

Behavioral and Neurobiological Assessments of Predator-Based Fear Conditioning and Extinction

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Abstract

Shock, immobilization, and exposure to predator-related stimuli have all been used to study fear conditioning in rodents, but they have never been used in conjunction in a single study. Experiment 1 compared the effects of these three reinforcers, alone and in various combinations, on the expression of long-term conditioned fear memory and extinction in adult male rats. Whereas foot shock conditioning, alone, was rapidly extinguished; the combination of immobilization and cat exposure, or all 3 stimuli together, produced a significant increase in the magnitude of fear conditioning and greater resistance to extinction, which persisted for at least 5 weeks post-training ($p < 0.05$). Experiment 2 assessed the role of the hippocampus in predator-based context and cued fear conditioning. Pharmacological suppression of hippocampal activity during fear conditioning produced a selective impairment of contextual, but not cued, fear memory. Experiment 3 investigated the effects of sleep deprivation prior to fear conditioning on the expression of fear memory. This experiment demonstrated that pre-training sleep deprivation blocked the expression of contextual (hippocampal-dependent), but not cued (hippocampal-independent), fear memory. Overall, this series of experiments has extended the use of predator exposure in conjunction with conventional reinforcers, such as foot shock and immobilization, to advance our understanding of the neurobiology of traumatic memory.

Keywords

Fear Conditioning, Predator Exposure, Hippocampus, Sleep Deprivation

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1. Introduction

Fear associations to environmental stimuli are part of an evolutionary defensive behavior system that protects individuals from danger [1]-[4]. Fear conditioning paradigms in a broad range of species [5]-[7] have investigated how environmental stimuli become associated with aversive events [8] [9], and the neural mechanisms underlying associative learning have been studied [10] [11]. Moreover, understanding the neurobiological basis of fear conditioning should enhance our understanding of how traumatic experiences produce pathologically intense and intrusive memories which underlie psychiatric disorders, such as post-traumatic stress disorder (PTSD).

A vast literature has demonstrated that the amygdala is necessary for fear-based memory associations to be made [12]-[16]. Lesions of the amygdala block Pavlovian fear conditioning [17] and prevent freezing expressed to contexts and cues associated with foot shock [18]-[23]. Lesions of the amygdala made one week before or up to one month after training can block memory-related (freezing) behavior [19]. More recent work targeting the molecular biology of amygdala neurons has confirmed the crucial role of the lateral and basolateral subdivisions of the amygdala in the formation and expression of fear memories [24]-[27].

Whereas the amygdala is necessary for fear conditioning to occur, the hippocampus is involved in the processing of the representation of the components of the environment (context) in which the fear experience occurred [13] [23] [28] [29]. Investigations of the role the hippocampus plays in Pavlovian contextual fear conditioning have shown that lesions of the dorsal hippocampus made prior to [23] [30]-[33] or soon after [34] conditioning block freezing upon re-exposure of the subject to the conditioning context. In hippocampus-lesioned animals, associations formed to discrete cues presented in conjunction with the aversive stimulus remain intact, as indicated by freezing to discrete stimuli, such as auditory cues. Parsons & Otto [35] used the GABA receptor agonist muscimol to demonstrate that temporary inactivation of the dorsal hippocampus produced context-specific fear memory deficits. In the past decade novel approaches employing optogenetic manipulation of cell activity have confirmed and extended our understanding of the necessary involvement of the hippocampus in the formation and consolidation of the contextual features of fear conditioned memory [24] [25] [36]-[38].

In an ethologically relevant approach to the study of the neurobiology of fear, numerous laboratories have assessed the effects of predator exposure, or predator-based cues, on rodent brain, behavior and memory [39]-[53]. One group, in particular, has conducted an extensive behavioral and anatomical assessment of predator-based fear conditioning. Canteras and co-workers have identified predator-specific fear circuitry in the brain [54] [55], with evidence that sub-nuclei in the amygdala, thalamus, periaqueductal grey and hypothalamus are responsive to sensory and memory-specific processing of predator cues [22] [53] [56]-[58].

Our group has also examined the influence of predator exposure on the brain and behavior, with an emphasis on memory-related functions of the amygdala and hippocampus. We have shown that cat exposure impairs rat hippocampus-dependent memory [59]-[61] and blocks hippocampal synaptic plasticity [62]. It is noteworthy that we have shown that the same stressor that blocks hippocampal synaptic plasticity (cat exposure) enhances synaptic plasticity in the amygdala [63]. In speculative reviews we have discussed how the formation of new fear memories with cat exposure can impair spatial (hippocampal) memory, and simultaneously produce strong hippocampal and amygdala-based memories of the traumatic experience [64]-[66].

In addition to our studies on acute predator exposure, synaptic plasticity and memory, we have developed a predator-based animal model of PTSD. We reported that rats exposed to a cat on two occasions, occurring within a 31-day period of chronic social instability, exhibited numerous behavioral and physiological outcomes which resemble the clinical symptoms of PTSD (for reviews see [67] & [68]). The most relevant observation from this work with regard to the current study is our finding that rats exhibit a long-term fear conditioned memory of the context and cue associated with the cat exposure component of chronic psychosocial stress [69]-[71] which persisted for at least 4 months [72].

The current series of experiments is an extension of the fear conditioning component of our PTSD model, with an emphasis on exploring factors that influence the magnitude and durability of the predator-based fear memory. Specifically, in the three experiments described here we have examined: 1) the magnitude of contextual and cued fear memory when the reinforcement was predator exposure, shock or immobilization, individually and when multiple reinforcers were presented together; 2) the rate of fear memory extinction when the conditioning stimuli were delivered over the course of 3 weeks of extinction training; 3) the influence of pharmacological inactivation of the dorsal hippocampus on contextual and cued predator-based fear conditioning; and 4)

the influence of sleep deprivation on the expression of contextual and cued predator-based fear conditioning.

