Operant overtraining leads to increased Fos expression in IL and PL that is unaffected by fear conditioning

by

Alisa Pajser

B.S., Drake University, 2014

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Psychological Sciences
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2019

Approved by:

Major Professor
Charles L. Pickens

Copyright

Abstract

The fear incubation task is an extended training procedure used to cause low fear soon after training that grows over time. However, the neurobiological basis of this effect, particularly the low fear observed soon after training, is unknown. One possibility is that extended training leads to habituation to the shocks, so that the tone cue is repeatedly paired with ineffective shocks. If so, then the overtraining may lead to an effect similar to extinction, and the low fear seen soon after extended training may be associated with increased neuronal activity in the infralimbic cortex (IL), an area involved in extinction learning, and decreased neuronal activity in the prelimbic cortex (PL), an area involved in fear expression. The current study examined whether low fear soon after extended training is associated with increased IL and decreased PL activation, compared with rats given a single day of fear conditioning or no fear training. Male Long-Evans rats acquired lever-pressing and then underwent fear training for 1 or 10 days. During each fear training session, while lever-pressing on a VI60 schedule of reinforcement, half of the animals in each group received 10 30-second tones co-terminating with a 0.5-second foot-shock pseudo-randomly throughout each 90-minute session and half of the animals received the same tones with no shock. Two days later, animals underwent a cued fear test in which fear was measured using conditioned suppression of lever pressing. Brain tissue was extracted 120 minutes after the beginning of the test and subsequently processed using immunohistochemistry to target Fos, a protein marker of neuronal activation. As is typical with fear incubation, rats that underwent 10 days of fear conditioning exhibited lower fear than those that underwent 1 day of fear conditioning. There was no effect of shock on IL or PL Fos expression in either the 1 day or 10 day groups. However, both groups that received extended training (10 days) showed higher levels of Fos expression in IL and PL than the limited training (1 day) groups, regardless of

whether the tones they experienced were paired with shock. My results suggest that, in our procedure, neither IL nor PL activity are associated with high fear after a single day of fear training or suppression of fear after extended fear training, or that any such association is undetectable with our procedures. However, our results also suggest that the extended lever-press training in our fear incubation procedure leads to increased IL and PL activity (possibly related to habit formation), and that the increased neuronal activity is unaffected by the co-occurring fear training and associated stress. Additional research will be needed to determine whether operant responding in our task would be insensitive to devaluation, and whether habit formation would be affected by co-occurring fear training. I will also analyze tissue (taken from rats in the current experiment) from the amygdala, a region known for its role in fear behaviors, for fear-related Fos expression.

Table of Contents

List of Figures vii
List of Tables x
Acknowledgements xi
Dedication xii
Chapter 1 - Introduction 1
Fear Conditioning 1
Animal Models of Fear Incubation 2
Extended Training Fear Incubation Paradigm 6
Research Methods and Findings in the Role of the Prefrontal Cortex in Fear Learning and Extinction 8
Measures of Neuronal Activity and Microstimulation in Rodents 9
Prelimbic activity 11
Infralimbic activity 12
Lesions and Inactivations 15
Prelimbic cortex 16
Infralimbic cortex 17
Behavioral Flexibility and Habit Formation 17
Overtraining Leads to Habit-Formation 19
PL in Flexible Decision-Making 20
IL in Habitual Responding 21
Hypotheses 22
Chapter 2 - Methods 24
Animals 24
Apparatus 25
Behavioral Training 25
Behavioral Measures 27
Immunohistochemistry 29
Statistical Analyses 30
Chapter 3 - Results 32

Behavioral Results 32
Behavioral training 32
Behavioral testing 34
Neurobiological Results 36
IL effects 37
PL effects 38
Chapter 4 - Discussion 39
Discussion of Behavioral Results 39
Discussion of Neurobiological Results 40
PL activity did not appear to be related to fear training 40
IL activity did not appear to be related to fear training 42
Increased neuronal activity in PL and IL may be indicative of goal-directed and habitual behavior, respectively 46
Methodological Considerations 48
Conclusions 52
Chapter 5 - Epilogue 53
References 54

List of Figures

Figure 1. Behavioral training data from the current experiment. A. Average precue lever-pressing (30-sec precue period) for each day. A comparison of precue lever-pressing across the training sessions showed that precue lever-pressing increased across the 10 days of training (* indicates a main effect of Day, significant at $p < 0.05$). A comparison of precue lever-pressing on the last day of training for each group showed that groups with 10 days of training showed higher precue lever-pressing than groups that received 1 day of training (# indicates a main effect of Training Length, significant at $p < 0.05$). B. Average suppression ratio per day for each training group. Higher suppression ratio values represent higher fear and lower values represent lower fear. A comparison of suppression ratios across training sessions showed that the 10 day-shock group had a high suppression ratio that decreased across training sessions (particularly Days 7-10 of training), while the 10 day-tone group had low suppression ratios throughout training (*† indicates a Shock X Day interaction, significant at $p < 0.05$). For Fig. 1A-B, groups that received 1 day of training are indicated with a triangle and groups that received 10 days of training are indicated with circles. Groups that received tone-shock pairings are indicated with black symbols and groups that received tone-only exposure during training are indicated with white symbols. C. Average suppression ratio on the last day of training for each training group. Black bars indicate groups that received tone-shock pairings, while white bars indicate groups that received tone-only exposure. A comparison of the last day of training for all groups, showed that Shock groups had higher suppression ratios than Tone-only groups (* indicates a main effect of Shock, significant at $p < 0.05$). 71

Figure 2. Behavioral test data from the current experiment. A. Average precue lever-pressing (30-sec precue period) for each day. A comparison of precue lever-pressing across test trials shows that groups that received 10 days of training had higher rates of precue lever-pressing than groups that received 1 day of training, regardless of shock exposure (# indicates a main effect of Training Length, significant at $p < 0.05$). B. Suppression ratio across test trials for each training group. High suppression ratio values indicate higher fear and low values indicate lower fear. A comparison of suppression ratios showed that suppression ratios decreased as training length increased in groups that received shock, while suppression

ratios started and stayed low for the tone-only groups (*# indicates a Training Length X Shock interaction, significant at $p < 0.05$). The same comparison also showed that suppression ratios decreased across test trials for groups that received shock, but suppression ratios started low and stayed low for the tone-only groups (*† indicates a Shock X Trial interaction, significant at $p < 0.05$). For Fig. 2A-B, groups that received 1 day of training are indicated with a triangle and groups that received 10 days of training are indicated with circles. Groups that received tone-shock pairings are indicated with black symbols and groups that received tone-only exposure during training are indicated with white symbols. For Fig. 2C, Black bars indicate groups that received tone-shock pairings, while white bars indicate groups that received tone-only exposure (*# indicates a Training Length X Shock interaction, significant at $p < 0.05$). 73

Figure 3. IL Fos expression in the 4 experimental groups. A. A comparison of the average Fos expression across the different training groups. Black bars indicate groups that received tone-shock pairings, while white bars indicate groups that received tone-only exposure. Groups that received 10 days of training showed higher Fos expression than groups that received 1 day of training, regardless of shock exposure (# indicates a significant main effect of Training Length at $p < 0.05$). B. Example images from each of the 4 experimental groups. 74

Figure 4. Correlations between IL Fos density (mm^2) and behavior (lever-press/min) during test. A. A correlation between IL Fos density and precue lever-pressing during test for all subjects. B. A correlation between IL Fos density and suppression ratio during test for all subjects. C. A correlation between IL Fos density and precue lever-pressing for subjects that received shock. D. A correlation between IL Fos density and suppression ratio for subjects that received shock. For Fig. 4A-D, none of the correlations were significant, $r = -0.16$-0.11. 75

Figure 5. PL Fos expression in the 4 experimental groups. A. A comparison of the average Fos expression across the different training groups. Black bars indicate groups that received tone-shock pairings, while white bars indicate groups that received tone-only exposure. Groups that received 10 days of training showed higher Fos expression than groups that received 1 day of training, regardless of shock exposure (# indicates a significant main

effect of Training Length at $p < 0.05$). B. Example images from each of the 4 experimental groups. 76

Figure 6. Correlations between PL Fos density (mm^2) and behavior (lever-press/min) during test. A. A correlation between PL Fos density and precue lever-pressing during test for all subjects. B. A correlation between PL Fos density and suppression ratio during test for all subjects. C. A correlation between PL Fos density and precue lever-pressing for subjects that received shock. D. A correlation between PL Fos density and suppression ratio for subjects that received shock. For Fig. 6A-D, none of the correlations were significant, r = -0.24--0.04. 77

List of Tables

Table 1 68
Table 2 69
Table 3 70

Acknowledgements

I would like to thank my advisor, Charles Pickens, for his guidance and support. I would also like to thank the members of the Comparative Learning and Pharmacology Lab who assisted with data collection, especially Hayley Fisher, Christian Foster, Brooke Fowler (Gaeddert), Alicia Lensing, Charday Long and Skyler Wikoff (Gilbert).

Dedication

In memory of my grandfather, Najil Šehić, who nurtured my curiosity from the time I was a young child. That, in addition to his love, encouragement, and chess lessons, led me to where I am today.

Chapter 1 - Introduction

The current study was designed to test the neurobiological mechanism underlying the extended training fear incubation paradigm. In particular, I was interested in whether an extinction-like process might be responsible for the initially low fear caused by this paradigm, so I begin this literature review by explaining the rationale behind that possibility. However, the data do not support this idea. Instead, I found evidence to suggest that the extended training fear incubation paradigm may lead to habit formation. Consequently, I go on to discuss the potential for the extended training fear incubation paradigm to lead to habit formation in the literature review.

Fear Conditioning

Pavlovian fear conditioning is a procedure commonly used to model fear and anxiety (Annau & Kamin, 1961; M. Davis, 1990; Iwata & LeDoux, 1988; Lang, Davis, & Ohman, 2000; Walters, Carew, & Kandel, 1981). In contextual fear conditioning, the context in which fear conditioning takes place serves as a configural cue that is associated with shock, and extinction and/or testing may take place in the same or a different context. In discrete cued fear conditioning, a discrete cue, often a tone in rodent studies, signals the delivery of an impending electrical shock. The length of the sessions, number of shocks delivered, and strength of the shocks vary depending on the researcher, but the basis of the paradigm remains the same across contextual and discrete cued fear conditioning. Fear is often tested in extinction (fear-related cues are present, but no shock is delivered) and measured in animals using freezing (no movement indicates high fear) or the conditioned emotional response (CER) measure (low or no lever pressing during a discrete cue when compared to baseline indicates high fear).

Testing normally occurs 1-2 days after the final training session but can be conducted after a delay. As a general rule, fear conditioning procedures result in fear responses that either decrease or are maintained over time. Depending on the procedure, fear may be maintained for days or even months before decreasing (Borghans & Homberg, 2015; LeDoux, 2007; Rasmusson & Charney, 1997). Interestingly, there are a few Pavlovian fear conditioning procedures that cause a long-lasting increase in fear to the conditioned cue across an extended training-free interval when compared to testing 24 hours after training. These procedures that cause increases in fear to the conditioned cue over time tend to be contextual fear conditioning models, but one discrete cued fear conditioning procedure that does result in increased fear over time is the extended training fear incubation paradigm (Pickens, Golden, Adams-Deutsch, Nair, & Shaham, 2009b). A unique application of this procedure and other incubation paradigms is modeling delayed-onset PTSD, in which symptoms increase in severity over time. Delayed-onset PTSD makes up approximately one-third of all PTSD cases (Andrews, Brewin, Stewart, Philpott, & Hejdenberg, 2009) yet this pattern of PTSD is not effectively modeled by the standard fear conditioning tasks used to model PTSD because they result in stable fear rather than fear that increases over time. The current study aimed to examine the neurobiological basis behind the increase in fear over time in this extended training paradigm by focusing on the reason fear is so low after training. Below I review animal models of fear incubation, with a special focus on the extended training fear incubation paradigm, as it is the model I used in the present experiment.

Animal Models of Fear Incubation

Most procedures do not cause a lasting increase in fear when comparing levels of fear at a test 24-48 hours after training to tests at later timepoints. Examining procedures that *do* produce increased fear expression from its initial levels 24-h after training may provide insight into the

mechanisms underlying fear expression that increases over time, with that expression being sustained. Below, I discuss rodent models in which fear increases over time from its initial levels 1-2 days after conditioning occurs.

A number of animal models of increasing fear are contextual fear conditioning paradigms. Generally, increasing the number of foot-shocks in contextual fear conditioning paradigms produces higher fear to fear-related cues when testing after an extended post-conditioning interval. However, contextual conditioning procedures also often result in generalization, or fear to other contexts or untrained cues that emerges over time, so this increase in fear is nonspecific, especially as the number of shocks administered increases.

While the majority of procedures result in fear that is maintained at the same level over time rather than increasing (Gale et al., 2004; Gleitman & Holmes, 1967; Hendersen, 1978; Poulos et al., 2016; Quirk, 2002), below I discuss the exceptions that may be more relevant, as they result in increased fear. In one demonstration, Poulos et al. (2016) conducted a contextual conditioning experiment in which rats received 0, 1, 2, or 5 foot-shocks and used freezing as a measure of fear. Animals were tested in extinction 1 or 28 days later in the training context, and the following day in a novel context. Fear to the training context was higher on day 28 than on day 1 in the animals that received 2 foot-shocks, while animals that received 1 or 5 foot-shocks maintained the same level of fear from day 1 to day 28. Fear to the novel context did not increase over time in the 0 and 1 shock groups and increased only slightly in the 2 and 5 shock groups (such that the main effect of shock number was not significant, and planned comparisons found no difference between day 1 and day 28 fear in any group). These findings suggest that the incubation effect was not due to generalization. This shows that fear incubation can occur under

the right conditions, but it is parameter-specific (in this case, only the animals given two shocks showed incubation).

Relatedly, Houston and colleagues (1999) fear conditioned rats from three different age groups (3, 9, or 27 months old) using tone-shock pairings in a distinct context and freezing as a measure of fear. A subset of rats from each age group was tested at a retention interval of 1, 20, 40, or 60 days for fear to the context associated with shock, fear to the tone that predicted shock (tested in a novel context), and fear to a novel context. Rats in all three age groups showed an increase in fear to the tone as the retention interval increased. They also all showed an increase in fear to the novel context (indicating generalization) as the retention interval increased, and the increase was greatest in the 3 month old rats, followed by the 9 month old rats, and was marginal in the 27 month old rats. Important to note, a ratio of tone fear to novel context fear (which can correct for whether the increase in fear to the tone is really due to increased fear to the novel context in the background) stayed constant throughout testing intervals, further suggesting there was fear generalization and the increased fear to the tone might simply be due to this generalization. Finally, 3 and 9 month old rats showed increased fear to the training context that the 27 month old rats did not exhibit. In summary, all rats showed increases in fear to the tone cue that predicted shock in this procedure, although this might be due to constant fear to the tone being inflated by increased fear to the context in the background. Additionally, younger rats tended to show increases in fear to the trained and novel contexts as well.

Lastly, Balogh and colleagues (2002) trained two commonly used strains of mice (B6 and D2) in a cued fear conditioning task and compared their behavior at different time points and in multiple contexts. Mice were trained with 2 30-second auditory cues, each followed by a foot shock. They were then tested in extinction at long intervals (2, 3, 7, 14, 30, or 60 days) with 24-

hour controls for comparison and freezing as a measure of fear. B6 mice exhibited increased fear to the training context (compared to the day 1 test) at the 14 day interval, but fear decreased to levels indistinguishable from day 1 on day 60. B6 mice also showed steady fear responding to the tone CS across all retention intervals that was unchanged from the day 1 test. In contrast, D2 mice exhibited steady fear to the training context at time points 1, 2, and 3, and subsequently decreased fear on days 7-60. D2 mice also exhibited steady fear to the tone CS that tended to decrease over time, with fear expression on day 60 lower than fear expression on days 1, 2, 3, and 14. In summary, B6 mice showed specific contextual fear incubation while D2 mice did not.

Additional contextual fear conditioning procedures have been called models of incubation, but do not fit the description quite as well as the Poulos et al. (2016), Houston et al. (1999), and Balogh et al. (2002) procedures. Frankland and colleagues (2004) context-conditioned 129P2/OlaHsd mice (a wild-type mouse sometimes used as a background for knockout mice) using 5 foot-shocks and tested them 1, 3, 18, or 36 days later. Fear was sustained across the testing intervals and showed a non-significant trend towards increasing with longer test intervals. Similarly, Siegmund and Wotjak (2007) contextually conditioned mice and tested them in either the same context or in a different context with a neutral cue 1, 14, or 28 days later. They found similar levels of freezing in the training context at each time interval, but an increase in freezing to the neutral cue in a different context on day 28, which represents generalization because fear was not increased to the trained cue. Tsuda and colleagues (2015) used the same model as described by Siegmund and Wotjak (2007), but they were able to find increased fear to the training context, unlike Siegmund and Wotjak (2007). The reason for the discrepant findings between the two studies is unclear, but one possible explanation is that the two groups used different strains of mice, with differing genetic backgrounds (Tsuda et al. used C57BL/6J mice

and Siegmund and Wotjak used C57BL/6N mice), as no other methodological differences were apparent.

Although the existing research shows that contextual fear incubation is possible, it is clear from the literature that this phenomenon is parameter-specific. The reviewed literature demonstrates that contextual fear incubation can differ based on the age and strain of the animals used as well as the number of shocks that are administered. Because of this, it is difficult to derive any general patterns that determine whether contextual fear incubation will occur.

