Prefrontal Cortex Circuitry in Sex Differences of Context-Mediated Renewal of Appetitive Pavlovian Conditioned Responding

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PREFRONTAL CORTEX CIRCUITRY IN SEX DIFFERENCES OF CONTEXT-MEDIATED RENEWAL OF APPETITIVE PAVLOVIAN CONDITIONED RESPONDING

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Title: Prefrontal Cortex Circuitry in Sex Differences of Context-Mediated Renewal of Appetitive Pavlovian Conditioned Responding

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Abstract: Learned associations are formed when cues from the environment are paired with biologically important events and can later drive appetitive and aversive behaviors. These behaviors can persist and reappear after extinction because the original learned associations continue to exist. In particular, cues previously associated with food can later stimulate appetite and food consumption in the absence of hunger. Renewal, or reinstatement, of extinguished conditioned behaviors may help explain the mechanisms underlying persistent responding to food cues and difficulty associated with changing unhealthy eating habits. The aim of this dissertation was to determine key components in the neural circuitry mediating renewal of responding to food cues. The main focus was on the ventromedial prefrontal cortex (vmPFC; includes the infralimbic (ILA) and prelimbic (PL) areas) because that region was selectively recruited during context-dependent renewal (Chapter 3). In all of the experiments, the behavior and neural substrates of male and female rats were compared. It was important to examine both males and females because sex differences in context-mediated renewal were recently established: males consistently show renewal responding while females fail to do so (Chapters 2 and 3).

The first study in this dissertation examined whether behavioral sex differences were driven by estradiol (Chapter 2) and whether the vmPFC is recruited during renewal responding (Fos induction; Chapter 3). Then, to establish the vmPFC is causal in driving the behavioral responding during renewal in a sex-specific way (Chapter 4), the vmPFC was silenced in males and stimulated it in females. This was accomplished using a chemogenetic methodology, DREADDs (Designer Receptors Exclusively Activated by Designer Drugs). Inhibiting the vmPFC in males blocks renewal responding. Reversely, stimulating the vmPFC in females resulted in renewal of responding. To determine key components of the vmPFC circuitry mediating renewal and whether these were different in males and females the experiments in Chapter 5 examined activation of PL inputs using a retrograde tract tracing combined with Fos detection design. The pathways to the PL from the ventral hippocampal formation (subiculum and CA1), the thalamus (anterior paraventricular nucleus), and the amygdala (anterior basolateral nucleus) were recruited in males and not recruited in females. This lack of recruitment could explain the lack of behavioral responding during renewal for females. Taken together, there are distinct and sex-specific circuitries recruited during context-mediated renewal. The findings from these experiments advanced our understanding of the neural mechanisms underlying sex differences in associative memory and contextual processing. They are also important for our understanding of the resilience of food cue to influence our consumption and diet choices.

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Chapter 1: General Introduction

Learned associations are formed when cues from the environment are paired with biologically important events, such as finding food or avoiding danger, and can later drive appetitive and aversive behaviors important for survival. However, these behaviors can persist and reappear after extinction because the original learned associations continue to exist, evidenced by spontaneous recovery and other forms of renewal of responding (for review see Bouton, 2004; Pavlov, 1927; Rescorla, 2004). In particular, cues previously associated with food can later stimulate appetite and food consumption in the absence of hunger, which can lead to maladaptive overeating behavior and obesity (for review see Martin & Davidson, 2014; Murray, Tulloch, Gold, & Avena, 2014; Petrovich, 2013). Renewal, or reinstatement, of extinguished conditioned behaviors may help explain the mechanisms underlying persistent responding to food cues and difficulty associated with changing unhealthy eating habits (Boutelle & Bouton, 2015; Calu, Chen, Kawa, Nair, & Shaham, 2014; Todd, Winterbauer, & Bouton, 2012).

The renewal phenomenon was originally shown by Bouton and King (1983) and has been replicated in appetitive and aversive conditioning preparations. In a context-based renewal preparation, a return to the context in which the initial learning occurred induces robust responding to the cues that were extinguished elsewhere. Most of the previous studies that investigated the neural substrates of renewal behavior focused on conditioned fear response (Frohardt, Guarraci, & Bouton, 2000; Lee et al., 2013). However, appetitive and

aversive renewal may not have the same underlying circuitry. A recent study found the amygdala substrates for appetitive conditioning differed from previously established aversive conditioning substrates (Cole, Powell, & Petrovich, 2013), highlighting that appetitive conditioning and renewal need to be investigated separately. In addition, the majority of appetitive renewal research has used drugs as reinforcers and instrumental tasks (Bouton, Todd, Vurbic, & Winterbauer, 2011; Willcocks & McNally, 2013) and only a few studies have used Pavlovian conditioning. An investigation into the neural mechanisms of Pavlovian renewal is important for our understanding of the resilience of food cue to influence our consumption and diet choices.

The aim of this dissertation was to determine key components in the neural circuitry mediating renewal of responding to food cues. The main focus was on the ventromedial prefrontal cortex (vmPFC; includes the infralimbic (ILA) and prelimbic (PL) areas) because that region was selectively recruited during context-dependent renewal. Furthermore, the vmPFC has been implicated in appetitive and aversive associative conditioning as well as renewal. The vmPFC is recruited during appetitive (tone-food) associative learning (Cole, Hobin, & Petrovich, 2015), is critical for feeding stimulated by appetitive contextual conditioned cues (Petrovich & Gallagher, 2007) and is associated with fear renewal in an aversive paradigm (Herry & Mons, 2004). The included experiments first examined whether behavioral sex differences were driven by estradiol (Chapter 2) and whether the vmPFC is recruited (Fos induction; Chapter 3). The results from these experiments provided background for the

Chapters 4 and 5 studies, which used chemogenetic technologies to target the vmPFC to selectively inhibit or excite neurons (Chapter 4), and used retrograding tracing to determine recruitment of key inputs to the PL during renewal responding (Chapter 5).

In all of the experiments, we compared the behavior and neural substrates of male and female rats. It was important to examine both males and females because we have established sex differences in context-dependent renewal: males consistently show renewal responding while females fail to do so (Chapters 2 and 3; Anderson & Petrovich, 2015, 2016). Additionally, in Chapter 5 we investigated whether acute estradiol effects on test day mediated sex differences in renewal (Anderson & Petrovich, 2015). There are also sex differences in learning and memory and in the control of feeding behavior and associated disorders (reviewed in Anderson & Petrovich, 2015).

The majority of extinction and renewal research has been conducted exclusively in males or with no comparison between the sexes (Bouton, 2004; Bouton et al., 2011). Female subjects are extremely underrepresented in behavioral and neural research as a whole (Zucker & Beery, 2010), and females are the minority in basic research and clinical trials even though anxiety, eating disorders and obesity afflict more women than men (McCarthy, Arnold, Ball, Blaustein, & De Vries, 2012; Ogden, Carroll, Kit, & Flegal, 2014). The assumption that females are intrinsically more variable, one of the major reasons given for excluding females, was shown to be false in a meta-analysis of almost 300 articles (Prendergast, Onishi, & Zucker, 2014). Including both males and

females and treating sex as a biological variable will both increase our knowledge about sex differences and also help broaden our understanding of mechanisms that are conserved across sex (Shansky & Woolley, 2016). In addition to the discrepancy between lack of research on females and morbidity of eating disorders and diseases in females, there is strong evidence of sex differences in associative and contextual learning (Maren, De Oca, & Fanselow, 1994; Reppucci, Kuthyar, & Petrovich, 2013; Shansky, 2015). Determining sex differences in neural systems underlying appetitive context-based renewal will provide valuable knowledge about how males and females process context and respond to appetitive cues.

One of the major goals of this thesis was to establish the vmPFC is causal in driving the behavioral responding during renewal (Chapter 4). A related goal was to establish the vmPFC functions differently in males versus females. These hypotheses were supported by our evidence that Fos induction in the vmPFC during context-mediated renewal is different in males and females in a way that correlates to the behavioral sex differences (Chapters 2 and 3). The Fos patterns suggested that the vmPFC activation mediates renewal in males but that this region is under-activated in females. To test this hypothesis, we proposed to silence the vmPFC in males and stimulate it in females. This was accomplished using a chemogenetic methodology, DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) (for more detail see: Chapter 4).

The second major goal was to determine key components of the vmPFC circuitry mediating renewal and whether these were different in males and

females (Chapter 5). Specifically, we identified direct inputs to the PL and determined if they were activated during renewal in males and females. The primary focus was on the ventral subiculum in the hippocampal formation (SUBv), as we hypothesized these projections to the vmPFC are mediating the contextual information during renewal, and in a sex specific way.

The hippocampal formation is critical for contextual processing (Holland & Bouton, 1999) and has been implicated in context-dependent renewal of fear (Orsini, Kim, Knapska, & Maren, 2011). Specifically, the SUBv has extensive direct connections to the vmPFC and there are also direct inputs from the adjacent ventral dorsal field CA1, Ammon's horn (Canteras & Swanson, 1992; Fanselow & Dong, 2010). Determining the role of this pathway is crucial for defining the appetitive renewal circuitry. While the SUBv-vmPFC input was the primary focus of these experiments, inputs from additional areas within the ventral hippocampus, amygdala and paraventricular nucleus that send direct pathways to the vmPFC were also examined. These areas were chosen based on activation (Fos induction) during renewal and support by prior work (see Chapter 3).

Determining activation of vmPFC inputs (Chapter 5) was accomplished using a retrograde tract tracing combined with Fos detection design. In these experiments, a retrograde tracer was used to identify inputs from the hippocampal formation, amygdala and paraventricular nucleus of the thalamus. Combining labeling of retrograde tracer and Fos induction allowed us to examine neurons in these regions that send direct projections to the vmPFC (by

retrograde tracer labeling) and determined whether they were specifically activated (by measuring Fos induction) during renewal.

The work in this dissertation explored the sex differences during renewal responding of appetitive behavior on both a behavioral and a neural level. Defining the neural circuitry of context-mediated renewal of responding to food cues after extinction in both males and females, and how those circuitries may differ to support different behaviors, improves our knowledge of both appetitive conditioned behaviors and sex differences. The work in this dissertation is also relevant to sex differences in learning and memory as well as the control of eating behavior and eating disorders.

Chapter 2: Renewal of conditioned responding to food cues in rats: Sex differences and relevance of estradiol *

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ABSTRACT: Cues associated with food can stimulate food anticipation, procurement, and consumption, independently of hunger. These and other behaviors driven by learned cues are persistent and can reappear after extinction, because the original learned associations continue to exist. Renewal, or reinstatement, of extinguished conditioned behavior may explain the inability to change maladaptive eating habits driven by food cues, similar to the mechanisms of drug use relapse. Here, we investigated sex differences in context-induced renewal of responding to food cues, and the role of estradiol in females in a Pavlovian conditioning preparation. We compared adult male and female rats because there is evidence for sex differences in learning and memory and in the control of feeding. Context-induced renewal involves conditioning and extinction in different contexts and the renewal of conditioned behavior is induced by return to the conditioning context ("ABA renewal"; experimental groups). Control groups remain in the same context during conditioning, extinction, and test. In Experiment 1, male and female rats were trained to associate a tone with food pellets during acquisition, and after

extinction with tone only presentations, were tested for renewal of responding to the tone. Learning was assessed through the expression of the conditioned response, which included approach and activity directed at food receptacle (food cup behavior). Males and females learned the acquisition and extinction of tone–food associations similarly, but there were sex differences during renewal of the conditioned responses to the food cue. Males showed robust renewal of responding, while renewal in intact females was inconsistent. Males in the experimental group had significantly higher food cup behavior compared to males in the control group, while females in both groups showed similar levels of food cup behavior during the tone. In Experiment 2, we examined a potential role of estradiol in renewal, by comparing intact females with ovariectomized females with, and without, estradiol replacement. Rats in all groups acquired and extinguished tone–food associations similarly. During the test for renewal, the ovariectomized rats with estradiol replacement in the experimental group showed renewal of responding, evidenced by significantly higher food cup behavior compared to the control group. Intact and ovariectomized rats in the experimental groups had similar rates of food cup behavior as their corresponding control groups. These results provide novel evidence for sex differences and relevance of estradiol in renewal of responding to food cues and more broadly in contextual processing and appetitive associative learning, potentially relevant to maladaptive eating habits and eating disorders.

1. Introduction

Learned associations have an important impact on our behavior, usually beneficial but sometimes negative, especially when they become persistent. These associations are readily formed when cues from the environment are paired with biologically important events, such as finding food or avoiding danger, and can later drive appetitive and aversive behaviors important for survival. However, behaviors driven by learned cues can persist and reappear after extinction, because the original learned associations continue to exist, evidenced by spontaneous recovery and other forms of renewal of responding (for review see Bouton, 2004; Pavlov, 1927; Rescorla, 2004). Persistent behaviors and thoughts, driven by learned cues, have been implicated in eating disorders and drug abuse, as well as anxiety disorders and post-traumatic stress disorder (Parsons & Ressler, 2013; Stewart, 2008; Stewart, de Wit, & Eikelboom, 1984).

In particular, cues previously associated with food can later stimulate appetite and food consumption in the absence of hunger, which can lead to maladaptive overeating behavior and obesity (for reviews see Martin & Davidson, 2014; Murray, Tulloch, Gold, & Avena, 2014; Petrovich, 2013). Renewal, or reinstatement, of extinguished conditioned behaviors may help explain the mechanisms underlying persistent responding to food cues and difficulty associated with changing unhealthy eating habits (Boutelle & Bouton, 2015; Calu, Chen, Kawa, Nair, & Shaham, 2014; Todd, Winterbauer, & Bouton, 2012). Indeed, the food reinstatement model was recently introduced as a framework to study mechanisms of relapse to palatable food seeking during dieting, similar to

the reinstatement model for relapse of drug use (Calu et al., 2014; Stewart et al., 1984; Torregrossa & Taylor, 2013; Volkow & Wise, 2005).

The current study examined context-dependent renewal of conditioned responding to Pavlovian food cues, using an adapted ABA protocol (Bouton & King, 1983). In a context-based renewal preparation a return to the context in which the initial learning occurred induces robust responding to the cues that were extinguished elsewhere. We compared behavior of adult male and female rats, because there are sex differences in learning and memory and in the control of feeding behavior and associated disorders. Women are more susceptible to severe obesity and eating disorders, and obese women show more impairments in food reward-associative learning (for review see Becker, Perry, & Westenbroek, 2012; S. Brooks, Prince, Stahl, Campbell, & Treasure, 2011; Ogden, Carroll, Kit, & Flegal, 2014; Zhang, Manson, Schiller, & Levy, 2014). Nevertheless, female subjects are underrepresented in basic and clinical research (Asarian & Geary, 2013; Zucker & Beery, 2010). Prior research on extinction and renewal has been conducted exclusively with male rats or with no comparisons between males and females (Bouton, Todd, Vurbic, & Winterbauer, 2011; Campese & Delamater, 2013). Notably, studies that have compared males and females found sex differences in associative learning and contextual processing (e.g., Dalla, Papachristos, Whetstone, & Shors, 2009; Maren, De Oca, & Fanselow, 1994; Reppucci, Kuthyar, & Petrovich, 2013).

In the first experiment we compared behaviors of intact males and females and found sex differences. To examine whether estradiol is important in these

sex differences, in the second experiment, we compared behavior of intact females, ovariectomized females (OVX) and ovariectomized females with estradiol replacement (OVX+E). Estradiol is important in the regulation of food intake and body weight in females, as well as an important modulator in learning and memory including food-associative learning and subsequent expression of learned behaviors (Almey et al., 2014; Asarian & Geary, 2013; Dalla & Shors, 2009; Eckel, 2011; Graham & Milad, 2013; Milad et al., 2010; Rey, Lipps, & Shansky, 2014; Somogyi et al., 2011). Therefore, we tested whether estradiol may be a modulator of renewed food seeking in a context-driven preparation.

2. Materials and Methods

2.1. Subjects

2.1.1. Experiment 1

32 adult male and female Long-Evans rats (Charles River Laboratories; Portage, MI), which weighed 250-275 grams at arrival, were individually housed and maintained on a 12 h light/dark cycle (lights on 07:00). Males and females were housed in separate colony rooms. After arrival, subjects were allowed one week to acclimate to the colony housing room before behavioral procedures began, during which they had *ad libitum* access to water and standard laboratory chow (18% Protein Rodent Diet #2018, Harlan Teklad Global Diets; Madison, WI), and were handled daily. All housing and testing procedures were in compliance 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.

2.1.2. Experiment 2

16 ovariectomized and 8 intact adult female Long-Evans rats (Charles River Laboratories; Raleigh, NC) weighing 250-300 grams at arrival were individually housed and maintained on a 12 h light/dark cycle (lights on 07:00). Subjects were allowed one week after arrival to acclimate to the colony room before capsule implantation surgery. During acclimation rats had *ad libitum* access to water and standard laboratory chow (18% Protein Rodent Diet #2018, Harlan Teklad Global Diets; Madison, WI), and were handled daily. Animals were given a week to recover post-surgery before behavioral procedures began, during which they were weighed and handled daily. All housing and testing procedures were in compliance 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. Surgical procedure and estradiol replacement for Experiment 2

Bilateral ovariectomies were performed at Charles River laboratories, and after recovery and acclimation to the Boston College colony, all rats (ovariectomized and intact) received subcutaneous placement of silastic capsules. Assembly and implantation of the silastic capsules followed the protocol outlined by Strom, Theodorsson, Ingberg, Isaksson, and Theodorsson (2012). The capsules were made using silastic tubing (1.6 mm inner diameter, 3.17 mm outer diameter; 3 cm in length; Fisher; Pittsburgh, PA) and sealed with 5 mm wooden dowels, filled either with 180 mg/ml estradiol (Sigma-Aldrich; Saint Louis, MO) in sesame oil, or vehicle (sesame oil). Half of the ovariectomized

animals received estrogen (OVX+E), while the other half, along with intact, received only vehicle (OVX and Intact). Animals were anesthetized with isoflurane (2-5% in oxygen) and the capsules were inserted through an incision made caudal to the shoulders. To verify estradiol release, one day after completion of behavioral testing trunk blood was collected, and estradiol serum levels were measuring using a Mouse/Rat Estradiol ELISA kit (Calbiotech, Spring Valley, CA). Serum estradiol levels were significantly higher in OVX+E rats compared to OVX rats (OVX+E: 5.14 ± 0.9 pg/ml; OVX: 1.06 ± 0.4 pg/ml; $t(28) = -4.414$, $p < 0.01$).

2.3. Apparatus

The behavioral training was conducted in identical behavioral chambers (30 x 28 x 30 cm; Coulbourn Instruments; Allentown, PA) located in a room different than the colony housing rooms. The chambers had aluminum top and sides, clear Plexiglas rear wall and front hinged door and a floor of stainless steel rods 5 mm thick spaced 15 mm apart. Chambers contained a recessed food cup (3.2 x 4.2 cm) and a 4 W house light. Each chamber was located in a sound- and light-attenuating cubicle (79 x 53 x 53 cm), which was equipped with a ventilation fan (55 dB) and video camera attached to a recording system (Coulbourn Instruments; Allentown, PA). The conditioned stimulus (CS) was a 10 second tone (75dB, 2kHz). The unconditioned stimulus (US) consisted of two food pellets (45 mg pellets, formula 5TUL; Test Diets, Richmond; IN, USA) delivered to the food cup. Chambers were modified in visual, tactile, and olfactory features, to create two distinct environments (Context A and Context B). In Context A, a

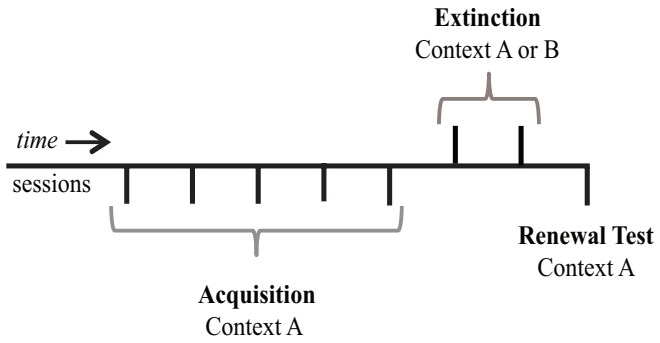

Figure 2.1 Experimental Design. Behavioral training consisted of three phases: conditioning (acquisition), extinction and renewal test. All rats received identical sessions throughout, except that experimental groups received extinction in a context different from the one in which the acquisition occurred, while the control groups remained in the same context throughout all training and testing ("ABA" design; counterbalanced across contexts). Rats received one session per day, and during each session there were presented with 8 tones (CSs) immediately followed by food (US) during acquisition sessions (CS-US pairings), but presented without USs during extinction and renewal.

black Plexiglas panel was placed on top of the grid floor (so that rats could not see or feel the grids), and the doors to the cubicles were closed. In Context B, a black Plexiglas panel was inserted diagonally across the side of the chamber creating a wall, and the doors to the cubicle were left open. For Context B, 1% acetic acid (Fisher Scientific; Fair Lawn, NJ) was sprayed onto the tray below the grid floor.

2.4. Behavioral Training Procedure

All behavioral training and testing occurred between 9:00 and 14:00. A week before start of training, rats were food deprived and their daily food allotment was restricted to gradually reach 85% of their body weight; they were maintained at this weight for the duration of the experiment. All rats received 1 g of the food pellets (US) in the home cage the day before the training started to familiarize them with the pellets. The training consisted of three phases: conditioning (acquisition), extinction, and renewal test (Figure 2.1). The training

protocol followed an “ABA” design where conditioning occurred in Context A, extinction occurred in Context B and the renewal test occurred in Context A (counterbalanced across contexts) (Bouton & King, 1983). Rats in the control condition remained in the same context across all training phases (AAA or BBB). During the acquisition phase, rats were trained for five days, with one 34-minute training session per day. During each session they received eight presentations of the tone (CS), each immediately followed with delivery of food pellets (US) into the food cup. The acquisition training occurred in Context A for half of the rats, and in Context B for the other half. During the extinction phase, rats received two 34-minute sessions (one session per day), each with eight presentations of the CS alone with no USs. Rats in the experimental condition received extinction training in a context different than the training context while rats in the control condition received extinction training in the same context as acquisition. The test for renewal was one 34-minute session with eight CS presentations with no USs, conducted in the conditioning (acquisition) context. All sessions were recorded and stored on DVDs for behavioral analysis.

2.5. Behavioral Observations

Trained observers, unaware of experimental condition or sex of the rats, analyzed animals’ behavior from the video recordings. The primary measure of conditioning (conditioned response, CR) was the expression of ‘food cup behavior’ during the CS. The food cup behavior was defined by distinct nose pokes into the recessed food cup, or by rats standing in front of and directly facing the food cup. Behavior was scored every 1.25 seconds during each 10

second preCS and CS periods. At each observation only one behavior was recorded (food cup or other). The number of food cup observations were summed and converted to a percentage of the total time during each period an animal spent at the food cup.

2.6. Statistical Analysis

Behavioral data (i.e., food cup behavior) were analyzed with ANOVAs, t-tests and Fisher's LSD *post hoc* tests as appropriate. In all cases, $p < 0.05$ was considered significant. SPSS software was used for all statistical analyses. Two subjects from Experiment 1 did not complete behavioral training and were removed from all statistical analyses, one due to poor health (a male in the control condition) and the other due to technical malfunction during testing (a female in the control condition). Two subjects from Experiment 2 did not complete behavioral training due to poor health (one OVX+E in the control condition and one OVX+E in the experimental condition).

3. Results

3.1. Experiment 1

3.1.1. Acquisition

Across training sessions during acquisition, all rats showed an increase in food cup responding (CRs) during CSs compared to their responding during preCSs which remained consistently low (Figure 2.2, on next page; CRs during CS presentations $F(24)=79.219$, $p < 0.001$; CRs during preCS $p > 0.05$). There were no sex or condition differences during acquisition ($p > 0.05$, both). During the last acquisition session (Acquisition 5), all rats showed high CRs during CSs (Males:

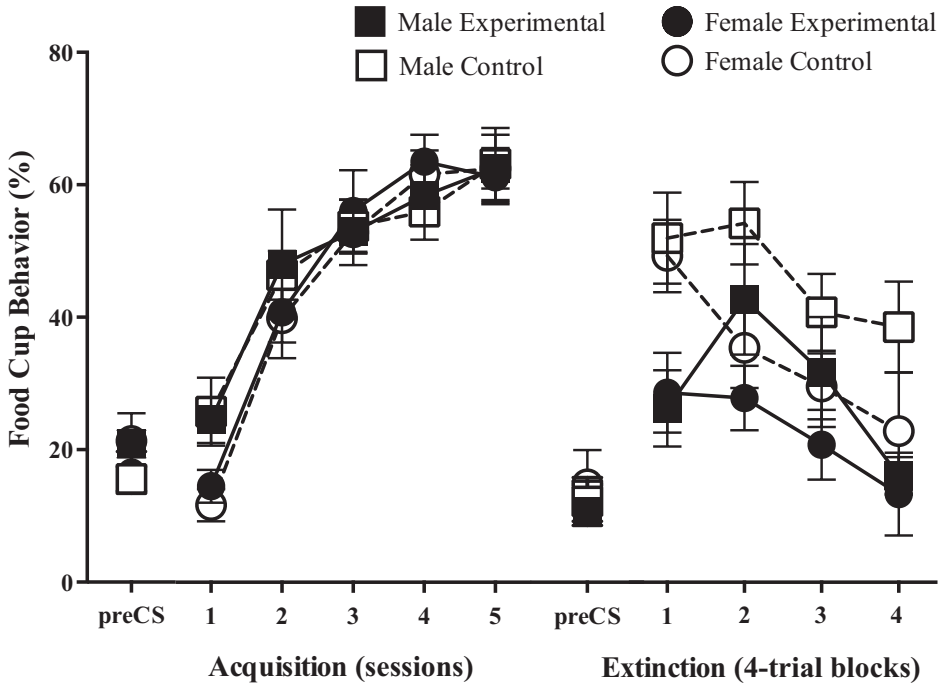

Figure 2.2 Conditioned responses during acquisition and extinction in Experiment 1. Percentage of time rats expressed food cup behavior (mean ± SEM) during the preCS and CS periods during training sessions. PreCS values are the average across all sessions for acquisition and extinction, respectively. Acquisition is shown as the average responding during each session. Extinction is shown as the average responding in 4-trial blocks (2 blocks per session; blocks 1&2 were trials during Session 1 and blocks 3&4 during Session 2).

62.60 ± 2.9%; Females: 61.56 ± 3.1%) compared to their responding during preCSs (Males: 19.79 ± 2.9%; Females: 17.50 ± 4.4%; t(29)=-18.613, $p < 0.001$). There was no effect of sex, condition, or sex by condition for CRs during CSs on Acquisition 5 ($p > 0.05$, both).

1.1.1. Extinction

All rats showed a decrease in conditioned responding due to extinction training (Figure 2.2). Rats in all groups (both sexes and conditions) had lower CRs to the CSs during extinction sessions compared to the last acquisition session (Table 2.1, on next page). This decrease was statistically significant between the total responding during last acquisition session (Acquisition 5) and second extinction

Table 2.1 Conditioned responses during extinction in Experiment 1. Percentage of time rats expressed food cup behavior (mean ± SEM) during the CSs on the final day of acquisition (session 5) and during each extinction session.

Group	Acquisition 5	Extinction 1	Extinction 2
Male Control	62.90 ± 5.4	52.90 ± 5.7	39.51 ± 5.5
Male Experimental	62.30 ± 3.0	34.38 ± 5.8	22.46 ± 4.2
Female Control	62.28 ± 5.1	42.19 ± 5.4	26.12 ± 6.6
Female Experimental	60.94 ± 4.0	28.13 ± 4.2	17.00 ± 5.4

session (Extinction 2) for males and females in both conditions ($t(29)=-12.767$, $p < 0.001$). We also compared responding during the first and last CSs of each extinction session. During the first CS of the first extinction session there was a significant effect of condition in CRs ($F(26,1)=25.564$, $p < 0.01$). Experimental male and female rats expressed significantly lower CRs compared to control male and female rats ($p < 0.05$). During the last CS of Extinction Session 1, as well as the first and last CS of Extinction Session 2, all groups had similar responding and there was no effect of condition, sex, or condition by sex ($p > 0.05$). CRs during preCSs remained consistently low across all sessions and did not differ between groups ($p < 0.05$).

