THE ROLE OF THE MEDIAL PREFRONTAL CORTEX IN THE CONTEXTUAL FEAR MEMORY OF RATS USING OPTOGENETICS

by

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ABSTRACT

The medial prefrontal cortex (mPFC) has previously been shown to play various roles in different phases of fear-learning and memory, particularly in extinction. While there has been substantial progress in understanding how the mPFC regulates fear to discrete cues (e.g., tones and lights), less is known about the mPFC's involvement in learning fear to a context. Therefore, this study examined the effects of mPFC inhibition during the acquisition phase of contextual fear conditioning on the long-term retention and extinction of contextual fear. Twenty-four rats received bilateral infusions of a pan-cellular optogenetic neural silencer CAG-ArchT-EGFP (ArchT, n=12) or a CAG-EGFP (control, n=12) virus. Three weeks later, optic fiber assemblies (OFAs) were bilaterally implanted over the injection site. After an additional week of recovery, rats were conditioned using a contextual fear conditioning paradigm administering 5 shocks, each 3 minutes apart on day one and tested for freezing to the context-alone on days two and three. The mPFC was optogenetically inhibited on day one during contextual fear acquisition. Optogenetic inhibition of mPFC during the acquisition phase of contextual fear conditioning (Day 1) did not significantly affect the increases in freezing to the context or the rate of acquisition. The control rats showed significantly decreased freezing levels compared to the sustained increased freezing levels of the ArchT rats during day 2 of the extinction retention test. On the second extinction retention test, there was a similar trend in decreased freezing of the control rats. In an alternate context, rats were subsequently reconditioned and extinction tested. ArchT rats showed significantly higher freezing levels before shocks began, but showed no significantly different training and extinction testing behavior compared to controls, indicating that optogenetic silencing did not cause permanent inhibition to the mPFC. These data suggest that inhibition of the mPFC activity during acquisition interferes with later extinction and facilitates fear generalization to a novel context.

Chapter 1

INTRODUCTION

Animals learn to predict threat through fear conditioning as an important mechanism for survival during danger. However, this form of associative learning can become pathological when perceived, expected, or even absent threats cause excessive behavioral and physiological reactions implicated in many anxiety disorders such as phobias, generalized anxiety disorder (GAD) and post-traumatic stress disorder (PTSD) (Giustino & Maren, 2015). The associative learning process and its many forms are mediated by specific brain regions and complex neural circuits, many of which are disrupted during these anxiety disorders and are not fully understood (Giustino & Maren, 2015; Rozeske, Valerio, Chaudun, & Herry, 2015). Since over 18% of the United States population may suffer from an anxiety disorder, it is imperative to make further advancements in the field (Gilmartin, Balderston, & Helmstetter, 2014; Kessler et al., 2005).

To gain a better understanding of neural fear circuits, laboratories utilize contextual fear conditioning models to simulate the associations between traumatic experiences and a specific context. At its simplest form, contextual Pavlovian fear conditioning is an associative learning paradigm. During fear training, an animal is placed in chamber (context conditioned stimulus (CS)), and given an aversive unconditioned stimulus (US) such as a shock, ultimately leading to a conditioned response (CR), such as freezing behavior. After a single or repeated shock, the CS alone elicits a CR. The process of pairing the US to the CS is known as fear

acquisition, while the display of the CR is known as fear expression. A second phase is the test of memory of the conditioning, where the animal is returned to the context and the CR is measured. In an additional testing phase, extinction, the CS is repeatedly presented in the absence of the US, causing a new extinction memory to diminish the expression of the CR (Bouton & Bolles, 1979; Gregory J Quirk et al., 2010).

A context is made up of multiple cues and features in contextual conditioning to account for the vast amount of stimuli an animal encounters in a natural environment. (J. W. Rudy, Huff, & Matus-Amat, 2004; Jerry W Rudy, 2009). Not only can a context be comprised of discrete cues and features (e.g., tones and lights that may be present), but it also may contain: spatial elements, such as the configuration of objects and features; temporal elements, such as duration and frequency of events; interoceptive elements, such as stress or thirst; cognitive elements, such as instructions; and social and cultural contexts; such as the individuals and special events within a context (Maren, Phan, & Liberzon, 2013). It is thought that once an animal forms a configural representation containing these elements (encoded context), animals can then associate this representation to the US (context conditioning) (Maren et al., 2013).

Within the neural fear circuit, there are fairly well defined roles of various brain regions in contextual fear conditioning. The basolateral amygdala (BLA) and central nucleus of the amygdala (CeA) are involved during the acquisition and expression of cued and contextual fear memories (LeDoux, 2012; Rozeske et al., 2015). The hippocampus, however, is only involved in contextual fear conditioning, not cued conditioning (J. J. Kim & Fanselow, 1992; Rozeske et al., 2015). The dorsal hippocampus (dHPC) is needed to form the configural representation to the context,

and more specifically the dentate gyrus (DG), Ca1, and Ca3 subregions are needed for context encoding (LeDoux, 2012; Rozeske et al., 2015). Both the amygdala and hippocampus play a major role in the fear circuit, however, all aspects of acquisition, expression, and extinction of contextual fear memories cannot be explained by just these two structures, thus more regions in the brain must be involved.