Extended Training Fear Incubation Paradigm

The animal models of contextual fear conditioning described above are not perfect. They generally do not cause an increase in fear to the trained cues over time (incubation), but rather high fear to the trained cues that either decreases or is sustained. When fear *does* increase, it is often associated with generalization, an increase in non-specific fear, making the assessment of incubation more difficult to interpret. However, previous research has demonstrated that a lasting increase in fear is possible, particularly after "overtraining" CS-US pairings (Millenson & Dent, 1971). Across the 60 conditioned fear training sessions in this study, one of the three subjects in this paper showed a decrease in fear expression during training, but there was an increase in fear expression in this rat after a 30-day post-training period without training. Rosas and Alonso (1997) also found fear incubation in an experiment where they fear conditioned rats for a total of 16 daily sessions and tested them either 3 or 20 days after training. Rats tested on day 20 showed higher fear than those tested on day 3. Because these animals received such extended training, it is possible they were overtrained and that is why fear increased from day 3 to day 20.

One of the few animal models that reliably causes a lasting increase in fear expression to a discrete cue is the extended training fear incubation model, which is unique in a number of

ways (Pickens et al., 2009b). It uses an extended training procedure, consisting of 10 CS-US pairings per day for 10 days of conditioned fear training while other, more common cued fear paradigms use limited training: just a handful of CS-US pairings on one day of conditioned fear training. Additionally, rats that have undergone a specific permutation (discussed in-depth in the methods section) of the extended-training fear incubation procedure display low levels of fear when tested soon after conditioned fear training and higher levels of fear when tested 1 and 2 months later (Pickens et al., 2009b). This is a highly reliable phenomenon, found when fear is measured using CER in the form of conditioned suppression of lever pressing as well as freezing (Pajser, Limoges, Long, & Pickens, 2019; Pickens et al., 2009a; Pickens, Navarre, & Nair, 2010). However, when J. D. Morrow, Saunders, Maren, and Robinson (2015) used this procedure, but categorized animals based on sign- and goal-tracking behaviors, the authors found that a subset of their subjects, the goal-trackers, did not show fear incubation. Importantly, when the same procedure is implemented, but training is reduced to just one session, fear is high immediately and does not incubate (Pickens et al., 2009a; Pickens et al., 2009b).

The reason why fear is low initially after extended fear training is unknown. One possibility is that the internal representation of fear is actually high, but rats are in a state of dissociation, and as a result are unable to produce a fear response (Morrow, Saunders, Maren, & Robinson, 2015). Alternatively, perhaps rats' pain sensitivity is decreased with extended fear training. Because fear is often sensitive to the current value of the US (Fanselow & Gale, 2003; J. D. Morrow, Maren, & Robinson, 2011; Rabinak & Maren, 2008; Rabinak, Orsini, Zimmerman, & Maren, 2009; Rescorla, 1973; Rescorla, 1974), a US rendered ineffective due to decreased pain sensitivity might result in low fear if rats are sensitive to the current value of the shock at the time of test. Yet another possibility is that rats are habituating to the foot-shocks

delivered during fear conditioning. This would mean that rats are undergoing a process akin to extinction (learning that a prior contingency is no longer in place due to the lack of US delivery), as tones are paired with an ineffective shock, which may be functionally similar to be being paired with no shock at all. This would cause low fear soon after extended training. The shocks administered in the extended training fear incubation procedure are mild, at only 0.5mA, so it is possible that mild shocks and the tone that predicts them would become irrelevant over the course of extended training (Annau & Kamin, 1961). In this instance, it might be that spontaneous recovery, an increase in behavioral expression due to the passage of time, is responsible for the increase in fear seen over time (Goode & Maren, 2014; Ma, Ma, & Yu, 2012; Pavlov, 1927).

The last of these possibilities can be tested by examining whether extended fear conditioning leads to similar brain area activity patterns to those observed after conditioned fear extinction. The infralimbic cortex (IL) is a subdivision of mPFC heavily involved in extinction (Quirk, 2000; Sierra-Mercado et al., 2011), suggesting this might be a promising area to investigate. Examining the activity of this area would test whether an extinction-like process driven by the IL might underlie the behavioral pattern seen in the extended training fear incubation procedure. If repeated shock presentations do indeed lead to an extinction-like process, then increased activity of PFC regions such as IL, which exhibits increased neuronal activity during extinction (Sierra-Mercado, Padilla-Coreano, & Quirk, 2011), may be seen.

Research Methods and Findings in the Role of the Prefrontal Cortex in Fear Learning and Extinction

Although the focus of this study is an extended fear conditioning procedure, this procedure is quite uncommon, so below I have outlined the role of prefrontal cortex regions in

more standard, limited fear conditioning paradigms (consisting of 1-2 days of conditioned fear training), which result in immediately high fear.

Measures of Neuronal Activity and Microstimulation in Rodents

In this section I will introduce and explain several methods used to measure or alter, without silencing, neuronal activity. Specifically, I will discuss immunohistochemistry, which indirectly measures neuronal activity via endogenous protein expression; electrophysiology, which measures electrical activity; microstimulation, which affects neuronal activity using electrical signals, and optogenetics, which affects neuronal activity using light stimulation.

Immunohistochemistry is a well-established tool for the visualization of various structures in tissue samples. Common targets of immunohistochemistry in brain tissue are proteins coded for by immediate early genes (IEG) such as *c-Fos, zif268, Arc, Homer*, and *c-jun*. Immediate early genes are thought to cause long-term changes in expression of other genes, producing lasting alterations in neuronal responding to the stimulus or stimuli that caused IEG expression in the first place (Sagar, Sharp, & Curran, 1988). They are minimally expressed in 'quiet' or inactive neurons, their transcription temporarily surges in response to neuronal stimulation, and the products of their transcription have short half-lives (Sheng & Greenberg, 1990). I will focus on the protein Fos (which the *c-Fos* gene codes for [Morgan & Curran, 1991]), as the timing of its expression works well for our task. Its peak expression is 90-120 minutes after neuronal activity and is a result of neuronal depolarization, meaning neurons exhibiting extensive excitatory signaling will express Fos (Hoffman, Smith, & Verbalis, 1993; Morgan & Curran, 1986). These properties conveniently allow the Fos protein to be used as an indirect marker of activation, if tissue is collected 90-120 minutes after the presentation of the target stimulus.

Electrophysiology is a technique that can be used as a direct measure of neuronal activity. Although a global measure of neuronal activity can be recorded using electroencephalography, animal research often uses single-unit recording, a specific type of electrophysiology, which allows researchers to measure the electrical activity of individual neurons (Delacour, Bassant, Onofrj, Santucci, & Kleinlogel, 1990). Global measures of neuronal activity generally sacrifice one type of temporal or spatial resolution for the other, but single-unit recording allows for both temporal and spatial precision. As a result, electrophysiology can be used to determine to what extent different brain regions are involved in any given task.

Microstimulation is a related technique in which a population of neurons is electrically or optogenetically stimulated in order to determine the role of the stimulated neurons (Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006). Optogenetic stimulation is performed by using lasers of specific wavelengths of light to increase or decrease the neural activity of a specific subset of neurons that are biomedically engineered to contain a light-sensitive protein via viral vector infection or the use of transgenic model organisms. This method of changing activity is highly specific, as target neurons contain a specific protein that is sensitive to the laser light being used, and other neurons should remain unaffected (Boyden, 2011; Zalocusky & Deisseroth, 2013). Stimulation, whether optogenetic or electrical, does not indicate whether the area being stimulated is required for a task, but it does inform researchers of what the area is capable of if it is activated. In that way, microstimulation is more related to the measurement methods used to examine brain function than the knockout methods. This allows researchers to speculate on the role of those neurons in a normally-functioning (i.e., not being microstimulated) brain without being able to say that it is required.

Prelimbic activity

Activity in the prelimbic cortex (PL) is associated with fear behavior. For example, Burgos-Robles and colleagues (2009) acquired single-cell recordings from rats in different phases of a limited training fear conditioning paradigm. The researchers found that PL activity was correlated with conditioned freezing and, specifically, the greatest activity occurred during acquisition (when fear is high), followed by early extinction (when fear is still high). Activity late in extinction and before any fear conditioning had occurred was similarly low, as was fear expression. When microstimulation was used to examine the role of PL during a fear extinction test, researchers found that PL microstimulation results in high fear expression when compared to control rats that did not receive any stimulation (Vidal-Gonzalez et al., 2006). In summary, it is difficult to define the exact role of PL in fear learning, expression, and extinction, but activity in PL is generally associated with fear learning and expression.

Immunohistochemistry has also been used to examine the role of PL in fear. High levels of Fos expression have been found in the rodent PL after fear acquisition, when compared to shock-naïve animals (Morrow, Elsworth, Inglis, & Roth, 1999). Relatedly, Santini and colleagues (2004) also examined Fos expression in rats that underwent fear conditioning and extended extinction (both within a single day) in comparison to several groups of rats that did not receive any prior fear conditioning, did not receive extinction training 90-120 minutes before sacrifice, or both. For the key experimental group, sacrifice occurred 4 hours after fear conditioning and 120 minutes after the beginning of extinction, allowing for neural activity during the beginning of extinction training to be visualized. Brain tissue was collected at a time that would capture brain activity at the beginning of extinction training, if given. The researchers found that rats that underwent extinction after fear conditioning had higher Fos expression in PL

than any of the comparison groups. Because the extinction group was the only group that experienced extinction of a conditioned fear memory, it is possible that the Fos expression seen in that group was indicative of fear extinction or fear expression. Knapska and colleagues (2012) implemented a similar approach, using a combination of Fos immunohistochemistry and anterograde tracing (a technique used to visualize projections from one brain region to another) to examine activity during a retrieval test. They injected an anterograde tracer into PL and examined Fos staining in BLA, allowing them to visualize the PL projections that synapsed onto active BLA neurons to determine that increased activity in the PL projections to the amygdala (an area associated with fear learning) is correlated with high freezing. Although the measurement is taken in the amygdala, the fact that only neurons receiving projections from PL would have the anterograde stain means this is a measurement of activity of neurons receiving information from PL, with that activity possibly caused by input from PL.

Infralimbic activity

Activity in the infralimbic cortex (IL) is generally associated with low fear expression. An electrophysiological study by Santini et al.(2008) examined IL neuron excitability in rats that underwent different phases of a cued fear conditioning paradigm. The researchers found that IL neurons showed a reduction in neuronal spiking (action potentials) after conditioning took place when compared to naïve rats. In contrast, IL neurons following the completion of extinction training showed spiking at similar rates to naïve animals. In addition, the researchers found a negative correlation between inter-spike interval (the shorter the interval, the more spiking occurs) and freezing during testing, suggesting that IL spiking promotes fear inhibition. Similarly, Soler-Cedeño and colleagues (2016) used patch-clamp electrophysiology to examine the activity of IL neurons in rats that underwent fear conditioning and subsequent extinction and

testing. IL neurons from rats that underwent contextual fear conditioning produced fewer spikes than those from control animals, which received either context exposure and no shocks or received shocks administered in rapid succession soon after being placed in the chambers, which has been shown to interfere with fear memory formation (Landeira-Fernandez, DeCola, Kim, & Fanselow, 2006). Additionally, the number of spikes was strongly negatively correlated with freezing to the context, suggesting that the decrease in spiking in IL neurons is related to contextual conditioned fear.

Stimulation studies also provide insight into the role of specific brain regions in a task. By sending signals to the neurons in a particular brain region, stimulation allows us to examine how behavior changes when that area is activated. In a task in which all rats underwent previous fear conditioning and then extinction testing, microstimulation of the IL during the extinction test resulted in low fear when compared to control rats that did not receive any stimulation (Vidal-Gonzalez et al., 2006). Optogenetic stimulation of IL during an extinction retention test also enhanced expression of extinction by decreasing fear, suggesting increased IL activity leads to decreased fear expression (Kim, Cho, Augustine, & Han, 2016).

Immunohistochemistry has also been used to examine the role of IL in fear. Morrow and colleagues (1999) found high Fos expression in IL following fear acquisition, moderate expression after fear extinction, and finally lowest expression in shock-naïve animals. Although it might seem surprising that the IL had the highest amount of Fos expression after acquisition rather than extinction, a study by Klavir and colleagues (2017) found that fear learning information is transmitted to IL as well as PL. Santini and colleagues (2004) also examined Fos expression in rats that underwent fear conditioning and extinction in comparison to rats that did not undergo extinction (one group had received just fear conditioning prior while the rest were

control groups that did not form an association between the cue and shock). The researchers found that rats that underwent extinction after fear conditioning had higher Fos expression in IL than any of the comparison groups which received no training or testing 90-120 minutes before sacrifice. Given that the extinction group was the only group that experienced extinction training, it is possible that the Fos expression seen in that group was indicative of fear extinction and/or expression. These results do not necessarily contradict the aforementioned Morrow et al. (1999) study in which fear conditioning led to high Fos expression in IL. Morrow and colleagues (1999) collected tissue at time points that were optimal for capturing neural activity related to specific parts of a fear conditioning task such as acquisition or extinction. In contrast, Santini and colleagues (2004) collected tissue at a timepoint optimal for visualizing neural activity related to fear expression during extinction or during its control procedures (homecage controls or no conditioning controls). Fear extinction tends to lead to fear expression at the beginning of extinction training, meaning only one group expressed fear within the appropriate timeframe for Fos visualization. As a result, it is not surprising that there was low Fos expression in all of the groups that did not receive extinction training (and therefore did not express fear at that time). It is possible that if Santini and colleagues (2004) also collected tissue at the same time points as Morrow and colleagues (1999), their results would be more similar. However, because of the timing of tissue collection is a critical component of experimental design when measuring Fos, and because these times differ, I am unable to directly compare the results of these studies.

Immunohistochemistry has also been used to examine IL functional connectivity with other brain regions. For example, Knapska and colleagues (2012) used a combination of Fos immunohistochemistry and anterograde tracing to determine that activity in the IL projection to the amygdala, largely separate from the PL projection, is correlated with low freezing. They

injected an anterograde tracer into IL and examined Fos staining in BLA, allowing them to visualize the IL projections that synapsed onto active BLA neurons to determine that increased activity in the IL projections to the amygdala is correlated with low freezing. Although the measurement is taken in the amygdala, the fact that only neurons receiving projections from IL would have the anterograde stain means that this is a measurement of activity of neurons receiving information from IL, with that activity possibly caused by input from IL. In summary, IL is known to be involved in fear extinction and extinction memory expression, but Fos expression suggests that it might also be activated during fear expression.

Lesions and Inactivations

Examining the effects of increased neuronal function is a useful tool for determining the role of brain regions, but it does not offer a complete picture. Assessing the effects of artificially decreasing brain activity in the same regions of interest offers an opportunity to determine more clearly what those regions do. Both lesions and inactivations allow researchers to assess what happens when a brain region is offline/inactivated, but because of the difference in the length of time in recovery from lesion surgery or inhibitory infusion/optogenetic manipulation into the brain and behavioral testing, they provide different information. Recovery from lesion surgery may last anywhere from days to weeks before animals begin behavioral training. This allows for the brain to potentially reorganize and compensate for the loss of the lesioned brain area by having a different region take over the functions that the lesioned area would typically perform. Recovery from cannulation surgery takes about as long as recovery from lesion surgery, but the damage caused by cannulation surgery is usually minimal. As such, brain function should be minimally affected until infusions or optogenetic inactivation occurs. Brain activity is dramatically impaired only once an animal receives an infusion of an inhibitory substance

(GABA agonists are a common choice) or optogenetic inactivation. If an inhibitory substance is infused, inhibition of brain activity may last anywhere from minutes to hours, but once the substance is cleared from the brain, activity returns to normal. If inactivations use optogenetic methods, the inhibition of brain activity can be tightly controlled: inhibition occurs while a laser is shining and discontinues when the laser turns off. As a result, when a lesion is performed, the brain has time to compensate for the loss of function, so impaired function as a consequence of a lesion shows that the lesioned area is absolutely necessary for the given task and this requirement cannot be circumvented by weeks of brain reorganization. Meanwhile, because inactivations (whether due to infusions or optogenetics) do not provide the opportunity for compensation of function, they show whether the inactivated region is normally necessary in a task (even if the brain may develop a compensatory response if given enough time).

Prelimbic cortex

Animal research has shown that PL activity is generally necessary for fear expression, but not fear acquisition. For example, Corcoran and Quirk (2007) inactivated PL in rats during the acquisition phase of a fear conditioning paradigm. Animals that had inactivations showed lower fear during acquisition than controls, but when tested the following day, the two groups showed comparable levels of fear. Conversely, PL inactivation during fear extinction results in low fear during extinction when compared to controls, but the inactivation group had comparable levels of fear to the control group during the extinction test, one day later when not inactivated (Sierra-Mercado et al., 2011). This shows that PL is required for expression, but not learning of fear. Optogenetic inactivation of PL during extinction also has no effect on fear behavior during an extinction retrieval test performed one day later when not inactivated (Kim et al., 2016). Because fear expression was reduced during training (when PL was inactivated), but fear memory and

extinction memory were still intact when tested occurred later, this suggests PL is involved in fear expression, not acquisition.