1.1.2. Renewal

During the test for renewal, only male groups showed differential conditioned responding (Figure 2.3, on next page). An ANOVA (sex and condition) revealed a significant effect of condition on CRs during CSs ($F(1,26)=10.097$, $p < 0.01$). There was no effect of sex ($p > 0.05$), however there was a trend towards significance for condition by sex interaction ($p = 0.062$). *Post hoc* tests confirmed sex differences. Experimental males spent significantly more time expressing CRs during CSs compared to control males (Male Experimental: 42.77 ± 3.2 %, Male Control: 17.19 ± 4.2%; $p < 0.01$). Experimental and control females showed

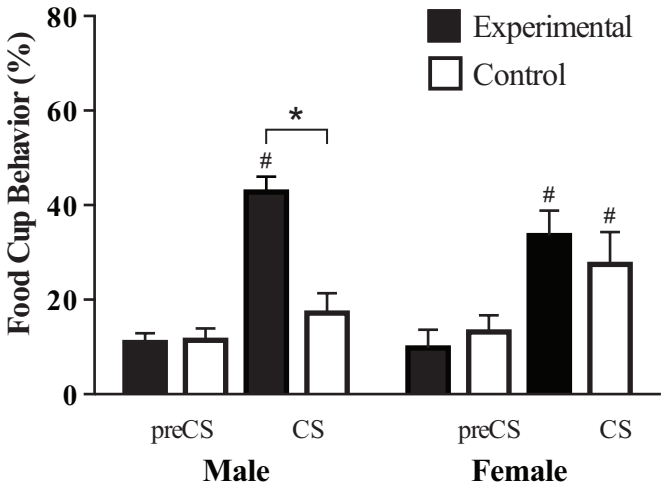

Figure 2.3. Conditioned responses during the test for renewal in Experiment 1. Percentage of time rats expressed food cup behavior (mean ± SEM) during preCS and CS periods; * indicates p < .001, # indicates within-group preCS vs. CS difference $p < 0.05$.

similar rates of responding, with no significant difference between conditions (Female Experimental: 33.59 ± 5.3%, Female Control: 27.46 ± 6.9%; $p > 0.05$). CRs during preCSs remained low and did not differ between groups ($p > 0.05$). We found similar results when preCS responding was subtracted from CS (elevation scores) to assess learning independent of individual variability in responding. There was a significant effect of condition ($F(1,26)=14.630$, $p < 0.01$), but no sex, or sex by condition effects ($p > 0.05$). Experimental males had significantly higher responding than control males (Male Experimental: 31.84 ± 3.5%, Male Control: 5.80 ± 4.9%; $p < 0.01$) while there was no significant difference between experimental and control females (Female Experimental: 23.83 ± 4.8%, Female Control: 14.29 ± 5.5%; $p > 0.05$). To further assess sex differences and lack of differential responding in females, we compared CRs during CS and preCS within each group during renewal tests. Paired *t*-tests confirmed the male experimental group had higher CRs during the CS compared

to the preCS ($t(7)=-9.147$, $p < 0.001$), while CRs in male control group remained low during preCS and CS periods ($p > 0.05$). Both female groups had higher CRs during the CS period compared to the preCS (Female Control: $t(6)=-2.611$, $p < 0.05$; Female Experimental: $t(7)=-5.007$, $p < 0.01$).

1.2. Experiment 2

1.2.1. Acquisition

Across training sessions during acquisition, all rats showed an increase in CRs during CSs compared to their responding during preCSs, which remained consistently low (Figure 2.4, on next page; CRs during CS presentations $F(4,172)=72.702$, $p < 0.001$; CRs during preCS $p > 0.05$). There were no condition (control, experimental) or treatment (Intact, OVX, OVX+E) differences during acquisition ($p > 0.05$ for all). During the last acquisition session (Acquisition 5), all rats showed significantly higher CRs during CSs (Intact Females: 56.34 ± 3.4%; OVX: 58.89 ± 6.9%; OVX+E: 65.40 ± 3.4%) compared to their responding during preCSs (Intact Females: 17.77 ± 2.9%; OVX: 19.34 ± 4.3%; OVX+E: 16.4 ± 3.7%; $F(1,43)=462.026$, $p < 0.001$). Rats in all groups had similar, low CRs during preCSs on Acquisition 5 ($p > 0.05$, both).

1.1.1. Extinction

All rats showed a decrease in conditioned responding due to extinction training (Figure 2.4). Rats in all groups had lower CRs to the CSs during extinction sessions compared to the last acquisition session (Table 2.2, on next page). This decrease was statistically significant between total responding during the last acquisition session and second extinction session (Extinction 2) for all

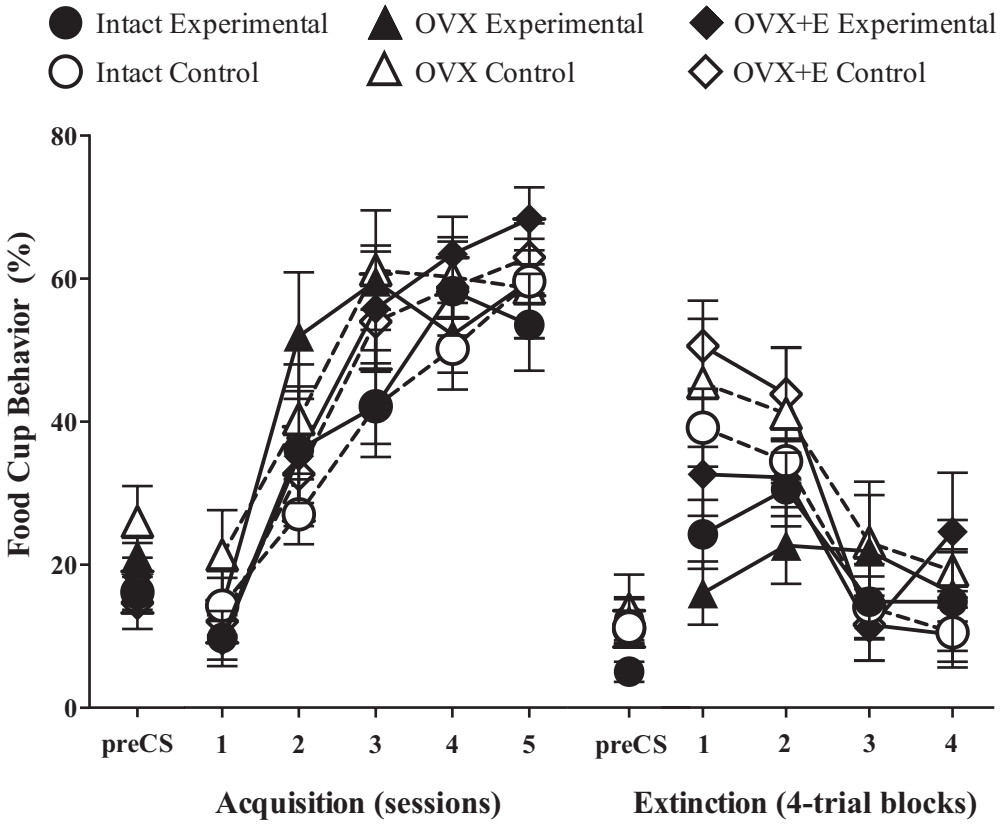

Figure 2.4. Conditioned responses during acquisition and extinction in Experiment 2. Percentage of time rats expressed food cup behavior (mean ± SEM) during the preCS and CS periods during training sessions. PreCS values are the average across all sessions for acquisition and extinction, respectively. Acquisition is shown as the average responding during each session. Extinction is shown in 4-trial blocks (2 blocks per session; blocks 1&2 were trials during Session 1 and blocks 3&4 during Session 2).

Table 2.2. Conditioned responses during extinction in Experiment 2. Percentage of time rats expressed food cup behavior (mean ± SEM) during the CSs on the final day of acquisition and during each extinction session.

Group	Acquisition 5	Extinction 1	Extinction 2
Intact Control	59.38 ± 2.0	36.72 ± 3.7	28.32 ± 6.4
Intact Experimental	53.32 ± 6.7	27.34 ± 3.7	17.38 ± 4.9
OVX Control	58.40 ± 6.9	43.16 ± 6.5	34.77 ± 8.5
OVX Experimental	59.38 ± 8.0	19.34 ± 2.9	22.46 ± 6.1
OVX+E Control	62.72 ± 5.3	47.10 ± 5.4	18.08 ± 5.0
OVX+E Experimental	68.08 ± 4.4	32.37 ± 2.6	29.24 ± 7.4

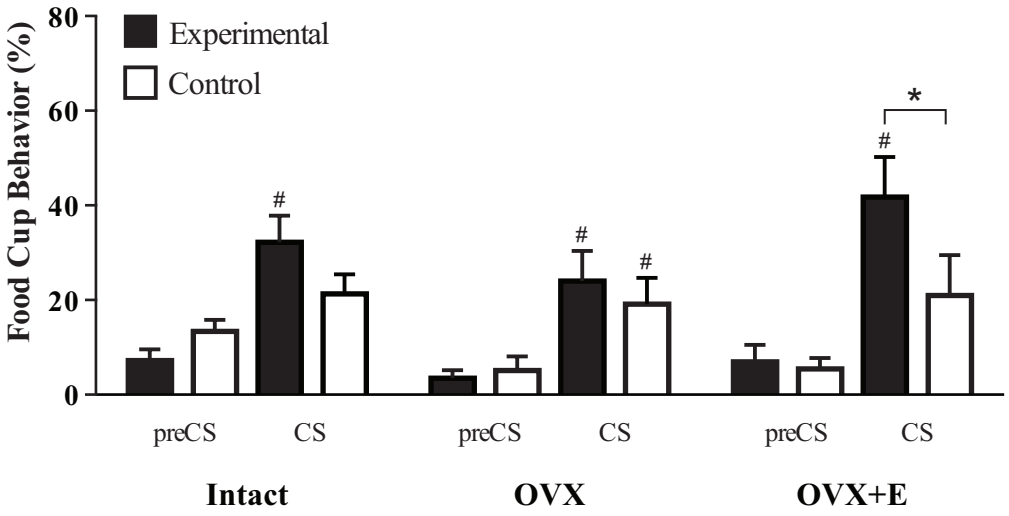

Figure 2.5 Conditioned responses during the test for renewal in Experiment 2. Percentage of time rats expressed food cup behavior (mean ± SEM) during PreCS and CS periods; * indicates $p < 0.05$, # indicates within-group preCS vs. CS difference $p < 0.05$.

groups ($F(1,40)=-154.189$, $p < 0.001$). During the first CS of the first extinction session there was a significant effect of condition in CRs during CSs ($F(1,40)=20.489$, $p < 0.001$), but no effect of treatment, or treatment by condition interaction ($p > 0.05$). During the last CS of Extinction Session 1, as well as the first and last CSs of Extinction Session 2, all groups had similar responding ($p > 0.05$). CRs during preCSs remained consistently low across extinction sessions and did not differ between groups ($p > 0.05$).

1.1.2. Renewal

During the test, OVX+E rats in the experimental group showed renewal of conditioned responding compared to OVX+E rats in the control group, while there were no differences between experimental and control groups in OVX rats. The pattern of Intact rats was more complex: the experimental group showed renewal when baseline responding was accounted for (elevation scores [CS-preCS] and CS vs. baseline [preCS]) even though their CRs were similar to that of intact

controls during the CS (Figure 2.5). An ANOVA (condition and treatment) revealed a significant effect of condition on CRs during CSs ($F(1,40)=5.337$, $p < 0.05$), and no effect of treatment, or condition by treatment effect ($p > 0.05$). *Post hoc* tests confirmed that OVX+E rats showed renewal. Experimental OVX+E rats had significantly more CRs compared to control OVX+E rats (OVX+E Experimental: 41.74 ± 8.5%, OVX+E Control: 20.98 ± 8.5%; $p < 0.05$). Intact and OVX rats had similar patterns, and within each, the experimental and control groups showed similar responding, with no significant differences (Intact Experimental: 32.23 ± 5.6%, Intact Control: 21.29 ± 4.1%; OVX Experimental: 24.02 ± 6.3%, OVX Control: 19.14 ± 5.6%; $p > 0.05$). Conditioned responding during preCSs remained low and did not differ between groups ($p < 0.05$). To assess learning independent of individual variability in non-specific responding, we analyzed elevation scores (baseline, or preCS, responding subtracted from CRs during CS). Similar to behavior during CSs above, there was a significant effect of condition ($F(1,40)=9.588$, $p < 0.01$), but no treatment, or condition by treatment effects ($p > 0.05$). *Post hoc* tests confirmed again that OVX+E experimental rats responded significantly higher than OVX+E control rats (OVX+E Experimental: 34.82 ± 7.9%, OVX+E Control: 15.63 ± 6.9%; $p < 0.05$). In addition, responding of Intact experimental rats was significantly higher than responding of Intact control rats (Intact Experimental: 25.00 ± 4.6%, Intact Control: 8.01 ± 5.1%; $p < 0.05$), while responding of OVX rats in both conditions remained similar (OVX Experimental: 20.51 ± 4.7%, OVX Control: 14.06 ± 4.6%; $p > 0.05$). As in Experiment 1, we compared CRs during CS and preCS within

each group during renewal tests. Paired *t*-tests showed the OVX+E experimental group had higher CRs during the CS compared to the preCS ($t(6)=-4.416$, $p < 0.01$), while CRs in the OVX+E control group remained low during preCS and CS periods ($p > 0.05$). Both OVX groups had higher CRs during the CS period compared to preCS (OVX Experimental: $t(7)=-4.320$, $p < 0.01$; OVX Control: $t(7)=-3.081$, $p < 0.05$). Intact rats showed a pattern similar to OVX+E: the experimental group had higher CRs during the CS compared to the preCS ($t(7)=-5.420$, $p < 0.01$), while the control group expressed similar, low CRs during preCS and CS periods ($p > 0.05$).

4. Discussion

The current study found sex differences in renewal of conditioned responding to food cues and established an important role of estradiol in this behavior in females. To accomplish this, we tested males and females in context-dependent renewal of appetitive Pavlovian conditioned responding. Much of recent work on appetitive renewal used instrumental conditioning, drugs as reinforcers, or examined only males (e.g., Bouton et al., 2011; Crombag & Shaham, 2002). The current study complements prior work with comparisons of responding to food cues in both sexes.

Here, we found sex differences specifically during the test for renewal, but not during learning acquisition or extinction of conditioned responses. The observed sex difference in renewal, therefore, was not due to differential learning acquisition or extinction of the tone-food association, or females' inability to distinguish between contexts. In Experiment 1, male rats displayed renewed food

seeking after extinction when tested in the acquisition context, while renewal in females was ambiguous. Males in the experimental groups had higher food cup behavior to the tone (CS) than the males in the control group, whose responding remained low during preCS and CS. Both female groups responded similarly and each expressed higher food cup behavior during the tone compared to the baseline (preCS) period. This high expression of conditioned responses in the control condition suggests poor extinction retention in females. Therefore it cannot be disambiguated whether food cup behavior in the experimental group, which was similar to controls, is due to specific context-induced renewal or poor extinction retention.

In Experiment 2, we examined the potential role of estradiol by comparing behavior of females with no gonadal steroid hormones (OVX) to those with estradiol replacement (OVX+E), along with intact females. The acquisition and extinction of conditioned responses was similar across the three groups. Interestingly, we found renewal of food seeking in rats with estradiol replacement, but not in ovariectomized females. OVX+E rats in the experimental groups had higher conditioned responding to the food cue than the OVX+E rats in the control group, whose responding remained low during preCS and CS. In contrast, both OVX groups responded similarly and each had higher responses during the tone (CS) compared to the baseline (preCS) period. This pattern was similar to that of intact rats in Experiment 1, and suggests poor extinction retention in OVX control group. Consequently, the responding in the experimental OVX group could reflect context-specific renewal and/or poor

extinction retention. Renewal in intact rats was again inconsistent. Similar to findings in Experiment 1, there were no differences in conditioned responses between the two groups of intact females; however, when responding was expressed as an elevation score (preCS subtracted from CS), it was significantly higher in the experimental compared to the control group. Furthermore, only the experimental group had higher food cup behavior during the tone (CS) compared to the baseline (preCS) confirming that this renewal was context-induced and not due to impaired extinction memory. It should be noted, during behavioral training and testing the amount of estradiol was constant in OVX+E, but was likely variable and insufficient in intact rats depending on the stage of estrous cycle, which may explain different the behavioral outcomes in Experiment 1 and 2.

This is the first evidence there are sex differences in renewal of extinguished food cue responding. Indeed, prior work has demonstrated successful context-induced renewal in female rats (Bouton et al., 2011; D. C. Brooks & Bouton, 1993; Campese & Delamater, 2013; Todd et al., 2012; Woods & Bouton, 2008). There were important procedural differences between the current and prior studies with Pavlovian conditioning that likely account for differential findings. In the current study, there was no pre-exposure to the behavioral apparatus (contexts) prior to conditioning, and there were fewer extinction sessions than in the prior work (Bouton & Peck, 1989; D. C. Brooks & Bouton, 1993; Woods & Bouton, 2008). Therefore, lack of context pre-exposure and shorter extinction is likely why females were impaired in our preparation.

Context-induced renewal involves complex processing across acquisition, extinction, and test, which depend on accurate encoding, memory, and use of different contexts. According to the role of context in 'setting the occasion' for reinforcement, or non-reinforcement, of the primary conditioned cue, one context is aiding retrieval of the CS-US association (the acquisition context) while the other context is aiding retrieval of the CS-no US association (the extinction context) (Bouton, 2004; Holland & Bouton, 1999). Therefore, behavioral renewal during tests in the acquisition context illustrates an ability to accurately retrieve and use context-dependent memory. It requires evaluation of the memory of each context and CS experience in it, and the current context interpretation is then used to guide responding—a function akin to decision-making. Sex differences in the conditioned responding during context-dependent renewal in the current study therefore might reflect differences in context interpretation (including extinction memory), or its use (context-induced renewal) to guide responding in males and females.

The current findings are in agreement with prior evidence for sex differences in associative learning and contextual processing (for review see Dalla & Shors, 2009). For example, male rats showed greater contextual conditioned fear, and faster acquisition of this learning, compared to female rats (Maren et al., 1994; but see other strains: Pryce, Lehmann, & Feldon, 1999). Another study using aversive conditioning and contextual cues found that male rats demonstrated context discrimination, as they avoided the fear conditioned context but not a novel context, up to seven days post conditioning, while female

rats properly expressed discrimination one day after training but later expressed fear avoidance in both contexts. This context generalization by the females was, in part, dependent on estrogen (Lynch, Cullen, Jasnow, & Riccio, 2013). Similarly, a study that compared food consumption in neutral and aversive contexts found that males showed appropriate fear-anorexia, they inhibited feeding only in an aversive conditioned context, while females inhibited feeding in both contexts (Reppucci et al., 2013). The lack of renewal of conditioned behavior in females in the current study might also be due to generalization in the use of contextual cues during testing, which might be improved with additional training, including context pre-exposure, as mentioned above.

These findings also agree with prior evidence that estradiol is an important modulator of learning and memory. The loss of estrogen due to menopause can have significant effects on executive functions in women (for review see Shanmugan & Epperson, 2014). Furthermore, working memory impairments due to the decline in estrogen during aging in monkeys was improved by treatment with cyclic estradiol (Rapp, Morrison, & Roberts, 2003). Estrogen is also important in Pavlovian conditioning and extinction (Barker & Galea, 2010; Gupta, Sen, Diepenhorst, Rudick, & Maren, 2001; for a review of gonadal hormones effect on fear extinction see Lebron-Milad & Milad, 2012)

Interestingly, estrogen enhances some forms of learning but impairs others, and this modulation in part depends on the amounts of circulating estrogen and whether it is continuous or cycling. For example, what memory strategy females use to solve a maze depends on estradiol levels; when high,

female rats are biased to use place memory but when low, they are biased to use response memory (Korol & Kolo, 2002). Fear extinction during proestrus (high estradiol and progesterone) was enhanced, compared to metestrus (low estradiol and progesterone) phase, and an estrogen receptor-beta agonist facilitated extinction recall in intact female rats (Lebron-Milad & Milad, 2012; Milad, Igoe, Lebron-Milad, & Novales, 2009). High levels of estradiol impaired performance on a long delayed alternation T-maze (non-spatial, working memory task) but low levels facilitated performance on the maze at a shorter delay (Wide, Hanratty, Ting, & Galea, 2004). Furthermore, cyclic, but not continuous, estradiol replacement facilitated acquisition of spatial place-learning in an open-field tower maze (Lipatova, Byrd, Green, & Toufexis, 2014). In the current study, estradiol treatment to ovariectomized rats was continuous and that was the only group that showed consistent renewal. These results suggest that females' responding to previously extinguished food cues depends on constant estradiol levels, either because it is needed during all stages of the task or because it was available during the critical stage. It might also be important that the estradiol treatment here continued for 22 days prior to testing. Chronic treatment improved radial arm maze performance in ovariectomized rats, while short-term estradiol treatment prior to testing did not alter performance (Luine, Richards, Wu, & Beck, 1998).

In addition to estradiol, progesterone is also secreted by the ovaries and fluctuates during estrous cycle, and has been shown to facilitate extinction recall and enhance spatial learning (Becker et al., 2005; Frye, Duffy, & Walf, 2007;

Milad et al., 2009). Therefore, progesterone may also be an important modulator of renewal of conditioned food seeking, and its insufficient amounts may have contributed to inconsistences in renewal in intact females observed here. Nevertheless, the current results demonstrated that after ovariectomy, estradiol treatment alone is sufficient to induce renewal after extinction. Future work could address if progesterone, or combined estradiol and progesterone, treatment could further enhance renewal in females, and whether normal cycling of these hormones modulates motivation to respond to food cues.

5. Conclusions

We found sex differences in context-dependent renewal of extinguished responding to food cues, using Pavlovian conditioned procedures. We also demonstrated estradiol is an important mediator of renewal processing in female rats. Mechanisms underlying renewal of suppressed responding to food cues are informative to our inability to resist palatable foods and change maladaptive eating habits, similar to the mechanisms of drug use relapse (Calu et al., 2014). The results presented here show that in addition to its well-known effects on consumption, estradiol is also important for renewed food seeking driven by food cues (for reviews see Asarian & Geary, 2013; Eckel, 2011).

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Chapter 3: Sex specific recruitment of a medial prefrontal cortex-hippocampal-thalamic system during context-dependent renewal of responding to food cues in rats.*

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ABSTRACT: Renewal, or reinstatement, of responding to food cues after extinction may explain the inability to resist palatable foods and change maladaptive eating habits. Previously, we found sex differences in context-dependent renewal of extinguished Pavlovian conditioned responding to food cues. Context-induced renewal involves cue-food conditioning and extinction in different contexts and the renewal of conditioned behavior is induced by return to the conditioning context (ABA renewal). Male rats showed renewal of responding while females did not. In the current study we sought to identify recruitment of key neural systems underlying context-mediated renewal and sex differences. We examined Fos induction within the ventromedial prefrontal cortex (vmPFC), hippocampal formation, thalamus and amygdala in male and female rats during the test for renewal. We found sex differences in vmPFC recruitment during renewal. Male rats in the experimental condition showed renewal of responding and had more Fos induction within the infralimbic and prelimbic vmPFC areas compared to controls that remained in the same context throughout training and testing. Females in the experimental condition did not show renewal or an

increase in Fos induction. Additionally, Fos expression differed between experimental and control groups and between the sexes in the hippocampal formation, thalamus and amygdala. Within the ventral subiculum, the experimental groups of both sexes had more Fos compared to control groups. Within the dorsal CA1 and the anterior region of the paraventricular nucleus of the thalamus, in males, the experimental group had higher Fos induction, while both females groups had similar number of Fos-positive neurons. Within the capsular part of the central amygdalar nucleus, females in the experimental group had higher Fos induction, while males groups had similar amounts. The differential recruitment corresponded to the behavioral differences between males and females and suggests the medial prefrontal cortex-hippocampal-thalamic system is a critical site of sex differences during renewal of appetitive Pavlovian responding to food cues. These findings provide evidence for novel neural mechanisms underlying sex differences in food motivation and contextual processing in associative learning and memory. The results should also inform future molecular and translational work investigating sex differences and maladaptive eating habits.

1. Introduction

Learned associations between cues from the environment and biologically important events can largely impact our behavior. Cues associated with food can stimulate appetite and food consumption independently of hunger (for review see Petrovich, 2013) and responding to food cues has been correlated with long-term weight gain (Boswell and Kober, 2016; Sun, Kroemer, Veldhuizen, Babbs, de Araujo, Gitelman, Sherwin, Sinha, and Small, 2015). Food cues can drive these behaviors even after extinction, because the original learned associations continue to exist, evidenced by spontaneous recovery and other forms of renewal of responding (Bouton, 2004; Rescorla, 2004). Renewal, or reinstatement, of responding to previously extinguished food cues may help explain the difficulty associated with changing unhealthy eating habits—persistent cravings and the inability to resist palatable foods even when eating is maladaptive (Boutelle and Bouton, 2015; Todd, Winterbauer, and Bouton, 2012). This model was recently introduced as a framework to study the relapse of palatable food seeking during dieting, based on the reinstatement model of relapse of drug use (Calu, Chen, Kawa, Nair, and Shaham, 2014).

Here, we sought to identify key neural systems underlying context-mediated renewal and sex differences by assessing Fos induction. We examined renewal of conditioned responding to Pavlovian food cues with an adapted ABA protocol (Bouton and King, 1983). In this preparation a return to the context in which the initial learning occurred induces robust responding to the cues that were extinguished elsewhere. Recently, we found sex differences in the ABA

protocol where male rats exhibited renewal of responding, while behavior of females was inconsistent and successful renewal depended on estradiol (Anderson and Petrovich, 2015). Males and females learned the acquisition and extinction of Pavlovian cue–food associations similarly, but only males showed robust renewal of responding to the food cue. A comparison of intact females with ovariectomized females with, and without, estradiol replacement found only the group with estradiol replacement exhibited renewal of responding. These behavioral sex differences are in agreement with accumulating reports of differences between males and females during associative learning and contextual processing (e.g. Dalla, Papachristos, Whetstone, and Shors, 2009; Farrell, Sengelaub, and Wellman, 2013; Maren, De Oca, and Fanselow, 1994; Reppucci, Kuthyar, and Petrovich, 2013).

We hypothesized the ventromedial prefrontal cortex (vmPFC) is critical during renewal and would be a site of sex differences due to its well-known executive function in decision-making and behavioral guidance (Dalley, Cardinal, and Robbins, 2004; O'Doherty, 2011) and its role in associative learning, including renewal (Eddy, Todd, Bouton, and Green, 2016; Willcocks and McNally, 2013). Additionally, we examined three areas connected with the vmPFC and important for associative learning, contextual processing, and the control of food consumption: the hippocampal formation, thalamus, and amygdala. The hippocampal formation is critical for contextual processing and body weight regulation and has been implicated in context-dependent renewal of aversive and appetitive behaviors (Benoit, Davis, and Davidson, 2010; Davidson,

Chan, Jarrard, Kanoski, Clegg, and Benoit, 2009; Fanselow, 2000; Holland and Bouton, 1999; Marinelli, Funk, Juzytsch, Li, and Le, 2007; Orsini, Kim, Knapska, and Maren, 2011). The thalamus, specifically the paraventricular nucleus (PVT), has been implicated in context-induced renewal (Hamlin, Clemens, Choi, and McNally, 2009) and is involved in the regulation of food consumption (Bhatnagar and Dallman, 1999; Cole, Mayer, and Petrovich, 2015b; Stratford and Wirtshafter, 2013). The amygdala is important for appetitive associative learning and subsequent control of behavior (Cole, Hobin, and Petrovich, 2015a; Cole, Powell, and Petrovich, 2013; Crombag and Shaham, 2002; Holland and Petrovich, 2005). The current study examined Fos induction during context-mediated renewal of responding to food cues in these key brain regions, and compared the patterns in male and female rats.

