Examination of Time-of-Day Differences in Fear-Related Memory Behavior in Male and Female Rats

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ABSTRACT

Post-traumatic stress disorder (PTSD) and other anxiety disorders are often characterized by impairment of the circadian system. Fear extinction learning is known to be modulated by the circadian system, but it is unclear if the time of extinction training or the time of extinction testing determines fluctuations in behavioral responses, or if these modulations are also present in female rats. Furthermore, it is unclear if fear relapse mechanisms show circadian rhythmicity. In experiment 1, rats were fear conditioned and extinction trained at zeitgeber time 4 (ZT4), ZT10, ZT16, or ZT22 and underwent five extinction testing sessions, separated by 6hr, beginning 24hrs after training. Rats trained at all times exhibited similar behavior at the time of extinction training. However, rats tested at ZT16 (active phase) demonstrated enhanced fear extinction recall memory and rats tested at ZT4 (inactive phase) demonstrated weaker fear extinction recall memory. In a follow-up experiment (experiment 3), results showed that male and female rats housed in constant darkness (DD) also exhibited time-of-day differences during fear extinction recall testing. These results suggest that fear extinction recall behavior may be regulated by the circadian system, and that molecular clocks in the prefrontal cortex may regulate molecular mechanisms associated with fear extinction memory retrieval. Follow-up experiments examined circadian modulation of fear relapse behavior. In experiment 2, rats trained at either ZT4 or Z16 underwent fear relapse testing 24, 36, or 48hrs later. No significant effects were observed; however, experiment 1, which tested rats for fear relapse 72hrs following extinction sessions, observed trending results that suggest that fear relapse behavior is enhanced at ZT16. For this reason, experiment 3 also examined fear relapse behavior 72hrs following extinction testing (as in experiment 1) and replicated the results of experiment 1 fear relapse.

These data suggest that the circadian system may also modulate of fear relapse behavior, particularly spontaneous recovery of fear responses.

1. INTRODUCTION

1.1 Overview

For patients suffering from anxiety disorders, exposure therapy is a treatment commonly used to improve emotion regulation in the face of fear-provoking environmental triggers. Understanding how to optimize human exposure therapy is a major goal of current research (Harvey et al., 2014). Rodent fear extinction paradigms are used to model exposure therapy protocols in humans (Milad and Quirk, 2012). Much research has been conducted to characterize brain mechanisms underlying rodent fear extinction, and recent evidence suggests that rodent fear extinction may be fundamentally regulated by the circadian system (Woodruff et al., 2015). This manuscript presents a brief review of the circadian system, behavioral phenomena surrounding rodent fear memory, and relevant brain areas. Then discussed are experiments that examined how rodent fear extinction and other related processes may be modulated by time of day. This report will conclude by considering how these experiments contribute to the existing literature and may inform clinical practice.

1.2 Circadian Rhythms, Protein Oscillation, and the Molecular Clock

Circadian rhythms are endogenous, near-twenty-four-hour cycles present in most organisms. These rhythms can be observed from the molecular to behavioral levels. In humans, the circadian system regulates many homeostatic processes like blood pressure, heart rate, body temperature, and higher cognitive functioning (Escobar et al., 2016; Potter et al., 2016).

Circadian rhythms serve a critical adaptive function, enabling an organism to synchronize its behavior and physiology with daily fluctuations in the external environment (Van Oort et al., 2005). For example, cortisol (corticosterone in rats; abbr. CORT) is an adrenal hormone controlled by circadian function. It is released daily near the time an organism awakens (Khani and Tayek, 2001). This exemplifies a molecular circadian rhythm, whilst a typical sleep/wake cycle exemplifies a behavioral circadian pattern.

Circadian rhythms in behavior and physiology arise from a molecular clock present in cells throughout the body. Molecular clocks are observed in most unicellular and eukaryotic cells, and depend on molecular transcription-translation feedback loops (TTFLs, Figure 1) lasting approximately 24 hours. This molecular TTFL consists of a positive arm, made up of the BMAL1 and CLOCK proteins; and a negative arm, made up of Cryptochrome (CRY1/2) and Period (PER1/2/3) proteins (Dunlap, 1999). BMAL1 and CLOCK proteins act as a transcription factor by forming heterodimers and binding the E-box enhancer regions of the CRY and PER genes (Fustin et al., 2009; Gekakis et al., 1998; Hogenesch et al., 1998; Yoo et al., 2004).

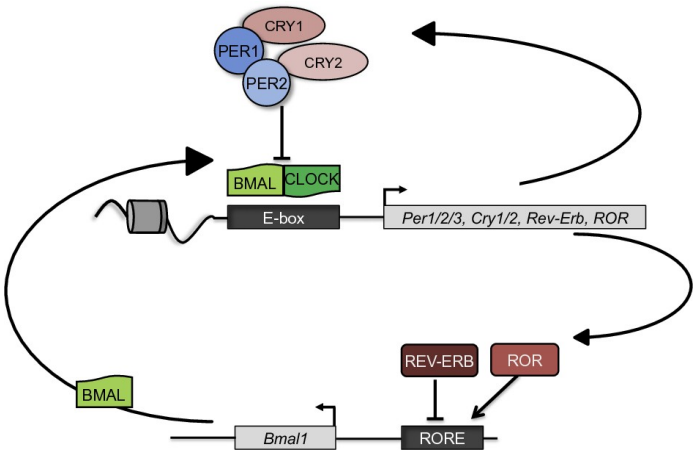

Figure 1: The oscillation of the BMAL/CLOCK, PER/CRY TTFL. This cycle operates on a 24hr basis and occurs in most cells of the body. This cycle is the foundation for many biological rhythms. (Adamovich et al., 2015).

Activation of the CRY and PER promoter upregulates transcription of those genes, and translation produces CRY and PER proteins, the negative arm of the TTFL.

PER and CRY proteins also act as transcription factors by heterodimerizing to each other, migrating back into the nucleus and preventing the BMAL1/CLOCK complex from binding the E-box (Kume et al., 1999; Zheng et al., 2001). PER and CRY are then targeted for phosphorylation by casein kinases 1δ and 1ε. This phosphorylation allows the ubiquitin proteasome system to degrade PER and CRY proteins (Camacho et al., 2001; Eide et al., 2005), allowing the BMAL1:CLOCK heterodimer to initiate E-box-mediated transcription once again. This oscillation of PER/CRY expression occurs with a period of approximately twenty-four hours and anchors cell functions to a twenty-hour cycle. How clock gene expression rhythms translate to rhythms in cell function remains unclear and is a major pursuit of current research.

1.3 Entrainment of the Suprachiasmatic Nucleus of the Hypothalamus by Light

Circadian clocks throughout the body are arranged in an anatomical hierarchy, headed by the Suprachiasmatic Nucleus of the Hypothalamus (SCN) (Honma, 2018). Though all cells exhibit oscillations in the expression of clock genes, the SCN has been identified as the "master clock" of the brain, unique in that its molecular clock can be entrained to exogenous signals, namely the light-dark cycle (Report & Weaver, 2002). The SCN also serves to relay exogenous entraining cues to other molecular clocks throughout the body using neuronal, hormonal, and behavioral signals. Animals with SCN lesions show dysfunctional circadian rhythms, often affecting their peripheral molecular clock synchronicity and behavior on a twenty-four hour scale (Moore & Eichler, 1972; Stephan & Zucker, 1972). This suggests that the rat SCN is required for synchronization of bodily rhythms to the daily light-dark cycle.

The effect of light as an entraining cue for the SCN leads to its designation as a "zeitgeber," German for "time giver." The time of light onset is called zeitgeber time 0, or

"ZT0," and represents the beginning of an individual's "subjective day." In diurnal species such as humans, the time of waking typically aligns with the beginning of the subjective day. In nocturnal species such as rats, however, the time of waking typically aligns with the beginning of the subjective night. For rats housed on a light-dark cycle with lights on for 12 hours and then off for 12 hours, ZT0 would correspond to the beginning of the light period (rest phase), and ZT12 would correspond to the beginning of the dark period (waking phase).

The retina has light receptors that directly innervate the SCN via the retinal-hypothalamic tract, and it is this pathway by which the retina informs the SCN of environment lighting information (Liu et al., 2007). Light cues function to synchronize clock gene expression in cells of the SCN which in turn are able to synchronize all of the clocks in cells throughout the rest of the body. Interestingly, SCN cells are able to maintain synchronicity in clock gene expression even in the absence of entraining cues (Ospeck et al., 2009). Contrarily, this is not true for peripheral cells, whose clock gene expression cycles desynchronize from each other if they cannot receive entraining signals from the SCN. Without the SCN, molecular clocks in cells could not synchronize their clock gene expression cycles with each other, or with environmental light cues.

1.4 SCN entrainment of Peripheral Clocks

Despite the literature describing the SCN as sufficient to synchronize circadian signaling in the body, the mechanisms by which the SCN coordinates peripheral circadian rhythms are not well-characterized. One of the proposed mechanisms by which the SCN could accomplish peripheral clock synchronicity is by controlling glucocorticoid hormone (cortisol in humans; corticosterone in rats; abbr. CORT in both) release from the adrenal cortex (Woodruff et al.,

2016). In both humans and rodents, CORT release surges at the beginning of the active phase as a result of hypothalamic-pituitary-adrenal (HPA) axis stimulation (Mohawk et al., 2007). CORT is a steroid hormone that circulates through the bloodstream, passively diffuses through cell membranes, and acts on glucocorticoid receptors (GRs) or mineralocorticoid receptors (MRs). MRs are mostly activated by aldosterone, another adrenal hormone; moreover, they are protected by an enzyme that inactivates CORT. CORT-GR complexes heterodimerize and act as transcription factors in the nucleus to regulate gene expression. All cells in the body express GR receptors except the SCN itself, which could explain how the SCN may use CORT to entrain other cells in the body without disrupting its own clock gene expression (Balsalobre et al., 2000; Rosenfeld et al., 1988).

