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Adrenal-dependent diurnal modulation of conditioned fear extinction learning

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Abstract

Post Traumatic Stress Disorder (PTSD) is associated with altered conditioned fear extinction expression and impaired circadian function including dysregulation of glucocorticoid hormone secretion. We examined in adult male rats the relationship between conditioned fear extinction learning, circadian phase, and endogenous glucocorticoids (CORT). Rats maintained on a 12 hr light:dark cycle were trained and tested across 3 separate daily sessions (conditioned fear acquisition and 2 extinction sessions) that were administered during either the rats' active or inactive circadian phase. In an initial experiment we found that rats at both circadian phases acquired and extinguished auditory cue conditioned fear to a similar degree in the first extinction session. However, rats trained and tested at zeitgeber time-16 (ZT16) (active phase) showed enhanced extinction memory expression during the second extinction session compared to rats trained and tested at ZT4 (inactive phase). In a follow-up experiment, adrenalectomized (ADX) or sham surgery rats were similarly trained and tested across 3 separate daily sessions at either ZT4 or ZT16. ADX had no effect on conditioned fear acquisition or conditioned fear memory. Sham ADX rats trained and tested at ZT16 exhibited better extinction learning across the two extinction

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sessions compared to all other groups of rats. These results indicate that conditioned fear extinction learning is modulated by time of day, and this diurnal modulation requires the presence of adrenal hormones. These results support an important role of CORT-dependent circadian processes in regulating conditioned fear extinction learning, which may be capitalized upon to optimize effective treatment of PTSD.

Keywords

Conditioned fear; extinction; circadian; glucocorticoid; PTSD

1.1 Introduction

The ability of organisms to form associations between innately dangerous events (unconditioned stimuli or US) and surrounding cues (conditioned stimulus or CS) serves an adaptive function—it permits organisms to better escape or avoid dangerous situations. Normally, conditioned fear responses diminish with repeated exposure to the CS in the absence of the US due to the acquisition of new competing conditioned fear extinction associations [1]. However, in many individuals with anxiety-related psychiatric disorders the expression of conditioned fear persists and becomes pathological [2,3]. Patients suffering from these maladies, as well as subjects in an animal model of post traumatic stress disorder (PTSD), show deficits in fear extinction learning [4,5]. Given the debilitating and persistent nature of PTSD, research leading to a better understanding of the underlying neural processes that regulate conditioned fear and extinction expression may lead to improved treatment strategies.

Conditioned fear is an aversive learning process that is highly sensitive to environmental conditions such as context and degree of stress [6]. Time of day has also been found to modulate various aspects of conditioned fear expression and extinction in rodent and human studies [7–10]. Glucocorticoid hormones (CORT, cortisol in humans and corticosterone in rats) are a well-known transducer of the biological effects of environmental stressors [11] and time of day [12–14]. There has been some examination of CORT-dependent modulation of auditory cue and contextual conditioned fear acquisition, memory, and extinction but virtually no examination of CORT effects on auditory cue conditioned fear extinction learning (reviewed in Rodrigues et al., 2009 [15]). Furthermore, no prior studies have examined whether CORT regulates various aspects of conditioned fear and extinction expression in a diurnal fashion. Subregions of the amygdala are central to conditioned fear and extinction learning, and the medial prefrontal cortex (mPFC) and hippocampus contribute critical modulation of these processes [16–18]. Neuroplasticity in the mPFC and hippocampus is sensitive to circadian modulation [8,19], and a number of related cognitive functions exhibit diurnal performance patterns [20–22].

The goals of our study were to determine if there was a time of day difference in conditioned fear and/or extinction learning in rats (experiment 1), and to assess whether the presence of adrenal hormones was necessary for any observed time of day differences (experiment 2). Emotionally driven memories are strongly modulated by the presence and/or timing of acute CORT administration [23]. Therefore, diurnal variations in conditioned fear learning and/or

extinction learning may be modulated by the presence or absence of endogenous CORT. For this study we assessed conditioned fear to a discrete auditory cue as well as extinction learning when it was presented in a context separate from the initial cue-shock pairing in order to assess the diurnal and adrenal-dependent effects on cue specific conditioned fear learning and extinction [24].

