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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 & Kober, 2016; Sun et al., 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 & Bouton, 2015; Todd, Winterbauer, & 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, & Shaham, 2014).

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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 & 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 & 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, & Shors, 2009; Farrell, Sengelaub, & Wellman, 2013; Maren, De Oca, & Fanselow, 1994; Reppucci, Kuthyar, & 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, & Robbins, 2004; O'Doherty, 2011) and its role in associative learning, including renewal (Eddy, Todd, Bouton, & Green, 2016; Willcocks & 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, & Davidson, 2010; Davidson et al., 2009; Fanselow, 2000; Holland & Bouton, 1999; Marinelli, Funk, Juzytsch, Li, & Le, 2007; Orsini, Kim, Knapska, & Maren, 2011). The thalamus, specifically the paraventricular nucleus (PVT), has been implicated in context-induced renewal (Hamlin, Clemens, Choi, & McNally, 2009) and is involved in the regulation of food consumption (Bhatnagar & Dallman, 1999; Cole, Mayer, & Petrovich, 2015; Stratford & Wirtshafter, 2013). The amygdala is important for appetitive associative learning and subsequent control of behavior (Cole, Hobin, & Petrovich, 2015; Cole, Powell, & Petrovich, 2013; Crombag & Shaham, 2002; Holland & 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 g 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 × 28 × 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 × 4.2 cm) and a 4 W house light. Each chamber was located in a sound- and light-attenuating cubicle (79 × 53 × 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 s tone (75 dB, 2 kHz), 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 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 (Fig. 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 & 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-min 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-min 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-min session with eight CS presentations and no USs, conducted in the conditioning (acquisition)

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

Fig. 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 min after the end of Renewal test and brains were collected for Fos induction detection by immunohistochemistry.

context. The inter-trial interval was 110–326 s 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 s during each CS and during 10 s 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 20–24 h 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 40 μm 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 h at room temperature in a blocking solution (0.02 M 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 h 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 min. 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).

2.7. Image acquisition and analysis

Images of the Fos-stained and adjacent thionin-stained sections were acquired (10× 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 Fig. 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 Fig. 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 −2.00 mm), 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., Cole et al., 2015; Li & Kirouac, 2012). Thus, images were taken from the anterior and posterior halves, and were analyzed separately, as anterior (PVTa) (Level 26, −1.78 mm) and posterior (PVTp) (Level 30, −3.25 mm) 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,

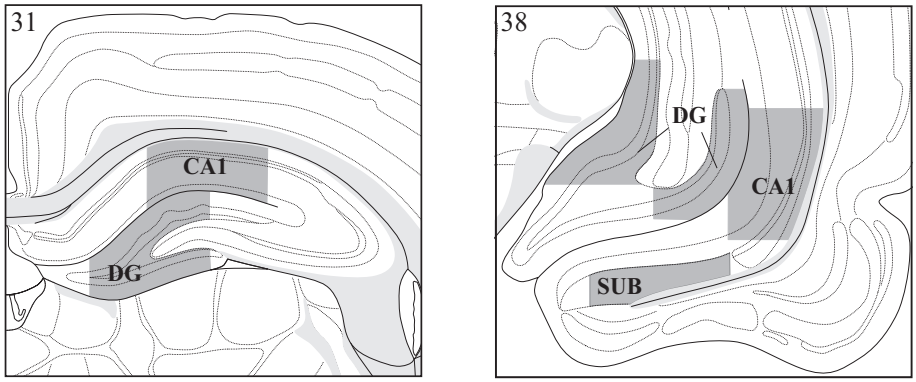

Fig. 2. Sampling areas in the hippocampal formation. Dark gray shading denotes sampling areas in dorsal (left) and ventral (right) hippocampal formation. Illustrations were made on modified templates from the Swanson atlas (2004), and numbers in the upper left corner of each denote atlas levels. Abbreviations: CA1 - field CA1, Ammon's horn; DG - dentate gyrus; SUB - subiculum.

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 (Fig. 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 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).

