MEDIAL PREFRONTAL CORTEX NEURONAL ENSEMBLES PLASTICITY DURING CONTEXT-MEDIATED RENEWAL OF RESPONDING TO FOOD CUES

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MEDIAL PREFRONTAL CORTEX NEURONAL ENSEMBLES PLASTICITY DURING CONTEXT-MEDIATED RENEWAL OF RESPONDING TO FOOD CUES

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Abstract: Cues existing in the surrounding environment repeatedly paired with biologically relevant events can exert a powerful drive over behavior. When learned cues recurrently signal consumption, this can lead to eating in the absence of hunger or physiological need. The difficulties associated with resisting palatable foods and maintaining healthy habits may be related to the neurobiological underpinnings of pervasive responding to food cues. Behavioral flexibility through updating information about formed reward associations is vital to appropriately adapt to the surrounding environment and physiological need. Studying the renewal of responding of extinguished food-seeking behaviors can help us better understand the mechanisms mediating behavioral control over responding to learned reward cues. This dissertation aimed to explore behavioral sex differences and the neural substrates of renewal of responding to food cues after extinction by utilizing a context-mediated renewal of responding paradigm.

The first chapter in this dissertation explored the effects of context habituation on context-induced renewal of responding to food cues in males and females. We investigated if increased familiarity with the behavioral contexts, and if presentation of food reward or not during these habituation sessions, would impact the strength of cue-food learning and renewal of responding after

extinction differently in males and females. We discovered that when males received context habituation paired with food prior to training they exhibited elevated food-seeking behaviors throughout conditioning, as well as strengthened renewal. This suggests that for males the context habituation with food had a lasting, amplifying effect on cue-food learning. For females, however, increased context familiarity did not improve renewal of responding and, moreover, these experiments revealed evidence for resistance to extinguishing food-seeking behaviors in females. Then, in Chapter 2, we found neural evidence for potential plasticity mechanisms in the prelimbic (PL) and infralimbic (ILA) subregions, which were both recruited during context-mediated renewal of responding to food cues. Our findings are in line with evidence demonstrating that the PL and ILA are both recruited during appetitive learning and possibly provide overlapping contributions to encoding and responding in context-based reward learning. Taken together, the experiments outlined in this dissertation add to existing evidence of sex differences in appetitive motivated behaviors and the intricacies of the roles of the PL and ILA in cue-food learning and contextual processing. The findings from these studies advance our understanding of persistent food-seeking behaviors and highlight the importance of elucidating the neural substrates mediating behavioral responding to learned reward cues.

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Although you are not here, you are always with me. You are my source of inspiration and my reason for wanting to leave the world better than I found it. It is to you that I dedicate this dissertation because I always have, and always will, dedicate everything I am to you.

GENERAL INTRODUCTION

Learned cues have a powerful relationship with the drive to seek rewards such as food and drugs of abuse. These associations are formed when cues existing in the external environment are paired with biologically relevant events, such as finding food or other forms of reward. When these cue and reward pairings repeatedly occur, this can increase the drive to obtain and consume food (for review see Petrovich, 2013). Unfortunately, these cues can produce persistent preoccupation with seeking these rewards, which for food can mean eating in the absence of hunger or physiological need, leading to the development of maladaptive eating behaviors, obesity, and even eating disorders (for reviews see Martin & Davidson, 2014; Petrovich, 2013). Renewal of responding can occur even after the cue-induced behavior has been extinguished because extinction processes do not erase the initially learned association, as demonstrated by spontaneous recovery and other types of renewal of responding (Bouton & King 1983; for review see Bouton, 2004; Pavlov, 1927; Rescorla, 2004). The renewal of learned associations after extinction demonstrates the power of the initial learning and researching the brain mechanisms underlying these processes is valuable to understanding how they contribute to overeating and drug-seeking.

Elucidating how these learned associations are acquired, extinguished, and renewed or reinstated is beneficial because it informs us of the mechanisms supporting pervasive responding to reward cues, as well as the difficulties

involved in mitigating maladaptive reward-seeking behaviors (Boutelle & Bouton, 2015; Todd, Winterbauer, & Bouton, 2012). Similarly, context-mediated renewal is valuable for investigating how context determines a discrete cue's meaning (Holland & Bouton, 1999). When a discrete cue (e.g., a tone) is associated with food delivery in one context but that association is extinguished in a different context, the cue possesses a different meaning in each of these two contexts. That is to say that the context sets the occasion for whether or not the cue will predict food delivery (Holland & Bouton, 1999). Given that these cues do not exist in isolation, but instead have an interacting or even compounding effect with the surrounding environment, it remains crucial to unveil the underlying neural mechanisms that allow context and discrete cues together to exert such control over behavioral drive.

In the experiments that comprise this dissertation, the paradigm used to study this context-mediated renewal of responding to food cues was an ABA renewal protocol. In this protocol, animals underwent Pavlovian conditioning in one context where they learned to associate a tone cue with food delivery. This is followed by extinction sessions in a different context where the tone is presented without food delivery and new learning occurs – extinction learning where the tone no longer predicts food delivery (Quirk, Garcia, & González-Lima, 2006). The *renewal effect* is observed when the subject is returned to the acquisition context where the extinguished food-seeking response reappears upon presentation of the tone cue (Bouton & Bolles, 1979). This type of learning, where the outcomes of the cue can change based on the surrounding

environment, requires brain plasticity mechanisms to help inform the changing meanings of the cue. As learning occurs and new information must be incorporated about the context-based implications of the cue, the brain must update the previously formed associations to continue guiding the appropriate behavioral response.

Initially, the majority of appetitive studies examining the renewal effect were conducted in males (Bouton, 2004), but more recently there is accumulating evidence for sex differences in renewal of responding. Prior work has established that, while male rats consistently demonstrate renewal of responding to a food cue, females' patterns of behavior remains inconsistent (Anderson & Petrovich, 2015, 2017), and similar findings have been reported using alcohol as a reinforcer (Segal, Valyear, & Chaudhri, 2021). Studying both males and females in these types of experiments remains important to not only determine the neurobiological basis of these sex differences, but also to document the aspects of learning that are conserved across both sexes, which together, will help effectively combat pervasive maladaptive reward-seeking behaviors in more individuals (for review see Shansky & Woolley, 2016). Therefore, one of the aims of the studies in this dissertation was to continue investigating sex differences in appetitive renewal of responding. The first set of the following experiments (Chapter 1) sought to determine whether habituation in the form of pre-exposure to the context in which conditioning of a tone-food association was to be learned, and to the context in which extinction would occur, would impact the strength of context-mediated renewal of responding to the food cue in males and females.

The second major goal of these studies was to contribute to the ongoing investigation of the complex neural circuitry supporting cue-food learning and context-induced renewal of responding to food cues after extinction. While the renewal phenomenon is prevalent in both the appetitive and aversive learning literature, until recently, the majority of studies regarding the renewal effect have focused on the conditioned fear response (Bouton & King, 1983; Fanselow, 1990). Moreover, the limited studies that have been conducted on appetitive conditioning tend to use instrumental conditioning or use drug reinforcers (Bouton, Todd, Vubric, & Winterbauer, 2011; Willcocks & McNally, 2013), meaning that the number of studies evaluating Pavlovian appetitive conditioning are even fewer still. Of particular interest, there are contradictory findings regarding the neurobiological basis of context-induced renewal of responding for the subregions of the ventromedial prefrontal cortex (vmPFC) between appetitive and aversive renewal behaviors. Although there is ample research on the functionally distinct roles of the subregions of the vmPFC in the aversive literature (Sierra-Mercado, Padila-Coreano, & Quirk, 2011), there is conflicting evidence in the specific involvement of the prelimbic (PL) and infralimbic (ILA) regions in the appetitive literature (Cole, Hobin, & Petrovich, 2015; Moorman, James, McGlinchey, & Aston-Jones, 2015), and very few studies regarding these subregions in terms of context-mediated renewal. There is established evidence that the mPFC is involved in cue-food learning and contextual appetitive associations (Eddy, Todd, Bouton, & Green, 2016; Petrovich, Ross, Gallagher, & Holland, 2007; Willcocks & McNally, 2013) and drug-seeking behaviors (Bossert

et al., 2012; Moorman, James, McGlinchey, & Aston-Jones, 2015), but investigation of the nuanced roles of the PL and ILA remains ongoing. While many studies provide support for dichotomous roles of the PL and ILA mediating the execution of and inhibition of behavior, respectively, in the aversive literature, the appetitive literature contains contradictory evidence as there are studies demonstrating that they are functionally distinct, as well as studies showing they are similarly recruited (Cole, Hobin, & Petrovich, 2015; for review see Kaminska, Caballero, & Moorman, 2021). These neural differences further emphasize the importance of studying how encoding and memory recall of appetitive associations occur so we can unveil their neurobiological underpinnings. By revealing if the same or different neuronal ensembles, or a group of distributed coactive neurons with coordinated activity, are critical for different stages across appetitive associative learning, extinction, and recall and renewal, we can better understand the crucial components of the neural network mediating persistent food-seeking behaviors.

Therefore, the second set of studies in this dissertation (Chapter 2) aimed to identify key neuronal ensembles in the PL and ILA and their roles in driving behavior during context-induced renewal of responding to a food cue. The main goal was to test if the PL and ILA are critical sites for plasticity during cue-food acquisition and renewal. Specifically, we tested the hypothesis that the PL, but not ILA, mediates cue-food acquisition and renewal, and that the same PL neuronal ensemble is recruited during both acquisition and renewal in the acquisition context. This was accomplished using the Daun02 selective

inactivation methodology in transgenic *Fos-LacZ* rats during our ABA renewal paradigm (for reviews see Cruz et al., 2013; Bashir & Banks, 2017). Fos is an immediate early gene marker of neuronal activation and, in the *Fos-LacZ* line of transgenic rats, the strongly activated cells that produce Fos co-express a protein called β-galactosidase (Koya et al., 2009). Intracranially infusing Daun02 into the brain allows the Daun02 to be converted by β-gal into daunorubicin, which then selectively silences only those neurons which had recently been active. This chemogenetic methodology allows researchers using various behavioral paradigms to demonstrate causal roles of specific neuronal ensembles (Fanous et al., 2012; Whitaker et al., 2017; for review see Bashir & Banks, 2017).

Overall, the aim of the studies in this dissertation was to explore how the surrounding environment and discrete cues interact to influence food-seeking behaviors. Understanding what mediates the persistent drive to seek food can help combat maladaptive eating behaviors. By pinpointing the neural substrates responsible for cravings and the inability to resist palatable foods, we will be better equipped to treat these matters. The results from this field of work are expected to have implications for potential therapeutic targets in regulating maladaptive eating habits, eating disorders, and drug relapse.

1.0 CHAPTER 1: Context-induced renewal of responding to food cues: The effects of context habituation in males and females *

* Chapter 1 was submitted in partial fulfillment of the requirements for the Masters of Arts at Boston College in December of 2021.

1.1 INTRODUCTION

Learned cues have a powerful relationship with the drive to seek rewards such as food and drugs of abuse. Unfortunately, these cues can produce persistent preoccupation with seeking these rewards. For food, this can mean eating in the absence of hunger or physiological need, leading to the development of maladaptive eating behaviors, obesity, and even eating disorders (for reviews see Martin & Davidson, 2014; Petrovich, 2013). Renewal of responding can occur even after the cue-induced behavior has been extinguished because extinction processes do not erase the initially learned association, as demonstrated by spontaneous recovery and other types of renewal of responding (Bouton & King 1983; for review see Bouton, 2004; Pavlov, 1927; Rescorla, 2004). The renewal of learned associations after extinction demonstrates the power of the initial learning and researching the brain mechanisms underlying these processes is valuable to understanding how they contribute to overeating and drug seeking. The findings from this field of work have translational value in helping us study what contributes to the persistent drive to seek food, maladaptive eating habits, eating disorders, and drug relapse.

Context-mediated renewal is valuable for investigating how context determines a discrete cue's meaning (Holland & Bouton, 1999). When a discrete cue (i.e., a tone) is associated with food delivery in one context, but that association is extinguished in a different context, the cue possesses a different meaning in each of these two contexts. That is to say that the context sets the occasion for whether or not the cue will predict food delivery (Holland & Bouton, 1999). In the current experiments, the protocol for context-mediated renewal of Pavlovian responding was the ABA paradigm, which begins with acquisition sessions in one context where rats learn to associate a tone cue with food delivery. This is followed by extinction sessions in a different context where the tone is presented without food delivery and a new learning occurs – extinction learning where tone no longer predicts food delivery (Quirk, Garcia, & González-Lima, 2006). The context-mediated renewal effect is observed upon returning to the acquisition context where the extinguished food seeking response reappears upon presentation of the tone cue (Bouton & Bolles, 1979) and is identified by higher conditioned responding during the CS periods in the acquisition context compared to the extinction context.

There are procedural variations across the literature for context-mediated renewal, particularly in the use of context habituation and whether it includes food presentations. Because our previous work used novel contexts at the start of training, we sought to systematically investigate the impacts of context habituation with and without food. Based on protocols from various renewal experiments (Bouton & Peck, 1989; Bouton, Todd, Vubric, & Winterbauer, 2011;

Campese & Delamater, 2013), we evaluated the effects of habituation to both acquisition and extinction training contexts, either with or without food presentations, to determine if it would facilitate the context-mediated CS-US outcome learning.

Prior work has found sex differences in renewal with food or alcohol where male rats consistently demonstrated renewal of responding to the reward cue but females' pattern of renewal of responding was inconsistent (Anderson & Petrovich, 2015, 2017; Segal, Valyear, & Chaudhri, 2021). A key difference in our lab's prior work compared to other reward-based renewal studies is that it used novel contexts that rats first encountered during the first session of acquisition and first session of extinction training. Given that the context switch is critical in this preparation, we presumed that familiarity with context is important because the outcome of the cue is entangled with the meaning of the context. Therefore, the current study aimed to test whether context familiarity impacts the strength of renewal. This was accomplished through habituation to each context prior to training. In Experiment 1, habituation included food delivery, and in Experiment 2, rats received context habituation alone.

Very few studies have compared males and females and, thus, it remains important to compare males and females to establish if they are similar or different in learning, extinction, and renewal of appetitive behaviors. The current research utilized both male and female rats to identify if habituation to the training contexts could help absolve the irregular renewal of responding patterns previously observed in females. We hypothesized that habituation to the

acquisition and extinction training contexts would improve context-specific learning and memory about tone-cue associations, which would result in better context-mediated renewal of responding after extinction. We predicted that additional time spent exploring and encoding information about each context prior to training would make it easier to later incorporate specific cue-food outcome information. We further theorized that habituation to the contexts paired with food would increase the strength of renewal in both sexes, because the food reward would enhance learning about contexts, and, in the case for females, thereby rescue the absence of the renewal effect that previous work in our lab has documented. Thus, in the present study, for Experiment 1, one group of male and female rats experienced habituation to both training contexts paired with food presentations, while the other group (control condition) remained in their homecage where they received exposure to the food (Bouton & Peck, 1989). For Experiment 2, the experimental group of male and female rats experienced habituation to both training contexts without food, while their control counterparts remained in their homecage (Campese & Delamater, 2013).

1.2 MATERIALS AND METHODS

1.2.1 Subjects

Experimentally naïve 32 male and 32 female adult Sprague-Dawley rats were used. These rats were bred from our transgenic *Fos-lacZ* colony, and were determined with genotyping as wild type. Rats were individually housed and maintained on a 12hr light/dark cycle (lights on at 07:00). Prior to food restriction and any behavioral testing, rats had *ad libitum* access to water and standard rat

chow (Prolab RMH 3000 5P00, LabDiet, St. Louis, MO), and were handled daily for 7 days before any experimental procedures began. All housing and testing procedures were compliant with the National Institutes of Health Guidelines for Care and Use of Laboratory Animals and approved by the Boston College Institutional Animal Care and Use Committee.

1.2.2 Apparatus

Behavioral training was conducted in the same set of identical behavioral chambers (30 x 28 x 30cm; Coulbourn Instruments, Allentown, PA) located in a room separate from the colony housing room. These chambers were composed of a clear Plexiglas rear wall and front hinged door, a floor of stainless-steel rods 5mm thick spaced 15mm apart, and aluminum top and sides, with one side containing a house light (4W) and a recessed foodcup (3.2 x 4.2cm, 5.7cm off the floor). Each chamber was contained within a sound- and light- attenuating cubicle (79 x 53 x 53cm) composed of monolithic rigid foam walls, equipped with a ventilation fan (55dB) and a video camera attached to a recording system (Coulbourn Instruments, Allentown, PA). The visual, tactile, and olfactory features of the behavioral chambers were modified to create two distinct contexts, which were counterbalanced across conditioning and extinction. For one context, a black Plexiglas panel was laid across the grate floor so the rats could not see or feel the grate floor, and the doors to the cubicle were closed. For the other context, the black Plexiglas panel was inserted up against the left wall of the chamber, the rats were able to see and touch the grate floor, the doors to the cubicle remained open, and a 1% acetic acid (Fisher Scientific, Fair Lawn,

NJ) solution was sprayed onto the tray below the grate floor. The conditioned stimulus (CS) was a 10s tone (75dB, 2kHz) and the unconditioned stimulus (US) was two food pellets (formula 5TUL, 45mg; Test Diets, Richmond, IN) delivered to the foodcup. A computer in an adjacent room controlled the stimuli and video cameras (GraphicState 3.0; Coulbourn Instruments, Allentown, PA).