2. Materials and Methods

2.1 Animals

32 adult male and female Long-Evans rats weighing 250-275 grams at arrival (Charles River Laboratories; Portage, MI) were individually housed and maintained on a 12 h light/dark cycle (lights on at 07:00). Males and females were housed in separate colony rooms. After arrival, subjects were allowed one week to acclimate to the colony room during which they had *ad libitum* access to water and standard laboratory chow (18% Protein Rodent Diet #2018, Harlan Teklad Global Diets; Madison, WI), and were handled daily. All housing and testing procedures were in compliance 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 Apparatus

The behavioral training was conducted in identical behavioral chambers (30 x 28 x 30 cm; Coulbourn Instruments; Allentown, PA) located in a room different from the colony housing rooms. The chambers had aluminum top and sides, clear Plexiglas rear wall and front hinged door and a floor of stainless steel rods 5 mm thick spaced 15 mm apart. Chambers contained a recessed food cup (3.2 x 4.2 cm) and a 4 W house light. Each chamber was located in a sound- and light-attenuating cubicle (79 x 53 x 53 cm), equipped with a ventilation fan (55 dB), and a video camera attached to a recording system (Coulbourn Instruments; Allentown, PA). The conditioned stimulus (CS) was a 10 second tone (75dB, 2kHz), and the unconditioned stimulus (US) consisted of two food pellets (45 mg pellets, formula 5TUL; Test Diets, Richmond; IN, USA) delivered to the food cup. Chambers were modified in visual, tactile, and olfactory features, to create two distinct environments (Context A and Context B). In Context A, a black Plexiglas panel was placed on top of the grid floor (so that rats could not see or feel the grids), and the doors to the cubicles were closed. In Context B, a black Plexiglas panel was inserted diagonally across the side of the chamber creating a wall, and the doors to the cubicle were left open. For Context B, 1% acetic acid solution (Fisher Scientific; Fair Lawn, NJ) was sprayed onto the tray below the grid floor.

2.3 Behavioral Training Procedure

All behavioral training and testing occurred between 9:00 and 14:00. A week

	Acquisition 5 sessions	Extinction 2 sessions	Renewal Test 1 session
Experimental	A+	B-	A-
Control	A+	A-	A-

Figure 3.1 Experimental design. **A** denotes training in Context A, **B** denotes training in Context B (contexts were counterbalanced). Each training session consisted of eight presentations of either CS-US (denoted as **+**), or CS alone (denoted as **-**). All animals were sacrificed 90 minutes after the end of Renewal test and brains were collected for Fos induction detection by immunohistochemistry.

before start of training, rats were food deprived and their daily food allotment was restricted to gradually reach 85% of their body weight; they were maintained at this weight for the duration of the experiment. All rats received 1g of the food pellets (US) in the home cage the day before the training started to familiarize them with the pellets. The training consisted of three phases: conditioning (acquisition), extinction, and renewal test (Figure 3.1). The training protocol followed an “ABA” design where conditioning and extinction occurred in different contexts while renewal occurred in the same context as conditioning (Bouton and King, 1983). Rats in the control condition remained in the same context across all training phases. During the acquisition phase, rats were trained for five days, with one 34-minute training session per day. During each session they received eight presentations of the tone (CS), each immediately followed with delivery of food pellets (US) into the food cup. The acquisition training occurred in Context A for half of the rats, and in Context B for the other half. During the extinction phase, rats received two 34-minute sessions (one session per day), each with eight presentations of the CS alone, with no USs. Rats in the experimental condition received extinction training in a context different than the training context (ABA or BAB), while rats in the control condition received extinction training in the same

context as acquisition (AAA or BBB). The test for renewal was one 34-minute session with eight CS presentations and no USs, conducted in the conditioning (acquisition) context. The inter-trial interval was 110-326 seconds and the length varied randomly across trials and training sessions. All sessions were recorded and stored on DVDs for behavioral analysis.

2.4 Behavioral Observations

Trained observers, unaware of experimental condition or sex of the rats, analyzed animals' behavior from the video recordings. The primary measure of conditioning, the conditioned response (CR) was the expression of 'food cup behavior' during the CS. The food cup behavior was defined by distinct nose pokes into the recessed food cup, or by rats standing in front of and directly facing the food cup. Behavior was scored every 1.25 seconds during each CS and during 10 seconds immediately prior to CS (preCS). At each observation only one behavior was recorded (food cup or other). The total number of food cup observations during each period (preCS or CS) were summed and converted to a percentage of the total time during each period an animal expressed food cup behavior.

2.5 Histological Procedures

Ninety minutes after the end of renewal tests, rats were anaesthetized with tribromoethanol (375 mg/kg body weigh, intraperitoneal injection) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M borate buffer. The brains were stored for 20-24hrs at 4°C in a paraformaldehyde and 12% sucrose mixture and then rapidly frozen in hexanes

cooled with dry ice and stored at -80°C. Brains were cut into 40μm coronal sections using a microtome and collected into three adjacent series. One tissue series was immediately processed with Fos immunohistochemistry, described below. Another series was mounted and stained with thionin for identification of cytoarchitectonic borders.

2.6 Fos Immunohistochemistry

Free-floating sections were incubated for 1 hour at room temperature in a blocking solution (0.02M potassium phosphate-buffered saline [KPBS] containing 2% normal goat serum [NGS], 0.3% Triton X-100 and 10% milk), and then incubated with rabbit antiserum against Fos (1:30,000, PC38; Calbiochem, CA) in the blocking solution for 72 hours at 4°C with gentle agitation. Sections were rinsed with KPBS, 2% NGS and 10% milk, incubated with biotinylated secondary antibody against rabbit (1:500, BA-1000; Vector Laboratories) in the blocking solution, rinsed in KPBS, incubated in avidin biotin complex (ABC, PK-6100; Vector Laboratories), rinsed in KPBS and recycled through the secondary antibody and ABC solutions with KPBS rinses in between, such that the total time in each incubation was 75 minutes. Finally, the tissue was processed with 3, 3'-diaminobenzidine (SK-4100; Vector Laboratories) to visualize Fos immunoreactivity. Sections were rinsed, mounted on SuperFrost slides (Fisher Scientific), dried at 45°C, dehydrated through graded alcohols, cleared in xylenes, and coverslipped with DPX Mountant (Electron Microscopy Services; Hatfield, PA).

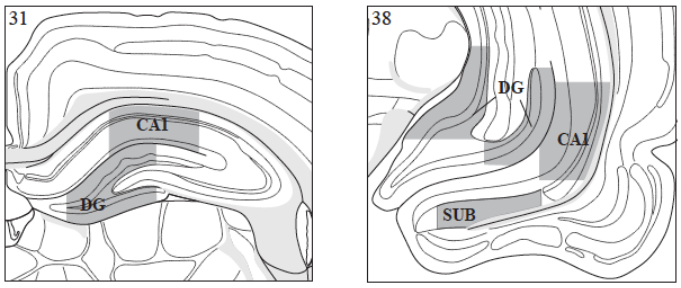

Figure 3.2 Sampling areas in the hippocampal formation. Dark gray shading denotes sampling areas in dorsal (left) and ventral (right) hippocampal formation. Illustrations were made on modified templates from the Swanson atlas (2004), and numbers in the upper left corner of each denote atlas levels. Abbreviations: CA1- field CA1, Ammon's horn; DG- dentate gyrus; SUB- subiculum.

2.7 Image Acquisition and Analysis

Images of the Fos-stained and adjacent thionin-stained sections were acquired (10x magnification) with an Olympus DP72 camera and DP2-BSW software (Olympus America Inc, Center Valley, PA, USA). Using Image J software (NIH) the images were stacked and transformed to 8-bit grayscale. The analysis followed parcellation and nomenclature as defined in the Swanson atlas (2004), except for the dorsal and ventral hippocampal formation. These exceptions are depicted in Figure 3.2 and described in detail below. On the image of thionin-stained sections, borders were drawn, or a rectangular template was placed, around cell groups of interest, then the borders were transferred to the adjacent Fos-stained section, and automated counting was performed within the borders. The threshold for counting Fos-positive cells was determined from an area on each section with no specific labeling (background). Fos-positive cells were identified based on the pre-set size and circularity parameters. Automated counting was performed consistently across sections and brains using the same criteria. The criteria were determined before the start of analysis and accuracy

was confirmed by comparing the automated counts with manual counts by a well-trained observer, unaware of the experimental condition. For each area analyzed, images were acquired bilaterally and Fos-positive cells within left and right hemispheres were summed, and these individual totals were then averaged for each group resulting in a mean total number of labeled cells.

2.7.1 Medial Prefrontal Cortex

Within the medial prefrontal cortex, three areas were analyzed: the dorsal part of the anterior cingulate area (ACAd), the prelimbic area (PL), and the infralimbic area (ILA) (Swanson atlas (2004) levels 8, 9 and 10, +3.20, +2.80, and +2.15 mm from Bregma respectively; all subsequent measurements refer to mm from Bregma).

2.7.2 Hippocampal formation

Five regions were analyzed within the hippocampal formation: dorsal field CA1, Ammon's horn (Level 31, -3.70 mm) dorsal dentate gyrus (DG; Level 31, -3.70 mm), ventral CA1 (Level 38, -5.65 mm), ventral subiculum (SUBv; Level 38, -5.65 mm), and ventral DG (the ventral area of the medial and lateral blade; Level 38, -5.65 mm). For these areas analyses were conducted within templates created in Image J, as shown in Figure 3.2.

2.7.3 Amygdala

Seven cell groups were analyzed within the basolateral and central amygdala. Within the basolateral area, the anterior part of the basolateral nucleus (BLAa, Levels 26 and 27, -1.78 and -2.00mm), the posterior part of the basolateral nucleus (BLAp, Level 30, -3.25 mm), the posterior part of the basomedial nucleus

(BMAp, Level 30, -3.25 mm), and the lateral nucleus (LA, Level 30, -3.25 mm) were analyzed. Within the central amygdalar nucleus (CEA), the medial part (CEAm), lateral part (CEAl) and capsular part (CEAc) (Level 26, -1.78 mm) were analyzed.

2.7.4 Thalamus

Within the thalamus, the paraventricular nucleus (PVT) was analyzed. The PVT is a large nucleus, and there are connectional and functional differences across its rostro-caudal extent (e.g., Li & Kirouac, 2012; Cole, Hobin & Petrovich, 2015). Thus, images were taken from the anterior and posterior halves, and were analyzed separately, as anterior (PVTa) (Level 26, -1.78mm) and posterior (PVTp) (Level 30, -3.25mm) respectively.

2.8 Statistical Analysis

Behavioral data (i.e., food cup behavior) and Fos-induction data were analyzed with ANOVAs, t-tests, Fisher's LSD *post hoc* tests, and Pearson correlations, as appropriate. For the ANOVAs, the factors of Group (Experimental, Control) and Sex (Male, Female) were used, unless otherwise stated. In all cases, $p < 0.05$ was considered significant. SPSS software was used for all statistical analyses. Three rats (male experimental, male control, and female control) were excluded from behavioral and histological analyses based on their high responding during preCS in the last acquisition sessions (preCS responding higher than 3 standard deviations from the mean). Fos induction data were not collected from the following due to tissue damage: one male experimental (ILA, PL, ACAd, CA1d, DGd, BLAp, BMAp, LA, PVTa); two male controls (CA1v, SUBv, DGv, PVTa),

one female experimental (BLAa, CEAm, CEAl, CEAc, PVTp); one female control (ILA, PL, ACAd, CA1d, DGd, CA1v, SUBv, DGv).

3. Results

3.1 Behavior

3.1.1 Acquisition

During acquisition (Figure 3.3), rats showed an increase in food cup responding (CR) across the training sessions during the tone (CS) presentations (Repeated Measures ANOVA, $F(22)=22.662$, $p < 0.01$), while their CRs during preCS remained consistently low ($p > 0.05$). There were no sex or group differences ($p > 0.05$, both). During the last acquisition session (Acquisition 5), all rats showed

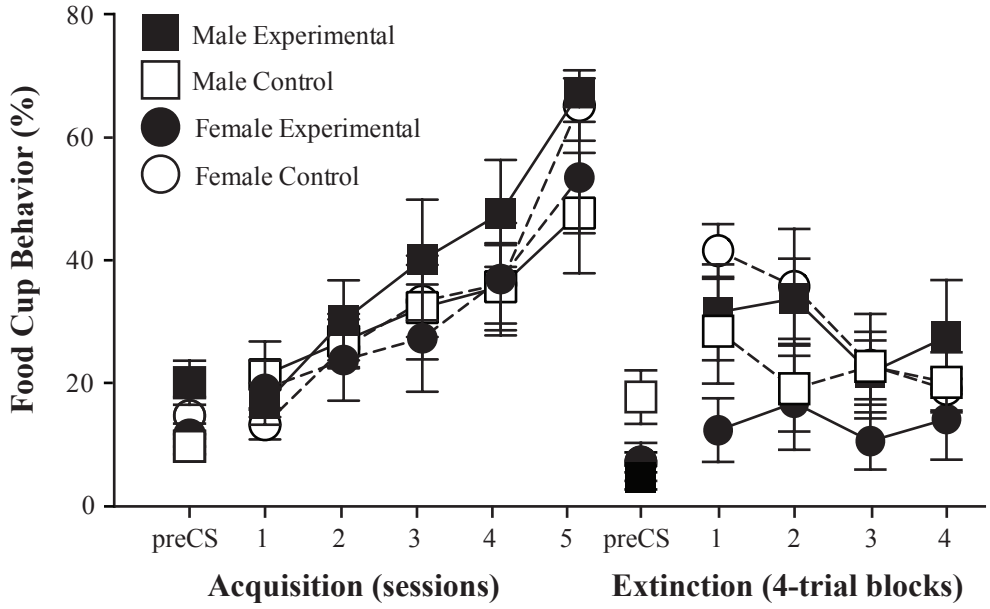

Figure 3.3 Conditioned responses during acquisition and extinction. Percentage of time rats expressed food cup behavior (mean ± SEM) during the preCS and CS periods during training sessions. PreCS values are the average across all sessions for acquisition and extinction, respectively. Acquisition is shown as the average responding during each session. Extinction is shown as the average responding in 4-trial blocks (2 blocks per session; blocks 1&2 were trials during Session 1 and blocks 3&4 during Session 2).

Table 3.1. Conditioned responses during extinction. Percentage of time rats expressed food cup behavior (mean ± SEM) during the CSs on the final day of acquisition (session 5) and during each extinction session.

Group	Acquisition 5	Extinction 1	Extinction 2
Male Control	47.14 ± 9.8	23.96 ± 5.5	21.61 ± 6.2
Male Experimental	66.74 ± 2.3	32.81 ± 4.4	24.78 ± 4.2
Female Control	64.65 ± 5.7	38.87 ± 5.7	21.09 ± 3.7
Female Experimental	52.90 ± 9.1	14.73 ± 3.4	12.50 ± 4.0

high CRs during CSs ($t(28)$=- 13.094, $p < 0.001$; Males: 58.37 ± 4.9; Females: 59.17 ± 5.3) compared to their low responding during preCSs (Males: 16.18 ± 2.9; Females: 11.77 ± 2.0). An ANOVA (Sex and Group) confirmed that there were no significant differences across groups in responding during CS or preCS ($p > 0.05$ for both). Body weights were similar between groups of the same sex (Male Experimental: 296.6 ± 3.7, Male Control: 301.1 ± 3.5, Female Experimental: 256.1 ± 4.4, Female Control: 256.1 ± 3.3; p > 0.05).

3.1.2 Extinction

All groups showed a decrease in conditioned responding due to extinction training (Figure 3.3, Table 3.1). This decrease was statistically significant between the total responding during CSs in the second extinction session (Extinction 2) compared to the last acquisition session (Acquisition 5) for males and females in both conditions ($t(28)$=-11.957, $p < 0.001$). During the second extinction session there were no differences in responding across groups ($p > 0.05$). During the first extinction session (Extinction 1), an ANOVA for Sex and Group found significant effect of Sex by Group interaction ($F(25,1)$=7.151, $p < 0.05$), which was due to significantly lower responding in females in experimental compared to control groups ($p > 0.05$). Responding was similar between males in

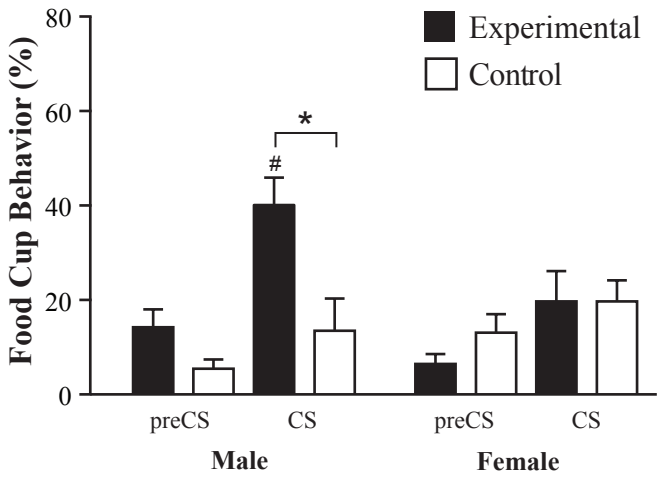

Figure 3.4 Conditioned responses during the test for renewal. Percentage of time rats expressed food cup behavior (mean ± SEM) during preCS and CS periods; * indicates p < .001, # indicates within-group preCS vs. CS difference $p < 0.05$.

control and experimental groups ($p > 0.05$). All groups showed similar, low responding in preCS periods during each extinction session ($p < 0.05$).

3.1.3 Renewal

During the test for renewal, only male groups showed differential responding (Figure 3.4). An ANOVA (Sex and Group) revealed a significant effect of Group by Sex on CRs during CSs ($F(1,25)=5.522$, $p < 0.05$) but no effect of Sex or Group ($p > 0.05$, both). *Post hoc* tests confirmed that the males in the experimental group had significantly more CRs compared to the males in the control condition (Male Control: 22.27 ± 7.6, Male Experimental: 40.18 ± 5.9; $p < 0.05$). Female rats in the control and experimental groups had similar low rates of responding, with no significant difference between the groups (Female Control: 19.73 ± 4.5, Female Experimental: 18.57 ±5.8; $p > 0.05$). Additionally, males in the experimental group had significantly higher responding than the females in the experimental group ($p < 0.01$), while controls of both sexes had similar, low

responding ($p > 0.05$). We also analyzed CRs during CS compared to preCS within each group. Paired *t*-test confirmed the male experimental group had higher CRs during the CS compared to the preCS ($t(6)=-3.779$, $p < 0.01$), while CRs in the male control group remained low during both preCS and CS periods ($p > 0.05$). Both female groups responded similarly low during preCS and CS ($p > 0.05$).

3.2 Fos Induction

Table 3.2 (on page 54) shows values for Fos induction in 17 cell groups analyzed within the medial prefrontal cortex, amygdala, hippocampal formation, and thalamus.

3.2.1 Medial Prefrontal Cortex

Fos induction patterns within the ILA and PL were similar and differed across conditions and sexes (Figure 3.6, on page 53). Within the ACA, there were no differences between groups ($p > 0.05$; Table 3.2). Within the ILA, an ANOVA for Sex and Group found significant effect for Sex by Group interaction ($F(23,1)=12.439$, $p < 0.01$; Figure 3.5, 3.6), but no effect of Sex or Group ($p > 0.05$, both). Total number of Fos-positive neurons in males was significantly higher in the experimental compared to the control group ($p < 0.05$). The pattern of Fos induction was opposite in females. Total number of Fos-positive neurons in the experimental group was significantly lower compared to the control group ($p < 0.05$). Furthermore, males in the experimental group had significantly higher Fos induction than females in the experimental group ($p < 0.05$) while male controls were no different compared to female controls ($p > 0.05$). Similar to the

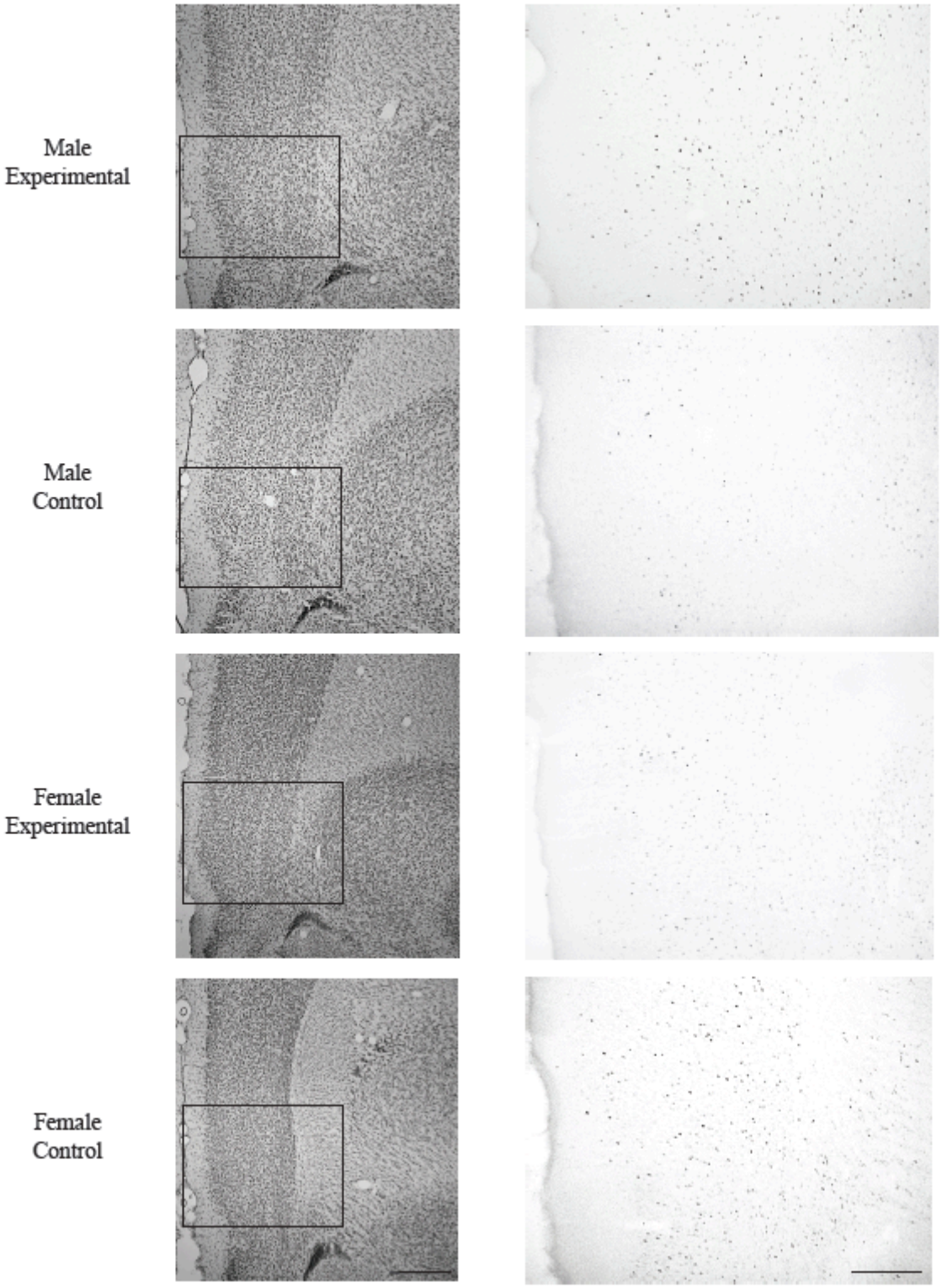

Figure 3.5 Fos induction in the medial prefrontal cortex. Representative photomicrographs of Fos induction in the infralimbic area (ILA) are shown on right (Scale bar = 200 μm), and thionin-stained adjacent sections are shown on left (level 9; Scale bar = 500 μm). Each box depicts the area shown in the corresponding Fos image.

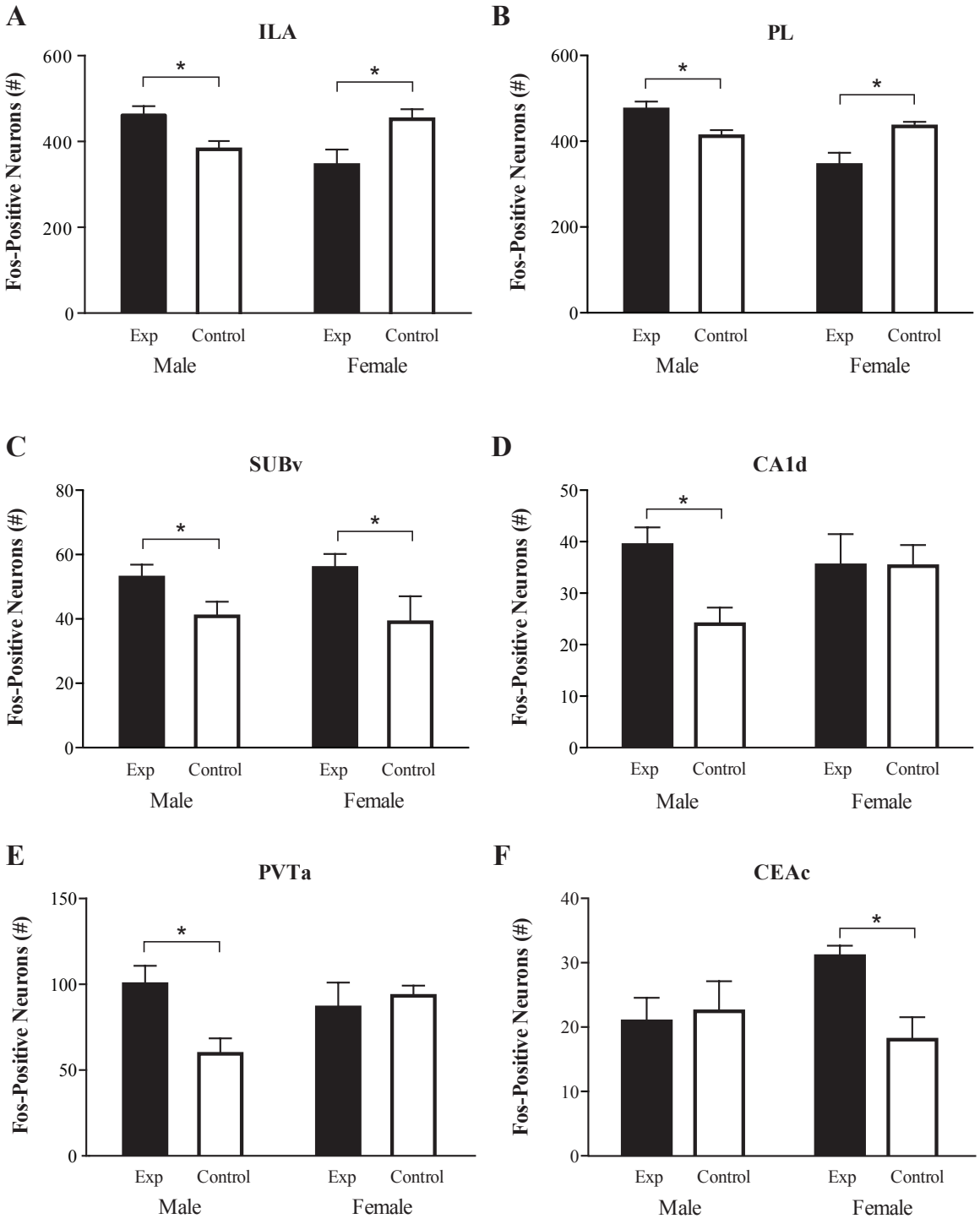

Figure 3.6 Fos induction. Total number of Fos-positive neurons in the infralimbic area, ILA (A), prelimbic area, PL (B), ventral subiculum, SUBv (C), dorsal CA1 (D), anterior part of the paraventricular nucleus, PVTa (E), capsular part of the central amygdalar nucleus, CEAc (F). * indicates $p < 0.05$.