2. Experiment 1

In Experiment 1, we assessed the effects of different aversive stimuli on context and cued fear conditioning and extinction. This experiment addressed the hypothesis that shock, immobilization, and predator exposure, alone or in combination, would result in a synergistic amplification of the magnitude, duration and resistance to extinction of the fear conditioned memory. Standard foot shock conditioning was used as an unconditioned stimulus (US) to compare its effects, as an established conditioning stimulus, with immobilization and cat exposure. Immobilization was utilized because restraint stress enhances fear conditioning and resistance to extinction [73]-[75]. Immobilization also produces alterations in neurotrophic factors, such as glutathione [76], brain-derived neurotrophic factor [77] [78] and the immediate early gene *c-fos* [79]. Furthermore, immobilization influences the hypothalamic pituitary adrenal (HPA) axis and adrenergic systems modulated by glucocorticoid receptors in the brainstem, hypothalamus, and locus coeruleus [80]. Therefore, immobilization, alone, was assessed as a US. Predator exposure was also used as a US, alone and in conjunction with foot shock and immobilization. Overall, Experiment 1 was designed to assess the potential combined effects of the delivery of combinations of immobilization, predator exposure and shock on the persistence and magnitude of fear conditioning memory, as well as its resistance to extinction.

2.1. Method

Animals. A total of 48 male Sprague-Dawley rats (Charles River Laboratories) approximately 8 weeks old and weighing 225 - 250 g upon arrival were used in this experiment. All rats were acclimated to the vivarium and cage cleanings for at least 7 days before any experimental manipulations were performed. Rats were housed 2 per cage in standard Plexiglas cages (46 × 25 × 21 cm) with tap water and rat chow available *ad libitum*. The animal housing room was maintained at 20°C ± 1°C with a humidity range of 60% ± 3% and a 12-hr light cycle (lights on at 700). All procedures were approved by the Institutional Animal Care and Use Committee at the University of South Florida.

Conditioning Apparatus and General Procedure. All animals received the same general treatment outlined as follows. Rats were transported in their home cage to the laboratory approximately 30 min before conditioning was initiated. The rats were removed from their home cages and placed inside a standard fear conditioning box (25.5 × 30 × 29 cm; Coulbourn Instruments; Allentown, PA) which was inside a separate larger sound attenuation chamber. The conditioning box consisted of aluminum sides, an aluminum ceiling, and a Plexiglas front and back covered with black plastic. The floor consisted of 18 stainless steel rods, spaced 1.25 cm apart. This box was also the context test apparatus; the cue test apparatus is outlined in the fear association section. After the rats were in the box for 2 min, they were presented with two 10-sec, 74-dB, 2500-Hz tones, separated by a 40-sec inter-stimulus interval. Thus, total exposure of the rats to the box lasted for 3 min.

As illustrated in **Figure 1**, there were six groups in this experiment (8 rats/group). In two groups of rats each tone presented during conditioning co-terminated with a 2-sec, 0.4 mA foot shock. In the remaining four groups, no foot shock was delivered. For two immobilization groups, immediately with the termination of the training trial, shocked rats (Shock/Cat/Immobilization) and non-shocked rats (No Shock/Cat/Immobilization) were immobilized using plastic Decapi Cones (Braintree Scientific; Braintree, MA). Within 2 min of being immobilized, rats in these two groups were brought to the cat housing room in the vivarium and were then placed individually in a triangle-shaped wedge (20 × 20 × 10 cm) within a pie-shaped Plexiglas enclosure (Braintree Scientific; 46 cm diameter × 8 cm height), which was then placed in a metal cage (61 × 53 × 51 cm) with an adult female cat for 30 min. In addition to these groups, one group of shocked rats (Shock Only) and one group of non-shocked rats (Box Only) were immediately returned to their home cages following the conditioning trial. Another non-shocked group (Immobilization Only) was immediately immobilized in plastic DecapiCones upon completion of the conditioning session. After being immobilized, the rats were placed in the pie-shaped Plexiglas enclosure described above in a room other than the cat housing room, where they remained for 30 min. The last non-shocked group (Cat Only) was placed in a small, unrestrictive, novel Plexiglas box (28 × 9 × 14 cm) after conditioning. Within 2 min of being put in the box, the rats were placed in a metal cage (61 × 53 × 51 cm) with an adult female cat for 30 min. In each group exposed to a cat, canned cat food was smeared on top of the Plexiglas enclosure to direct the cat's attention toward the rats.

Timeline and General Procedures

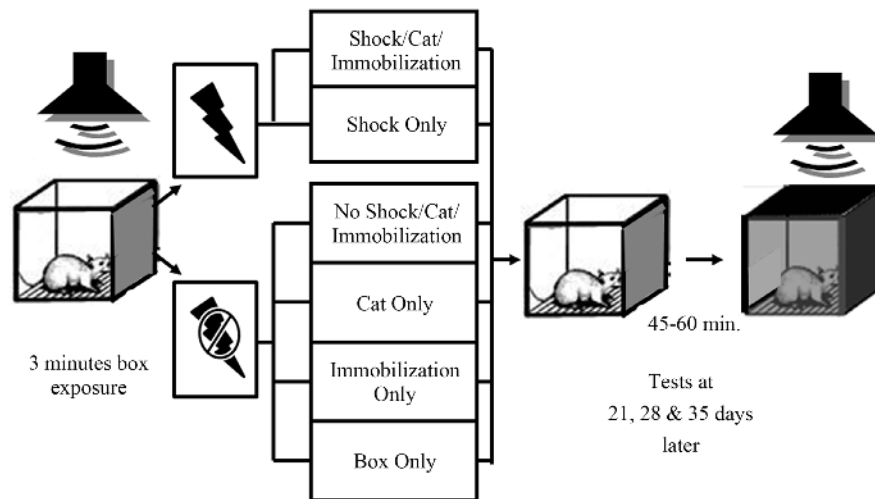


Figure 1. All groups were given 3 min of exposure to the conditioning chamber with 2 tones presented to the rats. Two of the groups received shocks paired with the tones (as illustrated by the lightning bolt symbol). The other 4 groups were not shocked (indicated by the “no” symbol through the lightning bolt). All of the groups received memory tests consisting of unreinforced re-exposure to the conditioning context and, in a different apparatus (indicated as the shaded box), the tone 21, 28 & 35 days after conditioning.