Infralimbic cortex

Although the exact role of the infralimbic cortex (IL) is less clear than that of PL, research has shown it is necessary for the storage of lasting fear extinction memories. Quirk and colleagues (2000) performed electrolytic or sham lesions of the vmPFC, focused on IL, and animals subsequently underwent fear acquisition and extinction and were tested for extinction memory. There was no difference in fear acquisition or within-session extinction between sham or lesion animals, but on test day sham animals displayed low fear while lesioned animals recovered fear responding (exhibiting high fear as if extinction had not occurred), suggesting long-term extinction memory was disrupted. Relatedly, Sierra-Mercado and colleagues (2011) inactivated IL during extinction in rats that had previously undergone fear acquisition. IL inactivation resulted in higher fear during within-session extinction when compared to controls, and higher fear during the extinction test (conducted one day later when not inactivated) than controls. Similarly, Kim and colleagues (2016) manipulated IL activity during different phases of a fear conditioning paradigm using optogenetics and found that inhibition of IL during extinction training impaired expression of extinction learning during a retention test a day later (resulting in high fear expression). These studies provide evidence that IL is required for extinction memory storage.

Behavioral Flexibility and Habit Formation

Important to note, however, is that PL and IL perform multiple functions. Although they are both involved in fear, they are also known to play roles in goal-directed behavior and habit formation (Killcross & Coutureau, 2003). Goal-directed behaviors can be described as those that

are sensitive to changes in the value of an outcome, and diametrically opposed to habits, which are inflexible regardless of the outcome (Adams, 1982).

The task most commonly used to study goal-directed behaviors and habit formation is devaluation, in which responses initially earn a food reward, but that food reward later has its value decreased (via selective satiety, taste aversion, etc.) (Adams, 1982; Colwill & Rescorla, 1985; Holland & Rescorla, 1975; Holland & Straub, 1979; Tinklepaugh, 1928). Devaluation tends to be conducted under extinction conditions, so that subjects must use their memory of the reinforcer earned by the operant response to guide behavior. If subjects respond for the devalued food reward then they are said to be performing habitually, while if the subject withholds responding for the devalued food reward, their behavioral flexibility is still intact, meaning they can still perform goal-directed behaviors.

As previously noted, one of the key differences between our fear conditioning procedure and most others is the extended training animals receive, which includes extended lever-press training as the rats are lever pressing for food throughout fear conditioning. Extended operant training is known to lead to habit formation, making animals' behavior inflexible so that they are unable to adjust their responding as their goals may shift (Coutureau & Killcross, 2003; Killcross & Coutureau, 2003, further discussed in the next section). As such, it quite possible animals that undergo the extended training fear incubation paradigm are responding habitually on the operant lever by the end of training, in contrast to animals that receive limited conditioned fear training. Neuronal activity related to goal-directed behavior in the limited training procedure and habitual behavior in the extended training procedure may interfere with our ability to observe differences in neuronal activity related to fear expression and suppression.

Overtraining Leads to Habit Formation

Notably, goal-directed behavior is flexible and allows for the voluntary control of behavior but requires effort. Conversely, habits are involuntary, require little to no effort, and are associated with extended training/overtraining (Adams, 1980; Adams, 1982; Coutureau & Killcross, 2003; Killcross & Coutureau, 2003). Using the devaluation task, Adams (1980) found that animals would still perform operant behaviors even when the associated reinforcer has been devalued via taste aversion, suggesting a possibility for habitual responding in the devaluation task. Similarly, Adams (1982) found that extended lever-press training (11 sessions) led to an insensitivity to devaluation (via taste aversion) that was not seen after limited lever-press training (2 sessions), which indicates that extended training may cause habit formation that limited training does not. Relatedly, Killcross and Coutureau (2003) compared rats that received limited (5 lever training sessions) and extended (20 lever training sessions) training in a selective-satiety devaluation task. They found that rats that received limited training were still sensitive to devaluation, while those that received extended training were insensitive to devaluation. Particularly relevant to the current study, Dickinson, Nicholas, and Adams (1983) found that animals trained on an interval (RI 60) schedule of reinforcement, in which reinforcement delivery is dependent on the performance of a behavior after a specified amount of time, were insensitive to devaluation via taste aversion. In contrast, animals with an identical amount of training on a ratio schedule (RR 15), in which reinforcement delivery is dependent on the performance of a behavior a specified number of times, remained sensitive to devaluation. This is especially relevant because our version of the extended training fear incubation paradigm maintains rats on a VI60 schedule, so it is plausible that the procedure may result in habit formation. In summary, habit formation (which can be induced using extended training and/or

interval schedules of reinforcement with one response and reinforcer) is associated with insensitivity to devaluation, while behavioral flexibility (often seen after limited operant training) allows for sensitivity to devaluation.

PL in Flexible Decision-Making

PL has been shown to be necessary for flexible decision-making. Killcross and Coutureau (2003) performed excitotoxic or sham lesions in PL in rats that went on to receive limited or extended training and subsequently underwent devaluation. Rats with PL lesions were insensitive to the value of the food reward regardless of training length, while only sham lesioned rats that received overtraining were insensitive, suggesting that PL is involved in behavioral flexibility. Corbit and Balleine (2003) also performed excitotoxic or sham PL lesions in rats that subsequently performed the devaluation task. When tested in extinction, lesioned rats did not show a devaluation effect, while sham rats did. Two related studies aimed to determine the timeframe during which PL is involved in goal-directed behavior. First, Ostlund and Balleine (2005) performed excitotoxic or sham PL lesions in rats either before or after extended training took place and examined behavior during a devaluation task using selective satiety to assess goal-directed behavior. Rats that received lesions prior to training showed impaired devaluation performance, while those that received lesions after training showed unaffected devaluation performance. Second, Tran-Tu-Yen and colleagues (2009) gave operant training and then induced a taste aversion in one group of animals while another group received no taste aversion, so that the group receiving taste aversion experienced reward devaluation while the other group remained unaffected. Testing took place in extinction. The researchers infused either muscimol (to inactivate) or vehicle into PL during either the operant acquisition phase or the test phase of the experiment. Animals with PL inactivations during acquisition had high responding during

testing whether or not they underwent reward devaluation, suggesting they were insensitive to devaluation. The comparable group that received vehicle infusions remained sensitive to devaluation. When infusions were given prior to testing, animals that received taste aversions responded less than animals that did not receive taste aversions regardless of PL inactivation, suggesting they were still sensitive to reward value. In summary, normal PL function during training, but not testing, is necessary for goal-directed action. This suggests that PL is necessary for the acquisition, but not expression, of goal-directed learning. If any goal-directed acquisition occurs during our conditioned fear test, then there may be goal-directed PL activity. Additionally, there should also be PL activity in animals that show fear expression, so it is possible that the neural activation related to fear expression and goal-directed behavior may interact.

IL in Habitual Responding

As previously noted, it has been shown that PL is involved in goal-directed behavior, while IL is necessary for habitual behavior. Killcross and Coutureau (2003) performed excitotoxic or sham lesions in IL in rats that went on to receive limited (up to 5 sessions) or extended (up to 20 sessions) operant training and subsequently underwent devaluation via selective satiety. They found that rats with IL lesions remained sensitive to the value of the food reward, even with extended training, suggesting that IL is involved in habit formation. In contrast, sham rats were not sensitive to the value of the reinforcer after extended training but were sensitive after limited training. Relatedly, Coutureau and Killcross (2003) infused rats with muscimol, an inactivating agent, or vehicle into IL before the testing phase of a devaluation task. In this task, animals had previously received extensive training in order to induce habitual responding. Inactivated animals regained sensitivity to reward value, while control animals did

not. This supports the idea that there is some goal-directed neural activity occurring even when animals behave habitually and IL is suppressing this goal-directed neural activity from controlling behavior. Similarly, Schmitzer-Torbert and colleagues (2015) performed neurotoxic or sham lesions to either IL or dorsolateral striatum (also involved in habits (Yin, Knowlton, & Balleine, 2004)) in rats. The researchers found that sham lesioned animals were sensitive to devaluation after extended training, but animals with neurotoxic lesions to either brain region maintained behavioral flexibility. This suggests that the neuronal activity for goal-directed behavior is still present, but that it may be suppressed by habit-related activity in IL. In sum, there may still be goal-directed neural activity even if habit-related neural activity is present in IL and habitual behavior is being expressed. Habit-related activity, if present, may overpower any goal-directed processes that take place.

Hypotheses

The current study was designed to test the neurobiological mechanism underlying the extended training fear incubation paradigm. It was based on the possibility that an extinction-like process might be responsible for the initially low fear caused by this paradigm, unlike others. In this case, I would expect low Fos expression in PL and high Fos expression in IL in animals that underwent the extended training fear incubation paradigm; high Fos expression in PL and low Fos expression in IL in animals that underwent limited conditioned fear training, and low Fos expression in both PL and IL of animals that underwent tone-only training, regardless of training length. I used 4 groups of animals to investigate this possibility: 10 day-shock, 10 day-tone only, 1 day-shock, and 1 day-tone only. The shock groups received 10 tone-shock pairings/session for the specified number of sessions, while the corresponding tone group received tone-only exposure for the same number of sessions. The tone groups served as controls for the experience

with the tones that the shock groups receive, but without the stress of experiencing the shocks. Notably, activity in the PFC can be affected by training length, particularly in the case of habit formation. If the extended training fear incubation paradigm induces habit formation, it is possible that the habit-related neuronal activity would be present in IL in the tissue of animals that received 10 days of training in comparison to those that received 1 day of training.

Chapter 2 - Methods

Animals

Male Long-Evans rats (*n*=60, Charles River, Kingston, NY) were used in this experiment. A power analysis based on the preliminary behavioral data determined that 60 animals should yield sufficient power. Because female rats have never been used in the extended training fear incubation procedure, preliminary research would have to be conducted in order to optimize the parameters of the procedure to work for female rats. As such, I used exclusively male rats, which prior research has shown perform consistently in our procedure. The rats were individually housed and held in a temperature- and humidity-controlled colony room on a 12-h reverse light-dark cycle with lights off at 7:30am. After acclimation to the facility, animals were food-restricted and fed to allow a growth in body weight of 1.5g/day for the duration of the experiment. This food restriction procedure allows the animals to grow in a controlled manner over time. Extended food restriction is a known stressor that activates the hypothalamic-pituitary-adrenal axis, which in turn has been shown to affect fear expression during testing (Rodrigues, LeDoux, & Sapolsky, 2009; Toth & Gardiner, 2000). Our food-restriction conditions are generous in an attempt to keep animals motivated to lever-press without adding stress that could affect fear conditioning or expression. Water was available *ad libitum*. Animals were run in two cohorts at two separate times. All procedures and animal care were in accordance with the Kansas State University Institutional Animal Care and Use Committee guidelines, the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and United States federal law.

Apparatus

Experiments were conducted in 12 operant chambers (Med Associates, St Albans, VT). Each chamber has two retractable levers 9 cm above the floor, but only one lever ("active," retractable lever) is extended into the chamber during behavioral sessions. Responding on this lever activates the pellet dispenser, delivering 45-mg precision pellets (#1811155, 5% fat, 66% carbohydrate, 20.3% protein; TestDiet, Richmond, IN). A red house light is located on the centered at the top of the back wall of the chambers. A tone generator that delivers a 2900 Hz, 20 dB above background tone is located directly to the right of the house light. The chambers have grid floors connected to electric shock generators that are capable of delivering a 0.5 mA scrambled foot shock.

Behavioral Training

There were 4 groups of animals in this experiment: 10 day-shock, 10 day-tone only, 1 day-shock, and 1 day-tone only. The 10 day-shock group received 10 days of conditioned fear training with tones paired with shocks, while the respective tone group received the same training but with tones only—no shock presentations. The 1 day-shock group received 1 day of conditioned fear training with tones paired with shocks, while the respective tone group received the same training but with tones only—no shock presentations. This resulted in a Training Length X Shock factorial design.

Table 1 contains a summary of the training and testing procedures. All animals underwent 5 days of lever-press training. Lever-press training consisted of: 1 session of magazine training in which a pellet was delivered every 125 seconds for 60 minutes; 2 sessions of FI1 in one day (fixed interval 1, the first lever-press after 1 second earned a reward), which ended when animals earned 50 pellets or 60 minutes were up, whichever came first; one 90-minute session of VI30

(variable-interval-30, pellet availability for lever-presses ranged from 1 to 59 seconds); and two 90-minute sessions of VI60 (variable-interval-60, pellet availability for lever-presses ranged from 1 to 119 seconds). All sessions were given in the order described above, and animals underwent one session per day for each session except the FI1 training, for which two sessions were given in one day.

Once lever-pressing was acquired, fear training groups underwent conditioned fear training sessions consisting of 10 30-second tones paired with 0.5-mA, 0.5-second foot shocks dispersed pseudo-randomly throughout a 90-minute session. Shocks of this strength are relatively mild, allowing fear to decrease across multiple days of training in a way it would not were the shocks stronger (Annau & Kamin, 1961; Witnauer & Miller, 2013). The tones and foot shocks always co-terminated. Control groups received 10 tone presentations without shock, also presented pseudo-randomly throughout a 90-minute session to account for experience with the tone cue and any effect that might have on behavior (Rodnick, 1937; Thompson & McConnell, 1955). This control was also meant to allow examination of the behavioral and neural data while avoiding the possibility of any inhibitory or excitatory conditioning that might occur to the tone although recent research findings suggest that this might not actually control for the possibility of inhibitory learning, as discussed in the discussion section (Lingawi, Holmes, Westbrook, & Laurent, 2018; Lingawi, Westbrook, & Laurent, 2017). The extended training groups received one of these sessions daily for 10 days while the limited training groups received just 1 such session. Ten sessions of conditioned fear training is necessary because all prior published work with the extended training fear incubation paradigm uses 10 conditioned fear training sessions, and a personal communication revealed that fear did not incubate when just 8 sessions were used (though there were other confounds) (C. L. Pickens, personal communication, February, 4,

2019). On the following day, all groups underwent lever reacquisition for one 90-minute session in which they were allowed to lever-press for food pellets on a VI60 schedule with no tones or shocks presented. This reacquisition session served to extinguish any contextual fear that may have been acquired, giving a relatively pure assessment of cued fear (Jacobs, Cushman, & Fanselow, 2010). The next day, each group underwent a cued fear test in which the training tone was presented 4 times without shock in a 35-minute VI60 session. Testing occurs in extinction such that shocks are not delivered so that fear memory is assessed rather than a physiological reaction to a physical stressor that is actually presented. Animals were deeply anesthetized using sodium pentobarbital (1mL, i.p.) 120 minutes after the beginning of the cued fear test (85 minutes after the end of the cued fear test) and transcardially perfused with 100mL 0.1 M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (in PBS; pH 7.4). The timing of the perfusions is within the ideal range to capture peak Fos expression throughout the cued fear test.

Behavioral Measures

The behavioral dependent measure is CER measured via a suppression ratio, calculated as ((Precue responding-Cue responding)/(Precue responding+Cue responding)) (Armony, Servan-Schreiber, Romanski, Cohen, & LeDoux, 1997). There were no trials in which the numerator and denominator were both zero, although this would be dealt with by averaging the suppression ratio for the trials immediately before and after the trial in question. The suppression ratio used in the current study is in contrast to the more well-known Kamin-Annau suppression ratio: Cue responding/(Precue responding+Cue responding) (Annau & Kamin, 1961). The two formulas are similar, but the main difference is that the subtraction of cue responding from precue responding in the numerator of our suppression ratio makes it so that a value near 0

signifies low fear while a value near 1 signifies high fear. In contrast, in the Kamin-Annau suppression ratio, values near 0.5 signify low fear while values near 0 signify high fear. Statistical analyses with the two forms of suppression ratios produce identical statistical results. The main reason for this deviation from this more popular suppression ratio is that I believe our suppression ratio more clearly communicates the data, with higher values indicating higher fear and lower values indicating lower fear.

The use of CER to measure fear offers two main advantages over other methods, such as conditioned freezing. First, because lever-presses are used to calculate the suppression ratio, the calculation of the suppression ratio is relatively automated in comparison to scoring freezing videos, for which a scorer blind to the condition of each rat would have to sit and score behavior for each trial of training and testing in a lengthy and time-consuming process. The automation of calculating a suppression ratio also removes the human error inherent in scoring freezing in videos. Second, the suppression ratio standardizes fear based on each individual rats' baseline lever-pressing, while freezing is typically reported without standardization. Standardization minimizes the effects of individual differences in baseline lever-press rate on the fear measure and decreases between-subjects variability due to baseline lever-press rate. Finally, the suppression ratio is a measure of fear that depends on at least some degree of movement (a lever-press). Freezing is considered an absence of movement except that which is required for breathing (Fanselow, 1980), so it is possible that an animal that is not moving for reasons other than fear (i.e., sleeping) could be scored as highly fearful if using freezing as the fear measure. The same is not the case if using the suppression ratio as a fear measure in hungry rats lever-pressing for food because movement is required to press a lever, encouraging rats to move unless they are afraid. Despite the differences between suppression ratios and freezing as fear measures,

the two show similar patterns (Bouton & Bolles, 1980; Mast, Blanchard, & Blanchard, 1982; Pickens et al., 2010).

Immunohistochemistry

Rats were perfused 120 minutes from the beginning of the cued fear test and brain tissue was extracted and postfixed (4% paraformaldehyde in PBS for 2 hours) and then cryoprotected (30% sucrose in PBS; until tissue sank in the solution). The tissue was frozen and sliced coronally into 40-μm thin slices using a cryostat at -18°C (Leica Microsystems, Wetzlar, Germany). Slices were stored in cryopreservant (20% glycerol and 2% dimethyl sulfoxide in 0.1 M phosphate buffer) and frozen at -80°C until immunohistochemical processing.

