a species that has been widely used in studies of agonistic and communicative behavior as well as of circadian rhythms (Albers, 2012; Albers et al., 2006; H Elliott Albers et al., 2002). We found that the anatomical distribution of PNNs in Syrian hamster is somewhat different from that reported in other lab rodents, such as mice (Mus musculus) and rats (Rattus norvegicus). In hamsters, PNN staining is observed mainly throughout the cortex while being low or absent in the hypothalamus, thalamus, and striatum. In mice and rats, expression of PNNs is also highest in cortical regions but is also more widely spread throughout the brain including subcortical structures like thalamic nuclei, amygdala, and striatum compared to hamsters (Ciccarelli

et al., 2021). Human studies on PNNs thus far have been restricted to preselected brain regions or regions of interest (ROI) within the cortex, hippocampus, or amygdala, so how the distribution of PNNs in hamsters compares to that in humans across the brain is yet to be determined. In marmosets, a non-human primate, the distribution of PNNs is also distinctly different than that observed in mice in many brain regions (Ciccarelli et al., 2021) but appears to more closely mimic the pattern observed in hamsters. Together, the data suggest marked differences in the regional expression of PNNs among species and suggest that the selection of model species could have an enormous impact on the conclusions drawn. Given that hamsters are more human-like in their physiology and susceptibility to a variety of diseases than are many more commonly used animal models (for a review, see (Fan et al., 2014)), it may be that hamsters are a valuable model for asking questions about PNN function, as well. The data certainly demonstrate that a comparative approach is going to be necessary to determine the range of functions subserved by PNNs.

We had hypothesized that PNN expression would be different at different phases of the light-dark cycle based on literature examining PNN expression across the day in mice (Pantazopoulos et al., 2020). Contrary to this hypothesis, however, we observed no significant difference in PNN expression between the beginning of the dark, or active, phase of the cycle compared to the beginning of the light, or inactive, phase in male hamsters. Again, we chose these two time points because they are the times when we have observed behavioral differences in the response to pharmacological manipulations in male hamsters. It is certainly possible that there could be daily changes in PNN expression that occur outside these two time points. Indeed, in mice

and rats, wherein differences in PNN expression across the daily light-dark have been reported, the significant differences were observed in the middle of the light or dark phase and not at the time of lighting transitions (Pantazopoulos et al., 2020). Future studies should further characterize the possible daily or circadian variation in PNN expression in hamsters across a wider range of times. Future studies across species should also determine if diurnal variation, if present, is a result of the daily light-dark cycle or if it is driven by the circadian clock. A limitation, which must be acknowledged given the sex differences discussed below, is that we included only males in this experiment. Future studies should determine whether there is diurnal variation in PNN expression in female hamsters. The current data, however, do not support our hypothesis that differences in PNN expression underlie behavioral differences that we have previously observed between the beginning of the dark versus light phases of the daily light-dark cycle.

In hamsters, PNNs do not appear to be particularly labile after social defeat stress given that we observed no significant changes in PNN expression following defeat stress in most of the brain regions sampled. The only brain area in which we observed a significant effect of defeat on the number of PNN-enwrapped cells was hippocampal region CA1 wherein there was a defeat by sex interaction. We have previously demonstrated that the hippocampus is required for the acquisition of behavioral responses to social defeat stress in Syrian hamsters (Markham et al., 2010) and cfos mRNA expression increases in this brain region after handling stress (Kollack-Walker et al., 1997). In addition, the hippocampus is required for recognizing novel and familiar conspecifics in hamsters (Lai et al., 2005). Given this, it is possible that the

observed decrease in number of WFA+ cells following defeat in females in the CA1 region of the hippocampus may contribute to neuronal plasticity or activity following exposure to stress. In addition, males may respond more strongly to social defeat than do females (Bath & Johnston, 2007; Faruzzi et al., 2005; Kim L. Huhman et al., 2003). Thus, it is possible that the decrease in WFA+ cells in CA1 might contribute to the relative resilience of females to social defeat. This possibility could be tested in future studies. Most of the other brain regions studied have also been implicated in response to social defeat stress and yet fail to show changes in perineuronal net expression. This could be due to the heterogeneity of cell types within these regions, especially within the cortical regions. A future study could examine the co-expression of WFA with other cellular markers to determine the phenotype of cells associated with PNNs and to discover if PNNs associated with a subset of these are more labile.

Relatedly, this study used only WFA antibody to quantify PNN expression. This is the most commonly used method for detecting PNNs (Hartig et al., 2022). PNNs, themselves, are heterogenous, containing multiple different chrondroitin sulfate proteoglycans (CSPGs) such as aggrecan, neurocan, and brevican (Galtrey & Fawcett, 2007), however, and WFA staining cannot differentiate between these CSPGs and stains anything containing an internal or terminal N-acetylgalactosamine. Future studies may be useful in comparing PNN component expression to WFA-labeled cells in Syrian hamsters to note any differences, but the data above provide a solid baseline for understanding the expression of PNNs throughout hamster brain.

