Published in final edited form as:

Behav Brain Res. 2021 September 24; 414: 113476. doi:10.1016/j.bbr.2021.113476.

Extended operant training increases infralimbic and prelimbic cortex Fos regardless of fear conditioning experience

Alisa Pajser, Christian Foster, Brooke Gaeddert, Charles L. Pickens*

Department of Psychological Sciences, Kansas State University, Manhattan, KS, 66506, USA

Abstract

Extended fear training can lead to initially low fear expression that grows over time, termed fear incubation. Conversely, a single fear conditioning session typically results in high fear initially that is sustained over time. Fear expression decreases across extended training, suggesting that a fear extinction-like process might be responsible for low fear observed soon after training. Because of the prominent role medial prefrontal cortex (mPFC) plays in fear conditioning and extinction, we decided to examine Fos expression resulting from a cued fear retrieval test to gain insight into possible mechanisms involved in extended training fear incubation. Male Long-Evans rats received 1 or 10 days of tone-shock pairings or tone-only exposure (while lever-pressing for food). Two days after the end of fear training, rats received a cued fear test, with perfusions timed to visualize Fos expression during test. As expected, the limited fear conditioning group exhibited higher fear in the test than any of the other groups (as measured with conditioned suppression of lever-pressing). Interestingly, we found that extended training animals (whether they received tone-shock pairings or tone-only exposure) expressed higher levels of Fos in both prelimbic and infralimbic cortices than limited training animals. There was no association between fear expression and mPFC Fos expression. These results suggest we may have visualized Fos expression related to operant overtraining rather than conditioned fear related processes. Further research is needed to determine the neurobiological basis of extended training fear incubation and to determine processes represented by the pattern of Fos expression we observed.

Keywords

Fear conditioning; Overtraining; Habit; Fos; Medial prefrontal cortex

1. Introduction

Pavlovian fear conditioning is a procedure commonly used to model fear and anxiety [1,2]. The pairing of a cue with shocks for a few trials in one or two sessions (limited training)

*Corresponding author at: Department of Psychological Sciences, 469 Bluemont Halls, Kansas State University, Manhattan, KS, 66506, USA. pickens@ksu.edu (C.L. Pickens).

Author statement

Alisa Pajser: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing- Original Draft, Writing-Review & Editing, Visualization, Supervision, Project Administration.

Christian Foster- Investigation, Writing- Review & Editing.

Brooke Gaeddert- Investigation, Writing- Review & Editing.

Charles Pickens: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Writing- Review & Editing, Supervision, Project Administration, Funding Acquisition.

typically leads to high fear in tests soon after the end of the conditioned fear training, and this level of fear is typically either maintained or decreases over time [3–7]. However, when a subject is given extended fear conditioning, such as 10 once-daily fear conditioning sessions, the level of fear is reliably low soon after training and then grows over time [8–12]. This is not merely due to use of different procedures than those used in limited training experiments, as one day of conditioned fear training results in high fear immediately that does not grow while ten days of otherwise identical training leads to low fear two days later that grows across the course of a month [8]. It is unclear why longer fear training leads low conditioned fear soon after the end of conditioned fear training, and this was the focus of the current study.

One possible explanation for the low fear observed soon after extended fear conditioning depends on the concept that animals' pain sensitivity may decrease with extended conditioned fear training due to habituation and/or stress-induced analgesia, rendering the US ineffective [13,14]. If the value of the shocks is decreased due to habituation or stress-induced analgesia, rats may undergo a process akin to extinction as tones are paired with ineffective shocks. In the current paper, we examine neuronal activity of medial prefrontal cortex (mPFC) areas implicated in fear expression and fear extinction to determine if they exhibit extinction-like activity after extended fear training.

The mPFC has been shown to be extensively involved in fear expression and extinction. The prelimbic cortex (PL) subregion is generally involved in and required for fear expression, but not acquisition [15–18]. The literature on the infralimbic cortex (IL) subregion is more mixed than that on PL, but IL is involved in and necessary for the long-term storage of extinction memory and expression of extinction learning [15,17,19–22]. More importantly for our purposes, lower fear during a conditioned fear extinction retention test (a test for extinction memory administered after extinction training), was associated with higher IL neuronal activity during that test [23,24]. Likewise, rats exhibiting low fear in an extinction test conducted in the same context as prior extinction exhibited higher IL Fos than rats exhibiting high fear due to context-induced renewal and compared to rats that had not received prior extinction [25]. Together, these findings suggest that fear suppression based on prior extinction is associated with greater IL activity. Conversely, PL activity is high under conditions where fear is high (ex: early extinction) and low under conditions with low fear (ex: pre-conditioning, later extinction, or in rats never receiving fear conditioning [26–28] (but see [25])).