2.1 Methods

2.1.1 Animals

For experiments 1 (n=6, N=12) and 2 (n=6, N=24) male Sprague Dawley rats (250–280g; Harlan Laboratories, Indianapolis, IN) were housed two per cage (polycarbonate tubs, 47 cm × 23 cm × 20 cm) and given food (Teklad Rodent Diet 8640; Harlan) and water *ad libitum*. For experiment 2, rats were given food and either tap water (Sham rats) or 0.9% saline (adrenalectomized, ADX rats) *ad libitum*. Rats were housed in two separate rooms within a suite of rooms that included the behavioral testing rooms. Each housing room had independent control of its light/dark cycle, and all rats were maintained on a 12 h light/dark cycle but with the phase adjusted so that behavioral testing occurred between 0900 and 1100h. All behavioral tests took place at either zeitgeber time (ZT; hours after light phase onset in rats housing room) 4±60 min or ZT16±60 min. Rats were given 2 weeks to adjust to their respective light/dark phase prior to behavioral testing. For experiment 2, the surgical procedure occurred in the middle of the 2 week adjustment period, allowing for 1 week of surgical recovery before the onset of behavioral testing. Experimental 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.

2.1.2 Adrenalectomy Surgery

For experiment 2 rats received either bilateral adrenalectomy (ADX) or Sham-ADX. During surgery rats were anesthetized with halothane. Bilateral incisions were made through the dorsal lateral skin and peritoneal wall near the kidney, and adrenal glands of ADX rats were isolated with forceps and excised. Sham rats went through the same procedure, but adrenal glands were left in place.

2.1.3 Fear Conditioning and Testing Apparatus

Conditioned fear acquisition took place in rectangular chambers (25.4×25.4×30.4cm) comprised of 3 stainless steel walls and a Plexiglas front with a shock grid floor comprised of stainless steel rods placed 2 mm apart (context A). Shock grid floors were connected to a current generator (Precision Regulated Animal Shocker, Whitehall, PA) used to provide a 2 second 0.8mA foot shock. Audio speakers (Coulbourn Instruments, Whitehall, PA) were located on the top of the shuttle boxes and designed to provide a 30 second 1 kHz 70dB tone. Extinction training took place in clear Plexiglas boxes (28.6×18.5×22cm) with wire mesh floors (context B). Extinction boxes were contained in sound attenuating icebox coolers containing the same type of audio speakers used in context A. Context B boxes, but not context A boxes, were cleaned with 70% ethanol and allowed to dry before and after each session. Context B boxes were scented with peppermint oil (Now Foods, Bloomingdale, IL) during the extinction sessions.

2.1.4 Behavioral Testing

Development of conditioned fear or conditioned fear extinction can be acquired within a single session, and memory of each can be directly assessed in a follow-up session. Each of these training and testing phases of conditioned fear expression can be effectively accomplished within a relatively short session (< 40 min), which allows for scheduling of these sessions at the same time each day for each experimental subject. Thus, we trained and tested rats over the course of 3 separate sessions that took place either during the rats' inactive (lights-on) or active (lights-off) circadian phase. For both experiments rats were trained and tested at either ZT4±60 min or ZT16±60 min (Fig 1). At ZT4 house lights remained on for testing. At ZT16 training and testing were performed under red light conditions. Session 1 (conditioned fear acquisition): rats were placed in context A for a 5 minute pre-exposure period after which they were trained to fear a 30 sec 1 kHz 70dB tone conditioned stimulus (CS) that co-terminated with a single 2 sec 0.8 mA foot shock unconditioned stimulus (US; one CS-US pairing). Rats remained in the testing chambers for 1 minute post-shock. Session 2 (conditioned fear recall and extinction training): 24 h after session 1 rats were pre-exposed to context B for 3 min followed by fifteen 30 sec 1 kHz 70dB tones unaccompanied by a foot shock (CS alone, random inter-trial interval 90–120 seconds). Freezing during the first 3 trials (trial block 1) was used as a measure of conditioned fear recall. Session 3 (additional extinction training): 24 h after session 2 rats were pre-exposed to context B for 3 min followed by fifteen 30 sec 1 kHz 70dB tones unaccompanied by a foot shock (CS alone, intertrial interval 90–120 sec). Freezing during the first 3 trials (trial block 1) was used as a measure of conditioned fear extinction recall, with the remaining trials serving as additional extinction training.