Fear Memory and Extinction Testing. Context and cued fear memory tests occurred 21, 28 and 35 days after conditioning. These time points were chosen to test long-term memory for the conditioning to potentially model intrusive traumatic memories that occur at great delays following a traumatic experience. On fear memory assessment days, the rats were returned to the laboratory and then tested 30 min later. Rats were placed in the same fear conditioning box as the one in which they were placed during conditioning. The immobility of each rat was monitored by computer for five minutes. Immobility data were analyzed using the time window after the first 30 sec until the beginning of the last minute of the 5-min chamber exposure. Thus, a total of 3.5 min were analyzed for immobility in the context. This served as a measure for contextual fear memory. Approximately 45 - 60 min after the contextual memory test, rats were individually placed in a novel, illuminated conditioning box (25 × 22.5 × 33 cm, Coulbourn Instruments; Allentown, PA) that consisted of two aluminum sides, an aluminum ceiling, and a Plexiglas front and back and a square metal floor (21.5 × 21.5 cm). The use of this second box reduced the similarities between the original conditioning chamber and the auditory cue testing box. The tone (74 dB; 2500 Hz) used during training was presented for the last 3 min of a 6-min test. Immobility during each assessment was measured by 24-cell infrared activity monitors (Coulbourn Instruments; Allentown, PA), mounted on the top of the boxes. Freezing was defined as continuous periods of immobility lasting at least 3 sec. A Microsoft Excel macro designed to analyze the percent time freezing calculated the total number of seconds spent freezing by each animal in 30-sec epochs.

Statistical Analyses. Mixed-model ANOVAs were used to detect significant differences between groups in freezing to the context and cue tests, with group serving as the between-subjects factor and test day serving as the within-subjects factor. Post hoc LSDs were employed when the omnibus F test indicated the presence of significant effects. Alpha was set at 0.05 for all analyses, and data points were considered outliers if they were more than 3 standard deviations from the exclusive means.

2.2. Results and Discussion

Context Memory and Extinction. The analysis of freezing during the context tests revealed a significant effect of group, $F(5, 42) = 13.18$, $p < 0.01$, with borderline significance for the Group × Test Day interaction, $F(10, 84) = 1.82$, $p = 0.07$. Across all test days, the Shock/Cat/Immobilization group froze significantly more than all other groups and the Shock Only group and No Shock/Cat/Immobilization group froze significantly more than the Cat, Immobilization and Box Only groups (see **Figure 2**). During the second context test, the Shock/Cat/

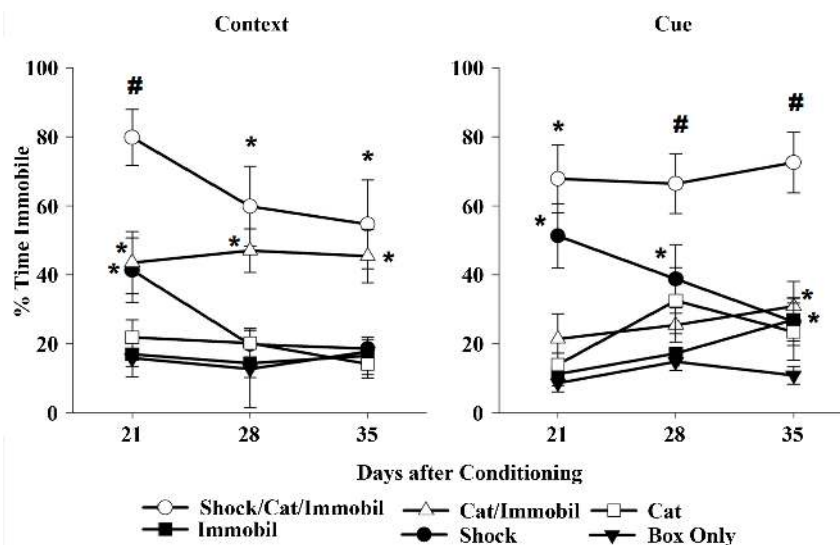


Figure 2. The left graph shows that the group receiving the combination of shock, cat and immobilization exhibited greater freezing in the context than any other group 21 days after conditioning. The no shock/cat/immobilization group also exhibited freezing to the context equivalent to the shock only group. Both the no shock/cat/immobilization and shock only groups spent significantly more time freezing in the context compared to cat, immobilization and box only groups 21 days after conditioning. The shock only group extinguished its freezing to the context, as indicated by reduced freezing on Days 28 and 35. Both the shock and no shock cat/immobilization groups maintained statistically equivalent freezing percentages on days 28 and 35 relative to their initial memory test. The right graph shows that the groups that received shocks spent more time freezing to the tone than groups that were not shocked, but only the group that was shocked, cat exposed and immobilized expressed extinction resistant freezing to the tone. # indicates $p < 0.05$ vs. all other groups, * indicates $p < 0.05$ vs. box only group.

Immobilization and No Shock/Cat/Immobilization groups froze significantly more than the Shock, Cat, Immobilization and Box Only groups. The third context test revealed that the Shock/Cat/Immobilization and No Shock/Cat/Immobilization groups froze significantly more than the Shock, Cat, Immobilization and Box Only groups.