This cycle of CORT may be a synchronizing signal used by the SCN to entrain extra-SCN cells; moreover, it could explain why stress-related disorders like depression and PTSD can disrupt circadian clocks. Stress can cause CORT levels to elevate (Hannibal & Bishop, 2014). Acute CORT due to stress may interfere with daily CORT secretion controlled by the SCN, interfering with peripheral molecular clock entrainment. This could explain why depression and PTSD are often paired with abnormal sleep cycles and other abnormal circadian rhythms. Several recent studies have attempted to explain circadian arrhythmia and the molecular and behavioral effects

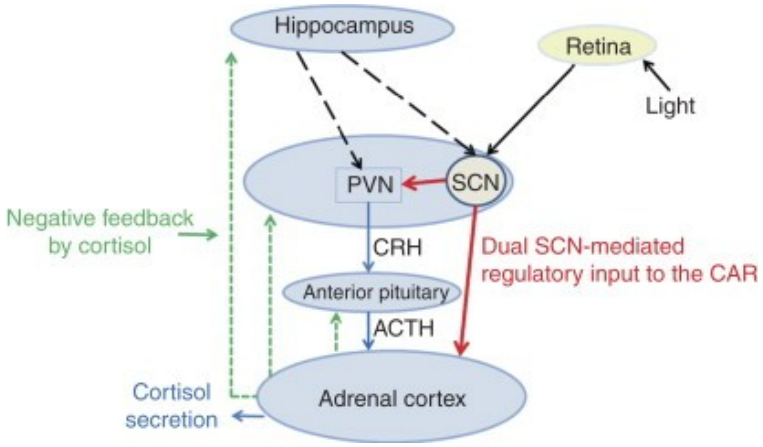

Figure 2: Light entrains the SCN via the retinal-hypothalamic tract, which then synchronizes the rest of the cells in the body's molecular clocks via cortisol release. (Clow et al. 2010)

associated with it, with CORT at the forefront of many experimental questions (Woodruff et al., 2015; Woodruff et al., 2016).

1.5 Fear Conditioning and Associated Brain Pathways

Classical conditioning is a paradigm first demonstrated by Ivan Pavlov (1849-1936). He showed that a conditioned stimulus (CS; something that would not normally provoke a behavioral response; e.g. the sound of a tone or a bell), could be paired with an unconditioned stimulus (US; something that would normally provoke a behavioral response; e.g. the presentation of food or a shock) so that eventually the CS could be presented alone and still provoke an unconditioned behavioral response (UR; e.g. salivation or freezing behavior). Initially used with dogs by ringing a bell to provoke salivation, the classical conditioning model can be implemented into methods for studying several neuropsychiatric disorders like generalized anxiety disorder (Fendt, 2001; Gewirtz et al., 1997) and post-traumatic stress disorder (PTSD) (Woodruff et al., 2015).

Classical conditioning methods (Figure 3) are a powerful tool that can be employed to study neurological and psychiatric disorders in which a strong emotional memory or event is involved. To study PTSD specifically, a version of classical conditioning called “cued fear conditioning” has been tested with rodents. In cued fear conditioning, the CS is typically a tone played directly prior to an aversive foot shock (US), to which the rodents express freezing behavior (UR). Eventually, the tone alone can elicit freezing behavior, as the animal anticipates

the shock from the tone. This model is thought to be analogous to the adverse events that can precede PTSD onset in humans, as certain conditioned stimuli can trigger episodes of panic and fear. Another version of fear conditioning called "contextual fear conditioning" implements a certain location as the CS, rather than an external stimulus (like the tone in cued fear conditioning). In rodents, fear conditioning has been shown to alter the neurocircuitry of many areas of the brain, including the hippocampus (Orsini and Maren, 2012), the basolateral nucleus of the amygdala (BLA), the basomedial nucleus of the amygdala (BMA), and the central nucleus of the amygdala (CeA) (Ehrlich et al., 2009; Tovote et al., 2016). The contextual fear conditioning model has been shown to emulate generalized anxiety disorder rather than PTSD (Luyten et al., 2011); moreover, contextual fear conditioning is hippocampus-dependent, unlike cued fear conditioning which is pre-frontal cortex (PFC)-dependent and emulates PTSD more closely.

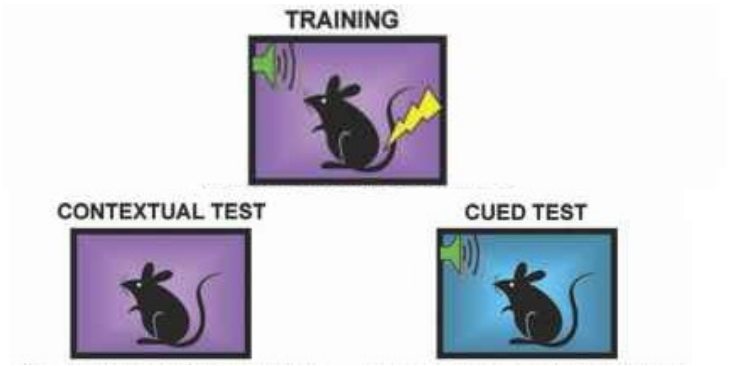

Figure 3: Basic fear conditioning models. During conditioning (training), an animal is played a tone (CS) and given a foot shock (US) to evoke freezing (UR). In a contextual test, the animal is placed in the same place it received the initial shock, associates the context with the shock, and freezes (CR). In a cued test, the animal is placed in a new context and played the tone (CS) to evoke freezing (CR). (Radiant Thinking Blog, May 2018, https://www.radiantthinking.us/memory-theory/box-4.html

1.6 Fear Extinction Learning and Associated Brain Areas

Conditioned fear is established when a person or animal exhibits a fear response (freezing in rats) in the presence of a conditioned stimuli (like the tone or bell mentioned previously), and without the presence of the unconditioned stimulus (the foot shock). This CS, however, can be disassociated from the US by a process called "fear extinction." In rodent experiments, this is

typically done by delivering the CS (the tone) repeatedly until the animal no longer predicts the US (the shock), therefore not performing the response (freezing) (Quirk & Mueller, 2007). Fear extinction protocols are performed by re-exposing the animals to the CS (e.g. tone) in a novel context in the absence of the US (e.g. shock). Upon repeated presentation of the CS, the rodent learns that the CS is not paired with the US in this context.

Whereas conditioned fear responses generalize across contexts, extinction learning does not. In other words, while an animal can learn to dissociate the US/CS pairing in one location, that same animal may fail to dissociate that connection in another location (Knapska and Maren, 2009). Typically, this is demonstrated by performing fear conditioning in "context A," extinction learning in "context B," and then performing a test to assess extinction recall in "context B."

The ventromedial prefrontal cortex (vmPFC), and specifically the infralimbic cortex (IL) of the vmPFC, has been identified as an area that plays a major role in extinction learning for auditory conditioned fear (Milad & Quirk, 2002; Peters et al., 2009). The IL projection to the BMA has been shown to modulate fear extinction and recall processes. Specifically, inhibition of the IL-BMA pathway leads to increased freezing behavior during extinction processes (Adhikari et al., 2015). Another important PFC-amygdala projection travels from the dorsomedial prefrontal cortex (dmPFC), and specifically the prelimbic cortex (PL) of the dmPFC, to the (BLA). This projection is important specifically for the expression of fear behavior in response to both unconditioned and conditioned stimuli (Milad & Quirk, 2002; Peters et al., 2009). Together, the vmPFC and dmPFC exhibit top-down control of fear memories and mediate fear extinction learning. Alternatively, the hippocampus likely regulates memory pertaining to context; therefore, the phenomenon that extinguished fear is context specific likely stems from the hippocampus (Orsini and Maren, 2012).

1.7 Fear Relapse

Fear extinction learning allows an animal to inhibit responses to the US/CS pairing; however, sometimes the CS still produces the CR following extinction. The general umbrella term for this phenomenon is "fear relapse." There are several subtypes of fear relapse that have been characterized: renewal, spontaneous recovery, reinstatement, and reacquisition (for review, see Goode & Maren, 2014). Components of the experiments described below examine fear renewal and spontaneous recovery, as other characterized versions of fear relapse are beyond the scope of this manuscript.

Fear renewal is the explicit circumstance in which an animal, following extinction, is presented the CS outside of the context of extinction, and the CR ensues (Maren et al., 2013; Maren et al., 2014). In a rodent's case, this renewal of fear is strongest when the animal is given the CS in the original context (in which fear conditioning occurred); however, a rodent will still exhibit fear when presented the CS in an entirely novel environment (Maren, 2014).

Spontaneous recovery, however, occurs when the CS can produce the previously extinguished CR from the passage of time alone (Bouton, 1993). This implies that after a given period, retrieval of the fear memory is more prominent than retrieval of the extinction training. Because spontaneous recovery occurs in the presence of the CS in any context after a period of time, it can often overlap with fear renewal phenomena (Bouton, 2002; 2004). The spontaneous recovery phenomenon is difficult for researchers to understand, especially in designing exposure therapy protocols, as it is unclear why a distant memory is recalled more easily than a recent one.

