Perirhinal Cortex Inactivation Produces Retrieval Deficits in Fear Extinction to a Discontinuous Visual Stimulus

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Words in Abstract: 139 (250 max)

Words in Manuscript: 7,689 (including captions and references)

Number of Figures: 3 (3figures max)

Number of Tables: 0

Running Title: Perirhinal Extinction Deficits

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Abstract

Several studies suggest that the perirhinal cortex (PER) may function to unitize stimulus components across time or modalities. While PER has been shown to be critical for fear acquisition to discontinuous stimuli, the role of the PER in fear extinction memory has not been evaluated. The current study assessed the involvement of the PER during fear extinction training to a continuous or discontinuous conditioned stimulus (CS). Rats were randomly assigned to one of four groups based on two factors: the CS type (a continuous or discontinuous light) and a pre-testing PER manipulation (muscimol inactivation or saline). Results showed that PER inactivation impaired fear memory to both CS types; however, PER inactivation had only impaired extinction memory to the discontinuous light. These results suggest the role of PER in stimulus unitization extends to supporting the acquisition of fear extinction memory.

Keywords: stimulus unitization, emotional communication, fear conditioning, extinction memory

The physiological response to an aversive stimulus alerts us that our environment is threatening, harmful, or dangerous. Threat detection is evolutionarily advantageous; however, in humans aberrant fear can lead to anxiety- and fear-related disorders. Exposure therapy is central to treatment and attempts to extinguish the maladaptive responses in these disorders (Bryant, Harvey, Dang, Sackville, & Basten, 1998; Kar, 2011). Generally, exposure therapy consists of re-exposing a person to the stimulus that was associated with a traumatic event, but in the absence of any danger. Fear extinction paradigms are a reliable model for examining therapeutic dysfunction, and predicting exposure therapy outcomes (Berry, Rosenfield, & Smits, 2009; Graham & Milad, 2011). Thus, understanding the neural mechanisms underlying fear extinction are critical to improving current treatments and to creating new avenues for treatments of anxiety-and fear-related disorders.

Pavlovian fear conditioning consists of pairing an emotionally neutral stimulus (conditioned stimulus, CS) with an innately aversive stimulus (unconditioned stimulus, US). After paired CS-US presentations, the CS begins to elicit a conditioned response (CR) in the absence of the US (Pavlov, 1927). In fear extinction studies, attention is also paid to the learning that occurs during testing sessions when only the CS is presented. Extinction is observed as a gradual decrease of the CR during CS-alone trials (Pavlov, 1927). It is generally thought that new "CS-no US" associations are formed during CS-alone extinction trials. That is, the memory of the original CS-US association is not unlearned, but instead it is overridden by a new association that the CS no longer predicts the US (Myers & Davis, 2007). Evidence of these competing memories is commonly observed with spontaneous recovery, renewal, and reinstatement paradigms suggesting the original CS-US association is preserved despite

extinction (Bouton & Bolles, 1979; Bouton & King, 1983; Pavlov, 1927; Quirk, 2002; Rescorla, 2001; Rescorla & Heth, 1975).

Over two decades of research have identified several brain regions involved in fear memory, including fear acquisition and fear extinction. There is broad agreement that fear memory involves the amygdala, the hippocampus, and the medial prefrontal cortex (mPFC; LeDoux, 2000; Maren & Quirk, 2004; Milad & Quirk, 2012; Tovote, Fadock, & Lüthi, 2015). The amygdala has an essential role in the acquisition, consolidation, and expression of conditioned fear (Choi & Brown, 2003; Kim & Davis, 1993; LeDoux, Cicchetti, Xagoraris, & Romanski, 1990; Pochiro & Lindquist, 2016). The mPFC has a division of function within its prelimbic and infralimbic subregions (Ongur & Price, 2000). Specifically, the prelimbic cortex modulates the consolidation of fear expression, or retrieval, of a fear memory, whereas the infralimbic cortex is required for both the consolidation and retrieval of an extinction memory (Bloodgood, Sugam, Holmes, & Kash, 2018; Laurent & Westbrook, 2009; Quirk, Russo, Barron, & Lebron, 2000; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011). Substantial evidence suggests the hippocampus has a modulatory role in contextual aspects of fear learning (Blanchard, Blanchard, & Fial, 1970; Kim & Fanselow, 1992; Phillips & LeDoux, 1992). Each of these brain regions are also implicated in human fear learning (Delgado, Nearing, Ledoux, & Phelps, 2008; Hartley, Fischl, & Phelps, 2011; LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998; Linnman et al., 2012; Milad et al., 2007a; Milad et al., 2007b; Raij et al., 2018), suggesting a preservation of function across species. Furthermore, dysfunction within these regions is observed in individuals with anxiety- and fear-related disorders and correlate with impaired fear learning (Milad et al., 2013; Milad et al., 2009).

Recent studies suggest that the perirhinal cortex (PER) may play a stimulus-specific role in fear memory. The PER is directly connected with the amygdala, hippocampus and mPFC (Agster & Burwell, 2009, 2013; Furtak, Wei, Agster, & Burwell, 2007; Hwang, Willis, & Burwell, 2018; Pitkanen, Pikkarainen, Nurminen, & Ylinen, 2000). In addition, pretraining PER lesions impair fear conditioning to both discontinuous auditory stimuli (Bang & Brown, 2009; Kholodar-Smith, Allen, & Brown, 2008; Lindquist, Jarrard, & Brown, 2004) and contextual stimuli (Bang & Brown, 2009; Bucci, Phillips, & Burwell, 2000; Bucci, Saddoris, & Burwell, 2002; Kholodar-Smith et al., 2008; Lindquist et al., 2004). One proposed hypothesis, the *Stimulus Unitization Hypothesis*, suggests that the PER unitizes stimuli across different modalities and feature space (including time) into a single stimulus representation (Kent & Brown, 2012). A similar unitization function of the PER has also been proposed by Murray and Kensinger (2013) described as the encoded association of multiple pieces of information. Murray and Kensinger (2013) argued that accessing multiple related stimuli all at once is beneficial. To date, no study has assessed whether the PER is critical in fear extinction in a similar stimulus-specific manner. In the present study, the PER was temporally inactivated during extinction training to a continuous or a discontinuous visual stimulus to test whether the Stimulus Unitization Hypothesis (Kent & Brown, 2012; Murray & Kensinger, 2013) extends to extinction memory.