1.2.3 Behavioral Training Procedure

1.2.3.1 Experiment 1: Context Habituation with Food

Experimental timeline is shown in Figure 1.1. All training and testing occurred between 07:00 and 15:00. A few days before the start of training, rats were gradually food restricted to reach 90% of their *ad libitum* body weight. They were maintained at this weight for the duration of the experiment. To familiarize rats with the cart transportation and the US, the day before training began, rats were transported from the colony room to the behavioral room where they remained for 10mins and received 1g of the food pellets (US) in their homecage. Training consisted of four phases: habituation to the behavioral contexts, conditioning (acquisition), extinction, and testing. Acquisition and extinction training occurred in two different contexts (the physical contexts used were counterbalanced, see above). For rats in the experimental condition, context habituation consisted of 38mins exposure to each context over two days (one day for each of the two contexts, counterbalanced). During the habituation session, rats received 16 deliveries of the US (32 food pellets total) on a random interval schedule. No CS presentations occurred during context habituation

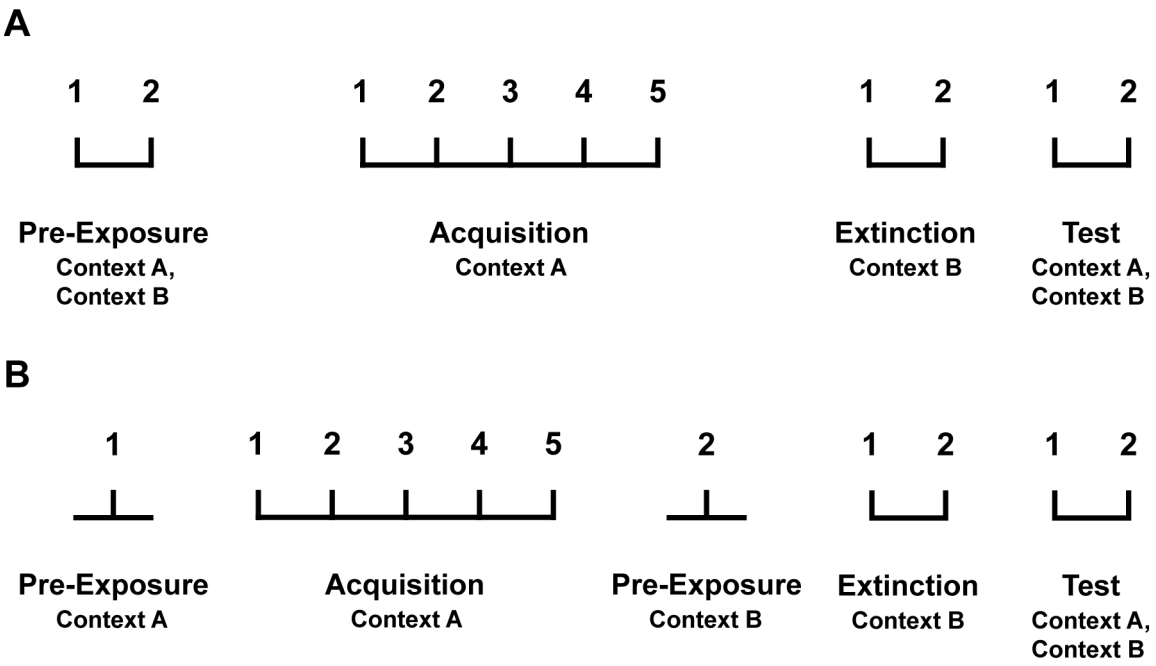

Figure 1.1 Timelines for experiments. Behavioral training consisted of four phases: context habituation (pre-exposure), conditioning (acquisition) training, extinction training, and testing. The pre-exposure procedures differed between experiments. **(A)** Experiment timeline for Experiment 1: Context Habituation with Food. **(B)** Experiment timeline for Experiment 2: Context Habituation without Food.

sessions. Rats in the control condition were transported to the behavioral room, but were not placed in the experimental chambers and instead remained in their homecage where they received 32 food pellets. During the acquisition phase, all rats were trained for 5 days with one 34min training session per day. In each session, rats received 8 CS-US pairings, in which presentations of the CS were immediately followed by delivery of the US to the foodcup. These CS-US pairings occurred on a 2-6min variable inter-trial interval schedule. Rats then experienced two days of extinction training, one session per day, in a context different from their acquisition training context. The extinction training sessions were 34mins

long consisting of 8 CS presentations with no US delivery. Testing was conducted over two days with one session per day, in the acquisition context and in the extinction context (order counterbalanced). These test sessions were 34mins long consisting of 8 CS presentations with no US delivery. All behavioral sessions were recorded and saved for behavioral analysis.

1.2.3.2 Experiment 2: Context Habituation without Food

Experimental timeline is shown in Figure 1.1. All training and testing occurred between 08:00 and 13:00. The procedures for Experiment 2 were the same as in Experiment 1 with the exception of context habituation conditions. Instead of receiving habituation to both contexts prior to any training, rats experienced habituation to the acquisition context prior to the start of acquisition training and habituation to the extinction context prior to extinction training. Additionally, there were no CS or US presentations during habituation sessions. Rats in the control condition were transported to the behavioral room, but were not placed in the experimental chambers and instead remained in their homecage. Acquisition training, extinction training, and testing for Experiment 2 were the same as described for Experiment 1.

1.2.4 Behavioral Observations

Behavior was analyzed from video recordings for all sessions by trained observers, blind to the experimental condition or sex of the rats. The primary measure of conditioning (conditioned responding, CR) was expression of “foodcup behavior,” defined as nosepokes into the recessed foodcup, or standing directly in front of and facing the foodcup. Behavioral observations were recorded

every 1.25s during the 10s CS periods, as well as during the 10s before the onset of the CS (PreCS). At each observation, only one behavior was recorded – foodcup or other. The number of foodcup observations were summed and converted to a percentage of the total time during each PreCS or CS period that the animal spent expressing foodcup behavior.

1.2.5 Statistical Analysis

Foodcup behavioral data were analyzed separately for males and females using two- and three-way mixed repeated measures analysis of variance (ANOVA) with experimental condition as a between-subjects factor, responding during PreCS and CS periods as a within-subjects repeated measure, and session or training block as an additional repeated measure. Simple effects and *post-hoc* Bonferroni tests were analyzed as appropriate, as well as *t*-tests for some analyses determined *a priori*. Elevation of responding was examined separately in order to evaluate learning independent of baseline differences, and was calculated by subtracting baseline (PreCS) responding from CS responding. Extinction training data were analyzed in 4 trial blocks; 2 blocks per session, where blocks 1 and 2 included trials during extinction training day 1 and blocks 3 and 4 were during extinction training day 2. We determined *a priori* to conduct *t*-tests for extinction block 1 and 4 to evaluate if each group successfully extinguished responding across training. Data were tested for sphericity, normality, equality of error variances, and homogeneity of variances and covariances. Appropriate corrections for sphericity violations and homogeneity of variances and covariances violations were used when necessary, as well as

corrected one-way ANOVAs using Welch's F when the equality of error variance assumption was violated. In cases where the assumption of normality was violated, results were checked using square root and log transformations. Additionally, in cases where data transformations could not correct normality violations, non-parametric tests were conducted to confirm the results of the ANOVA. Exceptions are reported in the results. SPSS (v.28) software was used for all statistical analyses, and the significance value was set at $p < 0.05$. Five subjects from Experiment 1 and three subjects from Experiment 2 were removed from statistical analyses because they did not meet pre-determined learning criteria for acquisition (during Acquisition Session 5, CS responding must be higher than PreCS responding, and higher than CS responding during Acquisition Session 1) or extinction (CS responding during Extinction Session 2 must be lower than CS responding during Acquisition Session 5). Furthermore, two outliers (1 experimental male, 1 control female) from Experiment 1 and four outliers (3 experimental males, 1 control female) from Experiment 2 were identified by box plots and were removed from all statistical analyses.

1.3 RESULTS

1.3.1 Experiment 1: Context Habituation with Food

1.3.1.1 Acquisition and Extinction Training

During acquisition training, male rats in both conditions increased foodcup behavior (conditioned responding; CR) during CS periods (Fig. 1.2). A three-way mixed repeated measures ANOVA of CR for condition (context habituation, control) with training day and PreCS and CS period as repeated measures found

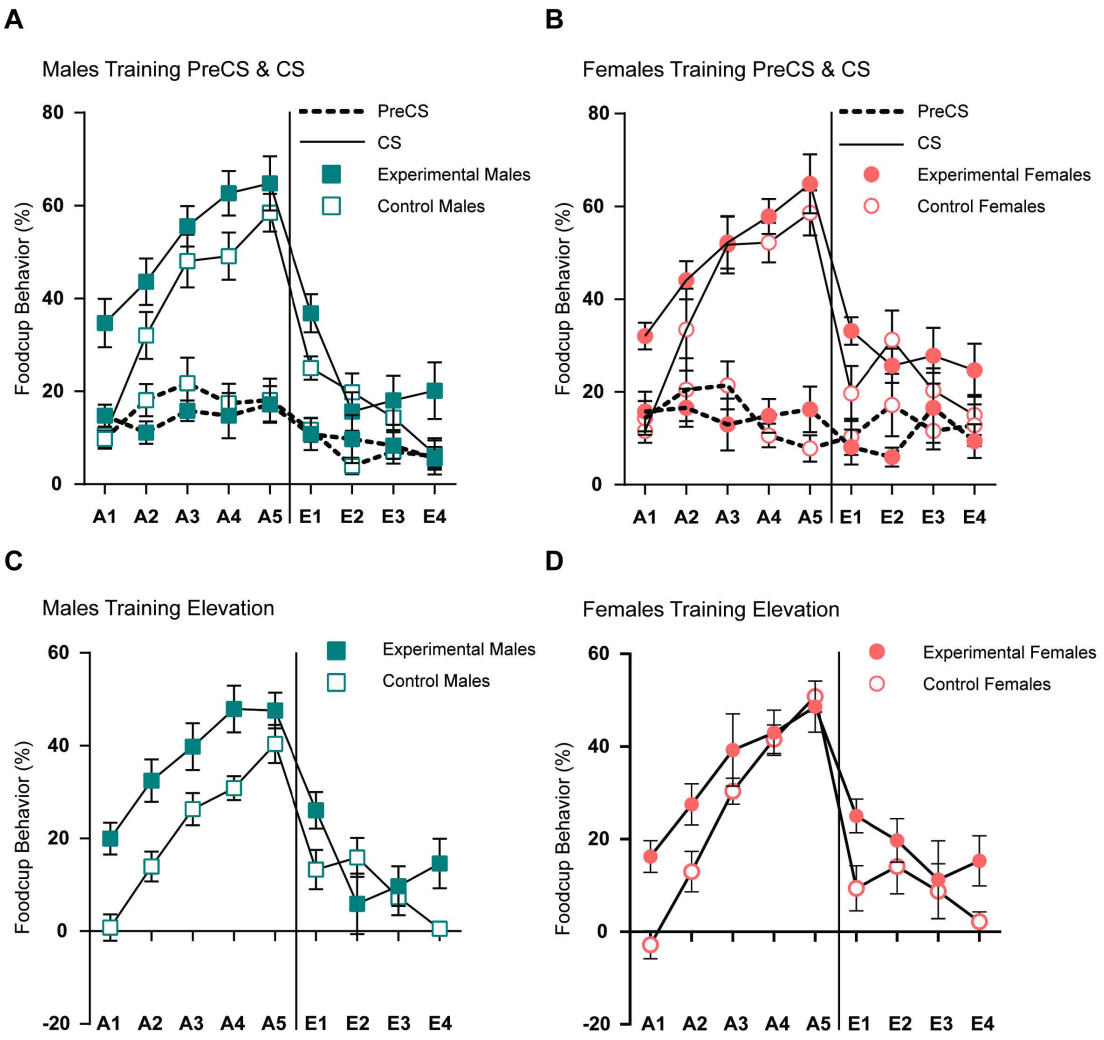

Figure 1.2 Conditioned responding during acquisition and extinction training for Experiment 1: Context Habituation with Food. **(A,B)** Males, and females, respectively, average percent of time (mean ± SEM) spent expressing foodcup behavior during PreCS and CS periods during each session across acquisition and extinction training. **(C,D)** Males, and females, respectively, average percent of time spend expressing foodcup behavior represented as an elevation score (CS responding minus PreCS [baseline] responding) across training sessions.

main effects of training ($F(4,76) = 15.670$, $p < 0.001$) and period ($F(1,19) = 268.346$, $p < 0.001$), but no effect of condition ($F(1,19) = 1.634$, $p = 0.216$). There was a training day by period interaction ($F(4,76) = 36.651$, $p < 0.001$) and a period by condition interaction ($F(1,19) = 16.975$, $p < 0.001$). Follow up simple

effect analyses indicated that CR during CS periods increased over training ($F(4,16) = 21.682$, $p < 0.001$), while CR during PreCS periods remained low ($F(4,16) = 0.798$, $p = 0.544$). Follow up analyses on the period by condition interaction found that male rats in the experimental condition had higher CR during CS periods compared to control rats across acquisition training ($F(1,19) = 5.949$, $p = 0.025$), whereas CR during PreCS was similar between conditions ($F(1,19) = 0.529$, $p = 0.476$). Simple effect analyses also confirmed that both groups had higher overall CR during CS periods compared to PreCS periods for acquisition training (Experimental $F(1,19) = 183.884$, $p < 0.001$; Control $F(1,19) = 87.696$, $p < 0.001$). Similar patterns were observed when elevation of responding (CS minus PreCS; see Methods) was analyzed (Fig. 1.2). Elevation for male rats increased across acquisition training sessions, as confirmed by a two-way mixed repeated measures ANOVA with condition as a between-subjects factor and training day as a within-subjects factor ($F(4,76) = 36.654$, $p < 0.001$). Male experimental rats had higher elevation of responding compared to controls ($F(1,19) = 16.976$, $p < 0.001$).

Across extinction training, males decreased CR (Fig. 1.2; Greenhouse-Geisser $F(2.247,42.686) = 9.440$, $p < 0.001$), but their responding was still overall higher during CS compared to PreCS periods (Pillai's Trace $F(1,19) = 76.885$, $p < 0.001$). There was no main effect of condition for PreCS and CS responding ($F(1,19) = 1.581$, $p = 0.224$). To confirm extinction learning, we carried out planned comparisons for CS responding during the first and last blocks of extinction. Both groups of males sufficiently decreased CR during CS periods

from extinction training block 1 to extinction training block 4 (Experimental $t(8) = 3.038$, $p = 0.016$; Control $t(11) = 4.868$, $p < 0.001$). Similarly, elevation of responding decreased across extinction training blocks (Fig. 1.2; Pillai's Trace $F(3,17) = 5.272$, $p = 0.009$), and there was no main effect of condition ($F(1,19) = 1.807$, $p = 0.195$), indicating both groups decreased responding similarly.

During acquisition training, females also increased responding across sessions (Fig. 1.2). The three-way mixed repeated measures ANOVA of CR indicated main effects of training day ($F(4,72) = 9.163$, $p < 0.001$) and period ($F(1,18) = 206.449$, $p < 0.001$), but no main effect of condition ($F(1,18) = 1.071$, $p = 0.314$). However, there were training day by period ($F(4,72) = 42.578$, $p < 0.001$) and condition by training day by period interactions ($F(4,72) = 2.783$, $p = 0.033$). Simple effect analyses showed that females increased CR during CS periods over training ($F(4,15) = 24.557$, $p < 0.001$), while PreCS responding remained low ($F(4,15) = 0.699$, $p = 0.612$). Follow up analyses also revealed that experimental females had higher CR during CS periods compared to control females, but only during Acquisition 1 ($F(1,18) = 27.941$, $p < 0.001$). Furthermore, experimental females responded more during CS periods compared to PreCS periods on each day of training (all $p < 0.001$), whereas controls responded similarly across periods during Acquisition 1 ($F(1,18) = 0.749$, $p = 0.398$) and had higher CR during CS periods compared to PreCS periods during Acquisition 2 and onward (Acquisition 2 $F(1,18) = 8.707$, $p = 0.009$; Acquisition 3-5 all $p < 0.001$). Similar patterns were observed when elevation of responding was analyzed (Fig. 1.2). Females showed an increase in elevation of

responding across acquisition training sessions ($F(4,72) = 42.572$, $p < 0.001$), and there was a trend toward a main effect of condition ($F(1,18) = 3.803$, $p = 0.067$). There was a significant condition by training day interaction ($F(4,72) = 2.782$, $p = 0.033$), because elevation of responding was higher for experimental than control females during Acquisition 1 ($F(1,18) = 17.215$, $p < 0.001$) and Acquisition 2 ($F(1,18) = 5.465$, $p = 0.031$).