Cue Memory and Extinction. The analysis of freezing during the cue tests revealed a significant effect of group, $F(5, 39) = 12.84$, $p < 0.01$, and a significant Group \times Test Day interaction, $F(2, 81) = 2.36$, $p < 0.05$. During the first cue test, the Shock/Cat/Immobilization and Shock Only groups froze significantly more during the tone than all other groups. During the second and third cue tests, the Shock/Cat/Immobilization group froze significantly more during the tone than all other groups. The Box Only group spent significantly less time freezing during the tone than the No Shock/Cat/Immobilization group, which was statistically equivalent to the Cat Only and Immobilization Only groups.

This experiment examined how three aversive stimuli, used alone or in various combinations, impacted memory. There are three novel findings from this experiment. First, the combination of immobilization with predator exposure in conjunction with standard foot shock conditioning produced greater persistence and magnitude of conditioned contextual and cued fear memory than any of these reinforcers delivered in isolation. The second finding is that the combination of immobilization, cat and shock produced a fear conditioned memory, for both context and cue, which was resistant to extinction, tested at weekly intervals from 3 - 5 weeks after conditioning. The third finding is that the combination of immobilization and predator exposure, in the absence of a standard foot shock US, produced intact contextual, but not cue, fear conditioning which was resistant to extinction training. Finally, immobilization or predator exposure, alone, resulted in a similar pattern to the Box Only group, which was the absence of an expression of fear memory 3 weeks post-conditioning.

One feature that is unique in this experiment is the considerable trace interval (approximately 2 minutes) between context exposure and immobilization to predator exposure, and yet the combination produced robust and extinction-resistant fear. Long trace intervals (more than 30 sec) reduce the associative qualities in Pavlovian conditioning [81] [82]. It is likely that the use of immobilization immediately after removal of the rats from the shock chamber was sufficiently strong reinforcement to bridge the 2 minute trace period between removal of the rat from the context and cat exposure. The relatively long trace period between the time that the rat was removed

from the conditioning box and placed in the presence of the cat (2 min without immobilization) is a likely explanation for the lack of conditioned freezing in the cat only group.

Overall, the results of this experiment indicate that immobilization combined with predator exposure and foot shock produced a powerful synergistic effect on fear memory which was resistant to extinction assessed with 3 weekly exposures to the training context and cue from 3 - 5 weeks post-conditioning. It therefore appears the combination of all three reinforcers may serve as the most effective stimulus to model the pathologically intense and extinction-resistant memories which are a hallmark feature of PTSD and related disorders [83] [84].

3. Experiment 2

Shapiro & Eichenbaum [85] hypothesized that the capacity of the hippocampus to receive and integrate information from different senses allows the hippocampus to generate a coherent representation of the context through the associations made between the information. Thus, the hippocampus is important for acquiring new declarative memories [86] [87] which can be either emotional or neutral in nature. In laboratory animals, damage to the hippocampus seven days before contextual learning [31] or muscarinic cholinergic receptor antagonism of the hippocampus fifteen minutes prior to learning [88] impairs performance on contextual fear conditioning. Rogers, Hunsaker, & Kesner [89] demonstrated that chemical lesions of the dorsal CA1 area of the hippocampus led to significantly less freezing in the conditioned context, yet the lesions did not significantly impair trace auditory cue conditioning. More recent work employing selective optogenetic manipulation of hippocampal cell activity has confirmed the role of the hippocampus in contextual fear conditioned memory [24] [25] [36]-[38]. Based on these findings, experiment 2 tested the hypothesis that inactivation of the hippocampus would result in impaired context, but not cue, predator-based fear expression.

3.1. Method

Design. We employed a 2×2 factorial design, with drug [vehicle (aCSF) or muscimol] and group [immobilization with cat exposure (Cat) or home cage (No Cat)] serving as the factors.

Animals and Surgery. A total of 38 male Sprague-Dawley rats (Charles River Laboratories) were used in this experiment and all aspects of care, age and weight were identical to those in Experiment 1. On the day of surgery, the rats were brought to the laboratory, where all surgical procedures were performed under aseptic conditions. Rats were deeply anesthetized using isoflurane. Their heads were shaved and then placed level in a stereotaxic device. After the skull was exposed, the topographical coordinates for the landmarks of bregma and lambda were recorded for targeting purposes. The target was the dorsal CA1 region of the hippocampus (coordinates, in reference to bregma: -3.8 mm AP, ± 3.0 L, -2.8 DV) and insertions were made with 26-gauge, stainless steel, guide cannula (Plastics One Inc., Roanoke, VA). Guide cannula were held in place by dental cement and anchored to the skull with four skull screws. Removable stylets projecting 1 mm from the tip of the guide cannula were inserted and held in place with a screw-on dust cap (Plastics One Inc., Roanoke, VA) to keep the cannula patent.

Intracerebral Infusions. All animals were given one week to recuperate from surgery before data collection. All infusion and behavioral procedures were performed between 900 - 1500 hours, and animals were tested on three consecutive days. On the first day, the dust cap was removed, and a mock injection tube was placed on the cannula pedestal. The second and third days consisted of removing the dust cap and stylet and gently placing the injectors (Plastics One) into the guide cannula. A Harvard Apparatus pump (Holliston, MA), connected to 25- μ l syringe injectors (Hamilton) by plastic tubing (Plastics One), infused aCSF at a rate of 0.1 μ l/min for 3 min on Day 2 (based on a pilot study optimizing flow rate and dose). After the infusion, the pump was turned off and the fluid was given 1 min to diffuse before the dummy cannula was replaced and dust cap was screwed back on the top of the pedestal. On the third day, aCSF or muscimol (1 μ g/ μ l dissolved in aCSF, a total of 0.3 μ g per infusion site) was administered; these infusion procedures were derived from Holt & Maren [90] and modified based on pilot testing results.