For each session the behavior of each rat was scored in real-time by an experimenter blind to the treatment conditions of each rat (Sham vs. ADX). Freezing was defined as lack of movement except for that due to breathing. Briefly, rats were assessed and scored as either freezing or not freezing every 10 seconds during the tone and the intertrial interval. The sessions were also video recorded, and a second researcher likewise blind to treatment conditions of each rat scored video recorded behavior for a sample of rats. For session 1 behavioral scores are given as percent time freezing in 60 second blocks for the 5 minute pre-exposure, percent time freezing for the 30 second tone, and percent time freezing during the 1 minute post-shock exposure period; for sessions 2 and 3 behavioral scores are reported as the percent of time spent freezing during and after the cue; The inter-rater reliability for percent time freezing was on the average greater than 95%. For sessions 1 (acquisition) freezing data are reported for individual trials. For clarity, freezing data for sessions 2 and 3 are reported in blocks of three collapsed trials.

2.1.5 Statistical Analysis

Mixed design analysis of variance (ANOVA) was used to test for within and between group treatment effects for each session. For the first experiment, time of day was a between groups factor and trial or trial block was a within group factor. In the case of significant ANOVA main effects and interactions, Student's t-test was used as a post hoc test for time of day differences within a specific trial or trial block. For the second experiment, time of day and CORT status (Sham vs. ADX) were between groups factors, and trial or trial block

was a within group factor. In the case of significant ANOVA main effects and interactions, Fisher's least significant difference (FLSD) was used as a post hoc test for between group differences within a specific trial or trial block. For sessions 2 and 3 pre-tone freezing was analyzed separately and was not included in the trial block by treatment group ANOVAs. The statistical package for the social sciences (SPSS, version 3.0 1992) was used for these analyses; $p < 0.05$ was considered significant. Data presented in figures are the mean $\pm$ SEM, and all significant effects are reported.

3.1 Results

3.1.1 Experiment 1: Rats exhibited better conditioned fear extinction learning when trained and tested at ZT16

Session 1 (Day 1) Conditioned Fear Acquisition—There was no effect of time of day on conditioned fear acquisition freezing behavior (Fig 2A). Rats trained at either ZT4 or ZT16 exhibited minimal freezing behavior before and during the tone and displayed high levels of freezing during the 1 minute post-shock observation period.

Session 2 (Day 2) Expression of Conditioned Fear and Extinction—Rats tested at either ZT4 or ZT16 exhibited comparable levels of freezing throughout the conditioned fear recall/extinction session (Fig 2B). Both groups of rats froze to a similar extent during block one trials, indicating they had similar 24 h recall of conditioned fear memory for the tone. There was a significant decrease in freezing across trial blocks ($F_{(4, 36)} = 9.2$, $p < 0.001$), but no significant interaction between trial block and time of day. This suggests that both groups of rats developed extinction of their conditioned fear response over the course of the session at the same rate and to the same extent.

Session 3 (Day 3) Additional Extinction—There was a significant interaction between trial block and time of day ($F_{(4, 36)} = 3.2$, $p = 0.023$) such that rats trained and tested at ZT16 exhibited less initial freezing and therefore better conditioned fear extinction learning compared to rats trained and tested at ZT4 (Fig 2C). Post hoc tests revealed a significant difference between groups in block 1 ($p = 0.042$), representative of superior extinction recall in rats trained and tested at ZT16.

3.1.2 Experiment 2: Adrenalectomy abolished time of day differences observed in conditioned fear extinction learning

Session 1 (Day 1) Conditioned Fear Acquisition—As in the first experiment, we found no significant differences between groups trained at either time of day in conditioned fear acquisition. We also observed no effect of CORT status (Sham vs. ADX) on session 1 baseline or post-shock freezing (Fig 3A). Similar to experiment 1, all rats exhibited minimal freezing before and during the tone, and high levels of freezing during the 1 minute post-shock observation period.