The medial prefrontal cortex (mPFC) has been previously investigated for its involvement in contextual fear conditioning. The mPFC can be broken up into four distinct sections in most dorsal to most ventral order: the medial precentral cortex (PrCm), anterior cingulate (AC), the prelimbic cortex (PL), and the infralimbic cortex (IL), the most ventral region (Courtin, Bienvenu, Einarsson, & Herry, 2013; Rozeske et al., 2015). In general, the mPFC contributes to contextual fear conditioning via emotional regulation, decision making, threat responsivity, and context encoding (Rozeske et al., 2015). More specifically, the more dorsal regions (PrCm and AC) are thought to regulate motor behaviors, while the more ventral regions (PL and IL) are more involved in emotional and cognitive processes, such as contextual fear conditioning (Heidbreder & Groenewegen, 2003; Rozeske et al., 2015).

The PL and IL are known to receive excitatory input from the thalamus, BLA, and hippocampus, as well as having strong outputs to the BLA (Giustino & Maren, 2015). Electrophysiology studies using local field potential (LFP) recordings have shown coordinated theta oscillations between the mPFC and BLA post-fear conditioning, indicating increased synaptic strength between the areas (Pape & Pare, 2010). The IL specifically is thought to inhibit fear behavior via its projections to the CeA and the BLA (Courtin et al., 2013). The PL is thought to promote fear expression via its projections to the BLA (Courtin et al., 2013; Pape & Pare, 2010).

Courtin et al summarize the results of many conflicting studies of cued and contextual conditioning via different methodological manipulations of the mPFC's subregions at different temporal points (Courtin et al., 2013). From lesion studies, the AC is shown to contribute to aversive memories, the PL is shown to mediate the expression of conditioned fear, and the IL is believed to contribute to extinction of conditioned fear (Corcoran & Quirk, 2007; Courtin et al., 2013; Do-Monte, Manzano-Nieves, Quiñones-Laracuente, Ramos-Medina, & Quirk, 2015; Sharpe & Killcross, 2014). Specifically, IL lesions within the vmPFC in rats extinguish the freezing response to tone within an extinction session, but excessive freezing spontaneously recovers the following day, suggesting failure of extinction recall (Lebrón, Milad, & Quirk, 2004; G J Quirk, Russo, Barron, & Lebron, 2000). In histological studies using immediate early genes (IEG), such as EGR-1, data suggest that the dorsal medial prefrontal cortex (dmPFC) is involved in expression of fear behaviors, while the ventral prefrontal cortex (vmPFC) is involved in the consolidation of fear extinction (Courtin et al., 2013). There is evidence that the PL could also be implicated in acquisition and consolidation of cued fear memories in addition to extinction using pharmacological manipulations (Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006).

While there is evidence for the role of the subregions of the mPFC in fear conditioning, there is still a wide range of discrepancies between the results and limitations in studies. For instance, studies that used lesions and inactivation had less consistency in results in those with sizeable lesions or inactivation of overlapping mPFC subregions (Courtin et al., 2013). Procedural factors also could explain the inconsistencies across lesion and inactivation studies such as lesion time, targeted

region, and training procedures (Chang, Berke, & Maren, 2010). Additionally, histological studies using IEGs were inherently flawed due to the uncertainty of information regarding the ability, meaning, and frequency, of IEG expression in all types of neurons (Courtin et al., 2013). Electrophysiology studies using LFPs are currently mostly limited just to theta oscillations only, so gamma and sleep slow oscillations would improve the study design by providing more information on cognitive tasks such as attention and working memory (Carlé N et al., 2011).

New optogenetic methods are being used more frequently in fear conditioning paradigms due to their ability to directly inhibit or excite specific neuronal populations and reverse the effect instantaneously in animals while undergoing behavioral tests via light sensitive protein infusion and illumination through fiber optic implants (Courtin et al., 2013; Tovote, Fadok, & Lüthi, 2015). The three main light sensitive proteins are the excitatory Channelrhodopsin (ChR2), the inhibitory Halorhodopsin (NpHR), and the inhibitory Archaerhodpsin (Arch) (Courtin 2013). ArchT is a light-sensitive outward proton pump of *Halorubrum* genus. It pushes protons (H+) out of the cell causing hyperpolarization of the cell. Techniques such as optogenetics can provide newer and more accurate experimental design due to the targeted inhibitory and excitatory of neural populations. For instance, Do-monte et al. integrated optogenetic inhibition methods to previous IL extinction research with cued fear conditioning (Do-Monte et al., 2015). Kim et al. took this one step further by not only observing optogenetic inhibition of the IL, but also inactivation of the PL during extinction (H.-S. Kim, Cho, Augustine, & Han, 2015).