1.8 Relevance of Fear Conditioning, Extinction, Recall, and Relapse in Stressor-Related and Anxiety Disorder Treatments

Post-Traumatic Stress Disorder (PTSD) is a disorder arising from the experience of extreme trauma, such as that occurring with military combat, a near death experience, or domestic or sexual abuse (Basille et al., 2004; Hoge et al., 2004; Lowe et al., 2014). Despite the variety of events that could precipitate PTSD, symptoms often include acute episodes of panic or stress that are triggered by stimuli related to the traumatic event (Bryant et al., 2013). Other symptoms can include insomnia, heightened partner conflict, and increased risk to other health problems like cardiovascular diseases (Boscarino 2008; Cohen et al., 2009). PTSD is extremely prevalent in the United States, affecting 7.3% of the population for life, 74% of those affected being women (Roberts et al., 2011).

One of the most efficacious treatments for anxiety disorders is exposure therapy. Exposure therapy is essentially a learning experience through which patients become desensitized to fear-inducing stimuli through repeated exposure to those stimuli. Experiments have utilized rodent fear conditioning as a model for trauma, as it is relatively benign, induces behavior associated with fear (freezing), and is repeatable (Woodruff et al., 2015). There are obvious issues in using this parallel. For instance, multiple types of events can induce PTSD in humans; however, research generally only uses tone-shock pairing to emulate traumatic events. Also, PTSD in humans can have late (months-years) onset following a traumatic event, whereas rats are typically given far less time to consolidate fear memories (Bryant et al., 2013).

Fear extinction learning is similar to exposure therapy (Craske et al., 2008), and because parallels have been drawn between rat and human brain activity during extinction, its optimization could lead to more effective treatments for those with PTSD (Milad and Quirk,

2012; Milad et al., 2006b). Exposure therapy has been shown to be effective in some humans, especially in combination with some medications (Foa et al., 2002), but relapse of fear responses often occurs following exposure learning (Craske et al., 2014). For this reason, identifying strategies to improve the retention of exposure memory may improve mental health outcomes after exposure therapy. Unfortunately, fear memories seem to generalize across contexts, while extinction memories do not (Maren, 2014). Optimization of these processes could be essential in treating PTSD.

1.9 Research Questions

The current manuscript is particularly concerned with rodent behavior in fear conditioning, fear extinction learning, fear extinction recall, and fear relapse, and their relationships to molecular clocks in the PFC. It has been demonstrated that fear extinction recall behavior has some connection to the circadian system. Studies (Woodruff et al., 2015, 2018) have shown that fear extinction recall is stronger when a rat is trained and tested during its active phase (ZT16). However, previous experiments were not sufficiently controlled to determine whether the time-of-day difference observed in fear extinction recall depends on the time of extinction training (learning) or the time of extinction testing (recall). Secondly, these past experiments did not examine whether this time-of-day differences operates on a circadian cycle or merely changes with the rat's active or inactive phase, as it only tested two points times (active versus inactive phase).

Previous experiments observed only male rats, and no experiments have examined whether this time-of-day difference is exhibited by female rats. Another open question regards whether the time-of-day difference in fear extinction recall fluctuates on a circadian basis--that

is, when entraining light cues are removed. Experiment 1 examines whether training time or testing time drives behavioral differences during fear extinction recall and whether this pattern is a circadian rhythm. Experiment 2 examines if there are time-of-day differences in fear relapse, and if these potential differences are dependent on training time or testing time. Experiment 3 examines whether behavioral rhythms in fear extinction recall are maintained in constant darkness, and whether fear extinction recall and fear relapse show consistent behavioral patterns between males and female rats. Together, these experiments seek to determine how fear-related behavior may be modulated by the circadian system.

We hypothesize that the time-of-day differences in fear extinction recall are due to the time of extinction training. Should this prove to be correct, animals trained and tested at ZT4 will freeze significantly more than animals trained at ZT16 during fear extinction recall testing. Secondly, because molecular clocks in the PFC are likely driving extinction training (Woodruff et al., 2018), this pattern in freezing behavior is likely a circadian rhythm. This means that animals trained at ZT10 and ZT22 will freeze at a level between the ZT4 and ZT16 rats, and that this rhythm should not be abolished if animals are housed in constant darkness.

Very little research has been conducted regarding circadian patterns related to fear relapse. For reasons similar to those mentioned in the previous paragraph, we hypothesize that animals trained at ZT4 will freeze significantly more than animals trained at ZT16 (Chun et al., 2015). Lastly, while male and female rats show comparable clock gene expression cycles in the PFC, female rats are known to freeze less than male rats (Gruene et al., 2015). Therefore, we expect that female rats will freeze less than their male counterparts, but the overall behavioral trends will remain consistent.

2. METHODS

2.1 Animals

All experiments used male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) housed two per cage (polycarbonate tubs, 47cm×23cm×20cm). Experiment 3 also used female rats (Harlan Laboratories, Indianapolis, IN) housed two per cage. All animals were provided food (Teklad Rodent Diet 8640; Harlan) and water *ad libitum*. In experiments 1 and 2, animals were housed on a 12:12hr light:dark cycle (LD). In experiment 3, male and female rats were housed in identical, separate suites, but both suites were maintained in constant darkness (DD) starting 36 or 48 hours (depending on ZT group) before fear conditioning. All behavioral tests occurred at zeitgeber time (ZT; hours after light phase onset) 4+/-60 minutes, 10+/-60 minutes, 16+/-60 minutes, or 22+/-60 minutes. For all experiments, animals were moved in red light from their suites into double-bagged black plastic bags (Hefty 55g bags; Reynolds Consumer Products, Inc.; Lake Forest, IL) before being transported to testing rooms. All behavioral tests were conducted in dim red light, and animals were returned to their respective suites in double-bagged black plastic bags following each test. All procedures were conducted in accordance with the ethical treatment of animals and were approved by the University of Colorado Institutional Animal Care and Use Committee.

2.2 Testing Apparatuses

All three experiments were conducted in contexts A and B. Context A was a rectangular chamber (29.2cm x 21cm x 25.4cm; Med Associates Inc.; EMV-008; St. Albans, VT) made up of two stainless steel walls and two plexiglass walls, with a stainless steel floor made up of rods spaced 2cm apart. This shock grid flooring in Context A was attached to a current generator

(Med Associates Inc.; ENV-414S; St. Albans, VT). Thirty-second, 1kHz, 70dB tones were produced through speakers (Med Associates Inc.; ENV-025F; St. Albans, VT) placed on top of the boxes and co-terminated with a two-second, 0.8mA foot shock. Context A was cleaned with 70% ethanol and allowed to dry following each group. Context B was a rectangular chamber (42.5cm x 42.5cm x 62.2cm; Med Associates Inc., PHY-102P; St. Albans, VT) made up entirely of plexiglass. Tones identical to those played in context A were produced from speakers (Med Associates Inc.; ENV-025F; St. Albans, VT) on the tops of the boxes. Each ceiling in context B was wiped with liquid peppermint extract (Kroeger; Cincinnati, OH) and 2mL peppermint extract was placed in an open plastic container next to small air holes in each box. Context B was cleaned with lemon-scented sanitizing wipes and allowed to dry following each group.

2.3 Fear Conditioning

All three experiments began with identical conditioned fear acquisition protocols. Following transportation, rats were placed into context A and allowed 5min of baseline exposure. A 30s tone (CS) sounded, the final 2s of which the animals were shocked (US). A 2min inter-trial interval (ITI) followed. An identical 30s tone and 2s shock occurred, and the session finished with 2min post-session exposure. An experimenter in the room live-scored freezing behavior (UR) of each rat every 10s throughout each session. Freezing behavior was defined as cessation of all movement but respiration for a 1s when the experimenter assessed behavior. Rats often "scan" when expressing freezing behavior by slowly moving their heads while maintaining an otherwise rigid posture; this behavior was scored as freezing behavior. Rats were then returned to their cages and transported to their respective housing suites.

2.4 Fear Extinction

Conditioned fear is established in a single session, and memory for conditioned fear can be tested in a following session. 24h after conditioned fear acquisition, rats were transported and placed in context B boxes. Rats were allowed 3min of baseline exposure, which preceded a series of 15 30s tones (identical to those presented during fear conditioning), separated by randomized ITIs between 90-120s. Following the final tone, freezing was scored for 2min more. Rats were then returned to their cages and transported back to their suites. All freezing behavior was scored by an experimenter in the room, noting behavior of each rat for about 1s every 10s for the entirety of each session.

2.5 Fear Extinction Recall

In experiments 1 and 3, additional extinction training sessions (fear extinction recall sessions) began exactly 24 hours following the respective initial extinction trainings and repeated every 6h a total of 5 times for each group of animals. In these additional trainings, animals were placed in Context B and allowed 3min of baseline exposure, followed by 2 30s tones (separated by a 120sec ITI), and finishing with 2min post-session exposure.