Since successful extinction leading to decreased fear is associated with high IL neuronal activity and low PL activity, we tested whether the extended conditioned fear training that leads to low fear initially (e.g. 2 days after the end of training) would lead to a similar pattern in a fear retention test. If so, this would suggest that an extinction-like process may be causing the low fear as tones are paired with ineffective shocks over the course of extended training. We chose to assess neuronal activity (with Fos) during a retention test rather than during a training session in which shocks were delivered for 2 reasons. First, while we hypothesize that stress-induced analgesia leads to decreased sensitivity to shocks and an extinction-like process during the training sessions, it is unclear whether this process is fully engaged on the first trial of each training session or if it develops on later trials after

delivery of the first shock in each session. If the stress-induced analgesia/extinction-like process was not fully engaged on the first trial, then examining Fos after a training session would assess both fear conditioning/shock exposure-related processing from the first trial and processing related to the hypothesized extinction-like process on trials 2–10, producing muddled results. Second, differences in Fos expression could be more clearly interpreted if all groups had identical stimulus presentations immediately before sacrifice. If we sacrificed the rats during fear conditioning training sessions, then any differences between the shock and tone-only groups could be explained as being due to the presence of shocks in the training session immediately before sacrifice rather than the rats' previous history. However, assessing Fos after a test session in which all rats were exposed to tones and no shocks would provide stronger evidence that these differences were based on the rats' prior history.

In the current examination, we investigated the neural activity in PL and IL evoked from a conditioned fear test in animals that received 1 or 10 days of fear conditioning or tone-only exposures. If a fear extinction-like process is indeed responsible for the low fear observed soon after extended fear training, we would expect increased IL neural activity in the 10 day fear conditioning group compared to the 10 day tone-only and both 1 day groups (as IL activity is required for both fear extinction learning and expression). Likewise, if PL supports fear expression, then we would expect increased neural activity in PL for the 1 day fear conditioning group (which exhibits high fear) compared to the 1 day tone-only and both 10 day groups. The data did not support these hypotheses. Instead we found that extended training (with or without shock deliveries) led to increased neuronal activity in both IL and PL compared to groups that only received limited training. This pattern suggests that the mPFC neuronal activity we observed was associated with the length of operant training, which is in accord with an alternative hypothesized role of IL in habit learning of operant responses for reward after extended operant training [29–33]. This interpretation will be discussed more fully in the Discussion section.

2. Methods

Male Long-Evans rats ($n = 60$, Charles River, Kingston, NY, purchased such that the cue test and sacrifice occurred on post-natal day 87–97) 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, rats were food-restricted and fed to allow a growth in body weight of 1.5 g/day for the duration of the experiment. These feeding conditions minimize the potential stress induced by a more severe food restriction procedure, which is known to affect fear expression [9,34]. Water was available ad libitum. 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.

Experiments were conducted in 12 operant chambers (Med Associates, St Albans, VT). Each chamber had two retractable levers 9 cm above the floor, but only the right lever was extended into the chamber during behavioral sessions. Responding on the lever activated the pellet dispenser, delivering 45-mg precision pellets (#1811155, 5% fat, 66 % carbohydrate,

20.3 % protein; TestDiet, Richmond, IN). A red houselight was located in the center at the top of the wall opposite to the side with the lever and foodcup. This houselight remained on throughout the duration of all training and testing sessions. A tone generator that delivered a 2900 Hz tone (20 dB above background) was located directly to the right of the houselight. The chambers had grid floors connected to electric shock generators that delivered scrambled foot-shock.

A graphical representation of the procedure can be found in Fig. 1A and the experimental design can be found in Fig. 1B. After acclimating to food restriction, rats were given a single 60-min food-cup (magazine) training session, with pellet deliveries every 125 s. Next, the rats received several lever-press training sessions in order to provide robust baseline lever-pressing for assessment of conditioned suppression of lever-pressing. The day after food-cup training, the rats received 2 sessions on a fixed interval-1 (FI-1) reinforcement schedule for lever-presses (lever-presses could earn a pellet each s) 2–4 h apart. These sessions ended when rats received 50 pellets (with a limit of 1 h/session). Starting the following day, the rats were given one 90-min session in which pellets were earned on a variable-interval-30 (VI-30) reinforcement schedule (pellet availability for lever-presses ranging from 1 to 59 s), and then 2 daily 90-min sessions on a VI-60 schedule (pellet availability ranging from 1 to 119 s). Rats were maintained on the VI-60 schedule for the remainder of the experiment.