While there has been substantial progress in understanding how the mPFC regulates fear to discrete cues, still less is known about the mPFC's involvement in

learning fear to a context, specifically during acquisition. Optogenetics serves as a good method for fear conditioning due to its ability to inhibit cells and reverse the effect instantaneously with illumination. Previous optogenetic inhibition fear conditioning studies have only looked at the effects of inhibition during extinction training and retrieval, not initial fear acquisition (Do-Monte et al., 2015; H.-S. Kim et al., 2015). Therefore, the present study examined the effects of optogenetic mPFC inhibition during acquisition on the long-term retention and extinction of contextual fear.

Chapter 2

METHODS

2.1 Subjects:

Subjects were 24 adult male Long-Evans rats 8-11 weeks old and weighing 250-275 grams at the start of experiment obtained from Harlan/Envigo Breeders (Indianapolis, IN). Rats were initially pair housed in opaque polypropylene shoebox cages with wood shavings and free access to food and water in a temperature-controlled colony room with a 12-hour light/dark cycle (lights on at 7:00AM). After arriving in the colony room, rats were left undisturbed for a 1-week period. All experiments were conducted between 8:00AM and 5:00PM. All procedures were in accordance with the *National Institute of Health Guide for the Care and Use of Experimental Animals* and approved by the University of Delaware's Institutional Animal Care and Use Committee (IACUC).

2.2 AAV Infusion:

Bilateral infusions of pan-cellular optogenetic neural silencer, CAG-ArchT-EGFP (ArchT) or a CAG-EGFP (control) in adeno-associated viruses (AAV) were performed in the respective experimental and control groups. The experiments and surgeries were performed on two cohorts of rats of 12 rats each. For the first cohort, 11 of the rats received ArchT and only one received the control. For the second cohort, 6 rats received the ArchT virus and 6 received the control.

Rats were anesthetized with an IP injection of ketamine/xylazine solution (85/15 mg/kg) in preparation for stereotaxic surgery. Bilateral infusions of ArchT or

the control were performed in the respective experimental and control groups. The infusions were placed directly into the PL (AP = +3.2 mm to bregma; ML = ± 0.8 mm; DV = -3.6 mm). A syringe pump infused 1μL of virus into each hemisphere consecutively, each at an infusion rate of 5 minutes. Rats were pair-housed again and given 1 week of recovery time.

2.3 Optic Fiber Assemblies (OFAs) and Testing

A fiber optic cable was cut and stripped so that the desired length of glass fiber was exposed to construct OFAs. The exposed fiber glass inserts were then cleaved to equal sections. For each OFA made, a small bead of epoxy was placed on the flat side of the ferrule and a section of the optic fiber was guided through until the fiber was slightly sticking out of the beveled end. An additional bead of epoxy was added to the beveled end and left to dry for at least 24-48 hours. The OFAs were cleaved to the desired length of 0.5 mm and then sanded down for optimal transmission through the optical fiber.

The OFAs were tested for power output testing. The laser light source was hooked up to the rotary joint at the top of the fear conditioning chamber by a fiber optic patch cord. Two fiber optic coupler cords are plugged into the rotary joint. After the laser is set, turned on, and powered up, each OFA is individually placed in the ferrule sleeve of one of the fiber optic coupler cords and tested for power output using a photodiode sensor and power meter. The power output of the OFA ranged from 10mW to 15mW. Each OFA was paired with another OFA that was close to the same power as the other and assigned to a rat to ensure each hemisphere of the brain received the same amount of laser stimulation.

2.4 OFA Placement Surgery

Rats were again anesthetized with an IP injection of ketamine/xylazine solution (85/15 mg/kg) in preparation for stereotaxic surgery. Paired OFAs were placed in the cannula holders of the stereotaxic arm. The OFAs were implanted into the PL at an angled approach of 18º (AP = +3.2 mm to bregma; ML = ± 1.8 mm; DV = -3.2 mm). The OFAs were fixed into the skull with dental cement. Rats are single housed post OFA placement surgery. Behavioral testing began one week after OFA placement surgeries.

2.5 Handling

Prior to behavioral experimentation, rats were transported to a holding room and handled for five minutes a day for three consecutive days by the same experimenter. During handling, rats were individually taken out of cage and held within arms and allowed exploration within the area during the allotted time period. The ferrule sleeves of two fiber optic coupler cords were placed on the implanted OFAs of the rats during handling to familiarize the rats with the sensation.

2.6 Multi-Trial Contextual Fear Conditioning and Retention and Extinction Testing

Contextual fear conditioning was conducted in one Plexiglas/metal chamber containing metal grid floors (Context A). Both groups were counterbalanced across days by alternating between the ArchT and control groups during the behavioral tests. During conditioning on the acquisition training day (Day 1), each animal's OFAs were plugged into the two fiber optic couple cords in the chamber for an 18-minute-long trial, receiving five 1s 0.6mA shocks, one every 180 seconds. As mentioned earlier, rats were run in two cohorts. For the first cohort (n=12), six animals with the ArchT

virus had the green laser turned on the duration of the acquisition training. The five other rats that received the ArchT virus and one that received the control virus were trained with the light turned off. For the second cohort, all 12 rats were trained with the light on (six ArchT virus rats and six control virus rats).