Method

Subjects

Thirty-nine male Sprague-Dawley derived rats (Simonsen Laboratories, Gilroy, CA; 250-350g; 56-90 days old at surgery) were used in the present study. Of these rats, 36 rats were randomly divided into Saline groups (n = 9, discontinuous CS; n = 8, continuous CS) and

Muscimol groups (n = 10, discontinuous CS; n = 9, continuous CS). The remaining three rats were used for the estimation of muscimol diffusion. Prior to surgery, animals were handled 3-5 times. Rats were maintained on a 16/8 hour light/dark cycle with free access to food and water. All procedures were approved and conducted in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee at California State University, Sacramento.

Surgical Procedure

Animals were anesthetized (Ketamine 100 mg/kg, Xylazine 6 mg/kg, Acetylpromazine 1 mg/kg, i.p.), placed in a stereotaxic apparatus on a heating pad and ophthalmic eye ointment was applied. Under aseptic conditions an incision was made to expose the dorsal surface of the skull. Four trephines were made and threaded with screws. Two additional trephines were made lateral to the ridge, and custom-made guide cannula (22 ga; Plastics One, Roanoke, VA) were implanted targeting the PER bilaterally (A/P: -4.5; D/V: -6.5; M/L: ±6.8, relative to Bregma; adapted from Burwell, 2001). Grip cement (Caulk Dentsply, Milford, DE) secured cannulas to the skull. Dummy cannulas that extended 1mm past the cannula tip (same depth as the injection needle) were inserted into the guide cannulas. Neosporin® was applied to the scalp, and a post-surgical analgesic was given (Ketafen 2-3 mg/kg, s.c.). Rats were given at least a week to recover prior to behavioral procedures. During recovery, rats were handled, and dummy cannulas were removed, cleaned, and replaced to maintain patency.

Behavioral Paradigm

A three-day fear extinction paradigm was adopted from Sierra-Mercado and colleagues (2011) to increase the comparability across studies. All stages were performed in a single context due to the context dependent characteristics of fear extinction. Animals were transported to the behavioral room in their home cage. All phases occurred in a standard Coulbourn chamber

(Coulbourn Instruments; Whitehall, PA) that contained aluminum side and top panels, Plexiglas front and rear panels, and an aluminum grid floor. The chamber was enclosed in a sound-attenuating cubicle. A fan was used to provide background noise and a small amount of vinegar was used as an odorant. The chamber was equipped with a camera (CB21; Circuit Specialists, Tempe, AZ) mounted on the ceiling to record behavior (29 fps).

On day one, Fear Acquisition, animals were placed in the Coulbourn chamber for 2-minute baseline period followed by five presentations of a discontinuous light (50 lux; 9.7 sec, inter-stimulus interval, ISI, = 300 msec; inter-trial interval, ITI, = 120 ± 20 sec) or a continuous light (50 lux; 9.7 sec, ITI = 120 ± 20 sec) that co-terminated with a foot shock US (0.8 mA, 0.5 sec). A visual stimulus is used for better alignment to human studies (Delgado et al., 2008; LaBar et al., 1998; Milad et al., 2007a; Milad et al., 2007b) .

On day two, animals received bilateral intracranial infusions of either 0.5 μl of muscimol (1 μg/μl in 0.9% sterile saline; Sigma, St. Louis, MO; Muscimol group), or 0.5 μl of 0.9% sterile saline (Saline group) at a rate of 0.25 μl/min. This volume was previously used for infusion into the PER and the spread of muscimol was verified with fluorescent muscimol (Ahn & Lee, 2015). The injection needle was left in place for 2 min after infusions to allow for diffusion. Approximately 40 mins later, a time period used previously to ensure the effectiveness of the drug (Allen et al., 2008), animals were returned to the Coulbourn chamber for Extinction Training. After a 2-min baseline, animals were given 21 presentations of the discontinuous or continuous light CS (ITI = 60 ± 10 sec), depending on CS group, without the US. On day three, Extinction Retrieval, animals once again were returned to the Coulbourn chamber. After a 2-min baseline period, animals received 17 presentations of the discontinuous or continuous light CS (ITI = 60 ± 10 sec) without the US.

Histology

At the conclusion of the experiment, animals were anesthetized (Beuthanasia®, 100 mg/kg, i.p.; Intervet Inc., Madison, NJ) and perfused. Brains were extracted and stored in formalin. Brains were transferred to 30% sucrose for cryoprotection. Coronal sections (50 μm) were collected using a freezing microtome (AO Reichert Scientific Instruments, Buffalo, NY), Nissl stained, and examined under a light microscope (Zeiss; Thornwood, NY). The section with the deepest cannula placement was reconstructed using a camera lucida, and anatomical boundaries were identified (Paxinos & Watson, 1998). Target locations were estimated to be 1mm past the end of the cannula track.

Estimation of Muscimol Diffusion

Three animals were used to estimate the diffusion of inactivation using a fluorescent muscimol probe (BODIPY™ TMR-X Conjugate Muscimol; Invitrogen; Carlsbad, CA). These animals underwent identical surgical procedures for cannula implantation. After a week post-surgical recovery time, which included handling and removing of dummy cannulas, animals were infused with fluorescent muscimol (0.5 μg/μl in 0.1M phosphate-buffered saline; PBS). Animals were infused with 0.5 μl fluorescent muscimol bilaterally at a rate of 0.25 μl/min, the needle was left in for 2 min to allow for diffusion. Animals were perfused 45 min post-infusion. Brains were transferred to a 30% sucrose solution for cryoprotection. Coronal sections were collected using a freezing microtome and mounted on slides with ProLong™ Gold antifade reagent (Invitrogen; Carlsbad, CA). Sections were imaged under an epifluorescence microscope.

