Effect of time of training and recall interval on behavior and neural activity under the trace conditioned fear paradigm

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1. Abstract

Circadian rhythms are endogenous 24-hour cycles that regulate behavioral and physiological processes. Disruptions to these rhythms can lead to a wide range of health deficits, including impairments in fear-based mental disorders. One form of fear conditioning that has been extensively studied is delayed fear conditioning. The neuroanatomy and neural connections have been mapped out, and circadian rhythms in behavior and mRNA expression have been reported by our lab and others. Here, we set out to explore time-of-day differences in behavior and underlying neurobiology in a related fear paradigm called trace fear conditioning. For our experiment, adult male rats were conditioned through multiple trials of exposure to a 15-second tone followed by a 10-second "trace" period that concluded with a foot shock and a 240-second intertrial interval. We analyzed freezing behavior and *c-Fos* and *Per1* mRNA data to see whether it was the time of training (ZT4-inactive phase or ZT16-active phase), the time of testing (ZT4 or ZT16), or the recall interval (24 hours, 36 hours, or 48 hours) that produced any significant differences. Freezing data was collected from previous work done in the lab. We then imaged and analyzed the basolateral amygdala, basomedial amygdala, central amygdala, medial amygdala, and the whole amygdala for mRNA expression of *c-Fos* (a common marker for neural activity) and *Per1* (a circadian gene that also acts as an immediate early gene). We found time-of-day differences in freezing behavior and *c-Fos* expression, indicating multiple interactions between time of training, time of testing, and the recall, but surprisingly no time-of-day differences in *Per1* expression. Taken together, our findings suggest that rats are biased toward fear recall during the inactive phase, potentially through an innate and unlearned suppression of fear behavior during the active phase. Such work has the potential to aid in the development of treatment methods and more specific diagnoses of fear-based mental disorders.

2. Introduction

Circadian Rhythms

Circadian rhythms are 24-hour cycles in behavior and physiological processes. These rhythms are crucial for time-sensitive daily functions, such as sleep, regulation of blood pressure, and regulation of body temperature (Bollinger and Schibler 2014). In turn, the disruption of these circadian rhythms may predispose people to many different diseases, including cancer, diabetes, and potentially a variety of mental disorders (Pluquet et al. 2014, Bass and Takahashi 2010, Menet and Rosbash 2011).

Circadian rhythms exist in virtually every cell in the body, arising from a transcriptional-translational negative feedback loop called the molecular clock (Takahashi 2016). This clock consists of a positive arm, including the clock genes called circadian locomotor output cycles kaput (*Clock*) and brain and muscle ARNT-like 1 (*Bmal1*), and a negative arm, including the clock genes period (*Per1*, *Per2*, and *Per3*) and cryptochrome (*Cry1*, *Cry2*). To begin one cycle of the molecular clock, the CLOCK and BMAL1 proteins heterodimerize and then bind to E-box sequences in the promoter regions of the *Per* and *Cry* genes, inducing the transcription of *Per* and *Cry* mRNAs. After *Per* and *Cry* mRNAs are translated into proteins, their protein products dimerize with one another and a complex of other proteins. This complex translocates back into the cell nucleus, where it inhibits the action of the BMAL1-CLOCK heterodimer on the E-box, thereby inhibiting further production of *Per* and *Cry* mRNA. The PER-CRY dimer goes on to be degraded by cellular homeostatic mechanisms, allowing BMAL1-CLOCK to act upon the E-box again. One complete cycle of the molecular clock takes around 24 hours to complete. Interactions between the cellular clock and other cellular processes in turn lead to 24-h cycles in many different cellular processes.

In the brain, the suprachiasmatic nucleus of the hypothalamus (SCN) is considered the "master clock" of the body. Cells of the SCN contain molecular clocks (Ralph et al. 1990, Yoo et al. 2004, Zucker 1980), just like other cells throughout the body (Reppert and Weaver 2002). But molecular clocks in cells of the SCN can be directly reset by periodic light exposure. After light enters the eye, it is transduced into a neural signal by intrinsically photosensitive retinal ganglion cells (Hattar et al. 2002). These specialized retinal ganglion cells pass along light information to the SCN, resulting in the induction of *Per1* gene expression in SCN cells (Reppert and Weaver 2001). This causes a daily resetting, or entrainment, of the molecular clock in SCN cells, ultimately aligning SCN circadian rhythms to periodic daily light exposure (Li and Androulakis 2021).

The entrainment and downstream effects of the SCN have been studied extensively, and the SCN is considered one of the most vital brain regions for survival. One reason is that circadian rhythms throughout the rest of the body appear to be entrained primarily by the SCN. A study examining the effects of lesioning the SCN in squirrel monkeys showed that the SCN is necessary for proper sleep-wake processes and regulation (Edgar and Dement and Fuller 1993). Another study lesioned the SCN in rats and observed that the SCN is responsible for proper brain temperature regulation and sleep rhythmicity (Eastman and Mistlberger and Rechtschaffen 1984). An additional study found that lesioning the SCN in rats led to the disappearance of circadian rhythms (Zucker et al. 1980). Even a modification of the SCN's cycle length led to a change in the length of daily rhythms (Ralph et al. 1990). It is for such reasons that the SCN has come to be thought of as a master regulator of circadian rhythms throughout the body.

The SCN uses a variety of signals to communicate circadian rhythms to the rest of the body. These signals appear to entrain molecular clocks in tissues outside the SCN. For example,

one of the ways the SCN achieves this is through control of the hypothalamic-pituitary-adrenal (HPA) axis. The SCN was found to be crucial for maintaining circadian rhythms in normal adrenal glucocorticoid release (Moore and Eichler 1972). Relatedly, both physical and psychological stress, which induce glucocorticoid release, have been shown to disrupt circadian function, highlighting an important relationship between stress and glucocorticoid secretion (Spencer et al 2018, Tahara et al, 2017). This effect appears to occur through the direct induction of *Per1* gene expression by glucocorticoids (So et al. 2009, Conway-Campbell et al. 2010).

SCN output signals such as glucocorticoids carry circadian timing information to molecular clocks in other tissues. These extra-SCN molecular clocks work together at the tissue level (just like molecular clocks in the SCN), generating tissue-level clocks that are sometimes called local clocks. Indeed, local clocks have been identified in many different brain regions, including the prefrontal cortex, hippocampus, PVN, and amygdala (Chun et al. 2015). Local clocks in the brain influence many cognitive processes, and we are especially interested in their role in memory due to an abundance of evidence showing that local clocks modulate synaptic plasticity (Hartsock and Spencer 2020). We believe that by better understanding the mechanisms of local clocks and their role in memory we will be able to diagnose and treat memory-related disorders more effectively.

Conditioned Memory Processes

Fear-based mental disorders include anxiety disorders, obsessive-compulsive disorders, and trauma- and stressor-related disorders, such as post-traumatic stress disorder (DSM-5, 2013). These disorders represent a major public health burden. For example, according to the National Institute of Health, about 31.1% of adults in the U.S. have experienced some form of anxiety

disorder in their life (Harvard Medical School 2007). Despite the high prevalence of fear-based mental disorders in society, optimal treatment strategies for these disorders are still debated.

An important mechanism underlying fear-based mental disorders is memory. For example patients with a history of PTSD or currently suffering from PTSD display elevated physiological responses to learned fear (Norrhold et al. 2011, Orr et al. 2000). Additionally, patients with a similar background suppress fear poorly (Acheson et al, 2015, Milad et al. 2009, Norrhold et al. 2011, Orr et al. 2000). Thus, the study of emotional memory processes may be useful for understanding fear-based mental disorders.

A common paradigm used to study memory is conditioning. Conditioning first debuted when Pavlov demonstrated that he could make dogs salivate to the sound of a bell (Pavlov 1927). In this study, there was a pre-existing unconditioned stimulus (presence of food) and a conditioned response (salivation). Pavlov took the unconditioned stimulus of food and presented it to the dog while he rang a bell. After multiple trials, the dog began to salivate at the sound of the bell, even when the food was not present. The bell thus became a so-called conditioned stimulus, capable of producing the unconditioned response (salivation) in the absence of the unconditioned stimulus (presence of food).

In our work with rats, we use a modified version of Pavlovian conditioning called fear conditioning. Foot shocks are substituted for food (unconditioned stimulus), and there is a tone played in lieu of a bell (conditioned stimulus). The rat displays freezing, a species-specific fear behavior (unconditioned response), after each foot shock. This freezing behavior is a common behavior among animals in response to aversive stimuli, and it is often regarded as a third possible response element alongside “fight or flight.” Through the association of the tone with the shock, the rat begins to freeze after the tone by itself. The fear conditioning paradigm can be

systematically broken down into four basic memory processes: fear learning, fear recall, fear extinction learning, and fear extinction recall.

The initial phase of the fear conditioning paradigm encompasses fear learning. During this portion of the protocol, the rat is set inside a behavioral testing chamber with a shock-grid floor. After giving the rat time to acclimate to its surroundings, the rat is exposed to multiple trials of a 30-sec auditory tone that co-terminates with a mild 2-sec foot shock. The rat learns quickly that the tone predicts the shock, and it exhibits freezing behavior when exposed to just the tone.