Twenty-four hours later during retention testing (Day 2), all 24 animals were placed in Context A with each rat's OFAs plugged into the two fiber optic coupler cords with no shocks and no laser stimulation for the 18-minute-long trial.

Twenty-four hours later (Day 3), the second cohort rats (n=12) were placed in Context A for an extinction test. This procedure was the same as Day 2 (i.e., each rat's OFAs plugged into the two fiber optic couple cords with no shocks and no laser stimulation for the 18-minute-long trial).

The chamber was cleaned with a 5% ammonium hydroxide solution between sessions. A camera placed in front of the chamber recorded the behavior of each rat and transmitted the signal to a computer running FreezeFrame software (Actimetrics, Wilmette, IL). Freezeframe was set to score freezing behavior as 0.75s bouts without changes in pixel luminance and was also manually verified offline by another experimenter.

2.7 Contextual Fear Re-training in an Alternate Context with Laser Illumination

Twenty-four hours later (day 4), the second cohort rats (i.e.,the rats with ArchT virus and six with the control virus) were re-trained in a different context of four Plexiglas chambers with metal grid floors with cameras monitoring the rat's behaviors above the chamber (Context B). This context had identical contextual fear

conditioning parameters as Day 1 without the laser stimulation (i.e., 18-minute-long trial, five 1s 0.6mA shocks 180 seconds apart).

Twenty-four hours later (day 5), the second cohort rats went through a retention test in Context B under the same retention testing parameters as Days 2 and 3 (e.g., 18-minute long trial, no shocks). The chambers were again cleaned with the 5% ammonium hydroxide and recorded with the same FreezeFrame (e.g. 0.75s bouts) parameters in Context B.

2.8 Brain Collection

At the end of final behavioral testing, animals were sacrificed by rapid decapitation. Brains were removed within two minutes, immediately frozen in isopentane on dry ice for 10 seconds, and then stored at -80°C until sectioned.

2.9 Brain Sectioning

Brains were cut at 40 µm on a cryostat and sections corresponding to OFA placement in the mPFC were stained with DAPI and coverslipped in darkness. After 24 hours, the slides were wrapped in desiccant and stored at -20°C until fluorescence was observed for placement of viral infusion and for OFA placement.

2.10 Statistical analysis

Freezing during conditioning and testing was statistically analyzed separately with a mixed model repeated measures ANOVAs (2 between x 6 within). The between effect was virus (control vs. ArchT) and the within measure was time after being placed in the chamber in six time bins of 3 minutes. For fear conditioning, the initial 3-min time measure ended just before the first shock and then the subsequent 5 bins were the 3-min period starting after a shock. For testing, there were six 3-min bins

without shock given. Significance level of $p<0.05$ selected. Post hoc tests were performed with a bonferroni corrected t-test.

Chapter 3

RESULTS

3.1 OFA Placement and Viral Spread in the mPFC

Figure 1A depicts the target region for OFAs in the PL. All 24 animals had both tips of the OFAs placed inside of this region and were included for statistical analysis. No animals were excluded due to misplacements.

Virus Figure 1 displays the spread of ArchT and the green shaded fluorescence within the mPFC. The darker green areas represent maximum expression, which is mostly within the PL. There are lighter shaded green areas representing the minimum expression, which spreads into the IL.

3.2 Acquisition

For day 1 fear training (acquisition) data of all 24 animals (Figure 2a), a repeated measures analysis of variance (ANOVA) showed no difference in freezing levels between ArchT and control groups across training trial blocks ($F(1,22) = 2.0$, ns). There was a significant main effect of trial blocks in all animals ($F(5,18) = 77.24$, $p<0.0001$). There was no interaction effect between group and minutes ($F(5,18) = 0.87$, ns).

3.3 Retention and Extinction Test

During day 2 retention testing of all 24 animals (Figure 2b), a repeated measures ANOVA showed no main group effect ($F(1,22) = 2.52$, ns). However, there were significant differences in freezing levels over the six 3-min time bins of the retention test ($F(5,18) = 3.52$, $p<0.021$). There also was an interaction effect of group

x minutes, indicating there were significant differences in groups over particular bins of 3 minutes ($F(5,18) = 3.30$, $p<0.027$).

Examining the graph of the retention test in Figure 2b, it appeared that freezing during bins 4 and 5 (minutes 10-12 and 13-15 minutes of the test, respectively) might be significantly different between the control and ArchT groups. T-tests were run on only those two bins. During minutes 10-12, and 13-15, the ArchT group froze more than the control group (minutes 10-12: $t(22) = 1.87$, $p<0.04$; minutes 13-15: $t(22)=3.61$, $p<0.002$). These data suggest that inhibition of the mPFC during acquisition inhibited within-test extinction during the retention test.

3.4 Extinction Retention

During day 3, the retention testing was repeated (extinction test) in the 12 animals from cohort 2 (Figure 3, Figure 4). A repeated measures ANOVA showed no main group effect ($F(1,10)=2.52$). However, there was a trend towards less freezing levels over the 18 minute retention test ($F(5,6)=3.6$, $p<0.075$). There was no interaction ($F(5,6)=0.92$, ns).