Data Analysis

Freezing behavior (Blanchard & Blanchard, 1969) was analyzed using custom video analysis software that detected movement (Freez & FreezR, courtesy of Dr. Thomas Brown at

Yale University; See Boguszewski, Leung, Zhao, & Brown, 2007). Brief cessations of movement (< 1 sec) were not included as freezing. Freezing was quantified in 1-min bins for each animal. Data was imported into SPSS (v25, IBM, Corporation; Armonk, NY) for statistical analysis using t-tests and repeated measures analysis of variance (ANOVA). In order to facilitate comparison to previous research, freezing was recalculated into 2-min time blocks throughout all experimental phases. If sphericity was not met in a repeated measure ANOVA, then a Greenhouse-Geisser correction was used (Howell, 2001). Significant main effects or interactions were followed by a post-hoc analysis using a Scheffe correction. All descriptive statistics are reported as mean ± 1 SEM.

Results

Histological location of cannula placements and estimated diffusion of muscimol are illustrated in Figure 1. Based on the diffusion of the fluorescent muscimol, the extent of muscimol diffusions is greater in a dorso-ventral direction than a medio-lateral direction (Fig. 1A). Histological analysis determined that four cannula placements were outside the borders of the PER. Therefore, a total of four rats were excluded from further analysis. Of these remaining 32 animals, cannulas were implanted between -3.24 and -5.88 mm caudal; -6.6 to -7.6 mm ventral; and -6.0 to -7.0 lateral, relative to bregma. Thus, cannula placements targeted the middle of the rostro-caudal extent of the PER (Fig. 1B).

Stimulus-Dependent Differences in Freezing Levels

Freezing levels were analyzed in the control infusions groups in each experiment phase to compare freezing levels with those observed in more typical Pavlovian fear paradigms that use auditory CSs, as compared to the visual CSs used here. This included the Saline groups fear conditioned to the continuous light CS (n = 7) and those conditioned to the discontinuous light

CS (n = 8). During Fear Acquisition, significant increases in freezing compared to baseline levels were observed in rats conditioned to a continuous light CS ($t_{(6)} = 29.62$, $p < .001$) and a discontinuous light CS ($t_{(7)} = 13.59$, $p < .001$). The mean freezing level during baseline and CS-US presentations were 13% (± 2%) and 75% (± 3%) in the continuous CS group, respectively, and 4% (± 3%) and 55% (± 7%) in the discontinuous light CS group, respectively. These findings indicate that both groups were able to learn the CS-US association with a light CS.

To verify that the Sham groups displayed successful extinction memory, the first minute of cue-elicited freezing was compared between Extinction Training and Extinction Retrieval. Within the continuous light CS group, there was a significant decrease in freezing to the CS in the first minute of Extinction Retrieval 58% (± 8%) compared to Extinction Training 90% (± 5%; $t_{(6)} = 5.01$, $p < .01$). Similarly, the discontinuous light CS group also displayed a significant decrease in cue-elicited freezing in the first minute of Extinction Retrieval 24% (± 12%) compared to Extinction Training 55% (± 13%; $t_{(7)} = 3.758$, $p < .01$). Collectively, these findings indicate successful retrieval of the extinction memory in both Sham groups.

Overall freezing levels across all phases were comparable to those previously reported with auditory CSs (Kholodar-Smith et al., 2008; Sierra-Mercado et al., 2011). However, the continuous light CS Saline group froze significantly more than the discontinuous light CS Saline group during Fear Acquisition ($t_{(13)} = 2.54$, $p < .05$), Extinction Training ($t_{(13)} = 4.00$, $p < .01$), and Extinction Retrieval ($t_{(13)} = 4.04$, $p < .001$). The average freezing level in the continuous light CS Saline group was 75% (± 3%) during Fear Acquisition, 71% (± 7%) during Extinction Training, and 46% (± 6%) during Extinction Retrieval. In the discontinuous light CS Saline group the average freezing levels were 55% (± 7%), 27% (± 8%), and 12% (± 6%), in Fear Acquisition, Extinction Training, and Extinction Retrieval, respectively. This likely suggests

differences in the efficacy of a continuous and discontinuous visual stimulus. Due to these stimulus-dependent differences in freezing levels of control animals, the continuous and the discontinuous CS groups were analyzed separately.

Fear Learning to a Continuous Light CS

A total of 16 animals underwent a fear extinction paradigm to a continuous light CS (Saline group, n = 7; Muscimol group, n = 9). A repeated measure ANOVA compared freezing during Fear Acquisition between Groups (Saline or Muscimol) over Time (2-min blocks during CS-US presentations; Fig. 2A). A Greenhouse-Geisser correction was used due to lack of sphericity. There was a significant main effect of Time ($F_{(1,14)} = 55.09$, $p < .001$). There was no significant difference in freezing levels between Groups ($F_{(1,14)} = .33$, $p = .575$), and no significant interaction between Time and Group ($F_{(1,14)} = 2.10$, $p = .141$). The average freezing levels during Fear Acquisition were 75% (± 3%) and 77% (± 4%) for the Saline and Muscimol groups, respectively. Results indicate that both groups froze at comparable levels throughout Fear Acquisition to the continuous light CS prior to the manipulation of PER inactivation.