The next day, the rat is placed in a new environment, or context, and is exposed to only the tone. At first, the rat freezes a lot, indicating a strong association of the tone with the shock. Thus, such behavior is interpreted as fear recall. The rat is then presented with multiple trials of just the tone. The objective of this is to teach the rat that the tone does not predict shock in this new context. It is crucial to understand that the rat is not erasing the fear memory that a tone predicts shock, but rather is creating a new memory that the tone does not predict shock in this new context. Through this process, the rat learns to no longer exhibit freezing behavior in response to the tone by itself. Hence, this process is called fear extinction.

The final phase of this paradigm is fear extinction recall. This is an additional phase to test how well the rat learned that the tone does not predict shock in the extinction context. The rat is placed back into the extinction context and is exposed again to just the tone. Very little freezing behavior during the extinction recall session suggests strong recall of the extinction memory (tone predicts no shock) compared to the fear memory (tone predicts shock).

The paradigm I have described above is called delayed fear conditioning. Delayed fear conditioning is the best-studied type of fear conditioning, and there is a lot known about its

neuroanatomical underpinnings. The three brain regions that have received the most attention are the amygdala, hippocampus, and prefrontal cortex. The amygdala is necessary for the learning of both fear and extinction memories (Sun, Gooch, and Sah 2020). The hippocampus plays a critical role in discriminating between the fear context and the extinction context (Bouton et al. 2021). Different subdivisions of the prefrontal cortex appear to be important for the recall of both fear and extinction memories (Gonzalez and Fanselow 2020, Woodruff et al. 2018).

The neural circuitry and connections of the amygdala underlying fear conditioning has been well-established. Information about the conditioned stimulus (tone) and unconditioned stimulus (foot shock) come together in the lateral and basolateral amygdala, collectively the basolateral amygdala (BLA). The signal is then sent to the central amygdala (CEA) where this pairing of stimuli is processed. Contextual information is also transferred to the central amygdala from the basomedial amygdala (BMA). Finally, the central amygdala takes all of this information and projects it to other brain regions to produce fear-related behaviors, such as freezing (Orsini and Maren 2012).

There is another type of fear conditioning, however, called trace fear conditioning (Han et al., 2003). The major difference between delayed and trace fear conditioning is that in delayed fear conditioning the tone is immediately followed by the shock, whereas in trace fear conditioning there is a short pause between the tone and shock. This one small change in protocol causes trace fear conditioning to have very different neuroanatomy. Although many studies have been conducted to explore the neuroanatomy of delayed fear conditioning, there is still a great deal that is unknown about trace fear conditioning.

As in delayed fear conditioning, the amygdala, hippocampus, and prefrontal cortex appear to be key players in trace fear conditioning. Unilateral injection of muscimol (a GABA

agonist) into either the ventral hippocampus or the amygdala was sufficient enough to produce trace fear conditioning impairment (Gilmartin, Kwapis, and Helmstetter 2012). Similarly, blocking amygdala AMPA receptors impairs both delayed and trace fear recall (Kwapis et al., 2013). Various methods of inactivation, such as lesions and muscimol injections, demonstrate that dorsal hippocampus inactivation leads to disruptions in the acquisition of trace fear conditioning (Raybuck and Lattal 2013). Lesions in the prelimbic prefrontal cortex disrupt both trace fear learning and recall (Park et al. 2022). In spite of such progress, however, the underlying mechanisms of trace fear conditioning have not been studied much compared to delayed fear conditioning.

Research Objectives

It is believed that disruptions to circadian rhythms may play a major role in fear-based mental disorders (Agorastos and Olff 2020). For example, patients with PTSD show a disrupted circadian regulation of glucocorticoids, sleep, and other circadian processes (Dayan et al. 2016). These impairments occur alongside disruptions in fear memory, as discussed above. Therefore, it is possible that disrupted circadian rhythms underlie disruptions in fear memory in fear-based mental disorders.

To this end, there is a local clock in the prefrontal cortex that appears critical for circadian regulation of memory processes in the delayed fear conditioning paradigm. The prefrontal cortex, particularly its infralimbic subdivision (IL) in rats, is important for the recall of conditioned fear extinction (Gonzalez and Fanselow 2020). Our lab has previously shown rhythmic cycles in the expression of clock genes, including *Per1*, in the prefrontal cortex (Chun et al 2015). Additionally, there is ample evidence of circadian modulation of conditioned fear

extinction in rodents (Chaudhury and Colwell 2002, Woodruff et al. 2015, Woodruff et al. 2018, Hartsock et al. 2022). Experiments with humans show enhanced fear extinction learning and recall in the morning, providing further evidence for the role of circadian rhythms in extinction memory (Pace-Schott et al. 2013). Moreover, intact prefontal cortex clock gene expression is necessary for optimal conditioned fear extinction recall in rats (Woodruff et al. 2018). Taken together, these findings provide strong evidence for a local clock in the prefrontal cortex that is crucial for the regulation of recall of conditioned fear extinction memories.

Given that the trace fear paradigm also depends on the prefrontal cortex, we hypothesized that trace fear conditioning might also exhibit circadian rhythms. For this reason, our laboratory previously conducted experiments that found time-of-day differences in trace fear recall and trace fear extinction recall. Specifically, rats showed significantly better trace fear recall during their inactive phase and significantly better trace fear extinction recall during their active phase. In a follow-up experiment, our laboratory explored whether it was the time of training or the time of testing that accounted for the differences observed in trace fear recall. These studies are described in the public online Honors Thesis of a former member of the Spencer Lab, Alyssa Bryce Fausnaught (Fausnaught 2021).

Here, I will describe the behavioral findings from this follow-up experiment and build upon the findings with a mechanistic characterization. In my project, I set out to examine the activity of specific brain regions during trace fear conditioning and whether or not there were time-of-day differences. My work has focused on the amygdala, considering that this region has been previously found to be important for trace fear conditioning. After rats underwent trace fear recall, I measured the abundance of amygdala mRNA for the clock gene *Per1* and the immediate early gene *c-Fos*, a common correlate of neuronal activity. The gene *c-Fos* is known as an

immediate early gene because its expression rapidly increases upon cellular stimulation, and it is a common marker used for detecting cellular activity (Abraham and Tate 1991, Kováks 2008). The gene *Per1* is one of the clock genes, but it also serves as a marker for other chemical cascades such as the CREB cycle (Brenna et al. 2021), and previous work in our lab has found that *Per1* can also act like an immediate early gene in the prefrontal cortex after stress (Chun et al,. 2018). For these reasons, I hypothesized that amygdala *Per1* and *c-Fos* mRNA expression might positively correlate with freezing behavior during trace fear recall only in the BLA, BMA, and CEA as these brain regions have already shown to be involved in fear learning and memory.

3. Methods and Materials

Rats

A total of thirty-six male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were pair-housed in polycarbonate cages measuring 47cm x 23cm x 20cm (Allentown Caging Equipment Company, Allentown, NJ) at the University of Colorado Boulder AAALAC-accredited animal vivarium. The rats were supplied with food (Tekland Rodent Diet 8640; Harlan) and water *ad libitum*. The rats were placed in separate rooms in 12:12 hour light:dark cycles and allowed to acclimate for two weeks. Behavioral tests conducted at zeitgeber time (ZT) 4 (the inactive phase: 4 hours after light onset) were conducted under white light. Behavioral tests conducted at ZT16 (the active phase: 4 hours after light offset) were conducted under dim red light conditions. To control lighting conditions between ZT4 and ZT16 rats during transportation to and from the testing suite, cages were transferred in black Hefty bags (Geft 55g bags; Reynolds Consumer Products, Inc.; Lake Forest, IL). The experimentation, treatment, and

handling of rats were in accordance with ethical animal treatment procedures and approved by the University of Colorado Institutional Animal Care and Use Committee.

Experimental Design

The aim of this experiment was to determine whether the time-of-day differences in trace conditioned fear recall were due to time of training or time of testing. Eighteen rats underwent Session 1 trace fear conditioning at ZT4, and the other eighteen rats underwent Session 1 trace fear conditioning at ZT16. Both groups were then further divided into different Session 2 testing groups for a total of six different groups: rats trained at ZT4 and tested 24 hours later at ZT4, rats trained at ZT4 and tested 36 hours later at ZT16, rats trained at ZT4 and tested 48 later hours at ZT4, rats trained at ZT16 and tested 24 hours later at ZT16, rats trained at ZT16 and tested 36 hours later at ZT4, and rats trained at ZT16 and tested 48 hours later at ZT16. Testing 24 hours later at the same ZT and testing 36 hours at the opposite ZT was to explore the possibility that the time-of-day differences in trace conditioned fear recall are due to either time of training or time of testing. Testing 48 hours later at the same ZT was in place to eliminate the possibility that differences were due to time of day differences and not simply time elapsed since training.