Immunohistochemistry was performed on free-floating sections according to procedures from Marchant and colleagues (2010) to stain for Fos. All washes and incubations included gentle agitation with a plate shaker (Thermo Fisher Scientific, Waltham, MA). Sections were washed in 0.1 M phosphate buffer (PB; pH 7.4) repeatedly to remove cryopreservant from the tissue before beginning the immunohistochemical processing. The sections then underwent 4 30-minute washes in the following order: PB; 50% ethanol in PB, 50% ethanol and 3% H_2O_2 in PB, and 5% normal horse serum (NHS) in PB. The tissue was then incubated in rabbit-anti-c-Fos serum (1:8,000, Cell Signaling Technology, Danvers, MA) diluted with 0.1% sodium azide, 0.2% Triton X-100, and 2% NHS in PB at room temperature for 48 hours. Unbound primary antibodies were washed off of the sections with 3 10-minute washes in PB. The sections were then incubated with donkey anti-rabbit-biotin (1:1,000, Jackson Immuno Research Laboratories, West Grove, PA) diluted with 0.2% Triton X-100, 2% NHS, and PB at room temperature for 2 hours. Unbound secondary antibodies were washed off with 3 additional 10-minute washes in PB. Sections were then be placed in the mixed avidin-biotin horseradish peroxidase complex

solution (ABC Elite Kit: 6μL/mL avidin and 6μL/mL biotin, Vector Laboratories, Burlingame, CA) for 2 hours. Visualization was achieved using a 10-minute incubation in a solution of 2% nickel sulfate, 0.25% 3,3'-diaminobenzidine, 0.04% ammonium chloride, and 0.02% D-glucose in a 0.1M acetate buffer solution (pH 6, all chemicals from Sigma-Aldrich, St. Louis, MO) followed by a 9-minute incubation in the same solution with the addition of 0.2μL/mL glucose oxidase. The reaction was stopped with acetate buffer and sections underwent 2 10-minute washes with PB and were stored at 4°C in PB until mounting.

Sections were mounted onto charged slides, dehydrated, and coverslipped with mounting medium (Permount, Fisher Scientific, Waltham, MA). Images were taken with a microscope and SPOT 5.1 Advanced Software at 10X by using an atlas for reference (Paxinos, 2009). Images at +3.24 and + 3.00 mm were acquired and used for analysis of Fos present in PL and IL to measure activity in sections of PL and IL examined in previous literature, based on architectural features (Knapska & Maren, 2009; LaLumiere, Niehoff, & Kalivas, 2010; B. A. Morrow et al., 1999; Quinones-Laracuente, Hernandez-Rodriguez, Bravo-Rivera, Melendez, & Quirk, 2015; Sharpe & Killcross, 2015; Sierra-Mercado et al., 2011). Neurons that are positive for Fos were counted using the multi-point tool in ImageJ, by an experimenter who was blind to treatment condition, and the number of Fos positive neurons were normalized for the size of the picture. The neurobiological dependent variable is the density of Fos-positive cells in PL and IL.

Statistical Analyses

Data were analyzed using mixed-effects models and ANOVAs in JMP Pro 14.1.0 (Cary, NC). Mixed effects models were used so that Trial and/or Day could be treated as a continuous variable. ANOVAs were used so that the data could be analyzed as they would be traditionally, allowing for comparison of the current data to the existing literature. Mixed-effects models used

were conducted using restricted maximum likelihood variance component estimates. Significant main effects and interactions were probed using Tukey's HSD test for all pairwise comparisons. The testing of two rats was compromised during data collection, so there was no behavioral test data or brain tissue available for them. Additionally, tissue from two rats were excluded from neural analysis because there were no usable sections from the medial prefrontal cortex due to cryostat complications. This resulted in 15 rats per group for the behavioral training data and 13-15 rats per group for the behavioral test data and the neurobiological data.

Chapter 3 - Results

Behavioral Results

Behavioral training

The general pattern found when examining precue lever-presses during training is that as training increases, so do precue lever-presses. More importantly for my analysis, during training, shock groups had high suppression ratios that decreased over days in the group given 10 days of training, while tone-only groups had low suppression ratios that did not change over time.

A mixed-effects model was run on the dependent variable, precue lever-pressing, with the fixed effects of Shock and Trial, and the interaction between the two, and the random effects of Subject and the Subject X Trial interaction. There was a significant main effect of Trial $F(1, 39.98) = 86.08$, $p < 0.01$. Pairwise comparisons of precue lever-pressing were conducted using Tukey's HSD test to probe the main effect of Trial at trials 1, 10, and 100, and found that precue lever-pressing increased as the Trial increased (Table 2). There were no other significant effects or interactions ($F = 0.00\text{-}2.66$, $p = 0.11\text{-}0.97$).

A secondary analysis using a more traditional mixed-factor ANOVA supported the findings of the primary analysis. The secondary analysis was conducted on the dependent variable, precue lever-pressing, with the between-subjects variable of Shock (Shock vs. Tone-only) and the within-subjects variable of Day (training days 1-10) for the animals that received 10 days of training. There was a significant main effect of Day, $F(9, 252) = 28.14$, $p < 0.01$, indicating that precue lever-pressing increased over the course of training (Fig. 1A). There was also a marginally significant Day X Shock interaction, $F(9, 252) = 1.87$, $p = 0.06$, suggesting that this increase in precue lever-pressing over the course of training was more pronounced in the 10 day-shock group than in the 10 day-tone only group, although these results must be interpreted

with caution because the alpha level of 0.05 was not achieved. The main effect of Shock was not significant ($F = 0.08$, $p = 0.78$).

An additional secondary analysis was conducted in order to compare animals on the last day of conditioned fear training they received, and this analysis supported the findings of the primary analysis. A 2X2 ANOVA was conducted on the dependent variable, precue lever-pressing, and the independent variables of Shock (Shock vs. Tone-only) and Training Length (1 vs. 10 days). There was a significant main effect of Training Length, $F(1, 56) = 23.69$, $p < 0.01$, indicating that precue lever-pressing was higher for groups that received 10 days of training compared to groups that received 1 day of training (Fig. 1A). There were no other significant effects or interactions ($F = 0.30$-0.77, $p = 0.38$-0.59).

A mixed-effects model was run on the dependent variable, suppression ratio, with the fixed effects of Shock, Trial, and the interaction between the two, and the random effects of Subject and the Subject X Trial interaction. There was a significant main effect of Shock, $F(1, 60.02) = 14.79$, $p < 0.01$, a significant main effect of Trial, $F(1, 34.36) = 9.85$, and a significant Shock X Trial interaction, $F(1, 34.36) = 6.20$, both $p < 0.05$. Pairwise comparisons of suppression ratio were conducted using Tukey's HSD test to probe the main effect of Trial and the interaction of Shock X Trial at trials 1, 10, and 100. The shock and tone-only groups initially differed significantly at trials 1 and 10, but eventually converged and no longer differ on trial 100 (Table 3).

A secondary analysis was conducted and supported the findings of the primary analysis. A more traditional mixed-factor ANOVA was conducted with the dependent variable, suppression ratio, and the between-subjects variable of Shock (Shock vs. Tone-only) and the within-subjects variable of Day (training days 1-10) for the animals that received 10 days of

training. There was a significant main effect of Day, $F(9, 252) = 2.71$, $p < 0.01$, and a significant main effect of Shock, $F(1, 28) = 11.92$, $p < 0.01$. There was also a significant Day X Shock interaction, $F(9, 252) = 2.87$, $p < 0.01$, reflecting that the suppression ratios for the 10 day-tone only group did not change over the 10 days of training, but the suppression ratios for the 10 day-shock group started high and tended to decrease over the 10 days of training (Fig. 1C). Specifically for the animals that experienced shock, suppression ratios for days 7-10 were significantly lower than for day 2, and the suppression ratio for day 9 was significantly lower than for day 3 (all $p < 0.05$).

A secondary analysis was conducted in order to compare animals on the last day of conditioned fear training they received and supported the findings of the primary analysis. A 2X2 ANOVA was run on the dependent variable, suppression ratio, and the independent variables of Shock (Shock vs. Tone-only) and Training Length (1 vs. 10 days). There was a significant main effect of Shock, $F(1, 56) = 14.54$, $p < 0.01$, indicating that the suppression ratio was increased for groups that received shock compared to groups that received tones only (Fig. 1D). There were no other significant effects or interactions ($F = 0.19$-0.26, $p = 0.61$-0.66).

Behavioral testing

As with the training data, the pattern found when examining precue lever-presses during testing is that animals that had more training showed higher precue lever-pressing. More importantly for my analyses, the 1 day-shock group had the highest fear expression during testing, while the 1 day-tone only, 10 day-tone only, and 10 day-shock groups all showed similarly low fear expression.

A mixed-effects model was run on the dependent variable, precue lever-pressing, with the fixed effects Shock (Shock vs. Tone-only), Training Length (1 vs. 10 days), Trial (the 4 test

trials), and all possible interactions, and the random effects of Subject and the Subject X Trial interaction. There was a significant main effect of Training Length, $F(1, 54) = 10.27$, $p < 0.01$, indicating that precue lever-pressing increased as Training Length increased (Fig. 2A). There were no other significant effects or interactions ($F = 0.05$-2.19, $p = 0.14$-0.83).

A secondary analysis using a more traditional method of analysis supported the primary findings of the primary analysis. A mixed-factor ANOVA was conducted on the dependent variable, precue lever-pressing, with the between-subjects variables of Shock (Shock vs. Tone-only) and Training Length (1 vs. 10 days) and the within-subjects variable of Trial (test trials 1-4). There was a significant main effect of Training Length, $F(1, 54) = 10.27$, $p < 0.05$, indicating that precue lever-pressing was increased for groups that received 10 days of training compared to groups that received 1 day of training (Fig. 2A). There were no other significant effects or interactions ($F = 0.04$-2.14, $p = 0.15$-0.84).

A mixed-effects model was run on the dependent variable, suppression ratio, with the fixed effects of Shock (Shock vs. Tone-only), Training Length (1 vs. 10 days), Trial (the 4 test trials), and all possible interactions, and the random effects of Subject and the Subject X Trial interaction. There was a significant main effect of Shock, $F(1, 54) = 4.09$, $p < 0.05$, a significant Training Length X Shock interaction, $F(1, 54) = 5.67$, $p < 0.05$, and a significant Shock X Trial interaction, $F(1, 54) = 15.72$, $p < 0.01$. Pairwise comparisons of suppression ratio were conducted using Tukey's HSD test to probe the Shock X Trial interaction and found that the shock groups differed from the no-shock groups on Trial 1, but not on subsequent trials (Fig. 2C). Pairwise comparisons of suppression ratio were conducted using Tukey's HSD test to probe the Training Length X Shock interaction and found that the 1 day-Shock group had a significantly higher suppression ratio than either the 1 day-Tone only or 10 day-Shock groups

(shown more clearly in Fig. 2D). There were no other significant effects or interactions (F = 0.00-2.19, p = 0.14-0.95).

A secondary analysis was run and supported the findings of the primary analysis. A mixed-factor ANOVA was conducted on the dependent variable, suppression ratio, and the between-subjects factors of Shock (Shock vs. Tone-only) and Training Length (1 vs. 10 days) and the within-subjects factor of Trial (the 4 test trials). There was a significant main effect of Shock, $F(1, 54) = 4.42$, $p < 0.05$, (Fig. 2C). There was also a significant Training Length X Shock interaction, $F(1, 54) = 5.67$, $p < 0.05$, and post-hoc tests indicated that the suppression ratio was higher (either significantly or barely missing significance) for the 1 day-shock group than the others and none of the other groups differed from each other (t = -0.63-2.58, p = 0.92-1.00). The 1 day-shock group had a significantly higher suppression ratio than the 1 day-tone only group and the 10 day-shock group (both $p < 0.05$), and a marginally significantly increased suppression ratio compared to the 10 day-tone only group, ($p = 0.059$, Fig. 2C). The Shock X Trial interaction was also significant, $F(1, 54) = 15.59$, $p < 0.01$, and pairwise comparisons using Tukey's HSD test found that the shock groups differed from the no-shock groups on Trials 1 and 2, but not on subsequent trials (Fig. 2B). No other effects or interactions were significant (F = 0.00-2.19, p = 0.14-0.95).

Neurobiological Results

Animals that received 10 days of training showed increased Fos expression in IL and PL, regardless of shock experience. Additionally, there was no association between Fos expression in either area and precue lever-pressing or suppression ratio.

IL effects

Increased training length was associated with increased Fos density in IL, but there was no association between IL Fos density and precue lever-pressing or suppression ratio. A mixed-effects model was run on the dependent variable, IL Fos density, with the fixed effects of Shock (Shock vs. Tone-only), Training Length (1 vs. 10 days), the interaction between the two, and the random effect of Subject. There was a significant main effect of Training Length, $F(1, 51.17) = 12.08$, $p < 0.01$. Tukey's HSD revealed that the 10 day groups exhibited significantly higher Fos expression in IL than the 1 day groups (Fig. 3). There were no other significant effects or interactions ($F = 1.15\text{-}2.53$, $p = 0.12\text{-}0.29$).

A secondary analysis was run on the dependent variable, IL Fos density, and the independent variables of Shock (Shock vs. Tone-only) and Training Length (1 vs. 10 days) using a more traditional ANOVA. There was a significant main effect of Training Length $F(1, 51) = 12.18$, $p < 0.01$), indicating that groups that received 10 days of training exhibited higher IL Fos density than groups that received 1 day of training (Fig. 3). There were no other significant effects or interactions ($F = 1.10\text{-}2.57$, $p = 0.12\text{-}0.30$). In addition, there were non-significant correlations between IL Fos density and average precue lever-pressing during testing, r (55) = -0.09, p = 0.54 (Fig. 4A) and IL Fos density and average suppression ratio during test, r (55) = 0.11, $p = 0.41$ (Fig. 4B). I conducted similar analyses, but with tone-only animals excluded in an attempt to isolate fear-related neural activity and there were non-significant correlations between IL Fos density and average precue lever-pressing during test, r (28) = -0.16, p = 0.41 (Fig. 4C) and IL Fos density and average suppression ratio during test, $r(28) = 0.06$, $p = 0.78$ (Fig. 4D).

PL effects

Increased training length is associated with increased Fos density in PL, but there was no association between PL Fos density and precue lever-pressing or suppression ratio. A mixed-effects model was run on the dependent variable, PL Fos density, with the fixed effects of Shock (Shock vs. Tone-only), Training Length (1 vs. 10 days), the interaction between the two, and the random effect of Subject. There was a significant main effect of Training Length, $F(1, 51.11) = 7.86$, $p = 0.01$. Tukey's HSD revealed that the 10 day groups exhibited significantly higher Fos expression in PL than the 1 day groups (Fig. 5). There were no other significant effects or interactions ($F = 0.77$-1.21, $p = 0.28$-0.39).

A similar analysis was run on the dependent variable, PL Fos density, and the independent variables of Shock (Shock vs. Tone-only) and Training Length (1 vs. 10 days) using a more traditional ANOVA. There was a significant main effect of Training Length, $F(1,51) = 7.87$, $p < 0.01$) indicating that groups that received 10 days of training exhibited higher PL Fos density than groups that received 1 day of training (Fig. 5). There were no other significant effects or interactions ($F = 0.75$-1.20, $p = 0.28$-0.39). In addition, there were non-significant correlations between PL Fos density and average precue lever-pressing during test, $r(55) = -0.04$, $p = 0.77$ (Fig. 6A), and PL Fos density and average suppression ratio during test, $r(55) = -0.10$, $p = 0.45$ (Fig. 6B). I conducted similar analyses, but with tone-only animals excluded in an attempt to isolate fear-related neural activity and there were non-significant correlations between PL Fos density and average precue lever-pressing during test, $r(28) = -0.06$, $p = 0.75$ (Fig. 6C) and PL Fos density and average suppression ratio during test, $r(28) = -0.24$, $p = 0.22$ (Fig. 6D).

Chapter 4 - Discussion

The purpose of this study was to examine the neurobiological mechanism underlying the behavior seen as a result of the extended training fear incubation paradigm: a lasting increase in fear from initial post-training levels (fear incubation). Previous research has investigated this model using pharmacological, physiological (food restriction manipulations), and behavioral methods (J. D. Morrow et al., 2015; Pajser et al., 2019; Pickens et al., 2009a; Pickens, Golden, & Nair, 2013; Pickens et al., 2010; Pickens & Theberge, 2014), but this model had not been investigated with measures of neuronal activity (direct or indirect) associated with differing levels of fear. I was interested in whether an extinction-like process might be the cause for the initially low fear caused by this paradigm, so I chose to investigate this question using Fos immunohistochemistry. This method allowed me to indirectly measure activity of an area of the brain associated with the low fear expression seen after extinction training, IL, and its counterpart which is generally necessary for fear expression, PL. I *did* find the expected pattern in the behavioral data such that the 1 day-shock group showed higher levels of fear during test than the 1 day-tone, 10 day-shock, and 10 day-tone groups (either significantly or marginally significantly). However, despite the rationale for the current study, I did not find a relationship between conditioned fear and Fos expression in either IL or PL. Instead, I found an effect of training length such that animals that received extended training (whether shocks were presented or not) showed an increased number of Fos-positive cells in IL and PL. The implications of these findings are discussed below.