2.6 Fear Relapse

Fear relapse sessions in experiments 1 and 3 occurred 72hrs after the final extinction recall training for each group (at the same respective ZT at which groups received fear conditioning and extinction training). In experiment 2, rats began fear relapse sessions either 24, 36, or 48hrs following their respective extinction trainings. For each relapse session, rats were placed back into context A and allowed 3min of baseline exposure. Following the pre-exposure,

a 30sec tone was played, the last two seconds of which a foot shock was delivered. A 2min ITI followed, and then another 30sec tone and 2sec foot shock. In experiment 1, relapse sessions only contained two tones. In experiments 2 and 3, there were 15 total tones. A 2min exposure session followed the trials, and animals were then transported back to their respective cages and suites.

2.7 Statistical Analysis

The Statistical Package for Social Sciences (SPSS, v. 25.0, 2017) was utilized to conduct mixed analysis of variance (ANOVA) on each data set. A two-way ANOVA was used in experiments 1 and 2. For these experiments, the within group factor was the trial (tone) of each session and the between group factor was the time of training or testing for fear conditioning, fear extinction, fear extinction recall (for experiment 1), or fear renewal (for experiment 2). For experiment 3, a three-way ANOVA was employed using the same group factors described for experiments 1 and 2, and sex was also included as a third between group factor. In the instances of significant ANOVA main effects or interactions, post hoc tests (Fisher's Least Significant Difference), was used to determine variance between individual groups, with $p<0.05$ marking significance.

2.8 Experiment 1: Circadian Patterns in Fear Extinction Recall

Male rats (n=6, N=24) were acclimated for two weeks to a 12h:12h light:dark cycle, with lights on at ZT0. Animals were then fear conditioned at ZT4, ZT10, ZT16, or ZT22 and extinction trained 24hrs later. Animals were then tested for extinction recall at five consecutive time points (separated by 6hr intervals), starting 24hrs after their respective extinction trainings (ex. ZT4 rats received conditioning at ZT4, extinction 24hrs later at ZT4, and 5 recall sessions

beginning 24hrs later at ZT4, with other recall sessions at ZT10, ZT16, ZT22, and the following ZT4). These experimental parameters were chosen to determine if time-of-day differences in freezing behavior during extinction recall depend on the time of extinction training or the time of extinction testing, and also whether freezing behavior appears to cycle in a smooth fashion over the course of the day.

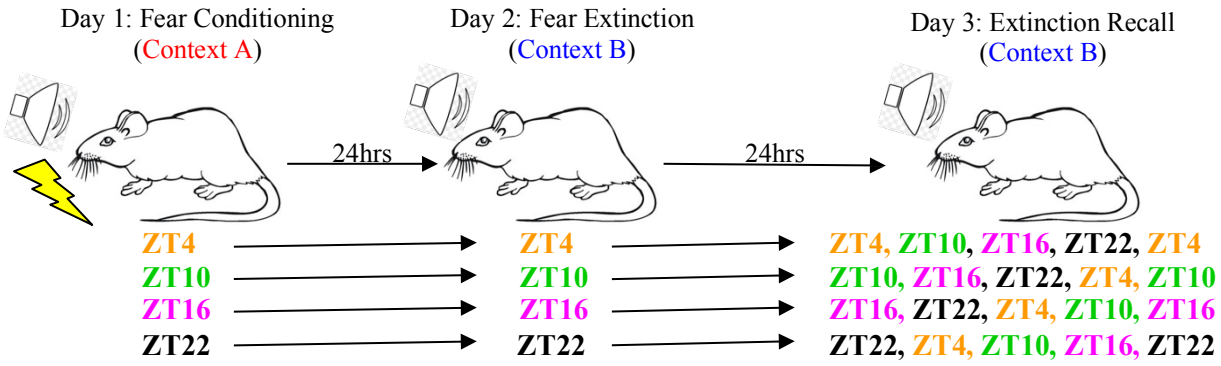

Figure 4: Experiment 1. Groups were fear conditioned at four different times (ZT4, ZT10, ZT16, and ZT22) and underwent fear extinction training exactly 24hrs following their respective conditioning sessions. 24hrs later, groups underwent the first of five fear extinction recall training sessions, separated 6hrs apart. Freezing behavior was measured for all sessions.

Following all necessary data collection for Experiment 1, the animals in this study underwent a pilot fear renewal process exactly 72hrs after recall tests terminated, detailed in sections 2.7 and 2.9.

2.9 Experiment 2: Circadian Patterns in Fear Renewal

The purpose of this experiment was to determine if any time-of-day differences could be observed during fear renewal. This experiment followed up on a pilot study conducted following experiment 1. In experiment 1, rats tested for fear relapse at ZT16 tended to freeze less than rats tested at ZT4, ZT10, or ZT22, but it was unclear if this time-of-day difference was dependent on the time of fear extinction training or the time of fear relapse testing.

This study was designed to control for both the time of extinction training and the time of fear relapse testing (Figure 5). Six groups of animals (n=6, N=36) were given conditioning and extinction (24hrs apart, as in experiment 1), either at ZT4 (three groups) or ZT16 (three groups). Each group then underwent fear renewal at either ZT4 or ZT16. To control for varying lapses in time between extinction and renewal, groups with specific intervals between extinction training and relapse testing were created, such that rats were fear relapse tested either 24hrs, 36hrs, or 48hrs after extinction training. All groups only received one fear renewal session following extinction, and no fear extinction recall tests occurred in this experiment.

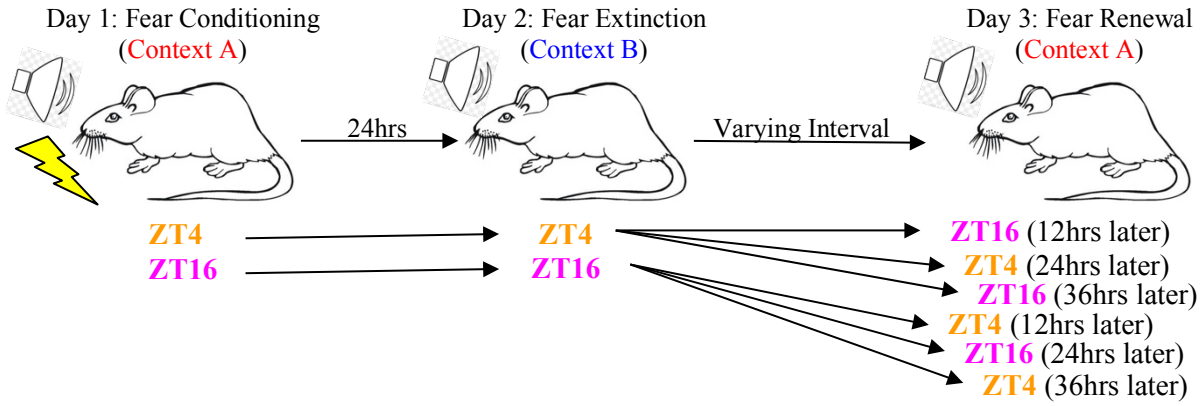

Figure 5: Experiment 2. Rats are fear conditioned and undergo extinction 24hrs apart (like in experiment 1). However, each group then undergoes fear renewal either 12, 24, or 36hrs following their respective fear extinction trainings. Freezing is measured throughout.

2.10 Experiment 3: Sex Differences in Circadian Patterns in Fear Extinction Recall

The purpose of this experiment was two-fold: 1) to compare males and females in their freezing responses to fear extinction recall, and 2) to determine if the time-of-day difference in extinction recall would persist in constant darkness. Experiment 3 (Figure 6) followed identical protocols to experiment 1, except both male and female rats were tested (n=6, N=24; 12 males,

12 females), and groups were only given fear conditioning/extinction at either ZT4 or ZT16 (with the same 24hr period between conditioning and extinction). Furthermore, animals were kept in constant darkness (DD) starting 36-48hrs before fear conditioning. Animals were then tested for recall in the same manner as in experiment 1 (five total recall sessions beginning 24hrs after extinction training). Starting 12-24hrs after fear relapse, rats were again maintained on a 12h:12h light:dark cycle. Fear relapse testing then occurred 72hrs after the final extinction recall testing session for each group. In this experiment, fear relapse consisted of 6 tone presentations for females or 15 tone presentations for males in context A. Females received only 6 tone presentations due to their exhibiting floor levels of freezing by tones 4-6.

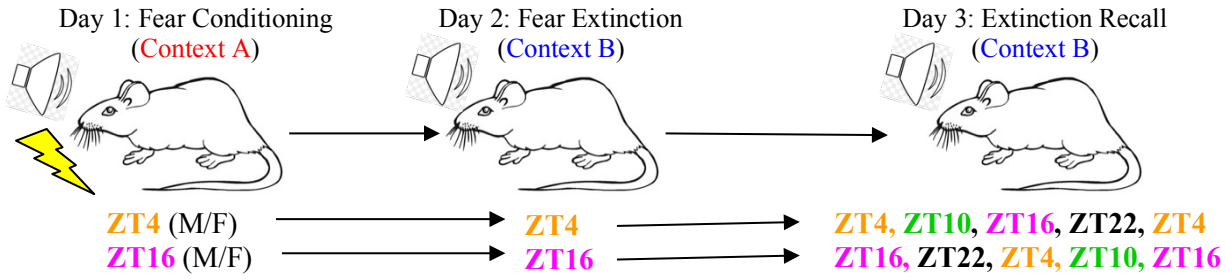

Figure 6: Experiment 3. This experiment follows the exact protocol as experiment 1, but no animals were fear conditioned or underwent extinction training at ZT10 or ZT22. Both male and female rats were used, and the animals were on a constant darkness (DD) cycle.