Across extinction training, unlike males, females did not show a main effect of extinction training block (Fig. 1.2; $F(3,54) = 0.490$, $p = 0.691$). However, there was a main effect of period ($F(1,18) = 43.652$, $p < 0.001$), and a period by condition interaction ($F(1,18) = 5.321$, $p = 0.033$), indicating group differences in PreCS and CS responding. Simple effect analyses showed that there were no effects of condition (PreCS $F(1,18) = 0.621$, $p = 0.441$; CS $F(1,18) = 2.412$, $p = 0.138$), but there were effects of period (Experimental $F(1,18) = 39.727$, $p < 0.001$; Control $F(1,18) = 9.246$, $p = 0.007$), suggesting that while both groups showed higher CR during CS periods compared to PreCS periods, the effect was more robust in the experimental group. To confirm the absence of extinction in females, we compared CS responding during the first and last blocks of extinction. Neither experimental nor control females sufficiently decreased CR during CS periods from extinction training block 1 to extinction training block 4 (Experimental $t(9) = 1.232$, $p = 0.249$; Control $t(9) = 0.596$, $p = 0.566$). Similarly, females did not significantly decrease elevation of responding across extinction blocks (Fig. 1.2; $F(3,54) = 1.397$, $p = 0.261$) and females in the experimental condition had higher elevation of responding compared to controls, although this

effect did not remain after a transformation to correct the distribution normality violation (Untransformed $F(1,18) = 5.320$, $p = 0.033$; Transformed $F(1,18) = 1.870$, $p = 0.188$). There were no other main effects or interactions (all $p > 0.05$).

1.3.1.2 Renewal Testing

During renewal testing for males, a three-way mixed repeated measures ANOVA of CR in the acquisition and extinction contexts during tests (Fig. 1.3) found a main a main effect period ($F(1,19) = 95.384$, $p < 0.001$), a context by period interaction ($F(1,19) = 32.027$, $p < 0.001$), and a period by condition interaction ($F(1,19) = 6.461$, $p = 0.020$). There was no effect of condition ($F(1,19) = 0.027$, $p = 0.872$), and the main effect of testing context did not withstand the transformation to correct a normality distribution violation (Untransformed $F(1,19) = 4.927$, $p = 0.039$; Transformed $F(1,19) = 3.250$, $p = 0.076$). Follow up simple effect analyses confirmed that CR was higher during CS compared to PreCS periods in both the acquisition and extinction context (Acquisition context test $F(1,19) = 81.686$, $p < 0.001$; Extinction context test $F(1,19) = 7.552$, $p = 0.013$) and that there were no group differences in overall PreCS ($F(1,19) = 2.248$, $p = 0.150$) nor CS responding ($F(1,19) = 0.619$, $p = 0.441$). Follow up analyses for the period by condition interaction indicated that both conditions had higher CR during CS periods compared to PreCS periods, but the effect was more robust in the experimental group (Experimental $F(1,19) = 66.279$, $p < 0.001$; Control $F(1,19) = 30.447$, $p < 0.001$).

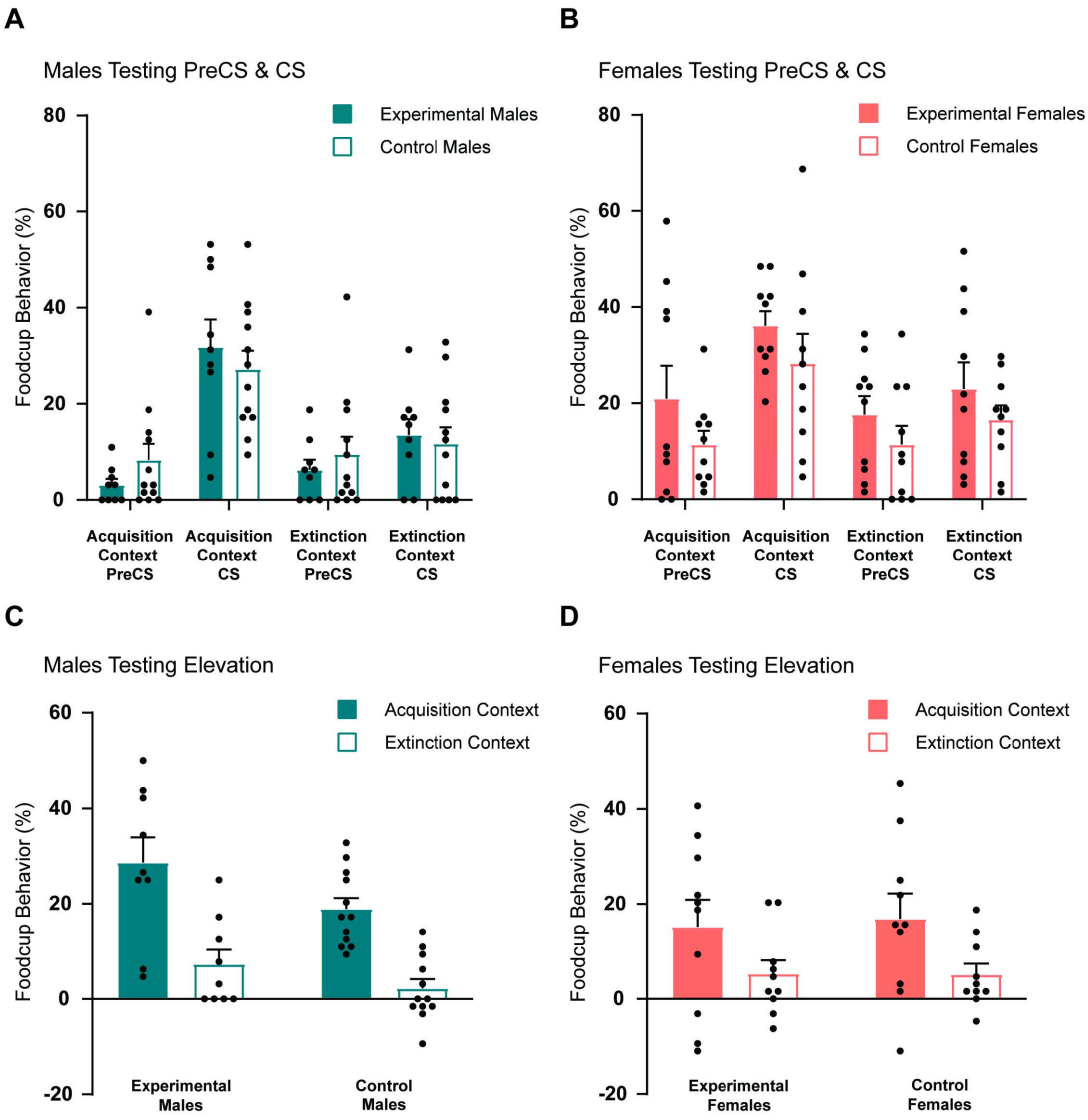

Figure 1.3 Conditioned responding during renewal testing for Experiment 1: Context Habituation with Food. **(A,B)** Males, and females, respectively, average percent of time (mean ± SEM) spent expressing foodcup behavior during PreCS and CS periods during the acquisition context test and extinction context test. **(C,D)** Males, and females, respectively, average percent of time spend expressing foodcup behavior represented as an elevation score (CS responding minus PreCS [baseline] responding) across testing.

Separate analyses for elevation of responding confirmed renewal effects with higher responding during the test in the acquisition compared to extinction contexts (Fig. 1.3; $F(1,19) = 32.023$, $p < 0.001$). Additionally, there was a

significant main effect of condition, as male rats in the experimental condition had overall higher elevation of responding compared to controls ($F(1,19) = 6.461$, $p = 0.020$).

Female rats also responded more during CS compared to PreCS periods during tests as indicated by a main effect of period (Fig. 1.3; $F(1,18) = 20.652$, $p < 0.001$), but there was no main effect of testing context ($F(1,18) = 3.130$, $p = 0.094$) or condition ($F(1,18) = 3.219$, $p = 0.090$). However, there was a significant context by period interaction ($F(1,18) = 7.622$, $p = 0.013$). Follow up simple effect analyses confirmed that females' CR was higher during CS compared to PreCS periods in each context test (Acquisition context test $F(1,18) = 16.796$, $p < 0.001$; Extinction context test $F(1,18) = 8.342$, $p = 0.010$), and higher during CS periods in the acquisition context compared to CS periods in the extinction context ($F(1,18) = 6.821$, $p = 0.018$). PreCS responding in both contexts was similar ($F(1,18) = 0.162$, $p = 0.692$). Separate analyses for elevation of responding indicated that it was higher during the test in the acquisition compared to the extinction context (Fig. 1.3; $F(1,18) = 7.623$, $p = 0.013$) and there was no effect of condition ($F(1,18) = 0.028$, $p = 0.869$).

1.3.2 Experiment 2: Context Habituation without Food

1.3.2.1 Acquisition and Extinction Training

Male rats increased CR across acquisition training (Fig. 1.4; $F(4,72) = 18.221$, $p < 0.001$) and exhibited higher CR during CS periods compared to PreCS periods ($F(1,18) = 334.238$, $p < 0.001$), and there was an acquisition day by period interaction ($F(4,72) = 48.043$, $p < 0.001$). There was no main effect of

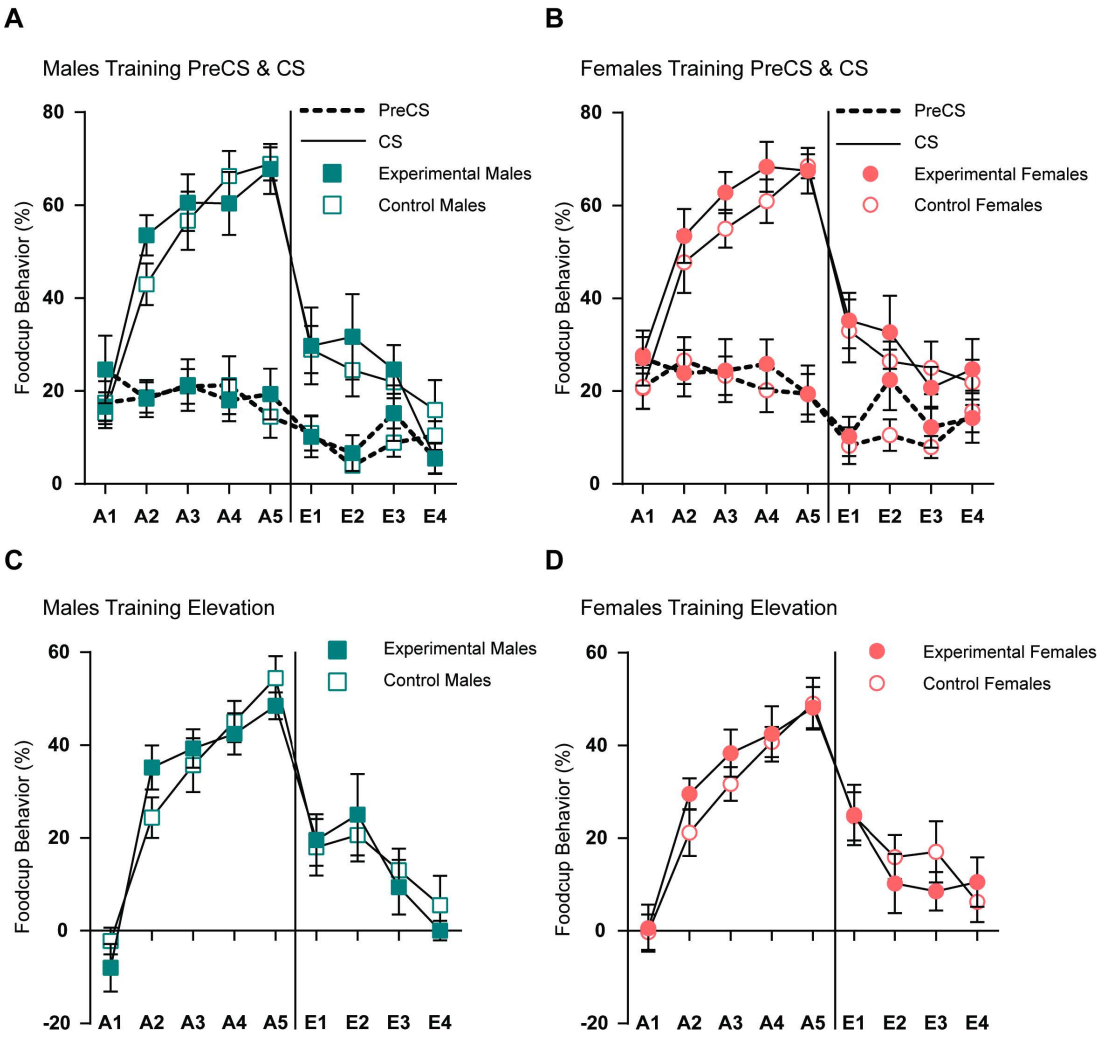

Figure 1.4 Conditioned responding during acquisition and extinction training for Experiment 2: Context Habituation without Food. **(A,B)** Males, and females, respectively, average percent of time (mean ± SEM) spent expressing foodcup behavior during PreCS and CS periods during each session across acquisition and extinction training. **(C,D)** Males, and females, respectively, average percent of time spend expressing foodcup behavior represented as an elevation score (CS responding minus PreCS [baseline] responding) across training sessions.

condition for PreCS and CS responding ($F(1,18) = 0.132$, $p = 0.721$). Follow up analyses confirmed that CR during CS periods increased across training ($F(4,15) = 81.274$, $p < 0.001$), but remained low during PreCS periods ($F(4,15) = 0.355$, $p = 0.836$). Separate analyses for elevation of responding showed that males

increased responding throughout acquisition training (Fig. 1.4; $F(4,72) = 48.050$, $p < 0.001$) and there was no effect of condition ($F(1,18) = 0.000$, $p = 0.997$).

During extinction, males decreased overall CR across training blocks (Fig. 1.4; $F(3,54) = 3.475$, $p = 0.022$), but continued to respond more during CS compared to PreCS periods ($F(1,18) = 23.499$, $p < 0.001$). There was also an interaction for training block by period ($F(3,54) = 5.636$, $p = 0.002$). Follow up analyses found that CR during CS periods was higher compared to PreCS periods for extinction training blocks 1, 2, and 3 (Extinction Block 1 $F(1,18) = 18.194$, $p < 0.001$; Extinction Block 2 $F(1,18) = 20.959$, $p < 0.001$; Extinction Block 3 $F(1,18) = 8.950$, $p = 0.008$), but CR during extinction block 4 was similar for PreCS and CS periods ($F(1,18) = 0.461$, $p = 0.506$). Furthermore, simple effect analyses also showed that overall PreCS responding across extinction training remained low ($F(3,16) = 1.610$, $p = 0.226$), while CS responding trended toward a decrease ($F(3,16) = 2.887$, $p = 0.068$). Experimental males sufficiently decreased CR during CS periods from extinction training block 1 to extinction training block 4 ($t(7) = 3.340$, $p = 0.012$), but controls did not ($t(11) = 1.489$, $p = 0.165$). Separate analyses for elevation also showed that males decreased responding across extinction training (Fig. 1.4; $F(3,54) = 5.637$, $p = 0.002$) and there was no effect of condition ($F(1,18) = 0.037$, $p = 0.849$).

During acquisition training, female rats increased CR across sessions (Fig. 1.4; Greenhouse-Geisser $F(2.883,71.783) = 12.748$, $p < 0.001$), and had higher CR during CS periods compared to PreCS periods ($F(1,20) = 259.877$, $p < 0.001$), but there was no effect of condition ($F(1,20) = 0.597$, $p = 0.449$). There

was also an acquisition day by period interaction ($F(4,80) = 39.204$, $p < 0.001$), with follow up analyses indicating CR increased during CS periods across training ($F(4,17) = 36.455$, $p < 0.001$), but remained low during PreCS periods ($F(4,17) = 0.620$, $p = 0.655$). Similarly, female rats increased elevation of responding throughout acquisition training (Fig. 1.4; $F(4,80) = 39.199$, $p < 0.001$) and there was no effect of condition ($F(1,20) = 0.322$, $p = 0.577$).

During extinction training, females continued to respond more during CS periods compared to PreCS periods (Fig. 1.4; $F(1,20) = 48.268$, $p < 0.001$) and there was no effect of training block ($F(3,60) = 1.539$, $p = 0.214$) or condition ($F(1,20) = 0.372$, $p = 0.549$). There was an interaction for extinction training block by period ($F(3,60) = 3.501$, $p = 0.021$) and follow up analyses showed that CR during CS periods was higher compared to PreCS periods for each of the four extinction blocks, although the effect was less robust by the end of extinction training (Extinction block 1 $F(1,20) = 35.291$, $p < 0.001$; Extinction block 2 $F(1,20) = 10.614$, $p = 0.004$; Extinction block 3 $F(1,20) = 10.751$, $p = 0.004$; Extinction block 4 $F(1,20) = 5.877$, $p = 0.025$. Elevation decreased across extinction training, but this effect did not withstand the transformation to correct a distribution normality violation (Fig. 1.4; Untransformed $F(3,60) = 3.502$, $p = 0.021$; Transformed $F(3,60) = 2.278$, $p = 0.089$) and there was no effect of condition ($F(1,19) = 1.581$, $p = 0.224$).