For Session 1 (trace fear conditioning), rats were placed in a box measuring 29.2cm x 21cm x 25.4cm, with a shock-grid floor made of stainless steel bars (Med Associates Inc.; EMV-800; St Albans, VT). The shock grid was connected to a current generator (Med Associates Inc.; ENV-414S; St Albans, VT) to control foot shock delivery and speakers (Med Associates Inc.; ENV-025F; St Albans, VT) to produce auditory tones. VideoFreeze Software version 3.0.0.0 (Med Associates Inc.; SOF-843; St Albans, VT) was used for scoring of freezing behavior. At the beginning of each session, a five-minute acclimation period was used to score baseline levels

of freezing. Then, six tones were presented, each 3kHz at 80dB for 15 seconds. Each tone was followed by a ten-second "trace" interval. A one-second 0.8 mA foot shock was then administered. Finally, an intertrial interval of 240 seconds followed each shock.

For Session 2 (trace fear recall), rats were placed in Context B after their assigned test interval: 24, 36, or 48 hours. Context B was the same box as Context A, but a white curved plexiglass insert covered the wall, and the shock grid was covered with the same white plexiglass. Peppermint oil was also used inside the box to further differentiate Context B from Context A. Three minutes were used for the acclimation period. Then, three tone presentations occurred, each followed by a trace period and interval but with no shock delivery.

Tissue Sectioning

A cryostat was used to slice 12 um coronal slices for further processing. To collect brain tissue throughout the amygdala, brain section collection onto Colorfrost Plus glass slides (VWR, Radnor, PA) using thaw mounting started at -2.40 mm posterior to bregma and ended at the ventral hippocampus at -5.04 mm posterior to bregma. All brain coordinates were used from Paxinos and Watson 2013 Brain Atlas.

Radioactive In Situ *Hybridization*

Radioactive *in situ* hybridization is a technique that allows for good spatial resolution of specific mRNA(s) of interest. We used this technique to visualize *Per1* mRNA (Figure 1). The first day consisted of first building the probe. Linearized DNA was added to serve as a template strand, and RNA polymerase was added to build the probe. DNAase was added to degrade the

template. We then dehydrated the tissue in a series of ethanol washes, adding the probe to the tissue, and allowed the tissue to incubate overnight at 54 degrees C. The next day, RNAase was added to break down any probe that was not bound to the Per1 mRNA, and we washed away any nonhybridized probe. We then dehydrated the slides in ethanol again and exposed the tissue to film for 5 weeks. ImageJ version 1.52q (NIH) was then used to calculate optical density in each region of interest – which included the BLA, BMA, CEA, and MEA – for the 3-4 darkest amygdala sections per brain.

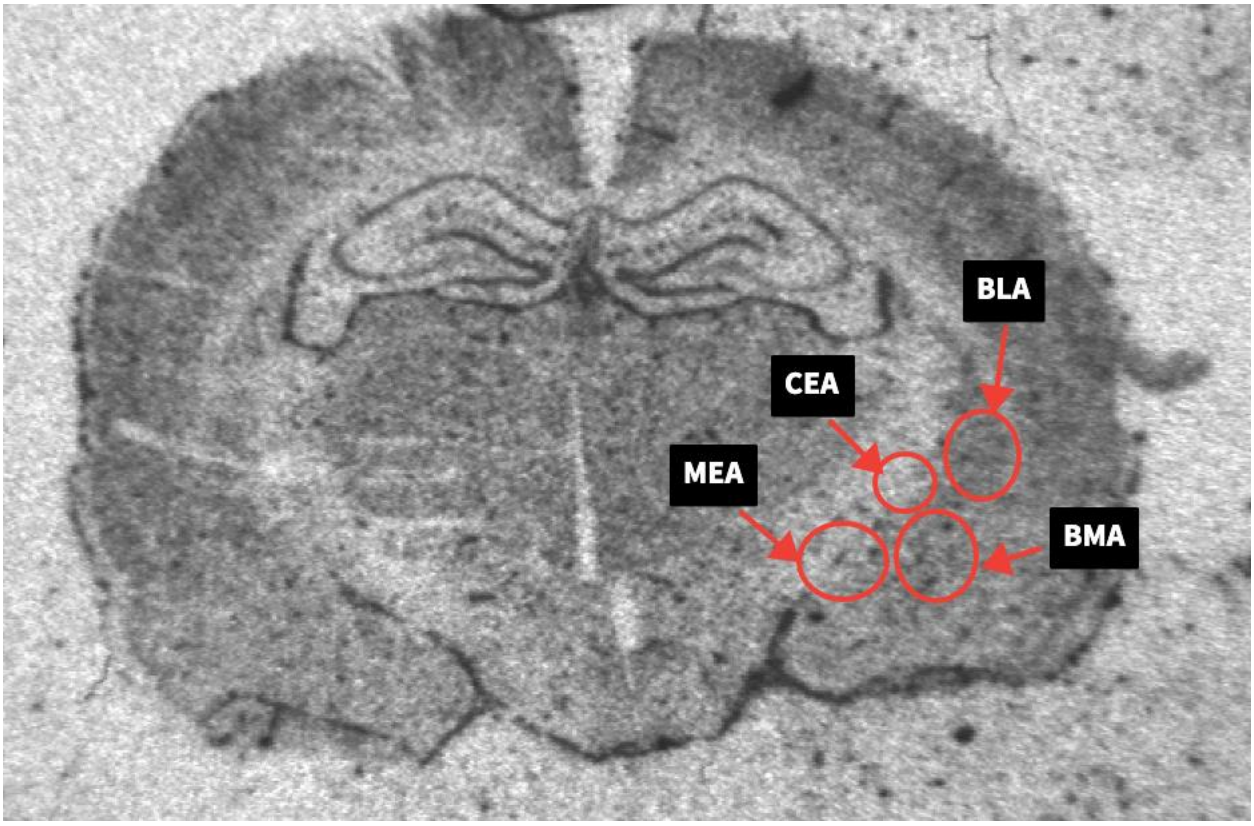

Figure 1: *A representative image of an amygdala section we captured and analyzed for* Per1 *optical density.*

Fluorescent In Situ *Hybridization*

Fluorescent *in situ* hybridization is a technique that also allows for excellent spatial resolution of specific mRNA(s) of interest. In this case, we used the technique to visualize the localization of *c-Fos* mRNA (Figure 2). The first day, much like radioactive *in situ*, consisted of making the probe and preparing the tissue. We fixed the tissue using paraformaldehyde, washed with sodium, saline citrate (SSC) to help the DNA stick together, and placed the tissue in hybridization chambers. The second day also consisted of multiple SSC washes. We also prepared and exposed the tissue to an RNase digestion solution to remove any single-stranded DNA remaining. On the third day, we first exposed the tissue to a blocking reagent that we had prepared. Then, we coverslipped the tissue with anti-digoxigenin-horseradish peroxidase (anti-dig-HRP) to bind our probe with the HRP, an enzyme that catalyzes the process of bringing the amplification reagent onto the tissue. We washed those coverslips off, and then we coverslipped them with a diluted Cy3 Fluorohpore Tyramide Amplification Reagent Stock Solution which is responsible for the actual deposition of fluorophore labels. After a series of incubations, we concluded by coverslipping the tissue with Prolong Gold with DAPI.

Imaging

We imaged all regions of interest for each brain using a Zeiss Axioimager M1 fluorescent microscope accompanied by ZenBlue software version 2.6 (Zeiss Microscopy, Jena, Germany). This process was repeated in each hemisphere for two brain sections showing the amygdala for a total of four images per rat. Sections were chosen for their inclusion of all regions of interest and optimal tissue quality, barring circumstances where tears and folds were present in every section available. These include the basolateral amygdala (BLA), basomedial amygdala (BMA), central

amygdala (CEA), medial amygdala (MEA), and the whole amygdala (AMY). QuPath, an automated cell counting software, was used to automatically count the number of cells positive for *c-Fos* in each region (Bankhead et al, 2017).

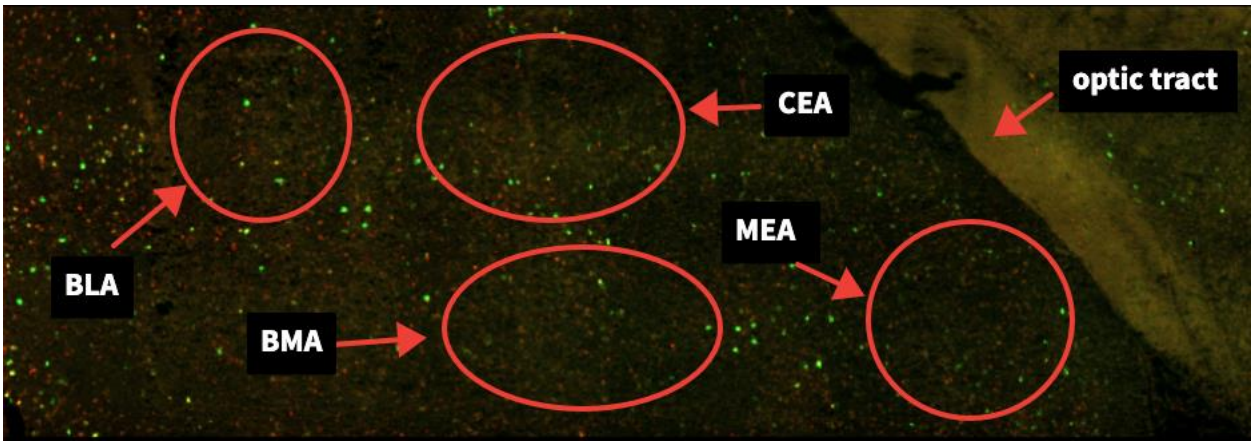

Figure 2: *A representative image of images we captured and analyzed for* c-Fos *positive cells. This shows the left hemisphere of one brain's amygdala. The green represents cells positive for* c-Fos *mRNA. The red represents cells positive for* Per1 *mRNA, but we did not analyze* Per1 *cell counts due to technical difficulties.*

Statistical Analyses

We used two-way ANOVAs to test for between-group effects on behavior, *Per1* mRNA expression, and *c-Fos* mRNA expression. The between-group factors were training ZT and recall interval. Fisher's Least Significant Difference post hoc tests were used for multiple comparisons. All data presented are the means with standard error of the mean (SEM). We considered a *p*-value of less than 0.05 to be significant.