3. RESULTS

3.1 Experiment 1: Fear extinction recall is enhanced during rat's active phase (ZT16)

Session 1: Conditioned Fear Acquisition

No time-of-day differences were observed in conditioned fear acquisition (Figure 6). Animals trained at all times (ZT4, ZT10, ZT16, ZT22) showed no freezing behavior before the tones or shocks, and all animals showed significantly elevated freezing after delivery of the first shock ($F_{1,22}$=836.359, p<0.001).

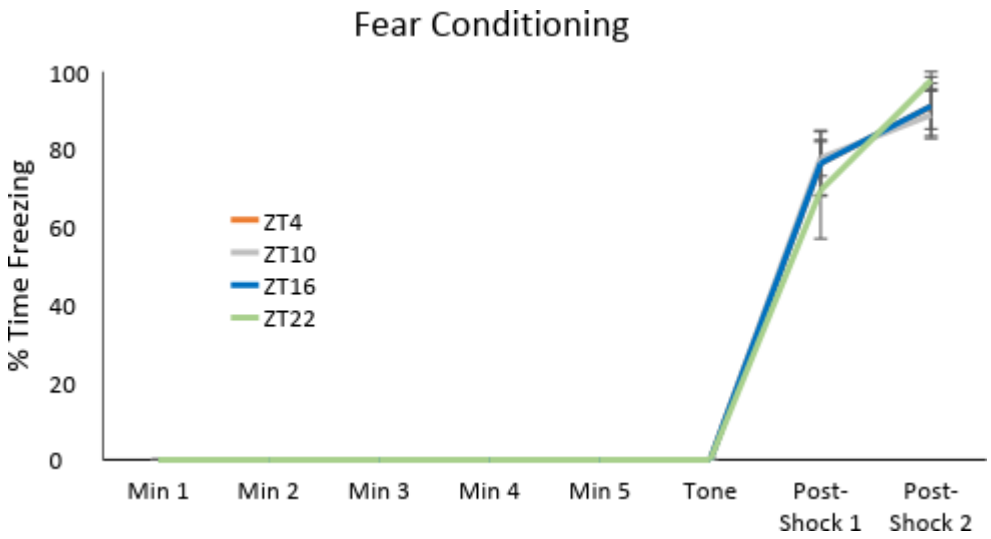

Figure 6: Rats were trained to fear a tone at multiple times (ZT4, ZT10, ZT16, ZT22) throughout the day. There was no time of day effect on freezing behavior resulting from training time. Freezing was significantly elevated following shock delivery.

Session 2: Conditioned Fear Expression and Extinction Training

Animals trained at each time of the day showed similar conditioned fear expression during tones 1-3, demonstrating that they exhibited similar 24hr recall for conditioned fear (Figure 7). No time-of-day effects were observed in extinction training (Figure 7). All rats displayed similar patterns of extinction learning, with ANOVA revealing no differences between groups. Further, all rats extinguished conditioned fear to the same extent by tones 13-15, regardless of time of day.

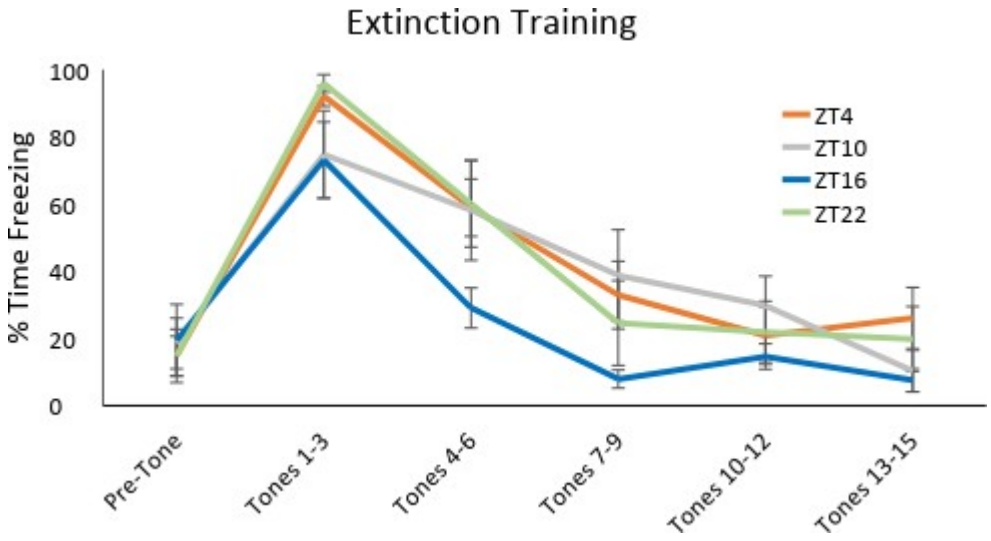

Figure 8: Rats received extinction training 24hrs after their respective conditioning sessions. All rats responded similarly during the fear extinction sessions.

Session 3: Extinction Recall Testing

There was a significant main effect of testing time ($F_{3,22}$=9.212, p<0.001) (Figure 9). Posthoc tests revealed that rats trained at ZT4 froze significantly more at ZT4 than at ZT10 (p<0.001), ZT16 (p<0.001) and ZT22 (p<0.001). Rats trained at ZT22 froze significantly more at ZT4 than at ZT16 (p<0.001) and ZT22 (p<0.05). An effect was also seen in which animals froze less at the repeated time point (e.g. ZT4 rats froze more at the first ZT4 testing time than the second), indicating a modest but significant effect of additional extinction learning that occurred through repeated recall testing.

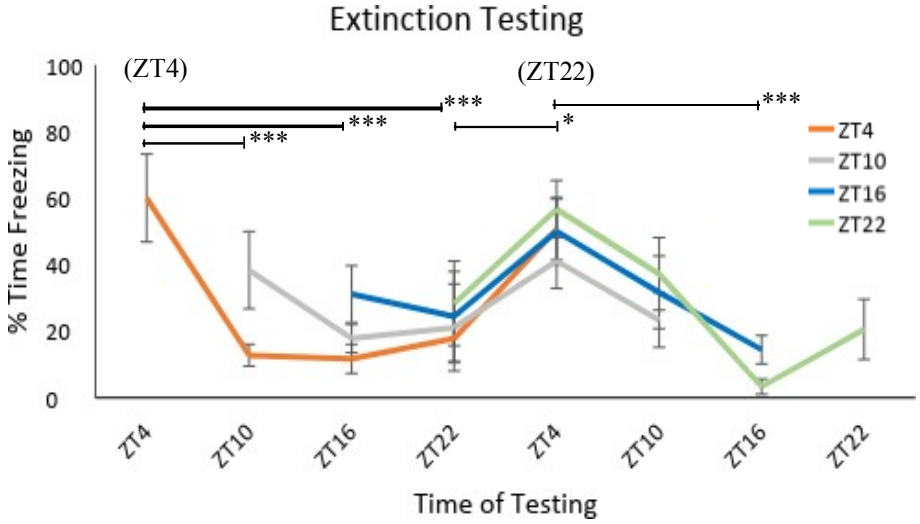

Figure 9: During extinction recall sessions, rats froze less during the early active phase (ZT16) and most at the early inactive phase (ZT4). Animals also froze less across the sessions, as evidenced by each group's repeated time points. (* denotes p<0.05, *** denotes p<0.001).

Session 4: Relapse of Fear

No significant effects were found between groups (Figure 7); however, trending data showed a trial x time interaction that ZT16 rats displayed less freezing behavior after the second tone ($F_{3,22}$=2.145, p=0.089). Though not significant, these data prompted subsequent relapse experiments.

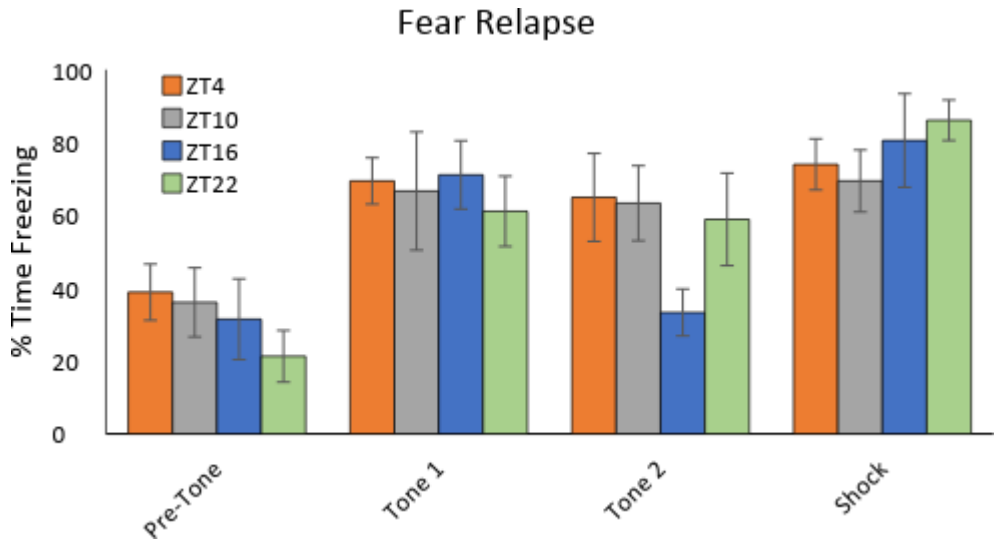

Figure 7: Session 4 fear relapse data. Though not significant, ZT16 rats tended to freeze less after the second tone than all other rats.