Discussion of Behavioral Results

My findings that the 1 day-shock group showed higher levels of fear during test than the 1 day-tone, 10 day-shock, and 10 day-tone groups (either significantly or marginally

significantly) are in accordance with my predictions and in accordance with the previous literature (J. D. Morrow et al., 2015; Pajser, Breen, Fisher, & Pickens, 2018; Pajser et al., 2019; Pickens et al., 2009a; Pickens et al., 2009b; Pickens et al., 2013; Pickens et al., 2010). Although research using the extended training fear incubation paradigm is limited, my finding that animals that received 10 days of conditioned fear training show low levels of fear at test adds to the literature suggesting that this phenomenon is robust. This suggests that the behavioral manipulation was strong enough to observe fear-related neuronal activity.

However, a curious finding in the present experiment is that shock and tone-only groups differed in suppression ratio on the very first trial of training. This means the groups differed before any shocks were delivered. The reason for this difference is unknown, as the groups should have had identical experiences up until the first shock was delivered, so this is a limitation of the present experiment that I cannot explain. This could complicate analysis of the neural result if we had found differences in Fos between the shock and no-shocks groups, as this might be explained by pre-existing differences in the groups rather than their different training conditions. However, as I found no effect or interaction of shock condition in the IL or PL Fos data, this is a limitation of the current data but it is unlikely that this difference on the first trial had effects on the Fos patterns observed

Discussion of Neurobiological Results

PL activity did not appear to be related to fear training

My neurobiological results were unexpected, and the existing fear conditioning literature does not explain my findings that the shock and no-shock groups did not differ in the evoked neural activity in PL at each training length. As a reminder, I expected high Fos expression in PL in animals that underwent limited conditioned fear training and low Fos expression in PL in

animals that underwent tone-only training, regardless of training length, or the extended training fear incubation paradigm.

A variety of research suggests that neural activity in PL should be higher as a result of early extinction in fear conditioned rats than as a result of tone exposure in animals never fear conditioned, likely due to fear expression rather than extinction itself (Burgos-Robles et al., 2009; B. A. Morrow et al., 1999; Santini et al., 2004). In this case, my 1-day and 10-day shock groups could be expected to exhibit a higher level of Fos expression in PL than either of the tone-only groups, but instead, I found that both 10-day groups had higher Fos expression in PL than either of the 1-day groups, regardless of shock experience. Additional literature suggests that increased PL activity should correspond to increased fear expression, however, the one group that *did* show high fear expression (1-day shock) did not show increased Fos expression in PL compared to the other 3 groups. Instead, it seems clear that the PL Fos expression I visualized does not appear to be related to fear conditioning.

It is possible that activity in the PL related to lever-pressing and food restriction may interfere with measurement of neuronal activity related to fear (Carr, Park, Zhang, & Stone, 1998). In addition to potentially facilitating habit formation because of the utilization of lever-pressing to measure fear, lever-pressing may interfere with the ability to detect pure fear-related activity. A similar preparation using non-food restricted, non-lever-pressing rats may be able to uncover fear-related neural activity in mPFC, which is discussed in the methodological considerations section below. Prior fear conditioning research using exclusively freezing as a measure of fear has indeed found fear-related activity in mPFC using a more direct measure of neuronal activity, electrophysiological recordings of action potentials. For example, Burgos-Robles et al. (2009) collected in-vivo single-unit recordings from freely behaving rats and found

that PL neurons show a sustained increase in excitatory responses during conditioned stimulus presentation after an animal undergoes fear conditioning. Fitzgerald, Giustino, Seemann, and Maren (2015) also collected in-vivo single-unit recordings and similarly found that fear conditioning increased spontaneous firing in PL neurons when compared to activity that occurred immediately prior to fear conditioning. However, prior research has also shown increased PL Fos expression resulting from extinction training in both food-restricted lever-pressing rats (Santini et al., 2004) and in free-fed rats that were not lever-pressing (B. A. Morrow et al., 1999), so the implications for the current study are unclear. There are a few key differences between the Santini et al. (2004) preparation and the current preparation. Although rats in both studies were food-restricted, Santini et al. (2004) restricted rats to 85% of their original weight, while my rats were simply restricted in growth such that they gained 1.5 grams per day. In addition, Santini et al. (2004) gave 80 minutes of extinction training 2 hours after conditioned fear training and collected brains 30 minutes after end of extinction training, while I gave an extinction test and collected brain tissue 2 days after a conditioned fear training session. As a result, it is possible that the Fos I visualized was indicative of a different process (such as recall) than the Fos that Santini et al. (2004) visualized (which may have been indicative of learning). It is possible that conditioned fear learning causes a stronger neural response that can overcome neural activity related to food-restriction, while fear recall does not. Additional research is needed to confirm either of these possibilities.

IL activity did not appear to be related to fear training

The pattern of neuronal activity found in IL was unexpected. As a reminder, I expected high Fos expression in IL in animals that underwent the extended training fear incubation paradigm and low Fos expression in IL in animals that underwent limited conditioned fear

training or tone-only training, regardless of training length (although I detail two other fear-related patterns in this paragraph). In contrast, I found high IL Fos expression in groups that received 10 days of training compared to groups that received 1 day of training, regardless of fear conditioning. Alternatively, a variety of research findings suggest that neural activity in IL should be higher as a result of early extinction in fear conditioned rats than as a result of tone exposure in animals never fear conditioned (Burgos-Robles et al., 2009; B. A. Morrow et al., 1999; Santini et al., 2004; Santini et al., 2008). In this case, my 1-day and 10-day shock groups should show a higher level of Fos expression in IL than either of the tone-only groups, but instead, I found that both 10-day groups had higher Fos expression in IL than either of the 1-day groups, regardless of shock experience. Other literature suggests that increased IL activity should correspond to decreased fear expression (Kim et al., 2016; Soler-Cedeno et al., 2016; Vidal-Gonzalez et al., 2006). However, of the three groups that showed low fear expression (10 day-Shock, 10 day-Tone only, and 1 day-Tone only), only the two 10-day groups exhibited increased IL Fos expression, regardless of shock experience. Instead, it seems clear that the Fos expression I visualized does not appear to be related to fear extinction.

As with PL, it is possible that activity in the IL related to lever-pressing and food restriction may interfere with measuring neuronal activity related to fear (Carr et al., 1998). However, prior research has shown increased IL Fos expression in rats that received fear conditioning and extinction when compared to controls in both food-restricted lever-pressing rats (Santini et al., 2004) and in free-fed rats that were not lever-pressing (B. A. Morrow et al., 1999), so the implications for the current study are unclear. Importantly, there are methodological differences between the Santini et al. (2004) study and mine to consider. Although rats in both studies were food-restricted, Santini et al. (2004) restricted rats to 85% of their original weight,

while my rats were simply restricted in growth such that they gained 1.5 grams per day. In addition, Santini et al. (2004) gave 80 minutes of extinction training 2 hours after conditioned fear training and collected brains 30 minutes after the end of extinction training, while I gave an extinction test and collected brain tissue 2 days after a conditioned fear training session, allowing for the possibility that the Fos I visualized was indicative of a different process (such as recall or extinction) than the Fos that Santini et al. (2004) visualized (which may have been indicative of learning). It is possible that conditioned fear learning causes a stronger neural response that can overcome neural activity related to food-restriction, while fear recall or extinction does not. The possibility that lever-pressing and food restriction may interfere with the measurement of neuronal activity related to fear and its implications are discussed more in-depth in the methodological consideration section below.

Although my neurobiological results in IL were also unexpected, there is some previous literature that could help explain my findings that the shock and no-shock groups did not differ in the evoked neural activity in IL at each training length. If the 10-day shock group is undergoing an extinction-like process over the course of 10 days of training leading to low fear on test day 2, my findings that they exhibit similar levels of IL activation to the 10-day tone-only group are in accordance with those of Santini et al. (2008), who found that IL excitability is similar in rats that underwent acquisition and extinction and rats that never acquired fear. My results of high IL Fos in the 10-day training rats that exhibited low fear are also not incongruent with those of Knapska et al. (2012), who found that an increase in activity in IL neurons that project to BLA is correlated with a decrease in freezing. However, none of these possibilities explain why activity between both 10-day groups would differ from the 1-day tone-only group.

One possibility is that the pattern observed reflects a general role for IL in inhibitory learning. Researchers have shown that IL is involved in more general inhibitory learning, such as that resulting from stimulus pre-exposure that causes latent inhibition, in addition to its prominent role in extinction memory storage (Lingawi et al., 2017). Lingawi et al. (2017) examined the possibility that there is a general inhibitory process involved in different types of inhibitory learning that is localized in IL. Using pharmacological inactivation methods, they examined the role of IL in extinction learning and latent inhibition, both of which require inhibitory learning. Lingawi et al. (2017) found that blocking NMDA receptors, receptors known to be involved in learning and memory, in IL during CS pre-exposure impaired latent inhibition and blocking NMDA receptors during extinction impaired extinction. This suggests that IL activity is more generally involved in the formation of inhibitory memories. These findings can help to reconcile my results that the 10 day-tone only group and the 10 day-shock group both show high levels of Fos expression in IL. It is possible that the 10-day tone-only group is learning inhibitory information about the tones throughout training (akin to the inhibitory learning that takes place during pre-exposure for latent inhibition), and it is possible that the 10 day shock group is learning extinction-like inhibitory information throughout training, as I initially hypothesized. Therefore, it is possible that this inhibitory learning that may be taking place in both 10 day groups is then expressed during behavioral testing and neurobiologically in IL.

The low levels of Fos expression seen in both of the 1-day groups, however, is more puzzling. It is possible that limited fear conditioning leads to lower overall mPFC activation than extended training does, which could explain the low levels of Fos expression in the 1-day Shock group. However, this does not explain why the 1-day Tone-only group would show similar levels

of Fos as the 1-day Shock group, although this result is not completely novel. Knapska and Maren (2009) found similar levels of Fos expression in IL in rats that received fear conditioning and extinction training and a group that did not receive any conditioning at all. These groups seem comparable to our 1-day Shock and 1-day Tone-only groups, respectively, and the reason for similar levels of Fos expression in the two groups is unclear, but it is in accord with the current findings. Nonetheless, the majority of the fear conditioning literature as it relates to IL activity, does not fit well with the current findings in that fear conditioning tends to lead to higher IL activity than no conditioned fear training (Burgos-Robles et al., 2009; B. A. Morrow et al., 1999; Santini et al., 2004). Thus, it is likely that keeping my rats food-restricted and using conditioned suppression of lever-pressing as a measure of fear interfered with my ability to observe the expected pattern of neural activation.

Increased neuronal activity in PL and IL may be indicative of goal-directed and habitual behavior, respectively

The differences I found between animals that received 1 or 10 days of training, regardless of shock, suggest the neural activity measured was related to length of training, specifically that as training length increased, so did Fos expression in both IL and PL. IL and PL are known for their roles in habitual and flexible behavior, respectively, and animals that received 10 days of training were maintained on the same schedule of reinforcement for 14 days (including lever-press training, conditioned fear training, and testing). As such, it is possible that the activity visualized by Fos immunohistochemistry was related to habitual and flexible behavior. This is particularly possible in light of previous research that has shown that animals trained on a VI60 schedule of reinforcement, the same schedule used in the current study, are prone to developing habitual behavior (Dickinson et al., 1983). This could explain increased activity seen in both IL

and PL, as the literature shows that the neural activity underlying flexible behavior (possibly found in PL) is still present or able to be evoked, even after the neural activity underlying habitual behavior develops (Coutureau & Killcross, 2003). If this is the case, my experiment is the first that I know of that has shown differential PFC Fos expression based on whether operant training was limited or extended.

Because PL and IL are involved in both fear conditioning/fear extinction and goal-directed learning/habit formation, it is possible that neural activity from habit learning is obscuring neural activity from fear expression or fear extinction. In order to examine neural activity more directly related to fear conditioning in the extended training fear incubation paradigm, I could remove the lever-press training and measure fear using freezing instead of conditioned suppression of lever-pressing. This would eliminate the possibility of the development of habitual responding, as there would be no concurrent task for the animals to perform alongside fear conditioning. Another possibility is to determine whether our limited-training procedure leads to goal-directed behavior and our extended-training procedure leads to habitual behavior using a devaluation task. This option is discussed in the next paragraph.

The possibility that I may have measured activity related to habitual or flexible behavior is congruent with the literature in that field, but the current study was not designed to investigate habitual or flexible behavior. In order to investigate this further, I could train animals in a limited- or extended-training procedure similar to our existing conditioned fear training procedures (Pajser et al., 2018; Pajser et al., 2019). Rats could be tested in a devaluation paradigm in which they are sated on rat chow (for the non-devalued control) or the precision pellet operant reinforcer (for the devaluation manipulation) and then tested in extinction on lever-pressing for the precision pellets. If our extended-training procedure induces habit

formation, but our limited-training procedure does not, rats that undergo extended training should be insensitive to precision pellet devaluation via satiation, while rats that undergo limited training should maintain this sensitivity. In particular, an experiment comparing training length (1 vs. 10 days) crossed with shock (shock vs. no shock, much like the current preparation) followed by devaluation testing could demonstrate whether the training parameters used in the current experiment are sufficient to lead to habit formation and whether fear conditioning alters the rate at which habits form. Once again, brain tissue could be collected at an optimal time to examine neural activity occurring during testing and I could perform immunohistochemistry for Fos (or other IEGs) on tissue from PL and IL, as well as other brain areas involved in goal-directed or habitual behavior. Not only would I be able to determine whether the neural pattern in PL and IL is the same as that in the current study, but I would also have tissue from additional areas related to behavioral flexibility and habitual responding, giving me a more complete picture of these neural systems. This might allow me to show that habitual behavior co-occurs with this high PFC Fos activity.

Methodological Considerations

As with any research conducted, there are limitations to the methodology used in this study. One limitation is my use of Fos as a marker of neuronal activity. There is more information available about the function that other IEGs perform when compared to Fos, which would allow me to make inferences about the activity visualized in PFC in a way I currently cannot. Additionally, food restriction has been shown to change basal brain activity as well as PFC activity in response to stimuli, which could be a confound in the current study. Finally, the exclusive use of lever-press suppression (CER) as a measure of fear is a limitation for a number of reasons: using multiple measures of fear in order to provide converging evidence is ideal,

CER may facilitate habit formation because it requires a task concurrent with fear conditioning, and it is possible that lever-pressing-related activity in PFC could interfere with the ability to detect fear-related activity. Each of these limitations is discussed below.

One important methodological consideration about the current experiment is the use of Fos as a measure of activity as opposed to other IEGs. A variety of other IEGs can be stained for, but as mentioned above, the timing of Fos expression worked well for my purposes, and our lab has an immunohistochemistry protocol worked out for Fos, but not for other IEGs. A limitation of using Fos immunohistochemistry is that little is known about the function Fos performs. It is commonly used as an indirect measure of neuronal activity because of its reliable expression in active neurons, but not much is known about the protein's function. More is known about the role of other IEGs. For example, Zif268 and Arc have been implicated in synaptic plasticity and memory formation (S. Davis, Bozon, & Laroche, 2003; Plath et al., 2006) and Homer is necessary for synaptic maintenance and plasticity (Hayashi, Ames, & Hayashi, 2006). Researchers have posited that Fos is involved in gene regulation, promotion of dimerization, and the encoding of transcription factors, among other functions (Hoffman et al., 1993; Sambucetti & Curran, 1986; Sheng & Greenberg, 1990), but knowing the definitive role of Fos in the nervous system could be particularly useful for data interpretation. In addition, performing similar experiments examining other IEGs (with appropriate time-courses) could provide converging evidence that the neurobiological results described in the current study are indicative of a true effect on neuronal activity.

Food restriction has been shown to increase basal activity levels in the rat brain (Carr et al., 1998) even with no other concurrent manipulation. Although Carr et al. (1998) did not examine PFC activity specifically, it is plausible that the same pattern found in the rest of the

brain would also be present in PFC. Conversely, Pagliusi, Tessari, DeVevey, Chiamulera, and Pich (1996) found no baseline difference between free-fed and food-restricted animals in PFC activity, suggesting that the pattern found elsewhere in the brain in Carr et al. (1998) might not occur in PFC. However, Pagliusi et al. (1996) did not differentiate between PL and IL, which often have opposing functions, and therefore differing patterns of activation. A notable difference between my experiment and that of Pagliusi et al. (1996) is that the free-fed and food-restricted animals being compared for PFC activity in the Pagliusi study were home-cage controls, while even my control animals experienced transportation to the operant chambers, lever-pressing, and other environmental stimuli. This means that food restriction might have different effects in my experiment compared to those with home-cage controls. Additional studies have found that food restriction increases responsiveness of PFC neurons to drugs of abuse (Carr, 2002; Carr & Kutchukhidze, 2000a; Carr & Kutchukhidze, 2000b; Pagliusi et al., 1996), so it is plausible that food restriction might be able to change PFC responsiveness to other stimuli (such as shock or cues associated with shock) as well. All of my animals were food restricted for a similar amount of time and they were on the same food restriction regimen, but I cannot exclude the possibility that food restriction may have changed PFC activity generally, or activity evoked in response to shock and/or cues associated with shock. Because conditioned suppression requires food motivation for lever-pressing, future studies could utilize freezing in free-fed rats as the exclusive behavioral measure of fear so that neural activation could be examined, unaffected by food restriction.