Once lever-pressing was acquired, animals received either 1 or 10 days of tone-shock or tone-only exposure training. Fear training groups underwent conditioned fear training sessions, each consisting of 10 30-s tones paired with 0.5-mA, 0.5-s foot shocks (adjusted for inter-box variability) dispersed pseudo-randomly throughout a 90-min session. The tones and foot shocks always co-terminated. Tone-only groups received sessions with the same tone presentations (at the same intervals) but without shock. The extended training groups received one of these sessions daily for 10 days, while the limited training groups received just 1 such session.

One day after fear or tone-only training was complete, all groups underwent context extinction for one 90-min session, in which they were allowed to lever-press for food pellets with no tones or shocks presented. This session served to extinguish contextual fear so that fear to the tone cue could be assessed and to increase the lever-press rate, to allow for a high baseline rate of pressing for our conditioned suppression measure. The next day, each group underwent a cued fear test in which the training tone was presented 4 times without shock in a 35-min session.

Animals were perfused according to procedures described in Fisher, Pajser [35] 120 min after the beginning of the cued fear test (85 min after the end of the cued fear test). This interval was chosen to visualize optimal Fos expression resulting from the cued fear test. Brain tissue was extracted and postfixed (4% paraformaldehyde in PBS for 2 h) and then cryoprotected (30 % sucrose in PBS; until tissue sank in the solution). The tissue was frozen and sliced coronally into 40- μ m 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 to stain for Fos was performed on free-floating sections based on procedures from Marchant and colleagues [36]. 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-min washes in the following order: PB; 50 % ethanol in PB, 50 % ethanol and 3% H₂O₂ in PB, and 5% normal horse serum (NHS) in PB. The tissue was then incubated in rabbit-anti-phospho-c-Fos serum (1:8,000, Ser32, 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 h. Unbound primary antibodies were washed off of the sections with 3 10-min washes in PB. The sections were then incubated with donkey anti-rabbit-biotin (1:1,000, 711-065-152, Jackson Immuno Research Laboratories, West Grove, PA) diluted with 0.2 % Triton X-100, 2% NHS, and PB at room temperature for 2 h. Unbound secondary antibodies were washed off with 3 additional 10-min washes in PB. Sections were then placed in the mixed avidin-biotin horseradish peroxidase complex solution (ABC Elite Kit: 6 µL/mL avidin and 6 µL/mL biotin, Vector Laboratories, Burlingame, CA) for 2 h. Visualization was achieved using a 10-min incubation in a solution of 2% nickel sulfate, 0.25 % 3, 3'-diaminobenzidine, 0.04 % ammonium chloride, and 0.02 % D-glucose in a 0.1 M acetate buffer solution (pH 6.0, all chemicals from Sigma-Aldrich, St. Louis, MO) followed by a 9-min incubation in the same solution with the addition of 0.2 µL/mL glucose oxidase. The reaction was stopped with acetate buffer and sections underwent 2 10-min 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 (based on architectural features) were acquired for both PL and IL and used for analysis of Fos. These coordinates were based on those examined in previous literature (Knapska & Maren, 2009; LaLumiere, Niehoff, & Kalivas, 2010; B. A. Morrow et al., 1999; Quinones-Laracuenta, Hernandez-Rodriguez, Bravo-Rivera, Melendez, & Quirk, 2015; Sharpe & Killcross, 2015; Sierra-Mercado et al., 2011). Neurons that were 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 was the density of Fos-positive cells in PL and IL.

Fear expression was measured using conditioned emotional responding measured via a suppression ratio, calculated as ((Precue responding - Cue responding)/(Precue responding + Cue responding)) [7,37]. The pre-cue period refers to the 30 s immediately prior to the CS onset and serves as a baseline to which to compare lever-pressing during the CS.

Several aspects of our procedure limit the effects of fear conditioning on contextual fear and pre-cue lever-press rates such that differences in baseline lever-press rates typically have a minimal effect on calculation of our suppression ratios. Rats experience extensive exposure to the operant chambers while receiving magazine (1 session) and lever-press training (5 sessions), before ever experiencing the CS or US, which could be considered

equivalent to the pre-exposure phase of a latent inhibition preparation. Such experience inhibits acquisition of conditioned fear to the pre-exposed stimulus (in our case, the context). In addition, the 90-min context extinction session serves to extinguish any contextual fear prior to the cue test. Finally, the lack of cue presentations or shocks during the first 5 min of the cued fear test serves to extinguish any lingering contextual fear.