3.2 Experiment 2: Fear relapse shows no time-of-day difference 24-48 hours after extinction training

Groups depicted in graphs are depicted by the time of training (ZT4 or ZT16), as well as the time interval (in hours) between extinction training and fear relapse (24, 36, or 48). For example, "ZT4-24" represents the group trained at ZT4 and tested for fear relapse 24hrs following extinction training.

Session 1: Conditioned Fear Acquisition

No time-of-day differences were observed in conditioned fear acquisition (Figure 10). Animals trained at ZT4 and ZT16 showed no freezing behavior before the tones or shocks, and all animals showed significantly elevated levels of freezing upon shock delivery ($F_{14, 21}$=0.618, p=0.009).

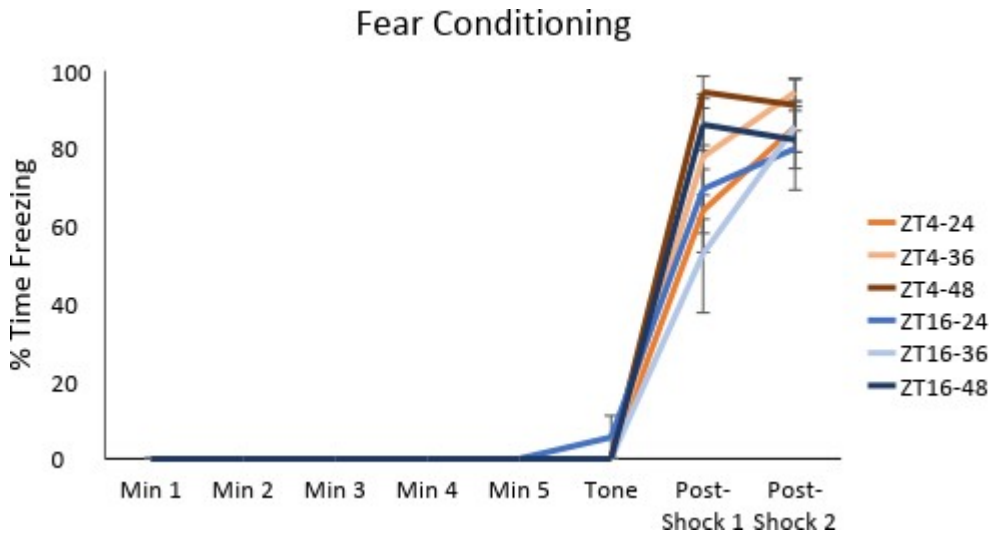

Figure 10: Groups undergoing conditioned fear acquisition showed similar freezing behavior, regardless of the time of day they received the training.

Session 2: Conditioned Fear Expression and Extinction Training

No time-of-day differences were observed in extinction training (Figure 11). Animals trained and tested at all times of the day showed similar conditioned fear expression during the first trial block (tones 1-3), demonstrating that each group exhibited similar 24hr recall to conditioned fear. They also displayed comparable decreases in freezing with repeated tone presentation, indicating that rats extinguished conditioned fear to the same extent, regardless of time of day.

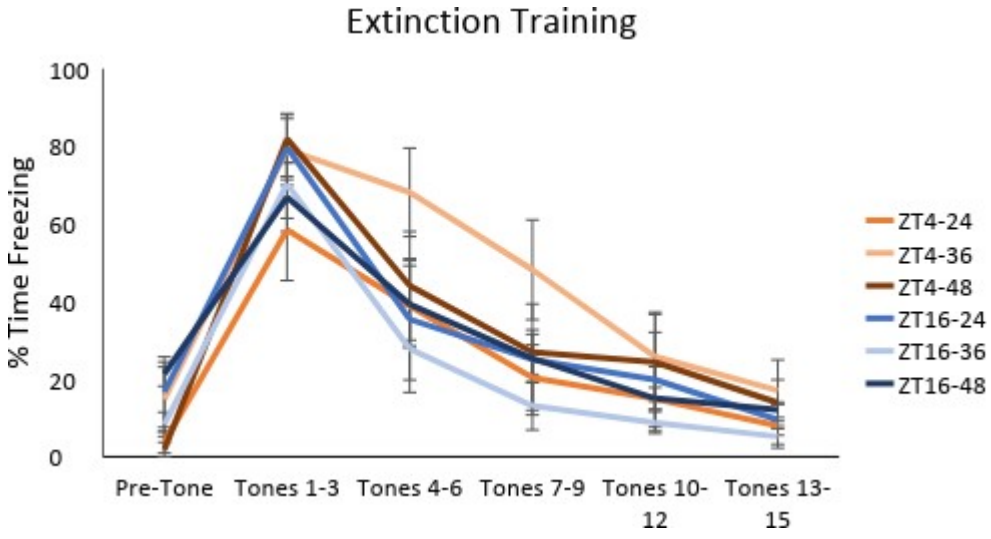

Figure 11: Groups that underwent conditioned fear extinction showed similar freezing patterns regardless of time of day.

Session 3: Relapse of Fear

No significant effect between groups was found (Figure 12); however, posthoc tests revealed a trending interaction between the ZT4-48 animals and some other groups during tones 1-3. ZT4-48 rats tended to freeze more than some other groups ($p=0.090$ vs. ZT4-24, $p=0.052$ vs. ZT16-24, $p<0.05$ vs. ZT16-36).

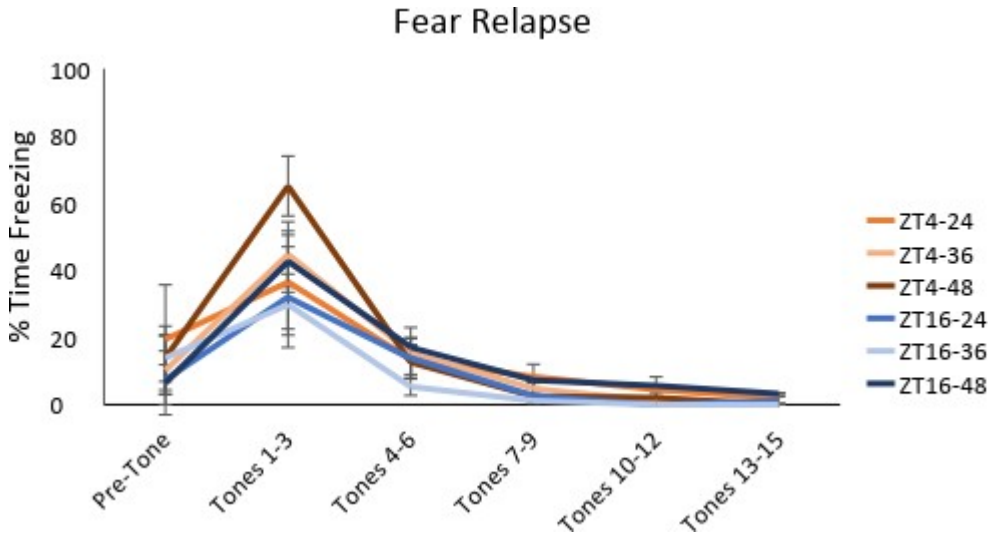

Figure 12: Groups had different ITIs between fear extinction learning and fear relapse. No significant effects or interactions were found but ZT4-48 animals tended to freeze more during tones 1-3.

3.3 Experiment 3: ZT4-F Rats Acquired Conditioned Fear at a Different Rate than Other Groups, Female Rats Freeze Less than Males Generally, ZT4-M Rats Froze More during Fear Relapse

Session 1: Conditioned Fear Acquisition

There was a significant interaction between sex, time of training, and within-session trial ($F_{7,21}$=11.495, $p<0.001$). Female rats trained and tested at ZT4 (ZT4-F) froze less than all other groups ($p<0.001$) after the first tone-shock pairing. After the second tone-shock pairing, ZT4-F also tended to freeze less than ZT4-M ($p=0.063$) and ZT16-F ($p=0.089$), and froze significantly less than ZT16-M ($p<0.05$).

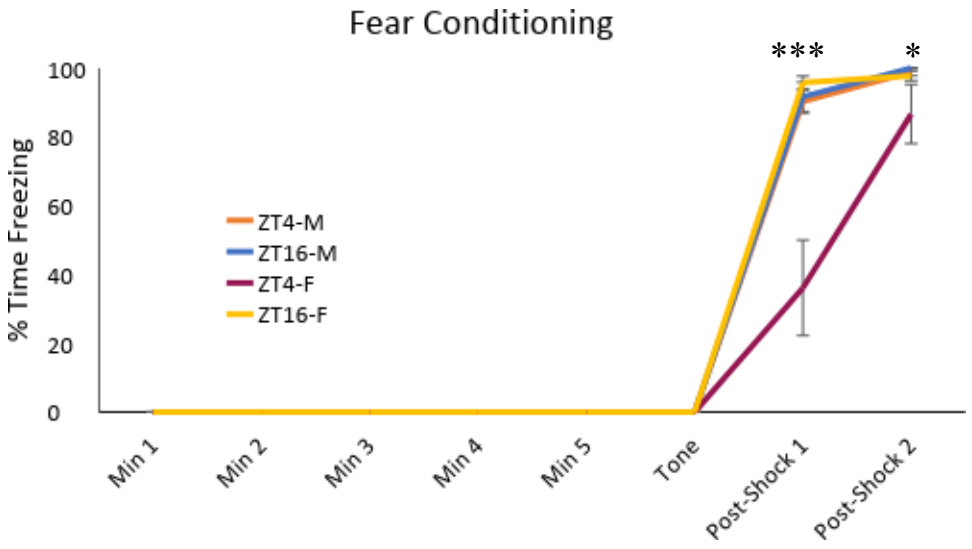

Figure 13: Male and female rates underwent conditioned fear acquisition at either ZT4 or ZT16. Females trained at ZT4 froze significantly less than all other groups after both shocks.