3.5 Alternate Context Acquisition

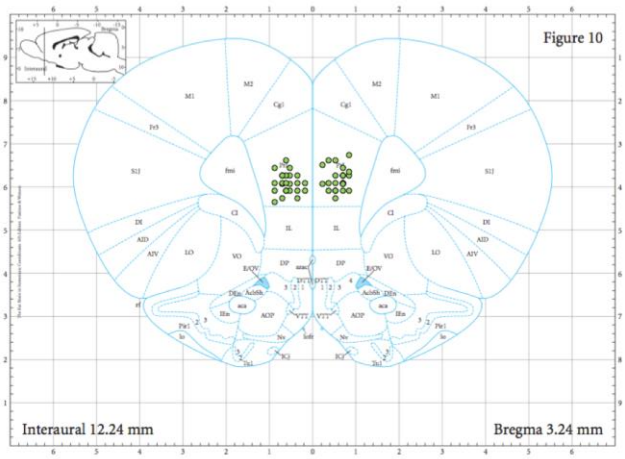
During day 4 re-training (alternate context acquisition) of the 12 animals from cohort 2 (Figure 5a), a repeated measures ANOVA showed no main group effect ($F(1,10) = 0.07$, ns). There was a significant increase in freezing levels as number of shocks increased in all animals ($F(5,6) = 5.6$, $p<0.029$). There was no interaction effect between group and increased freezing levels with additional shocks ($F(5,6) = 0.75$, ns). During baseline, before the first shock was given (minutes 0-3), the ArchT group froze significantly more than the control group ($t(10)= 2.59$), $p<0.027$). This last

piece of data suggests the effects of less extinction in the ArchT-light on group during acquisition transferred from context A to context B.

3.6 Alternate Context Retention Test

After retraining on Day 4 without laser illumination in an alternate context both group displayed similar levels of freezing in the retention test on Day 5 (Figure 5b). A repeated measures ANOVA showed no main group effect ($F(1,10)=0.12$, ns), no difference in freezing over the minutes ($F(5,6)=2.26$, ns), and no interaction effect of group x minutes ($F(5,6) = 1.95$, ns). The data demonstrate there was no long term effect from laser illumination on the ability to learn fear conditioning to a new context.

A



B

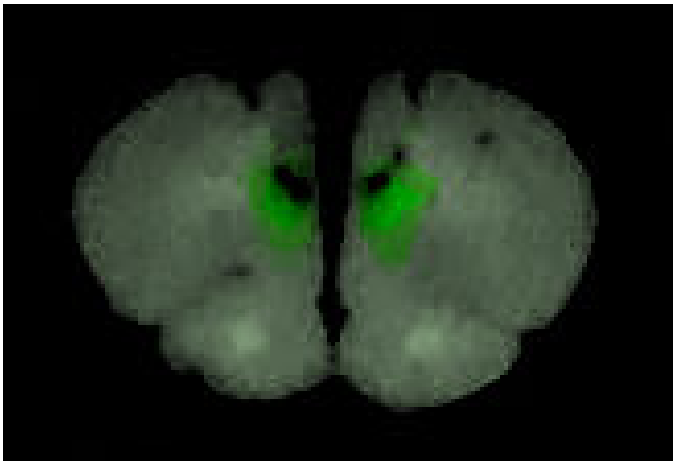


Figure 1 1A: Green dots represent OFA placement tips in the mPFC. All 24 OFAs were placed in the target region in the pre-limbic cortex (PL) of the mPFC. 1B: Green shaded areas represent fluoresence of ArchT within the mPFC. Darker green areas represent the maximum expression (mostly in the PL), while the lighter green areas represent the minimum expression (in the IL).