Due to an experimenter error during the cued fear test, we do not have behavioral test or neurobiological data for 2 rats (both in the 1 day–tone-only group). In addition, cryostat complications resulted in the loss of several brain tissue samples, so we do not have neurobiological data for 3 rats (1 in each the 10 day–tone-only, 10 day-shock, and 1 day-shock groups). Otherwise, data were analyzed using ANOVAs in Statistica 13.0 (Palo Alto, CA). The behavioral and neurobiological data were analyzed using ANOVAs, but the neurobiological data were transformed using the formula $\log(x+1)$ to make the count data amenable to analysis using ANOVAs. The factors used in the statistical analyses are described in the Results section for each analysis. Significant effects ($p < 0.05$) in the ANOVAs were followed by pairwise comparisons using post-hoc Tukey's HSD tests.

3. Results

3.1. Behavioral results

3.1.1. Behavioral training—Consistent with our previous findings, the extended training shock group had high suppression ratios that decreased over fear conditioning training days in the group given 10 days of training, while the extended training tone-only groups had low suppression ratios that did not change over time. In addition, conditioned fear did not differ on the last day of fear conditioning in the two shock groups (day 1 for the limited training group and day 10 for the extended training group).

Precue lever-pressing increased during fear/tone-only training in the groups given extended training (Fig. 2A). Within these groups, we ran a mixed-factor ANOVA on the precue lever-press measure with the between-subjects variable of Shock (Shock vs. Tone-only) and the within-subjects variable of Day (fear/tone-only training days 1–10). There was a significant main effect of Day $F(9, 252) = 28.14, p < 0.01$, and the Day X Shock interaction trended towards significance $F(9, 251) = 1.87, p = 0.057$. The effect of Shock was not significant ($p = 0.78$). Post hoc testing on the effect of Day indicated that precue lever-pressing on Days 3–10 increased significantly from Day 1. An additional analysis was conducted on animals in all groups in order to compare them on the last day of conditioned fear or tone-only training they received (day 1 for the limited training group and day 10 for the extended training group), and this analysis supported the findings of the primary analysis (Fig. 2B). A 2×2 ANOVA was conducted on the precue lever-press measure 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 fear/tone-only training compared to groups that received 1 day of training. There were no other significant effects or interactions ($F = 0.30\text{--}0.77, p = 0.38\text{--}0.59$).

Extended fear training led to levels of fear on training day 10 that were lower than their peak levels across the 10 days (on day 2 of training), although the fear levels on day 10 did not differ from fear on the first training day (in either the extended training or limited training groups). In the animals that received extended training, a mixed-factor ANOVA was conducted on the suppression ratio measure with the between-subjects variable of Shock (Shock vs. Tone-only) and the within-subjects variable of Day (fear/tone-only training days 1–10). There was a significant Day X Shock interaction, $F(9, 252) = 2.87$, $p < 0.01$, which reflected that the suppression ratios for the extended training tone-only group did not change over the 10 days of training, but the suppression ratios for the extended training shock group decreased from their peak (Fig. 2C). Specifically, suppression ratios on day 2 of fear conditioning were significantly higher than on day 10 of fear conditioning for the extended training shock group, a pattern not seen in the extended training tone-only group. An additional analysis on animals in all groups was conducted in order to compare them on the last day of conditioned fear or tone-only training they received (day 1 for the limited training groups and day 10 for the extended training groups) (Fig. 2D). A 2×2 ANOVA was conducted on the suppression ratio measure 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. There were no other significant effects or interactions ($F = 0.19$ – 0.26 , $p = 0.61$ – 0.66).

3.1.2. Behavioral testing—As with the training data, our test data were consistent with our prior findings. 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.

Animals that received extended fear/tone-only training had higher precue lever-press rates during testing than animals that received limited training (Fig. 2E). A mixed-factor ANOVA was conducted on the precue lever-press measure 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 extended fear/tone-only training groups compared to limited fear/tone-only training groups. There were no other significant effects or interactions ($F = 0.04$ – 2.14 , $p = 0.15$ – 0.84).