Session 2 (Day 2) Expression of Conditioned Fear and Extinction—As in the first experiment, rats tested at ZT4 or ZT16 showed comparable 24 h recall of conditioned fear memory (performance during first cue exposure trial block) (Fig 3B). There was an

overall significant decrease in freezing across trial blocks ($F_{(4, 80)} = 29.0$, $p < 0.001$) but no significant interaction between trial block and time of day or CORT status. Each of the 4 treatment groups exhibited a significant decline in freezing levels across the 5 trial blocks as assessed by separate repeated measures ANOVA for each treatment group ($p < .01$ for each treatment group). There was also an overall significant interaction between time of day and CORT status ($F_{(1,20)} = 6.6$, $p = 0.018$). Post hoc tests indicate that Sham ZT16 rats froze less than Sham ZT4 rats during trial blocks 2 and 5, however, this time of day difference was not present in ADX rats. There was also a difference in freezing between Sham ZT16 and ADX rats for the first trial block. Thus, it appears in this second experiment that adrenal-intact, but not ADX rats expressed extinction learning better during this first extinction training session when trained at ZT16 than at ZT4.

Session 3 (Day 3) Additional Extinction—As observed in the first experiment, adrenal-intact (Sham) rats trained and tested at ZT16 exhibited less initial freezing than rats trained and tested at ZT4 (Fig 3C), however, this reduced freezing was absent in ADX rats. There was an overall significant interaction between time of day and CORT status, $F_{(1, 20)} = 4.6$, $p = 0.044$. Post hoc tests indicate that Sham ZT16 rats had less freezing than all other rats in blocks 1, 2, and 4, whereas ADX rats trained and tested at either time of day exhibited levels of freezing comparable to those of Sham ZT4 rats in all trial blocks. We also found a trial block by time of day interaction ($F_{(4, 80)} = 2.9$, $p = 0.025$) primarily due to the slope difference across trials between Sham ZT16 rats and the other treatment groups. By block 5 there was no time of day difference, but post hoc analysis revealed a difference between Sham ZT16 rats and ADX ZT16 rats ($p = 0.019$).

To assess the overall treatment effects on the composite extinction learning across the 2 extinction training sessions we performed an ANOVA that included all 10 trial blocks as a repeated measure factor. There was an overall time of day effect ($F_{(1,20)} = 5.5$, $p = 0.029$), CORT status effect ($F_{(1,20)} = 6.0$, $p = 0.024$) and a time of day by CORT status interaction ($F_{(1,20)} = 8.86$, $p = 0.007$), indicating that there was an adrenal-dependent time of day effect on overall extinction learning across the two extinction training sessions.

4.1 Discussion

Our experiments revealed an adrenal-dependent time of day difference in auditory cue conditioned fear extinction learning. In contrast, neither adrenal status nor time of day influenced the acquisition of conditioned fear. Specifically, 24 h recall of auditory cue conditioned fear was comparable in adrenal-intact and ADX rats trained and tested at ZT16 (active phase) or ZT4 (inactive phase). However, adrenal-intact rats, but not ADX rats, trained and tested at ZT16 displayed reduced freezing during the two extinction training sessions compared to adrenal-intact and ADX rats trained and tested at ZT4.

Our finding of an absence of a time of day effect on the acquisition of cue conditioned fear is consistent with most studies [9,25,26], with the exception of one mouse study [7]. Importantly, our finding of superior cue conditioned fear extinction learning in rats trained and tested during their active phase is in good agreement with previous findings in both mice [7] and humans [10]. Chaudhury and Colwell [7] also found that the diurnal difference

in fear extinction learning persisted in mice maintained in constant darkness, suggesting that there is an underlying circadian rhythm of extinction learning performance. Further studies will be necessary to discern whether specific brain regions are responsible for the selective diurnal regulation of auditory cue conditioned fear extinction learning that does not extend to conditioned fear learning. Both the PFC and hippocampus modulate amygdala-dependent conditioned fear extinction learning and expression [18], so these are good targets for initial examination.