Histology. A total of 36 rats completed testing. Upon completing the behavioral tasks, all animals were euthanized with an overdose of ketamine and xylazine, and cresyl violet was infused into the cannula at a rate of 0.1 μ l/min for 5 min to allow visual inspection of cannula placement. The brains were extracted and flash frozen in methylbutane, and the tissue was stored at -80°C until it was sliced in coronal sections in 40- μ m increments on a Cryostat held at -16°C and mounted on microscope slides. There were 2 animals excluded from analysis

for cannula placement outside of the target area.

Cat Exposure Procedure. Approximately 15 min after the rats were infused with aCSF or muscimol on Day 3, they were placed in a fear conditioning chamber (as described in Experiment 1). Exposure to the chamber for 3 min terminated with the presentation of a single 30-sec, 74-dB, 2500-Hz tone, which served as the auditory cue. Animals in the Cat groups were immediately immobilized and then placed in close proximity to a cat and remained with the cat for 1 hr. Animals in No Cat groups were returned to their home cages. Three weeks later context and cue memory tests were conducted as described in experiment 1, but extinction memory was not tested.

Statistical Analyses. Data were analyzed with 2-way ANOVAs. A priori planned comparisons were tested with two-tailed Student's *t*-tests in each behavioral test of the experiment. Alpha was set at 0.05 for all analyses.

3.2. Results and Discussion

Context Memory. There were significant effects of group, $F(1, 28) = 4.46, p < 0.05$, and drug, $F(1, 28) = 5.95, p < 0.05$, but the Group \times Drug interaction was not significant, $F(1, 28) = 1.34, p = 0.26$. Planned comparisons showed that muscimol infused prior to the cat procedure significantly reduced ($p < 0.03$) freezing compared to aCSF (Figure 3).

Cue Memory. There was a significant effect of group, $F(1, 29) = 9.69, p < 0.01$, but the effect of drug, $F(1, 29) = 0.35, p > 0.05$, and Group \times Drug interaction, $F(1, 29) = 0.64, p > 0.05$, were not significant. Cat exposure resulted in animals freezing more to the cue, relative to the No Cat groups (Figure 4).

In this experiment, the role of the dorsal CA1 region of the hippocampus in Pavlovian predator-based contextual and cued fear conditioning was investigated. Previous work has shown that lesions or inactivation of the dorsal hippocampus prior to foot shock conditioning block the expression of contextual, but not cued, fear memory [23] [30]-[34]. The present experiment demonstrates that the dorsal hippocampus is necessary for predator-based context fear memory. Thus, there is aselective involvement of the hippocampus in contextual, but not cue, fear conditioning in our predator-based fear conditioned paradigm, as it is in conventional paradigms utilizing foot shock.

Pentkowski, Blanchard, Lever, Litvin, & Blanchard [91] presented results that implicated the ventral, but not dorsal, hippocampus in unconditioned and conditioned defensive responses. The results of their experiment suggest that the inactivation of the dorsal CA1 would not significantly affect behavior. However, the lesions in Pentkowski *et al.* [91] were made one week before behavioral testing and previous studies indicate that other brain structures can compensate for memory affected by dorsal hippocampal damage [29]. The results of Experiment 2 support the hypothesis that the dorsal CA1 area of the hippocampus plays a vital role in the conditioned contextual, but not auditory cue-based, associations formed during predator-based conditioning.

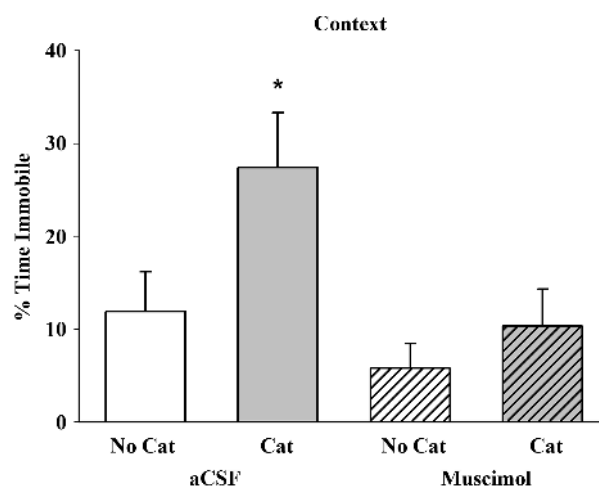


Figure 3. The aCSF-CAT group froze significantly more than the Muscimol-Cat group. Muscimol application to the dorsal CA1 area of the hippocampus blocked contextual memory in the cat-exposed group compared to the cat group administered aCSF. * indicates $p < 0.05$ vs. all other groups.

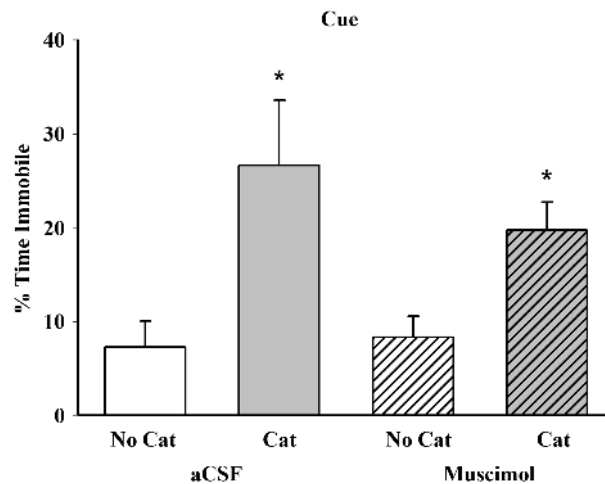


Figure 4. Predator exposure and immobilization resulted in significantly greater freezing to the tone than the no cat groups. Muscimol administration to the dorsal CA1 area of the hippocampus did not block the cued freezing in the cat-exposed group. * indicates $p < 0.05$ vs. respective no cat groups.