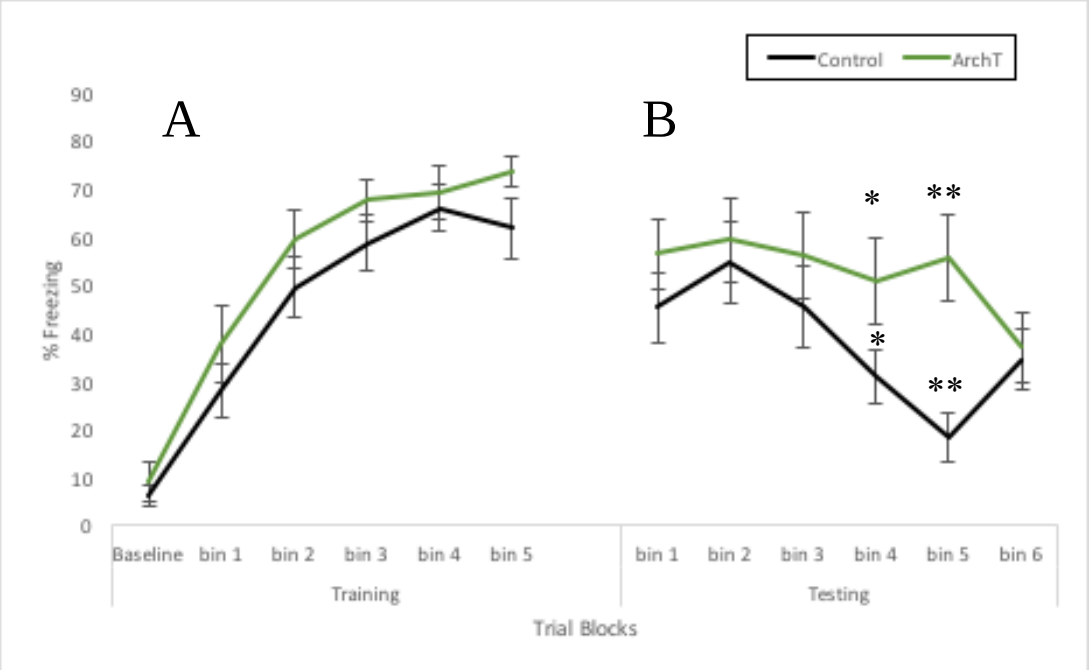


Figure 2 Context A bin freezing. ArchT and control rats are exposed to Context A chamber over a 2-day period and total freezing levels are observed. 2A: Day 1 training phase which consists of 5 shock training of 0.6mA with laser illumination freezing levels broken up into 3 minute bins. 2B: Day 2 retention and extinction test. There are no significant differences during any bins of the training phase (A); however, during testing, bins 4 and 5 (minutes 10-12 and 13-15), ArchT rats showed significantly higher freezing levels (B). (*$p<0.04$; **$p<0.002$).

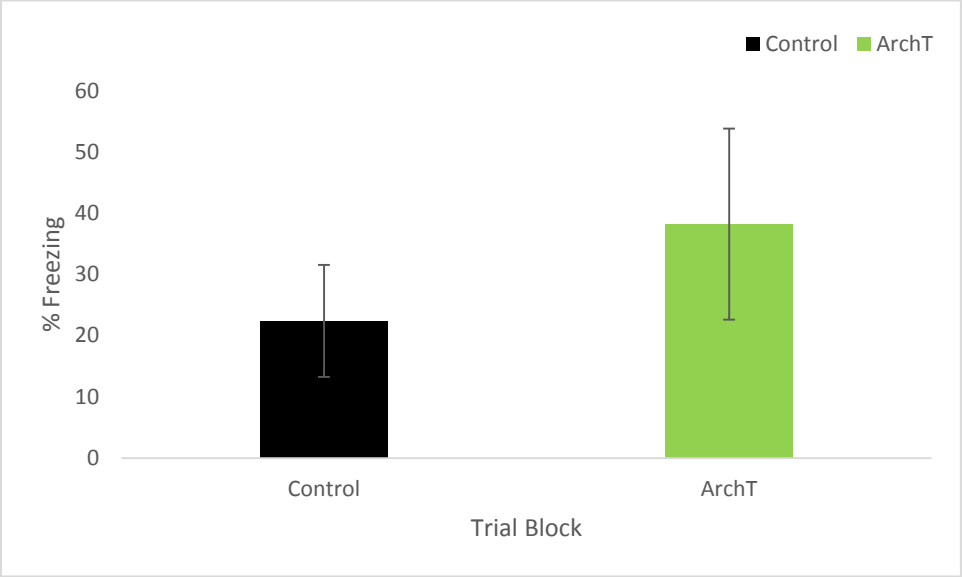


Figure 3 Context A Retesting. On day 3, animals were tested in Context A again. There was no significant main effect between groups during extinction testing for total freezing levels, but there was a trend towards differences between groups ($p<0.075$).