Finally, the exclusive use of CER as a fear measure is another limitation of the current study. Being able to use freezing as an additional measure of fear would be ideal, because if I measure both and both measures show the same pattern, this would provide converging evidence

that true fear and neural activity related to fear are being assessed. This is particularly true because freezing and CER are neurobiologically dissociable (Amorapanth, Nader, & LeDoux, 1999; McDannald, 2010; McDannald & Galarce, 2011). CER is also an indirect measure of fear, relying on food motivation to produce a response for an appetitive reinforcer (Mast et al., 1982). As a result, either an increase in fear or a decrease in motivation for food could lead to increased conditioned suppression of lever-pressing. In addition, the calculation of the suppression ratio depends on discrimination between the cue and non-cue periods, with which the 10-day groups have more experience (Mast et al., 1982). CER is also an aggregate measure comparing precue and cue lever-pressing, meaning that it is insensitive to differences in the baseline of values that are used in its calculation. As a result, looking at the raw data can help us to understand if differences represent changes in the baseline lever-press rate, the cue lever-press rate, or alterations in both. Finally, it is possible that activity in the PFC related to lever-pressing may interfere with measuring neuronal activity related to fear. In addition to potentially facilitating habit formation because of its utilization of lever-pressing, lever-pressing may interfere with the ability to detect pure fear-related activity. A similar preparation using non-food restricted, non-lever-pressing rats may be able to uncover fear-related neural activity in mPFC that the current preparation missed. Presumably, the rats in the current experiment also exhibit freezing as a fear response, although this cannot be assessed because the video recordings for many of the animals was lost due to a computer error. If fear-related Fos activity was observed in PFC resulting from a fear-conditioning task without concurrent lever-pressing, it would suggest that the neurobiology of fear conditioning (which leads to freezing responses, whether they are measured or not) may differ somewhat depending on whether there is a concurrent lever-press task. This has implications for labs that examine freezing with a concurrent lever-press task (Armony et al.,

1997; Burgos-Robles et al., 2009; Frankland et al., 2004; J. D. Morrow et al., 2015; Pajser et al., 2018; Pajser et al., 2019; Santini et al., 2004; Sierra-Mercado et al., 2011).

Conclusions

I have replicated previous findings that the extended training fear incubation procedure results in lower fear than 1 day of comparable fear conditioning. In addition, I have found an unanticipated pattern of neural activation in IL and PL as a result of differential training procedures. One possibility is that the neural activity observed may be indicative of habit formation. In this field of research, it is assumed that operant overtraining leads to increased IL activity, but this had not yet been examined. To the best of my knowledge, this is the first study to find evidence that operant overtraining does indeed increase neural activity in IL and PL, but further experimentation is necessary to confirm that possibility. For future research, additional tissue from the animals used in the current experiment was collected and processed and will be analyzed for Fos expression. Two regions that will be targeted in particular include the basolateral and central nuclei of the amygdala, both regions with well-established roles in fear-related behavior (Phelps & LeDoux, 2005; Quirk & Mueller, 2008). In addition, other experimental designs could be used to determine the relationship of brain activity in fear-related neurons (including those in PFC) and fear incubation.

Chapter 5 - Epilogue

Šuša, muša,

Magarac ko sluša.

Čiča, miča,

Gotova je priča.

References

Adams, C. D. (1980). Post-conditioning devaluation of an instrumental reinforcer has no effect on extinction performance. *The Quarterly Journal of Experimental Psychology, 32*(3), 447-458.

Adams, C. D. (1982). Variations in the sensitivity of instrumental responding to reinforcer devaluation. *The Quarterly Journal of Experimental Psychology, 34B*, 77-98.

Amorapanth, P., Nader, K., & LeDoux, J. E. (1999). Lesions of periaqueductal gray dissociate-conditioned freezing from conditioned suppression behavior in rats. *Learning & Memory, 6*(5), 491-499.

Andrews, B., Brewin, C. R., Stewart, L., Philpott, R., & Hejdenberg, J. (2009). Comparison of immediate-onset and delayed-onset posttraumatic stress disorder in military veterans. *The Journal of Abnormal Psychology, 118*(4), 767-777. doi:10.1037/a0017203

Annau, Z., & Kamin, L. J. (1961). The conditioned emotional response as a function of intensity of the US. *J Comp Physiol Psychol, 54*, 428-432.

Armony, J. L., Servan-Schreiber, D., Romanski, L. M., Cohen, J. D., & LeDoux, J. E. (1997). Stimulus generalization of fear responses: Effects of auditory cortex lesions in a computational model and in rats. *Cerebral Cortex, 7*(2), 157-165.

Balogh, S. A., Radcliffe, R. A., Logue, S. F., & Wehner, J. M. (2002). Contextual and cued fear conditioning in C57BL/6J and DBA/2J mice: context discrimination and the effects of retention interval. *Behavioral Neuroscience, 116*(6), 947-957.

Borghans, B., & Homberg, J. R. (2015). Animal models for posttraumatic stress disorder: An overview of what is used in research. *World Journal of Psychiatry, 5*(4), 387-396. doi:10.5498/wjp.v5.i4.387

Bouton, M. E., & Bolles, R. C. (1980). Conditioned fear assessed by freezing and by the suppression of 3 different baselines. *Animal Learning & Behavior, 8*(3), 429-434. doi:Doi 10.3758/Bf03199629

Boyden, E. S. (2011). A history of optogenetics: the development of tools for controlling brain circuits with light. *F1000 Biol Rep, 3*, 11. doi:10.3410/B3-11

Burgos-Robles, A., Vidal-Gonzalez, I., & Quirk, G. J. (2009). Sustained conditioned responses in prelimbic prefrontal neurons are correlated with fear expression and extinction failure. *The Journal of Neuroscience, 29*(26), 8474-8482. doi:10.1523/JNEUROSCI.0378-09.2009

Carr, K. D. (2002). Augmentation of drug reward by chronic food restriction: behavioral evidence and underlying mechanisms. *Physiology & Behavior, 76*(3), 353-364.

Carr, K. D., & Kutchukhidze, N. (2000a). Chronic food restriction increases Fos-like immunoreactivity (FLI) induced in rat forebrain by intraventricular amphetamine. *Brain Research, 861*(1), 88-96.

Carr, K. D., & Kutchukhidze, N. (2000b). Effect of chronic food restriction on Fos-like immunoreactivity (FLI) induced in rat brain regions by intraventricular MK-801. *Brain Research, 873*(2), 283-286.

Carr, K. D., Park, T. H., Zhang, Y., & Stone, E. A. (1998). Neuroanatomical patterns of Fos-like immunoreactivity induced by naltrexone in food-restricted and ad libitum fed rats. *Brain Research, 779*(1-2), 26-32.

Colwill, R. M., & Rescorla, R. A. (1985). Instrumental responding reamins sensitive to reinforcer devaluation after extensive training. *The Journal of Experimental Psychology: Animal Behavior Processes, 11*(4), 520-536.

Corbit, L. H., & Balleine, B. W. (2003). The role of prelimbic cortex in instrumental conditioning. *Behavioral Brain Research, 146*(1-2), 145-157.

Corcoran, K. A., & Quirk, G. J. (2007). Activity in prelimbic cortex is necessary for the expression of learned, but not innate, fears. *The Journal of Neuroscience, 27*(4), 840-844. doi:10.1523/JNEUROSCI.5327-06.2007

Coutureau, E., & Killcross, S. (2003). Inactivation of the infralimbic prefrontal cortex reinstates goal-directed responding in overtrained rats. *Behavioral Brain Research, 146*(1-2), 167-174.

Davis, M. (1990). Animal models of anxiety based on classical conditioning: The conditioned emotional response (CER) and the fear-potentiated startle effect. *Pharmacology & Therapeutics, 47*(2), 147-165.

Davis, S., Bozon, B., & Laroche, S. (2003). How necessary is the activation of the immediate early gene zif268 in synaptic plasticity and learning? *Behavioral Brain Research, 142*(1-2), 17-30.

Delacour, J., Bassant, M. H., Onofrj, M., Santucci, V., & Kleinlogel, H. (1990). Electrophysiological models for the study of cognition enhancers. *Pharmacopsychiatry, 23 Suppl 2,* 90-93. doi:10.1055/s-2007-1014541

Dickinson, A., Nicholas, D. J., & Adams, C. D. (1983). The effect of instrumental training contingency on susceptibility to reinforcer devaluation. *The Quarterly Journal of Experimental Psychology, 35B,* 35-51.

Fanselow, M. S. (1980). Conditioned and unconditional components of post-shock freezing. *The Pavlovian Journal of Biological Science, 15*(4), 177-182.

Fanselow, M. S., & Gale, G. D. (2003). The amygdala, fear, and memory. *Annals of the New York Academy of Sciences, 985,* 125-134.

Fitzgerald, P. J., Giustino, T. F., Seemann, J. R., & Maren, S. (2015). Noradrenergic blockade stabilizes prefrontal activity and enables fear extinction under stress. *Proceedings of the National Academy of Sciences of the United States of America, 112*(28), E3729-3737. doi:10.1073/pnas.1500682112

Frankland, P. W., Bontempi, B., Talton, L. E., Kaczmarek, L., & Silva, A. J. (2004). The involvement of the anterior cingulate cortex in remote contextual fear memory. *Science, 304*(5672), 881-883. doi:10.1126/science.1094804

Gale, G. D., Anagnostaras, S. G., Godsil, B. P., Mitchell, S., Nozawa, T., Sage, J. R., . . . Fanselow, M. S. (2004). Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats. *The Journal of Neuroscience, 24*(15), 3810-3815. doi:10.1523/JNEUROSCI.4100-03.2004

Gleitman, H., & Holmes, P. A. (1967). Retention of incompletely learned CER in rats. *Psychonomic Science, 7,* 19-20.

Goode, T. D., & Maren, S. (2014). Animal models of fear relapse. *ILAR J, 55*(2), 246-258. doi:10.1093/ilar/ilu008

Hayashi, M. K., Ames, H. M., & Hayashi, Y. (2006). Tetrameric hub structure of postsynaptic scaffolding protein homer. *The Journal of Neuroscience, 26*(33), 8492-8501. doi:10.1523/JNEUROSCI.2731-06.2006

Hendersen, R. W. (1978). Forgetting and conditioned fear inhibition. *Learning and Motivation, 9.*

Hoffman, G. E., Smith, M. S., & Verbalis, J. G. (1993). c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. *Frontiers in Neuroendocrinology, 14*(3), 173-213. doi:10.1006/frne.1993.1006

Holland, P. C., & Rescorla, R. A. (1975). The effect of two ways of devaluing the unconditioned stimulus after first- and second-order appetitive conditioning. *The Journal of Experimental Psychology: Animal Behavior Processes, 1*(4), 355-363.

Holland, P. C., & Straub, J. J. (1979). Differential effects of two ways of devaluing the unconditioned stimulus after Pavlovian appetitive conditioning. *The Journal of Experimental Psychology: Animal Behavior Processes, 5*(1), 65-78.

Houston, F. P., Stevenson, G. D., McNaughton, B. L., & Barnes, C. A. (1999). Effects of age on the generalization and incubation of memory in the F344 rat. *Learning & Memory, 6*(2), 111-119.

Iwata, J., & LeDoux, J. E. (1988). Dissociation of associative and nonassociative concomitants of classical fear conditioning in the freely behaving rat. *Behavioral Neuroscience, 102*(1), 66-76.

Jacobs, N. S., Cushman, J. D., & Fanselow, M. S. (2010). The accurate measurement of fear memory in Pavlovian conditioning: Resolving the baseline issue. *The Journal of Neuroscience Methods, 190*(2), 235-239. doi:10.1016/j.jneumeth.2010.04.029

Killcross, S., & Coutureau, E. (2003). Coordination of actions and habits in the medial prefrontal cortex of rats. *Cerebral Cortex, 13*(4), 400-408.

Kim, H. S., Cho, H. Y., Augustine, G. J., & Han, J. H. (2016). Selective control of fear expression by optogenetic manipulation of infralimbic cortex after extinction. *Neuropsychopharmacology, 41*(5), 1261-1273. doi:10.1038/npp.2015.276

Klavir, O., Prigge, M., Sarel, A., Paz, R., & Yizhar, O. (2017). Manipulating fear associations via optogenetic modulation of amygdala inputs to prefrontal cortex. *Nature Neuroscience, 20*(6), 836-844. doi:10.1038/nn.4523

Knapska, E., Macias, M., Mikosz, M., Nowak, A., Owczarek, D., Wawrzyniak, M., . . . Kaczmarek, L. (2012). Functional anatomy of neural circuits regulating fear and extinction. *Proceedings of the National Academy of Sciences of the United States of America, 109*(42), 17093-17098. doi:10.1073/pnas.1202087109

Knapska, E., & Maren, S. (2009). Reciprocal patterns of c-Fos expression in the medial prefrontal cortex and amygdala after extinction and renewal of conditioned fear. *Learning & Memory, 16*(8), 486-493. doi:10.1101/lm.1463909

LaLumiere, R. T., Niehoff, K. E., & Kalivas, P. W. (2010). The infralimbic cortex regulates the consolidation of extinction after cocaine self-administration. *Learning & Memory, 17*(4), 168-175. doi:10.1101/lm.1576810

Landeira-Fernandez, J., DeCola, J. P., Kim, J. J., & Fanselow, M. S. (2006). Immediate shock deficit in fear conditioning: effects of shock manipulations. *Behav Neurosci, 120*(4), 873-879. doi:10.1037/0735-7044.120.4.873

Lang, P. J., Davis, M., & Ohman, A. (2000). Fear and anxiety: Animal models and human cognitive psychophysiology. *The Journal of Affective Disorders, 61*(3), 137-159.

LeDoux, J. (2007). The amygdala. *Current Biology, 17*(20), R868-874. doi:10.1016/j.cub.2007.08.005

Lingawi, N. W., Holmes, N. M., Westbrook, R. F., & Laurent, V. (2018). The infralimbic cortex encodes inhibition irrespective of motivational significance. *Neurobiology of Learning and Memory, 150*, 64-74. doi:10.1016/j.nlm.2018.03.001

Lingawi, N. W., Westbrook, R. F., & Laurent, V. (2017). Extinction and latent inhibition involve a similar form of inhibitory learning that is stored in and retrieved from the infralimbic cortex. *Cerebral Cortex, 27*(12), 5547-5556. doi:10.1093/cercor/bhw322

Ma, X., Ma, Y. Y., & Yu, L. C. (2012). Distinct mechanisms mediate the reinstatement and spontaneous recovery of extinguished fear in rats. *Neurosci Lett, 510*(1), 34-37. doi:10.1016/j.neulet.2012.01.002

Marchant, N. J., Furlong, T. M., & McNally, G. P. (2010). Medial dorsal hypothalamus mediates the inhibition of reward seeking after extinction. *The Journal of Neuroscience, 30*(42), 14102-14115. doi:10.1523/JNEUROSCI.4079-10.2010

Mast, M., Blanchard, R. J., & Blanchard, D. C. (1982). The relationship of freezing and response suppression in a CER situation. *Psychological Record, 32*(2), 151-167.

McDannald, M. A. (2010). Contributions of the amygdala central nucleus and ventrolateral periaqueductal grey to freezing and instrumental suppression in Pavlovian fear conditioning. *Behavioral Brain Research, 211*(1), 111-117. doi:10.1016/j.bbr.2010.03.020

McDannald, M. A., & Galarce, E. M. (2011). Measuring Pavlovian fear with conditioned freezing and conditioned suppression reveals different roles for the basolateral amygdala. *Brain Research, 1374*, 82-89. doi:10.1016/j.brainres.2010.12.050

Millenson, J. R., & Dent, J. G. (1971). Habituation of conditioned suppression. *The Quarterly Journal of Experimental Psychology, 23*(Feb), 126-134. doi:Doi 10.1080/00335557143000130

Morgan, J. I., & Curran, T. (1986). Role of ion flux in the control of c-Fos expression. *Nature, 322*(6079), 552-555. doi:10.1038/322552a0

Morrow, B. A., Elsworth, J. D., Inglis, F. M., & Roth, R. H. (1999). An antisense oligonucleotide reverses the footshock-induced expression of Fos in the rat medial prefrontal cortex and the subsequent expression of conditioned fear-induced immobility. *The Journal of Neuroscience, 19*(13), 5666-5673.

Morrow, J. D., Maren, S., & Robinson, T. E. (2011). Individual variation in the propensity to attribute incentive salience to an appetitive cue predicts the propensity to attribute motivational salience to an aversive cue. *Behavioral Brain Research, 220*(1), 238-243. doi:10.1016/j.bbr.2011.02.013

Morrow, J. D., Saunders, B. T., Maren, S., & Robinson, T. E. (2015). Sign-tracking to an appetitive cue predicts incubation of conditioned fear in rats. *Behavioral Brain Research, 276*, 59-66. doi:10.1016/j.bbr.2014.04.002

Ostlund, S. B., & Balleine, B. W. (2005). Lesions of medial prefrontal cortex disrupt the acquisition but not the expression of goal-directed learning. *The Journal of Neuroscience, 25*(34), 7763-7770. doi:10.1523/JNEUROSCI.1921-05.2005

Pagliusi, S. R., Tessari, M., DeVevey, S., Chiamulera, C., & Pich, E. M. (1996). The reinforcing properties of nicotine are associated with a specific patterning of c-Fos expression in the rat brain. *The European Journal of Neuroscience, 8*(11), 2247-2256.