1.3.2.2 Renewal Testing

Male rats showed renewal of responding during the acquisition context test, as indicated by a main effect of testing context (Fig. 1.5; $F(1,18) = 29.994$,

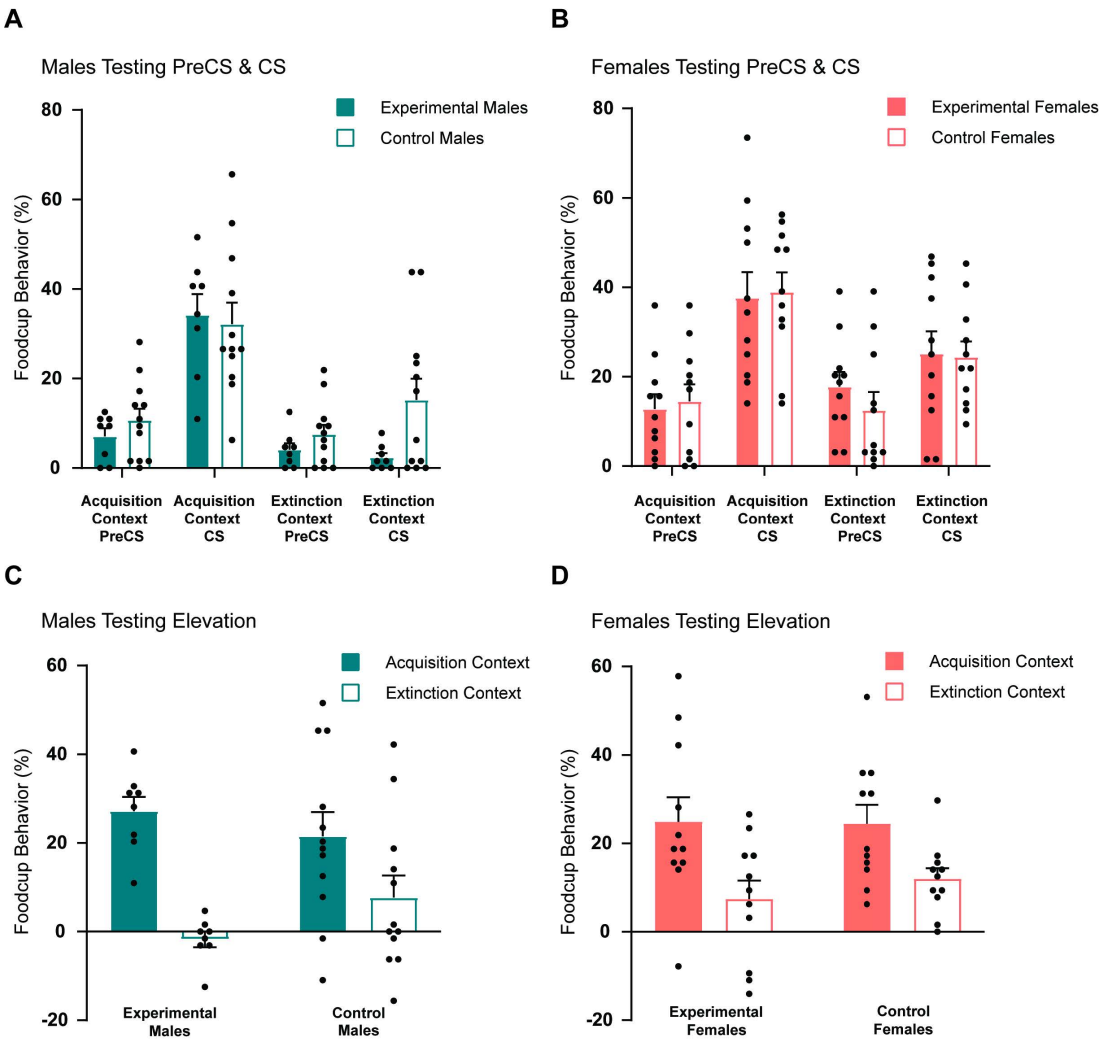

Figure 1.5 Conditioned responding during renewal testing for Experiment 2: Context Habituation without Food. **(A,B)** Males, and females, respectively, average percent of time (mean ± SEM) spent expressing foodcup behavior during PreCS and CS periods during the acquisition context test and extinction context test. **(C,D)** Males, and females, respectively, average percent of time spend expressing foodcup behavior represented as an elevation score (CS responding minus PreCS [baseline] responding) across testing.

$p < 0.001$), a main effect of period ($F(1,18) = 29.477$, $p < 0.001$), and a context by period interaction ($F(1,18) = 21.781$, $p < 0.001$). There was no effect of condition on renewal testing ($F(1,18) = 2.438$, $p = 0.136$), nor were there any interaction effects (all $p > 0.05$). Follow up simple effect analyses indicated that males

showed more CR to the CS during acquisition context test compared to extinction context test ($F(1,18) = 28.607$, $p < 0.001$), whereas PreCS period CR was similar across both tests ($F(1,18) = 4.146$, $p = 0.057$). Furthermore, males had selectively higher CR during the CS in the acquisition context test (Acquisition $F(1,18) = 44.889$, $p < 0.001$; Extinction $F(1,18) = 0.884$, $p = 0.360$). Similarly, the separate analyses for elevation of responding showed that males responded more during the test in the acquisition compared to the extinction context (Fig. 1.5; $F(1,18) = 21.781$, $p < 0.001$), and there were no condition or interaction effects (both $p > 0.05$).

For females, there were main effects of testing context (Fig. 1.5; $F(1,20) = 5.624$, $p = 0.028$) and period ($F(1,20) = 61.266$, $p < 0.001$), and a test context by period interaction ($F(1,20) = 13.125$, $p = 0.002$). Simple effect analyses showed that females had higher CR during CS periods when tested in the acquisition context compared to the extinction context ($F(1,20) = 20.029$, $p < 0.001$), whereas PreCS period CR was similar across both tests ($F(1,20) = 0.181$, $p = 0.675$). Females exhibited higher CR during CS compared to PreCS periods during both tests (Acquisition context test $F(1,20) = 49.493$, $p < 0.001$; Extinction context test $F(1,20) = 15.848$, $p < 0.001$). The analyses for elevation indicated that females responded more during the test in the acquisition compared to extinction context (Fig. 1.5; Females $F(1,20) = 13.127$, $p = 0.002$), but there was no effect of condition ($F(1,20) = 0.221$, $p = 0.643$) or any other main effects or interactions (all $p > 0.05$).

1.4 DISCUSSION

The present study examined whether habituation to the training contexts, with or without food (US) presentations, prior to learning impacts context-mediated renewal of responding to food cues (CS) in male and female rats. We found sex differences in renewal of responding and, interestingly, even more robust differences in extinction training. During extinction, when the CS was no longer followed by food, males successfully decreased responding to the CS by the end of training, but females did not. Context habituation with food presentations improved tone-food acquisition in both sexes compared to controls that were habituated to food in their home cages, although more strongly in males. Moreover, for males, context habituation with food presentations further strengthened the renewal effect. Neither context alone nor context habituation with food helped females unambiguously show renewal, as female groups in both experiments had impairments during extinction.

We sought to determine if habituation to context could help absolve the irregularity of renewal of responding patterns previously observed in females with food (Anderson & Petrovich, 2015, 2017) or alcohol as reward (Segal, Valyear, & Chaudhri, 2021). The results of the current study, together with previous findings from our lab, suggest that females cannot disambiguate the context-specific meaning of the cue, and that this is the reason for extinction learning impairments. In our prior work, control groups of females were able to acquire and extinguish tone-food responding when both training phases occurred in the same context (Anderson & Petrovich, 2015, 2017). Bouton (1993) suggests that

during acquisition training, context information about the CS-US relationship is not necessarily initially attended to, but only becomes relevant when the CS no longer reliably predicts the US delivery, as in extinction training. It is then that contexts are recalled in order to disentangle the cue's meaning. Thus, it is reasonable to expect that context familiarity would facilitate learning about the meaning of discrete cues. For example, as shown in the conditioned taste aversion paradigm, previous experience with behavioral contexts impacts conditioning and memory recall (León, Callejas-Aguilera, & Rosas, 2012). Context could be a direct mediator of the CS-US relationship, or provide information about the meaning of the CS, or the likelihood of delivery of the US (León, Callejas-Aguilera, & Rosas 2012). Research on the role of contextual learning and the effect of context pre-exposure is much more extensive in fear conditioning literature compared to appetitive and reward studies. Despite there being many differences between appetitive and aversive paradigms, it is not unreasonable to expect that there are still overlapping mechanisms and, in instances where we seek to elucidate mechanisms of appetitive conditioning, we may turn to insights from the fear literature. Contextual fear learning research on the immediate shock deficit has shown that context familiarity helps associative learning. When shock is given immediately upon placement, rats do not learn the association between the shock and contextual stimuli, but permitting the rat to explore the context before conditioning attenuates the immediate shock deficit (Fanselow 1986 & 1990). The reason for this effect is that, unlike a discrete cue, the context is a composite of many stimuli presented together that require time to

explore and unify; after this, the context can then be jointly encoded in an associative representation with the CS (Fanselow 1986 & 2010; Rudy & O'Reilly, 2001). With these studies in mind, we sought to explore if more substantial experience with context before training would improve context-mediated renewal, potentially through stronger context-mediated cue-outcome representations.

In the present study, we expected that habituation to each context before training would increase renewal of responding at testing, because it would strengthen both context-specific memories, tone-food memory in the acquisition context, and tone-no food memory in the extinction context. We also predicted that this habituation would rescue the absence of the renewal effect that previous work in our lab observed in females (Anderson & Petrovich, 2015, 2017). Lastly, we anticipated that delivery of the US during habituation in each context would enhance tone-food memory associations, although we suspected it may interfere with extinction. Because context influences memory retrieval, we hypothesized that increasing the familiarity of the context would improve context-dependent memory retrieval during renewal. Since context has such strong associative strength, it can act as a robust memory retrieval cue (Bouton, 1993). Meaning, context can control whether the cue-food or cue-no food memory is retrieved. Supported by the contextual fear learning literature, we conjectured that habituation to the two different training contexts would promote familiarity of each context and would provide an enhanced memory basis on which to incorporate the new memory representations for cue-food and cue-no food.

In the current study, we examined potential sex differences during the acquisition and extinction cue-food learning and memory and context mediated renewal. We found sex differences during extinction training, and we confirmed prior findings that males and females acquire tone-food associations similarly. Furthermore, experimental groups of both sexes that had context habituation with food learned faster, although the effect was more robust in males. Because this was an overall responding and across training effect for males, it suggests that habituation with food enhanced the tone-food association for males throughout the course of acquiring the tone-food memory. Females groups only differed during the first acquisition session, when the experimental group already had high CS compared to PreCS responding. Therefore, while it did not necessarily enhance the overall strength of the tone-food memory by the end of training, this suggests that context familiarity and the expectation of food initially increased the rate of acquiring the tone-food association.

All males in both experiments extinguished responding to the tone by the end of extinction training. Across both experiments, males showed successful extinction when overall responding was evaluated, which was confirmed, for all groups with exception of the control condition in Experiment 2, when analyzing the CS-specific decrease in responding from the first block to the last block of extinction training. Of particular importance, despite having experienced food in their extinction context during habituation, males still showed successful extinction learning. In contrast, all females in both experiments, regardless of the condition, had impaired extinction. Female rats did not show a consistent

decrease in conditioned responding across extinction training. This highlights a key sex difference, suggesting behavioral rigidity in females in terms of food-seeking responses.

For females in Experiment 1, where rats were habituated to context with food (US) presentations, neither elevation nor overall conditioned responding decreased across extinction training. Females in Experiment 1 who had US presentations during habituation to their extinction context had higher elevation of responding across extinction training compared to the homecage control group. This is perhaps unsurprising because the extinction learning would need to be extremely salient in order to overcome the memory of having food in that context prior to extinction training. In Experiment 2, where rats were habituated to context alone, females did demonstrate reduced elevation of responding across extinction training, but their overall conditioned responding (during PreCS and CS) did not sufficiently decrease, and there was no difference between the experimental and control group. This suggests that while females are encoding the discrete cue's meaning, they are not incorporating it with the context-based implications of the CS outcome. Taken together, the inconsistencies of females' behavior during extinction impact the interpretation of renewal behavior upon testing in the current study.

As very few studies compare males and females in appetitive learning, extinction, and renewal, our goal was to elucidate if this is due to weaker tone-food association, weaker context association, or weaker extinction recall. For males in Experiment 1, habituation to both contexts with food presentations

enhanced overall CS-specific responding, regardless of context, during both acquisition training and testing phases. That suggests that, during habituation, context-US associations were formed in both contexts for rats in the experimental condition compared to control. Furthermore, when rats were habituated to context with food, it created ambiguity in terms of determining the discrete cue's outcome since each (acquisition and extinction) context was initially paired with food.

In Experiment 2, all males showed robust renewal as seen by higher responding during the acquisition context compared to the extinction context test. All males in Experiment 2 demonstrated similarly high CS-specific responding during the acquisition context test, coupled with lower CS responding during the extinction context test and low PreCS responding during both tests. This exemplifies context-mediated renewal behavior because, not only are they demonstrating that they only expect food delivery to occur in conjunction with the discrete cue, but they also show that they understand the context-dependent meaning of the cue, in that food presentation would only follow the cue in the acquisition context.

Females across both experiments performed similarly during testing. All females had higher CS period responding compared to PreCS responding in both testing contexts, and higher CS responding during the acquisition context test compared to the extinction context test. Although this is a demonstration of cue-specific responding, it is difficult to claim that that was evidence of renewal of

responding, because females did not adequately extinguish conditioned responding by the end of extinction training.

The current study examined intact adult females and, of note, their circulating hormones may have influenced their conditioned responding, particularly during extinction. Previously, Anderson & Petrovich 2018 did not find differences between high and low estrogen groups during renewal testing and had hypothesized that estrogen levels were likely important during extinction. Indeed, estrogen was later shown to impact context-specific extinction memory during renewal (Hilz et al., 2019). Similarly, estrogen and progesterone have also been shown to facilitate extinction recall in fear learning (Lebron-Milad & Milad, 2012; Milad, Igoe, Lebron-Milad, & Novales, 2009). It is possible that circulating hormones could have impacted females' context-dependent encoding of the tone-food association and subsequent memory.

The results of the current study provide evidence for females' resistance to extinguishing food-seeking behaviors, and may suggest behavioral rigidity when it comes to appetitive drive. Future work should explore sex differences in the neural mechanisms of context-mediated extinction and renewal to food cues, as well as explore external conditions and internal factors such as circulating hormones, that may enhance females' extinction and recall during appetitive motivated learning and memory.

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2.0 CHAPTER 2: Investigation of the medial prefrontal cortex neuronal ensembles plasticity during context mediated renewal of responding to food cues

2.1 INTRODUCTION

Research to elucidate the neural substrates underlying the expression of food-seeking behaviors remains ongoing. Renewal of responding following extinction of learned cue-food associations can help us understand the neurobiological basis of eating independently of hunger and persistently seeking palatable foods (for reviews see Petrovich, 2013; Calu, Chen, Kawa, Nair, & Shaham, 2013). Appetitive conditioning, extinction learning, and the renewal of responding to food cues are complexly encoded (Bossert et al., 2012; Cole, Powell, & Petrovich, 2013). Cole, Powell, and Petrovich's 2013 study unveiled that distinct amygdalar nuclei were recruited differently across various training time points of appetitive conditioning, suggesting that this appetitive association and the information about this task was likely encoded across wider neural circuitry as training endured. One of the areas implicated in these processes was the medial prefrontal cortex (mPFC) and, more specifically, the prelimbic (PL) and infralimbic (ILA) subregions. This highlights the importance of deeper investigation into the neuronal ensembles mediating encoding, memory recall, and behavioral expression of these appetitive associations and, furthermore, determining if the same or different neural sites of this distributed network are crucial during different stages. Therefore, the current study sought to investigate

neuronal ensembles in the PL and ILA and their role in context-mediated renewal of responding to food cues after extinction.

It is well established that the mPFC is implicated in appetitive associations, both for cue-food learning and for contextual appetitive associations (Eddy, Todd, Bouton, & Green, 2016; Petrovich, Ross, Holland, & Gallagher, 2007; Willcocks & McNally 2013). Prior work demonstrated the critical involvement of a neuronal ensemble within the mPFC in cue-potentiated feeding, highlighting the importance of the mPFC and its plasticity during cue-food learning and expression of cue-induced feeding behaviors (Cole, Keefer, Anderson, & Petrovich, 2020). Furthermore, there is overlapping evidence that context-activated neuronal ensembles within mPFC subregions contribute to drug seeking (Bossert et al., 2012) and evidence showing mPFC μ-opioid receptor stimulation can induce dysregulated food seeking behaviors (Mena, Sadeghian, & Baldo, 2011; Mena, Selleck, & Baldo, 2013). There is substantial support indicating that the PL and ILA subregions of the mPFC are functionally distinct in aversive associative learning and may support differential encoding of contextual food cues (Rhodes & Killcross, 2007), but there is also conflicting evidence that they are similarly recruited (Cole, Hobin, & Petrovich, 2015; Kaminska, Caballero, & Moorman, 2021; Moorman, James, McGlinchey, & Aston-Jones, 2015). For example, selective inactivation of the PL or ILA in alcohol-seeking during context-induced reinstatement produced differing effects. Inactivation of PL weakened reinstatement whereas inactivation of ILA did not show these effects, but instead induced increased latency to respond in the

extinction context (Willcocks & McNally, 2013). Due to these complexities, we aimed to investigate these subregions separately in context-mediated appetitive learning. This was accomplished with a chemogenetic method, utilizing Daun02 to selectively inactivate neuronal ensembles in transgenic *Fos-lacZ* rats (for reviews see Cruz et al., 2013; Bashir & Banks, 2017). In these *Fos-lacZ* rats, the enzyme β-galactosidase (β-gal) is produced selectively in *Fos*-expressing neurons. Once Daun02 is infused, β-gal catalyzes the inactive prodrug Daun02 into its active form, daunorubicin, which is then responsible for inactivating only those cells which had been strongly active, and thus had produced Fos, during our behavioral task. Therefore, this method allows selective targeting of recently activated neuronal ensembles and permits manipulations at a specific time point within the behavioral protocol to determine if plasticity of the PL and ILA are critical for the expression of food-seeking behavior in our paradigm.