Table 3.2 Fos induction for all brain regions analyzed. Results are displayed as a mean total number of Fos-positive neurons in each area ± SEM. * denotes significant difference compared to same sex control, $p < 0.05$.

Brain Region	Male		Female	
	Experimental	*Control*	*Experimental*	*Control*
Prefrontal Cortex				
ILA	461 ± 22*	381 ± 20	345 ±36*	452 ± 23
PL	474 ± 19*	412 ± 14	344 ± 11*	434 ± 11
ACAd	365 ± 24	372 ± 30	401 ± 37	412 ±35
Hippocampus				
SUBv	53 ± 4*	41 ± 5	56 ± 4*	39 ± 8
CA1v	36 ± 3	40 ± 2	45 ± 3	42 ± 3
CA1d	39 ± 3*	24 ± 3	35 ± 4	35 ± 4
DGv	46 ± 5	40 ± 7	46 ± 7	51 ± 6
DGd	36 ± 4	34 ± 3	45 ± 6	45 ± 7
Amygdala				
BLAa	93 ± 4	96 ± 5	83 ± 4	93 ± 3
LA	39 ± 3	39 ± 2	41 ± 4	38 ±4
BLAp	13 ± 3	11 ± 2	17 ±4	14 ±1
BMAp	29 ± 3	34 ± 5	30 ± 2	27 ± 4
CEAm	11 ± 3	8 ± 2	9 ± 2	10 ± 2
CEAl	5 ± 1	3 ± 1	6 ± 2	6 ± 2
CEAc	20 ± 4	22 ± 5	30 ± 2*	18 ± 3
Thalamus				
PVTa	100 ± 10*	60 ± 9	87 ± 14	93 ± 6
PVTp	62 ± 5	49 ± 9	62 ± 9	66 ± 11

ILA, within the PL, there was a significant effect for Sex by Group interaction ($F(23,1)=12.439$, $p < 0.01$) and main effect of Sex ($F(23,1)=7.743$, $p < 0.05$), but there was no effect of Group ($p > 0.05$.) Within the PL total number of Fos–positive neurons was significantly higher in the experimental compared to the control group for males ($p < 0.05$), but was significantly lower in the experimental compared to the control group for females ($p < 0.05$). As in the ILA, males in the experimental group had significantly higher Fos induction than females in the

experimental group ($p < 0.01$) while male controls were no different compared to female controls ($p > 0.05$).

3.2.2 Hippocampal Formation

Two regions within the hippocampal formation had different Fos induction patterns across conditions or sexes: SUBv and the dorsal CA1 (Figure 3.6, Table 3.2). We found no differences in Fos induction patterns within the CA1v, DGv and DGd ($p > 0.05$ all; Table 3.2). Within the SUBv, experimental groups had higher Fos induction than controls for both sexes (Figure 3.6). An ANOVA of Fos induction by Sex and Group found a significant effect for Group ($F(22,1)=6.338$, $p < 0.05$), but no effect for Sex or Sex by Group interaction ($p > 0.05$, both). Males in the experimental group showed significantly higher responding than controls ($p < 0.05$), and there was a trend towards significance ($p = 0.078$) for female experimental group. Within the dorsal CA1, experimental males had higher Fos induction than control males, while there was no difference between female groups (Figure 3.6). An ANOVA of Fos induction by Sex and Group found no significant effects for Sex, ($p > 0.05$), while Group and Sex by Group were approaching significance (Group: $F(23,1)=2.933$, $p= 0.100$; Sex by Group: $F(23,1)=2.800$, $p = 0.108$). A *post hoc* test confirmed that experimental males had significantly higher Fos induction than control males ($p < 0.05$), while females in the experimental and control groups had similar levels of Fos induction ($p > 0.05$). Experimental males and females as well as control males and females did not differ ($p > 0.05$).

3.2.3 Amygdala

Within the amygdala we analyzed seven cell groups within the basolateral and central nuclei and found similar Fos induction patterns across experimental conditions and sexes in all areas (BLAa, CEAm, CEAl, LA, BLAa or BMAp, $p > 0.05$ for all; Table 3.2), except for the CEAc (Figure 3.6). Within the CEAc, the ANOVA approached significance (Sex by Group: $F(24,1)=3.945$, $p = 0.059$) and a *post hoc* test found this was due to higher Fos induction in experimental female compared to control female groups ($p < 0.05$). There were no differences in Fos induction between male experimental and control groups ($p > 0.05$). Females in the experimental group had significantly higher Fos than males in the experimental group ($p < 0.05$), while male controls were no different compared to female controls ($p > 0.05$).

3.2.4 Thalamus

Within the PVT, there were differences in Fos induction patterns between groups in PVTa (Figure 3.6) but not in PVTp ($p > 0.05$; Table 3.2). Within the PVTa, experimental males had higher Fos induction than control males, while there was no difference between female groups. An ANOVA for Sex and Group found a significant effect for Sex by Group interaction ($F(23,1)=5.242$, $p < 0.05$). There were no significant effects for Sex or Group ($p > 0.05$). A *post hoc* test confirmed that experimental males had significantly higher Fos induction than control males ($p < 0.05$), while females in the experimental and control groups had similar levels of Fos induction ($p > 0.05$). Males in the control group had significantly lower Fos than females in the control group ($p < 0.05$), while experimental males and females were not significantly different ($p > 0.05$).

3.2.5 Correlations

A Pearson correlation coefficient was computed for each region that had significant differences in Fos activation (ILA, PL, PVTa, SUBv, CA1d, CEAc) to assess the relationship between conditioned responding and Fos induction during test. Comparisons were made for responding during preCS and during CS, for each sex. In males, there was a significant positive correlation between responding during CS and Fos induction in the ILA ($r = 0.730$, $p < 0.01$) and PVTa ($r = 0.770$, $p < 0.01$). In females, the only significance was a negative correlation between CS responding and Fos induction was SUBv ($r = -0.795$, $p = 0.001$). There were no significant correlations between responding during preCS and Fos induction in any area for either sex, except in the SUBv, were responding in males was positively correlated ($r = .738$, $p > 0.01$) and females were negatively correlated ($r = 0.742$, $p > 0.01$).

4. Discussion

Here, we examined context-dependent renewal of conditioned responding to food cues and determined the recruitment of key forebrain regions in male and female rats. To accomplish this, we assessed Fos induction of 17 cell groups within areas important in associative learning and contextual processing: the medial prefrontal cortex, hippocampal formation, thalamus, and amygdala. We compared Fos induction patterns in males and females because a prior study found sex differences in this behavior. Male rats consistently exhibited context-dependent renewal of responding, while renewal in female rats was inconsistent in this preparation (Anderson & Petrovich, 2015). In the current study, we found

the vmPFC was recruited during renewal of responding in a sex specific way. Additionally, Fos expression differed between experimental and control groups and between the sexes in the hippocampal formation, thalamus and amygdala. Furthermore, sex specific Fos induction patterns suggest that during renewal a distinct medial prefrontal cortex-hippocampal-thalamic system is recruited differently in males and females.

4.1 Prefrontal Cortex

Within the vmPFC, we found selective recruitment of the PL and ILA, but not ACA, in a sex specific way. The patterns of Fos induction were well matched to the behavioral sex differences. Male rats in the experimental group that showed renewal of responding had more Fos induction in the PL and ILA, while females in the experimental condition did not show renewal or an increase in Fos induction. These results indicate that the vmPFC may be critical for context-dependent renewal in both sexes, and that differential recruitment in females may underlie the lack of behavioral responding. In that regard, in females there was more Fos induction in the control compared to the experimental groups. This suggests that during renewal the vmPFC is utilized differently in female than in male rats. Notably, for the animals in the control condition, the test for renewal is an additional extinction session and thus the impairment in the renewal in females may reflect impairments in extinction recall (Farrell et al., 2013; Lebron-Milad and Milad, 2012).

The current findings in males are in agreement with prior evidence that the medial prefrontal cortex is critical in appetitive tasks with food and drug reward,

including renewal of responding (for review see Moorman, James, McGlinchey, and Aston-Jones, 2015). Inactivation of the vmPFC disrupted context-mediated reinstatement of alcohol and drug seeking (Bossert, Stern, Theberge, Cifani, Koya, Hope, and Shaham, 2011; Willcocks and McNally, 2013), and reduced ABA renewal of instrumental responding to a sucrose reinforcer (Eddy et al., 2016). Specific ILA lesions enhanced renewal of responding compared to sham-lesioned animals (Rhodes and Killcross, 2007). Additionally, the PL and the ILA, but not the ACA, were recruited during appetitive (tone-food) associative learning (Cole et al., 2015a) and this area is also critical for feeding stimulated by contextual food cues (Petrovich, Ross, Gallagher, and Holland, 2007). Finally, the medial PFC has been implicated in renewal of conditioned fear (Herry and Mons, 2004; Knapska and Maren, 2009).

The vmPFC is structurally and functionally complex and there is strong support for distinct functions of its subregions in aversive associative behaviors, the PL in the expression and the ILA in the suppression of conditioned fear (Quirk, Russo, Barron, and Lebron, 2000). Whether their functions are similarly dissociable in appetitive associative tasks, particularly with food reward, however, is much less clear. In addition to this ‘go/stop’ framework there is evidence for their dissociable roles in goal-directed (PL) vs. habitual (ILA) tasks (for review see Balleine and O'Doherty, 2010; Smith, Virkud, Deisseroth, and Graybiel, 2012). As discussed in the recent, comprehensive review, their functions in reward-mediated behaviors are more complex than either dichotomy model predicts (Moorman et al., 2015).

There is also evidence for differential roles of the PL and ILA during extinction and renewal of responding. In an appetitive Pavlovian task, inactivation of the ILA, but not the PL, facilitated extinction (Mendoza, Sanio, and Chaudhri, 2015) and studies of renewal with aversive tasks found differences between the PL and ILA (Knapska and Maren, 2009). Selective manipulations of the PL or ILA during context-induced reinstatement of alcohol seeking suggest the PL may be required for retrieval of the learned associations underlying responding, while the ILA is important for contextual information processing (Willcocks and McNally, 2013). Inactivation of PL, but not ILA, attenuated reinstatement and augmented cue-drug reacquisition, while inactivation of the ILA had no effect on reinstatements or reacquisition but increased responding latency in the extinction context (Willcocks and McNally, 2013).

In the current study, we observed similar Fos induction across the PL and ILA, which suggests a common function. In agreement, stimulation of μ-opioid receptors in the neurons across the PL and ILA regions induced feeding (Mena, Selleck, and Baldo, 2013), while lesions that encompassed similar area abolished feeding stimulated by contextual appetitive cues (Petrovich et al., 2007). In addition to appetitive renewal, similar patterns of activation in PL and ILA inputs from the ventral hippocampal neurons were found during fear renewal (Wang, Jin, and Maren, 2016). Nevertheless, similar recruitment of the PL and ILA during context-mediated renewal of responding to food cues in the current study may be due to different functions, extinction recall or expression of renewed conditioned responding. Furthermore, whether the observed Fos

induction patterns were due to causal functions of each area, and whether the same types of neurons were recruited (projecting glutamatergic, or local inhibitory) is unknown and will require further investigation.

4.2 Hippocampal formation

In the hippocampal formation, we found Fos induction differences within the ventral and dorsal regions. Within the SUBv, Fos induction patterns were similar in males and females. In both sexes, experimental groups had more Fos compared to control groups. Within the dorsal CA1, in males, the experimental group had higher Fos induction compared to the control, while both females groups had similar amounts of Fos induction. These findings for differential recruitment within the SUBv and CA1s is important given the structural and functional distinction along the dorso-ventral axis of the hippocampal formation (Fanselow and Dong, 2010). The dorsal parts of the hippocampal formation are more directly connected to areas related to cognitive processes of learning and memory, while the ventral parts are more connected to areas mediating motivational and emotional behavior. The ventral hippocampal formation has previously been implicated in context-induced fear renewal (Hobin, Ji, and Maren, 2006). Furthermore, the SUBv has direct connections to the vmPFC, specifically the PL (Canteras and Swanson, 1992; Fanselow and Dong, 2010), and ventral hippocampal neurons, including SUBv, that project to both the vmPFC and amygdala were specifically activated during fear renewal (Herry, Ciocchi, Senn, Demmou, Muller, and Luthi, 2008; Jin and Maren, 2015). The current findings for similar SUBv recruitment in males and females together with

differential recruitment of the vmPFC, suggests SUBv-vmPFC connections may be crucial in renewal of appetitive responding in a sex specific way.

The dorsal hippocampal formation, including the dorsal CA1, has been implicated in renewal of appetitive and aversive conditioned responding. Interestingly, spontaneous recovery, but not context-depended renewal, of appetitive conditioned responding was impaired by inactivation of this area (Campese & Delamater, 2014). Nevertheless, lesions and inactivation of the dorsal hippocampal formation, including the dorsal CA1, impaired context-dependent renewal (ABA, or ABC) of conditioned fear responses in some studies (Corcoran and Maren, 2001; Ji and Maren, 2005) while in another study lesions impaired reinstatement induced by exposure to the US, but not context-dependent renewal, of fear (Frohardt, Guarraci, and Bouton, 2000). The current findings suggest the dorsal CA1 may be important in appetitive context-mediated renewal. Furthermore, differences in males and females suggest that the dorsal CA1 may be influencing the vmPFC, directly and via SUBv, in a sex specific way during renewal of responding (Cenquizca and Swanson, 2007).

4.3 Thalamus

Within the PVT we analyzed its anterior and posterior parts separately and found significant activation and sex differences specifically in the anterior part. In males, Fos induction was higher in the experimental compared to the control males, similar to Fos patterns in the vmPFC. In females, both groups had similar amounts of Fos induction. These findings are in agreement with prior evidence for the role of the PVT in appetitive renewal. The PVT was recruited during

context-dependent and cue-induced reinstatement of alcohol seeking (Marchant, Furlong, and McNally, 2010; Wedzony, Koros, Czyrak, Chocyk, Czepiel, Fijal, Mackowiak, Rogowski, Kostowski, and Bienkowski, 2003) and lesions or inactivation of the PVT prevented context-dependent renewal of alcohol seeking (Hamlin et al., 2009). In addition, the PVT is important in the regulation of food consumption. PVT lesions increased food consumption and body weight (Bhatnagar and Dallman, 1999), and the PVTa was selectively recruited, along with the vmPFC, when cue-induced feeding was blocked with ORX antagonist (Cole et al., 2015b).

4.4 Amygdala

Within the amygdala, the CEAc was the only area analyzed with differential Fos induction. Females in the experimental group had higher Fos induction compared to the controls, while males groups had similar amounts of Fos induction; however, the overall number of cells in the CEAc was low, and thus these results should be considered with caution. Interestingly, this is the only area were we found differential induction in females but not in males. The CEAc receives direct projections from the SUBv (Canteras and Swanson, 1992), where we found high Fos induction in experimental groups of both sexes. Whether the SUBv-CEAc connections are differently recruited in males and females and their role in appetitive renewal circuitry in female rats remains to be determined.

Lack of differential recruitment within the basolateral area of the amygdala was surprising given its importance in various appetitive conditioning tasks, including renewal (Cole et al., 2015a; Cole et al., 2013; Crombag, Bossert, Koya,

and Shaham, 2008; Holland and Petrovich, 2005; Petrovich, 2013; Wassum and Izquierdo, 2015). Furthermore, the basolateral area of the amygdala is part of the circuitry with the ventral hippocampal formation and the PL required for the renewal of extinguished fear (Herry et al., 2008; Orsini et al., 2011). It is important to note that the current study examined recruitment of distinct nuclei of the basolateral amygdala but not specific cell types or their connections, which may be the reason for the lack of significant differences in recruitment. Even though overall Fos induction patterns did not differ, specific pathways including the connections with the vmPFC, SUBv, and PVTa may be critical in context-mediated renewal of responding to food cues.

4.5 Circuitry

Importantly, the areas specifically recruited during renewal form a circuitry via complex, interconnected pathways (Figure 3.7 on next page). The vmPFC has distinct, topographically organized connectional patterns with the amygdala, the ventral hippocampal formation, and PVT (Fanselow and Dong, 2010; Hoover and Vertes, 2007; Li and Kirouac, 2012; Moga, Herbert, Hurley, Yasui, Gray, and Saper, 1990; Petrovich, Canteras, and Swanson, 2001; Pitkanen, Pikkarainen, Nurminen, and Ylinen, 2000; Reppucci and Petrovich, 2015; Sesack, Deutch, Roth, and Bunney, 1989; Swanson and Petrovich, 1998). For example, while both the PL and ILA have outputs to PVTa, the projections from the PL to the PVTa are denser (Li and Kirouac, 2012). Additionally, SUBv projects to both the

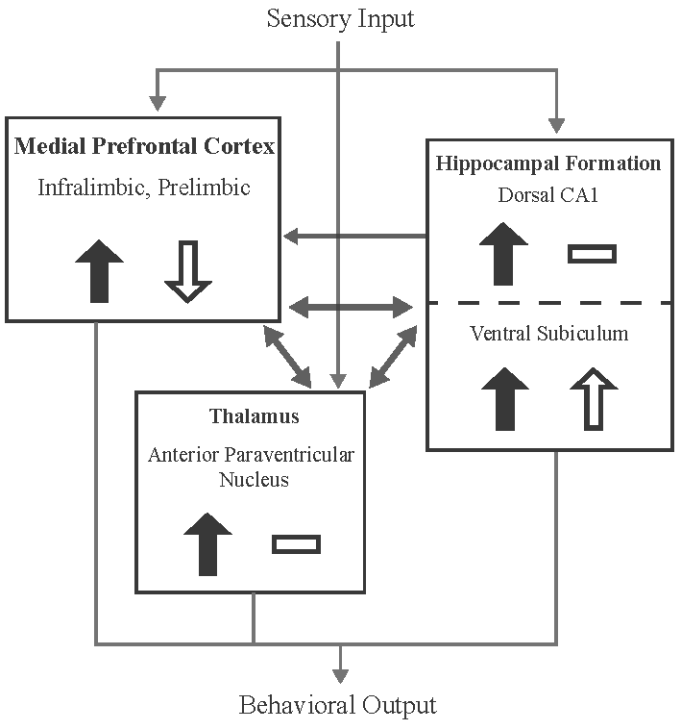

Figure 3.7 Medial prefrontal cortex-hippocampal-thalamic system recruitment during context-mediated renewal of conditioned responding to food cues. Summary of major Fos induction differences in males and females are shown on a connectional diagram. Males are represented by filled arrows and females are represented by open arrows. Arrow up indicates higher Fos induction compared to same sex control, arrow down indicates lower Fos induction, line indicates no change. See text for details and for additional areas, including amygdala.

PL and ILA (Canteras and Swanson, 1992). The SUBv also has direct projections to the PVT (Canteras and Swanson,1992), which in turn projects to the vmPFC (Hoover and Vertes, 2007). Therefore, the PVTa is well positioned to communicate with the vmPFC and SUBv via reciprocal connections (Hoover & Vertes, 2007; Li & Kirouac, 2012). There are also sparser projections to the PL (Hoover and Vertes, 2007). Given the well-known function of the hippocampal formation in contextual processing (Fanselow, 2000; Holland and projections from the dorsal CA1 to the ILA, with slightly Bouton, 1999) the recruitment we observed during the test for renewal may reflect contextual information relay

through direct SUBv projections to the PVTa and vmPFC. Together, the current findings suggest this circuitry is recruited in a sex specific way during appetitive context-dependent renewal.

4.6 Correlations between CS-specific behavior and Fos induction

In the current preparation, inherent to the ABA design, the experimental group experiences “context switch”, in order to induce renewal at test. Experimental groups are returned to the acquisition context at test, after extinction in a novel context, while control groups remain in the same context for all training and testing. Importantly, both experimental and control groups are tested in the same context (the acquisition context) and are given the same number of CSs. Thus, the behavioral differences in renewal of conditioned responding during the test are the most perspicuous cause for differences in Fos induction. Nevertheless, Fos induction due to the context switch alone cannot be ruled out completely. To assess whether Fos induction was specific to renewal behavior, we examined correlations between conditioned responding during preCS and CS and Fos induction in each sex. Specific renewal behavior (CRs during CSs) was positively correlated with Fos induction in the medial prefrontal cortex (ILA) and PVTa in males. Females did not show this behavior, or an increase in Fos in these areas, and there was only a negative correlation between the CRs and Fos induction in the SUBv. There were no correlations between Fos induction and the preCS responding in any area, except the SUBv, where there were sex-specific patterns (positive correlations in males, and negative correlations in females).

These findings suggest the most robust Fos induction in the current study (ILA and PVTa) reflects renewal of responding.

4.7 Sex Differences

This is the first evidence of neural sex differences in appetitive Pavlovian renewal. Nevertheless, these findings are in agreement with prior evidence for sex differences in associative learning and contextual processing and there is an overlap in the underlying neural substrates between current and prior findings (for review see Dalla and Shors, 2009). Sex differences were found in contextual fear conditioning and those differences were correlated with changes in hippocampal long-term potentiation (Maren et al., 1994). Males showed greater levels of conditioned contextual fear and faster acquisition of this learning in comparison to females, and had higher magnitude of long-term potentiation induction in the hippocampus, specifically in the perforant path. In an aversive learning task that is regulated differently by stress in males and females, neuronal activity in the medial prefrontal cortex was necessary to induce stress mediated suppression of conditioned eye blinking in females, but was not necessary for stress effects on learning in males (Maeng, Waddell, and Shors, 2010). This function depends specifically on the PL and its connections with the amygdala (Maeng and Shors, 2013).

Previously, we found estradiol mediates context-dependent renewal in females. Related to the current findings, the vmPFC, hippocampal formation and PVT all contain estrogen receptors (Almey, Cannell, Bertram, Filardo, Milner, and Brake, 2014; Khayum, de Vries, Glaudemans, Dierckx, and Doorduin, 2014;

Simerly, Chang, Muramatsu, and Swanson, 1990). Estradiol can affect memory via changes in dendritic spine density in the vmPFC and the dorsal hippocampal formation (for review see Frankfurt and Luine, 2015; Inagaki, Frankfurt, and Luine, 2012; Wallace, Luine, Arellanos, and Frankfurt, 2006). Estradiol administration in the dorsal hippocampal formation increased spine density on the pyramidal vmPFC neurons (Tuscher, Luine, Frankfurt, and Frick, 2016). Another mechanism of estradiol action in renewal may be through changes in the efficacy of dopamine transmission in the PFC (Rey, Lipps, and Shansky, 2014), as D1 receptors within the ILA are critical to successful extinction (Hikind and Maroun, 2008). Lastly, estradiol was shown to change serotonergic neural transmission in the PVT in females (Krajnak, Rosewell, Duncan, and Wise, 2003), which may be another mechanism underlying sex differences in renewal behavior and the Fos induction differences observed here.

5. Conclusions

We identified distinct, sex specific recruitment of a medial prefrontal cortex-hippocampal-thalamic system during context-dependent appetitive renewal. The differential recruitment corresponds to the behavioral differences between males and females during renewal of appetitive Pavlovian responding to food cues and suggests the vmPFC-SUBv-PVTa system is a critical site of sex differences. These findings should improve our understanding of the fundamental neural mechanisms underlying sex differences in food motivation and contextual processing in associative learning and memory. The results should also have a broad impact on future molecular and translational work investigating sex

differences and maladaptive eating habits.

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Chapter 4: Ventromedial prefrontal cortex mediates sex-specific persistent cognitive drive for food*

**Submitted Manuscript:*
Anderson, L.C. & Petrovich, G.D. (2017) Ventromedial prefrontal cortex mediates sex-specific persistent cognitive drive for food. Scientific Reports.

Abstract: Contemporary environments are saturated with food cues that stimulate appetites in the absence of hunger, which leads to maladaptive eating. These settings can induce persistent drive to eat, as learned behaviors can reappear after extinction. Renewal of responding paradigms provide a valuable framework to study how food cues contribute to the inability to resist palatable foods and change maladaptive eating habits. Here, using a rat model for this persistent food motivation, we determined a sex-specific causal function for the ventromedial prefrontal cortex (vmPFC) during context-mediated renewal of responding to food cues. Previously, we found sex differences in renewal (males exhibited renewal of responding, females failed to do so) and differential recruitment within the vmPFC (increased Fos induction in males but decreased in females). We used DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) to silence vmPFC neurons in males and to stimulate vmPFC neurons in females specifically during renewal. Silencing vmPFC neurons in males disrupted renewal of responding to a food cue, while stimulating vmPFC neurons in females induced this behavior. The findings demonstrate a sex-specific function of the vmPFC in a model of food seeking relevant to environmentally driven appetites and sex differences in obesity and other eating disorders.

Introduction

Persistent food cravings and the inability to resist palatable foods even when eating is maladaptive is a hallmark of overeating dysregulation. Food cues are an important contributor to these excessive drives [1]. Cues previously associated with food can stimulate appetite and food consumption in the absence of hunger, which can lead to maladaptive overeating [2-5]. Food cue exposure, and associated cravings, significantly influence eating behavior and weight gain in humans [6] and neural responding to food cues in sated states has been correlated with long-term weight gain [7]. Cognitive processes mediating learned behaviors after extinction, such as renewal, might explain the difficulty associated with changing unhealthy eating habits and weight control in our environment [1,8].

The renewal (reinstatement) model was recently introduced as a framework to study mechanisms of relapse to palatable food seeking, similar to the reinstatement model for relapse of drug use [1,9,10]. One important renewal mechanism is mediated by context; Environments (contexts) previously associated with food can later stimulate consumption as conditioned stimuli [11]. Importantly, these contexts also mediate memory formation and later recall of discrete cues (e.g., a tone or light), particularly when those cues have different meaning in different contexts. When a cue is paired with food in one context but not in another, context is 'setting the occasion' for cue's meaning—signaling food or not signaling food [12]. In that preparation acquisition of a cue-food association and extinction of that association occurs in different contexts, and then the renewal of conditioned behavior (food seeking) is induced by return to the

acquisition context.

Context-mediated renewal is a well-suited behavioral model for persistent food motivation, relevant to our contemporary lifestyle and insatiable appetites. A sharp rise in obesity has occurred over the last 30 years, and during the same period there have been profound changes in the locations where we consume food. The number of meals consumed outside the home has substantially increased, and they occur in distinct places such as fast-food restaurants [13]. Palatable foods are now so widely available that a very few locations in our environments are not associated with food and eating.