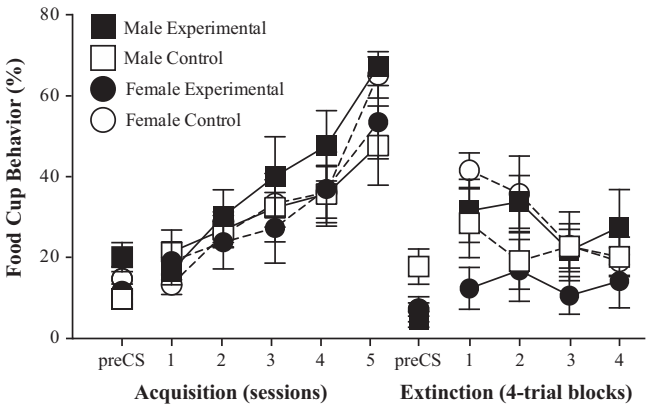

Fig. 3. Conditioned responses during acquisition and extinction. Percentage of time rats expressed food cup behavior (mean ± SEM) during the preCS and CS periods during training sessions. PreCS values are the average across all sessions for acquisition and extinction, respectively. Acquisition is shown 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).

3.1.2. Extinction

All groups showed a decrease in conditioned responding due to extinction training (Fig. 3, Table 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 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 (Fig. 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

Table 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

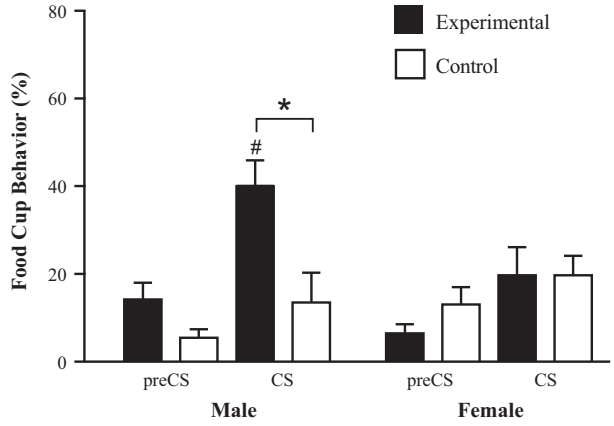

Fig. 4. Conditioned responses during the test for renewal. Percentage of time rats expressed food cup behavior (mean ± SEM) during preCS and CS periods; ∗ indicates p < 0.001, # indicates within-group preCS vs CS difference $p < 0.05$.

and CS periods ($p > 0.05$). Both female groups responded similarly low during preCS and CS ($p > 0.05$).

3.2. Fos induction

Table 2 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 (Fig. 6). Within the ACA, there were no differences between groups ($p > 0.05$; Table 2). Within the ILA, an ANOVA for Sex and Group found significant effect for Sex by Group interaction ($F(23,1) = 12.439$, $p < 0.01$; Figs. 5 and 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 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 (Fig. 6, Table 2). We found no differences in Fos induction patterns within the CA1v, DGv and DGd ($p > 0.05$ all; Table 2). Within the SUBv, experimental groups had higher Fos induction than controls for both sexes (Fig. 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 (Fig. 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

Table 2
Fos induction for all brain regions analyzed. Results are displayed as a mean total number of Fos-positive neurons in each area ± SEM.

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

* Significant difference compared to same sex control, $p < 0.05$.