The behavioral protocol used in this study was a context-mediated renewal of Pavlovian conditioned responding to a food cue after extinction. This ABA renewal paradigm consisted of three phases – acquisition of the tone-food (conditioned stimulus-unconditioned stimulus; CS-US) association, extinction of this learned association, and renewal of responding testing. The acquisition and extinction training occurred in distinct contexts that differ in olfactory, visual, and tactile features. Renewal of responding was tested by return to the acquisition context, where the extinguished food seeking response reappears upon presentation of the tone cue. In these experiments, we inactivated either PL or ILA neuronal ensembles that were active during the acquisition phase of our ABA

renewal protocol with the goal to pinpoint the causal neuronal ensemble for context-specific cue-food memory. Based on the existing literature and previous work from our lab, we predicted that the PL was a critical site of neuronal ensemble plasticity across cue-food learning and renewal (Eddy, Todd, Bouton, & Green, 2016; Willcocks & McNally 2013). We hypothesized that Daun02 inactivation of the PL would impair recall of the cue-food memory upon testing in the acquisition context, which would be evidenced by a lack of renewal of conditioned responding. We also performed the same procedures targeting the ILA, for which we predicted that Daun02 inactivation of the ILA would not impair the cue-food memory upon testing in the initial acquisition context, and that, therefore, we would see no impact on the renewal of conditioned responding. This prediction was based on prior evidence that the ILA is critical for extinction recall and our expectation that distinct neuronal ensembles mediate cue-food acquisition as opposed to extinction learning (Rhodes & Killcross, 2007). Together, these findings could contribute to our better understanding of the neural mechanisms underlying context-induced food seeking and help us illuminate what contributes to maladaptive eating habits, eating disorders, and drug relapse.

2.2 Materials and Methods

2.2.1 Subjects

The subjects of these experiments were 61 experimentally naïve adult male *Fos-lacZ* Sprague-Dawley rats. These rats were bed from our transgenic *Fos-lacZ* colony and were identified as transgenic via genotyping. Rats were

group housed until 6 days prior to surgical procedures, at which time they were individually housed and handled by the experimenter for 3 days leading up to surgical cannula implantation with an additional 7 days of handling prior to behavioral testing. All rats were between 12 and 14 weeks of age at the time of surgical manipulations, with the exception of 13 rats that were 17 weeks old due to shutdown procedures during the Covid-19 pandemic. Prior to surgical cannula implantation and any behavioral testing, rats had *ad libitum* access to water and standard rat chow (Prolab RMH 3000 5P00; LabDiet, St. Louis, MO) and were maintained on a 12hr light/dark cycle (lights on at 07:00). All housing and testing procedures were compliant with the National Institutes of Health Guidelines for Care and Use of Laboratory Animals and approved by the Boston College Institutional Animal Care and Use Committee.

2.2.2 Surgical Procedure

For surgical implantation of intracranial cannula, rats were anesthetized using isoflurane inhalant (Aerrane®, up to 5% in O2 during induction then 1-3% maintenance; Baxter, Deerfield, IL) and given a pre-operative subdermal line block local anesthetic (LidoJect Lidocaine 2%, 10mg/kg; Sparhawk Laboratories, Lenexa, KS). Rats received a subcutaneous injection of anesthetic, carprofen (Rimadyl by Pfizer, 50mg/mL, Covetrus; Portland, ME) in a sterile saline solution (4.4mg/kg), and were bilaterally implanted with 23-gauge guide cannula (PlasticsOne Inc.; Roanoke, VA) targeting the PL or ILA for Experiment 1 or Experiment 2, respectively. Coordinates were measured from flat-skull bregma and cannula were implanted at a 10-degree angle, using skull screws and dental

cement (Jet Brand, Lang Dental; Wheeling, IL) to anchor them to the skull. The coordinates for PL were +3.0mm anteroposterior (AP), ±1.3mm mediolateral (ML), and -3.4mm dorsoventral (DV) and the coordinates for ILA were +2.5-2.7mm AP, ±1.5mm ML, and -4.2-4.4mm DV according to the rat brain atlas of L. W. Swanson (2004). Immediately post-operatively, rats received subcutaneous injections of prophylactic antibiotic (PenJect, 22,000IU/kg 0.07mL/kg, Covetrus; Portland, ME) and warmed Lactated Ringers solution (5mL/hour of surgery, Henry Schein; Waltham, MA), and triple antibiotic ointment was applied around the cannula cap. Rats were given ingestible Rimadyl (Rodent's MD's™, 2mg/1 chewable tablet/100g body weight; Bio-Serv; Flemington, NJ) post-operatively and for two days after surgery, as well as an additional dose of antibiotic 24hrs after surgery. Application of triple antibiotic ointment continued daily as needed and rats were monitored and weighed daily for at least 4 days. Aside from during intracranial infusions, the guide cannulae were protected by obturators throughout the remainder of the experiment.

2.2.3 Intracranial Infusions

Rats were lightly anesthetized 90mins following the start of the final acquisition training session, obturators were removed, and 30-gauge injector cannula (PlasticsOne Inc.) were inserted into the guide cannula. These injector cannulae extended 1mm beyond the end of the guide cannula, and were connected via polyethylene tubing to 10µL Hamilton syringes (Hamilton Company; Reno, NV) mounted onto an infusion pump (Pump '11' Plus; Cambridge, MA). Bilateral infusions of either Daun02 or a vehicle solution

occurred over 1min with a volume of 0.5µl/side. The vehicle solution consisted of 89% phosphate buffered saline, 6% TWEEN®-80 (Sigma-Aldrich; Saint Louis, MO), and 5% dimethyl sulfoxide (DMSO, Sigma-Aldrich). The Daun02 solution was the same as the vehicle but with the Daun02 (MedChemExpress; Monmouth Junction, NJ) first dissolved in the 5% DMSO (2 µg/0.5µL Daun02 in DMSO). Injectors were left in place for an additional 1min after the infusion to allow for diffusion before being removed, obturators were reinserted, and the rats were returned to their homecage.

2.2.4 Apparatus

Behavioral training was conducted in the same set of identical behavioral chambers (30 x 28 x 30cm; Coulbourn Instruments, Allentown, PA) located in a room separate from the colony housing room. These chambers were composed of a clear Plexiglas rear wall and front hinged door, a floor of stainless-steel rods 5mm thick spaced 15mm apart, and aluminum top and sides, with one side containing a house light (4W) and a recessed foodcup (3.2 x 4.2cm, 5.7cm off the floor). Each chamber was located within a sound- and light- attenuating cubicle (79 x 53 x 53cm) composed of monolithic rigid foam walls, equipped with a ventilation fan (55dB) and a video camera attached to a recording system (Coulbourn Instruments). The visual, tactile, and olfactory features of the behavioral chambers were modified to create two distinct contexts, which were counterbalanced across conditioning and extinction. For one context, a black Plexiglas panel was laid across the grate floor so the rats could not see or feel the grate floor, and the doors to the cubicle were closed. For the other context,

the black Plexiglas panel was inserted up against the left wall of the chamber, the rats were able to see and touch the grate floor, the doors to the cubicle remained open, and a 1% acetic acid (Fisher Scientific; Fair Lawn, NJ) solution was sprayed onto the tray below the grate floor. The conditioned stimulus (CS) was a 10 s tone (75dB, 2kHz) and the unconditioned stimulus (US) was two food pellets (formula 5TUL, 45mg; Test Diets, Richmond, IN) delivered to the foodcup. A computer in an adjacent room controlled the stimuli and video cameras (GraphicState 3.0; Coulbourn Instruments). The apparatus and behavioral procedures were the same as in Chapter 1.

2.2.5 Behavioral Training Procedure

Figure 2.1 outlines the experimental timeline. Rat were given 8-12 days to recover from intracranial implantation surgery before food restriction began. Across 6 days leading up to the start of behavioral training, rats were food restricted to gradually reach 85% of their *ad libitum* body weight and were maintained at this weight for the remainder of the experiment. All training and testing occurred between 07:30 and 14:30. To familiarize rats with the cart transportation and the food pellets (US), the day before training began, rats were transported from the colony room to the behavioral room where they remained for 10mins and received 1g of the US in their homecage. Behavioral training consisted of three phases: conditioning (acquisition training) of the tone-food association, extinction training, and renewal testing. Acquisition and extinction training occurred in two different contexts (the physical contexts used were

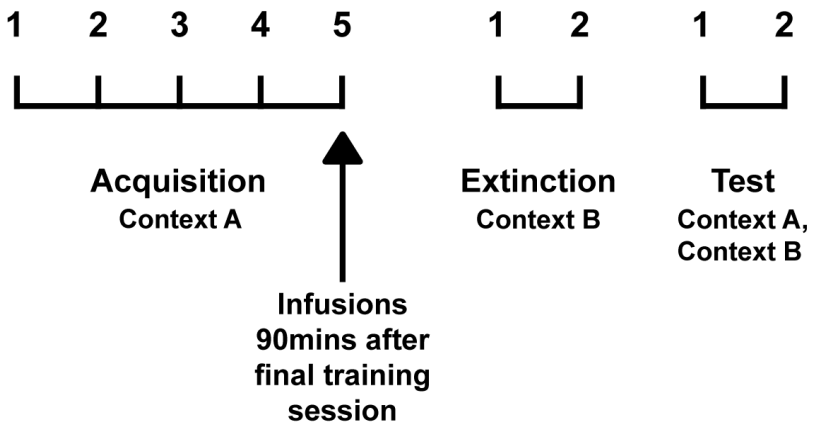

Figure 2.1 Timeline for experiments. Behavioral training experiment timeline for both Experiments 1 and 2 consisted of three phases: conditioning (acquisition) training, extinction training, and testing. Infusions of Daun02 or Vehicle were in PL (Experiment 1) or ILA (Experiment 2).

counterbalanced, see above). During the acquisition phase, all rats were trained for 6 days with one 34min training session per day. In each session, rats received 8 CS-US pairings, in which presentations of the CS were immediately followed by delivery of the US to the foodcup. These CS-US pairings occurred on a 2-6min variable inter-trial interval schedule. The final acquisition training session also served as the induction session for Daun02 infusions. The intracranial infusions occurred 90mins after the start of the final acquisition session in order to induce Fos, and therefore β-gal, in the neuronal ensembles activated during this session. Rats received bilateral infusions of either Daun02 or vehicle as described above. Rats then had two days of extinction training, one session per day, in a context different from their acquisition training context. The extinction training sessions were 34mins long consisting of 8 CS presentations with no US delivery. Testing was conducted over two days with one session per day, in the acquisition context and in the extinction context (order

counterbalanced). These test sessions were 34mins long consisting of 8 CS presentations with no US delivery. All behavioral sessions were recorded and saved for behavioral analysis.

2.2.6 Behavioral Observations

Behavior was analyzed from video recordings for all sessions by trained observers, blind to the experimental condition of the rats, as in the studies in Chapter 1. The primary measure of conditioning (conditioned responding, CR) was expression of “foodcup behavior,” defined as nosepokes into the recessed foodcup, or standing directly in front of and facing the foodcup. Behavioral observations were recorded every 1.25s during the 10s CS periods, as well as during the 10s before the onset of the CS (PreCS). At each observation, only one behavior was recorded: foodcup or other. The number of foodcup observations were summed and converted to a percentage of the total time during each PreCS or CS period that the animal spent expressing foodcup behavior.

2.2.7 Histological Procedures and Immunohistochemistry

Brain tissue was collected 90mins after the start of the second testing session in order to analyze Fos and β-gal expression as well as cannula placement. A lethal dose of Fatal-Plus (0.1mL/100g body weight, Vortech Pharmaceuticals; Dearborn, MI) was administered intraperitoneally and rats were perfused transcardially with 0.9% physiological saline followed by ice cold 4% paraformaldehyde (Sigma-Aldrich) in a 0.1M borate buffer. Brains were removed and post-fixed in a 12% sucrose paraformaldehyde solution at 4°C overnight (18-

24hrs), then blocked and rapidly frozen in hexanes (Thermo Fisher Scientific; Waltham, MA) cooled over dry ice, then stored at -80°C.

Using a sliding microtome (Leica Biosystems; Deer Park, IL), frozen brains were sliced into four serially adjacent series of 30µm coronal sections containing the cannula placements. One series of tissue was mounted onto gelatin-coated slides, dried at 45°C, dehydrated through graded alcohols, stained with thionin (Fisher Scientific), cleared in xylenes, and coverslipped with DPX Mountant (Electron Microscopy Sciences; Hatfield, PA). This series was used to identify neuroanatomical borders and cannula placements by use of a light microscope and a rat brain atlas (Swanson, 2004).

A second series was processed immunohistochemically to analyze Fos and β-gal induction and to verify the Daun02 inactivation method. This series was incubated for 72hrs at 4°C with an anti-*c-fos* primary antibody raised in rabbit (1:10,000; 226 003, Synaptic Systems; Goettingen, Germany) and an anti-β-gal primary antibody raised in mouse (1:2,000; sc-65670, Santa Cruz Biotechnology Inc.; Dallas, TX) in a potassium phosphate buffered saline (KPBS) solution also containing 2% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories Inc.; West Grove, PA), and 0.3% Triton® X-100 (Sigma-Aldrich). Tissue was rinsed in KPBS before incubation in a secondary fluorescent antibody solution containing Alexa Fluor™ 488 anti-rabbit raised in donkey (1:200; A21206, Invitrogen Thermo Fisher Scientific, Waltham, MA) and Alexa Fluor™ 594 anti-mouse raised in donkey (1:200; A21203, Invitrogen Thermo Fisher Scientific) in KPBS, NDS, and Triton X-100 for 1 hr in semi-darkness. Tissue was

rinsed in KPBS again then mounted in semi-darkness onto Superfrost™ slides (Fisher Scientific), allowed to air-dry, and coverslipped with Vectashield® Hard+Set™ mounting medium with DAPI (Vector Laboratories, Inc.; Burlingame, CA). Slides were stored at 4°C until imaged.

2.2.8 Image Acquisition and Analysis

The thionin stained tissue was used identify neuroanatomical borders and evaluate cannula placements. To account for infusion dispersion, the optimal cannula placement location was either just above the region of interest, or within the top half of the region. Both of the bilateral cannulae implants needed to be successfully placed, and remnants of a successful infusion deposit needed to be visually verified. Subjects with cannula placements that did not fulfill these criteria were excluded from the analyses. Exclusions are listed in section 2.9.

In order to verify colocalization of β-gal and Fos expression, images of the PL and ILA in the area of the cannula placements were acquired using a Zeiss Axio Image Z2 fluorescence microscope (Carl Zeiss Microscopy GmbH; Jena, Germany) equipped with a Hamamatsu ORCA-R2 camera (Bridgewater, NJ) using Zen software. Images were pseudo-colored with green for Fos, red for β-gal, and blue for DAPI. These images were processed using ImageJ in order to quantify single Fos-labeled and single β-gal-labeled neurons, as well as double-labeled (Fos + β-gal) neurons. The counts for each were then summed for each rat to calculate the total number of Fos, β-gal, and double-labeled labeled neurons. The percentage of β-gal-labeled cells that were also Fos-labeled was

determined. Mean counts for Fos-labeled, β-gal-labeled, and double-labeled cells were averaged for each infusion group.

2.2.9 Statistical Analysis

Behavioral data were analyzed using mixed repeated measures analysis of variance (ANOVA) with infusion type (Daun02, Vehicle) as between-subjects factors, and responding during PreCS and CS periods, and during different training sessions or test days, as within-subjects measures. For all behavioral training, the dependent variable was the percentage of time rats spent performing foodcup behavior. Simple effects and *post-hoc* Bonferroni tests were analyzed as appropriate.

Fos and β-gal expression data were analyzed using univariate tests with infusion type as a between-subjects factor, as well as univariate tests with infusion type and final context test day as a between-subjects factor (Daun02 group perfused after acquisition context test, Daun02 group perfused after extinction context test, Vehicle group perfused after acquisition context test, and Vehicle group perfused after extinction context test). Follow up pairwise comparisons were reported as appropriate.