Our final experiment was a small pilot to determine whether the selection of the 4 hr time delay was perhaps too late to observe potential differences in PNN expression.

That time window was justified by the existing literature (Banerjee et al., 2017), but the time course of PNN responses has certainly not been studied exhaustively. Thus, our pilot study examined two additional time points following defeat. Despite the comparisons being underpowered, we observed a trend in IL towards a decrease in PNN expression at one hour that had rebounded by two hours after defeat, and there were medium to large effect sizes of defeat in some of the other brain regions, such as the infralimbic and prelimbic cortices. For the most part, however, the “time course” experiment suggested that the absence of widespread changes in PNNs following defeat in Experiment 3, was not due to the timing of sampling. Of course, it is entirely possible that we still missed the ideal time point or that PNNs change significantly in brain regions that we did not examine. The current data do suggest that further studies investigating the time course of PNN expression after social defeat may be warranted. Interestingly, it should also be noted that PNNs have been shown to exhibit hemispheric differences in response to behaviors such as maternal care (Lau et al., 2020), suggesting the possibility to that we could have missed effects of defeat or sex on PNNs that are hemisphere-dependent. Future studies could thus evaluate hemispheric differences after social defeat stress.

Although most of the experiments in the current study included only males, it is important to note that we did include both sexes in Experiment 3, which revealed that there may be important sex differences in number of PNN-enwrapped cells in the basolateral amygdala and the somatosensory cortex that are independent of defeat. There may be other sex differences not captured by the current study, such as in hormonally-responsive brain regions like the central amygdala or the bed nucleus of

stria terminalis (BNST) (Ciccarelli et al., 2021). Future studies should examine potential sex differences more broadly. The present data strongly suggest that studies examining PNNs should include both sexes and be adequately powered to detect sex differences. In addition, in the present study, we chose to evaluate PNN expression on Diestrus Day 1 of the estrous cycle to try to minimize potential hormonal variation among the females. It is certainly possible that there is variation in PNNs over the estrous cycle and that there could be varying sensitivity to stress among females at different stages of the cycle that could influence the PNN response. These factors should certainly be considered in future studies seeking to fully understand sex differences in PNN expression.

In conclusion, we characterized perineuronal net expression in Syrian hamster brain and suggest that hamsters may exhibit a unique distribution of PNNs compared to other commonly studied laboratory rodents. In addition, we have shown that there are marked sex differences in PNN expression in brain regions that are known to be involved in stress-responding and perhaps in disordered responses to stress in patients with neuropsychiatric illnesses (Shin & Liberzon, 2009). Furthermore, we have demonstrated that PNN expression in hippocampal area CA1 may be sensitive to social stressors in a sex-dependent manner. Together, these data suggest that Syrian hamsters are a valuable model organism with which to examine the potential functional role of PNNs in behavioral responses to stress in males and females.

## 3.5 References

Albers, H.E. and C.F. Ferris, *Neuropeptide Y: Role in light-dark cycle entrainment of hamster circadian rhythms.* Neuroscience Letters, 1984. **50**: p. 163-168.

Albers, H.E., K.L. Huhman, and R.L. Meisel, *6-Hormonal Basis of Social Conflict and Communication*, in *Hormones, Brain and Behavior*. 2002, Academic Press. p. 393-433.

Albers, H.E., et al., *Role of V1a vasopressin receptors in the control of aggression in Syrian hamsters.* Brain Research, 2006. **1073-1074**: p. 425-430.

Albers, H.E., *The regulation of social recognition, social communication and aggression: Vasopressin the social behavioral network.* Hormones and Behavior, 2012. **61**(3): p. 283-292.

Almeida, D.M., *Resilience and vulnerability to daily stressors assessed via diary methods.* Current Directions in Psychological Science, 2013. **14**: p. 64-68.

Banerjee, S.B., et al., *Perineuronal Nets in the Adult Sensory Cortex Are Necessary for Fear Learning.* Neuron, 2017. **95**(1): p. 169-179.

Bath, K.G. and R.E. Johnston, *Dominant-Subordinate relationships in Hamsters: Sex Differences in Reactions to Familiar Opponents.* Hormones and Behavior, 2009. **51**(2): p. 258-264.

Bjorkqvist, K., *Social defeat as a stressor in humans.* Physiology & Behavior, 2001. **73**(3): p. 435-42.

Bosiacki, M., et al., *Perineuronal Nets and Their Role in Synaptic Homeostasis. .* International journal of molecular sciences, 2019. **20**(17).

Browne, C.A., et al., *Editorial: Perineuronal Nets as Therapeutic Targets for the Treatment of Neuropsychiatric Disorders.* Frontiers in Synaptic Neuroscience, 2022. **14**.