4. Results

Behavior

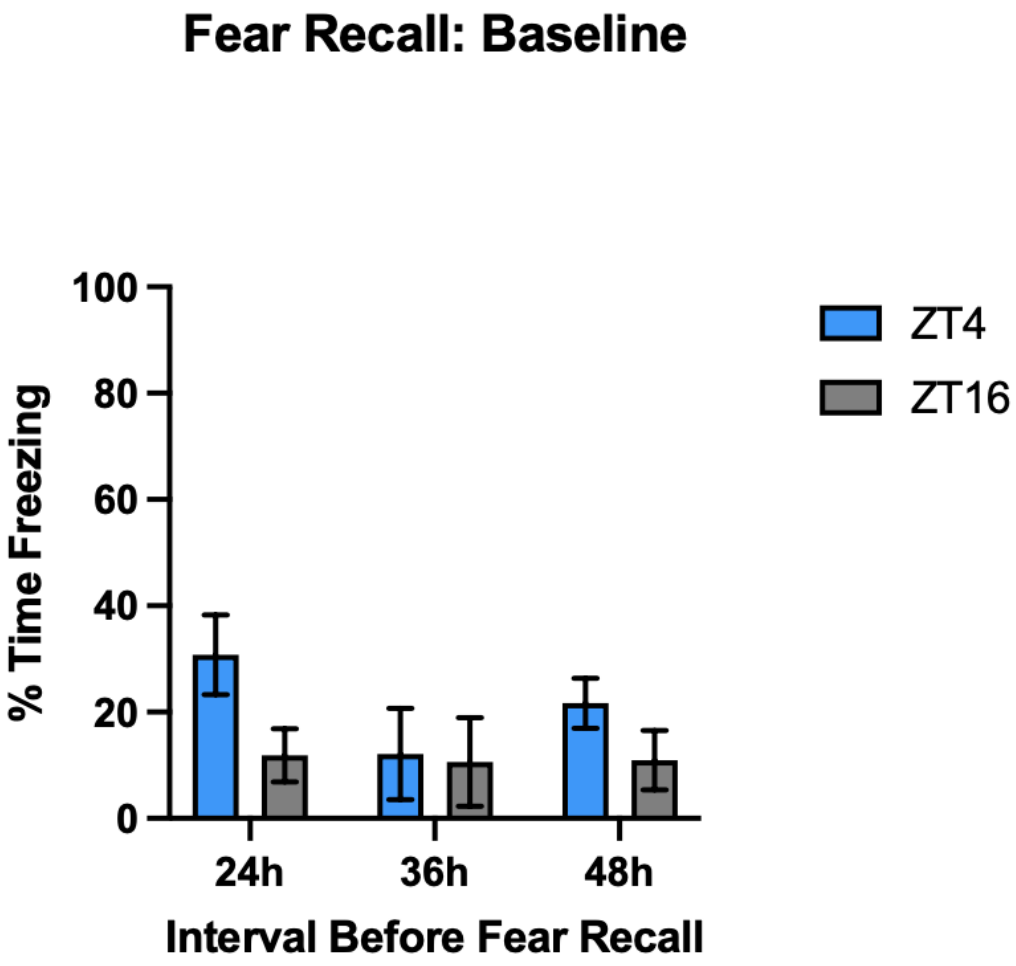

Figure 3: *Freezing behavior during the baseline period.*

Baseline freezing levels were measured during the first three minutes the rats were placed in Context B. All rats displayed minimal freezing levels and showed no significant differences between groups.

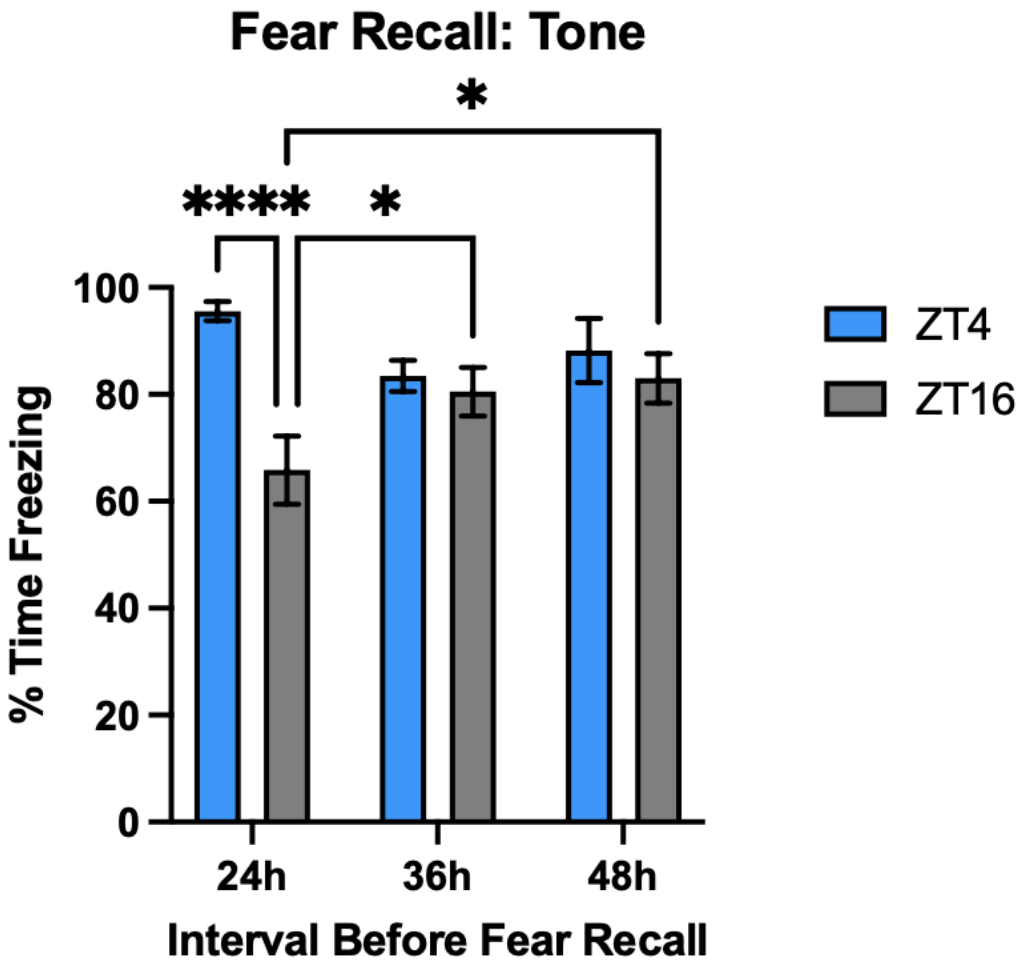

Figure 4: *Freezing behavior during tone presentation.*

During tone presentation, we observed a statistically significant interaction between time of training and recall interval ($F(2, 30)=5.050$, $p<0.05$). Post hoc tests revealed that this interaction was driven by an increase in freezing across intervals only in the ZT16-trained rats and a significant time-of-day difference at the 24-hour recall interval, with ZT16 rats showing reduced freezing compared to ZT4 rats.