On day two, animals were infused with either saline or muscimol prior to Extinction Training, when they received CS-alone presentations. A repeated measure ANOVA compared freezing between Groups (Saline or Muscimol) over Time (2-min blocks during CS presentations; see Fig. 2A). A Greenhouse-Geisser correction was used due to lack of sphericity. While not reaching significance, there was a downward trend of Time ($F_{(1,14)} = 2.30$, $p = .081$). However, there was a significant main effect of Group ($F_{(1,14)} = 29.65$, $p < .001$), and a significant interaction between Time and Group ($F_{(1,14)} = 4.09$, $p < .01$). The average freezing levels during Extinction Training for the Saline group was 71% (± 7%) compared to 34% (± 3%) for the Muscimol group. Post-hoc analyses revealed that freezing levels in the first ten blocks of

time were significantly different between Saline and Muscimol groups ($p < .05$; Fig. 2A). This demonstrates that PER inactivation prior to Extinction Training reduced the expression of conditioned freezing early in Extinction Training to a continuous light CS.

The next day, Extinction Retrieval, animals again received CS-alone presentations. A repeated measure ANOVA compared freezing between Groups (Saline or Muscimol) over Time (2-min blocks during CS presentations; Fig. 2A). A Greenhouse-Geisser correction was used due to lack of sphericity. There was a significant main effect of Time ($F_{(1,14)} = 2.71$, $p < .05$), suggesting all animals displayed within session extinction and froze at decreasing levels throughout Extinction Retrieval. There was no significant main effect of Group ($F_{(1,14)} = .89$, $p = .362$), and no significant interaction between Time and Group ($F_{(1,14)} = 1.05$, $p = .389$). The average freezing levels during Extinction Retrieval for the Saline group and Muscimol group was 46% (± 6%) and 53% (± 5%), respectively. Thus, there was no impairment in Extinction Retrieval following PER inactivation during Extinction Training when the CS is continuous in nature.

In order to assess whether these results were due to cue-elicited freezing or contextually driven freezing, an additional analysis compared the amount of freezing during the baseline period compared to freezing levels during the first 2-min of continuous light CS presentations. During Extinction Training, there was a significant effect of time, indicating significant increases in freezing during the onset of CS presentations ($F_{(1,14)} = 91.239$, $p < .001$). There was also a significant effect of group ($F_{(1,14)} = 53.371$, $p < .001$), and a significant interaction of time by group ($F_{(1,14)} = 17.748$, $p = .001$). The mean freezing level during baseline and the first 2-min block of continuous light CS presentations were 37% (± 18%) and 92% (± 12%) in the Saline group and 12% (± 6%) and 33% (± 17%) in the Muscimol group, respectively. During Extinction

Retrieval, there was a significant effect of time, indicating again a significant increases in freezing to the onset of CS presentations compared to baseline levels ($F_{(1,14)} = 46.949$, $p < .001$). There was a trend emerging for a difference between groups but it failed to reach significance ($F_{(1,14)} = 3.822$, $p = .071$), and there was no interaction between time and group ($F_{(1,14)} = .043$, $p = .838$). The mean freezing level during baseline and the first 2-min block of continuous light CS presentations, respectively, were 19% (± 12%) and 58% (± 17%) in the Saline group and 31% (± 17%) and 72% (± 22%) in the Muscimol group. Overall, these analyses suggest a significant increase across all animals during CS presentations during both Extinction Training and Extinction Retrieval.

Fear Learning to a Discontinuous Light CS

A total of 16 animals underwent a fear extinction paradigm to a discontinuous light CS (Saline, n = 8; Muscimol, n = 8). Due to lack of sphericity across all phases of the experiment, repeated measure ANOVA results are reported with a Greenhouse-Geisser correction. A repeated measure ANOVA compared freezing during Fear Acquisition between Groups (Saline or Muscimol) over Time (2-min blocks during CS-US presentations) during Fear Acquisition (Fig. 2B). There was a significant main effect of Time ($F_{(1,14)} = 43.70$, $p < .001$). There was no significant difference in the freezing levels between Groups ($F_{(1,14)} = .78$, $p = .392$), and no significant interaction between Time and Group ($F_{(1,14)} = 1.36$, $p = .274$). The average freezing levels across Fear Acquisition for the Saline and Muscimol group was 55% (± 7%) and 65% (± 7%), respectively. Thus, results suggest that there were no group differences during Fear Acquisition to the discontinuous light CS prior to the manipulation of PER inactivations.

On day two, animals were infused with either saline or muscimol prior to Extinction Training. A repeated measure ANOVA compared the level of freezing between Groups (Saline

or Muscimol) over Time (2-min blocks during CS presentations; Fig. 2B). There was no significant main effect of Time ($F_{(1,14)} = .67$, $p = .589$). While not reaching significance, there was a trend for a difference between Groups ($F_{(1,14)} = 1.22$, $p = .080$). There was a significant interaction between Time and Group ($F_{(1,14)} = 4.73$, $p < .001$). This interaction effect indicates that the Saline group displayed fear expression early during Extinction Training and within session extinction. In contrast, the Muscimol group failed to show fear expression early in Extinction Training and did not display changes in freezing levels within Extinction Training. The average freezing during CS presentations for the Saline group and Muscimol group was 27% (± 8%) and 16% (± 5%), respectively. A post-hoc analysis was performed to investigate the significant interaction between Time and Group, which found that time blocks 1-3 significantly different between groups (Fig. 2B). This result shows that PER inactivation prior to Extinction Training reduced the expression of conditioned freezing early in Extinction Training to a discontinuous light CS.

During Extinction Retrieval on Day 3, animals received CS-alone presentations. A repeated measure ANOVA compared freezing between Groups (Saline or Muscimol) over Time (2-min blocks during CS presentations; Fig. 2B). There was a significant main effect of Time ($F_{(1,14)} = 3.34$, $p < .05$), suggesting all animals displayed within session extinction and froze at decreasing levels. There was a significant main effect of Group ($F_{(1,14)} = 4.88$, $p < .05$), indicating a significant impairment in the retrieval of extinction training indicated by elevated freezing levels in the PER inactivation group. There was no significant interaction between Time and Group ($F_{(1,14)} = .87$, $p = .464$). The average freezing levels across Extinction Retrieval for the Saline and Muscimol groups was 12% (± 6%) and 31% (± 6%), respectively. Post-hoc analyses revealed that there was a significant difference between the Saline and Muscimol groups in

blocks 2-4 (Fig. 2B). These results indicate a significant impairment in Extinction Retrieval following PER inactivation during Extinction Training when the CS is discontinuous in nature.