Burgoon, P.W., P.T. Lindberg, and M.U. Gillette, *Different patterns of circadian oscillation in the suprachiasmatic nucleus of hamster, mouse, and rat.* Journal of Comparative Physiology, 2004. **190**: p. 167-171.

Ciccarelli, A., et al., *Sexually dimorphic perineuronal nets in the rodent and primate reproductive circuit.* Journal of Comparative Neurology, 2021. **529**: p. 3274-3291.

Cornez, G., et al., *Timing of perineuronal net development in the zebra finch song control system correlates with developmental song learning.* Proceedings Biological Sciences, 2018. **285**(1883).

Day, D.E., et al., *NR2B subunit of the NMDA receptor in the basolateral amygdala is necessary for the acquisition of conditioned defeat in Syrian hamsters.* Behavioral Brain Research, 2011. **217**: p. 55-59.

Fan, Z., et al., *Efficient Gene Targeting in Golden Syrian Hamsters by the CRISPR/Cas9 System.* PLoS one, 2014. **9**(10).

Faruzzi, A.N., et al., *Gonadal hormones modulate the display of submissive behavior in socially defeated female Syrian hamsters.* Hormones and Behavior, 2005. **47**(5): p. 569-575.

Galtrey, C.M. and J.W. Fawcett, *The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system.* Brain Research Reviews, 2007. **54**(1): p. 1-18.

Harkness, J.A., et al., *Diurnal changes in perineuronal nets and parvalbumin neurons in the rat medial prefrontal cortex.* Brain Structure and Function, 2021. **226**: p. 1135-1153.

Härtig, W., et al., *Update on Perineuronal Net Staining With Wisteria floribunda Agglutinin (WFA).* Frontiers in Integrative Neuroscience, 2022. **16**.

Huh, J., et al., *Treatment of generalized anxiety disorder: a comprehensive review of the literature for psychopharmacologic alternatives to newer antidepressants and benzodiazepines.* Primary Care Companion for CNS Disorders, 2011. **13**(2).

Huhman, K.L., et al., *Conditioned defeat in male and female Syrian hamsters.* Hormones and Behavior, 2003. **44**: p. 293-299.

Huhman, K.L., *Social conflict models: Can they inform us about human psychopathology?* Hormones and Behavior, 2006. **50**(4): p. 640-646.

Jasnow, A.M., et al., *Memory of social defeat is facilitated by cAMP response element-binding protein overexpression in the amygdala.* Behavioral Neuroscience, 2005. **119**: p. 1125-1130.

Kessler, R.C., et al., *Lifetime prevalence and age-of-onset distributions of DSM-IV in the National Comorbidity Survey Replication.* Archives of General Psychiatry, 2003. **62**(6): p. 593-602.

Kollack-Walker, S., S.J. Watson, and H. Akil, *Social stress in hamsters: defeat activates specific neurocircuits within the brain.* Journal of Neuroscience, 1997. **17**(22): p. 8842-55.

Lai, W.-S., et al., *Recognition of Familiar Individuals in Golden Hamsters: A New Method and Functional Neuroanatomy.* Journal of Neuroscience, 2005. **25**(49): p. 11239-11247.

Lau, B.Y.B., et al., *Lateralized Expression of Cortical Perineuronal Nets during Maternal Experience is Dependent on MECP2. LID - ENEURO.0500-19.2020 [pii] LID - 10.1523/ENEURO.0500-19.2020 [doi].* eNeuro, 2020. **7**(3).

Luckett, C., A. Norvelle, and K.L. Huhman, *The role of the nucleus accumbens in the acquisition and expression of conditioned defeat.* Behavioural Brain Research, 2012. **227**(1): p. 208-214

Markham, C.M., C.A. Luckett, and K.L. Huhman, *The medial prefrontal cortex is both necessary and sufficient for the acquisition of conditioned defeat.* Neuropharmacology, 2012. **62**(2): p. 933-999.

Markham, C.M., S.L. Taylor, and K.L. Huhman, *Role of amygdala and hippocampus in the neural circuit subserving conditioned defeat in Syrian hamsters.* Learning and Memory, 2010. **17**(2): p. 109-116.

Morin, L.P. and R.I. Wood, *A Stereotaxic Atlas of the Golden Hamster Brain*. 2001: Academic Press.

Mueller, A.L., et al., *Distribution of N-Acetylgalactosamine-Positive Perineuronal Nets in the Macaque Brain: Anatomy and Implications.* Neural Plasticity, 2016. **2016**: p. 6021428.

Newman, S.W., *The Medial Extended Amygdala in Male Reproductive Behavior A Node in the Mammalian Social Behavior Network.* Annals of the New York Academy of Sciences, 1999. **877**(1): p. 242-257.

Ottenweller, J.E., et al., *Adrenal, thyroid, and testicular hormone rhythms in male golden hamsters on long and short days.* American Jornal of Physiology, 1987. **253**: p. R321-8.