Session 2: Conditioned Fear Expression and Extinction Training

There was a significant interaction between within-session trial block and sex ($F_{4,21}$=3.218, $p<0.05$), such that females froze significantly less than males for the first three trial blocks (tones 1-9; Figure 14). Posthoc tests revealed that, before the first tone, ZT16-M froze significantly more than ZT4-M ($p<0.05$), ZT4-F ($p<0.001$), and ZT16-F ($p<0.05$). During tones 1-3, ZT16-F froze significantly less than ZT4-M ($p<0.01$) and ZT16-M ($p<0.05$), while ZT4-F tended to freeze less than ZT4-M ($p=0.052$) and ZT16-M ($p=0.068$). During tones 4-6, ZT16-F froze significantly less than ZT4-M ($p<0.05$) and ZT16-M ($p<0.05$). During tones 7-9, ZT4-F froze significantly less than ZT4-M ($p<0.05$) and ZT16-M ($p<0.01$) while ZT16-F froze significantly less than ZT16-M ($p<0.05$) and tended to freeze less than ZT4-M ($p=0.061$).

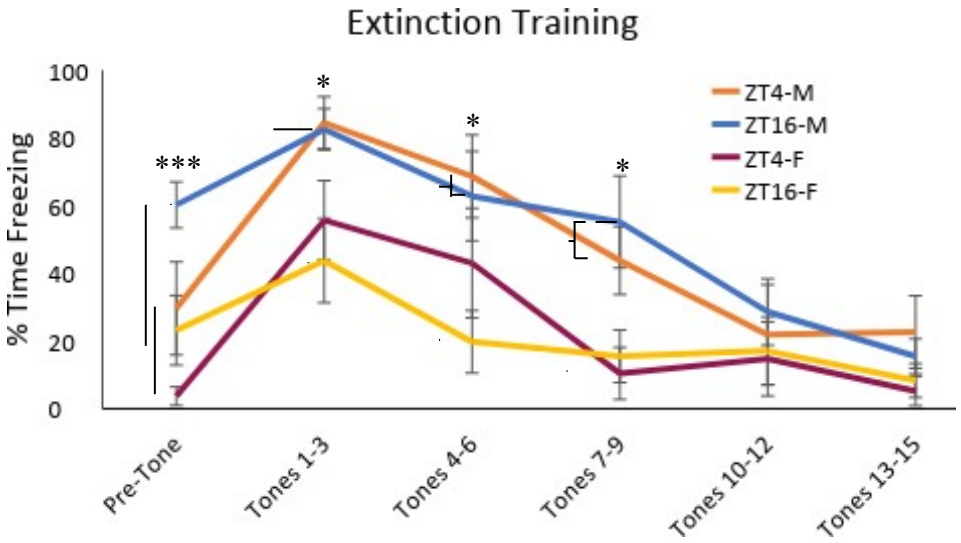

Figure 14: Sex differences in rats undergoing fear extinction learning. ZT16-M rats froze more than all other groups before tones began, whilst female groups (ZT4-F, ZT16-F) froze less than male groups (ZT4-M, ZT16-M) following tones 1-9. (*** indicates $p<0.001$, * indicates $p<0.05$).

Session 3: Fear Extinction Recall Testing

There was a significant main effect of testing time ($F_{3,22}$=7.162, p<0.001). These data show that rats, despite training at different times, showed significant differences in time of testing during fear extinction recall.

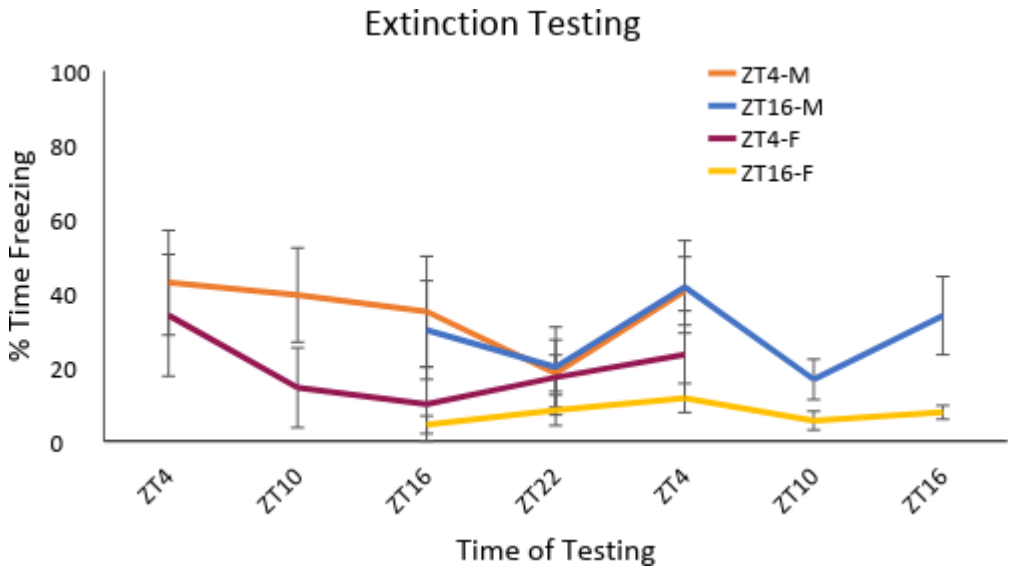

Figure 15: Fear extinction recall testing in male and female rats who underwent conditioned fear acquisition and extinction learning at either ZT4 or ZT16. A main effect of testing time was observed, but no between group effects or interactions were seen.

Session 4: Relapse of Fear

There was a significant interaction between time of training and sex ($F_{1,21}$=4.515, p<0.05), such that ZT4-Males froze significantly more than all other groups from the beginning of the session until tone 6 (Figure 16). Before tones began, ZT4-M froze more than ZT16-M (p=0.004), ZT4-F (p<0.001) and ZT16-F (p<0.001). From tones 1-3, ZT4-M froze more than ZT4-F (p<0.05) and ZT16-F (p<0.01). From tones 4-6, ZT4-M froze more than ZT16-M (p<0.01), ZT4-F (p<0.01), and ZT16-F (p<0.01). Due to floor levels of freezing, testing was terminated for ZT4-F and ZT16-F groups after tone 6.

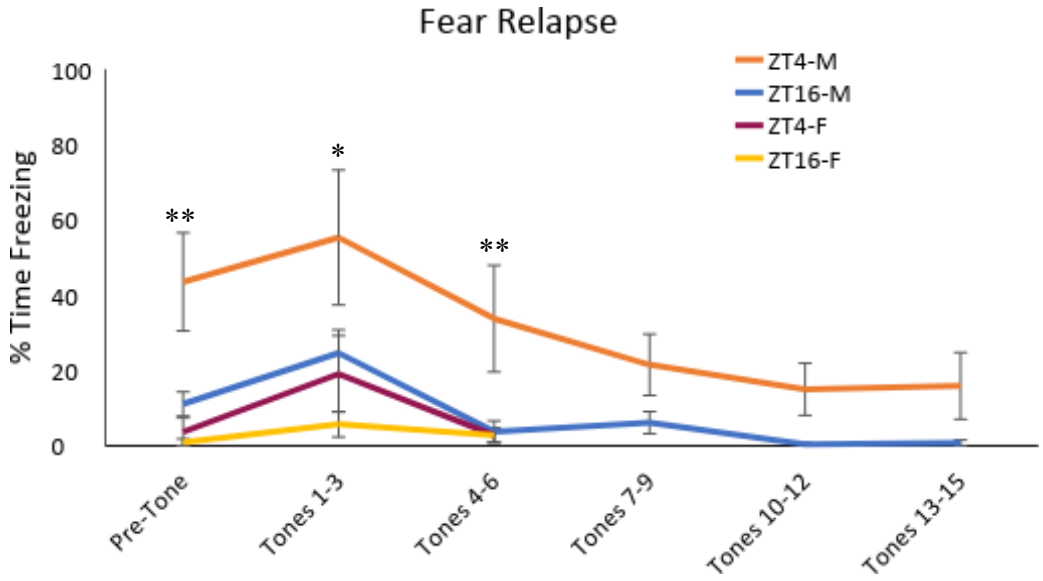

Figure 16: Sex and training time differences in rat fear relapse. ZT4-M rats froze more than all other groups from the beginning of the session until tone 6. (** indicates p<0.01, * indicates p<0.05).

4. DISCUSSION

4.1 Overview of Findings

Our experiments revealed a time-of-day difference in fear extinction recall. This time-of-day difference depended on the time animals were tested for extinction recall, not when animals underwent extinction training. Animals froze most in the inactive period (ZT4) and froze least in the active period (ZT16). Furthermore, a time-of-day effect was maintained in animals housed in DD, suggesting that the rhythm remains in DD conditions.

A trending rhythm was seen in experiment 1 in which animals that underwent conditioning and extinction at ZT16 tended to freeze less than other animals during fear relapse. This rhythm was seen in experiment 3 with ZT16 males (and both female groups) freezing less than ZT4 males.