In order to assess whether these results were due to cue-elicited freezing or contextually driven freezing, an additional analysis compared the amount of freezing during the baseline period compared to freezing levels during the first 2-min of discontinuous light CS presentations. During Extinction Training, there was a significant effect of time, representing a significant increase in freezing to the onset of the CS presentations compared to baseline levels ($F_{(1,14)} = 8.744$, $p = .01$). There was a significant effect of group ($F_{(1,14)} = 11.273$, $p < .01$), as well as a significant interaction of time by group ($F_{(1,14)} = 8.536$, $p < .05$). The mean freezing level during baseline and the first 2-min block of discontinuous light CS presentations were 22% (± 22%) and 54% (± 36%) in the Saline group and 6% (± 8%) and 6% (± 8%) in the Muscimol group, respectively. During Extinction Retrevial, there was also a significant effect of time, demonstrating a significant increases in freezing to CS presentations compared to baseline levels ($F_{(1,14)} = 30.492$, $p < .001$). There was no significant difference between groups ($F_{(1,14)} = .556$, $p = .468$). However, there was a significant interaction between time and group ($F_{(1,14)} = 9.706$, $p < .01$). The mean freezing level during baseline and the first 2-min block of discontinuous light CS presentations were 13% (± 18%) and 23% (± 36%) in the Saline group and 7% (± 8%) and 45% (± 20%) in the Muscimol group, respectively. These results suggest that the impairments observed in perirhinal inactivation is driven by cue-elicited freezing behavior.

Discussion

In this first study to examine a stimulus-specific role of the PER in fear extinction, it was predicted that the PER would be involved in fear extinction when a discontinuous visual stimulus was used as a CS, requiring stimulus unitization across time, but not when a continuous visual

stimulus was used as a CS. Results demonstrate that PER inactivation during extinction training caused a deficit in the subsequent retrieval of the extinction memory to a discontinuous, but not a continuous, light CS. Interestingly, and unexpectedly, PER inactivation also resulted in reduced fear expression during extinction training regardless of the CS type. These findings are discussed in relation to current literature on stimulus processing during fear learning.

Previous research has demonstrated the importance of the PER in fear conditioning in a stimulus-specific manner. For example, the PER is involved in fear conditioning to discontinuous auditory stimuli, such as an ultrasonic vocalizations or discontinuous tones, but not to continuous pure tones (Kholodar-Smith et al., 2008; Lindquist et al., 2004). These findings led to the Stimulus Unitization Hypothesis that posits the PER provides a representation of stimuli requiring unitization across time, modality, or feature elements (Kent & Brown, 2012). We further tested this hypothesis by inactivating the PER in a fear extinction paradigm to address if this function of unitization may be a more global function of the PER. Results from the current study provides evidence in support of the PER involvement in stimulus unitization during fear extinction. When a continuous visual stimulus was utilized, animals with PER inactivation did not demonstrate impairment during retrieval of the extinction memory. In contrast, PER inactivation produced significantly higher levels of freezing during retrieval of the extinction memory when the CS was a discontinuous light CS. Given these findings we propose a working model that extends the ideas proposed in the Stimulus Unitization Hypothesis. Within this theoretical framework the PER is hypothesized: 1) to be necessary when a stimulus requires unitization across time, modality or features; 2) to make this unitized stimulus representation available to task-specific regions through direct and reciprocal connections (i.e. the amygdala during fear acquisition and to the mPFC during fear extinction); and 3) to update the unitized

stimulus representation with relevant emotional and motivational information as new information becomes available.

Unexpectedly, conditioned freezing was impaired during extinction training for all animals that underwent PER inactivation no matter if the visual stimulus was continuous or discontinuous in nature. Previous research has suggested that the amygdala receives CS information from two distinct pathways (Boatman & Kim, 2006). The first, a thalamo-amygdalar pathway, was proposed to be a rapid pathway used to supply the amygdala with a simple representation of the CS; while the second, a thalamo-cortico-amygdalar pathway, was suggested to support richer, more complex representations of stimuli (LeDoux, 2000, 2007). Building off this research Brown and colleagues (2012), has suggested that the cortical-based pathway, including the PER, is used when the CS is discontinuous in nature. However, previous research has found that when auditory conditioning occurs within an intact brain, retention of the fear memory becomes dependent on the thalamo-cortico-amygdalar pathway. Thus, this may be a preferred pathway even if it is not an essential pathway (Boatman & Kim, 2006). The current results suggest that this preference for cortical-based processing may also exist with a continuous visual stimulus and would explain the unexpected impairment observed here in fear expression to both a continuous and discontinuous stimulus when the PER was inactivated during extinction training. A previous single unit study by Furtak and colleagues (2007), which examined PER neuronal firing during fear conditioning, suggests the PER may the preferred pathway to support fear conditioning continuous and discontinuous auditory stimuli consistent with the current findings. Future studies are necessary to elucidate this further.

Alternatively, this observed deficit in fear expression could result if the infusions of muscimol extended outside the borders of the PER to the anatomically adjacent amygdala.