Pantazopoulos, H., et al., *Extracellular matrix-glial abnormalities in the amygdala and entorhinal cortex of subjects diagnosed with schizophrenia.* Archives of General Psychiatry, 2010. **67**(2): p. 155-66.

Pantazopoulos, H., et al., *Circadian Rhythms of Perineuronal Net Composition.* eNeuro, 2020. **7**(4).

Rogers, S.L., et al., *Normal Development of the Perineuronal Net in Humans; In Patients with and without Epilepsy.* Journal of Neuroscience, 2018. **284**: p. 350-360.

Ross, A.P., et al., *Social housing and social isolation: impact on stress indices and energy balance in male and female Syrian hamsters.* Physiology & Behavior, 2017. **177**: p. 264-269.

Seeger, G., et al., *Mapping of perineuronal nets in the rat brain stained by colloidal iron hydroxide histochemistry and lectin chemistry.* Neruoscience, 1994. **58**(2): p. 371-88.

Solomon, M.B., et al., *Social defeat and footshock increase body mass and adiposity in male Syrian hamsters.* American Journal of Physiology, 2006. **292**(1).

Sorg, B.A., et al., *Casting a Wide Net: Role of Perineuronal Nets in Neural Plasticity.* Journal of Neuroscience, 2016. **36**(45): p. 11459-11468.

Shin, L.M. and I. Liberzon, *The Neurocircuitry of Fear, Stress, and Anxiety Disorders.* Neuropsychopharmacology, 2010. **35**(1): p. 169-191.

Toyoda, A., *Social defeat models in animal science: What we have learned from rodent models.* Animal Science Journal, 2017. **88**(7): p. 944-952.

Wood, S.K. and S. Bhatnagar, *Resilience to the effects of social stress: Evidence from clinical and preclinical studies on the role of coping strategies.* Neurobiology of Stress, 2015. **1**: p. 164-173.

# 4 CONCLUSION

## 4.1 Summary of Current Findings

The most important take-home finding of this dissertation is that time of day of pharmacological intervention and behavioral testing can have major impacts on the behavioral effect of a particular drug. Here, we have shown that the same drug given at the same dose immediately after social defeat stress produces opposite effects on stress-induced behavior depending on when during the light-dark cycle it is administered. Activation of the brain-derived neurotrophic factor (BDNF) receptor, tropomyosin kinase receptor B (TrkB) using 7,8-dihydroxyflavone (7,8-DHF), reduced the behavioral response to social defeat stress when given in the dark. However, when given in the light, 7,8-DHF enhanced the behavioral response to social defeat stress, resulting in a significant drug by lighting interaction (shown in **Aim 1**). The ultimate goal of the current research was to help identify potential novel treatment options for individuals suffering from stress-related neuropsychiatric disorders. Manipulation of BDNF signaling has received attention as a promising potential target. The current data demonstrate, however, that no simple assessment of this target as either promoting or preventing stress-induced behavioral responses is possible. More importantly, the data underscore that behavioral pharmacologists, as well as scientists as a whole, need to pay much more attention than we do currently in the field to how, and particularly when, we are intervening to alter brain and behavior. We must also think much more critically about how we design psychopharmacological and behavioral studies to determine the optimal way to examine a particular question.

When we observed this important effect of time-of-day on the response to a BDNF-active drug, we next sought to identify whether key nodes in the brain circuit that mediates social defeat-induced behavioral responses also respond differently to defeat or to drugs depending on time-of-day. To test this, we selected brain regions shown to be critical for the behavioral response social defeat stress that were also those where BDNF is known to affect behavioral responses to stress. These regions were the medial prefrontal cortex (and its subregions: anterior cingulate cortex, ACC; prelimbic cortex, PL; and infralimbic cortex, IL) (Laubach et al., 2018; Markham et al., 2012), the nucleus accumbens (NAc) (Gray et al., 2015; Luckett et al., 2012), and the basolateral amygdala (BLA) (Jasnow & Huhman, 2001; Jasnow et al., 2005; Markham et al., 2010; Rosenhauer et al., 2019). I hypothesized that different regions may be differentially activated, or even quiescent, in the light versus in the dark. Other labs have focused on the mesolimbic dopamine circuit—including the ventral tegmental area and NAc—as critical nodes for social defeat stress, while our lab mainly focuses on BLA and mPFC. We have previously demonstrated that infusion of BDNF into BLA but not NAc decreases behavioral responses to social defeat stress in mice and hamsters (Rosenhauer et al., 2019). Thus, we tested whether there is a time-of-day-dependent difference in cellular activation, as assess by the immediate-early gene product CFos, in these nodes of the neural circuit responding to social stress. Unfortunately, we did not observe a significant effect of time-of-day on cellular activation within these nodes following defeat or manipulation of BDNF-signaling, however, we did observe a trend towards statistical significance ($p=0.0533$) for a defeat x lighting interaction in the ACC. This suggests that the ACC could be an important node in the circuit wherein time-of-