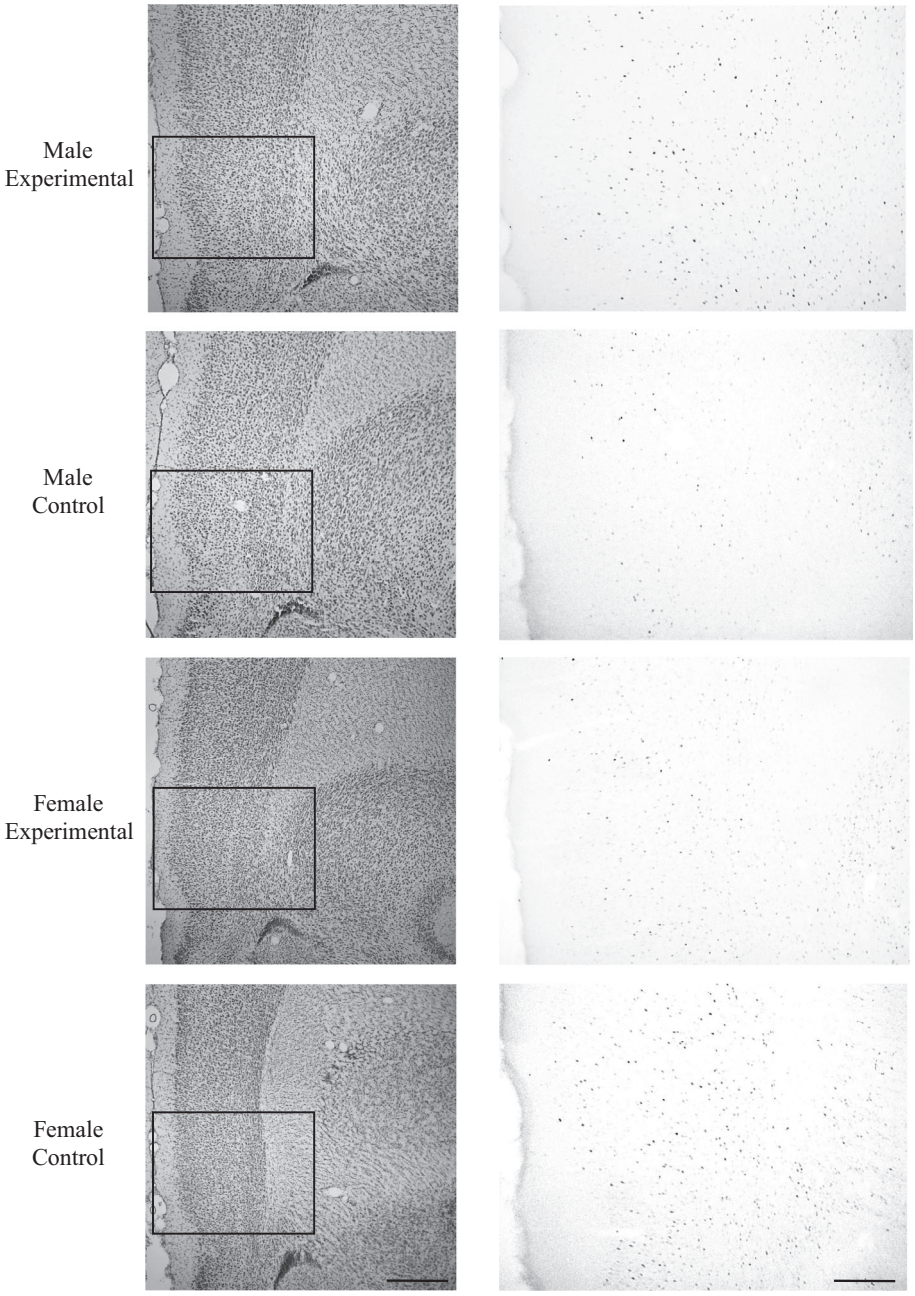

Fig. 5. Fos induction in the medial prefrontal cortex. Representative photomicrographs of Fos induction in the infralimbic area (ILA) are shown on right (Scale bar = 200 μm), and thionin-stained adjacent sections are shown on left (level 9; Scale bar = 500 μm). Each box depicts the area shown in the corresponding Fos image.

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 2), except for the CEAc (Fig. 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 (Fig. 6) but not in PVTp ($p > 0.05$; Table 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