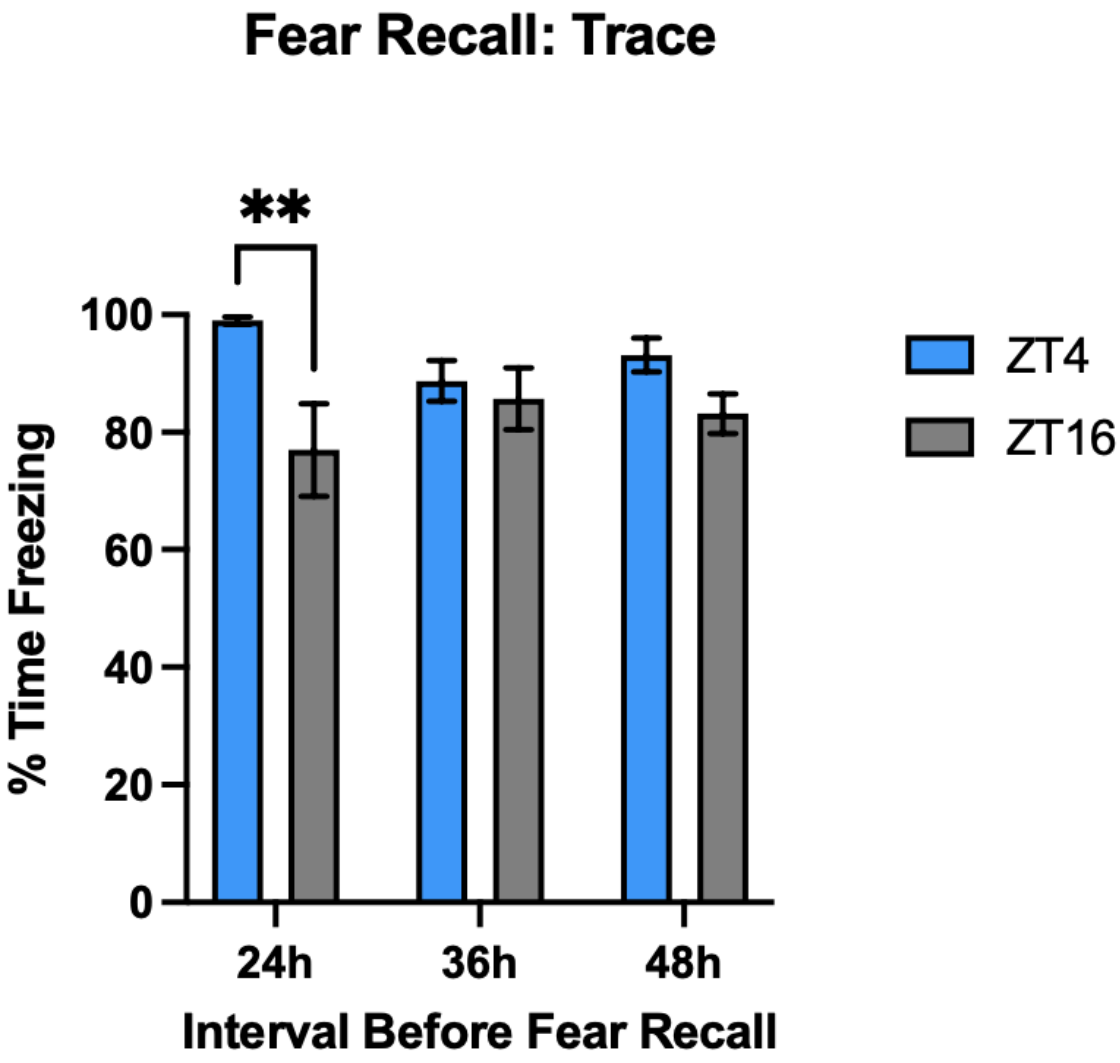

Figure 5: *Freezing behavior during the trace period.*

We observed a main effect of time of training on freezing levels during the ten-second trace period at which ZT4-trained rats froze significantly more than ZT16-trained rats ($F(1, 30)=10.08$, $p<0.01$). Post hoc tests showed that this effect was driven by ZT4-trained rats freezing significantly more than ZT16-trained rats, but only at the 24-hour recall interval.

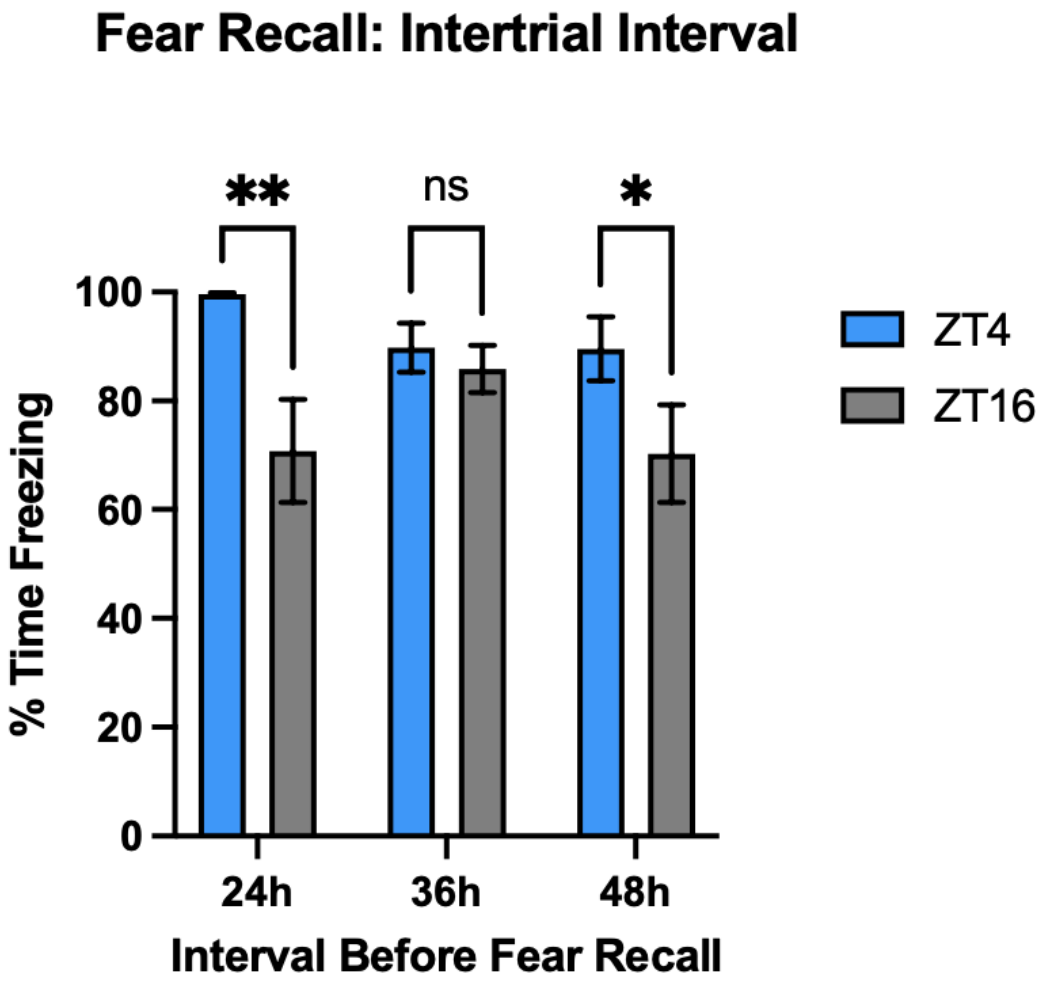

Figure 6: *Freezing behavior during the intertrial interval.*

Additionally, we observed a main effect of time of training of freezing levels during the 240-second intertrial interval ($F(1, 30)=11.11$, $p<0.01$). This interaction, as shown by post hoc tests, was driven by a statistically significant increase in freezing in the ZT4-trained rats compared to the ZT16-trained rats at the 24-hour recall interval and 48-hour recall interval.