day might play a role, a possibility that certainly should be examined further. In the PL and BLA we observed a statistically significant effect of drug or defeat (PL), or drug x defeat (BLA), findings that are consistent with the known role of these nodes in the circuit mediating responses to social stress. We next infused BDNF directly in BLA or mPFC, but unfortunately did not observe any effect on the social avoidance produced by social defeat stress in either the light or the dark. It is important to note that 7,8-DHF is a specific TrkB agonist, while BDNF protein is not; BDNF can bind to TrkA, TrkC, and p75NTR. It could be that the observed effects of lighting were specifically mediated by TrkB and that effect was washed out when other receptors were also activated by BDNF in the infusion study. Future studies will hopefully explore this possibility. Together, however, the data from both studies consistently fail to implicate the PL and the BLA in the observed time-of-day variation in the behavior and drug effects.

In addition to evaluating whether different brain regions underly the observed differences in behavioral response to BDNF-signaling and social defeat stress, we also assessed whether there were molecular changes with the BDNF-signaling pathway that could account for the observed difference in drug action based on time of day. We tested whether there was a difference in mRNA expression of *ntrk2* and *gad1,* the genes encoding TrkB and glutamate decarboxylase (an enzyme only present in GABAergic cells), respectively, using RNAscope™ *In Situ* hybridization. There was an interesting decrease in the number of cells expressing both *ntrk2* and *gad1* in the BLA during the light phase compared to the dark. This decrease was not associated with a decrease in the number of *gad1* puncta, which corresponds to relative expression of that mRNA. The ACC showed a significant increase in number of *ntrk2* puncta in the

light compared to the dark. These data taken together highlight the importance of BLA and mPFC for behavioral response to social defeat stress and pharmacological manipulation of TrkB. However, again we did not identify a specific brain region responsible for the interesting lighting effect observed in the behavioral response to social stress following manipulation of TrkB signaling.

Perineuronal nets (PNNs) are extracellular matrix proteoglycans that primarily surround parvalbumin-expressing interneurons but have been found on other neuronal subtypes as well as glia (Bruckner et al., 1993; Sorg et al., 2016). PNNs inhibit synaptic plasticity and develop postnatally and are postulated to at least partially underlie closing of so-called critical periods during development. More recently it has been shown that PNNs are labile in adulthood, and expression can be modulated in response to fear conditioning. Importantly, in rats and mice, they also show variation between the light and the dark (Harkness et al., 2021; Pantazopoulos et al., 2020). Thus, we hypothesized that PNNs may vary across the light:dark cycle to potentially alter response to drugs and to change in response to social defeat stress, again perhaps differentially in the light versus the dark phase of the cycle. We first sought to establish where in Syrian hamster brain PNNs are expressed and to compare this distribution to other common laboratory animal models and to humans. To our knowledge, no one has characterized the distribution of PNNs in hamster brain. PNNs in Syrian hamsters show similar patterns of expression to other rodent species studied, such as mice and rats, in cortical regions. However, mice and rats show extensive expression on PNNs in subcortical regions, namely the striatum and hypothalamus, while Syrian hamsters do not express PNNs significantly in these regions. We did not observe circadian variation

in PNNs, at least in the time points included in this study. Interestingly, PNNs show a significant interaction of defeat x sex in hippocampal region CA1. Defeat decreased PNN expression in males but increased it in females. BLA and somatosensory cortex (SSC) also showed significant effects of sex on PNN expression, with males showing significantly less expression compared to females in the BLA, regardless of defeat status. In SSC, males exhibited significantly higher expression of PNNs compared to females. It is not clear what these differences mean functionally at this point. We chose to assess PNN expression 4 hours after based on a study that showed maximum change in PNN expression in rats following fear conditioning (Banerjee et al., 2017). It is possible, however, that hamsters have a different response time from rats and that the 4-hour time point was insufficient to capture potential changes in PNNs following social defeat. In an effort to further understand the time course for the dynamic regulation of PNNs following social defeat, we did run a small pilot study to examine PNN expression at 1 and 2 hours after defeat. In this pilot, we did observe a trend towards a decrease in PNN expression in the IL at one hour after defeat which appeared to rebound by the 2 hr time point. This suggests that time courses of PNN responses may differ among species or stressors, a possibility that should be considered for future studies. The current pilot may have also been somewhat underpowered, however, so conclusions are limited.

### 4.2 Chronopharmacology

Chronopharmacology is the study of the interaction between time of day and pharmacological actions. Most biological processes are regulated in a circadian or time of day manner, therefore studying how time of day plays a role in pharmacology seems

obvious. However, it is an often-overlooked variable in experimental design. The majority of basic psychopharmacological research is done using rodent animal models, particularly inbred strains of mice. Almost all commonly used laboratory rodent species are nocturnal. Despite this, most experiments involving behavioral and/or pharmacological assays are conducted during the light phase of the daily cycle, when these animals are typically inactive. We maintain that this experimental design is not ideal and may lead to erroneous or incomplete conclusions based on data collected in an ethologically irrelevant time period.