We posit renewal is a decision-making process that involves retrieval, evaluation, and use of context-dependent cue memory to guide responding and that this function requires ventromedial prefrontal cortex (vmPFC) activation. Here we aim to establish the necessity of vmPFC and that lack of vmPFC activation underlies sex differences in context-dependent renewal of responding to Pavlovian food cues. Identifying the vmPFC as a critical site of sex-specific function will further our fundamental understanding of contextual processing and learning and memory in both sexes, as well as clinical treatment of excessive cognitive drive to eat. Women are more likely than men to be overweight and obese, and obese women show greater impairments in food associative learning [14-17]. There are also sex differences in animal models of associative learning, particularly with contextual processing [18-23] that underscores the importance of determining mechanisms in both sexes. Still, female subjects are underrepresented in both basic and clinical research [24,25]. Therefore, recently we

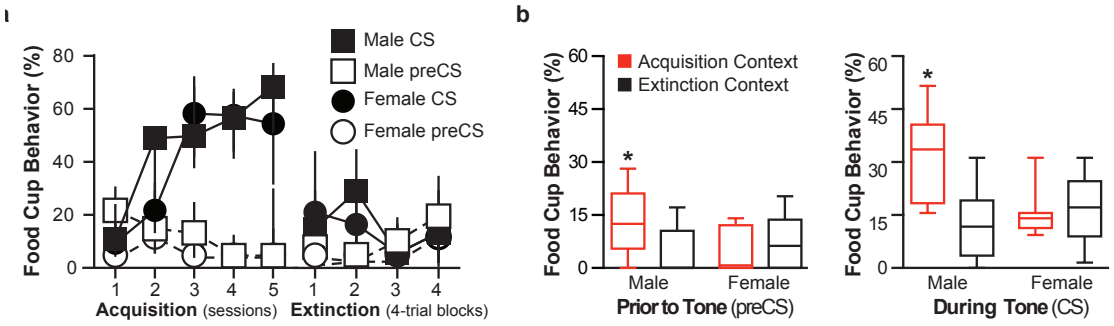

Figure 4.1 Conditioned responses during acquisition, extinction and tests for renewal in Experiment 1. (**a**) Percentage of time rats expressed food cup behavior (median, error bars=inner quartiles) during the preCS (open) and CS (filled) periods across training sessions. Acquisition is shown as the average responding during each session. Extinction is shown as the average responding in 4-trial blocks (2 blocks per session; blocks 1&2 were trials during Session 1 and blocks 3&4 during Session 2). Acquisition and extinction training occurred in different contexts. Squares represent males; circles represent females. (**b**) Percentage of time rats expressed food cup behavior (box plots showing min, inner quartiles, median, and max) during the period prior to the tone (preCS) and during the tone (CS) in the Acquisition (shown in red) and Extinction Contexts (shown in black). n=8 for both males and female groups.

began to investigate both sexes. We found sex differences in context-mediated renewal of responding to Pavlovian food cues [22], and distinct recruitment of the vmPFC in male and female rats. Increased Fos induction within the infralimbic and prelimbic areas in the vmPFC was associated with renewal behavior in males, while females did not show renewal or an increase in Fos induction. These distinct patterns suggest sex-specific vmPFC activation mediates behavioral sex differences; however, Fos induction does not address causality.

Results

Experiment 1

To directly test the causal function of the vmPFC, we used DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) [26] to silence vmPFC neurons in males and activate vmPFC neurons in females during renewal tests.

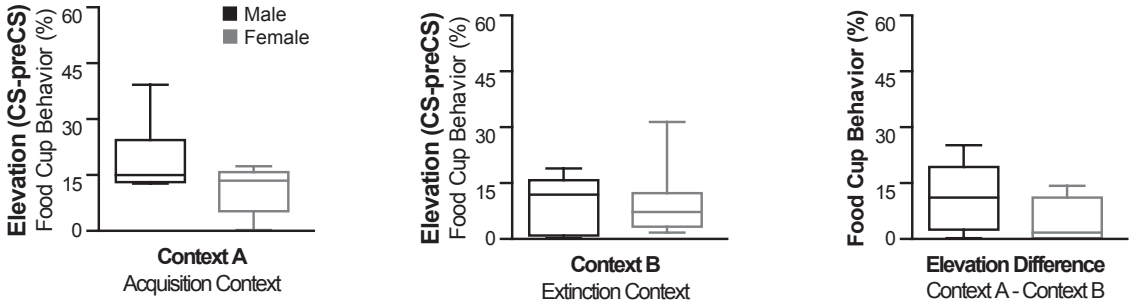

Figure 4.2 Conditioned responding in male and female rats during renewal tests in Experiment 1. Elevation scores represent responding (food cup behavior) during CS minus preCS in the Acquisition Context, Extinction Context and the Elevation Difference (Elevation score in the Acquisition context minus Elevation score in the Extinction context). There were no differences between the sexes ($p > 0.05$). n=8 for both males (shown in black) and females (shown in gray). Box plots show min, 25th percentile, median, 75th percentile and max.

We first established a within-subjects ABA renewal procedure (Methods, Fig. 4.1 on previous page, 4.2). All rats received acquisition training in a distinct context, consisting of tone (conditioned stimulus; CS) and food (unconditioned stimulus; US) pairings and then extinction training, consisting of CS presentations alone in a different context. Rats were then tested for food seeking conditioned responding (CR; food cup behavior) during CSs in each context. Higher responding during the CS in the acquisition context (vs. the extinction context) indicates successful renewal. We replicated the sex differences found with a between-subjects ABA paradigm [22,23]. Both sexes showed similar acquisition and extinction learning (Fig. 4.1a). At test, males showed significantly higher responding in the acquisition context compared to the extinction context during the CS, while females showed similar low responding in both contexts (Fig. 4.1b). A mixed-design ANOVA (repeated (context) and between (sex)) found a significant effect for Context by Sex interaction ($F(1,14)=19.483$, $p=.001$)). *Post hoc* two-tailed *t*-test determined males showed significantly higher responding in

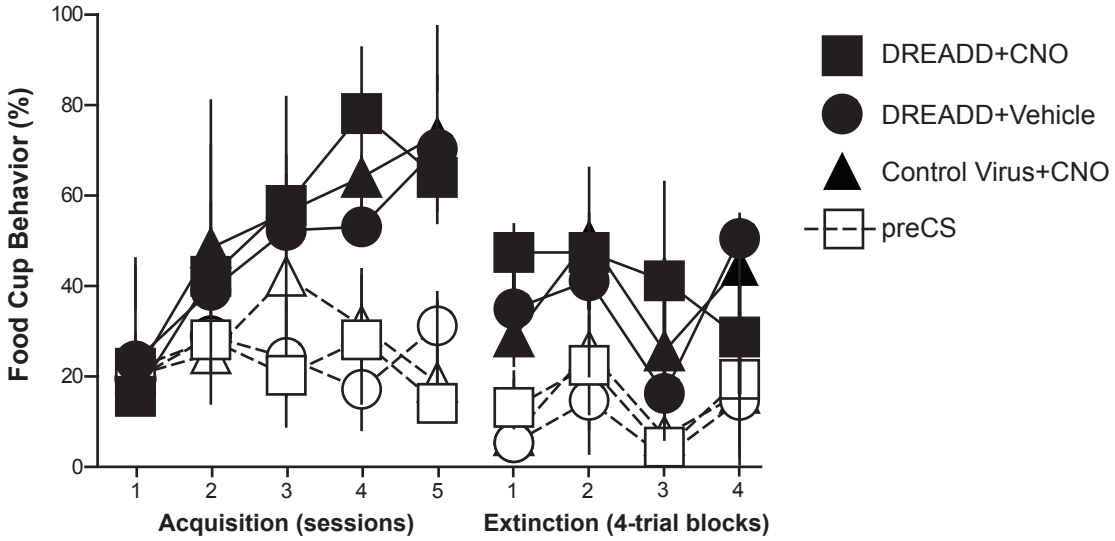

Figure 4.3 Conditioned responses during acquisition and extinction in Experiment 2. Percentage of time male rats expressed food cup behavior (median, error bars=inner quartiles) during the preCS (open) and CS (filled) periods across training sessions. Acquisition is shown as the average responding during each session. Extinction is shown as the average responding in 4-trial blocks (2 blocks per session; blocks 1&2 were trials during Session 1 and blocks 3&4 during Session 2). Acquisition and extinction training occurred in different contexts. DREADD+CNO group (n=7) represented with squares, DREADD+vehicle (n=6) represented with circles, Control Virus+CNO (n=5) represented with triangles. There were no group differences during Acquisition or Extinction (p>0.05).

the Acquisition context ($p < 0.05$). Males also responded significantly higher during preCS in the acquisition compared to extinction context, while females had low responding in both contexts (mixed-design ANOVA (repeated (context) and between (sex)) significant for Test Context by Sex interaction ($F(1,14)=10.286$, p=.006); *post hoc* two-tailed t-test ($p < 0.05$)). Additional two-tailed t-test determined males showed significantly higher responding during the CS compared to preCS period in Acquisition context ($t(7)=-4.257$, p=0.004). The elevation (CS-preCS responding) difference in responding in the acquisition vs. extinction context was also higher for males compared to females but that difference was not statistically significant (Fig. 4.2).

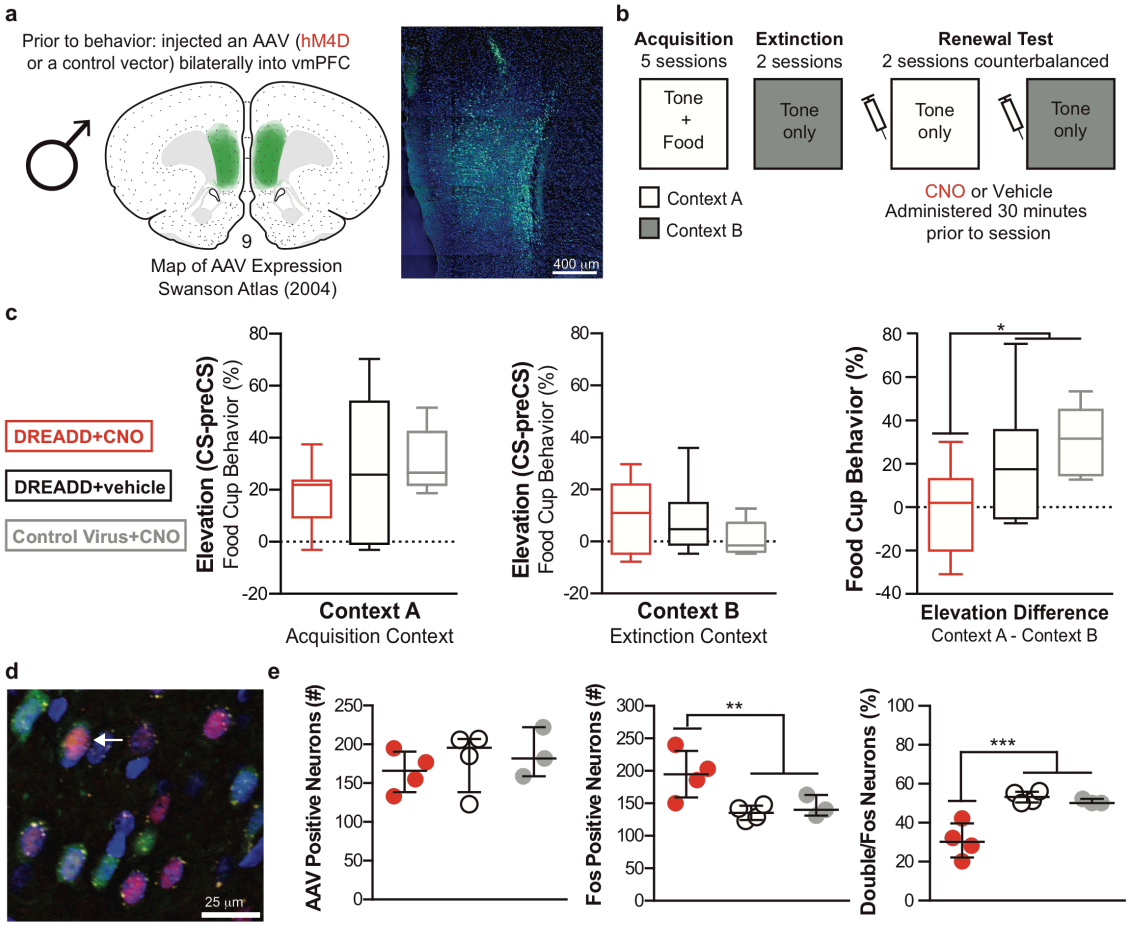

Figure 4.4 Chemogenetic vmPFC silencing disrupts renewal responding in males. (**a**) Expression of AAV5-hSyn-HA-hM4D-IRES-mCitrine or AAV5-hSyn-EGFP (Control Virus) into the vmPFC of male rats. Modified image from Swanson atlas (2004) (**b**) Experimental design. (**c**) Conditioned responding during renewal tests (**d**) Representative image of AAV positive (green), Fos positive (magenta), and double labeled cells (arrow). (**e**) Quantification of AAV and Fos positive neurons in vmPFC. DREADD+CNO group (n=7 behavior, n=4 Fos) in red, DREADD+vehicle (n=6 behavior, n=4 Fos) in black, Control Virus+CNO (n=5 behavior, n=3 Fos) in gray. Box plots show min, 25th percentile, median, 75th percentile and max. Scatterplots show individual data points (dots) with median and inner quartiles (line and error bars). *p < 0.05, **p < 0.01, ***p < 0.001.

Experiment 2

To silence the vmPFC in males, we infused an adeno-associated virus (AAV) containing the gene for an inhibitory Gi-coupled hm4Di receptor into the vmPFC (prelimbic and infralimbic areas) to express DREADD receptors (Fig. 4.4a). DREADD-selective, biologically inert ligand, clozapine-N-oxide (CNO) was

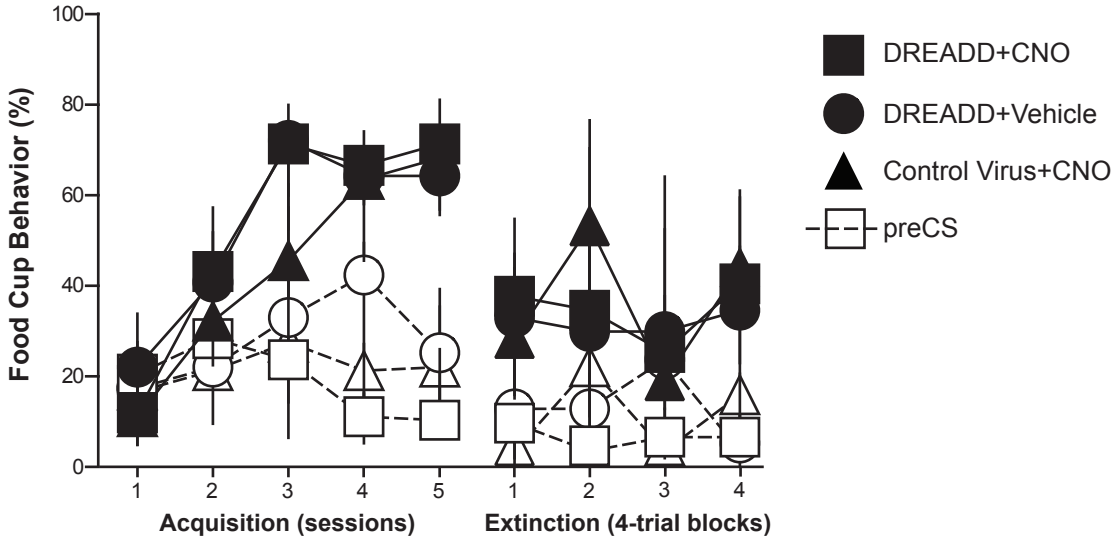

Figure 4.5 Conditioned responses during acquisition and extinction in Experiment 3. Percentage of time female rats expressed food cup behavior (median, error bars=inner quartiles) during the preCS (open) and CS (filled) periods across training sessions. Acquisition is shown as the average responding during each session. Extinction is shown as the average responding in 4-trial blocks (2 blocks per session; blocks 1&2 were trials during Session 1 and blocks 3&4 during Session 2). Acquisition and extinction training occurred in different contexts. DREADD+CNO group (n=8) represented with squares, DREADD+vehicle (n=5) represented with circles, Control Virus+CNO (n=6) represented with triangles. There were no group differences during Acquisition or Extinction (p>0.05).

injected i.p. prior to testing, which selectively silenced the infected neurons. Rats in the 3 groups: DREADD+CNO, DREADD+Vehicle, Control Virus (AAV with EGFP-only)+CNO (Fig. 4.4b) acquired and extinguished CRs to the tone similarly (Fig. 4.3 on page 81). On tests, the DREADD+CNO group failed to show renewal responding with similar low responding to CSs in both contexts, while DREADD+Vehicle and Control Virus+CNO groups showed renewal responding with higher induction of CRs to the CSs in the acquisition context (Fig. 4.4c). A planned orthogonal contrast of the elevation difference (t(15)=2.248, p= 0.040) confirmed that silencing vmPFC neurons disrupted renewal behavior in males.

Responding during preCS remained low and did not differ between groups (p > 0.05). We verified hM4Di inhibition of vmPFC neurons with a significantly lower percentage of double-labeled AAV+Fos neurons/Fos labeled neurons in the DREADD+CNO group (Fig. 4.4d-e; planned orthogonal contrast, t(8)=5.616, p=0.001). Interestingly, the DREADD+CNO group also had significantly higher total number of Fos positive neurons (Fig. 4.4e; planned orthogonal contrast, t(8)=-3.429, p=0.009) possibly due to disinhibition of neurons receiving inhibitory inputs from the hM4Di infected neurons.

Experiment 3

To activate vmPFC neurons in females, we infected vmPFC neurons with AAV containing hM3Dq vector and then trained the rats in the same behavioral design as described above (Fig. 4.6a on next page; Experiment 3). All rats acquired and extinguished CRs similarly (Fig. 4.5 on previous page). At test, the DREADD+CNO group had higher CRs to the CSs in the acquisition context (Fig. 4.6c; planned orthogonal contrast of the elevation difference t(16)=-2.528, p=0.022.), while control groups had similar responding to the CSs in both contexts. Responding during preCS remained low and did not differ between groups (p > 0.05). Stimulating vmPFC neurons in females induced renewal of behavior, thereby eliminating behavioral sex differences. We verified hM3Dq effectiveness with a significantly higher percentage of double AAV+Fos labeled neurons as a proportion of total AAV-labeled neurons in the DREADDs+CNO group compared to controls (Fig. 4.6d-e; planned orthogonal contrast, t(7)=-4.787, p=0.002.), as well as significantly higher number of double-labeled

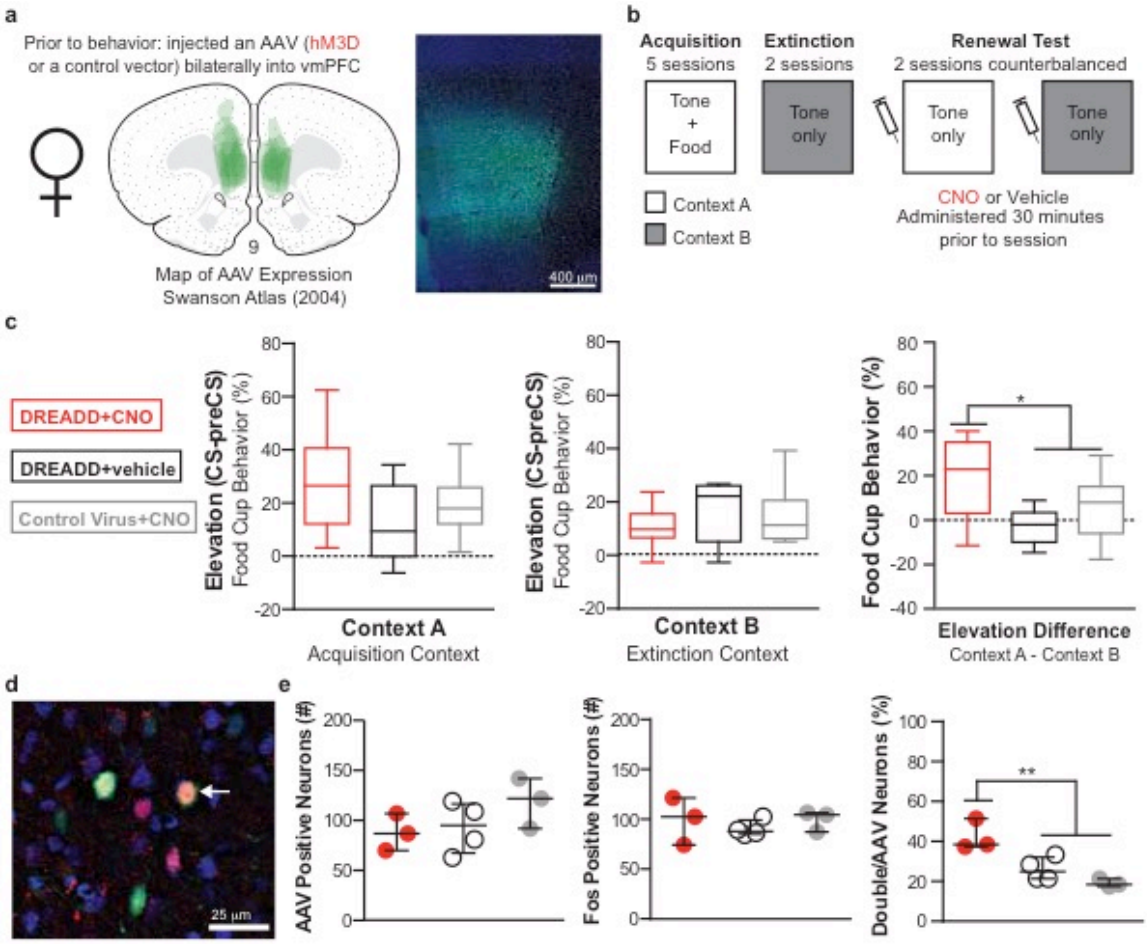

Figure 4.6 Chemogenetic vmPFC stimulation induces renewal responding in females. (**a**) Expression of AAV5-hSyn-HA-hM3D-IRES-mCitrine or AAV5-hSyn-EGFP (Control virus) into the vmPFC of female rats. Modified image from Swanson atlas (2004) (**b**) Experimental design. (**c**) Conditioned responding during renewal tests; (**d**) Representative image of AAV positive (green), Fos positive (magenta), and double labeled cells (arrow). (**e**) Quantification of AAV and Fos positive neurons in vmPFC. DREADD+CNO group (n=8 behavior, n=3 Fos) in red, DREADD+vehicle (n=5 behavior, n=4 Fos) in black, Control Virus+CNO (n=6 behavior, n=3 Fos) in gray. Box plots show min, 25th percentile, median, 75th percentile and max. Scatterplots show individual data points (dots) with median and inner quartiles (line and error bars). *p < 0.05, **p < 0.01.

neurons (planned orthogonal contrast, $t(7)$=- 3.649, p=0.008), and a higher

percentage of double labeled neurons as a proportion of total Fos-labeled

neurons (planned orthogonal contrast, $t(7)$=-2.526, p=0.039).

Discussion

The current findings are the first evidence that manipulating the vmPFC activity can impact behavioral sex differences. Chemogenetic silencing vmPFC neurons in males disrupted context-mediated renewal of cue driven food seeking while stimulating vmPFC neurons in females induced renewal of this behavior demonstrating the vmPFC activity is crucial for renewal in both sexes. These results reveal a sex-specific function of the vmPFC in context-mediated renewal, a model of persistent food seeking relevant to environmentally driven appetites. This function is in agreement with prior evidence for the vmPFC's role in the control of appetitive behaviors by associative cues in males. The vmPFC is recruited during cue-food associative learning [27], is critical for context-mediated renewal of instrumental responding for drug and food [28-30], and feeding stimulated by contextual food cues [31]. These novel results are expected to propel future molecular and translational work investigating sex differences in associative learning and memory and how learned cues contribute to maladaptive eating habits and eating disorders. This work points to the vmPFC as a novel neural target for therapeutic intervention and sex-specific treatment relevant to sex differences in obesity and maladaptive eating behaviors [16]. More generally, this work suggests dissociable vmPFC functioning in males and females in decision-making processes that guide our behavior[32,33].

Methods

Subjects. Adult male and female Long-Evans rats (250-275 g at arrival; total n=88; Charles River Laboratories) were individually housed and maintained

on a 12 h light/dark cycle (lights on at 7:00). Males and females were housed in separate colony rooms. 16 rats (8 males, 8 females) were used for Experiment 1. 36 males were used for Experiment 2; 36 females were used for Experiment 3. 35 rats total were excluded for the following reasons: poor health (1 male from Experiment 2, 1 female from Experiment 3), misplaced/insufficient viral expression (17 males from Experiment 2, 15 females from Experiment 3), or high preCS responding (1 female from Experiment 3; preCS responding higher than 3 standard deviations from the mean). Subjects in Exp. 2 and 3 underwent surgery after they were acclimated to the colony room for two days. Animals were given a week to recover post-surgery during which they were weighed and handled daily. All rats (Exp. 1-3) were given *ad libitum* access to standard laboratory chow (18% Protein Rodent Diet #2018, Harlan Teklad Global Diets, Madison, WI) and water, except as otherwise noted. All housing and testing procedures were in compliance with the National Institute of Health Guidelines for Care and Use of Laboratory Animals and were approved by the Boston College Institutional Animal Care and Use Committee. None of the animals were used in prior experimental procedures unrelated to these studies. Animals were randomly assigned to experimental condition and renewal test day order (see Behavioral Training Procedure).

Surgical Procedure. Viral stocks of adeno-associated virus (AAV) carrying constructs AAV5-hSyn-HA-hM4D-IRES-mCitrine (Experiment 2), AAV5-hSyn-HA-hM3D-IRES-mCitrine (Experiment 3), and AAV5-hSyn-EGFP (Experiment 2 and 3) were purchased from the Vector Core at University of North

Carolina, Chapel Hill. Animals were briefly anesthetized with isoflurane (5%; Baxter Healthcare Corporation, Deerfield, IL, USA), then deeply anesthetized with an intramuscular injection of a mixture (1 ml/kg body weight) of ketamine (50 mg/mL; Fort Dodge Animal Health, Fort Dodge, Iowa) and xylazine (10 mg/mL; LLOYD Laboratories, Shenandoah, IA, USA). While under anesthesia, animals received bilateral stereotaxically placed injections of the AAVs into the ventromedial prefrontal cortex (vmPFC; volume 0.75 µl; flow rate 0.25 µl/min; titer 1x10e12, relative to bregma anterior-posterior [AP]:+2.8mm, mediolateral [ML]: +/- 0.7mm, dorsoventral [DV]: -4.7mm). A 10 µl Hamilton syringe with 32 gauge cannula driven by a motorized stereotaxic injector (Stoelting, Wood Dale, IL) was used to deliver microinjections. Stereotaxic surgeries were performed according to the procedures for aseptic technique in survival surgery and postoperative care approved by Boston College IACUC. The Biosafety Committee at Boston College has approved all protocols with AAVs. Behavior started three weeks after surgery to allow for recovery and sufficient expression of the viruses.

Apparatus. The behavioral training was conducted in identical behavioral chambers (30 x 28 x 30 cm; Coulbourn Instruments, Allentown, PA) located in a room different from the colony housing rooms. The chambers had aluminum top and sides, clear Plexiglas rear wall and front hinged door and a floor of stainless steel rods 5 mm thick spaced 15 mm apart. Chambers contained a recessed food cup (3.2 x 4.2 cm) and a 4 W house light. Each chamber was located in a sound- and light-attenuating cubicle (79 x 53 x 53 cm), which was equipped with

a ventilation fan (55 dB) and video camera attached to a recording system (Coulbourn Instruments, Allentown, PA). The conditioned stimulus (CS) was a 10 second tone (75dB, 2kHz). The unconditioned stimulus (US) consisted of two food pellets (45 mg pellets, formula 5TUL; Test Diets, Richmond, IN, USA) delivered to the food cup. Chambers were modified in visual, tactile, and olfactory features, to create two distinct environments. For Context 1, a black Plexiglas panel was placed on top of the grid floor (so that rats could not see or feel the grids), and the doors to the cubicles were closed. For Context 2, a black Plexiglas panel was inserted diagonally across the side of the chamber creating a wall, and the doors to the cubicle were left open, and 1% acetic acid in water solution (Fisher Scientific, Fair Lawn, NJ) was sprayed onto the tray below the grid floor.