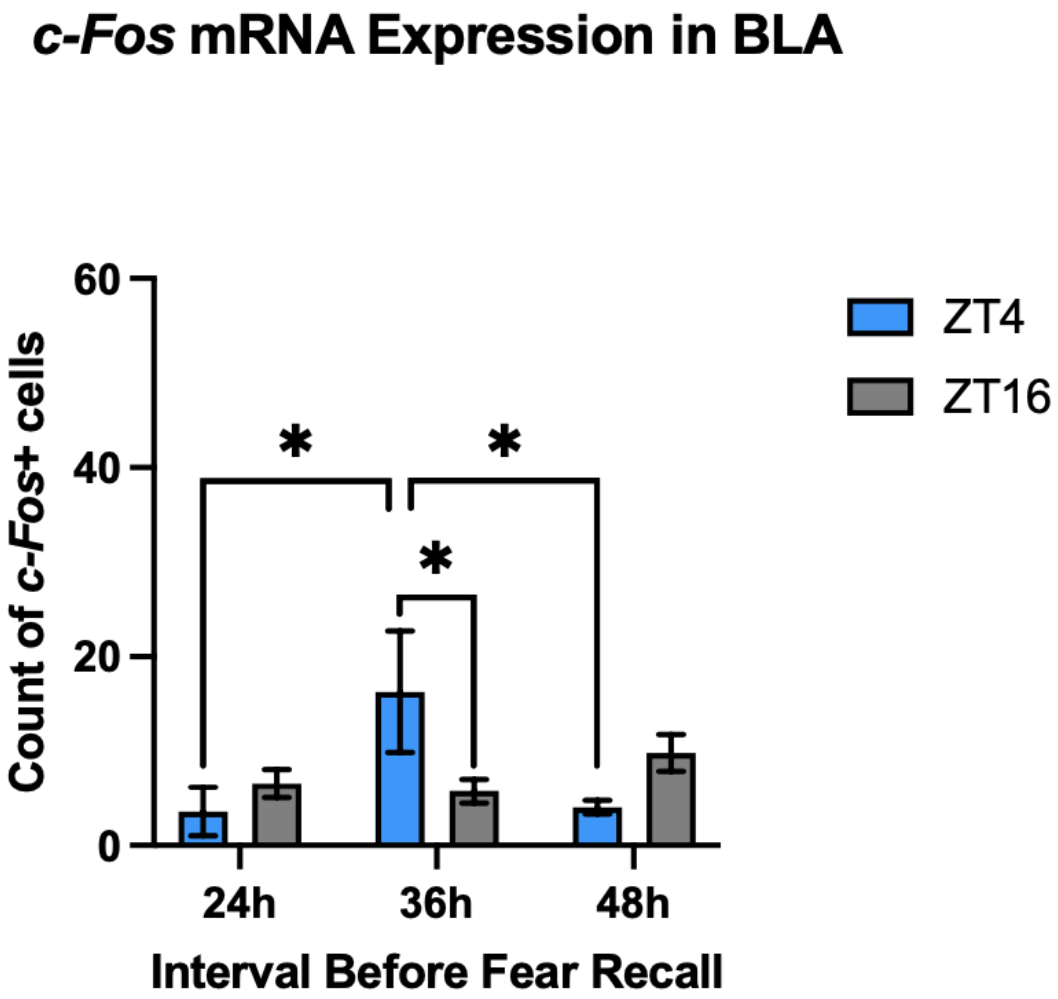

Figure 7: c-FOS *mRNA expression in the BLA.*

In the BLA, we observed a trend towards significance in the interaction between time of training and recall interval ($F(2, 24)=3.014$, $p=0.0680$). Unprotected post hoc tests showed us that this trend towards significance was driven by significantly higher *c-Fos* mRNA levels in the ZT4-trained rats at the 36-hour recall interval compared to the 24-hour and 48-hour recall intervals. At the 36-hour recall interval, the ZT4-trained rats also displayed significantly higher *c-Fos* mRNA than the ZT16-trained rats.

Figure 7: c-FOS *mRNA expression in the BLA.*

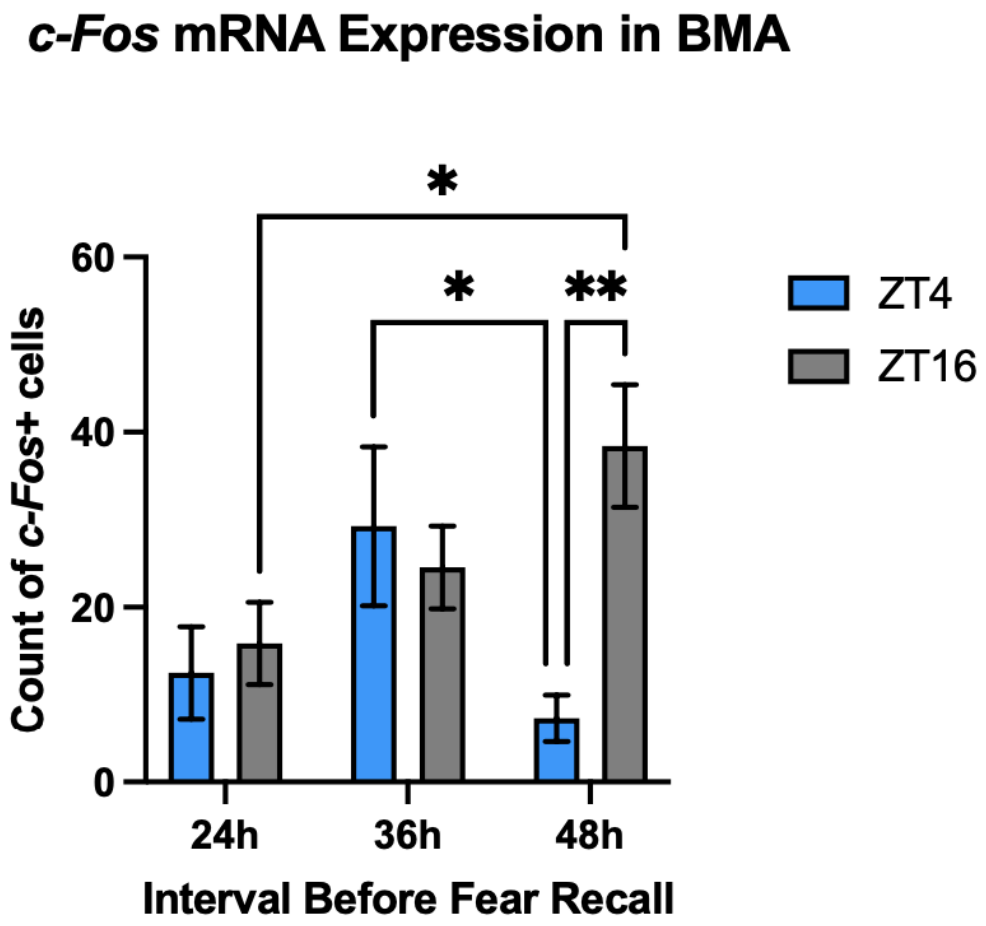

Figure 8: c-FOS *mRNA expression in the BMA.*

In the BMA, there was a statistically significant interaction between time of training and recall interval ($F(2, 24)=4.198$, $p<0.05$). Post hoc tests revealed that this interaction was driven by a few factors. First, ZT16-trained rats at the 24-hour recall interval displayed significantly lower *c-Fos* mRNA expression than ZT16-trained rats at the 48-hour recall interval. Second,

ZT4-trained rats at the 36-hour recall interval displayed significantly higher *c-Fos* mRNA expression than ZT4-trained rats at the 48-hour recall interval. Lastly, ZT16-trained rats showed significantly higher *c-Fos* mRNA levels than ZT4-trained rats at the 48-hour recall interval.

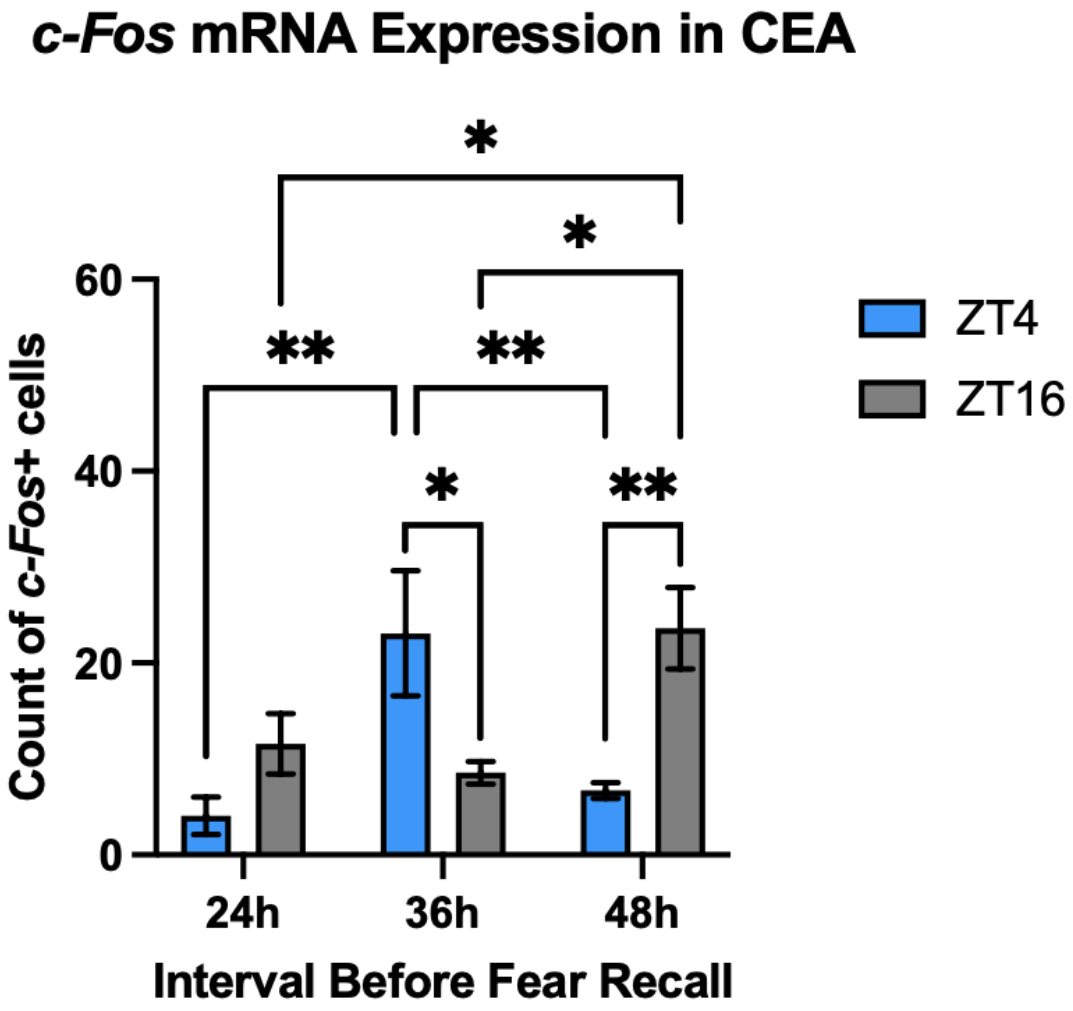

Figure 9: c-FOS *mRNA expression in the CEA.*

In the CEA, we observed a significant interaction between time of training and recall interval ($F(2, 24)=7.187$, $p<0.01$). Post hoc tests revealed that this interaction was driven by

significant time of day differences at the 36-hour and 48-hour recall intervals, a significantly higher level of *c-Fos* mRNA expression in the ZT4-trained rats at the 36-hour recall interval compared to the ZT4-trained rats at the 24-hour and 48-hour recall intervals, and ZT16-trained rats at the 48-hour recall interval displaying significantly higher *c-Fos* mRNA levels than ZT16-trained rats at the 24-hour and 36-hour recall intervals.