Multiple studies have employed the use of muscimol for brain inactivations (Ahn & Lee, 2015; Gilmartin, Kwapis, & Helmstetter, 2012). Similar to the estimate of muscimol diffusion in the current study, previous studies of fear learning have verified the spread of inactivations using fluorescent muscimol (Ahn & Lee, 2015; Allen et al., 2008). However, this may be an inaccurate representation of the diffusion. The diffusion range of the fluorophore-conjugated muscimol molecule can be quite different than the non-conjugated muscimol due to the larger molecular weight of the flurophore than the standard muscimol compound (Allen et al., 2008). In the current study, two arguments can be made supporting the conclusion that the muscimol infusions were limited to the PER. First, the estimated diffusion seen in Figure 1 suggests that the diffusion of muscimol is great in the dorso-ventral extent, making the likelihood of amygdala inactivation low. In addition, it has been argued that white matter between the amygdala and the PER may also act as a boundary that limits the diffusion of muscimol (Allen et al., 2008). Previous published studies that have infused muscimol directly into the amygdala have argued it is this white matter that prevents the muscimol from entering the PER or entorhinal cortices (Gilmartin et al., 2012). Second, the behavioral patterns observed over the fear extinction paradigm employed in the current study are dissimilar to previously reported patterns observed with inactivation of the amygdala, the hippocampus or the mPFC with continuous CSs (Sierra-Mercado et al., 2011). This latter argument is expanded below.

Sierra-Mercado and colleagues (2011), examined contributions of the basolateral complex of the amygdala, the prelimbic cortex, the infralimbic cortex, and the hippocampus during a fear extinction paradigm using a continuous tone CS. In the same manner as the current study, muscimol inactivation occurred prior to extinction training into one of the four brain regions. Sierra-Mercado and colleagues (2011) found that inactivation of all regions except for

the infralimbic cortex caused impairments in fear expression during Extinction Training with the muscimol groups showing reduced freezing levels with approximately 15%, 45%, and 50% of control animals in the basolateral complex of the amygdala, the prelimbic cortex, and the hippocampus, respectively (Sierra-Mercado et al., 2011). In addition, the inactivation of a subset of regions also showed significant deficits in the retrieval of the extinction memory the following day, indicated by significantly higher levels of freezing compared with the control group. Inactivation of the basolateral complex of the amygdala, the infralimbic cortex, and the hippocampus resulted in elevated freezing levels with approximately 225%, 250%, and 175% of control animals, respectively (Sierra-Mercado et al., 2011). Collectively, the findings suggest that impairments in both fear expression and extinction retrieval are seen with inactivation to either the basolateral complex of the amygdala or the hippocampus. While the prelimbic cortex appears exclusively involved in fear expression, and the infralimbic cortex seem to be specifically engaged in the retrieval of the extinction memory.

To facilitate a comparison across studies (Sierra-Mercado et al., 2011), Figure 3 graphs freezing levels of the Muscimol group as a percent of freezing levels observed in the Saline group during fear expression (the first 2-min block of Extinction Training; Fig. 3A) and during extinction memory (the first two 2-min blocks of Extinction Retrieval; Fig. 3B) for animals conditioned to the continuous CS and the discontinuous CS. When considering animals conditioned to a continuous light CS, the PER is impaired only in fear expression. This finding only aligns with the profile of prelimbic inactivation when compared across the studies (Sierra-Mercado et al., 2011). The prelimbic cortex and the PER are anatomically so distal, it would be highly unlikely, for the muscimol to have spread such a great distance. In contrast, PER inactivation in animals conditioned to a discontinuous CS have impairments in both fear

expression and extinction memory, a pattern similar to inactivation of the basolateral complex of the amygdala. However, if this was due to a spread of muscimol, then it would be unlikely for it only to affect animals in the discontinuous CS group but not affect animals in the continuous CS group. Again, arguing against a suggestion that the muscimol injections may have breached the anatomical boarders of the PER. Of course, additional studies with more sophisticated inactivation methods are required to verify the precise location of inactivations. For example, the novel chemogenetic technique referred to as designer receptors exclusively activated by designer drugs (DREADDs) would allow for the accurate verification of anatomical location and are ideal for elongated brain structures such as the PER (Smith, Bucci, Luikart, & Mahler, 2016). Future research using DREADDs will allow for a more precise inactivation profile and aid in the understanding of the temporal contributions of individual brain regions to fear learning.

Additionally, previous studies have suggested that while permanent lesions to the PER produce an increase in general locomotion and exploratory behaviors, inactivation of the PER may produce decreases in freezing behaviors. Given the current study, it is important to distinguish between these phenomena. Wiig and Bilkey (1994) found that general locomotion was increased when the PER was lesioned prior to place navigation testing. Perirhinal lesioned rats swam significantly faster than the control rats, swimming the same distance. However, an increase in exploratory behavior was not seen when the PER was lesioned. Perirhinal lesioned rats swam only on the periphery of the pool while control rats explored the center of the maze during the first trial during place navigation tasks (Wiig & Bilkey, 1994). When rats are fearful and cannot escape their environment, as in the current study, freezing occurs. Therefore, locomotion cannot be taken into account when looking at behavior when the PER is inactivated, as the rats have nowhere to move to. Furthermore, other studies have found no effect on

exploratory behavior in rats with PER lesions (Albasser et al., 2015). In this study, results suggest normal levels of exploration in perirhinal lesioned rats during novel object recognition tasks, when compared to control animals. Therefore, it can be concluded that inactivations of the PER prior to Extinction Training are suggested to be a reduction in expressed conditioned freezing, and not an increase in general locomotion and exploratory behaviors.

In conclusion, previous research suggests the PER functions to unitize stimuli across time or modality. Results from the current study suggest the role of PER in stimulus unitization extends to supporting the acquisition of fear extinction memory, and thus may be a more global function of the PER. Deficits in fear conditioning and fear extinction associated with the PER is an important step in understanding how brain regions outside of the commonly accepted fear circuit may be engaged with more complex stimuli, which likely contribute to stimulus representations during traumatic events associated with fear learning in humans.

Acknowledgements: This research was funded by a Research Creativity Award from Calfornia State University Sacramento to S.C.F. and NSF IOS award 1755111 to S.C.F.