Pajser, A., Breen, M., Fisher, H., & Pickens, C. L. (2018). Individual differences in conditioned fear are associated with levels of adolescent/early adult alcohol consumption and instrumental extinction. *Behavioral Brain Research, 349*, 145-157. doi:10.1016/j.bbr.2018.04.020

Pajser, A., Limoges, A., Long, C., & Pickens, C. L. (2019). Individual differences in voluntary alcohol consumption are associated with conditioned fear in the fear incubation model. *Behavioral Brain Research, 362,* 299-310. doi:10.1016/j.bbr.2019.01.027

Pavlov, I. P. (1927). *Conditioned reflexes*. London: Oxfod University Press.

Paxinos, G., Watson, C. (2009). *The Rat Brain in Stereotaxic Coordinates* (6th ed.): Elsevier Inc.

Phelps, E. A., & LeDoux, J. E. (2005). Contributions of the amygdala to emotion processing: from animal models to human behavior. *Neuron, 48*(2), 175-187. doi:10.1016/j.neuron.2005.09.025

Pickens, C. L., Adams-Deutsch, T., Nair, S. G., Navarre, B. M., Heilig, M., & Shaham, Y. (2009a). Effect of pharmacological manipulations of neuropeptide Y and corticotropin-releasing factor neurotransmission on incubation of conditioned fear. *Neuroscience, 164*(4), 1398-1406. doi:10.1016/j.neuroscience.2009.09.079

Pickens, C. L., Golden, S. A., Adams-Deutsch, T., Nair, S. G., & Shaham, Y. (2009b). Long-lasting incubation of conditioned fear in rats. *Biological Psychiatry, 65*(10), 881-886. doi:10.1016/j.biopsych.2008.12.010

Pickens, C. L., Golden, S. A., & Nair, S. G. (2013). Incubation of fear. *Current Protocols in Neuroscience, Chapter 6,* Unit 6 27. doi:10.1002/0471142301.ns0627s64

Pickens, C. L., Navarre, B. M., & Nair, S. G. (2010). Incubation of conditioned fear in the conditioned suppression model in rats: Role of food-restriction conditions, length of conditioned stimulus, and generality to conditioned freezing. *Neuroscience, 169*(4), 1501-1510. doi:10.1016/j.neuroscience.2010.06.036

Pickens, C. L., & Theberge, F. R. (2014). Blockade of CB1 receptors prevents retention of extinction but does not increase low preincubated conditioned fear in the fear incubation

procedure. *Behavioural Pharmacology, 25*(1), 23-31. doi:10.1097/FBP.0000000000000020

Plath, N., Ohana, O., Dammermann, B., Errington, M. L., Schmitz, D., Gross, C., . . . Kuhl, D. (2006). Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. *Neuron, 52*(3), 437-444. doi:10.1016/j.neuron.2006.08.024

Poulos, A. M., Mehta, N., Lu, B., Amir, D., Livingston, B., Santarelli, A., . . . Fanselow, M. S. (2016). Conditioning- and time-dependent increases in context fear and generalization. *Learning & Memory, 23*(7), 379-385. doi:10.1101/lm.041400.115

Quinones-Laracuente, K., Hernandez-Rodriguez, M. Y., Bravo-Rivera, C., Melendez, R. I., & Quirk, G. J. (2015). The effect of repeated exposure to ethanol on pre-existing fear memories in rats. *Psychopharmacology, 232*(19), 3615-3622. doi:10.1007/s00213-015-4016-9

Quirk, G. J. (2002). Memory for extinction of conditioned fear is long-lasting and persists following spontaneous recovery. *Learning & Memory, 9*(6), 402-407. doi:10.1101/lm.49602

Quirk, G. J., & Mueller, D. (2008). Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology, 33*(1), 56-72. doi:10.1038/sj.npp.1301555

Quirk, G. J., Russo, G. K., Barron, J. L., & Lebron, K. (2000). The role of ventromedial prefrontal cortex in the recovery of extinguished fear. *The Journal of Neuroscience, 20*(16), 6225-6231. doi:20/16/6225 [pii]

Rabinak, C. A., & Maren, S. (2008). Associative structure of fear memory after basolateral amygdala lesions in rats. *Behavioral Neuroscience, 122*(6), 1284-1294. doi:10.1037/a0012903

Rabinak, C. A., Orsini, C. A., Zimmerman, J. M., & Maren, S. (2009). The amygdala is not necessary for unconditioned stimulus inflation after Pavlovian fear conditioning in rats. *Learning & Memory, 16*(10), 645-654. doi:10.1101/lm.1531309

Rasmusson, A. M., & Charney, D. S. (1997). Animal models of relevance to PTSD. *Annals of the New York Academy of Sciences, 821*, 332-351.

Rescorla, R. A. (1973). Effect of US habituation following conditioning. *The Journal of Comparative and Physiological Psychology, 82*(1), 137-143.

Rescorla, R. A. (1974). Effect of inflation of the unconditioned stimulus value following conditioning. *The Journal of Comparative and Physiological Psychology, 86*(1), 101-106.

Rodnick, E. H. (1937). Does the interval of delay of conditioned responses possess inhibitory properties? *The Journal of Experimental Psychology, 20*, 507-527.

Rodrigues, S. M., LeDoux, J. E., & Sapolsky, R. M. (2009). The influence of stress hormones on fear circuitry. *The Annual Review of Neuroscience, 32*, 289-313. doi:10.1146/annurev.neuro.051508.135620

Rosas, J. M., & Alonso, G. (1997). The effect of context change upon long-term memory of CS duration. *Behavioural Processes, 39*(1), 69-76.

Sagar, S. M., Sharp, F. R., & Curran, T. (1988). Expression of c-Fos protein in brain: Metabolic mapping at the cellular level. *Science, 240*(4857), 1328-1331.

Sambucetti, L. C., & Curran, T. (1986). The Fos protein complex is associated with DNA in isolated nuclei and binds to DNA cellulose. *Science, 234*(4782), 1417-1419. doi:10.1126/science.3491427

Santini, E., Ge, H., Ren, K., Pena de Ortiz, S., & Quirk, G. J. (2004). Consolidation of fear extinction requires protein synthesis in the medial prefrontal cortex. *The Journal of Neuroscience, 24*(25), 5704-5710. doi:10.1523/JNEUROSCI.0786-04.2004

Santini, E., Quirk, G. J., & Porter, J. T. (2008). Fear conditioning and extinction differentially modify the intrinsic excitability of infralimbic neurons. *The Journal of Neuroscience, 28*(15), 4028-4036. doi:10.1523/JNEUROSCI.2623-07.2008

Schmitzer-Torbert, N., Apostolidis, S., Amoa, R., O'Rear, C., Kaster, M., Stowers, J., & Ritz, R. (2015). Post-training cocaine administration facilitates habit learning and requires the infralimbic cortex and dorsolateral striatum. *Neurobiology of Learning and Memory, 118*, 105-112. doi:10.1016/j.nlm.2014.11.007

Sharpe, M., & Killcross, S. (2015). The prelimbic cortex uses contextual cues to modulate responding towards predictive stimuli during fear renewal. *Neurobiology of Learning and Memory, 118*, 20-29. doi:10.1016/j.nlm.2014.11.005

Sheng, M., & Greenberg, M. E. (1990). The regulation and function of c-Fos and other immediate early genes in the nervous system. *Neuron, 4*(4), 477-485.

Siegmund, A., & Wotjak, C. T. (2007). A mouse model of posttraumatic stress disorder that distinguishes between conditioned and sensitised fear. *The Journal of Psychiatric Research, 41*(10), 848-860. doi:10.1016/j.jpsychires.2006.07.017

Sierra-Mercado, D., Padilla-Coreano, N., & Quirk, G. J. (2011). Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. *Neuropsychopharmacology, 36*(2), 529-538. doi:10.1038/npp.2010.184

Soler-Cedeno, O., Cruz, E., Criado-Marrero, M., & Porter, J. T. (2016). Contextual fear conditioning depresses infralimbic excitability. *Neurobiology of Learning and Memory, 130*, 77-82. doi:10.1016/j.nlm.2016.01.015

Thompson, R., & McConnell, J. (1955). Classical conditioning in the planarian, Dugesia dorotocephala. *The Journal of Comparative and Physiological Psychology, 48*(1), 65-68.

Tinklepaugh, O. L. (1928). An experimental study of representative factors in monkeys. *The Journal of Comparative Psychology, 8*(3), 197-236.

Toth, L. A., & Gardiner, T. W. (2000). Food and water restriction protocols: Physiological and behavioral considerations. *Contemporary Topics in Laboratory Animal Science, 39*(6), 9-17.

Tran-Tu-Yen, D. A., Marchand, A. R., Pape, J. R., Di Scala, G., & Coutureau, E. (2009). Transient role of the rat prelimbic cortex in goal-directed behaviour. *The European Journal of Neuroscience, 30*(3), 464-471. doi:10.1111/j.1460-9568.2009.06834.x

Tsuda, M. C., Yeung, H. M., Kuo, J., & Usdin, T. B. (2015). Incubation of fear is regulated by TIP39 peptide signaling in the medial nucleus of the amygdala. *The Journal of Neuroscience, 35*(35), 12152-12161. doi:10.1523/JNEUROSCI.1736-15.2015

Vidal-Gonzalez, I., Vidal-Gonzalez, B., Rauch, S. L., & Quirk, G. J. (2006). Microstimulation reveals opposing influences of prelimbic and infralimbic cortex on the expression of conditioned fear. *Learning & Memory, 13*(6), 728-733. doi:10.1101/lm.306106

Walters, E. T., Carew, T. J., & Kandel, E. R. (1981). Associative learning in aplysia: Evidence for conditioned fear in an invertebrate. *Science, 211*(4481), 504-506.

Witnauer, J. E., & Miller, R. R. (2013). Conditioned suppression is an inverted-U function of footshock intensity. *Learning & Behavior, 41*(1), 94-106. doi:10.3758/s13420-012-0088-0

Yin, H. H., Knowlton, B. J., & Balleine, B. W. (2004). Lesions of dorsolateral striatum preserve outcome expectancy but disrupt habit formation in instrumental learning. *The European Journal of Neuroscience, 19*(1), 181-189.

Zalocusky, K., & Deisseroth, K. (2013). Optogenetics in the behaving rat: Integration of diverse new technologies in a vital animal model. *Optogenetics, 1*, 1-17.

Tables

Table 1

Experimental design.

Lever-press training	Conditioned fear training	Context extinction	Cued fear test
Magazine training	100 T → Shock	1 session VI 60	4 T → No Shock
2 sessions FI1 right	100 T → No shock		
1 session VI 30	10 T → Shock		
2 sessions VI 60	10 T → No Shock		

Note. The experiment utilized a between-subjects design in order to examine the neural activity related to different training experiences. Rats experienced identical sessions in all phases of the experiment except the "conditioned fear training" phase. In this phase, rats differed in the number of trials they received (10 were given per day- rats that received 10 trials had 1 day of training and rats that had 100 trials received 10 days of training) and also in whether the tone (T) was paired with shock. Sacrifice took place 120 minutes from the start of the test session.

Table 2

Multiple comparisons for the mixed effect model examining precue lever-pressing during training.

Trial		Difference	Standard Error	P value
1	10	1.30	0.20	0.01*
1	100	14.26	2.17	0.01*
10	100	12.96	1.98	0.01*

Note. Pairwise comparisons of trials 1, 10, and 100 were conducted to assess the changes in precue lever-pressing across trials. Significant differences at $p < 0.01$ are indicated with *.

Table 3

Multiple comparisons for the mixed effect model examining suppression ratio during training.

Trial	Shock	Tone-only	Standard Error	P value
1	0.28	-0.00	0.04	0.01*
10	0.26	-0.00	0.04	0.01*
100	0.09	-0.02	0.05	0.57

Note. Pairwise comparisons of shock and tone-only groups at trials 1, 10, and 100 were conducted to assess the changes in suppression ratio across trials. Significant differences at $p < 0.01$ are indicated with *.

Figures

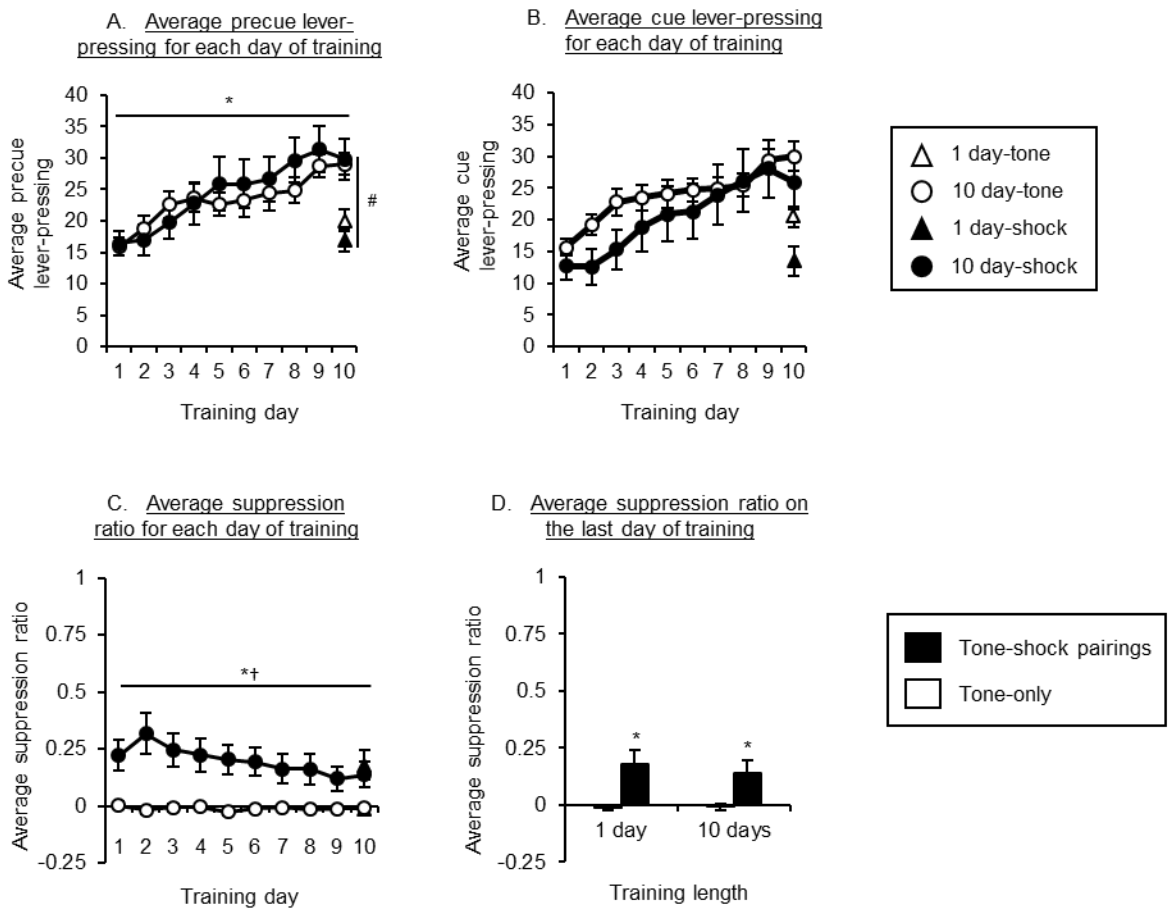

Figure 1. Behavioral training data from the current experiment. A. Average precue lever-pressing (30-sec precue period) for each day. A comparison of precue lever-pressing across the training sessions showed that precue lever-pressing increased across the 10 days of training (* indicates a main effect of Day, significant at $p < 0.05$). A comparison of precue lever-pressing on the last day of training for each group showed that groups with 10 days of training showed higher precue lever-pressing than groups that received 1 day of training (# indicates a main effect of Training Length, significant at $p < 0.05$). B. Average cue lever-pressing (30-sec cue period) for each day. C. Average suppression ratio per day for each training group. Higher suppression ratio values represent higher fear and lower values represent lower fear. A comparison of suppression ratios across training sessions showed that the 10 day-shock group had a high suppression ratio that decreased across training sessions (particularly Days 7-10 of training), while the 10 day-tone group had low suppression ratios throughout training (*† indicates a Shock X Day interaction, significant at $p < 0.05$). For Fig. 1A-C, groups that received 1 day of training are indicated with a triangle and groups that received 10 days of training are indicated with circles. Groups that received tone-shock pairings are indicated with black symbols and groups that received tone-only exposure during training are indicated with white symbols. D. Average

suppression ratio on the last day of training for each training group. Black bars indicate groups that received tone-shock pairings, while white bars indicate groups that received tone-only exposure. A comparison of the last day of training for all groups, showed that Shock groups had higher suppression ratios than Tone-only groups (* indicates a main effect of Shock, significant at $p < 0.05$).