The groups also differed in their conditioned fear. A mixed-factor ANOVA was conducted on the suppression ratio measure with the between-subjects factors of Shock (Shock vs. Tone-only) and Training Length (1 vs. 10 days) and the within-subjects factor of Trial (test trials 1–4). There was a significant Training Length X Shock interaction, $F(1, 54) = 5.67$, $p < 0.05$ (Fig. 2F), and post-hoc tests indicated that the suppression ratio was higher (either significantly or barely missing significance) for the limited training shock group than the others (all $p < 0.06$), and none of the other groups differed from each other ($t = -0.63$ – 2.58 , $p = 0.92$ – 1.00). The limited training shock group had a significantly higher suppression ratio than the limited training tone-only group and the extended training shock group (both $p < 0.05$), and a marginally significantly increased suppression ratio compared to the extended training tone-only group, ($p = 0.059$). The Shock X Trial interaction was

also significant, $F(3, 162) = 4.44$, $p < 0.01$, and pairwise comparisons using Tukey's HSD test found that the shock groups differed from the tone-only groups on Trials 1 and 2, but not on subsequent trials. No other effects or interactions were significant ($F = 0.00$ – 2.19 , $p = 0.14$ – 0.95). Although the Shock X Training Length X Trial interaction was not significant, we performed an exploratory analysis of the differences between groups trial-by-trial with Tukey's HSD tests. We found that the limited training shock group had significantly higher suppression ratios than the limited training tone-only group on trials 1–2, but the extended training shock group did not exhibit significantly higher suppression ratios than the extended training tone-only group on any trial. The limited training shock group had a significantly higher suppression ratio than the extended training shock group on trial 2 only, although this difference (along with trends towards a higher suppression ratio for the limited training shock group on trial 1) was strong enough to drive a significant difference between the two groups in average suppression ratios across the entire test.

3.2. Neurobiological results

The particular areas where Fos was counted are shown in Fig. 3.

3.2.1. IL results—Contrary to our predictions, we found that Fos expression was higher in both extended training groups than in both limited training groups, with no effect of prior fear conditioning (Fig. 4A, example images in Fig. 4D). A 2×2 ANOVA was conducted on the transformed average Fos count measure with the factors of Shock (Shock vs. Tone-only) and Training Length (1 vs. 10 days). There was a significant main effect of Training Length $F(1, 51) = 18.96$, $p < 0.01$, with the groups that received 10 days of fear/tone-only training exhibiting higher levels of Fos in IL than the groups that received 1 day of fear/tone-only training. The effect of Shock and the Shock X Training Length interaction were not significant ($F = 0.06$ – 1.39 , $p = 0.24$ – 0.94).

There was no relationship between Fos expression in IL and precue lever-pressing or fear expression during testing. When all rats in all groups were included in a correlation analysis, there were non-significant correlations between IL Fos density and average precue lever-pressing during testing, $r(53) = -0.09$, $p = 0.54$ and IL Fos density and average suppression ratio during testing, $r(53) = 0.11$, $p = 0.41$ (Fig. 4B). We conducted similar analyses, but with tone-only animals excluded (but still including rats in the extended training and limited training groups) in an attempt to isolate fear-related neural activity. With these analyses, there were non-significant correlations between IL Fos density and average precue lever-pressing during test, $r(26) = -0.16$, $p = 0.41$ and IL Fos density and average suppression ratio during test, $r(26) = 0.06$, $p = 0.78$ (Fig. 4C). In addition, rats in the limited training groups differed in their total number of lever-presses prior to the cue test (from FI1 training to context extinction, 4,128–13,841 lever presses) and rats in the extended training groups differed in their total number of lever-presses prior to the cue test (from FI1 training to context extinction, 9,882–47,210 lever presses). We correlated the total number of presses prior to the cue test or during the cue test with IL Fos. We found no significant correlations with either total in either the limited training or extended training groups (Table 1, all $p > 0.05$).

3.2.2. PL results—Consistent with our findings in IL, but in contrast to our predictions, increased training length was associated with increased Fos expression in PL, and shock experience increased Fos expression in the extended training group, but not the limited training group.

Contrary to our hypotheses, we found the pattern of Fos expression in PL to be similar to that in IL, with Fos expression that was higher in both extended training groups than in both limited training groups, with no effect of prior fear conditioning (Fig. 5A, example images in Fig. 5D). A 2×2 ANOVA was conducted on the transformed average Fos count measure with the factors of Shock (Shock vs. Tone-only) and Training Length (1 vs. 10 days). There was a significant main effect of Training Length $F(1, 51) = 10.39, p < 0.01$, with the groups that received 10 days of fear/tone-only training exhibiting higher levels of Fos in PL than the groups that received 1 day of fear/tone-only training. The effect of Shock and the Shock X Training Length interaction were not significant ($F = 0.08$ – $0.71, p = 0.40$ – 0.77).