Circadian rhythms are present throughout the animal kingdom, and in mammals are controlled by the “master clock,” the suprachiasmatic nucleus of the hypothalamus (SCN). There are a few key transcription factors that influence circadian clocks—CLOCK1 and BMAL—that activate or repress transcription of multiple genes (For review see, (Dallman et al., 2014). In fact, one-tenth of transcripts in every mouse tissue are regulated in a circadian fashion (Panda et al., 2002; Storch et al., 2002). This variation could obviously affect how organisms respond to drugs during different phases of the daily cycle.

Chronopharmacological behavioral studies are quite limited, but other scientific research areas are starting to consider time-of-day as a critical variable. The blood brain barrier appears to show circadian variation in its permeability. It has also been found that the chemotherapeutic drug (paclitaxel) had increased concentrations in brain tissue in the dark (Zeitgeber Time (ZT)14 and ZT17) compared to brain tissue from mice given the drug in the light (ZT0 and ZT5) (Walker et al., 2021). They also found that paclitaxel reduced number of cells of breast cancer brain metastases in the dark more than when

given in the light (Walker et al., 2021). Thus, the current findings have potential translational implications for human health outcomes and should certainly receive more careful consideration when assessing potential new therapeutic targets for any disease or disorder.

### 4.3 Limitations of Aim 1 and Future Directions

These studies were limited by multiple factors. Namely, the molecular and genetic tools available for Syrian hamsters still leave a lot to be desired. I originally wanted to investigate whether phosphorylated TrkB (pTrkB, the active form of this BDNF receptor) is different in the light and the dark. I hypothesized that differential activation of TrkB in the light and the dark could underlie the observed behavioral differences resulting from manipulation of BDNF signaling pathways after social defeat. Using western blotting, I initially wanted to assess amount of pTrkB in specific brain regions in the light and the dark. This would have been a better measure than looking at the transcript for TrkB, which is unable to reveal active versus inactive forms of the receptor. Ultimately, there simply weren't antibodies that worked successfully in hamster, so I had to pivot to studying mRNA expression of *ntrk2*, the gene that encodes TrkB. However, this switch did enable me to have better spatial resolution of *ntrk2* expression, as well as allowed me to assess cell type in cells expressing *ntrk2*. These data revealed that *ntrk2* expression in the ACC increases in the light compared to the dark, supporting the previously-described trend observed in the CFos study towards a defeat x lighting interaction in the ACC. Perhaps the increase in *ntrk2* expression in the light is related to the increase in CFos expression observed in animals that were not defeated. This observation certainly merits further investigation into the relationship

among ACC, time-of-day, and social defeat. An obvious next step would be to perform RNAScope again with defeated as well as non-defeated animals in the light and the dark to determine whether the phase of the cycle interacts with defeat status.

The CFos study could have been greatly improved by using tissue clearing and whole brain imaging to investigate activation simultaneously in every brain region, not just the five regions of interest that we preselected. As we performed it, animals were defeated in the light or the dark and sacrificed 90 minutes after the end of the defeat. I only assessed CFos immunoreactivity in the ACC, PL, IL, NAc, and BLA. While these regions have been certainly been shown to be critical for behavioral responses to social stress, they of course do not capture the whole picture. The current CFos data did not reveal a specific region that responds to lighting alone, but it is entirely possible that other brain regions or patterns of activation among regions that may be differentially regulated by time of day, stress, and BDNF-signaling that were missed. During this project, we initiated collaborations to attempt this whole brain examination of CFos in hamsters and were successful in clearing hamster brains, but an atlas to align hamster brain to the analysis software was not completed in time for this study. A future study, when possible, could use this technology to better understand which brain regions are regulated in a time-dependent manner in response to BDNF-signaling and social stress. I think the suprachiasmatic nucleus of the hypothalamus is an excellent starting point for this study, as we know it controls circadian rhythms based on lighting.

Beyond just time of day, there is another key difference between studies showing BDNF promotes responses to social stress and studies showing it prevents this response and that is the duration or intensity of the defeat. The current research used a

brief agonistic encounter with a single aggressor to induce social defeat stress. Importantly, many other labs use chronic social defeat stress, a protocol that involves 10-14 days of aggression and and constant exposure to the aggressor for the duration of the stressor. This latter protocol is clearly much more severe and, in fact, often results in injury to the defeated mouse. The duration of the aggression and defeat may strongly influence molecular mechanisms underlying the response to social stress and to manipulations of BDNF and other pathways. Again, the labs employing chronic social defeat stress have largely focused on brain regions in the mesolimbic dopamine system while studying effects of BDNF. Our lab, using acute social defeat stress, has demonstrated that BDNF infusion directly into NAc following acute defeat has no effect on the behavioral response to social defeat stress (Rosenhauer et al., 2019). It is possible that there is some sort of shift from BLA-mPFC signaling in acute defeat towards activation of mesolimbic dopamine system in chronic defeat. It would be worthwhile to investigate differences in response to BDNF-related pharmacological manipulation in hamsters that were acutely defeated compared to those exposed to chronic defeat stress to determine whether the duration of the defeat alters the direction of the BDNF effect.