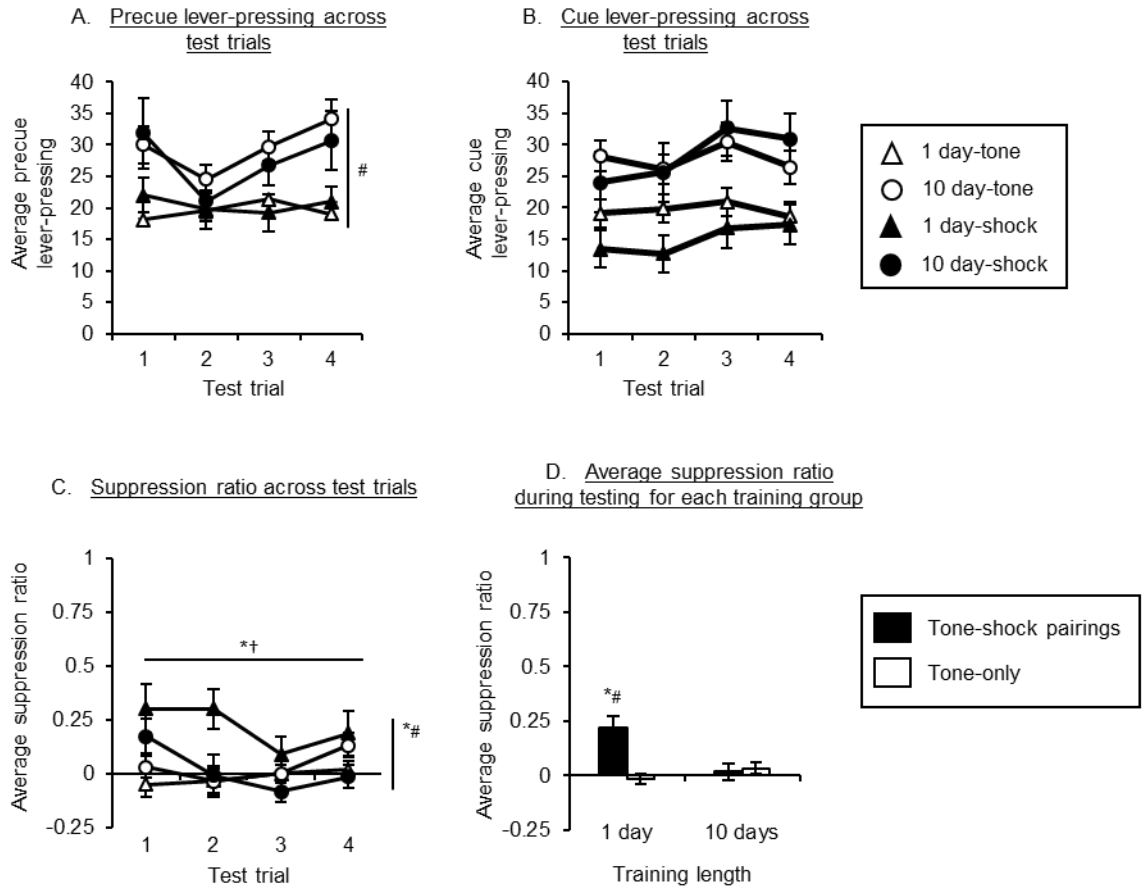

Figure 2. Behavioral test data from the current experiment. A. Average precue lever-pressing (30-sec precue period) for each trial. A comparison of precue lever-pressing across test trials shows that groups that received 10 days of training had higher rates of precue lever-pressing than groups that received 1 day of training, regardless of shock exposure (# indicates a main effect of Training Length, significant at $p < 0.05$). B. Average cue lever-pressing (30-sec cue period) for each test trial. C. Suppression ratio across test trials for each training group. High suppression ratio values indicate higher fear and low values indicate lower fear. A comparison of suppression ratios showed that suppression ratios decreased as training length increased in groups that received shock, while suppression ratios started and stayed low for the tone-only groups (*# indicates a Training Length X Shock interaction, significant at $p < 0.05$). The same comparison also showed that suppression ratios decreased across test trials for groups that received shock, but suppression ratios started low and stayed low for the tone-only groups (*† indicates a Shock X Trial interaction, significant at $p < 0.05$). For Fig. 2A-C, groups that received 1 day of training are indicated with a triangle and groups that received 10 days of training are indicated with circles. Groups that received tone-shock pairings are indicated with black symbols and groups that received tone-only exposure during training are indicated with white symbols. D. Average suppression ratio on the last day of training for each training group. Black bars indicate groups that received tone-shock pairings, while white bars indicate groups that received tone-only exposure (*# indicates a Training Length X Shock interaction, significant at $p < 0.05$).

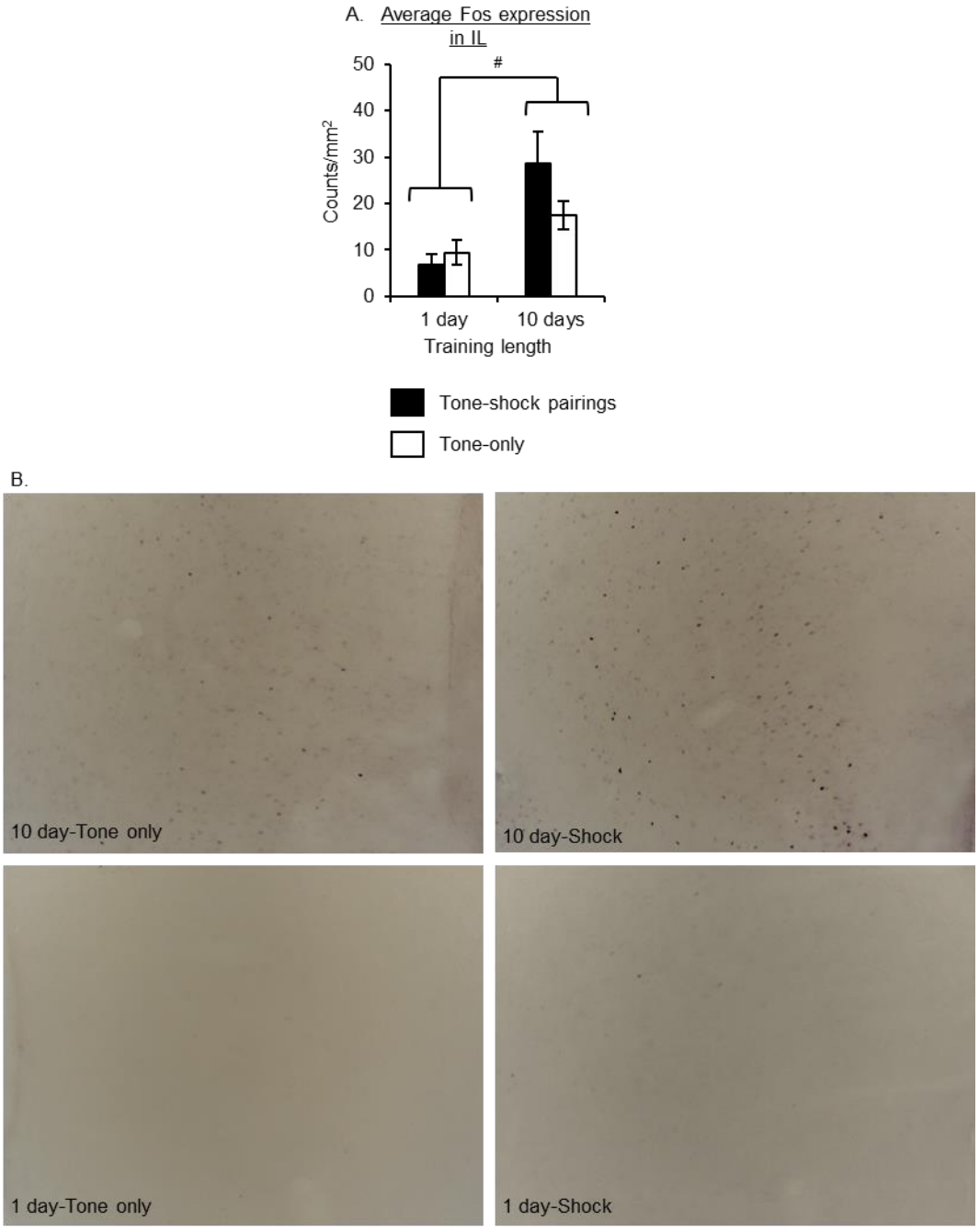

Figure 3. IL Fos expression in the 4 experimental groups. A. A comparison of the average Fos expression across the different training groups. Black bars indicate groups that received tone-shock pairings, while white bars indicate groups that received tone-only exposure. Groups that received 10 days of training showed higher Fos expression than groups that received 1 day of training, regardless of shock exposure (# indicates a significant main effect of Training Length at $p < 0.05$). B. Example images from each of the 4 experimental groups.

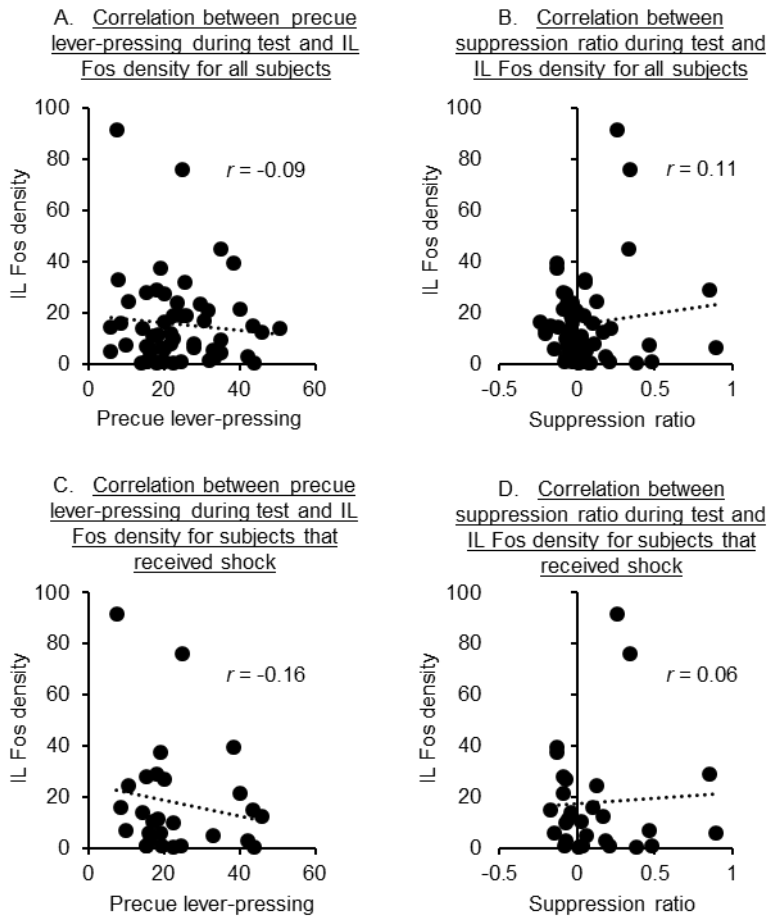

Figure 4. Correlations between IL Fos density (mm^2) and behavior (lever-press/min) during test. A. A correlation between IL Fos density and precue lever-pressing during test for all subjects. B. A correlation between IL Fos density and suppression ratio during test for all subjects. C. A correlation between IL Fos density and precue lever-pressing for subjects that received shock. D. A correlation between IL Fos density and suppression ratio for subjects that received shock. For Fig. 4A-D, none of the correlations were significant, r = -0.16-0.11.

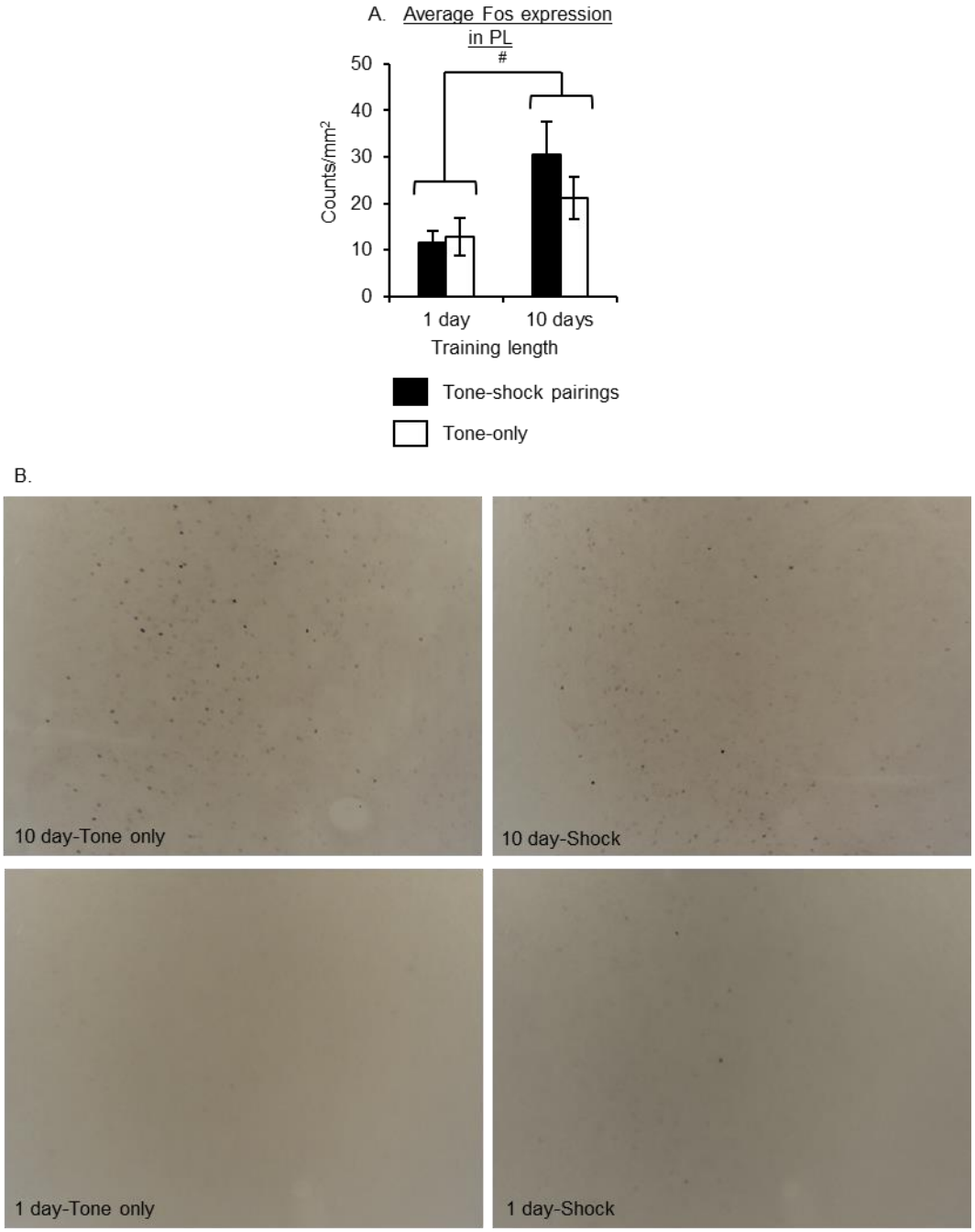

Figure 5. PL Fos expression in the 4 experimental groups. A. A comparison of the average Fos expression across the different training groups. Black bars indicate groups that received tone-shock pairings, while white bars indicate groups that received tone-only exposure. Groups that received 10 days of training showed higher Fos expression than groups that received 1 day of training, regardless of shock exposure (# indicates a significant main effect of Training Length at $p < 0.05$). B. Example images from each of the 4 experimental groups.

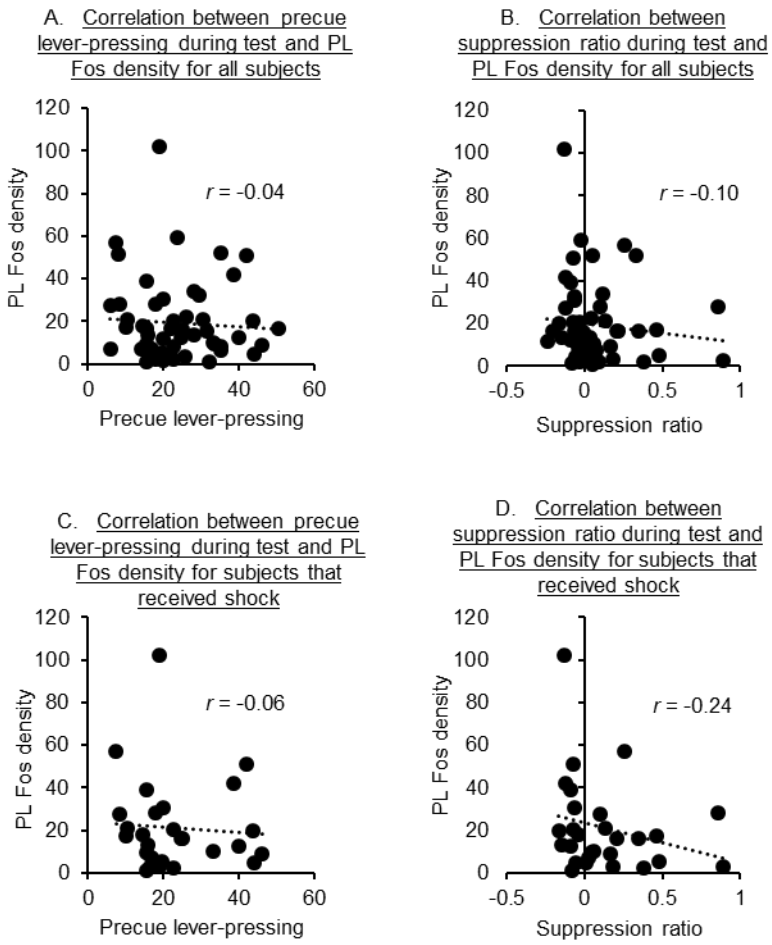

Figure 6. Correlations between PL Fos density (mm^2) and behavior (lever-press/min) during test. A. A correlation between PL Fos density and precue lever-pressing during test for all subjects. B. A correlation between PL Fos density and suppression ratio during test for all subjects. C. A correlation between PL Fos density and precue lever-pressing for subjects that received shock. D. A correlation between PL Fos density and suppression ratio for subjects that received shock. For Fig. 6A-D, none of the correlations were significant, r = -0.24--0.04.