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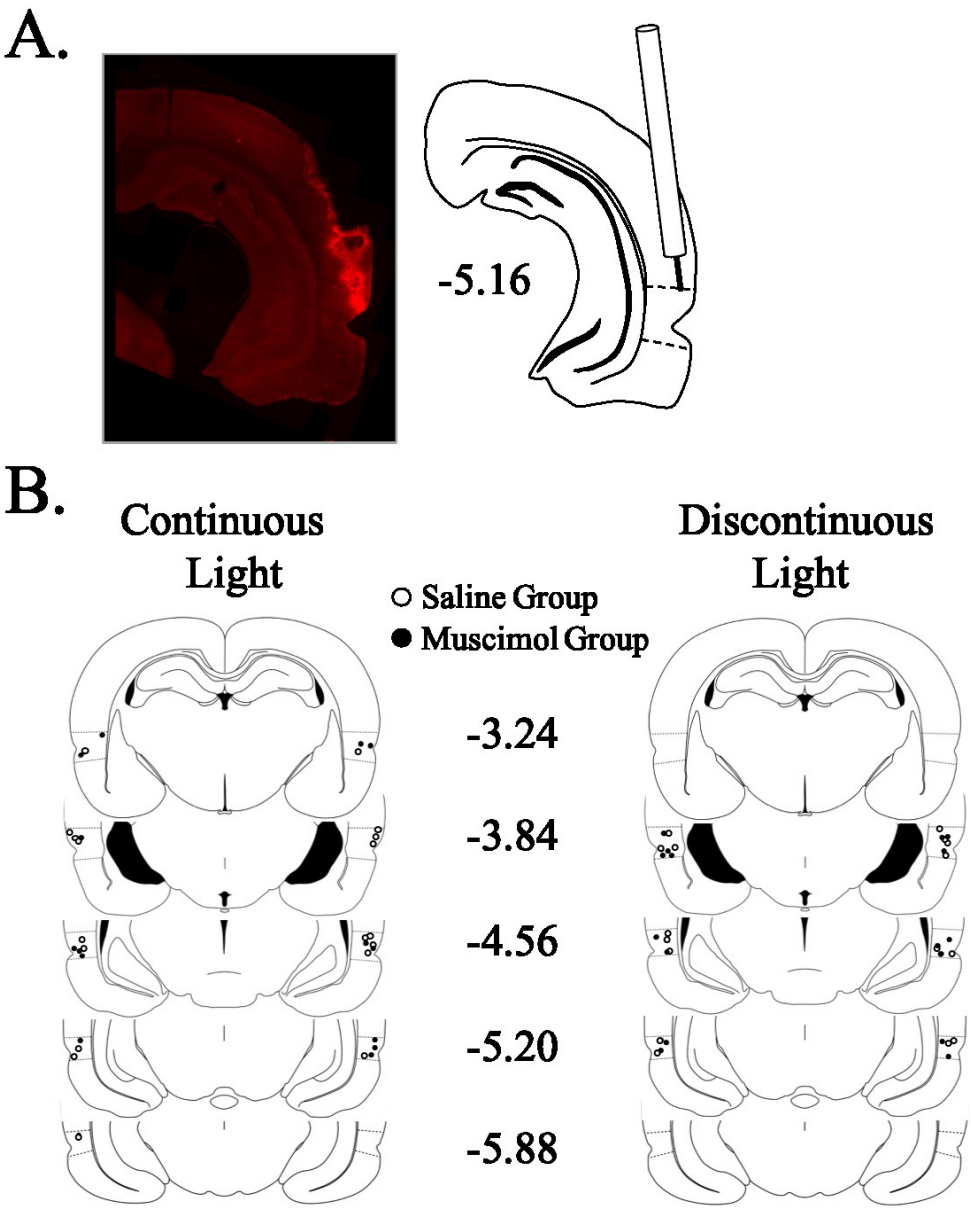

Figure 1. **Estimated spread and location of bilateral cannula implantation.** *A) Estimated spread of infusions.* Fluorescently conjugated muscimol was used to estimate the spread of musimol. *B) Histological verification of cannula tip placement.* Nissl-stained coronal sections of the rat brain were collected and examined. Cannula tip placement shown for animals conditioned to a continuous light CS (n = 7, Saline; n = 9, Muscimol), and discontinuous light CS (n = 8, Saline; n = 8, Muscimol). The location of each coronal section is given relative to bregma.