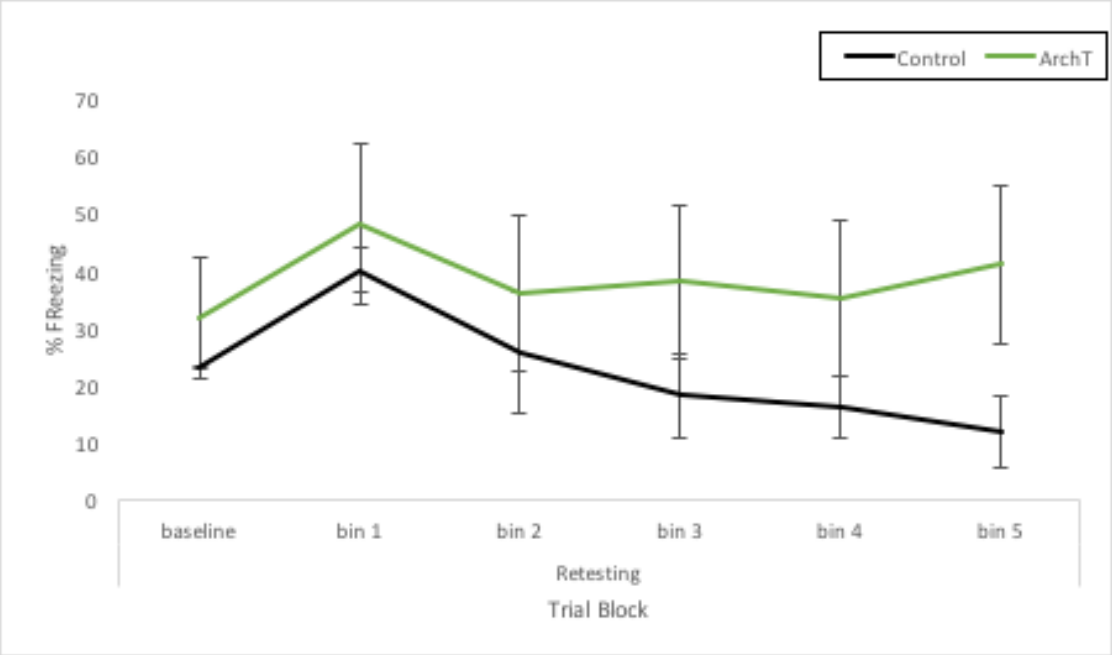


Figure 4 Context A bin freezing testing. On day 3, animals were tested in Context A again. There was no significant difference between bins across groups, but there was a trend of differences in freezing during bin 5 (minutes 16-18, $p<0.075$).

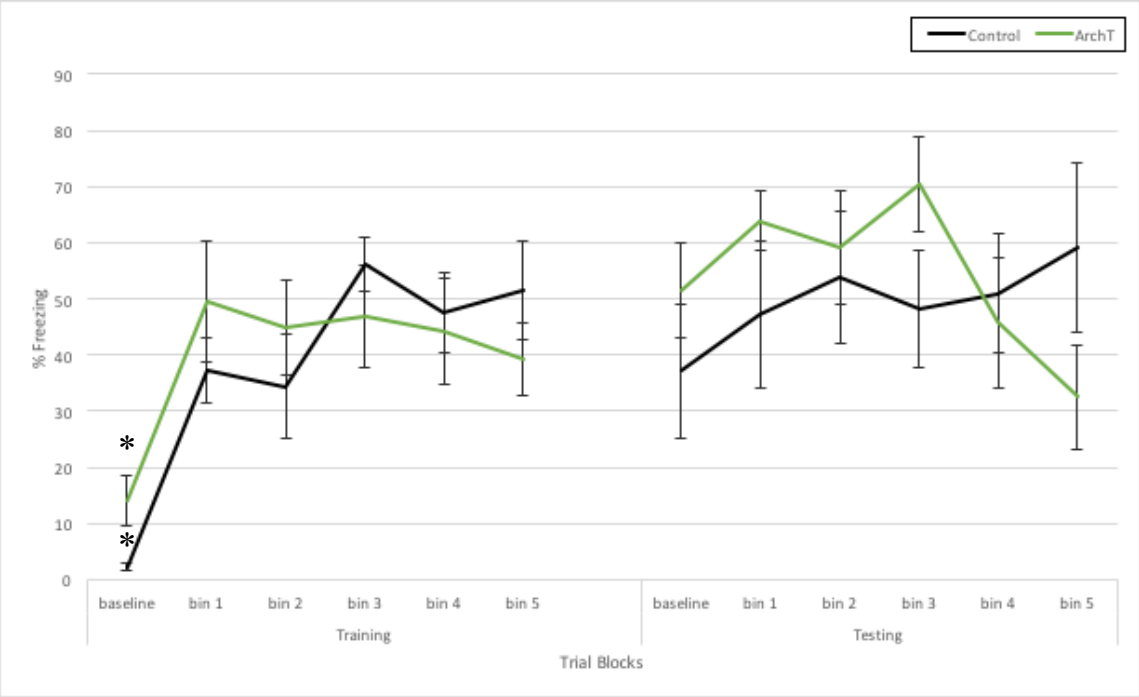


Figure 5 Context B bin freezing. On day 4 (5A), animals were placed in an alternate context, Context B, and were trained with 5 shock 0.6mA over the 18 minute protocol and freezing levels were observed between ArchT and control rats. On day 5 (5B), rats were tested in Context B for 18 minutes with no shocks. During baseline (minutes 0-3) the ArchT group froze significantly more than the control group (*$p<0.027$), suggesting the generalization of fear from contexts A to context B. There were no significant differences in bin freezing between groups during training and testing across bins in an alternate context.

Chapter 4

DISCUSSION

This study showed acquisition of the fear memory occurred in rats at an equal rate between groups with or without optogenetic inhibition of the mPFC. However, this inhibition during the training phase of fear conditioning impaired extinction during a retention test 24 hours later. This significant effect occurred during minutes 10-15 of the retention test, suggesting that a long test was needed to see the disruptive effects on extinction. The following day in the same context, the ArchT animals showed the same trend of impaired extinction expression during the final minutes of the trial. When the animals were placed in a new context, the ArchT group initially expressed more freezing at baseline than the control group, while acquisition without laser illumination and retention/extinction were normal. The data suggest that inhibiting the mPFC during contextual fear acquisition interfered with subsequent extinction and may have facilitated generalization of fear to another context.