The extent to which time of day modulated both within session extinction acquisition and the subsequent consolidation and retrieval of extinction memory cannot be readily resolved from our study. There is precedence from other studies for better within session extinction learning of mice and humans during the active phase compared to the inactive phase [7,10]. In our first experiment rats trained and tested at either time of day exhibited comparable levels of freezing during session 2 extinction training. However in experiment 2, Sham rats in the ZT16 condition displayed somewhat lower freezing levels during the first extinction training session than Sham (but not ADX) rats in the ZT4 condition. It also bears mentioning that there was overall greater freezing levels of rats during extinction training in experiment 2 compared to experiment 1. This could be simply a cohort difference. Alternatively, overall decreased rate of extinction learning in experiment 2 could be a result of residual effects of prior surgery. Surgery stress has been shown to have long lasting effects on other learning processes [27]. Regardless of these possibilities, the same general diurnal pattern of results was observed in both experiments.

An important question raised by our results is how does the presence of adrenal hormones influence conditioned fear extinction learning in a diurnal fashion? Although both adrenal catecholamine and CORT hormone secretion may be responsible for this adrenal-dependent effect, several considerations point to CORT as providing an important contribution. First, CORT, in contrast to adrenal catecholamines, crosses the blood brain barrier. Second, CORT has a very prominent circadian rhythm of secretion [11]. Third, many studies find effects of acute CORT manipulations on conditioned fear learning, although no prior studies have examined CORT modulation of auditory fear extinction learning (reviewed in [15]). It is not clear whether acute effects of CORT on conditioned fear and extinction learning, as previously reported, would be differentially engaged at ZT4 or ZT16. Most rodent studies have examined the acute effects of CORT manipulations on memory consolidation/retrieval only during the experimental subjects' inactive phase [15]. It is noteworthy that in our study, ADX only affected auditory extinction learning and relapse in rats trained and tested during their active phase.

Given the likelihood that CORT is a critical adrenal factor responsible for our pattern of results, how could the presence of endogenous CORT modulate auditory conditioned fear extinction learning in a diurnal fashion? One consideration is that the variation in basal CORT levels present in rats at the two training/testing times of day contribute to differential consolidation, retrieval, and performance. In rats, basal plasma CORT levels peak around the onset of the dark phase (ZT12) and steadily decline throughout the dark phase [11]. This diurnal profile is closely correlated with free CORT levels present in brain tissue [28]. Consequently, in our study CORT levels prior to training and testing were likely higher in

adrenal-intact rats trained and tested at ZT16 compared to rats trained and tested at ZT4. An elevation of CORT prior to memory retrieval tends to impair retrieval [29]. Thus, it is unlikely that elevated CORT levels per se influenced test performance; if this were the case then retention of the fear extinction memory (as indicated by block 1 freezing levels in session 3) for rats in the ZT16 condition should have been worse than for rats in the ZT4 condition.

A second consideration is that there may be different acute elevations of CORT levels elicited by the training and test sessions at these two times of day. Others find that CORT treatment immediately after contextual and auditory conditioned fear training results in improved 24 h memory [30]. We expect that each daily auditory conditioned fear training or testing session elicited a robust acute endogenous CORT response, as has been seen in other studies [31]. However, these elicited CORT levels were likely similar at both times of day, as stress-induced CORT levels in general do not vary with time of day [11].