4. Experiment 3

Sleep loss is associated with negative impacts on mood, motor function, and cognitive performance [92]. Sleep deprivation (SD) is mentally and physically stressful [93] and impairs cognitive functioning [94]-[99]. In humans, chronic mild sleep restriction, of 2 - 6 hours of sleep per night, and complete acute SD impair cognitive performance compared to non-sleep deprived individuals [100].

The cognitive impairments associated with SD are found in episodic and declarative memory, two forms of memory that involve hippocampal function. Research using rodent models indicates that SD interferes with learning and memory associated with the hippocampus. For example, contextual (hippocampus-dependent), but not cued (hippocampus-independent), fear conditioning is impaired in SD rats [101]-[104]. These findings indicate that SD targets hippocampus-dependent processing. We therefore tested the hypothesis that sleep deprivation would impair hippocampus-dependent contextual, but not amygdala-mediated cue, fear memory. Specifically, we hypothesized that 24 hr SD prior to predator-based fear conditioning would impair contextual fear, while sparing auditory cue fear memory.

4.1. Method

Animals and Sleep Deprivation. A total of 44 male Sprague-Dawley rats (Charles River Laboratories) were used in this experiment and all aspects of care, age and weight were identical to those in Experiments 1 and 2. The rats were randomly assigned to receive sleep deprivation (SD) or no sleep deprivation (NSD). Standard Plexiglas cages were modified to accommodate the flower pot technique and allow the availability of food and water throughout the 24 hr before conditioning [105]. The SD cages had a vertical extension such that when 4 platforms (polypropylene jars, PCG Scientific, 9 cm high \times 6 cm diameter) were placed in the cage, rats could move freely and access food and water from the standard wire lid. The cages were filled with room temperature tap water raised to within 1 cm of the top of the platforms. The NSD rats were housed in Standard Plexiglas cages for 24 hr prior to conditioning in same room that the SD procedure took place. All rats were continually housed with their regular cage mates throughout the 24 hr prior to conditioning.

Fear Conditioning. The same conditioning apparatus used in Experiments 1 and 2 were utilized. Rats were randomly assigned to receive immobilization and predator exposure (Cat) or home cage (No Stress). Rats were placed in the conditioning chambers and allowed 3 min to explore the context. During the last 30 sec of conditioning a 74-dB, 2500-Hz auditory tone was presented. Immediately after the cessation of the tone, rats in the Cat groups were immobilized and placed in the presence of a cat (as described in Experiment 1) for 10 min. After the 10 min in the presence of the cat, rats were placed either in their home cage or in the SD apparatus for 25 min before another identical conditioning trial occurred. This was repeated for a total of 3 conditioning trials. Testing for contextual and cued fear, as previously described, was performed 72 hr after conditioning.

Analyses. Separate 2-way ANOVAs were used to detect differences in freezing behavior in response to the context, novel environment and cue. SD and Cat served as the between-subjects factors for these analyses. Alpha was set at 0.05 for all analyses.

4.2. Results and Discussion

Context Memory. There were significant effects of SD, $F(1, 40) = 15.23, p < 0.01$, and Cat, $F(1, 40) = 9.25, p < 0.01$, and a significant SD \times Cat interaction, $F(1, 40) = 9.85, p = 0.01$. The NSD-Cat group froze significantly more in the conditioning context than all other groups.

There were no significant differences in freezing behavior in the novel environment. Analyses of the data for freezing to the cue revealed a significant main effect of Cat, $F(1, 39) = 5.05, p = 0.03$. The Cat groups spent significantly more time freezing during the cue than the No Cat groups. The effect of SD, $F(1, 39) = 2.69, p = 0.11$, and the SD \times Cat interaction, $F(1, 39) = 0.60, p = 0.81$, were not significant (Figure 5).

This experiment replicated and extended the predator-based Pavlovian fear conditioning paradigm developed in the previous experiments. Sleep deprivation before predator-based fear conditioning impaired contextual fear conditioning as indicated by the significant sleep deprivation and predator stress interaction. Notably, sleep deprivation had no effect on predator-based fear conditioning to the auditory cue. Therefore, these findings support the hypothesis that sleep deprivation is detrimental to contextual (hippocampus-dependent), but not cued (hippocampus-independent), fear conditioning [101]-[104]. The findings further suggest a commonality of mechanisms of adverse effects of sleep deprivation on shock and predator-based contextual fear conditioning, such as a diminished glucocorticoid response and a reduction of the phosphorylation of the transcription factor 3',5'-cyclic AMP response-element binding protein (CREB) [106].

5. General Discussion

The use of a live cat or cat odor to produce a strong innate fear reaction, as well as conditioned fear memory, in rats has been documented by numerous laboratories (for reviews see [41] [55] [67] [68] [107]-[111]). In the current work we assessed the magnitude and extinction of a rat's fear conditioned memory of a live predator (cat), alone and in combination with immobilization and foot shock. In Experiment 1 we found that either cat exposure or immobilization, in isolation, was ineffective as an unconditioned stimulus. In contrast, the combination of immobilization and predator exposure generated a long-lasting (3 - 5 week) contextual fear memory. The combination of all three reinforcers (cat exposure, immobilization and foot shock) produced the greatest magnitude and persistence of fear conditioned memory, as well as resistance to extinction, for both context and cue. In Experiment 2 we focused on the role of the hippocampus in predator-based fear memory. Pharmacological inactivation of the hippocampus produced a selective blockade of contextual, but not cued, fear conditioning. Experiment 3 extended the predator-based fear conditioning paradigm further by assessing the effects of sleep deprivation