Data were tested for normality, equality of error variances, and homogeneity of variances and covariances. Appropriate corrections for homogeneity of variances and covariances violations were used when necessary, as well as corrected one-way ANOVAs using Welch's F when the equality of error variance assumption was violated. In cases where the assumption of normality was violated, results were checked using square root

transformations. SPSS (v.28) software was used for all statistical analyses, and the significance value was set at $p < 0.05$.

For Experiment 1, seven rats were removed from the experiment for failure to consume food pellets during acquisition training. Additionally, two rats were excluded from analyses due to off-target cannula placements, and two rats were excluded because one of their bilateral infusion deposits could not be confirmed. Only one subject was removed from statistical analyses for not meeting the pre-determined learning criteria for acquisition (during Acquisition Session 6, CS responding must be higher than PreCS responding, and higher than CS responding during Acquisition Session 1) or extinction (CS responding during Extinction Session 2 must be lower than CS responding during Acquisition Session 6). The final number of subjects for this experiment were n=9 for the Vehicle infusion group and n=8 for the Daun02 infusion group.

Five rats were removed from Experiment 2 due to failure to consume food pellets during acquisition training. Five rats were excluded from analyses due to improper cannula placement, and an additional 3 rats were excluded because their infusions could not be confirmed. The final number of subjects for this experiment were n=9 for the Vehicle infusion group and n=10 for the Daun02 infusion group.

2.3 RESULTS

2.3.1 Experiment 1: Prelimbic Neuronal Ensemble Inactivation

2.3.1.1 Behavioral Analyses

2.3.1.1.1 Acquisition and Extinction Training

All rats in this experiment were implanted with PL-targeting cannula, but infusions did not occur until the completion of acquisition training and, therefore, no group (Daun02 and Vehicle) differences were expected for training. Across acquisition training, all rats increased foodcup behavior (conditioned responding; CR) during CS periods (Fig. 2.2). A two-way mixed repeated measures ANOVA of CR for group comparing the first and last days of acquisition training (Acquisition 1 and Acquisition 6) as repeated measures, as well as PreCS and CS period, showed main effects of training ($F(1,15) = 90.256$, $p < 0.001$) and period ($F(1,15) = 78.675$, $p < 0.001$), as well as a training by period interaction ($F(1,15) = 94.407$, $p < 0.001$). Follow up analyses showed that responding during CS periods increased from Acquisition 1 to Acquisition 6 ($F(1,15) = 151.547$, $p < 0.001$) while CR during PreCS periods remained low ($F(1,15) = 0.045$, $p = 0.835$), and that CR during PreCS and CS periods was similar on Acquisition 1 ($F(1,15) = 0.586$, $p = 0.456$), but CS responding was higher than PreCS by Acquisition 6 ($F(1,15) = 119.809$, $p < 0.001$). There was no effect of infusion group ($F(1,15) = 0.350$, $p = 0.563$), nor any other interactions (all $p > 0.05$).

Successful extinction training was analyzed by comparing CR on the last day of extinction training (Extinction 2) to Acquisition 6. From Acquisition 6 to Extinction 2, all rats decreased overall responding (Pillai's Trace $F(1,15) = 27.088$, $p < 0.001$), although a main effect of period indicated that their responding was still higher overall during CS compared to PreCS periods (Pillai's

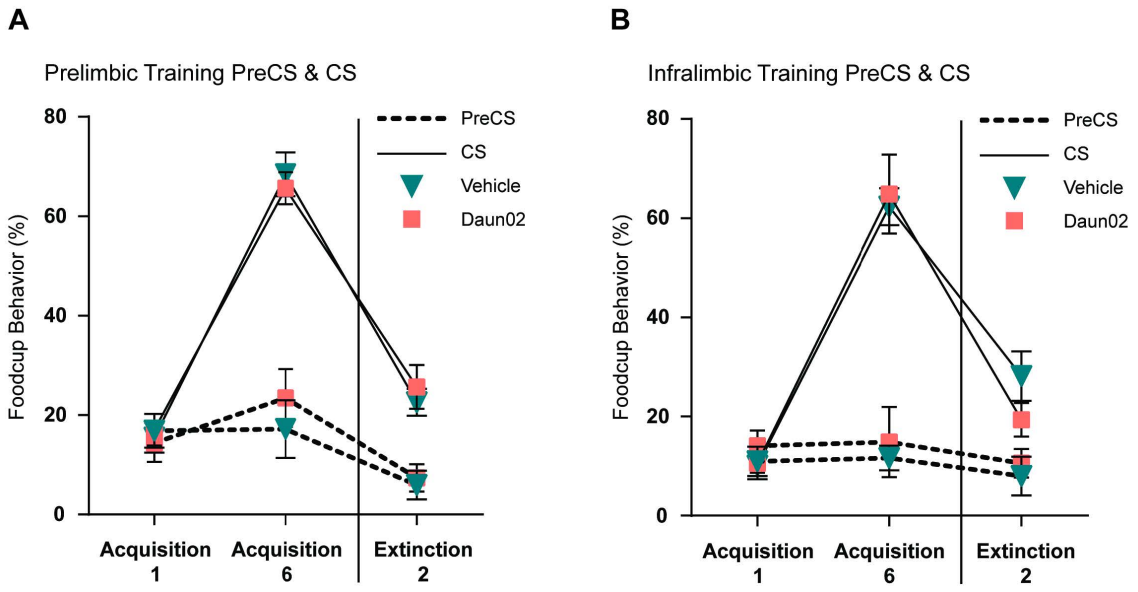

Figure 2.2 Conditioned responding during acquisition and extinction training. Average percent of time (mean ± SEM) spent expressing foodcup behavior during PreCS and CS periods on the first and last days of acquisition training, and the last day of extinction training. **(A)** Training data for rats with PL cannula implantations (Experiment 1). **(B)** Training data for rats with ILA cannula implantations (Experiment 2).

Trace $F(1,15) = 129.973$, $p < 0.001$). There was no main effect of infusion group ($F(1,15) = 0.001$, $p = 0.973$) indicating both groups decreased responding similarly, and there were no other interactions (all $p > 0.05$). There was a training by period interaction (Pillai's Trace $F(1,15) = 41.957$, $p < 0.001$) and follow up analyses indicated that CR during CS periods decreased from Acquisition 6 to Extinction 2 ($F(1,15) = 49.682$, $p < 0.001$) whereas PreCS period CR remained low ($F(1,15) = 0.838$, $p = 0.374$).

2.3.1.1.2 Renewal Testing

Rats in both the Daun02 and Vehicle infusion groups demonstrated renewal of responding during testing (Fig. 2.3). A two-way mixed repeated

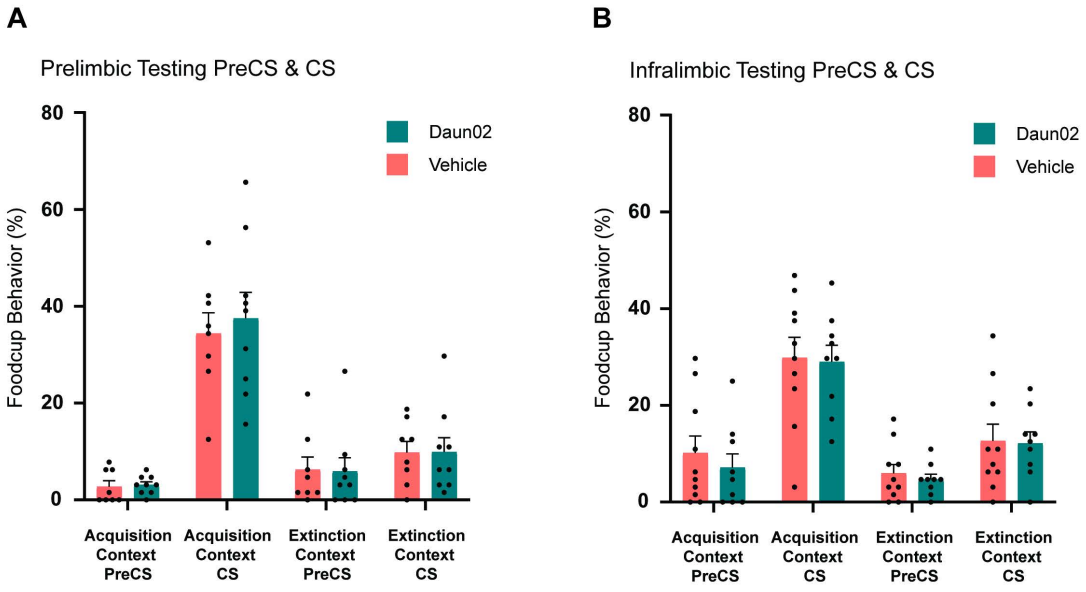

Figure 2.3 Conditioned responding during renewal testing. Average percent of time (mean ± SEM) spent expressing foodcup behavior during PreCS and CS periods during the acquisition context test and extinction context test. **(A)** Testing data for rats with PL cannula implantations (Experiment 1). **(B)** Testing data for rats with ILA cannula implantations (Experiment 2).

measures ANOVA analyzing CR with acquisition and extinction testing contexts and PreCS and CS periods as repeated measures found a main effect of testing context ($F(1,15) = 19.412$, $p < 0.001$), a main effect of period ($F(1,15) = 74.921$, $p < 0.001$), a testing context by period interaction ($F(1,15) = 47.190$, $p < 0.001$), and no effect of infusion group ($F(1,15) = 0.192$, $p = 0.668$). Follow up simple effect analyses confirmed that responding during CS periods was higher during the test in the acquisition compared to the test in the extinction context ($F(1,15) = 38.817$, $p < 0.001$), whereas CR during PreCS periods was similar during both tests ($F(1,15) = 2.209$, $p = 0.158$).

2.3.1.2 Histology

2.3.1.2.1 Cannula Placements

Both infusion groups received similar cannula placements, which were either directly above or within PL across levels 6-8 of the Swanson rat brain atlas (Fig. 2.4; +3.20 to +4.20mm from Bregma). Rats were perfused following their second test, which was in either their acquisition or their extinction context (test order was counterbalanced). After verifying cannula placements, the final number of subjects for the Vehicle infusion group was n=9 (n=4 for acquisition context test and n=5 for extinction context test) and the final number for the Daun02 group was n=8 (n=4 for acquisition context test and n=4 for extinction context test).

2.3.1.2.2 Fos- and β-gal-Labeled Neurons

The counts of single Fos- and single β-gal-labeled cells are shown in Figure 2.5. An additional quantification of double-labeled cells for all rats that received PL infusions showed that 84.000% ± 12.918% of the β-gal-labeled neurons were also Fos-positive (Fig. 2.4). This <100% colocalization of Fos- and β-gal-labeling is in agreement with other published Daun02 studies (Bossert et al., 2011; Fanous et al., 2012; Funk et al., 2016) and is likely a result of the slight differences in the time courses of peak Fos and β-gal expression, or differing antibody sensitivity in double-label fluorescence immunohistochemistry assays. Fos expression data patterns of effects matched the β-gal analyses in this experiment. We found higher Fos and β-gal induction in the Daun02 group, which was unexpected. Univariate tests for Fos and β-gal expression with infusion group as a between-subjects factor showed that rats that received PL Daun02 infusions had higher numbers of Fos and β-gal-labeled neurons compared to

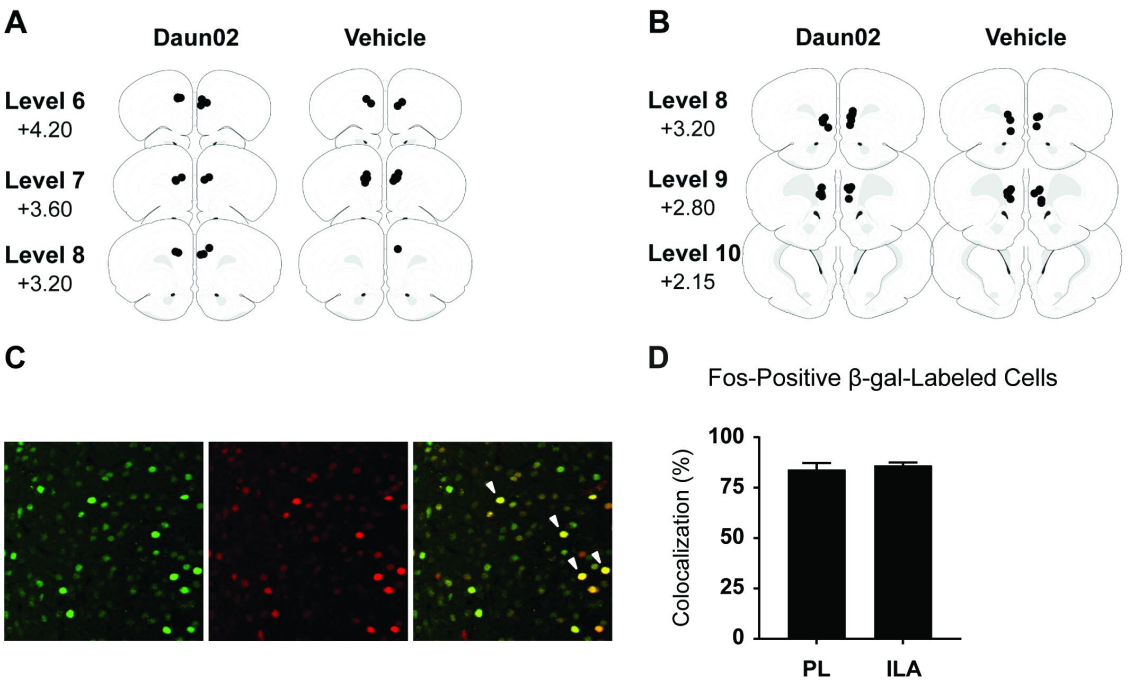

Figure 2.4 Cannula placements and Fos and β-gal expression. **(A)** All PL cannula placements are shown on Swanson atlas templates. **(B)** All ILA cannula placements are shown on Swanson atlas templates. **(C)** Representative images for Fos, β-gal, and double-labeled neurons (white arrows). **(D)** Percent colocalization of Fos and β-gal calculated after quantifying the number of β-gal-labeled cells that were also Fos-positive.

those that received vehicle infusions (Fig. 2.5; Fos $F(1,15) = 6.971$, $p = 0.019$; β-gal $F(1,15) = 25.005$, $p < 0.001$). Because the final test that induced Fos and β-gal expression was either in the acquisition or extinction context, additional univariate tests were conducted in order to analyze the context on test day as a between-subjects factor. For both Fos- and β-gal-labeled cells, there was a main effect of infusion group (Fig. 2.5; Fos $F(1,13) = 8.211$, $p = 0.013$; β-gal $F(1,13) = 22.460$, $p < 0.001$), but no main effect of test context nor an interaction for either cell-labeling (all $p > 0.05$). The main effect of infusion group indicated more positive-labeled neurons for the Daun02 groups (Fos $M = 186.875$, $SEM =$

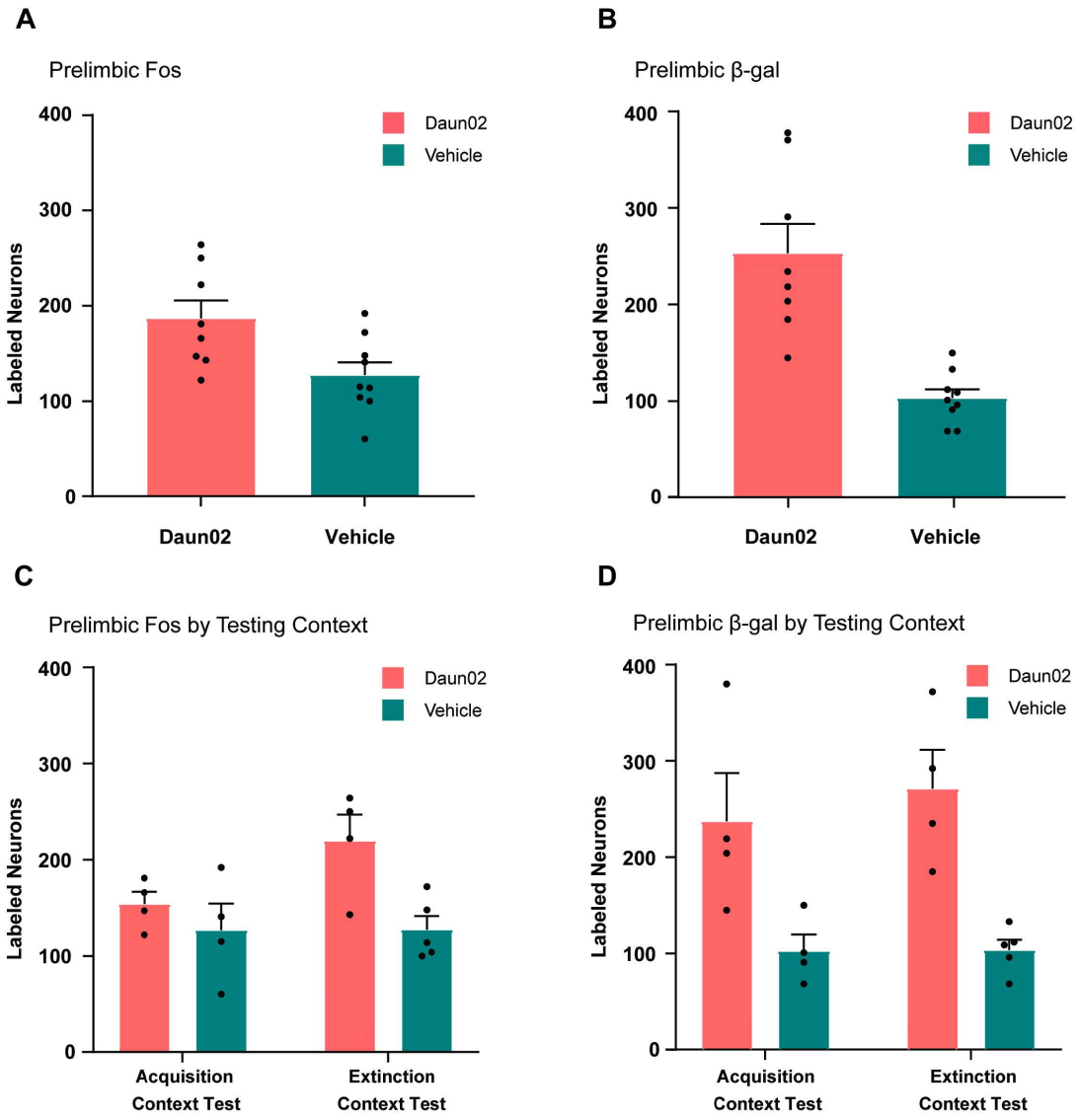

Figure 2.5 Fos and β-gal expression following renewal testing for rats that received PL infusions **(A)** Total number of Fos-labeled neurons for Daun02 and Vehicle infusion groups. **(B)** Total number of β-gal-labeled neurons for Daun02 and Vehicle infusion groups. **(C)** Total number of Fos-labeled neurons for each infusion group dependent on their testing context. **(D)** Total number of β-gal-labeled neurons for each infusion group dependent on their testing context.