It is also important to note that BDNF signaling is highly complex and still poorly understood. BDNF has multiple isoforms and splice variants that have slightly different actions on TrkB so that they can phosphorylate different tyrosine residues, for example. When purchasing commercial recombinant BDNF, the splice variant(s) are not specified. When I infused BDNF into two brain regions, it could have been a combination of these and that could have impacted the signaling mechanisms and

subsequent behavior. BDNF is synthesized and packaged as pre-proBDNF that can be cleaved within the vesicle into proBDNF. ProBDNF binds to a separate receptor called pan-neurotrophin receptor 75 (p75NTR). When activated, p75NTR acts through signaling cascades to promote long term depression and apoptosis, the opposite of what TrkB activation does. Many studies showing that BDNF is pro-depressant use genetic targeting of BDNF, meaning that it changes pre-proBDNF, proBDNF, and mature BDNF (mBDNF, the molecule that binds to TrkB with highest affinity). Our studies have been used pharmacological manipulations of BDNF that are more specific to TrkB signaling activation or inhibition. Further studies should tease apart differences in proBDNF/p75NTR and mBDNF/TrkB signaling mechanisms and their influence on stress response.

### 4.4 Limitations of Aim 2 and Future Directions

The perineuronal net studies were also limited. We characterized PNN expression throughout hamster brain and assessed whether PNN expression changes over the light:dark cycle or following social defeat stress. We found that PNNs do not change over the light:dark cycle, at least between the two timepoints chosen. Hippocampal area CA1 showed a sex x defeat effect, and the BLA and somatosensory cortex (SSC) showed a significant sex difference. We only investigated the commonly used antibody to visualize PNNs, *Wisteria floribunda* agglutinin (WFA), which might overlook other types of PNNs with components that may not have an epitope for the WFA antibody. These experiments would also improve if tissue clearing and whole brain imaging were used—there could be regions in which WFA expression changes following social defeat stress or in a circadian manner that were not captured by the

current study. It may also be worthwhile to assess PNN expression throughout the light:dark cycle; this study only examined samples collected at the onset of the light and the onset of the dark, the times when we had previously observed effects of drugs and social stress. In mice, however, maximal circadian differences in PNNs were observed 4-6 hours after the onset of the light and the dark (Pantazopoulos et al., 2020), suggesting that there could be other time points wherein PNNs could exhibit daily variation. Another future direction could be to inject chondroitinase, an enzyme that acutely degrades PNNs, in select cortical regions (like ACC) immediately before social defeat stress or avoidance testing to assess whether the presence of PNNs is necessary for the behavioral response to social defeat stress. PNNs may not be labile in response to social defeat stress, but they could still play an important role in responding to social stress.

### 4.5 Conclusions

The failure to reproduce rigorous studies is a problem throughout scientific disciplines. A major finding from this dissertation is the importance of time as a biologically significant variable that might explain the often-regretted failure of replication or translation. Originally, the goal of this research was to investigate some of the molecular mechanisms underlying social defeat stress and to assess the potential for manipulation of BDNF signaling as a target for the development of new interventions for individuals suffering from social stress-related neuropsychiatric disorders. Importantly, the current data suggest that the effect of manipulating BDNF signaling may vary, or even be opposite, depending on when during the daily light-dark cycle the manipulation is done. This suggests that a lot more work needs to be done to understand how BDNF

regulates responses to social stress. The data also emphasize the importance of considering time of day as a biological variable in all experimental designs, which is perhaps the most important take-away from this study.

### 4.6 References

Banerjee, S. B., Gutzeit, V. A., Baman, J., Aoued, H. S., Doshi, N. K., Liu, R. C., & Ressler, K. J. (2017). Perineuronal Nets in the Adult Sensory Cortex Are Necessary for Fear Learning. *Neuron*, *95*(1). https://doi.org/10.1016/j.neuron.2017.06.007

Bruckner, G., Brauer, K., Hartig, W., Wolff, J. R., Rickmann, M. J., Derouiche, A., Delpech, B., Girard, N., Oertel, W. H., & Reichenbach, A. (1993). Perineuronal Nets Provide a Polyanionic Glia-Associated Form of Microenvironment Around Certain Neurons in Many Parts of the Rat Brain. *Glia*, *8*, 183-200.