Only half of the rats (cohort 2, n=6 ArchT-light, n=6 control-light) were tested for extinction a second time. Although there were no significant differences between the ArchT and control rats in the second extinction test, it seems the ArchT rats might still have had a deficit in extinction compared to controls (trend of $p<0.075$). The lack of statistical differences is likely due to the low number of rats per group and low power. Addition of another six rats per group should provide sufficient power as it did for data of the retention test when there were 12 rats per group.

Rats from cohort 2 were also retrained for contextual fear conditioning in an alternate context (Context B) with no laser illumination given. Similar to the initial conditioning with laser illuminate, ArchT and control rats showed no difference in freezing during acquisition. However, this time freezing in ArchT and control rats did not differ during the retention test, showing that the earlier laser inhibition did not cause permanent cellular damage that impacted new context learning. Interestingly, ArchT rats showed enhanced freezing during the baseline period of acquisition (first 3 minutes before shocks were given, see Figure 5) to the alternate context relative to controls, suggesting that the deficit in extinction after two days of extinction was generalized to a new environment.

The data suggest that the mPFC activity at the time of acquisition is important for the future extinction of contextual fear memories. It also suggests previous deficits in extinction induced by mPFC inhibition during initial fear conditioning produces sustained conditioned fear in one environment generalizes to a new environment. However, we don't know how much of the two environments have shared features that can elicit residual fear behavior from the first environment during exposure to a new environment. Further testing of this phenomenon would be necessary to draw a firmer conclusion of generalized conditioned fear.

The results are important additions to previous findings in the literature. Similar to the present study, Morgan and LeDoux found when the PL was lesioned, before training, there was enhanced contextual fear learning and blocked contextual fear extinction (Morgan & LeDoux, 1995). Lacroix et al. also found lesions of the vmPFC before training blocked extinction of cued fear conditioning (Lacroix, Spinelli, Heidbreder, & Feldon, 2000).

During extinction of cued conditioning with optogenetic methods, Do-Monte et al. showed that eNpHR silencing of vmPFC (IL) neurons during extinction tone exposure and found no impact on fear expression, but did impair retrieval of extinction the following day (Do-Monte et al., 2015). In a different trial, eNpHR or mucimol inactivation of IL neurons during the retrieval test did not impair retrieval of extinction, even when these subsequent groups' IL neurons were re-silenced 7 days later (Do-Monte et al., 2015). Kim et al. showed that the inhibition of IL impairs the expression of fear extinction when inhibited during extinction training. In contrast, this effect was not seen when the PL was optogenetically silenced (H.-S. Kim et al., 2015). Overall, these studies, in concert with ours, show that optogenetic inhibition methods can be utilized effectively during all phases of learning (initial fear conditioning or extinction learning) and yield significant effects on retention and extinction.

In the two studies discussed above on optogenetic inhibition, the main difference when compared to the present study is that neither of the two studies inhibited the mPFC during initial fear acquisition, but instead only during extinction. Both of these studies were also utilizing cued conditioning rather than contextual conditioning. It would be interesting to see during a follow up of these studies if there is the same effect of impaired extinction when the PL is optogenetically inhibited during contextual conditioning.

One of the main strengths of the present study is the use of optogenetic inhibition in the mPFC. This method is an improvement on other silencing techniques such as lesions or pharmacological inhibition because of targeted inhibition and reversal of specific cell types. Another strength of the study was the testing and counterbalancing of the OFAs as explained in the methods. All OFAs were tested for

power output and paired with similar OFAs to make sure each side of the brain was stimulated the same amount. Due to the small range in power output as mentioned previously (approximately 10-15mW), OFA pairs were then placed in order of power and then alternatively assigned to each group (ArchT or control) in ascending order. Another advantage of this method in general, but was not used in the present study, is the laser can be turned on or off instantaneously specifically during any desired time length, duration, and phase in learning and memory. By utilizing a method that can exactly pinpoint when the inhibition of the mPFC is occurring, less temporal confounds exist.

One limitation of the study was that only 6 rats per group were assessed during extinction test in Context A, as well as during trials in Context B. Increasing the number of animals would bring down the variance and would increase the power. Another limitation is that this study did not differentiate between the subregions of the mPFC. Targeting the IL or the PL exclusively in future studies will be useful. A third limitation of the study was the difference between control groups of each cohort. In the first cohort, the animals all received the ArchT virus and the laser was switched off during their trial so no silencing of the mPFC would occur, while in the second cohort, the control animals received a different GFP virus that did not inhibit the mPFC, but also had the light on during acquisition. This had the potential of illuminating the test chamber differently in the first cohort of rats, but the data suggest this did not seem to have an effect on extinction.

In conclusion, optogenetic inhibition of the mPFC during training produced a disruption in extinction. When placed in an alternate context, ArchT rats froze more at baseline possibly due to generalization of fear from the previous context, however,

showed no overall difference in freezing during training or retention testing. The findings are novel because of its focus on inhibition of mPFC neurons during fear memory acquisition. Extinction can be considered a measure of strength of conditioning. (Gregory J. Quirk, 2002). By further investigating the specific roles of the subregions of the mPFC during the different learning phases of contextual fear conditioning, it will further help to understand the neural mechanisms behind debilitating anxiety related disorders and ultimately provide proper treatments.

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