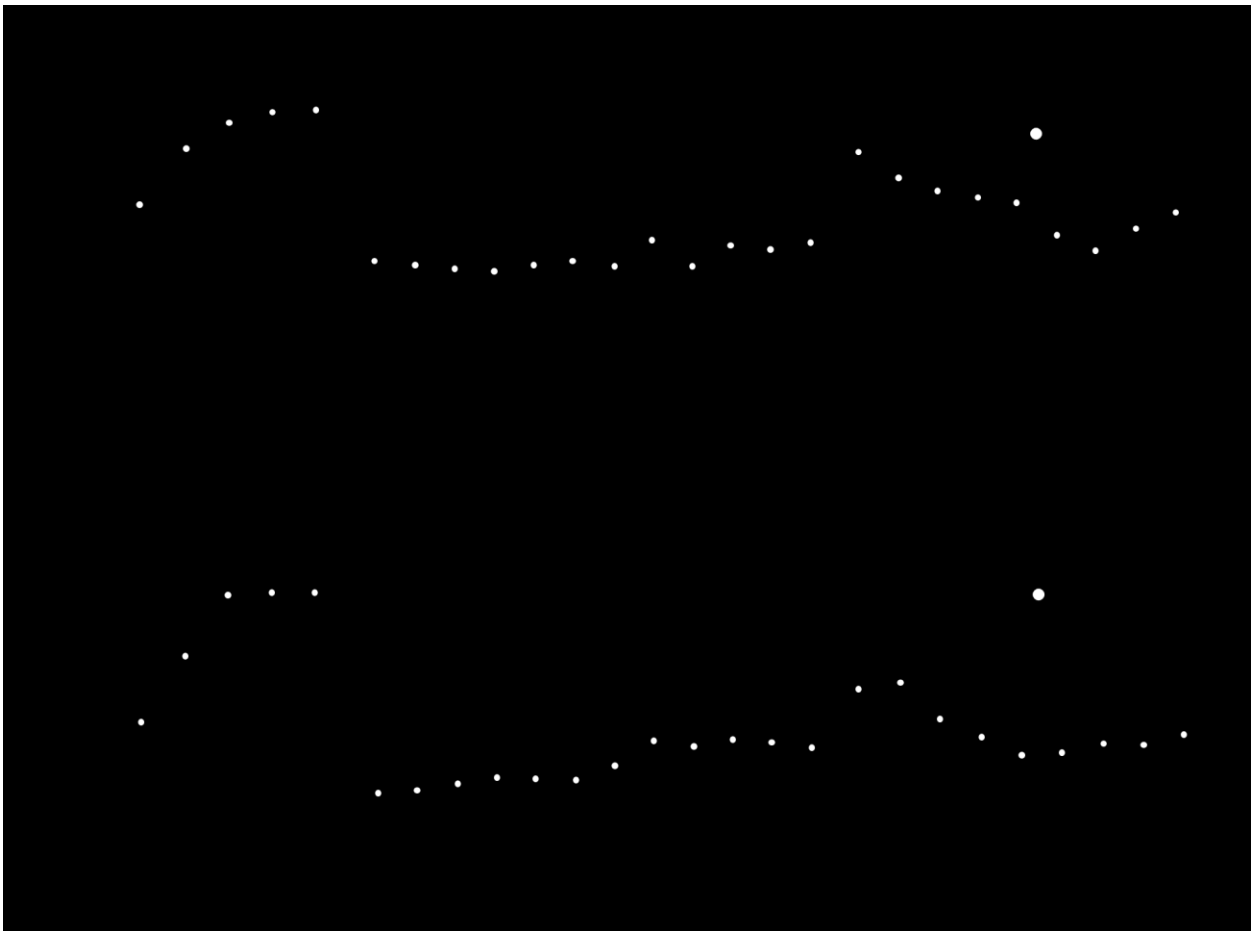

Figure 2. **Freezing levels during fear extinction to a continuous and discontinuous CS.** Prior to infusions, noted by arrows, there were no group differences in either CS condition. *A) Continuous Light CS.* During Extinction Training, there was a significant difference in freezing levels between Saline and Muscimol groups ($p < .05$). Post-hoc analyses indicated that blocks 1-10 were significantly different (noted by asterisk). No group differences existed in Extinction Retrieval. *B) Discontinuous Light CS.* There was a significant interaction of time block and group (Saline vs. Muscimol, $p < .05$) in Extinction Training with post-hoc analyses indicating blocks 1-3 were significantly different. On Day 3, a significant difference in the retrieval of the extinction memory was observed between Saline and Muscimol groups ($p < .05$) with post-hoc analyses indicated that blocks 2-4 were significantly different. Data presented as mean ± 1 SEM.

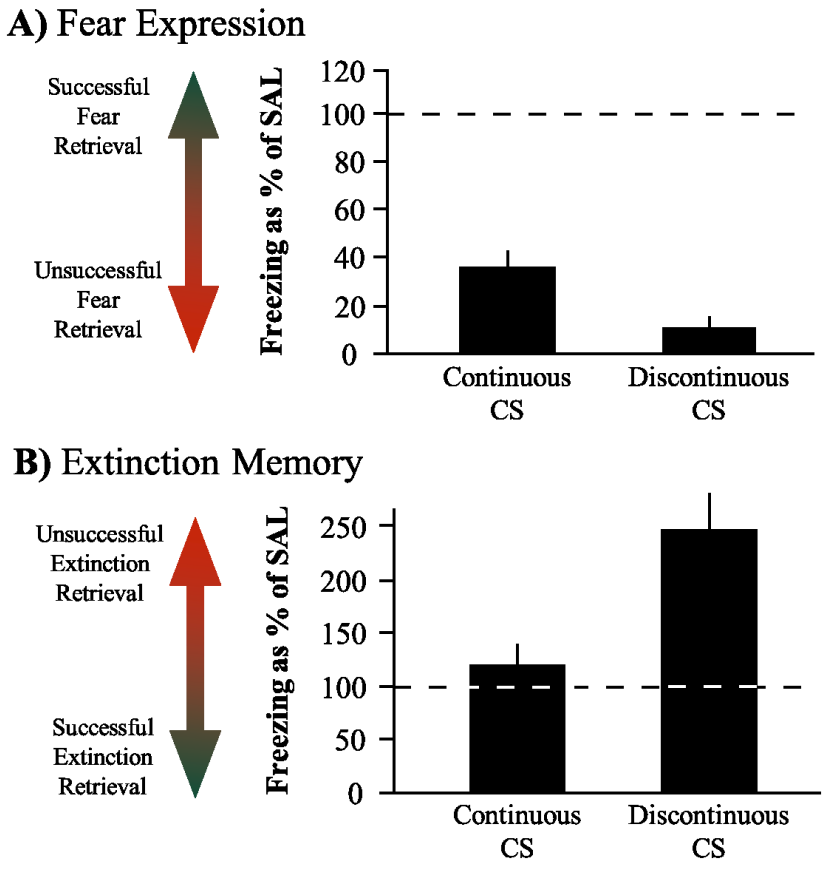

Figure 3. **Freezing levels of the Muscimol groups conditioned to a continuous or discontinuous light CS.** Freezing is plotted as a percent of the Saline group over the first 2-min block of either Extinction Training, A, or Extinction Recall, B. *A) Fear Expression.* Deficits in fear expression are seen in both Muscimol groups regardless of the CS type. *B) Extinction Memory.* Deficits in extinction memory are seen in Muscimol group conditioned to the discontinuous light CS, but not the continuous CS.