15.083; β-gal *M* = 254.000, *SEM* = 23.107) compared to the Vehicle groups (Fos

M = 127.300, *SEM* = 14.309; β-gal *M* = 103.050, *SEM* = 21.921).

2.3.2 Experiment 2: Infralimbic Neuronal Ensemble Inactivation

2.3.2.1 Behavioral Analyses

2.3.2.1.1 Acquisition and Extinction Training

For this experiment, all rats were implanted with ILA-targeting cannula and infusions occurred at the end of acquisition training, and, therefore, no group differences were expected across training. All rats in this experiment increased responding during CS periods by the end of acquisition training (Fig. 2.2). The two-way mixed repeated measures ANOVA of CR indicated main effects of training ($F(1,17) = 82.076$, $p < 0.001$) and period ($F(1,17) = 161.489$, $p < 0.001$), as well as a training by period interaction ($F(1,17) = 131.483$, $p < 0.001$). Follow up analyses confirmed that responding during CS periods increased from Acquisition 1 to Acquisition 6 ($F(1,17) = 331.630$, $p < 0.001$) while CR during PreCS periods remained low ($F(1,17) = 1.163$, $p = 0.296$), and that CR during PreCS and CS periods was similar on Acquisition 1 ($F(1,17) = 0.080$, $p = 0.780$), but CS responding was higher than PreCS by Acquisition 6 ($F(1,17) = 218.092$, $p < 0.001$). As expected, there was no effect of infusion group ($F(1,17) = 0.022$, $p = 0.884$), nor any other interactions (all $p > 0.05$).

From Acquisition 6 to the Extinction 2, all rats decreased CR ($F(1,17) = 87.516$, $p < 0.001$), although their responding was still higher during CS compared to PreCS periods ($F(1,17) = 196.190$, $p < 0.001$). There was a training by period interaction ($F(1,17) = 42.349$, $p < 0.001$). Follow up analyses indicated that CR during PreCS and CS periods decreased from Acquisition 6 to Extinction 2 although the effect was more robust for CS periods (PreCS $F(1,17) =13.197$, p

= 0.002; CS $F(1,17) = 9.009$, $p < 0.001$). There was no main effect of infusion group ($F(1,17) = 0.249$, $p = 0.624$) indicating both groups decreased responding similarly, and there were no other interactions (all $p > 0.05$).

2.3.2.1.2 Renewal Testing

All rats that received ILA infusions demonstrated renewal of responding (Fig. 2.3). The two-way mixed repeated measures ANOVA showed a main effect of testing context ($F(1,17) = 15.184$, $p = 0.001$), a main effect of period ($F(1,17) = 69.530$, $p < 0.001$), and a context by period interaction ($F(1,17) = 14.368$, $p = 0.001$). Simple effect analyses confirmed that responding during CS periods was higher during the test in the acquisition compared to the test in the extinction context ($F(1,17) = 18.971$, $p < 0.001$), while responding during PreCS periods was similar across both tests ($F(1,17) = 2.262$, $p = 0.151$). There was no effect of infusion group ($F(1,17) = 0.343$, $p = 0.566$) and there were no other significant interactions (all $p > 0.05$).

2.3.2.2 Histology

2.3.2.2.1 Cannula Placements

Rats for both infusion groups received similar cannula placements either directly above or within ILA across levels 8-10 of the Swanson rat brain atlas (Fig. 2.4; +2.15 to +3.20mm from Bregma). The final number of subjects in the Vehicle infusion group was n=9 (acquisition context test n=4; extinction context test n=5) and the final number in the Daun02 group was n=10 (acquisition context test n=6; extinction context test n=4).

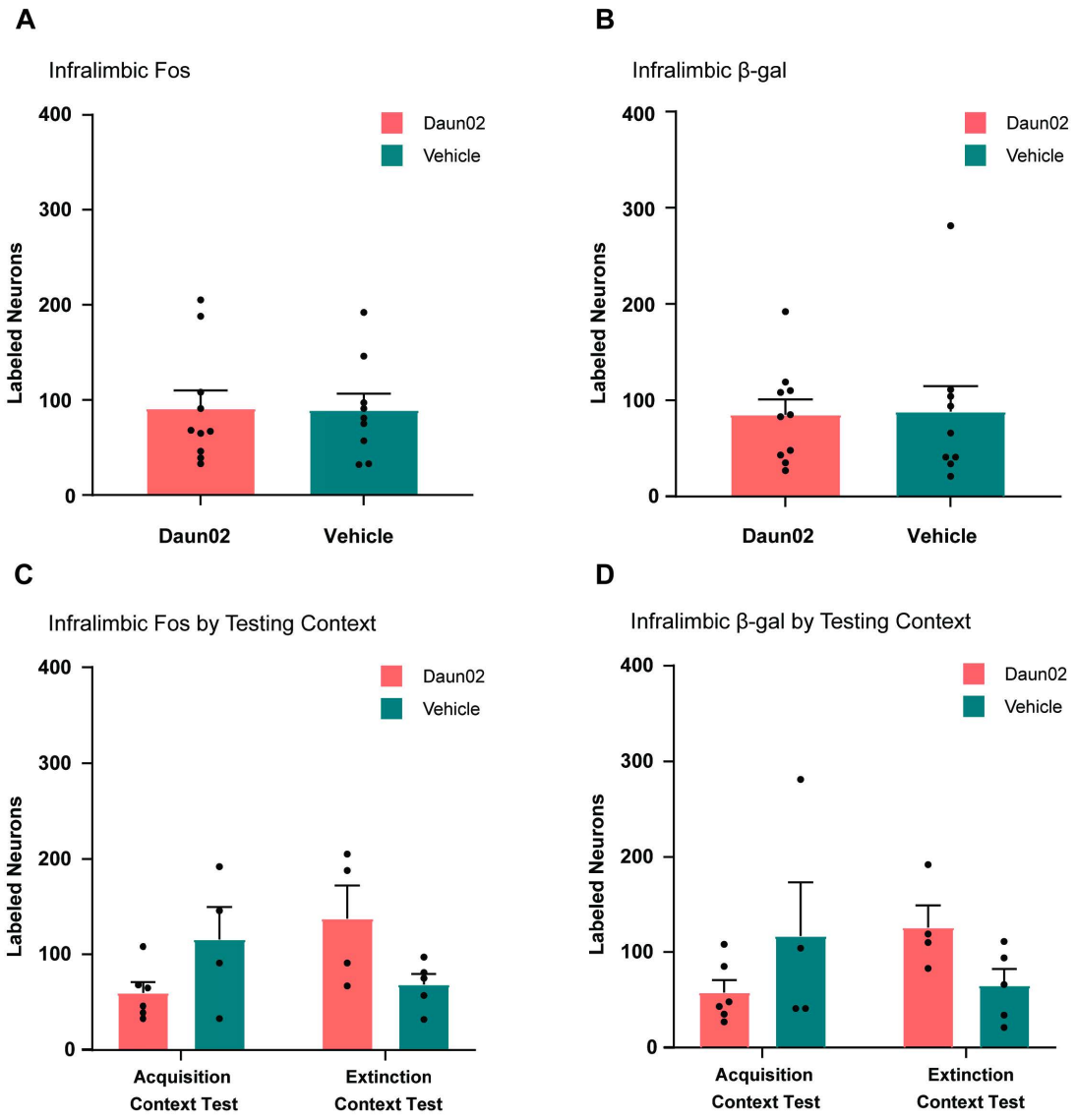

Figure 2.6 Fos and β-gal expression following renewal testing for rats that received ILA infusions **(A)** Total number of Fos-labeled neurons for Daun02 and Vehicle infusion groups. **(B)** Total number of β-gal-labeled neurons for Daun02 and Vehicle infusion groups. **(C)** Total number of Fos-labeled neurons for each infusion group dependent on their testing context. **(D)** Total number of β-gal-labeled neurons for each infusion group dependent on their testing context.

2.3.2.2.2 Fos and β-gal Labeled Neurons

The counts of single Fos- and single β-gal-labeled cells are shown in Fig. 2.6,

and quantification of double-labeled cells for all rats that received ILA infusions

showed that 85.990% ± 6.404% of the β-gal-labeled neurons were also Fos-positive (Fig. 2.4). The univariate tests for Fos- and β-gal-labeled neurons with group as a between-subjects factor revealed that the counts for each were similar (Fig. 2.6; Fos-labeled $F(1,17) = 0.004$, $p = 0.950$; β-gal-labeled $F(1,17) = 0.011$, $p = 0.919$). Because the final test inducing Fos and β-gal was either in the acquisition or extinction context, the univariate tests to analyze Fos and β-gal expression dependent on context test indicated a group by testing context interaction for Fos (Fig. 2.6; $F(1,15) = 7.804$, $p = 0.014$), and a trending interaction for β-gal ($F(1,15) = 4.440$, $p = 0.052$), with no other main effects or interactions (all $p > 0.05$). The follow up analyses indicated that for the groups tested in their extinction context, the Daun02 groups had more Fos-labeled neurons compared to their Vehicle counterparts ($F(1,15) = 4.625$, $p = 0.048$), whereas the Daun02 and Vehicle groups tested in their acquisition context had similar numbers of Fos-labeled neurons ($F(1,15) = 3.218$, $p = 0.093$). Additionally, the Vehicle groups had similar numbers of Fos-labeled cells regardless of testing context ($F(1,15) = 2.133$, $p = 0.165$), but of the rats that received Daun02, those tested in their extinction context had a higher number of Fos-positive cells than those tested in their acquisition context ($F(1,15) = 6.305$, $p = 0.024$).

2.4 DISCUSSION

This study sought to identify critical neuronal ensembles in the PL and ILA subregions of the vmPFC that support context-mediated recall of cue-food

associations. Using the Daun02 selective neuronal ensemble inactivation method, we specifically inactivated either PL or ILA neuronal ensembles that were active during the last training session of the Pavlovian cue-food acquisition phase of an ABA renewal paradigm. After extinction of this learned association in a different context, we expected to see a renewal of responding effect upon return to the initial context where cue-food acquisition occurred for rats who received Vehicle infusions into either the PL or ILA. For rats that received Daun02 selective neuronal ensemble inactivation in the PL, we expected to see an impairment of the renewal of responding effect compared to the Vehicle control group, and we did not expect to see any effects in the ILA Daun02 infusion group. Instead, we observed robust renewal of responding in all groups. We did, however, find interesting patterns of neuronal activation upon analyzing Fos and β-gal expression after final testing sessions that suggest that new ensembles were recruited in the rats that received Daun02 infusions. Such neural plasticity mechanisms may be aimed to preserve context-relevant food-seeking behaviors when the neuronal ensemble that was active during encoding of the learned association is not available. The results of the current study taken together with previous research support the growing idea that the roles of these subregions during appetitive motivated behaviors are more complex than simple behavioral output categorizations such as initiation of reward seeking versus behavioral inhibition (Rhodes & Killcross, 2007; Anderson & Petrovich, 2018; Quintana-Feliciano et al., 2021).

The neural analysis results of the current study support that the PL and ILA regions are critical in context-mediated renewal of responding to food cues. We found higher induction of Fos and β-gal in both the PL and ILA Daun02 infusion groups compared to the control groups with Vehicle infusions. These Fos and β-gal patterns indicate that more neurons were recruited during testing in the Daun02 groups, which suggest a possibility that a new neuronal ensemble is recruited to compensate for the absence of the ensemble that was inactivated by Daun02. Hence, the observed robust renewal of responding effect in all groups.

These behavioral results were unexpected because our main hypothesis was that Daun02 infusions into the PL would impair renewal of responding to the food cue, which we predicted would be manifested in decreased responding during testing in the acquisition context. We expected that this behavioral outcome would be the result of Daun02 selectively incapacitating the neuronal ensemble supporting the context-dependent tone-food memory, which, based on the existing literature, including previous results from our lab, we hypothesized was located in the PL (Bossert et al., 2012; Cole, Hobin, & Petrovich, 2015; Whitaker et al., 2017). Instead, we observed a complex pattern of results such that there was a null behavioral effect accompanied with differences in neural activation dependent on infusion group and the testing context. Due to the inactivating nature of the Daun02 methodology, we had expected that silencing a neuronal ensemble responsible for a behavior would result in a decrease of neural activation and, thus, a decrease in Fos-labeled and β-gal-labeled neurons.

However, for Experiment 1, we observed robust renewal of behavioral responding coupled with increases in neural activation in the PL for rats that received Daun02 inactivating infusions compared to Vehicle infusions. This suggests that, while the original neuronal ensemble formed during acquisition of the tone-food memory might be preferred, it is not the only neuronal ensemble capable of supporting memory recall and this appetitive behavior. Moreover, the increased neural activation in comparison to the Vehicle groups suggests that a new neuronal ensemble is recruited, potentially with more cells than initially required in order to compensate for the absence of the ensemble that was inactivated by Daun02. It is possible that during extinction training, an unsuccessful attempt was made to recall the original cue-food neuronal ensemble in order to update it with new information about the context-based implication of the cue's outcome. Considering that Daun02 was already present in the brain and taking effect while extinction training was occurring, when the original neuronal ensemble could not be re-activated due to the effects of Daun02, perhaps a new one was recruited to take its place and facilitate adapted learning about the appetitive association. This finding is valuable, as the neural activation patterns suggest that the PL is a site of neuronal plasticity involved in context-dependent tone-food learning and subsequent recall, despite not implicating that a single, specific neuronal ensemble in the PL needs to be recruited across both acquisition and recall.

For ILA, our initial hypothesis was based on prior evidence linking the ILA in extinction recall, and thus, we predicted that the ILA would not be required in

the recall of cue-food memory (Rhodes & Killcross, 2007; Warren et al., 2016). We did not expect to observe an effect on renewal of cue-food responding during testing after Daun02 infusions into the ILA, nor did we expect any differences in neural activation between our Daun02 and Vehicle groups. While there were no effects of ILA Daun02 infusions on renewal behavior, we did find unexpected patterns of neural activation. Though the Vehicle control group and Daun02 ILA inactivated group had similar Fos expression following their acquisition context test, the Daun02 group had higher neural activation after their extinction context test compared to the Vehicle group. This suggests that when the ILA inactivated neuronal ensemble was no longer available following Daun02 inactivation, new neuronal recruitment took place in order to continue supporting the appropriate suppression of food-seeking behavior during the extinction context test. The similar numbers of Fos- and β-gal-labeled neurons in the ILA Vehicle group during testing in either context suggests that the ILA may be recruited to a similar extent in order to inform the expression or inhibition of food-seeking behavior in a context-dependent manner. These results implicate the ILA as being capable of intensive plasticity mechanisms in order to continue supporting execution of, or inhibition of, food-seeking behavioral responses. Taken together, the increases in Fos- and β-gal-positive neurons in the Daun02 infusion groups indicate that neuronal participation from each the PL and ILA subregions is necessary in order to appropriately guide food-seeking behavior in either context.