There was no relationship between Fos expression in PL and precue lever-pressing or fear expression during testing. When all rats in all groups were included in a correlation analysis, there were non-significant correlations between PL Fos density and average precue lever-pressing during test, $r(53) = -0.04, p = 0.77$, and PL Fos density and average suppression ratio during test, $r(53) = -0.10, p = 0.45$ (Fig. 5B). We conducted similar analyses, but with tone-only animals excluded (but still including rats in the extended training and limited training groups) in an attempt to isolate fear-related neural activity. With these analyses, there were non-significant correlations between PL Fos density and average precue lever-pressing during test, $r(26) = -0.06, p = 0.75$ and PL Fos density and average suppression ratio during test, $r(26) = -0.24, p = 0.22$ (Fig. 5C). In addition, rats in the limited training and extended training groups differed in their total number of lever-presses prior to the cue test. We correlated the total number of lever-presses prior to the cue test or during the cue test with IL Fos. We found no significant correlations with either total in either the limited training or extended training groups (Table 1, all $p > 0.05$).

4. Discussion

The purpose of this study was to examine the neurobiological mechanism underlying one aspect of the behavior exhibited as a result of the extended training fear incubation paradigm: fear suppressed during the initial test conducted 1–2 days after extended fear conditioning. Previous research has investigated this model using pharmacological, physiological (food restriction manipulations), and behavioral methods [8–12,38], but this model had not been investigated with measures of neuronal activity (direct or indirect) associated with differing levels of fear. We were interested in whether an extinction-like process might be the cause for the initially low fear caused by this paradigm, so we used Fos immunohistochemistry to indirectly measure activity in areas of the brain associated with the acquisition, expression, and extinction of conditioned fear. We 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, we did not observe a relationship between conditioned fear and Fos expression in either IL or PL.

Instead, we 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 both IL and PL. The possible implications of these findings are discussed below.

4.1. Discussion of behavioral results

During training, we found that the 1 day- and 10 day-shock groups exhibited higher levels of fear than the 1 day- and 10 day-tone only groups, with fear decreasing across training in the 10 day-shock group. During testing, we found that the 1 day-shock group exhibited higher fear expression (either significantly or marginally significantly) than the 1 day-tone only, 10 day-tone only, and 10 day-shock groups, which all showed similarly low fear expression. These findings are in accordance with our predictions and the existing literature [7–9]. Although research using the extended training fear incubation paradigm is limited, our findings that rats 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 if it was expressed.

4.2. Discussion of neurobiological results

Our neurobiological results were unexpected, and the existing fear conditioning literature does not explain the findings that the shock and tone-only groups did not differ in the evoked neural activity in IL or PL at each training length. As a reminder, we expected high Fos expression in IL in animals that underwent the extended training fear incubation paradigm (if an extinction-like process had occurred) and low Fos expression in IL in animals that underwent limited conditioned fear training or tone-only training, regardless of training length. We also expected high Fos expression in PL in animals that underwent limited conditioned fear training (as is often observed in situations where conditioned fear is high) and low Fos expression in PL in animals that underwent tone-only training, regardless of training length, or the extended training fear incubation paradigm.

Our pattern of Fos expression in PL was unexpected. A variety of research findings suggests that neural activity in PL should be higher during early extinction in fear conditioned rats than during tone exposure in animals never fear conditioned, possibly due to fear expression rather than extinction itself [26–28] (but see [25]). In this case, 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 we visualized was not related to conditioned fear.

Our pattern of Fos expression in IL was also unexpected. Prior research suggests that neuronal activity in IL should be higher during early extinction in fear conditioned rats than during tone exposure in animals never fear conditioned [27,28]. Extending these findings to make predictions about the expected pattern in our experiment, one could expect that our 1 day- and 10 day- shock groups should exhibit a higher level of Fos expression in IL than either of the tone-only groups (as our cue test was also a brief extinction session with 4 tones presented without shocks). Alternatively, lower fear during a conditioned fear extinction

retention test was associated with higher IL neuronal activity during that test [23,24] and rats exhibiting low fear in an extinction test conducted in the same context as the prior extinction exhibited higher IL Fos than rats exhibiting high fear due to context-induced renewal and compared to rats that had not received prior extinction [25]. Based on this prior pattern, *our* expected results were that a putative extinction-like processes (such as having the tone paired with an ineffective shock in the 10 day-shock group) might lead to higher IL Fos in the 10 day-shock group than in the other 3 groups. Instead, we found that both 10 day groups had higher Fos expression in IL than either of the 1 day groups, regardless of shock experience. Once again, it seems clear that the Fos expression we visualized does not appear to be related to fear extinction or expression.