Dallman, R., Brown, S. A., & Gachon, F. (2014). Chronopharmacology: New Insights and Implications. *Annual Review of Pharmacology and Toxicology*, *54*, 339-361. https://doi.org/https://doi.org/10.1146/annurev-pharmtox-011613-135923

Gray, C. L., Norvelle, A., Larkin, T., & Huhman, K. L. (2015). Dopamine in the nucleus accumbens modulates the memory of social defeat in Syrian hamsters (Mesocricetus auratus). *Behavioral Brain Research*, *286*, 22-28. https://doi.org/10.1016/j.bbr.2015.02.030

Harkness, J. H., Gonzalez, A. E., Bushana, P. N., Jorgensen, E. T., Hegarty, D. M., Di Nardo, A. A., Prochiantz, A., Wisor, J. P., Aicher, S. A., Brown, T. E., & Sorg, B. A. (2021). Diurnal changes in perineuronal nets and parvalbumin neurons in the rat medial prefrontal cortex. *Brain Structure & Function*, *226*(4), 1135-1153. https://doi.org/https://doi.org/10.1007/s00429-021-02229-4

Jasnow, A. M., & Huhman, K. L. (2001). Activation of GABA(A) receptors in the amygdala blocks the acquisition and expression of conditioned defeat in Syrian hamsters. *Brain Research*, *920*(1-2), 142-150. https://doi.org/https://doi.org/10.1016/S0006-8993(01)03054-2

Jasnow, A. M., Shi, C., Israel, J. E., Davis, M., & Huhman, K. L. (2005). Memory of social defeat is facilitated by cAMP response element-binding protein overexpression in the amygdala. *Behavioral Neuroscience*, *119*(4), 1125-1130. https://doi.org/10.1037/0735-7044.119.4.1125

Laubach, M., Amarante, L. M., Swanson, K., & White, S. R. (2018). What, If Anything, Is Rodent Prefrontal Cortex. *eNeuro*, *5*(5). https://doi.org/10.1523/ENEURO.0315-18.2018

Luckett, C., Norvelle, A., & Huhman, K. L. (2012). The role of the nucleus accumbens in the acquisition and expression of conditioned defeat. *Behavioural Brain Research*, *227*(1), 208-214.

Markham, C. M., Luckett, C. A., & Huhman, K. L. (2012). The medial prefrontal cortex is both necessary and sufficient for the acquisition of conditioned defeat. *Neuropharmacology*, *62*(2), 933-999.

Markham, C. M., Taylor, S. L., & Huhman, K. L. (2010). Role of amygdala and hippocampus in the neural circuit subserving conditioned defeat in Syrian hamsters. *Learning and Memory*, *17*(2), 109-116.

Panda, S., Antoch, M. P., Miller, B. H., Su, A. I., Schook, A. B., Straume, M., Schultz, P. G., Kay, S. A., Takahashi, J. S., & Hogenesch, J. B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell*, *109*(3), 307-320. https://doi.org/10.1016/s0092-8674(02)00722-5

Pantazopoulos, H., Gisabella, B., Rexrode, L., Benefield, D., Yildiz, E., Seltzer, P., Valeri, J., Chelini, G., Reich, A., Ardelt, M., & Berretta, S. (2020). Circadian Rhythms of Perineuronal Net Composition. *eNeuro*, *7*(4). https://doi.org/10.1523/ENEURO.0034-19.2020

Rosenhauer, A. M., Beach, L. Q., Jeffress, E. C., Thompson, B. M., McCann, K. E., Partrick, K. A., Diaz, B., Norvelle, A., Choi, D. C., & Huhman, K. L. (2019). Brain derived neurotrophic factor signaling mitigates the impact of acute social stress. *Neuropharmacology*, *148*, 40-49.

Sorg, B. A., Berretta, S., Blacktop, J. M., Fawcett, J. W., Kitagawa, H., Kwok, J. C., & Miquel, M. (2016). Casting a Wide Net: Role of Perineuronal Nets in Neural Plasticity. *Journal of Neuroscience*, *36*(45), 11459-11468. https://doi.org/10.1523/JNEUROSCI.2351-16.2016

Storch, K., Lipan, O., Leykin, I., Viswanatha, N., Davis, F. C., Wong, W. H., & Weitz, C. J. (2002). Extensive and divergent circadian gene expression in liver and heart. *Nature*, *417*(6884), 78-83. https://doi.org/10.1038/nature744

Walker, W. H., Sprowls, S. A., Bumgarner, J. R., Liu, J. A., Melendez-Fernandez, O. H., Walton, J. C., Lockman, P. R., DeVries, A. C., & Nelson, R. J. (2021). Circadian Influences on Chemotherapy Efficacy in a Mouse Model of Brain Metastases of Breast Cancer. *Frontiers in Oncology*, *11*. https://doi.org/https://doi.org/10.3389/fonc.2021.752331