Behavioral training procedure. All behavioral training and testing occurred between 9:00 and 14:00. A week before start of training, all rats were food deprived and their daily food allotment was restricted to gradually reach 85% of their body weight; they were maintained at this weight for the duration of the experiment. All rats received 1 g of the food pellets (Unconditioned Stimulus, US) in the home cage the day before the training started to familiarize them with the pellets. The training consisted of three phases: conditioning (acquisition), extinction, and renewal tests. The conditioning and extinction occurred in two different contexts and the testing was conducted in both contexts (test order counterbalanced). The behavioral chambers used as distinct contexts for conditioning and extinction were counterbalanced (the acquisition context for half

of the rats was in Context 1 and for the other half was in Context 2). During the acquisition phase, rats were trained for five days, with one 34-minute training session per day. During each session they received eight presentations of the tone CS, each immediately followed with delivery of food pellets (US) into the food cup. During the extinction, rats received two 34-minute sessions (one session per day), each with eight presentations of the tone CS alone with no USs. Rats were tested for renewal of responding with CS-only presentations in Context A and Context B, counterbalanced for order, on separate days (order randomly assigned). The tests for renewal were 34-minute sessions with eight CS presentations with no USs. All sessions were recorded and stored on DVDs for behavioral analysis. In Experiments 2 and 3, prior to the tests for renewal, clozapine-N-oxide (CNO; Enzo Life Sciences, Farmingdale, NY) was injected i.p. in half of the animals who received AAV5-hSyn-HA-hM4D-IRES-mCitrine (Experiment 2) or AAV5-hSyn-HA-hM3D-IRES-mCitrine (Experiment 3), the other half received saline. All of the animals that received the control viral vector (AAV5-hSyn-EGFP) received CNO. This resulted in 3 groups: DREADDs+CNO, DREADDs+Vehicle, Control Virus+CNO. CNO was administered at a dose of 3 mg/kg (1mg/ml).

Behavioral Measures. Trained observers, 'blind' to experimental condition or sex of the rats, analyzed animals' behavior from the video recordings. The primary measure of conditioning (conditioned response, CR) was the expression of 'food cup behavior' during the CS. The food cup behavior was defined by distinct nose pokes into the recessed food cup, or by rats standing in

front of and directly facing the food cup. Behavior was scored every 1.25 seconds during each 10 second preCS and CS periods. At each observation only one behavior was recorded (food cup or other). The number of CRs was summed and converted to a percentage of the total time during each period an animal expressed food cup behavior.

Histological Procedures. After the end of renewal tests in Exp. 2 and 3, rats were anaesthetized with tribromoethanol (375 mg/kg body weigh, intraperitoneal injection) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M borate buffer. A subset of rats (n=4 per group) was perfused ninety minutes after the end of renewal test to be used for Fos induction detection. The brains were stored for 20-24hrs at 4°C in a paraformaldehyde and 12% sucrose mixture and then rapidly frozen in hexanes cooled with dry ice and stored at -80°C. Brains were cut into 40µm coronal sections using a microtome and collected into three adjacent series. For all brains, one tissue series was mounted unstained for identification of viral expression in the vmPFC. Another series was mounted and stained with thionin for identification of cytoarchitectonic borders. For the brains used for Fos detection, one tissue series was immediately processed with Fos immunohistochemistry, described below.

Tissue Processing and Immunohistochemistry. Immediately following slicing, sections were incubated for 72 h at 4 C in a blocking solution [KPBS containing 0.3 % Triton X-100, 2 % normal donkey serum (017-000-121; Jackson ImmunoResearch, West Grove, PA, USA)], with the primary antibody: c-Fos goat

primary (1:2,000; sc-52-G; Santa Cruz, Dallas, TX). After rinses in KPBS, tissue was incubated for 1 h in the dark in the blocking solution containing the secondary antibody: Alexa 546 anti-goat (1:200; A11056; Invitrogen, Carlsbad, CA, USA). Following rinses in KPBS, in semidarkness tissue was mounted onto slides (SuperFrost Plus), dried, coverslipped with Vectashield HardSet Mounting Medium with DAPI (H-1500; Vector Labs, Burlingame, CA, USA), and stored at 4 C until analysis. This immunohistochemistry procedure was successfully repeated 8 times in the current study, and has been used successfully numerous additional times within our laboratory.

Image acquisition and analysis. The acquisition of images was conducted with a Zeiss Axio Image Z2 fluorescence microscope (Carl Zeiss Microscopy GmbH; Jena, Germany) and attached Hamamatsu ORCA-R2 camera (Bridgewater, NJ, USA). To determine the location and extent of the AAV expression, images of the areas with viral expression and the adjacent thionin-stained tissue were acquired at 10x. Neuroanatomical borders were drawn onto the thionin-stained image and then transposed to the adjacent fluorescently image using ImageJ software (NIH). The spread of AAV expression were then drawn on computerized versions of the standard rat brain atlas templates [34] using illustration software (Adobe Illustrator CS5.5). Based on this analysis, well-defined and localized AAVs expressions were identified and included for data analysis (at least 50% in the prelimbic and infralimbic regions at bregma AP +3.60, +3.20 or +2.80; with less than 25% of the injection spread outside this target region). Only these subjects were included in behavioral data analysis. For

Fos analysis, 2x2 tiled images were taken at 20X (tissue area approximately 820x630µm) in the prelimbic area of the vmPFC (+3.20mm AP from bregma). Three brains were excluded from Fos analysis (one male Control Virus+CNO from Experiment 2, one female DREADD+CNO and one female Control Virus+CNO from Experiment 3) due to inadequate viral expression in the prelimbic area at bregma +3.20mm AP. Images were pseudocolored with green for DREADDs, red for Fos and blue for DAPI (nuclear counterstain). Single AAV, single Fos and double-labeled (AAV and Fos) cells were determined based on characteristic nuclear (Fos) or cytoplasmic (AAV) labeling by an investigator 'blind' to experimental condition. Single (AAV or Fos) and double-labeled (AAV and Fos) cells were counted. Double-labeled cells were expressed as a percentage of total AAV cells and total Fos cells.

Statistical Analysis. Behavioral data analysis was conducted to compare CRs using a mixed-design ANOVA (repeated (context) and between (sex)) and significant main effects and interaction effects ($p < 0.05$) were followed by *post hoc* two-tailed t-tests. In addition, in Experiments 2 and 3, *a priori* planned orthogonal contrasts were used. All statistical analyses were conducted in SPSS. Data are presented as median and inner quartiles, as well as minimum and maximum when indicated. Statistical details of the experiments can be found in the figure legends and figures. Exclusion of subjects was determined based on previously described exclusion criteria (Experimental Model and Subject Details, Method Details). Sample sizes were determined based on previous research in

our lab [22] [23]. All relevant data from the current study are available from the corresponding author upon request.

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Author Contributions: G.D.P. conceptualized the study, L.C.A. and G.D.P. conceived experimental design, L.C.A carried out the experiments and data analysis, L.C.A and G.D.P. co-wrote the manuscript.

Competing Financial Interests: The authors declare no competing financial interests.

Chapter 5: Distinct recruitment of the hippocampal, thalamic, and amygdalar prelimbic-projecting neurons during context-mediated renewal of responding to food cues in male and female rats*

**Manuscript in Preparation*
Anderson, L.C. & Petrovich, G.D. (2017)

Abstract: Persistent responding to food cues may help explain the difficulty associated with adapting healthy eating habits. Renewal of responding after extinction is a model of persistent food seeking that can be used to study the underlying neural mechanisms. In context-mediated renewal, a return to the context in which the initial cue-food learning occurred induces robust responding to the cues that were extinguished elsewhere. Previously, we found sex differences in context-mediated renewal and showed differential ventromedial prefrontal cortex (vmPFC) recruitment is causal during renewal of responding in a sex-specific way. Males exhibited renewal of responding to food cues and had higher Fos induction in the prelimbic area (PL) of the vmPFC compared to males in the control group, and silencing the vmPFC disrupted this behavior. In contrast, females failed to exhibit renewal of responding, had lower Fos induction in the PL compared to females in the control group, and stimulating the vmPFC in females induced renewal responding. In the current study, the main goal was to determine key components of the vmPFC circuitry mediating renewal. To accomplish this goal, we used a retrograde tracer (Alexa Fluor-594 conjugated cholera toxin B) to identify PL-projecting neurons and Fos induction to identify whether they were activated during context-mediated renewal responding in male and female rats. Specifically, we sought to determine whether neurons from the

ventral hippocampal formation, the paraventricular nucleus of the thalamus and the basolateral area of the amygdala that send direct projections to the PL are selectively activated during renewal in males and females. Within the ventral hippocampal formation, we found recruitment of PL-projecting neurons in both the SUBv and CA1v activated during renewal responding, selectively only in male rats that showed renewal. Similarly, the PL-pathway neurons in the PVTa and BLAa were activated in the male experimental group—the only group that showed renewal behavior. The recruitment of the SUBv-PL, CA1v-PL, PVTa-PL and BLAa-PL pathways in males, and the lack of recruitment in females, suggests these pathways mediate renewal behavior in males and are under activated in females.

1. Introduction

Persistent responding to food cues may help explain the difficulty associated with adapting healthy eating habits (Boutelle & Bouton, 2015). Cues associated with food can stimulate food seeking and consumption independently of hunger (for review see Petrovich, 2013), and these eating behaviors can continue to occur even after extinction because the original learned associations continue to exist (Bouton, 2004). Renewal of responding after extinction is a model of persistent food seeking that can be used to study the relapse of avoidance of palatable foods (Calu, Chen, Kawa, Nair, & Shaham, 2014). In context-mediated renewal, a return to the context in which the initial learning occurred induces robust responding to the cues that were extinguished elsewhere (Bouton & King, 1983). The neural substrates underlying context-mediated renewal of responding to food cues are still being elucidated. The ventromedial prefrontal cortex (vmPFC) is a crucial area in appetitive and fear renewal responding (Eddy, Todd, Bouton, & Green, 2016; Knapska & Maren, 2009; Orsini, Kim, Knapska, & Maren, 2011; Rhodes & Killcross, 2007; Wang, Jin, & Maren, 2016; Willcocks & McNally, 2013). Previously, we found sex differences in context-mediated renewal and showed differential vmPFC recruitment (Anderson & Petrovich, 2016) is causal (Anderson & Petrovich, 2017) during renewal of responding in a sex-specific way. Males exhibited renewal of responding to food cues, had higher Fos induction in the prelimbic area (PL) of the vmPFC compared to males in the control group, and silencing the vmPFC in males disrupted renewal responding. In contrast, females failed to exhibit

renewal of responding, had lower Fos induction in the PL compared to females in the control group, and stimulating the vmPFC in females induced renewal responding.

In the current study, the main goal was to determine key components of the PL circuitry mediating renewal. To accomplish this goal, we combined retrograde tracing and Fos induction detection to identify PL-input neurons that are activated during the test for context-mediated renewal in male and female rats. The PL has distinct connections with the amygdala, the ventral hippocampal formation, and the paraventricular nucleus of the thalamus (PVT) (Fanselow & Dong, 2010; Hoover & Vertes, 2007; Li & Kirouac, 2012; Moga et al., 1990; Petrovich, Canteras, & Swanson, 2001; Pitkanen, Pikkarainen, Nurminen, & Ylinen, 2000; Reppucci & Petrovich, 2015; Sesack, Deutch, Roth, & Bunney, 1989; Swanson & Petrovich, 1998). Importantly, these regions were recruited (Fos induction) during renewal in a sex-specific way that suggested their inputs to the PL may be causal in this behavior (Anderson & Petrovich, 2016).

All three of these areas have been implicated in appetitive learning. The hippocampal formation is critical for contextual processing, including context-dependent renewal (Fanselow, 2000; Holland & Bouton, 1999; Marinelli, Funk, Juzytsch, Li, & Le, 2007; Orsini et al., 2011), the PVT is important for context-induced renewal and is involved in the regulation of food consumption (Anderson & Petrovich, 2016; Bhatnagar & Dallman, 1999; Cole, Mayer, & Petrovich, 2015; Hamlin, Clemens, Choi, & McNally, 2009), and the amygdala is important for appetitive associative learning (Cole, Hobin, & Petrovich, 2015; Crombag &

Shaham, 2002; Holland & Petrovich, 2005). Thus, we examined whether neurons in these areas that send direct projections to the PL are selectively activated during renewal and whether they are differentially recruited in males and females.

In these experiments we tested females in different phases of the estrous cycle to determine if estradiol at test mediates sex differences in renewal responding. Previously, estradiol replacement to ovariectomized rats induced renewal responding, while ovariectomized females without estradiol replacement did not exhibit renewal responding (Anderson & Petrovich, 2015).

Determining which PL-projecting neurons are specifically recruited during renewal behavior and how they differ in male and female rats will provide understanding of the fundamental neural circuit mechanisms of reward driven behaviors and potential sites of sex differences. Knowledge about the neural mechanisms of Pavlovian renewal is important for our understanding of the resilience of food cue to influence our consumption and diet choices.

2. Methods

2.1 Subjects

16 adult male and 32 female Long-Evans rats (250-275g at arrival; Charles River Laboratories) were individually housed and maintained on a 12 h light/dark cycle (lights on at 7:00). Males and females were housed in separate colony rooms. Subjects were acclimated to the colony room for two days prior to brain surgery to receive a retrograde tracer. Animals were given a week to

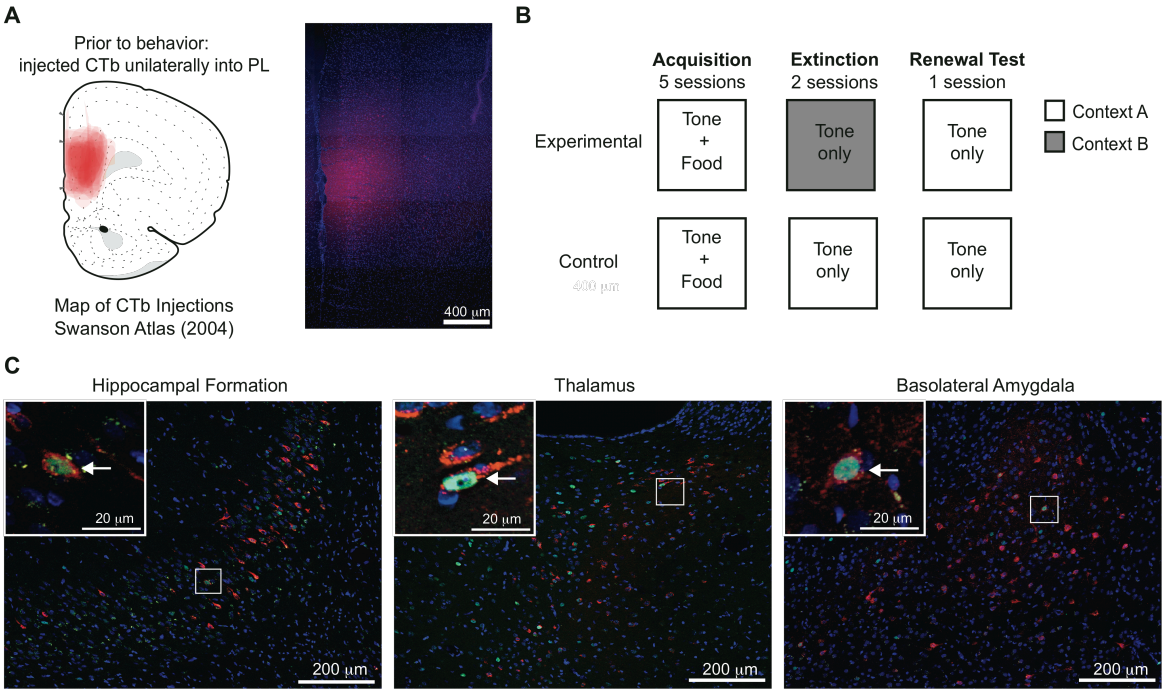

Figure 5.1 Experimental Design. (**A**) Map of all CTb injections in the PL of male and female rats (left) and a representative image (right). (**B**) Between-subjects context-dependent renewal protocol. (**C**) Representative images of CTb and Fos labeling in the hippocampal formation (CA1v), paraventricular nucleus of the thalamus (PVTa) and the basolateral amygdala. Insets in upper left of each image show magnified area in corresponding rectangles. Fos-positive (green), CTb-positive (red), double-labeled (CTb+Fos) neurons (arrow).

recover post-surgery during which they were weighed and handled daily and were given *ad libitum* access to standard laboratory chow (18% Protein Rodent Diet #2018, Harlan Teklad Global Diets, Madison, WI) and water, except as otherwise noted. Vaginal smears of the female rats were obtained daily between 15:00-16:00 to ensure normal estrous cycling starting four days prior to behavioral testing. All housing and testing procedures were in compliance with the National Institute of Health Guidelines for Care and Use of Laboratory Animals and were approved by the Boston College Institutional Animal Care and Use Committee.

2.2 Surgical Procedure

Animals were briefly anesthetized with isoflurane (5%; Baxter Healthcare Corporation, Deerfield, IL, USA), then deeply anesthetized with a mixture (1 ml/kg body weight) of ketamine (50 mg/mL; Fort Dodge Animal Health, Fort Dodge, Iowa) and xylazine (10 mg/mL; LLOYD Laboratories, Shenandoah, IA, USA; delivered via intramuscular injection). While under anesthesia, animals received unilateral stereotaxically placed infusions into the prelimbic area of the ventromedial prefrontal cortex (PL) of Alexa Fluor-594 conjugated cholera toxin B (CTb; Life Technologies, Carlsbad, CA; Figure 5.1a on previous page) delivered at a rate of 0.1µl/min for 4 minutes (0.4 µl totally volume; 5 µg/µl; relative to bregma anterior-posterior [AP]:+2.8mm, mediolateral [ML]: +/- 0.7mm, dorsoventral [DV]: -4.7mm). The injector remained in the site for 10 minutes post-infusion to allow for the diffusion of CTb. A 10 µl Hamilton syringe with 32 gauge cannula driven by a motorized stereotaxic injector (Stoelting, Wood Dale, IL) was used to deliver microinjections. Stereotaxic surgeries were performed according to the procedures for aseptic technique in survival surgery and postoperative care approved by Boston College IACUC. Behavioral experiments started one week after surgery to allow for recovery and sufficient transport of the tracer.

2.3 Apparatus

The behavioral training was conducted in identical behavioral chambers (30 x 28 x 30 cm; Coulbourn Instruments, Allentown, PA) located in a room different from the colony housing rooms. The chambers had aluminum top and sides, clear Plexiglas rear wall and front hinged door and a floor of stainless steel

rods 5 mm thick spaced 15 mm apart. Chambers contained a recessed food cup (3.2 x 4.2 cm) and a 4 W house light. Each chamber was located in a sound- and light-attenuating cubicle (79 x 53 x 53 cm), which was equipped with a ventilation fan (55 dB) and video camera attached to a recording system (Coulbourn Instruments, Allentown, PA). The conditioned stimulus (CS) was a 10 second tone (75dB, 2kHz). The unconditioned stimulus (US) consisted of two food pellets (45 mg pellets, formula 5TUL; Test Diets, Richmond, IN, USA) delivered to the food cup. Chambers were modified in visual, tactile, and olfactory features, to create two distinct environments. For Context 1, a black Plexiglas panel was placed on top of the grid floor (so that rats could not see or feel the grids), and the doors to the cubicles were closed. For Context 2, a black Plexiglas panel was inserted diagonally across the side of the chamber creating a wall, and the doors to the cubicle were left open, and 1% acetic acid in water solution (Fisher Scientific, Fair Lawn, NJ) was sprayed onto the tray below the grid floor.

2.4 Behavioral training procedure

All behavioral training and testing occurred between 9:00 and 14:00. A week before start of training, all rats were food deprived and their daily food allotment was restricted to gradually reach 85% of their body weight; they were maintained at this weight for the duration of the experiment. All rats received 1 g of the food pellets (Unconditioned Stimulus, US) in the home cage the day before the training started to familiarize them with the pellets. The training consisted of three phases: conditioning (acquisition), extinction, and renewal test (Figure 5.1b). The training protocol followed an “ABA” design where conditioning

acquisition and extinction occurred in different contexts and renewal occurred in the same context as acquisition. During the acquisition phase, rats were trained for five days, with one 34-minute training session per day. During each session they received eight presentations of the tone (CS), each immediately followed with delivery of food pellets (US) into the food cup. The acquisition training occurred in Context A (Context 1 for half of the rats, and in Context 2 for the other half). During the extinction phase, rats received two 34-minute sessions (one session per day), each with eight presentations of the CS alone, with no USs. Rats in the experimental condition received extinction training in a context different than the training context (ABA), while rats in the control condition remained in the same context across all training phases (AAA). The test for renewal was one 34-minute session with eight CS presentations and no USs, conducted in the acquisition context. The intertrial interval was 110-326 seconds and the length varied randomly across trials and training sessions. All sessions were recorded and stored on DVDs for behavioral analysis. After the second extinction session, females rats in the experimental and control groups were divided into two separate groups based on estrous cycle phase so that half of the females were in the a high estradiol phase (Proestrus; High E) while half of the females were in a low estradiol phase (Metestrus or early Diestrus; Low E) during the renewal test. This resulted in four female groups: High E Control, High E Experimental, Low E Control, Low E Experimental.

2.5 Behavioral Measures

Trained observers, ‘blind’ to experimental condition or sex of the rats, analyzed animals’ behavior from the video recordings. The primary measure of conditioning (conditioned response, CR) was the expression of ‘food cup behavior’ during the CS. The food cup behavior was defined by distinct nose pokes into the recessed food cup, or by rats standing in front of and directly facing the food cup. Behavior was scored every 1.25 seconds during each 10 second preCS and CS periods. At each observation only one behavior was recorded (food cup or other). The number of CRs was summed and converted to a percentage of the total time during each period an animal expressed food cup behavior.

2.6 Histological procedures

Ninety minutes after the end of renewal tests, rats were anaesthetized with tribromoethanol (375 mg/kg body weigh, i. p.) and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M borate buffer. The brains were stored for 20-24hrs at 4°C in a paraformaldehyde and 12% sucrose mixture and then rapidly frozen in hexanes cooled with dry ice and stored at -80°C. To determine the location and spread of the CTb injections, brain tissue containing the prefrontal cortex was cut into 30μm coronal sections using a microtome and collected into four adjacent series. One series was mounted unstained for identification of CTb location in the PL. Another series was mounted and stained with thionin for identification of anatomical borders as defined in Swanson (2004). Of those brains that had well-defined and localized injections (described below), the remaining part of the brains were cut into 30μm

coronal sections and collected into four adjacent series. One series was used to identify Fos expression with an anti-Fos antibody and fluorescent-conjugated secondary antibody (AlexaFluor-488) following a standard immunohistochemistry protocol, described below (Figure 5.1c). Another series was mounted and stained with thionin for identification of anatomical borders.

2.7 Immunohistochemistry

Immediately following slicing, sections were incubated for 72 h at 4°C in a blocking solution [KPBS containing 0.3 % Triton X-100, 2 % normal donkey serum (017-000-001; Jackson ImmunoResearch, West Grove, PA, USA)], with the primary antibody: c-Fos rabbit primary (1:10,000; Millipore, Billerica, MA). After rinses in KPBS, tissue was incubated for 1 h in the dark in the blocking solution containing the secondary antibodies: Alexa 488 anti-rabbit (1:200; A21206; Invitrogen, Carlsbad, CA, USA). Following rinses in KPBS, in semidarkness tissue was mounted onto slides (SuperFrost Plus), dried, coverslipped with Vectashield HardSet Mounting Medium with DAPI (H-1500; Vector Labs, Burlingame, CA, USA), and stored at 4°C until analysis.

2.8 Image acquisition and analysis

The acquisition of images was conducted with a Zeiss Axio Image Z2 fluorescence microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and attached Hamamatsu ORCA-R2 camera (Bridgewater, NJ, USA). To determine the location and extent of CTb injection sites, images of the areas with tracer deposits and the adjacent thionin-stained tissue were acquired at 10x.

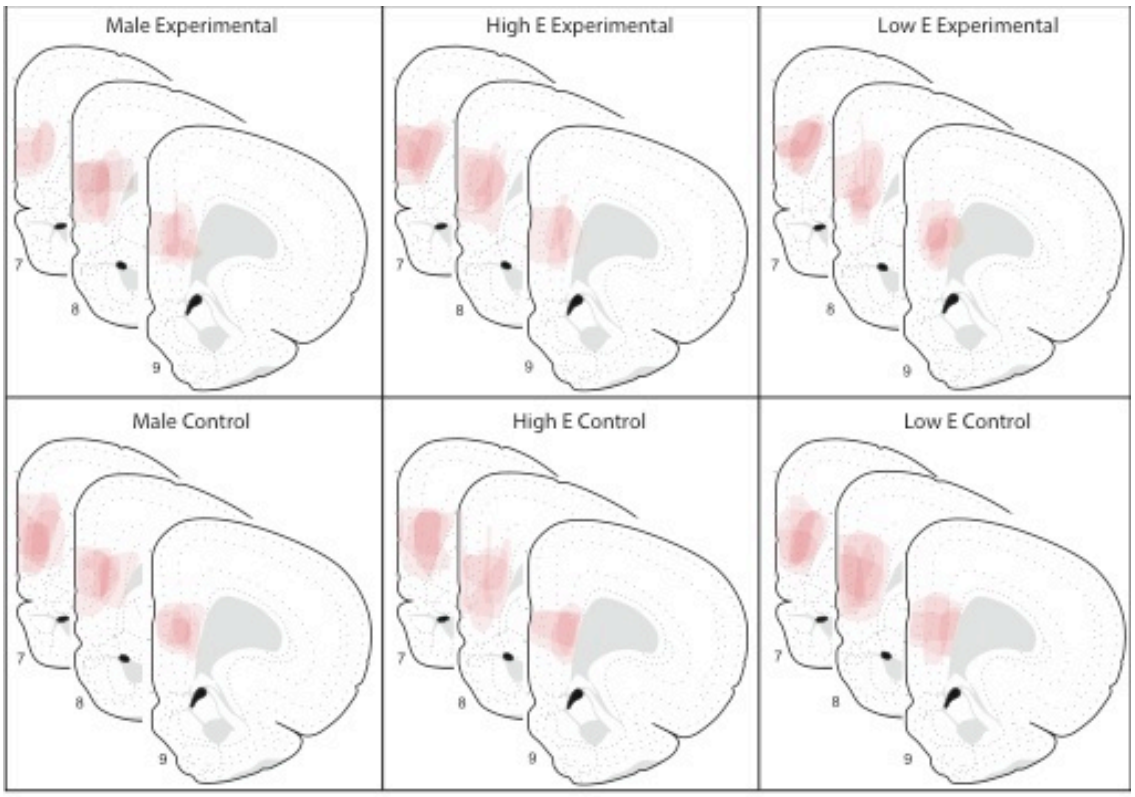

Figure 5.2 Illustration of CTb injection sites in the PL across groups. The extent of CTb injections in the PL for each group is shown on modified Swanson atlas templates (Swanson (2004); atlas levels 7, 8, 9; +3.6, +3.2 and +2.8 mm from bregma respectively).

Neuroanatomical borders were drawn onto the thionin-stained image and then transposed to the adjacent fluorescently image using ImageJ software (NIH). The injection sites were then drawn on computerized versions of the standard rat brain atlas templates (Swanson, 2004) using illustration software (Adobe Illustrator CS5.5). All CTb injections were analyzed and well-defined and localized injections were identified based on the following criteria: at least 50% of the target region contained CTb, with less than 25% of the injection spread outside the target region (Figure 5.2). Based on this selection, CTb and Fos labeled neurons in those brains were analyzed. Retrograde labeling and Fos were analyzed within the ventral hippocampus, paraventricular nucleus of the

thalamus, and the basolateral area of the amygdala (Figure 5.1c). Within the hippocampal formation, the ventral field CA1, Ammon's horn (Levels 34-39 as defined in the Swanson atlas (2004), -4.45mm to -6.60mm from Bregma respectively; all subsequent level refer to Swanson atlas, all subsequent measurements refer to mm from Bregma) and ventral subiculum (Levels 34-39, -4.45mm to -6.60mm) were analyzed. Within the paraventricular nucleus of the thalamus, the anterior (PVTa; Levels 24-27, -1.33mm to -2.00mm) and posterior (PVTp; Levels 28-32, -2.45mm to -3.90mm) parts were analyzed separately. Within the basolateral amygdala, the anterior part of the basolateral nucleus (BLAa; Levels 25-29, -1.53mm to -2.85 mm), the posterior part of the basolateral nucleus (BLAp; Levels 28-34, -2.45mm to -4.45mm), the posterior part of the basomedial nucleus (BMAp; Levels 28-32, -2.45mm to -4.20mm) and the lateral nucleus (LA; Levels 25-32, -1.53mm to -3.90mm) were analyzed. The analysis followed parcellation and nomenclature as defined in the Swanson atlas (2004). Consecutive images were taken at 20X through the regions of interest (one image per atlas level). Images were pseudocolored with red for CTb, green for Fos, and blue for DAPI (nuclear counterstain). Single CTb, single Fos and double-labeled (CTb and Fos) neurons were determined based on characteristic nuclear (Fos) or cytoplasmic (CTb) labeling. Single (CTb or Fos) and double-labeled (CTb and Fos) neurons were counted. Double-labeled neurons were expressed as a percentage of total CTb neurons.