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

The differences we observed 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, as operant training length increased, so did Fos expression in both IL and PL. Our experiment is the first that we know of that has shown differential PFC Fos expression based on whether operant training was limited or extended. IL and PL are known for their roles in habitual and flexible goal-directed operant behavior, respectively [29–33,39–42], and animals that received 10 days of training were maintained on the same schedule of reinforcement for lever-pressing 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 operant lever-press behavior, rather than fear expression or extinction. This is particularly true 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 [43].

Prior research suggests that IL would be active after extended operant training under interval schedules. IL lesions or inactivations cause maintained goal-directed action under extended training conditions that generally lead to habitual behavior [29,30] (but see [44]). Likewise, there are alterations in IL neuronal activity during operant behavior under conditions that promote habit learning (specifically, training with interval schedules) rather than conditions that promote goal-directed action [32]. Conversely, other evidence suggests that IL controls flexible goal-directed behavior after extended training [44], which suggests that the important factor determining which subregion of mPFC is controlling behavior is not whether a habit has formed, but the degree of training given. This explanation would be consistent with our findings.

The increased Fos in PL after extended training requires somewhat more explanation, but it does not explicitly conflict with prior literature (to the best of our knowledge). PL lesions lead to the loss of goal-directed action in several versions of the devaluation task, a procedure often used to assess habitual and/or flexible behavior [30,39,41], with this behavioral impairment apparent compared to sham rats after limited training when sham rats exhibit intact devaluation sensitivity. Similarly, other evidence suggest that PL controls flexible behavior after limited training (but not extended training) [44]. However, it is

entirely possible that PL exhibited increased neuronal activity after extended training in these prior experiments, and that this PL activity simply was not *necessary* for proper goal-directed action. In addition, although extended training can lead to impaired devaluation under normal conditions, IL inactivation leads to the return of goal-directed action even after extended training, suggesting that the neural activity underlying flexible behavior (possibly found in PL) is still present or able to be evoked [29].

4.4. Methodological considerations

There are at least two methodological limitations to consider in the current study. First, the rats used were food restricted and food restriction has been shown to alter mPFC neuronal activity in response to stimuli. For example, food deprivation lasting 12–36 h can increase Fos expression in mPFC [45] (but see [46] for no effect of weaker food restriction on mPFC Fos). Because conditioned suppression requires food motivation for lever-pressing, future studies could utilize freezing in free-fed rats (with no concurrent behavioral task) as a behavioral measure of fear so that neural activation could be examined, unaffected by food restriction. Second, the exclusive use of lever-press suppression (CER) as a measure of fear is a limitation for a number of reasons and measuring conditioned freezing as an additional measure of fear would have been ideal. The use of multiple fear measures would provide converging evidence that true fear and neural activity related to fear are being assessed. CER is also an indirect measure of fear, relying on food motivation to produce a response for an appetitive reinforcer. As a result, either an increase in fear or a decrease in motivation for food could affect the expression of fear [47,48]. Finally, it is possible that activity in the PFC related to lever-pressing may interfere with measuring neuronal activity related to fear. A similar preparation using non-food restricted, non-lever-pressing rats may be able to uncover fear-related neural activity in the mPFC that the current preparation missed.

4.5. Conclusions

The purpose of this experiment was to determine the neurobiological underpinnings of low fear exhibited 1–2 days after training in the extended training fear incubation procedure. Our behavioral findings were expected, with 1 day-shock animals showing higher fear expression than the 10 day-shock animals or either of the tone-only groups. In contrast, our neurobiological findings were unexpected. We found increased Fos expression in IL and PL of animals that received 10 days of training compared to those that received 1 day of training, regardless of shock exposure. Although we cannot say conclusively what the Fos expression in mPFC we visualized represents, our findings are consistent with the possibility that extended operant training leads to greater IL activity related to habit formation. Further research is needed to assess this possibility. In addition, further research, such as examining conditioned freezing as the fear measure in non-food restricted rats, will be conducted to address the limitations of the current study to better examine the neurobiological underpinnings of the low fear observed soon after training with the extended training fear incubation procedure.