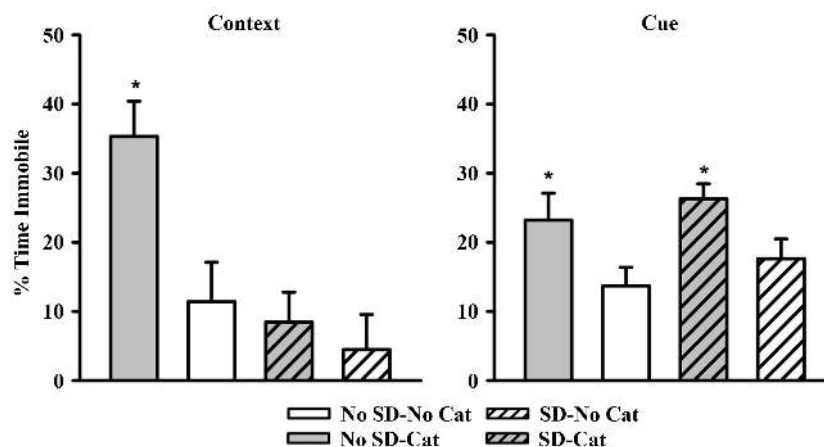


Figure 5. Sleep deprivation (SD) significantly reduced freezing to the context (hippocampal-dependent) aspect of predator based memory tested 72 hr after conditioning (left). Freezing to the auditory cue was not significantly affected by sleep deprivation (right). *indicates $p < 0.05$.

prior to fear conditioning on fear memory expression. This approach provided an effect which was equivalent to inactivation of the hippocampus, with the selective impairment of the expression of contextual, but not cued, fear memory.

One explanation for the findings in Experiment 1 is that the expression of conditioned fear is expressed as a function of the magnitude on the intensity of the aversive stimulus. That is, presenting different US stimuli (shock, cat and immobilization) as a combined US is analogous to increasing the intensity of shock in conventional fear conditioning to produce a greater magnitude of fear conditioned memory [112] [113]. Thus, the multiple types of aversive stimuli administered during fear conditioning could account for the more robust memory we observed. From this perspective, the predator or immobilization manipulations, alone, were insufficiently intense to reach a hypothetical threshold necessary to form the fear association, just as very low shock intensities do not produce fear conditioning [114]. In theory, the combination of immobilization and predator exposure was sufficiently intense to reach the conditioning threshold. The intensity of the stimulus interpretation also accounts for the augmented memory in the group that received all three aversive stimuli.

An alternative interpretation of a subset of the findings in Experiment 1 is that cat exposure, alone, was ineffective at producing a fear-conditioned memory because the 2 minute delay period between removal of the rat from the chamber and exposure to the cat was an excessively long trace period between the CS and US. Long trace intervals (more than 30 sec) reduce the associative qualities in Pavlovian conditioning [81] [82]. Therefore, immobilization immediately after removal of the rats from the shock chamber may have served as a partial US to bridge the trace period between removal of the rat from the explicit CS (chamber) and US (immobilization and cat exposure). Additional work employing immobilization only during the 2 minute trace period may be of value to determine whether immobilization can serve solely to link the CS with the cat as the US.

It is important to note that unlike conventional classical conditioning training, in the current work involving only immobilization and cat exposure as the combined US, the CS (context/cue) and US (immobilization during cat exposure) occurred in completely different locations. That is, in traditional classical conditioning, the CS and US are always presented together in the *same* context. For example, in typical fear conditioning training, rats are administered foot shock and a tone in the same context [115] [116]. A rat's memory for the shock is then tested by observing the rat's behavior when it is returned to the same environment where the shock occurred. We have shown here that rats can associate two neutral stimuli (CSs; chamber and tone) with an aversive experience (US; immobilization during predator exposure) that occurred in two different places. That is, the rats were removed from the chamber and brought to another room, where cat exposure occurred, and yet, the rats exhibited fear when they were returned to the chamber or exposed to the tone. Thus, our demonstration that rats can associate cues that occurred across time and space may be relevant toward understanding how traumatic stress can produce context-independent intrusive memories in people [117]-[120].

When all three reinforcers were delivered in conjunction, *i.e.*, shock in the chamber followed by immobilization and cat exposure outside the chamber, the fear conditioned memory exhibited its greatest magnitude, persistence and resistance to extinction. Thus, multiple aversive stimuli in one training session amplified the potency of context and cued fear memory. Related work has demonstrated that the use of a single (2 hour) restraint stress session 2 days prior to fear conditioning enhanced contextual freezing, but not freezing to an auditory cue [121]. In addition, research on stress (shock) prior to fear conditioning enhanced the expression of fear memory [122]-[124]. Our work is novel in that qualitatively different reinforcers generated a form of fear memory which was expressed 3 weeks after the conditioning session, which continued to be expressed following 3 weekly extinction sessions. This approach may serve as a global model for the great persistence and resistance to extinction in people with PTSD [83] [84] [125]

In related work, Corley *et al.* [126] demonstrated augmented foot shock conditioning with the use of predator odor. The design of their experiments involved shocking the rats in one context (acute stress) and then placing them in a standard housing cage and exposing them to an auditory cue (clicking noises). While in the standard housing cage, a predator-odor laden cloth was placed on top of the cage for 30 sec midway through the auditory conditioning; this procedure was repeated for a total of 5 training trials. To test extinction of conditioned fear, the rats were placed in a "runway with hide box" and re-exposed to the auditory cues for 5 consecutive days. The results of their experiments demonstrated that stress-induced fear conditioning led to persistent freezing to the cue over the 5 days of testing in the runway-hide box. Their work demonstrates the great value in the use of heterogeneous and ethologically relevant reinforcers in the assessment of fear learning.