2.9 Statistical Analysis

Behavioral (i.e. food cup behavior) and pathway data analysis were conducted with ANOVAs and *post hoc t*-tests as appropriate. For the ANOVAs, the factors of Group (Experimental, Control) and Sex (Male, Female) were used unless otherwise stated. In all cases, $p < 0.05$ was considered significant. SPSS software was used for all statistical analyses. All data are presented as means ± SEM. 2 male rats were excluded due to poor health. Based on tracer injection sites, the final group sizes were n=5 per group: Male Control, Male Experimental, High E Control, High E Experimental, Low E Control, Low E Experimental.

3. Results

3.1 Behavior

3.1.1 Acquisition

During acquisition (Figure 5.3 on next page), all rats showed an increase in food cup responding (CR) across the training sessions during the tone (CS) presentations (Repeated measures ANOVA Greenhouse-Geisser correction, $F(3.1,89.887)=76.387$, $p=0.000$). There was also a significant effect in responding during preCS across training sessions (Repeated measures ANOVA Greenhouse-Geisser correction, $F(3.719, 107.858)=3.492$, $p=0.012$). However, even though there was a difference across training session, preCS responding remained low and the first session was not significantly different from the last acquisition session (Paired *t*-test, $p > 0.05$). There were no Sex or Group differences ($p > 0.05$, both). During the last acquisition session (Acquisition 5), all

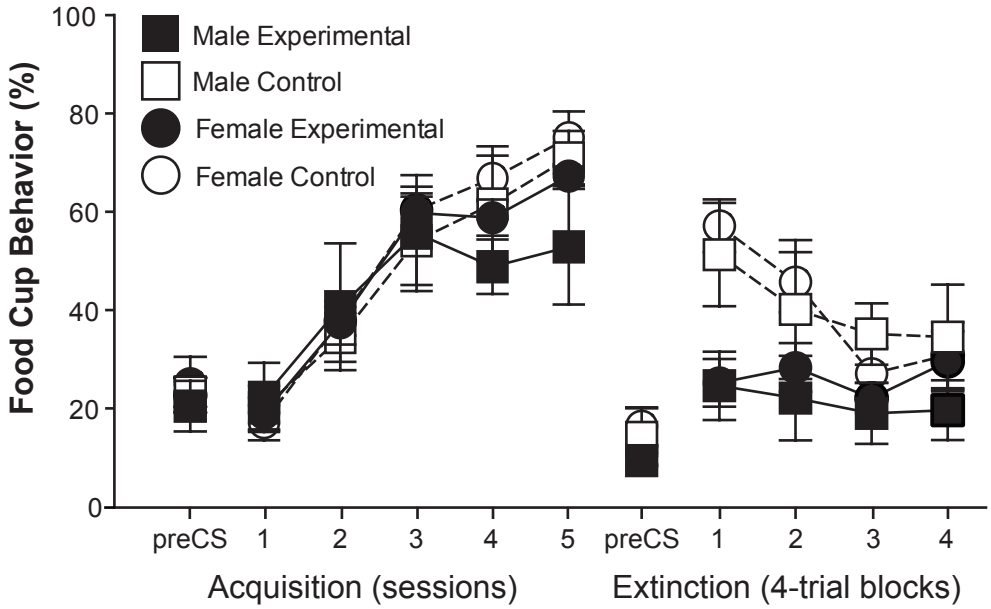

Figure 5.3 Conditioned responses during acquisition and extinction. Percentage of time rats expressed food cup behavior (mean ± SEM) during the preCS and CS periods during training sessions. PreCS values are the average across all sessions for acquisition and extinction, respectively. Acquisition is shown as the average responding during each session. Extinction is shown as the average responding in 4-trial blocks (2 blocks per session; blocks 1&2 were trials during Session 1 and blocks 3&4 during Session 2).

rats showed high CRs during CSs compared to their low responding during preCS ($t(29)=-17.418$, $p=0.000$). An ANOVA (Sex and Group) confirmed that there were no significant differences across groups in responding during CS or preCS ($p > 0.05$, both).

3.1.2 Extinction

All rats showed a decreased in CRs due to extinction training by the second extinction session (Figure 5.3). There was a statistically significant decrease in the total responding during CSs when comparing responding across the last acquisition session, the first extinction session, and the last extinction session for males and females in both groups (Repeated measures ANOVA Greenhouse-Geisser correction, $F(1.946,48.638)=118.662$, $p=0.000$). There were no effects

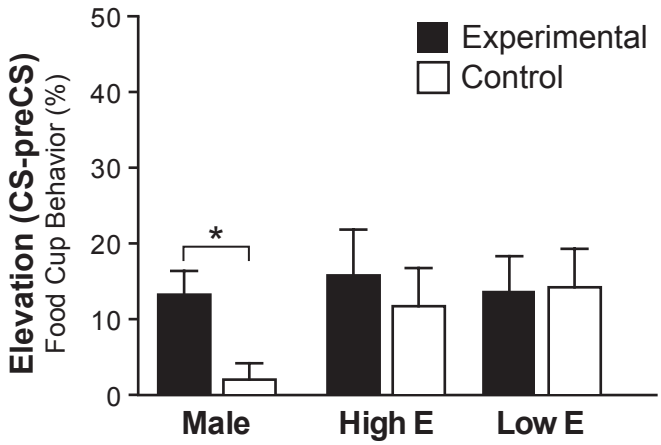

Figure 5.4 Conditioned responses during the test for renewal. Elevation scores represent percentage of time rats expressed food cup behavior during CS minus preCS during the test for renewal. Bars are mean ± SEM. * indicates p < 0.01.

Table 5.1 Conditioned responses during the test for renewal. Percentage of time (mean ± SEM) rats expressed food cup behavior during preCS and CS. * indicates difference from same-sex control. $p < 0.05$.

	Male		Female	
	Exp	**Control**	**Exp**	**Control**
preCS	17.2 ± 5	7.8 ± 5	9.8 ± 2	17.5 ± 4
CS	30.3 ± 3*	9.7 ± 4	24.4 ± 3	30.3 ± 5

of Sex (p > 0.05) but there was an effect of Group ($F(1.946,48.638)=3.969$, $p=0.026$). The group effect was driven by higher responding in the control groups (tested in the acquisition context) during the first extinction session (One-way ANOVA on Extinction 1 CR, $F(1,26)=12.379$, $p=0.002$). This group effect was transient and during the second extinction session there were no differences in responding across groups (One-way ANOVA on Extinction 2 CR, $p > 0.05$). All groups showed similar, low responding in preCS periods during each extinction session ($p > 0.05$).

3.1.3 Renewal

During the test for renewal, only male groups showed differential responding (Figure 5.4; Table 5.1). There was no effect of Estradiol condition for

females responding (Figure 5.4; ANOVA (Estradiol Condition by Group); $p > 0.05$)), and therefore High and Low Estradiol were analyzed together. An ANOVA (Sex and Group) revealed a significant effect of Sex by Group interaction on CRs during CSs ($F(1,26)=10.570$, p=0.003; Table 5.1) but no effect of Sex or Group ($p > 0.05$, both). *Post hoc* tests confirmed that the males in the experimental group had significantly more CRs compared to the males in the control condition ($t(6.984)=-4.501$, p=0.003) while females were not significantly different ($p > 0.05$). Additionally, there was a significant effect of Sex by Group interaction on CRs during preCSs ($F(1,26)=4.822$, p=0.037), which may be due to opposite patterns in males and females (Table 5.1, higher preCS responding in males in the experimental group and females in the control group compared to males in the control group and females in the experimental group). *Post hoc t*-tests revealed no significant differences between groups however ($p > 0.05$). To account for the difference in preCS responding, we examined Elevation Scores (CS-preCS responding) by group (Figure 5.4). Males in the experimental group had significantly higher elevation scores compared to the males in the control group ($t(7.160)=-2.963$, $p=0.020$). Females in the experimental group and control groups did not show differential responding ($p > 0.05$). Interestingly, both female groups showed high responding, similar to the experimental male group ($p > 0.05$).

3.2 Neuronal Analysis

3.2.1 Retrograde Tracer Injection Sites

The location and spread of CTb injection sites were analyzed throughout the rostro-caudal extent of the prelimbic cortex (PL) based on the Swanson brain atlas (Swanson, 2004). Acceptable injections were confined predominately within the PL (n=30) and were centered within the mid rostro-caudal extent of the PL (Figure 5.2; Swanson atlas levels 7, 8 and 9; +3.6, +3.2 and +2.8 from bregma, respectively).

3.2.1 Fos Induction

Retrograde labeling and Fos were analyzed within the ventral hippocampal formation (SUBv, CA1v), paraventricular nucleus of the thalamus (PVTa, PVTp), and the basolateral area of the amygdala (BLAa, BLAp, BMAp, LA). Single (CTb or Fos) and double-labeled (CTb and Fos) neurons were counted (Table 5.2 on next page). Double-labeled neurons were also expressed as a percentage of total CTb neurons in each animal. Because there were no behavioral differences between High and Low Estradiol groups for all statistical analyses these two groups were collapsed together by group.

3.2.2 Hippocampal formation

3.2.3.1 SUBv

In the SUBv, there was overall more total Fos induction in males compared to females and more Fos induction the experimental groups compared to control groups (Figure 5.5 on page 117). This was supported by an ANOVA, which found Sex ($F(1,26)=52.523$, $p=0.000$) and Group ($F(1,26)=5.517$, $p=0.027$) but no Sex by Group interaction effects ($p > 0.05$). *Post hoc* comparisons confirmed females in the experimental group had significantly higher Fos induction compared to

Table 5.2 Counts for all brain regions analyzed. Total number of Fos-positive, CTb-positive, double-labeled (CTb + Fos) neurons, and percentage of CTb neurons that were double-labeled. Results are displayed as a mean ± SEM. Abbreviations: BLAa- anterior part of the basolateral nucleus of the amygdala, BLAp- posterior part of the basolateral nucleus of the amygdala, BMAp- posterior part of the basomedial nucleus of the amygdala, CA1v- ventral field CA1, Ammon's horn, LA- lateral nucleus of the amygdala, PVTa- anterior part of the paraventricular nucleus of the thalamus, PVTp- posterior part of the paraventricular nucleus of the thalamus, SUBv- ventral subiculum. $^{\#}$ denotes significant main effect of Sex and $^{+}$ denotes significant main effect of Sex by Group. * denotes significant difference compared to same-sex control. $p < 0.05$.

Brain Region		**Male**		**Female**	
		Experimental	*Control*	*Experimental*	*Control*
SUBv	Fos^{+}	97 ± 17	74 ± 4	42 ± 2*	36 ± 2
	CTb$^{\#}$	45 ± 6	52 ± 8	65 ± 9	75 ± 9
	Double$^{\# +}$	14 ± 3	7 ± 2	4 ± 1	7 ± 1
	Percentage$^{\# +}$	33 ± 6*	13 ± 2	8 ± 1	10 ± 2
CA1v	Fos$^{\#}$	145 ± 8	145 ± 8	88 ± 8	95 ± 9
	CTb	129 ± 32	173 ± 29	193 ± 20	206 ± 23
	Double$^{\#}$	38 ± 11	31 ± 10	17 ± 2	17 ± 2
	Percentage$^{\# +}$	28 ± 3*	16 ± 4	10 ± 1	9 ± 1
PVTa	Fos$^{\# +}$	261 ± 23	203 ± 9	171 ± 18	197 ± 13
	CTb$^{\#}$	127 ± 10	144 ± 13	181 ± 15	168 ± 15
	Double	45 ± 6	30 ± 7	32 ± 3	36 ± 5
	Percentage^{+}	35 ± 5	20 ± 4	20 ± 3	22 ± 2
PVTp	Fos$^{\#}$	250 ± 11	226 ± 31	168 ± 14	185 ± 17
	CTb	143 ± 24	143 ± 21	189 ± 15	153 ± 13
	Double	34 ± 4	27 ± 3	35 ± 5	34 ± 6
	Percentage	25 ± 4	20 ± 3	19 ± 3	21 ± 3
BLAa	Fos$^{\# +}$	68 ± 10	44 ± 5	39 ± 5	42 ± 3
	CTb	99 ± 24	93 ± 18	101 ± 12	83 ± 4
	Double	12 ± 2	8 ± 1	7 ± 1	7 ± 1
	Percentage$^{\#}$	14 ± 3	10 ± 2	7 ± 1	8 ± 1
BLAp	Fos	50 ± 7	39 ± 3	35 ± 4	38 ± 4
	CTb	105 ± 24	157 ± 30	160 ± 18	179 ± 20
	Double	6 ± 1	7 ± 1	8 ± 1	9 ± 1
	Percentage	6 ± 1	6 ± 2	5 ± 1	5 ± 1
BMAp	Fos$^{\#}$	41 ± 8	36 ± 6	24 ± 2	30 ± 3
	CTb	50 ± 16	66 ± 13	80 ± 7	80 ± 10
	Double	5 ± 1	4 ± 1	4 ± 1	5 ± 1
	Percentage^{+}	10 ± 2	6 ± 2	6 ± 1	7 ± 1
LA	Fos$^{\#}$	51 ± 7	41 ± 5	27 ± 2	34 ± 5
	CTb	43 ± 11	50 ± 13	68 ± 10	66 ± 14
	Double	4 ± 2	3 ± 1	4 ± 1	5 ± 1
	Percentage	8 ± 3	7 ± 2	5 ± 1	6 ± 1

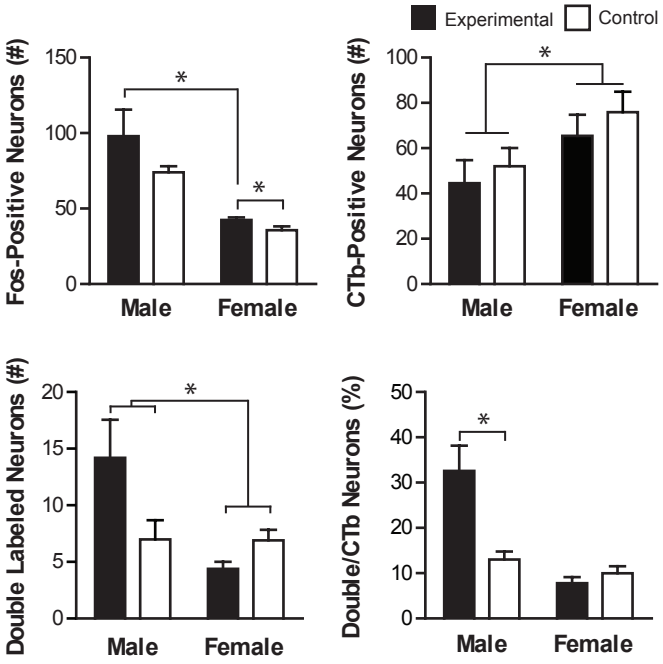

Figure 5.5 Fos induction in SUBv-PL projecting neurons. Shown are mean values (± SEM) for total number of Fos-positive, CTb-positive, and double-labeled (CTb + Fos) neurons, as well as percentage of CTb neurons that were double-labeled. * indicates $p < 0.05$.

females in the control group (t(15.850)=-2.216, p=0.042), while higher Fos in male experimental group compared to male controls was not significant ($p > 0.05$). Males in the experimental group were significantly different from females in the experimental group however ($t(4.073)=3.160$, $p=0.033$) as well as from females in the control group ($t(4.160)=3.517$, $p=0.023$).

There were more CTb neurons in the female groups compared to the male groups (Figure 5.5). This was confirmed with a significant effect of Sex on the number of CTb-positive neurons ($F(1,26)=4.607$, $p=0.041$), and there was no effect of Group or Sex by Group interaction ($p > 0.05$).

There were overall more double-labeled (Fos+CTb) neurons in males compared to females and the highest number was in the experimental males (Figure 5.5). There was a significant effect of Sex and Sex by Group on the

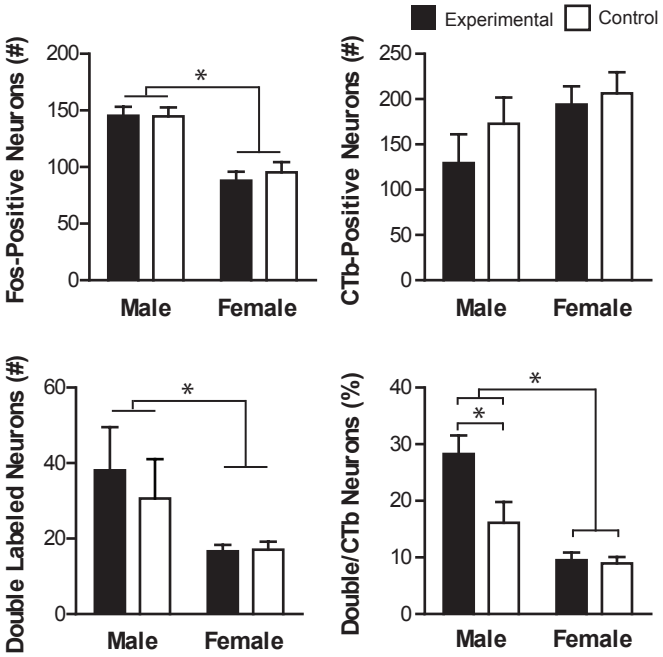

Figure 5.6 Fos induction in CA1v-PL projecting neurons. Shown are mean values (± SEM) for total number of Fos-positive, CTb-positive, and double-labeled (CTb + Fos) neurons, as well as percentage of CTb neurons that were double-labeled. * indicates $p < 0.05$.

number of double-labeled neurons (Sex: $F(1,26)=10.722$, $p=0.003$, Sex by Group: $F(1,26)=0.293$, $p=0.004$) but no effect of Group ($p > 0.05$). *Post hoc t*-tests found that the number of double-labeled neurons in the males in the control and experimental groups were not significantly different ($p > 0.05$). However, the number of Fos+CTb labeled neurons was significantly higher in the males in the experimental group compared to females in the experimental group ($t(4.274)=2.875$, $p=0.042$) but not compared to females in the control group ($p > 0.05$).

The proportion of total CTb neurons that were double-labeled (Fos+CTb) was the highest in the males in the experimental group compared to all other groups (Figure 5.5). There were significant effects of Sex, Group, and Sex by Group on the percentage of double-labeled neurons (Sex: $F(1,26)=31.414$,

p=0.00, Group: *F*(1,26)=12.256, *p*=0.002; Sex by Group: *F*(1,26)=19.212, *p*=0.000; Figure 5.5). These effects were driven by a statistically higher percentage in males in the experimental group compared to males in the control group (*t*(4.762)=-3.323, *p*=0.023). Female groups did not differ (*p* > 0.05).

3.2.3.2 CA1v

In the CA1v, there was more overall Fos induction in the male groups compared to the female groups (Figure 5.6 on previous page). This was confirmed with a significant effect of Sex on the number of Fos-positive neurons (*F*(1,26)=32.160, *p*=0.000), but no effect of Group or Sex by Group (*p* > 0.05).

There were no differences between groups in the number of CTb-positive neurons (*p* > 0.05).

There were more double-labeled neurons in the male groups compared to the female groups (Figure 5.6). This was confirmed with a significant effect of Sex on the number of double-labeled neurons (*F*(1,26)=9.662, *p*=0.005) , and there was no effect of Group or Sex by Group interaction (*p* > 0.05).

The proportion of double-labeled neurons was the highest in the males in the experimental group compared to all other groups (Figure 5.6). There were significant effects of Sex, Group and Sex by Group on the percentage of double-labeled neurons (Sex: *F*(1,26)=38.765, *p*=0.00, Group: *F*(1,26)=9.261, *p*=0.005; Sex by Group: *F*(1,26)=7.762, *p*=0.010). These effects were driven by a statistically higher percentage in males in the experimental group compared to males in the control group (*t*(7.902)=-2.483, *p*=0.038). Males in the experimental group were also significantly different from

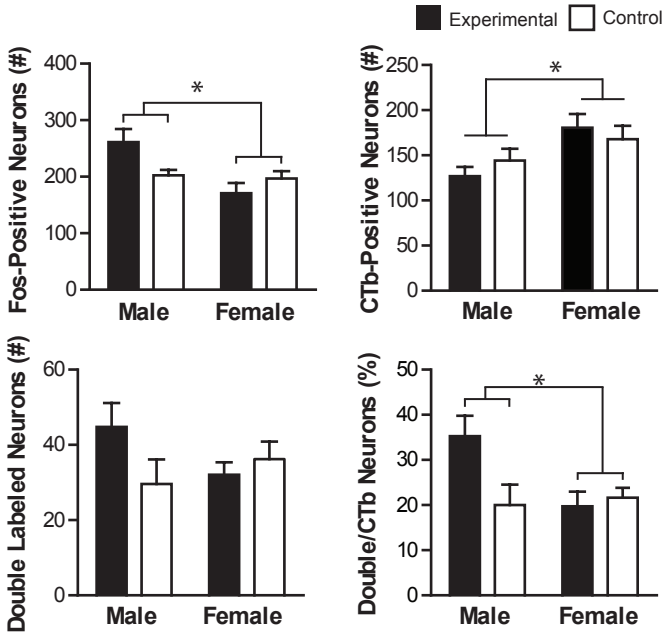

Figure 5.7 Fos induction in PVTa-PL projecting neurons. Shown are mean values (± SEM) for total number of Fos-positive, CTb-positive, and double-labeled (CTb + Fos) neurons, as well as percentage of CTb neurons that were double-labeled. * indicates $p < 0.05$.

females in the experimental (t(5.429)=5.318, p=0.002) and control (t(4.934)=5.613, p=0.003) groups. Female groups did not differ ($p > 0.05$).

3.2.3 Paraventricular Nucleus of the Thalamus

3.2.4.1 PVTa

Within the PVTa, Fos induction was highest in males in the experimental group (Figure 5.7). This was confirmed with significant effects of Sex and Sex by Group (Sex: F(1,26)=7.142, p=0.013, Sex by Group: F(1,26)=5.522, p=0.027), but no effect of Group ($p > 0.05$). Males in the experimental group had higher Fos induction than males in the control group, but this trend did not reach significance (t(5.335)=-2.377, p=0.060). Males in the experimental group had significantly higher Fos induction from females in the experimental (t(8.875)=3.118, p=0.013)

and control (t(6.711)=2.456, p=0.045) groups.

There were more CTb neurons in the female groups compared to the male groups (Figure 5.7). There was a significant effect of Sex on the number of CTb-positive neurons (F(1,26)=5.651, p=0.025) but no effect of Group or Sex by Group (p > 0.05). The effect of Sex was driven by higher number of CTb-positive neurons in females compared to males (t(27.379)=-2.898, p=0.007).

There were no differences between groups in the number of double-labeled neurons (p > 0.05).

The percentage of CTb-positive neurons that were double-labeled with Fos was highest in the male experimental group (Figure 5.7). There was a significant effect of Sex by Group (F(1,26)=4.924, p=0.035), and there was no effect of Group or Sex (p > 0.05). *Post hoc* t-test comparison found that difference between male experimental and control groups did not reach significance (t(7.882)=-2.080, p=0.072), however males in the experimental group were significantly different from females in the experimental group (t(8.052)=2.761, p=0.024).

3.2.4.2 PVTp

Within the PVTp, total Fos induction was higher in males compared to females (Figure 5.8 on next page). There was a main effect of Sex on total amount of Fos induction (F(1,26)=9.816, p= 0.004), and no effect of Group or Sex by Group interaction (p > 0.05), which was driven by higher Fos in males compared to females (t(17.685)=3.152, p= 0.006).

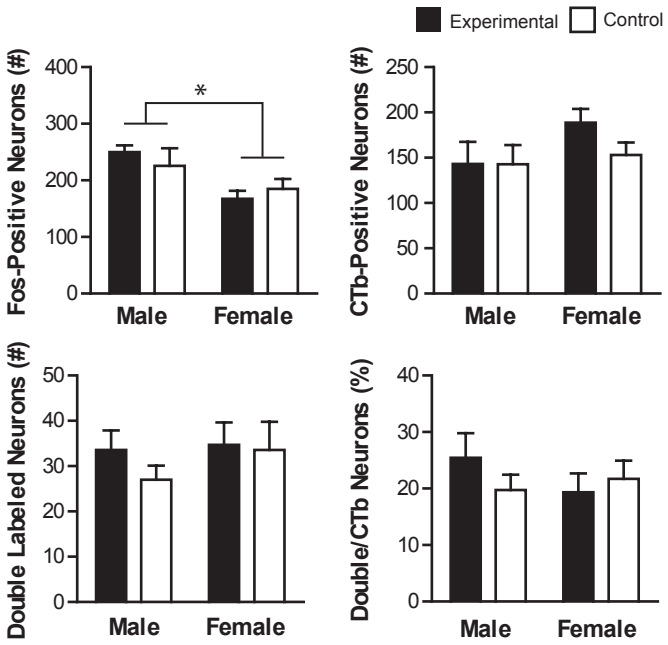

Figure 5.8 Fos induction in PVTp-PL projecting neurons. Shown are mean values (± SEM) for total number of Fos-positive, CTb-positive, and double-labeled (CTb + Fos) neurons, as well as percentage of CTb neurons that were double-labeled. * indicates $p < 0.05$.

There were no other differences between groups in the PVTp for number of CTb-positive neurons, double-labeled neurons or percentage of double-labeled neurons ($p > 0.05$).

3.2.4 Basolateral Area of the Amygdala

3.2.5.1 BLAa

Within the BLAa, Fos induction was highest in males in the experimental group (Figure 5.9 on next page). This was confirmed with an ANOVA with a main effect of Sex and Sex by Group (Sex: $F(1,26)=7.446$, $p=0.011$, Sex by Group: $F(1,26)=5.554$, $p=0.026$) , and there was no effect of Group ($p > 0.05$). A *post hoc t*-test found that the difference in Fos induction between males in the control experimental groups did not reach significance ($p > 0.05$). Males in the experimental group were significantly different from females in the experimental

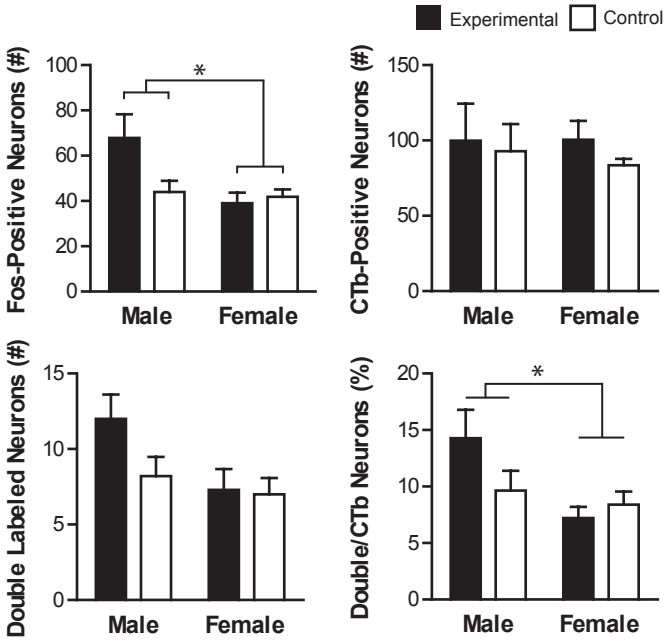

Figure 5.9 Fos induction in BLAa-PL projecting neurons. Shown are mean values (± SEM) for total number of Fos-positive, CTb-positive, and double-labeled (CTb + Fos) neurons, as well as percentage of CTb neurons that were double-labeled. * indicates $p < 0.05$.

group (t(5.563)=2.506, p=0.049) but not from females in the control group (t(4.777)=2.356, p=0.067).