Acknowledgements

We would like to thank Hayley Fisher, Skyler Gilbert, Paige Kallen-berger, Alicia Lensing, Aaron Limoges, Mallory Line, Lauren Londeen, and Charday Long for technical assistance. We also thank Hayley Fisher for helpful comments on an earlier version of the manuscript. There are no conflicts of interest arising from this work.

Funding

This project was supported in part by grant GM113109 from the National Institute of General Medical Science of the National Institutes of Health.

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Fig. 1.

The behavioral procedure for the current experiment. A. The procedure with experimental details for each session. B. The experimental design. All rats received the same lever-press training consisting of a magazine session, FI1 training, VI30 lever-press training, and VI60 lever-press training. In the conditioned fear training phase, rats received either 1 or 10 training sessions of fear conditioning or tone-only presentations. Lever-pressing was measured during the 30-s tone presentations (the cue period) and the 30-s immediately before the tone (the precue period). These are used to calculate the suppression ratio $((\text{Precue responding} - \text{Cue responding}) / (\text{Precue responding} + \text{Cue responding}))$ we use as a measure of fear. In the test phase of the experiment, rats received context extinction session during which they lever-press with no tone presentations and a cued fear test with 4 tone presentations. Sacrifice took place 120 min from the start of the test session. T = tone.

Fig. 2.

Behavioral data from the current experiment. A. Average precue lever-pressing (30-s precue period) for each fear/tone-only training day in the extended training groups. 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 $p < 0.05$. B. Average precue lever-pressing (30-s cue period) on the last day of fear/tone-only training for all groups. # indicates a main effect of Training Length, significant at $p < 0.05$. C. Average suppression ratio per fear/tone-only training day in the extended training groups. 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, while the 10

day-tone group had low suppression ratios throughout training. *† indicates a Shock X Day interaction, significant at $p < 0.05$. D. Average suppression ratio on the last day of fear/tone-only training for all groups. Higher suppression ratios represent higher fear. † indicates a main effect of Shock, significant at $p < 0.05$. E. Average precue lever-pressing (30-s precue period) for each test trial. # indicates a main effect of Training Length, significant at $p < 0.05$. F. Suppression ratio across test trials for each training group. Higher suppression ratio values indicate higher fear and lower values indicate lower fear. *# indicates a Training Length X Shock interaction, significant at $p < 0.05$. *† indicates a Shock X Trial interaction, significant at $p < 0.05$.

Fig. 3. The location where Fos-positive neurons were counted in prelimbic and infralimbic cortex. A 10x image was taken of the areas within the black boxes, and Fos within those areas was counted and analyzed. Brain images are from Paxinos and Watson [49].

Fig. 4.

IL Fos expression in the 4 experimental groups. A. A comparison of the average Fos expression across the different training groups. Groups that received 10 days of fear/tone-only training showed higher Fos expression than groups that received 1 day of fear/tone-only training, regardless of shock exposure. # indicates a significant main effect of Training Length at $p < 0.05$. Fig. 4B–C. Correlations between IL Fos density and suppression ratios during test. B. A correlation between IL Fos and the suppression ratio during test for all subjects. C. A correlation between IL Fos and the suppression ratio for subjects that received shock. D. Example images from each of the 4 experimental groups.

Fig. 5.

PL Fos expression in the 4 experimental groups. A. A comparison of the average Fos expression across the different training groups. Groups that received 10 days of fear/tone-only training showed higher Fos expression than groups that received 1 day of fear/tone-only training, regardless of shock exposure (# indicates a significant main effect of Training Length at $p < 0.05$). Fig. 5B–C. Correlations between PL Fos density and suppression ratios during test. B. A correlation between PL Fos and the suppression ratio during test for all subjects. C. A correlation between PL Fos and the suppression ratio for subjects that received shock. D. Example images from each of the 4 experimental groups.

Table 1

Correlation coefficients (Pearson's r values) for mPFC Fos and total lever presses prior to the cue test or during the cue test.

	Limited training groups		Extended training groups	
	All training before cue test	Cue test	All training before cue test	Cue test
IL Fos	0.04	-0.21	-0.23	-0.35#
PL Fos	-0.01	-0.24	0.05	-0.11

Critical values are $r = +0.37$ for limited training groups ($n = 27$) and $r = +0.36$ for limited training groups ($n = 28$). # represents $p = 0.07$.