4.1 Fear Conditioning

Experiments 1 and 2 did not find any time of day differences in fear conditioning (Figures 6 and 10). Animals did not freeze upon presentation of the CS, only following foot shock. Upon delivery of the second foot shock, all animals in experiments 1 and 2 froze almost completely. These data are consistent with our lab's recent data (Woodruff et al., 2015) as well as several other studies (Hopkins and Bucci, 2010; Valentinuzzi et al., 2000) that did not show a time of day difference in conditioned fear acquisition. In one mouse study (Chaudhury et al., 2002), researchers found a time of day difference in conditioned fear acquisition, a finding that was not observed in the present experiments.

In experiment 3, however, female rats undergoing conditioned fear at ZT4 froze much less than the other animals (Figure 13). This effect was an interaction between sex and time of

day, suggesting that freezing behavior is reduced during conditioned fear acquisition in females at time ZT4. It has been shown that female rats tend to freeze less than their male counterparts but females in the current experiments spent time not freezing exploring the boxes rather than displaying active avoidance behaviors, as shown in other studies (Gruene et al., 2015). Other data has shown time-of-day differences in conditioned fear acquisition (Chaudhury et al., 2002) but in male mice. Further testing is required to better understand this phenomenon in female rats. Nevertheless, all animals in experiment 3 exhibited conditioned fear to the same extent, especially after the second tone-shock pairing, which implies that conditioned fear can be obtained even in constant darkness.

A fundamental difference between fear conditioning in this study and our lab's previous studies (Woodruff et al., 2015) is that the present experiments employed two tone/shock pairings, whereas the previous studies only used a single pairing. Woodruff et al., (2015) consistently found that a single pairing could produce almost complete freezing responses. The present experiments did not observe the same amounts of freezing; therefore, a second tone/shock pairing was incorporated into the conditioned fear acquisition protocol. Due to the variability of freezing behavior (in both individual and cohorts of rats) in response to a single shock, the second shock was added to ensure strongly conditioned fear prior to extinction training. It is unclear whether the effects of this extra tone/shock pairing influenced the outcomes of the fear extinction, fear extinction recall or fear relapse sessions.

4.2 Fear Extinction Learning

All animals showed the ability to learn fear extinction regardless of what time of day they underwent training (Figures 8, 11, 14). These data are consistent with our lab's previous studies

(Woodruff et al., 2015; Woodruff et al., 2018). A sex difference was seen in experiment 3 (Figure 14) in which female rats froze less than male rats in the first part of fear extinction training. This could be because female rats typically freeze less than male rats (Gruene et al., 2015), but previous reports conflict with our observation that female rats exhibit exploratory behavior rather than active fear behavior during when not freezing. It is also important to note that all animals were able to learn fear extinction, regardless of the LD or DD light cycles they were housed in. These data suggest that fear extinction learning is not reliant on a normal LD cycle and therefore may be generated endogenously.

Another question remains: does fear extinction learning depend in some way on the time of prior fear conditioning? All of the present experiments, as well as our lab's recent experiments, have all separated conditioned fear acquisition from fear extinction learning by 24hrs. Further testing should be conducted to determine if the time of conditioned fear affects fear extinction learning, and if the time between conditioned fear acquisition and fear extinction affects fear extinction, recall, or fear relapse.

4.3 Fear Extinction Recall

Experiment 1 showed that rats froze least at ZT16 and most at ZT4. These results are consistent with previous data (Woodruff et al., 2015, 2018), and expand on it, as previous studies did not determine whether the time of extinction training or the time of extinction testing determined the strength of extinction recall. After experiment 1, it was determined that the time of an animal's fear extinction training does not influence the freezing of an animal during fear extinction testing. The time of the animal's fear extinction testing, however, does determine the relative amount of freezing (Figure 9). Furthermore, time-of-day effects were also seen in fear

extinction recall testing in DD conditions (Figure 15), suggesting that synchronization of PFC clocks was maintained and allowed this rhythm to persist in the absence of light entrainment cues. Fear extinction recall behavior appears to be regulated by the circadian system, suggesting that the molecular clock may regulate molecular mechanisms associated with fear extinction memory retrieval.

The IL-BMA pathway is thought to modulate fear extinction memory (Adhikari et al., 2015). Because there is a behavioral pattern in fear extinction recall testing that oscillates with the time-of-day, the molecular clock is likely required for this rhythm to occur. Without synchronized molecular clocks in the PFC, freezing rhythms in fear extinction recall testing cease, and average freezing behavior increases (Woodruff et al., 2018). Not only is an intact molecular clock in an individual PFC cell necessary for optimal fear extinction recall testing, it is likely that it must also be synchronized with other clocks around it.

Our previous work suggests that CORT may be an entrainment factor controlling this circadian rhythm in freezing behavior. CORT is released in a circadian fashion and is a steroid hormone, which allows it to cross the blood-brain barrier (Dallman et al., 1987). Also, the SCN has no GR receptors, suggesting that its entrainment by light would not be affected by CORT. Experiments have shown that adrenalectomized (ADX; removing an animal's source of CORT) rats show disruptions in PFC clock gene expression (Woodruff et al., 2016) Additionally, ADX animals injected with CORT in-phase to their normal CORT rhythm reestablished PFC clock gene expression rhythms, whereas ADX animals injected with antiphasic CORT did not regain rhythmicity (Woodruff et al., 2016). These data further suggest that CORT may be used by the SCN to entrain molecular clocks in the PFC.

Acute CORT is known to have modulatory effects on conditioned fear acquisition processes, though it is unclear if CORT has differing behavioral effects at ZT16 or ZT4. Previous research has shown that adrenalectomized rats do not show time of day differences in fear extinction recall; however, acute CORT following extinction testing restores the time of day difference in freezing behavior (Woodruff et al., 2016). Secondly, rats with a local molecular clock disruption (PER1/2 knockdown) also did not exhibit time of day differences in freezing behavior (Woodruff et al., 2018). These data further support the proposition that a functional molecular clock in the PFC, entrained by the SCN via CORT, is required for enhanced fear extinction recall in a rat's active phase.

These results have been observed in humans (Pace-Schott et al., 2013). Fear extinction is optimized in humans if learned and recalled during the day. This study by Pace-Schott and colleagues suffers from confounds similar to those presented by Woodruff et al. (2015) in that it does not determine if the time of fear extinction training or time of fear extinction testing determines time-of-day differences in fear extinction recall. However, the results between this human study and the present rodent study agree in that fear extinction recall performance is best in the active phase of the respective species. Further human experiments should be conducted to solve this issue.

4.4 Fear Relapse

A pilot fear relapse study following experiment 1 (Figure 7) showed a trending effect in which rats trained and tested for fear relapse at ZT16 showed less freezing behavior following the second tone in context A. However, similar to the issues raised in Woodruff et al. (2015) regarding fear extinction recall testing, these data did not describe whether the trending effect

was a result of the time of extinction training, or if it was an effect of the time of testing for fear relapse. Experiment 2 addressed these issues but failed to observe similar trending results.

Initially, we hypothesized that the animals in experiment 1 were experiencing fear renewal, the relapse of fear when the CS is presented outside the extinction context (reviewed in Goode and Maren, 2014). However, fear extinction training and testing finished a full three days prior to the pilot fear relapse study, whereas experiment 2, in which we saw no time-of-day difference in fear relapse, tested for fear relapse only 24hrs following extinction training. We now believe that in experiment 1, spontaneous recovery, the relapse of fear due to the passage of time alone, may have accounted for the freezing behavior seen in the rats (for review, see Goode and Maren, 2014). Because 72hrs passed between the final extinction recall tests and the fear relapse tests in experiments 1 and 3, spontaneous recovery is likely impacting freezing behavior more than in experiment 2, where fear relapse occurred only 24hrs after fear extinction sessions.

4.5 Clinical Implications

Stressor-related and anxiety disorders are associated with dysregulated prefrontal cortex, circadian, and glucocorticoid hormone function. This could correlate to humans undergoing exposure therapy protocols for stressor-related disorders like PTSD. If fear extinction learning can be compared to exposure therapy sessions, then fear extinction recall is analogous to a person applying those techniques to real-life situations, trying to regulate fear responses to environment triggers. We speculate that time of day may determine the persistence of memory for exposure therapy.

Nevertheless, fear often returns in humans following exposure therapy, a major concern with the efficacy of exposure therapies for patients with stressor-related and anxiety disorders. Further testing of fear relapse, its circadian nature, and associated brain pathways is necessary to

reduce fear relapse in humans, potentially reducing episodes of panic or anxiety in patients struggling with stressor-related and anxiety disorders.

Our lab hypothesizes that exogenous light cues entrain the SCN, which in turn entrains molecular clocks within the PFC via CORT. Our data suggests that a functional clock in the PFC, entrained by CORT, is necessary for optimal fear extinction recall performance. Many stressor-related anxiety disorders are characterized by circadian disruption, suggesting that molecular clocks in the PFC do not function optimally in patients with these disorders. This circadian misalignment in humans could lead to a decreased ability to recall exposure therapy treatment and could increase chances of fear relapse. Likewise, an intact, synchronized molecular clock could lead to enhanced exposure therapy recall, optimizing exposure therapy and providing a better quality of life for those suffering from PTSD and other anxiety disorders.

5. Acknowledgements

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