There were no significant differences between groups in the number of CTb-positive neurons or double-labeled neurons ($p > 0.05$, all).

The males in the experimental group had the highest percentage of CTb neurons that were double labeled (Figure 5.9). There was a effect of Sex (F(1,26)=7.575, p=0.011) but no effects of Group or Sex by Group interaction ($p > 0.05$) on the percentage of double-labeled neurons as a portion of the total number of CTb-positive neurons. This was driven by a significantly higher percentage in males in the experimental group compared to females in the experimental group (t(5.309)=2.608, p=0.045).

3.2.5.2 BLAp

Within the BLAp, there were no differences between groups for Fos induction, CTb-positive neurons, double-labeled neurons or percentage of double labeled neurons ($p > 0.05$ for all; Table 5.2).

3.2.5.3 BMAp

Within the BMAp, there was more Fos induction in the male groups compared to the female groups (Table 2). There was an effect of Sex ($F(1,26)=6.414$, $p= 0.018$) but not of Group or Sex by Group ($p > 0.05$) on total amount of Fos induction. However, *post hoc* t-tests revealed no significant differences between groups ($p > 0.05$ for all).

There were no significant differences between groups in the number of CTb-positive neurons or double-labeled neurons ($p > 0.05$, all).

The percentage of CTb neurons that were double-labeled was highest in males in the experimental group (Table 5.2). There was a significant effect of Sex by Group ($F(1,26)=4.979$, $p=0.034$) but not of Sex or of Group ($p > 0.05$). However, *post hoc t*-tests revealed no differences between groups.

3.2.5.4 LA

Within the LA, there was more Fos induction in the male groups compared to the female groups (Table 5.2). This was confirmed with an effect of Sex on total amount of Fos induction ($F(1,26)=9.458$, $p= 0.005$). There were no effects of Group or Sex by Group ($p > 0.05$).

There were no other differences between groups for number of CTb neurons, number of double-labeled neurons or percentage of CTb neurons (p > 0.05 for all; Table 5.2).

4. Discussion

Here, we identified recruitment of key pathways to the PL during context-dependent renewal of conditioned responding to food cues in male and female rats. Specifically we examined PL-projecting neurons within areas important in associative learning and contextual processing: the hippocampal formation, thalamus and basolateral area of the amygdala. To accomplish this, we used a retrograde tracer (CTb) to identify PL-projecting neurons in each area and Fos induction to examine their recruitment during context-mediated renewal. We found projections to the PL from the ventral hippocampal formation (SUBv and CA1v), thalamus (PVTa) and basolateral area of the amygdala (BLAa) were recruited during renewal in a sex-specific way.

Within the ventral hippocampal formation, we found selective recruitment of PL-projecting neurons in both the SUBv and CA1v during renewal responding. In the SUBv, male rats in the experimental group that showed renewal had significantly higher Fos induction in the SUBv-PL pathway (CTb-positive) neurons. Females in both conditions had similar behavior and similar SUBv-PL pathway recruitment. In addition to higher specific Fos induction in the PL-projecting neurons in the experimental group, males also had significantly higher total Fos induction than females. This is in contrast to prior findings that both males and females in the experimental groups had higher Fos induction than

their sex-matched controls in the SUBv (Anderson & Petrovich, 2016). A methodological difference in sampling is potentially the reason why these results differ. Anderson & Petrovich (2016) examined the entire dorso-ventral and medio-lateral extent within the sampling area, while in the current study the total Fos was counted within a more constrained area with substantial PL projections.

In the CA1v, as in the SUBv, males in the experimental group had the highest percentage of PL-projecting neurons that were recruited during renewal compared to all other groups. In addition, males had higher overall Fos induction and higher numbers of PL-projecting neurons with Fos compared to females. Taken together, the SUbv and CA1v results suggest that the ventral hippocampal formation to PL pathways are important mediators of renewal responding. These pathways were selectively activated in males that showed renewal but under activated in both groups of females, which likely influenced the differential behavioral responding observed in males and females. These activation patterns supports the hypothesis that the ventral hippocampal pathways to PL mediate contextual information during renewal of responding in a sex-specific way.

These findings are in agreement with previous evidence the ventral hippocampal formation is important for context-dependent aversive and appetitive renewal (Anderson & Petrovich, 2016; Herry et al., 2008; Hobin, Ji, & Maren, 2006; Jin & Maren, 2015). Additionally, the hippocampal formation is a documented site of sex differences in associative conditioning (Chang et al., 2009; Gresack, Schafe, Orr, & Frick, 2009; Maren, De Oca, & Fanselow, 1994; Matsuda et al., 2015). The hippocampal formation is a likely mediator in the

expression of sex differences in associative learning under the influence of estradiol and potentially other hormones. Different estradiol levels possibly influence the hippocampal formation responsiveness to associative cues, which in turn would aid or hinder associative learning (Gupta, Sen, Diepenhorst, Rudick, & Maren, 2001; Khayum, de Vries, Glaudemans, Dierckx, & Doorduin, 2014; Shors, Chua, & Falduto, 2001; Shors, Falduto, & Leuner, 2004). This is in part why we examined females during different stages in the estrous cycle on test day. However there were no behavioral differences between females in High and Low estradiol groups, which indicates previously observed estradiol effects (with chronic estradiol replacement) are not mediated acutely during test.

In the PVTa, similar to the SUBv, males in the experimental group had the highest percentage of PL-projecting neurons that were recruited during renewal. Males in the experimental group had higher total Fos induction compared to female groups, which suggests that, similar to the ventral hippocampal formation, the PVTa pathways are either under activated or inhibited in the females. These findings build upon prior evidence the PVT is recruited and necessary during appetitive renewal. The PVT and its inputs are important for context-dependent and cue-induced reinstatement of alcohol seeking (Hamlin et al., 2009; Marchant, Furlong, & McNally, 2010; Wedzony et al., 2003).

It should be noted that in the SUBv and PVTa, there were sex differences in the number of CTb neurons. In both regions, there were more CTb labeled neurons in females compared to males. This could be due to differential injections sizes or different connectional patterns in males and females.

However, as shown in Figure 2, the injection sites were evenly matched in size and distribution across sex and experimental conditions. This suggests an intriguing possibility that in females more neurons in SUBv and PVTa send inputs to PL than in males; however, that would need to be validated in future work. Here, to account for individual differences in the number of CTb labeled neurons we expressed double-labeled (CTb+Fos) neurons as a percent of total CTb cells.

The Fos induction patterns in the BLAa were similar to those in the PVTa and SUBv. The males in the experimental group had a significantly higher percentage of CTb+Fos positive neurons compared to females in the experimental group, suggesting that BLAa-PL pathways are recruited in a sex-specific manner. The male experimental group also had higher total Fos induction compared to females in the experimental group. Interestingly, prior work (Anderson & Petrovich, 2016) did not find Fos induction differences in the BLAa between conditions or sexes. As discussed above this is likely due to methodological difference in sampling areas between the studies. The current findings are in general agreement that the basolateral area of the amygdala is important in context-dependent renewal of drug or fear cues (Crombag, Bossert, Koya, & Shaham, 2008; Orsini et al., 2011) and more broadly in appetitive conditioning tasks (Cole, Hobin, et al., 2015; Cole, Powell, & Petrovich, 2013; Crombag et al., 2008; Holland & Petrovich, 2005; Petrovich, 2013; Wassum & Izquierdo, 2015).

Notably, there was less activation of PL-pathways from the SUBv/CA1v, PVTa, and BLAa in the experimental females compared to experimental males,

which suggest either a lack of recruitment or an inhibition of these pathways may have been the reason of lack of renewal behavior in female rats. Interestingly, in addition there was less total Fos induction in females, both in the areas where there were differences in PL-pathway specific Fos induction (SUBv, CA1v, PVTa, BLAa) and in other areas where there were no specific induction in the Pl-pathways (PVTp, BMAp and LA). These patterns suggest overall less activation in females. Taken together, the work here has elucidated key sites of neural sex differences in renewal: the recruitment of CA1v-PL, SUBv-PL, PVTa-PL and BLAa-PL pathways in males and the lack of recruitment in females.

Previously, we found the behavior of females in context-mediated renewal of responding was inconsistent and successful renewal depended on estradiol. Ovariectomized females with a steady dosage of estradiol delivered via silastic capsule exhibited renewal responding but intact and ovariectomized females without estradiol replacement did not (Anderson & Petrovich, 2015). In the current study, we tested females for renewal at both high and low estradiol stages in the estrous cycle in order to determine if acute estradiol was mediating responding at test. We found no behavioral differences between the two groups, and neither group showed renewal compared to their matched controls, but both groups had high responding that was similar to males in the experimental group. This suggests that estradiol may not be critical for renewal acutely at test, instead it may be important during extinction learning, especially considering that estradiol has an established impact on fear extinction learning. Fear extinction during proestrus (high estradiol and progesterone) is enhanced, compared to

metestrus (low estradiol and progesterone) phase, and an estrogen receptor-beta agonist facilitated extinction recall in intact female rats (Lebron-Milad & Milad, 2012; Milad, Igoe, Lebron-Milad, & Novales, 2009). Estradiol and progesterone administered together before or immediately after extinction training increased the rate of extinction learning (Milad et al., 2009). It is likely that estradiol has similar modulatory effects in appetitive extinction, which would explain why estradiol had an impact when given continuously throughout training and testing (Anderson & Petrovich, 2015) but not when tested acutely the day of test. It may even explain why both female groups exhibited high responding similar to males in the experimental group. For example, if the majority of the females were in metestrus during the extinction sessions, this could have impaired extinction learning that would have resulted in high responding on test day.

The current study focused on the PL however the ILA was similarly recruited during renewal of responding (Anderson & Petrovich, 2016) and it is likely the ILA is also important, possibly in a different way. There is evidence for differential roles of the PL and ILA during extinction and renewal responding of fear (Knapska & Maren, 2009; Mendoza, Sanio, & Chaudhri, 2015; Willcocks & McNally, 2013). However, the similar recruitment of the PL and ILA we previously found suggests a common function in agreement with recent work showing similar patterns of activation in PL and ILA inputs from ventral hippocampal formation neurons during fear renewal (Wang et al., 2016). Further work is necessary to determine if pathways to the ILA are similarly recruited and if there

is a functional dissociation in between the PL and ILA circuitries during renewal responding.

5. Conclusions

These results provide evidence that PL inputs are recruited differently in males and females that is likely underlying the behavioral sex differences in renewal responding. We identified sex-specific recruitment of SUBv-PL, CA1v-PL, PVTa-PL and BLAa-PL pathways during context-dependent appetitive renewal. This specific recruitment of projections to the PL corresponds to the behavioral differences between males and females, and suggest under activation in females. These findings add to our understanding of the neural circuit mechanisms underlying sex differences in associative learning and memory with respect to appetitive learning. More broadly, there are implications for future translational work investigating maladaptive eating habits in males and females.

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Chapter 6: General Discussion

The main goal of the research in this dissertation was to define the neural circuitry of appetitive context-dependent renewal in male and female rats. First, we established there are behavioral and neural sex differences during renewal responding (Chapters 2 and 3). We then determined the ventromedial prefrontal cortex (vmPFC) is critical for renewal responding in males and females in a sex-specific way (Chapter 4). Lastly, we determined that neurons projecting to the vmPFC, and specifically the prelimbic area (PL), are differentially activated during renewal responding in males and females (Chapter 5).

In Chapter 2, we first established the behavioral sex differences during context-mediated renewal: males exhibited renewal of responding while females failed to do so. We then tested if estradiol has a modulatory role in renewal responding and found renewal was induced in ovariectomized females with estradiol replacement. In Chapter 5 we tested if acute estradiol levels on test day impacts renewal responding and found it does not. Taken together with the well-established role of estradiol on extinction learning, it is most likely that estradiol is important during extinction learning, either impairing or enhancing extinction learning depending on high or low estradiol levels, which then impacts renewal responding.

In Chapter 3 we identified, using Fos induction, key regions important during renewal responding. Most importantly, we established in context-mediated renewal of responding the vmPFC is recruited differently in males and females.

This differential recruitment is in agreement with the behavioral differences where males exhibit renewal responding and females fail to do so.

By manipulating the vmPFC in Chapter 4, we showed not only is the vmPFC recruited during renewal in males, but inhibiting the vmPFC in males blocks renewal responding. Reversely, stimulating the vmPFC in females resulted in renewal of responding; suggesting females' failure to show renewal responding is due to a lack of recruitment of the vmPFC. This lack of recruitment could be due to inputs from other areas to the vmPFC, so we next examined pathways to the vmPFC that are recruited during renewal responding.

By examining the projections to the PL in Chapter 5, we determined during the test for renewal pathways to the PL from the ventral hippocampal formation, thalamus and basolateral area of the amygdala were recruited in males and not recruited in females. These results confirm that activational differences between males and females during renewal responding in the vmPFC, hippocampal formation, thalamus and basolateral amygdala are most likely due to differences in the recruitment of specific pathways to the vmPFC.

The vmPFC, hippocampal formation, thalamus and basolateral amygdala have well established roles in associative learning (Cole et al., 2015; Corcoran & Maren, 2001; Holland & Petrovich, 2005; Marchant, Furlong, & McNally, 2010; Orsini et al., 2011; Petrovich, Canteras, & Swanson, 2001; Petrovich & Gallagher, 2007; Shansky et al., 2010; Wassum & Izquierdo, 2015; Wedzony et al., 2003). The research in this dissertation defined how these regions are important for context-mediated renewal responding. In males, the vmPFC is

causal in renewal responding and the evidence from Chapter 5 showed inputs from the ventral hippocampal formation, paraventricular nucleus of the thalamus (PVT) and basolateral area of the amygdala may be critical. Comparably, in females, the vmPFC, PVT and basolateral area of the amygdala as well as the pathways to the PL are not recruited as they are in males. There is an overall lack of recruitment, either from lack of activation or possibly inhibition, in the circuitry for females. This lack of recruitment could explain the lack of behavioral responding during renewal for females.

As discussed in Chapter 5, we chose to investigate the projections to PL based on the strong inputs from the ventral hippocampal formation, but projections to the ILA of the vmPFC are likely also recruited during renewal responding. Further investigation would be required to determine if projections from the hippocampal formation, thalamus and basolateral amygdala to the PL and ILA have similar or dissociable roles during renewal responding. Additionally, the vmPFC has reciprocal connections with the ventral hippocampal formation, thalamus and basolateral amygdala (Canteras & Swanson, 1992; Cenquizca & Swanson, 2007; Fanselow & Dong, 2010; Hoover & Vertes, 2007; Moga et al., 1990; Pitkanen, Pikkarainen, Nurminen, & Ylinen, 2000; Reppucci & Petrovich, 2015; Sesack, Deutch, Roth, & Bunney, 1989; Swanson & Petrovich, 1998; Vertes & Hoover, 2008), and thus the projections from the vmPFC to these areas could be equally relevant as projections to the vmPFC from these areas. Importantly, the vmPFC is critical for decision-making processes that guide behavior (Dalley, Cardinal, & Robbins, 2004; Lopez-Ramos, Guerra-Narbona, &

Delgado-Garcia, 2015; O'Doherty, 2011), which would suggest it may be critical during the decision regarding whether to respond to the extinguished cue during the renewal test. This, along with the evidence that the vmPFC is recruited during renewal as described in Chapter 3, strongly suggests the vmPFC is the key mediator in the circuitry.

There are other areas connected with the vmPFC that could potentially be important for renewal responding, in particular the nucleus accumbens and the lateral hypothalamus for their roles in reward and decision-making (for reviews see Floresco, 2015; Salgado & Kaplitt, 2015) and in energy and metabolism regulation, respectively (for reviews see Jennings, Rizzi, Stamatakis, Ung, & Stuber, 2013; Swanson, 2000). The nucleus accumbens is involved in the relapse of alcohol seeking (Marchant & Kaganovsky, 2015). The lateral hypothalamus is critically involved in associative food leaning (Cole et al., 2015; Sharpe et al., 2017) as well as the relapse of alcohol and drug seeking (Hamlin, Clemens, & McNally, 2008; Marchant et al., 2010). The vmPFC mu-opioid system and pathways to the vmPFC, including the hypothalamus and nucleus accumbens, are important for controlling non-homeostatic feeding (Selleck & Baldo, 2017). The drug addiction and food motivation circuitries overlap (Tomasi & Volkow, 2013; Volkow & Wise, 2005), further supporting these areas as potentially being important for renewal of responding to food cues.

In addition, what types of neurons are being recruited in the vmPFC during renewal responding is still undetermined. In Chapter 4, we used a human synapsin-1 promoter that targeted all neurons. We had hypothesized that

glutamatergic neurons were critical and intended to test this hypothesis using a CamKII promoter that would have targeted these glutamatergic neurons but due to technical difficulties were not able to do so. The vmPFC's function in renewal may require either glutamatergic pyramidal neurons or inhibitory interneurons or both (Gabbott et al., 2012; Sesack et al., 1989). Recent work has suggested there is heterogeneity in the vmPFC control of feeding behavior based on differing results of blocking AMPA-type and NMDA-type glutamate receptors (Baldo, Spencer, Sadeghian, & Mena, 2016). Blocking AMPA-type receptors mimicked the effect of muscimol treatment by increasing the length of feeding bouts regardless of whether eating was due to hunger by food deprivation or due to the palatability of the food. Blocking by an NMDA-receptor antagonist did not have any effect on feeding behavior. Similarly, in cocaine-cue extinction, there was a significant increase in GluA1, an AMPA receptor subunit, protein expression after male rats underwent extinction training sessions (Nic Dhonnchadha et al., 2013).

GABAergic cells are also potentially important for vmPFC mediation of renewal. GABA, but not glutamate, transmission in the medial prefrontal cortex increased due to activation of inhibitory interneurons when male rats were placed in a cocaine-associated environment (Chefer, Wang, & Shippenberg, 2011). This inhibition was blocked by basolateral area of the amygdala inactivation. However, the large majority of work on GABAergic and glutamatergic neurons in the vmPFC has been completed using male rats, so it is unknown if these results are similar for female rats. There are documented sex differences in GABAergic

activity. A study that examined GABAergic activity found a GABA-A receptor subunit immunoreactivity due to maternal separation found maternal separated females had lower immunoreactivity in the hippocampal formation and vmPFC compared to female controls while maternal separated males had lower immunoreactivity in the amygdala and vmPFC compared to male controls (Leon Rodriguez & Duenas, 2013). It has been hypothesized that GABAergic mechanisms within the hippocampal formation influence resilience or vulnerability to activity-based anorexia in female mice (Aoki, Chowdhury, Wable, & Chen, 2017). Since it appears there is either a lack of recruitment or active inhibition of areas within the female brain, it could be that inhibitory GABAergic neurons are crucial during renewal behavior, but future work is required to confirm.

There were consistent behavioral sex differences across experiments in this thesis. In context-dependent renewal, higher responding in the acquisition context compared to responding in the extinction context after extinction learning is renewal of responding. In a between-subjects design, this comparison is made between separate experimental and control groups that experience the test for renewal in the acquisition or extinction contexts, respectively. In a within-subjects design, this comparison is made by comparing responding of the same rat in the acquisition and extinction contexts. Male rats showed renewal of responding after extinction either when tested in with-subjects or between-subjects designs. Intact female rats were variable in their responding, but consistently never exhibited this difference in either paradigm (e.g. whether compared to a female control

group or responding of the same rat in both contexts). It should be noted that in females in some instances we have observed low responding, comparable to males in the control condition, while in other instances females' responding was high, similar to males in the experimental condition. These differences in renewal responding were likely due to estradiol-mediated differences in extinction learning that then impacted responding on test day. We were only able to induce renewal responding in females in experimental groups by stimulating the vmPFC or supplying estradiol replacement to ovariectomized females. The lack of behavioral responding is therefore due to at least partially to under activation of the vmPFC and modulation by estradiol. Based on the results of the PL-inputs pathway analysis, it is likely that recruiting the under active pathways in females, in particular the SUBv/CA1v to PL pathways, would induce renewal responding in females.

Here we have established that males and females exhibit different context-mediated appetitive conditioned behavior after extinction. Context-mediated renewal responding has been well defined behaviorally as an increase in conditioned responding after extinction induced by contextual information (Bouton & Bolles, 1979; Bouton & King, 1983). By this definition, males are exhibiting the "correct" response and females are failing to do so. However, the lack of renewal responding in females is not necessarily a failure. Males seem to be using context as an "occasion setter" for reinforcement of the primary conditioned cue (Bouton, 2004; Holland & Bouton, 1999), and distinguishing learning in the acquisition and extinction contexts. Females may be generalizing

more than males, and thus relying more on the extinguished cue-food association than contextual information. Neither of these are inherently poor responses nor do they imply lack of learning or poor learning. Both males and females acquire and extinguish appetitive associations correctly, and both can correctly distinguish between contexts. It appears there are basic sex-differences in the neural circuitry that is recruited during context-mediated renewal that modulates this difference in responding. Based on the evidence presented in this thesis, the vmPFC and pathways to the vmPFC are under recruited in females compared to males. This has large implications for sex differences in learning and memory, as well as translation and clinical research.

The behavioral and neural sex differences in renewal responding are in agreement with the ever growing amount of evidence of sex differences in basic research, especially related to associative conditioning and contextual processing (Gruene, Roberts, Thomas, Ronzio, & Shansky, 2014; Keiser et al., 2017; Lynch, Cullen, Jasnow, & Riccio, 2013; Maeng & Shors, 2013; Maren et al., 1994; Matsuda et al., 2015; Reppucci et al., 2013) as well as sex differences in the prevalence of anxiety, eating disorders and obesity (Flegal, Carroll, Kit, & Ogden, 2012; McCarthy et al., 2012; Ogden et al., 2014). Female rats and mice are not intrinsically more variable (Becker, Prendergast, & Liang, 2016; Prendergast et al., 2014) and need to be included in both basic and translation research (Shansky & Woolley, 2016; Zucker & Beery, 2010). It is true that sex hormones influence both the brain and behavior in important ways (for reviews see Cover, Maeng, Lebron-Milad, & Milad, 2014; Frankfurt & Luine, 2015; Geary,

1998; Lebron-Milad & Milad, 2012) but that is not a reason to exclude females from research. The role of hormones on behavior is another biological variable to consider.

Understanding sex differences in eating behaviors is of particular importance when considering the obesity epidemic we are currently experiencing. Obesity is on the rise and females are more likely to be overweight or obese (Ogden et al., 2014). Environmental influences, including high prevalence of food cues, are an important contributor (Berthoud, Munzberg, & Morrison, 2017; Levitsky, 2005; Petrovich, 2013; Schachter, 1968). Maladaptive eating habits being a factor in obesity highlights the importance of understanding learned eating behaviors. The prevalence of obesity is an unfortunate example of how difficult it can be to change persistent behaviors. The difficulty of changing maladaptive eating habits cued by the environment is modeled in persistent responding to food cues such as renewal. This difficulty of changing learned eating habits is a problem; trying to inhibit eating of palatable foods during dieting, and subsequent eating, is comparable to attempt to stop drug use and subsequent relapse (Calu et al, 2014; Stewart, De Wit & Eikelboom, 1984; Torregrossa & Taylor, 2013; Volkow & Wise, 2005). Understanding the circuitry important for renewal of responding to food cues increases our knowledge about learned eating, and can hopefully advance research on this important topic. Current therapies for addiction rely on extinguishing negative learned behavior, but extinction is not unlearning, rather it is a new learning that can depend on context (Bouton, 2004), and do not treat males and females differently. It may be

that trying to alter unhealthy eating and drug use behaviors needs to be considered separately for males and females.

While the exact circuitries will differ, the current results can also provide insights into the circuitries underlying renewal of other behaviors. What areas are recruited in the drug responding circuitry may differ in males and females as well, though in a different way than renewal of responding to food cues. Behaviorally, female rats tend to have higher cocaine seeking and equal or enhanced cocaine intake compared to males (Kippin et al., 2005; Lynch & Taylor, 2004; Zhou et al., 2014). During cocaine conditioning, females also had higher Fos induction compared to males in multiple regions including the ventral hippocampal formation and nucleus accumbens (Zhou et al., 2014). Additionally, while Fos induction levels did not differ between groups, Fos induction in the PL was correlated with cue-induced cocaine seeking for both males and females. Therefore, in drug seeking females exhibited higher amounts of conditioned behavior and higher Fos induction compared to renewal of responding to food cues where females exhibited lower amounts of conditioned behavior and lower Fos induction. There are also clear sex differences in drug use, both in basic and clinical research (Bobzean, DeNobrega, & Perrotti, 2014; Evans & Reynolds, 2015; Venniro, Zhang, Shaham, & Caprioli, 2017). The drug reward circuitry includes a dopamine systems of the ventral tegmental area, nucleus accumbens, bed nucleus of the stria terminalis as well as the prefrontal cortex (Gardner, 2011) which is recruited differently in males and females and is influenced by ovarian hormones (Bobzean et al., 2014). In the nucleus accumbens, medium

spiny neuron density is higher in drug-naive females compared to males and females had a higher cocaine-induced increase in spine density (Wissman, McCollum, Huang, Nikrodhanond, & Woolley, 2011). In drug seeking the circuitry seems to be under recruited in males not females. This under recruitment may be mechanistically similar to renewal of food cues but with males being under recruited instead of females.

Sex differences in renewal also have implications for fear learning; differences in the vmPFC and hippocampal formation suggest an overlap in the sex differences of the fear and food circuitries (Maeng & Milad, 2015). There are sex differences in the acquisition and extinction of learned fear (Maren et al., 1994; Pryce, Lehmann, & Feldon, 1999; Ribeiro et al., 2010), as well as in anxiety and PTSD in humans (McCarthy et al., 2012; Shansky, 2015). The fear learning and fear extinction network, similar to the food learning and extinction network, includes the vmPFC, amygdala and hippocampal formation (Furini, Myskiw, & Izquierdo, 2014). Extracellular signal-related kinase (ERK) phosphorylation, a mediator of memory formation (Cestari, Rossi-Arnaud, Saraulli, & Costanzi, 2014), was increased in the ventral hippocampal formation in male mice but not females following contextual fear conditioning (Gresack, Schafe, Orr, & Frick, 2009). This lack of ERK phosphorylation in female mice is similar to the under activation in female rats observed in Chapters 3 and 5. Lesioning of the medial prefrontal cortex resulted in females freezing more than sham females; specifically female rats froze more during fear conditioning than sham females, with no differences in behavior between lesioned or sham males

(Baran, Armstrong, Niren, & Conrad, 2010). Lesioning of the mPFC impaired extinction learning by increasing freezing in females, compared to normal extinction in sham females, and lesioned and sham males (Baran et al., 2010). Injections of estradiol or an estradiol receptor agonist into the hippocampal formation enhanced retrieval of contextual fear extinction in female rats (Chang et al., 2009). These results suggest the sex differences observed in the vmPFC, hippocampal formation and amygdala in the renewal of appetitive conditioned behaviors are potentially also occurring in fear extinction and renewal.

In summary, the findings from experiments described in this dissertation advanced our understanding of the neural mechanisms underlying sex differences in associative memory and contextual processing. We determined key components in the neural circuitry mediating renewal of responding to food cues, valuable for understanding the fundamental mechanisms of reward driven behaviors and important for future translational and clinical studies. This investigation into the neural mechanisms of Pavlovian renewal was important for our understanding of the resilience of food cue to influence our consumption and diet choices.

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