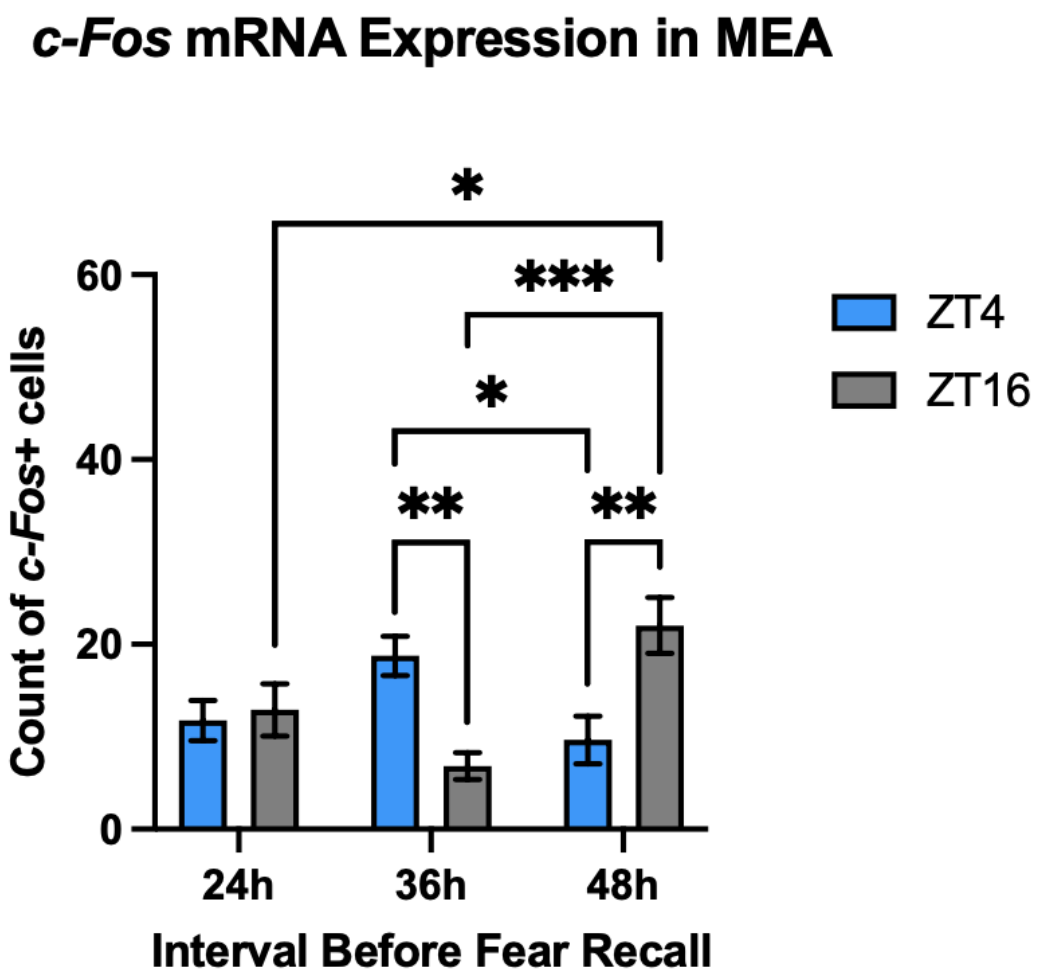

Figure 10: c-FOS *mRNA expression in the MEA.*

In the MEA, we observed a significant interaction between time of training and recall interval ($F(2, 24)=10.83$, $p<0.001$). Post hoc tests revealed that this interaction was driven by significant time-of-day differences at the 36-hour and 48-hour recall intervals, with significantly higher *c-Fos* mRNA levels in the ZT16-trained rats at the 36-hour recall interval compared to ZT16-trained rats at either the 24-hour or 36-hour recall interval, and ZT4-trained rats at the 36-hour recall interval showing significantly higher *c-Fos* mRNA expression than the ZT4-trained rats at the 48-hour recall interval.

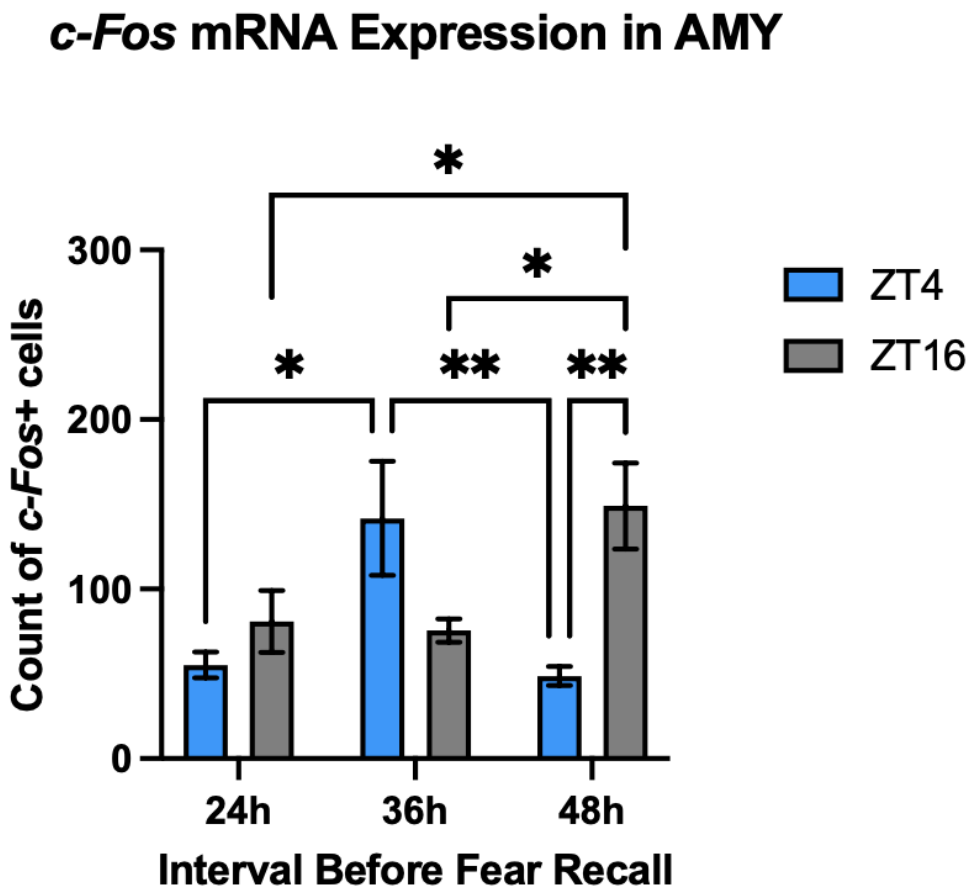

Figure 11: c-FOS *mRNA expression in the AMY.*

To get a sense of the general *c-Fos* mRNA expression, we also measured mRNA levels in the entire amygdala. We observed a statistically significant interaction between time of training and recall interval ($F(2, 24)=6.566$, $p<0.01$). This interaction, as calculated by post hoc tests, was driven by ZT4-trained rats in the 36-hour recall interval showing significantly higher *c-Fos* mRNA expression compared to the ZT4-trained rats in the 24-hour and 48-hour recall interval, ZT16-trained rats in the 48-hour recall interval showing significantly higher *c-Fos* expression that ZT16-trained rats in the 24-hour and 36-hour intervals, and a significant time of day difference at the 48-hour recall interval.

Per1

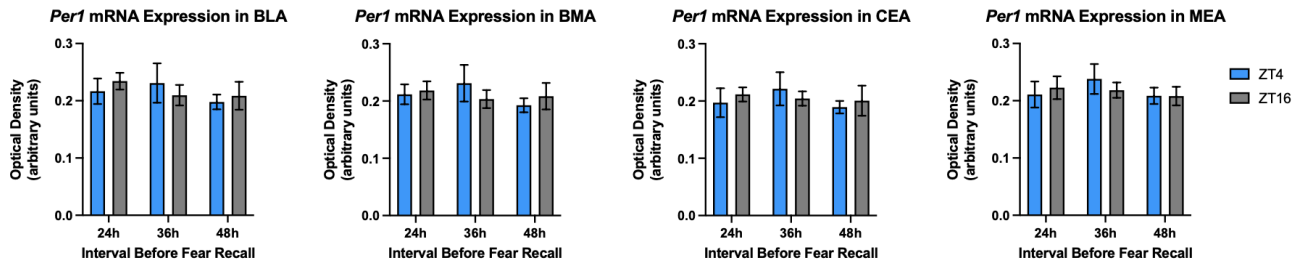

Figure 12: Per1 *mRNA expression in the BLA, BMA, CEA, and MEA.*

In the BLA, BMA, CEA, and MEA, we observed no main effects or significant interactions of time of training or recall interval on *Per1* mRNA expression.