We propose a third novel consideration by which CORT could lead to enhanced extinction learning at ZT16. The diurnal rhythm of circulating CORT could serve to entrain the normal circadian oscillation of clock gene expression in neurons located in brain regions that support fear extinction, such as the PFC, hippocampus, and amygdala. By this account, clock-controlled gene products regulate neuronal plasticity in a diurnal fashion and thereby differentially support the acquisition and/or retrieval of fear extinction memory [32]. Disruption of the normal oscillation of clock gene expression would interfere with these processes. Several facts are consistent with this hypothesis. Treatment of ADX rats with CORT in the drinking water, which approximates diurnal variations in basal CORT levels [33] is sufficient to normalize ADX-induced deficits in contextual fear memory [34] or PFC-dependent working memory [35]. Disruption of the expression of circadian rhythms by alteration of light/dark cycles impair contextual conditioned fear memory, PFC neuronal dendritic architecture, and associated cognitive flexibility [36,37]. Mice with genetically impaired clock gene expression have impaired conditioned fear memory [38,39], spatial learning [40] and emotional expression [41]. Diurnal CORT is capable of entraining the molecular clock in many different peripheral tissues such as the liver, kidney, and cornea [13]. We recently found that ADX disrupts normal clock gene oscillation in the PFC, and this disruption is normalized when CORT is replaced at the normal circadian peak time (Woodruff unpublished observations and [42]). Thus, an intriguing prospect is that the absence or improper timing of daily endogenous CORT secretion can lead to impaired extinction learning by disruption of the normal oscillation of clock gene expression within relevant brain areas. Regardless of which of these considerations underlies the adrenal-dependent diurnal effect on conditioned fear extinction learning, our results indicate that the effect was not due to an overall reduced extinction expression in the absence of those hormones. Further, the ADX by time of day interaction reduces the prospect that time of day variation in auditory cue extinction learning was simply due to impaired performance at ZT4 as a result of decreased activity levels or sleep disruption [43].

PTSD is a highly persistent disorder characterized by substandard extinction expression [44,45]. Clinicians and researchers have been vexed by the problem of finding a successful treatment for this disorder. To date, exposure therapy is one of the most widely used

treatments [46] although long-term success remains limited. Perhaps one of the most challenging obstacles to successful PTSD therapy is maintaining conditioned fear extinction expression outside of the clinical setting [46]. Altered function of both glucocorticoids and circadian rhythms are associated with PTSD [47,48]. New mechanistic understanding of how these two systems interact to modulate neuronal function associated with PTSD may yield important new therapeutic strategies.

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Highlights

- Conditioned fear extinction learning shows a robust time of day difference.
- This effect is dependent on the presence of adrenal hormones, likely glucocorticoids.
- This study is relevant to research aimed at optimizing therapy for anxiety related disorders.

Figure 1.

Experimental timeline. Rats were trained for auditory cue conditioned fear at either ZT4 or ZT16 and tested for extinction learning at the same ZT. For experiment 2 rats received either adrenalectomy or sham surgery 1 wk into acclimation.

Figure 2.

Experiment 1: Rats exhibited enhanced conditioned fear extinction learning if trained and tested at ZT16. A: Conditioned fear acquisition was not dependent on time of day. Rats were trained to fear a tone when paired with a mild foot shock at either ZT4 or ZT16. B: Conditioned fear (CF) recall was not dependent on time of day (Trial block 1). Conditioned fear extinction was also not dependent on time of day. Rats received extinction training at either ZT4 or ZT16, 24h after session 1 acquisition. C: Conditioned fear extinction recall was dependent on time of day (Trial block 1). Rats trained and tested at ZT16 show enhanced extinction learning compared to those trained and tested at ZT4 (significant time of day by trial block interaction, $p < 0.05$). Rats received additional extinction training at either ZT4 or ZT16, 24h after session 2 extinction. * time of day difference, $p < 0.05$, $n = 6$.

Figure 3.

Experiment 2: Adrenalectomy abolished time of day differences observed in conditioned fear extinction learning. A: Conditioned fear acquisition was not dependent on time of day or adrenal status. Sham and adrenalectomized (ADX) rats were trained to fear a tone when paired with a mild foot shock at either ZT4 or ZT16. B: Conditioned fear (CF) recall was not dependent on time of day or adrenal status (Trial block 1). Adrenal-intact (Sham) but not ADX rats exhibited somewhat better extinction performance when trained at ZT16 compared to ZT4. Sham and ADX rats received extinction training at either ZT4 or ZT16, 24h after session 1 acquisition. C: Conditioned fear extinction recall (Trial block 1) and additional learning (Trial blocks 1–5) were dependent on time of day and adrenal status. Adrenal-intact rats trained and tested at ZT16 showed enhanced extinction learning compared to all other groups. Rats were tested for additional extinction learning at either ZT4 or ZT16, 24h after session 2 extinction training. + denotes $p < .05$, Sham ZT16 less freezing than all other groups; * denotes $p < .05$, Sham ZT16 less freezing than Sham ZT4; # denotes $p < .05$, Sham ZT16 less than ADX ZT16.