In other related work, Cohen and colleagues [127]-[130] incorporated a fear conditioning-like component into

their animal model of PTSD. These investigators exposed rats to well-soiled cat litter (predator scent stress) and subsequently measured their freezing behavior in response to fresh, unused cat litter. They found that predator scent stress-exposed rats exhibited significant freezing behavior upon exposure to unused cat litter, which suggested that the rats had a fear-provoking memory of the predator scent stress experience. Their work provides insight into how a fear-provoking experience produces a long-term change in rat behavior. However, their approach did not provide conclusive evidence that the stressed rats had formed an explicit association between a previously neutral cue (CS) and a biologically relevant, arousing stimulus (US), which is an essential feature of classical conditioning [131]. The increased freezing their stressed rats exhibited in response to clean litter may have developed as a result of increased generalized fear to all novel stimuli. Thus, the possibility that the predator scent stress experience produced a non-associative sensitization of stress responses was not addressed in their work.

As with work from other laboratories using predators, or predator-related stimuli, the fear-based associations we have demonstrated here are likely to involve conjoint amygdala and hippocampal processing [22] [126] [132]. However, it is noteworthy to consider that additional brain structures are likely to have been involved in predator-specific based memory processing. Specifically, Canteras and co-workers have demonstrated that sub-nuclei in the amygdala, thalamus, periaqueductal grey and hypothalamus are responsive to sensory, as well as memory-specific processing of predator cues [22] [53] [56]-[58]. In theory, these nuclei not only contribute to sensory-specific cue responses, they may have also contributed to the powerful augmentation of fear conditioned memory and the resistance to extinction we observed when cat exposure was combined with immobilization and shock.

Experiment 2 investigated the role of the dorsal CA1 region of the hippocampus in predator-based contextual and cued fear conditioning. The CA1 region of the hippocampus theoretically is integral to memory because it receives input from various modalities and outputs to the cortex [133]-[136]. Moreover, CA1 has been shown to be activated during fear conditioning [137]-[139], is important for contextual fear memory formation [140] and optogenetic inhibition of CA1 impairs contextual fear memory retrieval [141]. Therefore, Experiment 2 tested the hypothesis that a transient inactivation of the dorsal CA1 region of the hippocampus would selectively block contextual, but not cued, fear memory formation and expression. This experiment demonstrated that, just as in foot shock conditioning paradigms, the predator-based context, but not cue, fear memory is dependent on a functioning CA1 region of the hippocampus.

Finally, Experiment 3 provided support for the hypothesis that sleep deprivation would impair hippocampus-dependent contextual, but not amygdala-mediated cue, fear memory. This finding is consistent with other work that demonstrates that sleep deprivation targets contextual, but not cued, fear memory with shock as reinforcement [101] [104] [106] [142].

The observations on sleep deprivation and fear conditioning are relevant to clinical research which demonstrates that sleep disturbances immediately prior to a traumatic event are associated with an increase in the risk of psychiatric disorders [143]. In their meta-analysis, Bryant *et al.* [143] pointed out that sleep disturbance predicted clinical disorders, such as PTSD and major depression, better than age, gender, severity of trauma and previous psychiatric disturbances. The authors acknowledged that there are likely common underpinnings for the relationship among the disorders; however, they recognized that there could be disorder-specific mechanisms posed by sleep disturbances prior to trauma. One explanation proposed for their finding is that sleep impairment reduces emotional, cognitive, and physical resources that would otherwise mitigate the aftermath of trauma exposure. Fatigue, a common outcome with impaired sleep [144], produces cognitive impairment, such as reduced attention and concentration [145]. Thus, individuals who are deprived of sleep and have depleted capacity to deal with trauma could develop the type of fragmented, dissociative memories that are common with trauma [125].

The ability to appraise events in a larger social and contextual network, along with heightened prefrontal cortex functioning, could buffer a traumatized person against the development of clinical disorders, such as PTSD [67] [146]. The buffering mechanisms, however, are reduced by sleep deprivation, thereby rendering sleep-deprived traumatized individuals prone to the development of PTSD [93] [147] [148].

The clinical disorders implicated in these studies have been theorized to involve the amygdala [149]. This line of research would suggest that an interaction between sleep deprivation and trauma would enhance amygdala processing and, by extension, auditory fear conditioning. Evidence from fMRI studies of increased amygdala activation and reduced amygdala-PFC functional connectivity as a result of sleep deprivation suggest that indi-

viduals are less able to adapt to trauma after sleep deprivation than non-sleep-deprived individuals [150].

One hypothesis addressing stress-induced insomnia posits that hyperarousal is at the core of the inability to sleep [151]. Therefore, sleep deprivation before trauma could add to the magnitude of fear conditioning. However, the findings of the current study do not support this hypothesis. Fear conditioning to an auditory cue occurred as a result of the predator-based stimulus, as indicated by the significant main effect of cat exposure. However, the magnitude of the cued fear memory expression was no greater in sleep-deprived animals than in non-sleep deprived animals. Nevertheless, an imbalance of the fear memory with a bias towards a representation in the amygdala without a hippocampal representation may be expressed as an abnormal, fragmented, and perhaps excessively intrusive memory trauma [125]. Research on traumatized people with brain imaging may resolve the issue of a putative imbalance between hippocampal and amygdala representation of trauma as the basis of intrusive memories.

6. Conclusion

In summary, this series of experiments centered on a predator-based fear conditioning paradigm provides a model for studying the neurobiology of fear memory with an ethologically relevant reinforcer. The findings indicate that predator-based fear conditioning and extinction involve an overlap of neural structures involved in conventional foot shock-based fear conditioning, as in the distinction between hippocampal (contextual) and amygdaloid (cue-based) fear conditioning. However, life-threatening stimuli appear to activate a unique neural circuitry in rodents [55], and perhaps in humans, as well [152]. Therefore, the hybrid approach of utilizing shock- with predator-based fear conditioning may provide the ideal approach toward modeling the neurobiology of traumatic memory, as well as in developing more effective pharmacotherapy for PTSD.

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