5. Discussion

Looking at the behavior data, baseline freezing levels did not offer any unexpected results, with all of the groups of rats freezing minimally. During fear recall, however, a

significant overall pattern for behavior is evident where ZT4-trained rats displayed ceiling levels of freezing regardless of recall interval, and ZT16-trained rats displayed increasingly higher levels of freezing at greater recall intervals. Notably, this effect appeared similar during both the tone and trace, although a significant interaction was present only during the tone (Figures 4 and 5). This might mean that ZT4-trained rats consolidate fear immediately while ZT16-trained rats need additional time to reinforce the fear memory. Another interesting pattern in the data is freezing during tone presentation and the trace period compared to freezing during the intertrial interval. The ZT4-trained rats still froze at ceiling levels, but the ZT16-trained rats froze at relatively low levels (about 70%) at the 24-hour and 48-hour recall intervals (Figure 6). The tone and trace period represent danger given that, during the fear conditioning session, they occur just before the shock. On the other hand, the intertrial interval represents temporary safety. Thus, it is notable that ZT16 rats freeze less than ZT4 rats at 48 hours during the intertrial interval. One possible explanation could be that ZT16-trained rats are better at discriminating when it is safe and when it is not. Interestingly, this effect also depends on the time of testing because even the ZT16 rats freeze near ceiling levels at the 36-hour recall interval (that is, when rats trained at ZT16 are tested at ZT4). These findings from trace conditioned fear align with our previous findings from delayed conditioned fear extinction in the sense that, in both learning paradigms, rats have a bias for fear at ZT4 and a bias for safety at ZT16.

Based on previous research highlighting the importance of the basolateral amygdala (BLA), basomedial amygdala (BMA), and central amygdala (CEA) on fear learning and recall (Orsini and Maren 2012), we measured *c-Fos* mRNA levels in all of these regions in addition to the medial amygdala (MEA), which is involved less strongly in conditioned fear, and the whole amygdala (AMY). With this information at hand, we expected to see differences in *c-Fos*

expression in just the BLA, BMA, and CEA. Surprisingly, we saw differences in all of these regions, including the MEA (Figures 7, 8, 9, and 10). The current mapped-out neural circuitry of fear memory is based largely on studies of delayed fear, and our results suggest that trace fear may recruit the MEA in addition to the rest of the brain regions. These differences may also be due to different neurons increasing their activity based on time of day, and other studies have shown that there are different neurons, fear and extinction neurons, within the amygdala that play a role in fear (Orsini and Maren 2012).

Another interesting finding in the *c-Fos* data is an effect we saw only in the BMA. At the 36-hour recall interval, there were no differences in *c-Fos* mRNA expression between the ZT4 and ZT16 rats. Comparing the BMA to our other amygdala regions, this effect appears to owe to increased *c-Fos* expression in ZT16-trained rats tested at 36 hours. Most notably, the *c-Fos* expression in the BMA is most reflective of the behavior data in ZT16 rats. The behavior data shows that, during the tone and trace, ZT16-trained rats increased freezing as the recall interval increased (Figures 4, 5, and 6). The BMA *c-Fos* data, likewise, shows expression in the ZT16-trained rats increasing across recall intervals and a time-of-day difference at the 48-hour recall interval. This is different from our other regions of interest, wherein *c-Fos* expression remains low at 36 hours. (Figure 8). One possible explanation for this is that while the BLA, MEA, and CEA serve supporting roles, the BMA is integral in the output of fear behavior in the trace fear conditioning paradigm. Thus, we see relatively high *c-Fos* expression in ZT16-trained rats tested at 36 hours in the BMA but not in other amygdala subregions.

Previous work in the delayed fear paradigm has shown that delayed fear extinction forms a new extinction memory that competes with a previously learned fear memory to modulate freezing behavior (Orsini and Maren 2012). In addition, following delayed fear extinction, there

is a gradual shift over time from the extinction memory winning to the fear memory winning, a phenomenon known as spontaneous recovery (Rescorla 2004). In our studies on circadian rhythms in delayed fear extinction, we have shown that rats tested for extinction recall at ZT16 show less freezing behavior than rats tested at ZT4, and we have interpreted this to indicate stronger extinction recall in ZT16 rats (Woodruff et al. 2015, 2018, Hartsock et al. 2022). Here, our *c-Fos* findings might suggest a more complex story. In our experiment, rats did not undergo extinction learning, yet the ZT16-trained rats at the 24-hour recall interval still show low *c-Fos* expression comparable to the ZT4-trained rats (Figures 7, 8, 9, 10, and 11). A compatible interpretation is that some sort of innate inhibition occurs at ZT16 without extinction learning even taking place. If this is the case, the ZT16-trained rats may have to undergo additional memory consolidation before they can overcome this innate inhibition, resulting in higher freezing behavior at longer recall intervals. Over time, this inhibition decays, and the rats begin to remember fear better, as seen by the gradual increase in freezing across intervals in ZT16-trained rats (Figures 7, 8, 9, 10, and 11). Our *c-Fos* findings support this second interpretation. In general, our rats tend to exhibit higher *c-Fos* expression when tested at ZT16. This is true for all groups except the ZT16 group tested at 24 hours, which showed a trend for higher *c-Fos* expression than the ZT4 group, but there is no statistically significant difference. These findings could be interpreted as circadian rhythms in *c-Fos* expression in the amygdala, with suppressed expression of *c-Fos* in ZT16 rats tested at 24 hours. In other words, the general pattern of *c-Fos* we see in other groups leads us to think that the ZT16 group tested at 24 hours is unique, potentially pointing to inhibited amygdala activity in ZT16 rats tested at 24 hours. In this way, the *c-Fos* pattern suggests an active suppression of the amygdala and fear behavior at the 24-hour recall interval in ZT16 rats.

In all of the brain regions of interest – the BLA, BMA, CEA, and MEA–, we did not see any time-of-day differences in *Per1* mRNA expression. While on its own this may not seem significant, this data is extremely exciting because of what the literature says about *Per1* expression. Previous studies have shown time-of-day differences in basal *Per1* expression in the CEA, MEA, and BLA, where we observe peak expression around ZT16 and trough expression around ZT4 (Chun et al., 2015). In our experiment, after trace fear conditioning, we no longer observe this time-of-day difference. This might be because *Per1* is acting as an immediate early gene. In our case, fear recall may be a stimulus capable of inducing *Per1* gene expression. If this were the case, we might not expect to see any increases in *Per1* expression in ZT16-trained rats, as expression is already near ceiling-levels from circadian regulation. However, in our experiment, ZT4-trained rats also display near-ceiling levels of expression when we would expect those levels to be much lower (Figures 7, 8, 9, 10, and 11). We know that these results are not due to a faulty probe or any other errors since we saw time-of-day differences in the prefrontal cortex, and our amygdala tissue and the mentioned prefrontal cortex tissue were run together, providing evidence that our assay worked well. Thus, perhaps trace fear recall induced *Per1* expression in ZT4 rats to levels observed in ZT16 rats, effectively getting rid of the time-of-day difference in amygdala *Per1* that we have shown in our published work.

Building on the idea that the amygdala has a local biological clock that shows peak *Per1* mRNA levels in the early active phase and the lowest levels in the early inactive phase (Chun et al, 2015), our experiment provides evidence that when rats undergo a fear paradigm, this circadian rhythm may be abolished. In turn, *Per1* mRNA is expressed at ceiling levels regardless of the time of day (Figure 12). This change in *Per1* levels across the day could lead to circadian impairments in amygdala function that lead to adverse downstream effects. For example, the rats

may begin to show disruptions in fear-related learning and memory behaviors, such as altered responses to future dangers or changes in fear learning and memory.

One future follow-up experiment could be one that looks at all of the same treatment groups and measures, but with a control group that receives no foot shocks. This would allow us to see whether the time-of-day differences we observe are due to time-of-day differences in general neural activity versus the fear recall task. Another future experiment could be one that looks at *c-Fos* expression in subpopulations of neurons, such as fear neurons and extinction neurons (Orsini and Maren 2012). This would shed light on the specific neural circuitry behind trace fear conditioning and also potentially help explain some of the data presented by our experiment. Based on our data, we hypothesize that the BMA may be a brain region necessary for fear behavior output in the trace fear paradigm. To test this hypothesis, we could lesion or inhibit the activity of the BMA and see whether fear behavior persists or is extinguished. Another follow-up experiment to run would be to replicate everything except using fluorescent *in situ* hybridization to count *Per1*-positive cells. This would enable us to see if *c-Fos* and *Per1* expression occur in the same cells.

Ultimately, the main purpose of our experiment was to provide novel information for the literature that is used to develop new treatment methods and improved diagnosis techniques for fear-based mental disorders. The differences between our findings and those of delayed fear conditioning may encourage other researchers to see which neural connections might be useful for further characterization of trace fear conditioning. The time-of-day differences we observed might lead to more individualized treatment that accounts for the time of day at which the initial traumatic experience happened and when the treatment should be administered, for example in PTSD. If the hypothesis we proposed of an innate inhibition mechanism were to hold up,

treatment methods that sustain the inhibition could be developed. Other work in our lab suggests a circadian contribution of the prefrontal cortex. All in all, our data has tremendous downstream clinical relevance and countless opportunities for more exploration.

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