The present studies contrast other studies utilizing the Daun02 inactivation method because, typically, there are observed behavioral effects accompanied

with decreases in Fos and β-gal expression following Daun02 inactivation (Funk et al., 2016; Whitaker et al., 2017; Cole, Keefer, Anderson, & Petrovich, 2020). Although those prior studies did not examine context-dependent renewal of Pavlovian responding, still the results of the current experiments were unexpected and the reason for these differences is unclear. There are important procedural differences between our protocol and other prior studies. One notable difference between the current experiments and a prior study from our lab is that, here, we explored differences pertaining to acquiring and extinguishing a tone-food association across different contexts, whereas our prior study investigated cue-potentiated feeding utilizing the same context across training and testing (Cole, Keefer, Anderson, & Petrovich, 2020). Another important procedural difference between our paradigm and other renewal paradigms is the timing of the Daun02 infusions. Other studies utilized an induction day after all training—both acquisition and extinction—was completed in order to target the neuronal ensemble of interest, then waited two days as Daun02 took effect before final testing (Fanous et al., 2012; Warren et al., 2016; for review see Cruz et al., 2013). In the current studies, we chose to target the neuronal ensemble activated during the last day of acquisition and, therefore, the extinction training took place over the time course of Daun02 taking effect. It is possible that during this time, while new extinction learning about the cue-food association was occurring, the initial cue-food neuronal ensemble was becoming inactive and a new population was recruited. Our prediction based on prior work was that different neuronal ensembles would be recruited for cue-food and cue-no food learning and

memory and that they would be dependent on the acquisition and extinction context, respectively. However, if these neuronal ensembles overlap in any capacity, then it stands to reason that the Daun02 inactivation occurring across the course of extinction training would impact ongoing cell recruitment changes. Plasticity mechanisms over these few days may have allowed for new neuronal recruitment in order to incorporate the new context-dependent cue-no food extinction learning in conjunction with the previously learned tone-food association. The rationale for the timing in our experiment was that we specifically wanted to target the neuronal ensemble responsible for tone-food learning in the acquisition context, before any extinction training, to determine if that initial neuronal ensemble would be recruited after extinction during renewal testing in the same context. Intriguingly, the results of the current study suggest that the same neuronal ensemble is not required, or at least not comprised of only those initially recruited cells. Instead, our research could even provide support for the idea that context does not necessarily become relevant in terms of the context-based cue-food association until the context switch occurs and the cue no longer reliably predicts food delivery, as in extinction training (Bouton, 1993; Holland & Bouton, 1999). This could explain why the neuronal ensemble active at the end of acquisition training is not necessarily needed in its identical form in order to for the appropriate food-seeking behavioral output to occur upon testing. It is possible that the original neuronal ensemble undergoes plasticity mechanisms as new information about the context-based implications of the cue are being incorporated during extinction training anyway. This would also help

explain why we saw robust expression of the context-induced renewal of responding to the food cue even after neuronal ensemble inactivation in the subregions of the vmPFC, despite contrary findings in the literature (Whitaker et al., 2017; Quintana-Feliciano, et al., 2021). The higher number of Fos- and β-gal-labeled neurons in the Daun02 groups may even suggest compensatory plasticity mechanisms to reform a neuronal ensemble capable of supporting the appropriate food-seeking behaviors in the case where the original neuronal ensemble, the preferred network of cells, is no longer available. Furthermore, the results of the current study taken together with prior research contribute to evidence that the PL and ILA are both required in appetitive motivated behaviors. Our results suggest that compensatory plasticity within both the PL and ILA can support crucial survival mechanisms, such as food-seeking behaviors, even when the neural basis a cue-food association is disrupted. However, this also provides a neural mechanism of how palatable food-seeking in the absence of hunger or physiological need can be a pervasive problem.

As future studies continue to investigate the vmPFC in appetitive associative learning, emphasis should be placed on the critical timepoints at which the PL and ILA exert execution or inhibition of behavioral output, as well as their overarching influence of context on food- and drug-seeking. It remains imperative to elucidate the neural mechanisms underlying context-induced renewal of responding to food cues. Findings from this type of research can help us understand and combat maladaptive behaviors that contribute to overeating and obesity, eating disorders, and drug abuse.

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GENERAL DISCUSSION

The major aims of the research in this dissertation were to explore sex differences and neural substrates of renewal of responding to food cues after extinction. Regarding this first goal, we determined that context habituation with a palatable food reward further strengthened the renewal effect in males, but not females (Chapter 1). Notably, in Chapter 1 we uncovered impairments in females' ability to extinguish responding during extinction training, which ultimately impacts the interpretability of the renewal effect. Then, regarding the second goal, we found neural evidence for potential plasticity mechanisms that contributed to the accumulating research that the PL and ILA are both recruited during context-mediated renewal of responding to food cues, and that their involvement is more complex than the dichotomous categorizations of behavioral control specified in the aversive literature (Chapter 2).

The first set of experiments in this dissertation work (Chapter 1) determined that pre-exposure (habituation) to the training contexts in an ABA renewal preparation differentially affected males and females in their acquisition of the cue-food association, extinction of that association in another context, and the renewal of responding effect upon returning to the initial reward learning context. Although the effect was stronger in males, we did observe improved tone-food acquisition in both sexes when context habituation occurred with food compared to controls that were habituated to the same palatable food reward in their home cages. Regarding the renewal effect, we established that context habituation with food further strengthened renewal of responding after extinction

for males, but neither context habituation alone nor with food decidedly impacted the renewal effect for females. Furthermore, the present studies recapitulated prior research from our lab and others that there are sex differences in renewal of responding where males consistently demonstrate the renewal effect, but females' patterns of behavior are inconsistent (Anderson & Petrovich, 2015, 2017; Binette, Totty, & Maren, 2022; Segal, Valyear, & Chaudhri, 2021). Adding to this established observation, the studies in this dissertation revealed another sex difference; that females showed evidence of resistance to extinguishing food seeking behaviors. This is consistent with prior predictions from other studies involving females in appetitive learning where researchers postulated that estradiol levels during extinction may be a key factor in why females exhibit high rates of responding regardless of context (Anderson & Petrovich, 2018; Hilz et al., 2019). These extinction learning results taken together with the prior studies regarding females' conditioned responding during renewal testing suggest behavioral rigidity in females in terms of appetitive motivated behaviors.

For the experiments in Chapter 2, after selectively silencing neuronal ensembles in the subregions of the vmPFC that had been active during the last tone-food acquisition training session in our ABA renewal protocol, we found no behavioral effects. Interestingly, however, the neural analyses provided support that both the PL and ILA are meaningfully involved during context-mediated renewal of responding to food cues. The Fos and β-gal expression following the final testing sessions was higher in the Daun02 groups suggesting new neuronal recruitment may have occurred to compensate for the absence of the original

neuronal ensemble. The increases in Fos- and β-gal-positive neurons in the Daun02 infusion groups illustrate that neuronal participation from both the PL and ILA are essential in order to facilitate the appropriate initiation of, or inhibition of, food-seeking behavior in the cue-food or cue-no food associated contexts, respectively.

The experiments that comprise this dissertation support the complexity of the neurobiological substrates underlying context-mediated renewal of responding to Pavlovian appetitive cues. In our ABA renewal experimental design, rats underwent a conditioning phase in which they acquire a Pavlovian tone-food association in one context, then, in a different context, the tone-food learning is extinguished, followed by testing in each of the two contexts. By using a within-subjects design, we were able to test and compare renewal of responding for the same rat in both the context used for their acquisition training, as well as the context used for their extinction training. The experiments in Chapter 1 sought to establish if additional familiarity with the context through habituation would improve learning about the meaning of the discrete cue once training commenced. Context-mediated renewal of responding after extinction is a well-defined phenomenon (Bouton & Bolles, 1979; Bouton & King, 1983), and research has shown that experience with the surrounding context impacts conditioning and memory recall (León, Callejas-Aguilera, & Rosas, 2012). Prior work showed that males and females can both acquire and extinguish appetitive associations successfully, but that females show inconsistent patterns of renewal compared to males whose conditioned responding robustly returns after

extinction once they are reintroduced to the initial conditioning context (Anderson & Petrovich, 2015, 2017). Context has compelling associative strength as a memory retrieval cue (Bouton, 1993) and, because it is a composite of several stimuli presented together, a context takes reasonably more time to encode than a single discrete cue (Fanselow 1986 & 2010; Rudy & O'Reilly, 2001). We anticipated that increased familiarity with the behavioral contexts via context habitation, would provide an enhanced memory basis for associative learning, particularly when food was presented. Indeed, for both males and females, there was evidence that this increased familiarity with the context when paired food enhanced their cue-specific responding during early conditioning. For females, this effect seemed to only impact the initial rate of acquiring the cue-food association, as they demonstrated increased cue-specific more quickly than their control counterparts who did not have the additional familiarity with the behavioral contexts through context habituation. Interestingly, males that received context habituation with food exhibited elevated food-seeking behaviors compared controls throughout the course of conditioning. Coupled with the strengthened renewal effect they displayed, this suggests the context habituation with food had a lasting, amplifying effect on the cue-food learned association for males. Intriguingly, these findings are also in line with context familiarity research in the aversive literature where studies have shown that permitting the subject to explore the context before conditioning attenuates the immediate shock deficit (Fanselow 1986 & 1990), similarly supporting that context familiarity can bolster associative learning. Although the are many fundamental differences in encoding

and behavioral responding to aversive compared to appetitive associations, there is also mounting evidence of similarities between them and we can benefit in studying both in terms discovering how context and discrete cues interact in associative strength and, ultimately, how behavioral output is controlled.

The most interesting finding from the studies in Chapter 1 was the extinction learning impairments observed in females. With or without context habituation, females in these experiments did not adequately extinguish conditioned responding to the food cue by the end of extinction training. On the scale of renewal studies, this pattern of responding during extinction should inform how we interpret their behavior during renewal testing. If the subjects did not sufficiently extinguish responding, then it is unreasonable to deem behavior during renewal testing as “renewal” because if the behavior was not extinguished in the first place this is simply continued cue-specific responding. On a larger scale beyond the scope of this immediate study, these findings have significant implications in understanding the persistent drive to seek food and rewards for females in their surrounding environment.

The observed behavioral differences in females compared to males in these experiments aligns with the mounting reports of sex differences in the associative learning and contextual processing literature (Hilz et al., 2019; Hilz et al., 2021; Lebron-Milad & Milad, 2012; Milad, Igoe, Lebron-Milad, & Novales, 2009; for review see Shansky & Woolley, 2016). One often suspected reason for this is the role of circulating hormones. Although prior work from our lab showed no sex differences in renewal of responding between high and low estrogen

groups, it did suggest that estrogen may be critical during extinction (Anderson & Petrovich, 2018). Other labs have shown that estrogen levels can impact recall of extinction memory in appetitive renewal (Hilz et al., 2019), and research from the fear learning literature highlights that estrogen and progesterone also impact extinction recall (Lebron-Milad & Milad, 2012; Milad, Igoe, Lebron-Milad, & Novales, 2009). Too few studies compare males and females in appetitive learning, extinction, and renewal, but the findings from these experiments add to the growing support that it remains crucial to investigate the sex differences in these appetitive motivated behaviors. These intricacies warrant further research because they may provide insight as to why it can be so difficult for all individuals, not just females, to change persistent food-seeking behaviors. It remains important to uncover why it can be such a struggle to change learned reward-seeking habits, particularly in the case of palatable foods when it comes to the obesity epidemic, but also translationally to halting drug abuse and relapse (Calu, Chen, Kawa, Nair, & Shaham, 2013).

It is clear that, like males, females are capable of effectively encoding the discrete cue's meaning, and that they are very capable of performing food-seeking behaviors when prompted by external cues. However, what these results do suggest is that females, unlike males, may not be incorporating the discrete cue's meaning with the context-based implication of that cue's outcome when it comes to extinction learning. This is interesting because it suggests sex differences in way the context-based implication of a discrete cue's outcome is encoded and recalled, as well as how this ultimately drives behavior. This sex

difference likely lies in the neural circuitry that modulates food- and reward-seeking behavioral execution and suppression.

In order to continue advancing the research on learned appetitive associations, emphasis should be placed on the neurobiological underpinnings of reward learning, extinction, and renewal or reinstatement. A vast collection of works has established that the vmPFC participates considerably in appetitive associative learning (Balleine & O'Doherty, 2020; Cole, Keefer, Anderson, & Petrovich, 2020; Moorman, James, McGlinchey, & Aston-Jones, 2015; Petrovich, Ross, Holland, & Gallagher, 2007), generally implicating it in decision-making processes that critically inform behavior (O'Doherty, 2011). Specifically of current interest to the field are the distinct functions of the PL and ILA subregions of the vmPFC. Presently, there is evidence that the PL and ILA differentially support associative learning (Rhodes & Killcross, 2007; Eddy, Todd, Bouton, & Green, 2016; Willcocks & McNally. 2013), but this is also support for them being similarly recruited (Cole, Hobin, & Petrovich, 2015; Kaminska, Caballero, & Moorman, 2021; Moorman, James, McGlinchey, & Aston-Jones, 2015). These complexities warrant further investigation to help us better understand the neural substrates that inform reward-associated behavioral responding, and the mechanisms underlying context-mediated renewal of responding to food-cues, specifically.

The results from the experiments in Chapter 2 of this dissertation lend additional evidence not only to the literature supporting the recruitment of PL and ILA in context processing related to food-cue learning, but also to the idea that there are robust neural plasticity mechanisms serving to persistently support

food-seeking responses. Despite the ample support for the roles of the PL and ILA in appetitive learning, the cellular mechanisms and plasticity at a circuit level mediating behavior during associative learning paradigms has yet to be unveiled. Evidence from operant conditioning studies demonstrate causal roles for the vmPFC in expressing and inhibiting reward-seeking (Warren et al., 2016; Whitaker et al., 2017), but less research is available on Pavlovian appetitive motivated behavior. Our experiments utilizing the Daun02 neuronal ensemble inactivation method showed that both the PL and ILA were ultimately necessary in renewal of responding after extinction. Although we expected behavioral impairments, we instead observed robust renewal of responding coupled with increases in neural activation in the groups that had received Daun02 infusions compared to our Vehicle control groups. This increase in Fos and β-gal induction in rats who could no longer reactivate the original tone-food learning neuronal ensemble suggests neural plasticity mechanisms serving to recruit new neurons, or perhaps an entirely new neuronal ensemble, in order to compensate for the inactivated tone-food neuronal ensemble from the cue-food learning phase. This finding highlights the remarkable tenacity the vmPFC subregions in persistently supporting food-seeking behaviors.

Despite not implicating a specific, single neuronal ensemble in the PL as causal for context-based cue-food learning and renewal, the findings of the experiments in Chapter 2 have significant implications. Following the aversive literature supporting functional dichotomy of the PL and ILA (for review see Quirk, Garcia, & Gonzalez-Lima, 2006) and evidence from other studies that

these regions may be functionally distinct in their contribution to the expression and inhibition of appetitive behaviors (Eddy, Todd, Bouton, & Green, 2016; Rhodes & Killcross, 2007), we theorized that there existed separate neuronal ensembles in the PL and ILA for cue-food and cue-no food learning, respectively. However, our findings are more in line with evidence demonstrating that the PL and ILA are both recruited and possibly provide overlapping contributions to encoding of and responding in context-based reward learning (Bossert et al., 2011; Warren et al., 2016). Considering that both the PL and ILA regions showed evidence of recruiting more neurons to compensate for the loss of the original neuronal ensemble that was active during cue-food learning, this provides support that both regions are critical for context-mediated renewal of responding to food cues. The Daun02 infusion effect on Fos and β-gal induction provide support that both the PL and ILA were recruited during both cue-food learning and context-induced renewal of responding upon returning to the acquisition context after extinction training. Interestingly, the current findings might also suggest that both regions are implicated during the extinction training phase of our behavioral paradigm. Due to our behavioral design, it is possible that Daun02 neuronal ensemble inactivation and new cell recruitment were occurring simultaneously during extinction training. Meaning, as new learning was occurring in the extinction training context, reactivation of the cue-food neuronal ensemble from acquisition training was required in order to update it with the information regarding the discrete cue's outcome. However, as that neuronal ensemble was likely gradually becoming unavailable due to the Daun02 infusion

taking effect, we suggest that plasticity mechanisms during extinction learning were responsible for simultaneously recruiting more neurons. In turn, this suggests that the PL and ILA were both undergoing neural plasticity mechanisms related to the new learning in the extinction context that interfered with complete cue-food neuronal ensemble inactivation. There is some support in the literature that PL and ILA neurons similarly respond to reward-predictive cues during both reward-seeking and extinction because they are helping inform appropriate context-based outcome contingencies (Moorman & Aston-Jones, 2015). The current findings potentially align with this observation because they demonstrate that both the PL and ILA are involved across learning and updating of the context-based meaning of a discrete cue. These insights highlight the pervasiveness of food-seeking behavioral output and the difficulties associated with changing palatable food-seeking behaviors and, likely, other reward-seeking behaviors.

The combination of results from this dissertation work might help explain the difficulties underlying pervasive food-seeking behaviors that can be resistant to modification. The PL and ILA experiments demonstrated plasticity mechanisms that support perpetuated food-seeking behaviors, and the experiments in Chapter 1 highlighted that context familiarity can strengthen appetitive behaviors in males, and that females may be more resistant to extinguishing appetitive behaviors. In summary, the findings from the studies that comprise this dissertation advanced the our understand of context familiarity, sex differences, and the roles of the PL and ILA in context-mediated renewal of

responding to food cues. These findings and continuation of this work are critical to progressing our understanding of how context and discrete cues interact to drive appetitive motivated behaviors. Elucidating the neural substrates of reward learning, extinction, and renewal or reinstatement of behavior is paramount in order to effectively combat maladaptive eating behaviors that contribute to obesity and, translationally, to drug abuse.

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