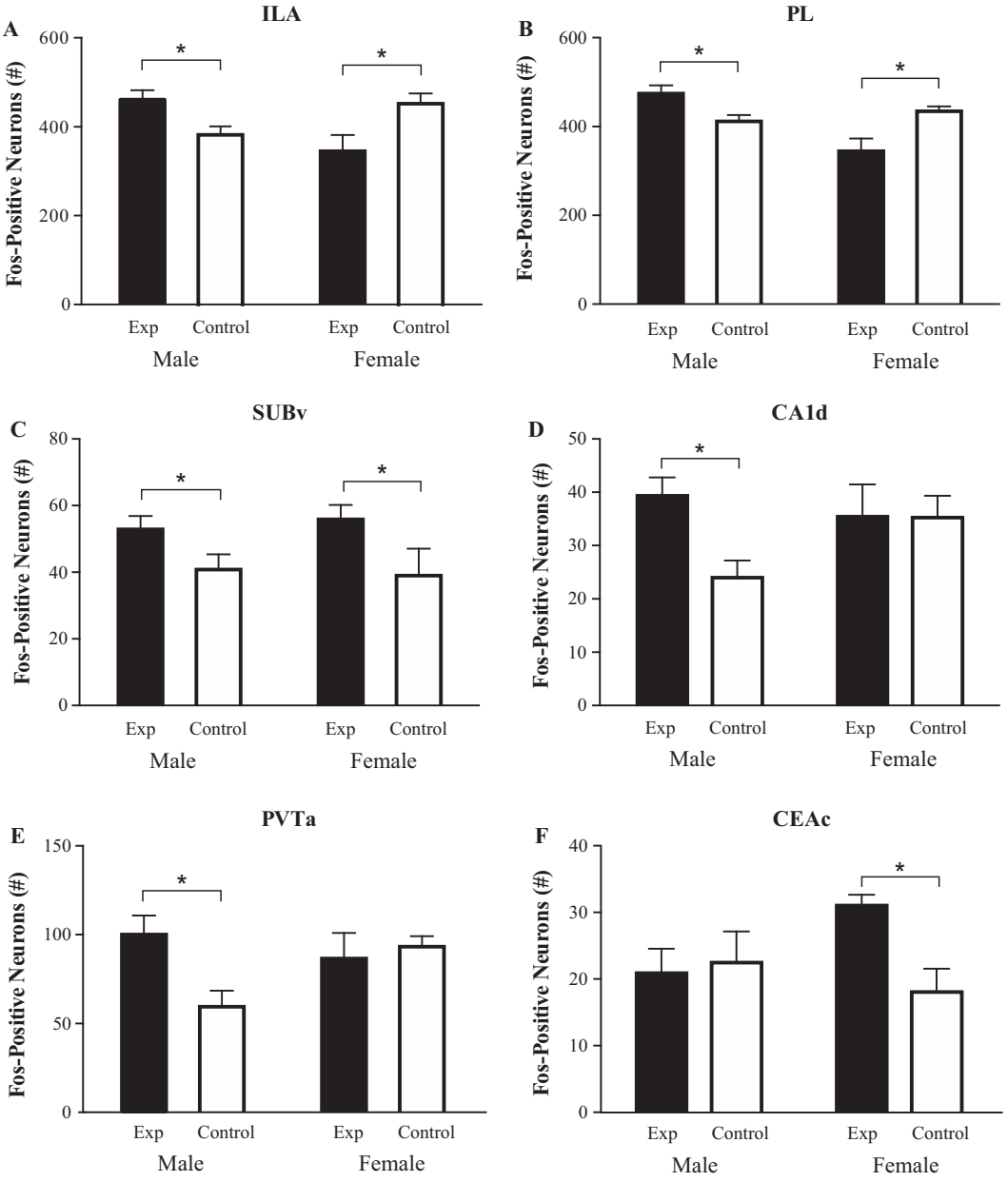

Fig. 6. Fos induction. Total number of Fos-positive neurons in the infralimbic area, ILA (A), prelimbic area, PL (B), ventral subiculum, SUBv (C), dorsal CA1 (D), anterior part of the paraventricular nucleus, PVTa (E), capsular part of the central amygdalar nucleus, CEAc (F). ∗ indicates $p < 0.05$.

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 in the 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 ($r = 0.738$, $p > 0.01$) 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 & 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, & Aston-Jones, 2015). Inactivation of the vmPFC disrupted context-mediated reinstatement of alcohol and drug seeking (Bossert et al., 2011; Willcocks & 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 & Killcross, 2007). Additionally, the PL and the ILA, but not the ACA, were recruited during appetitive (tone-food) associative learning (Cole et al., 2015) and this area is also critical for feeding stimulated by contextual food cues (Petrovich, Ross, Gallagher, & Holland, 2007). Finally, the medial PFC has been implicated in renewal of conditioned fear (Herry & Mons, 2004; Knapska & 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, & 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 & O'Doherty, 2010; Smith, Virkud, Deisseroth, & 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, & Chaudhri, 2015) and studies of renewal with aversive tasks found differences between the PL and ILA (Knapska & 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 & 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 & 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, & 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, & 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 are important given the structural and functional distinction along the dorso-ventral axis of the hippocampal formation (Fanselow & 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, & Maren, 2006). Furthermore, the SUBv has direct connections to the vmPFC, specifically the PL (Canteras & Swanson, 1992; Fanselow & Dong, 2010), and ventral hippocampal neurons, including SUBv, that project to both the vmPFC and amygdala were specifically activated during fear renewal (Herry et al., 2008; Jin & 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 & Maren, 2001; Ji & Maren, 2005) while in another study lesions impaired reinstatement induced by exposure to the US, but not context-dependent renewal, of fear (Frohardt, Guarraci, & 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 & 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, & McNally, 2010; Wedzony et al., 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 & Dallman, 1999), and the PVTa was selectively recruited, along with the vmPFC, when cue-induced feeding was blocked with ORX antagonist (Cole et al., 2015).

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 & 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., 2013, 2015; Crombag, Bossert, Koya, & Shaham, 2008; Holland & Petrovich, 2005; Petrovich, 2013; Wassum & 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 (Fig. 7). The vmPFC has distinct, topographically organized connectional patterns with the amygdala, the ventral hippocampal formation, and 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). For example, while both the PL and ILA have outputs to PVTa, the projections from the PL to the PVTa are denser (Li & Kirouac, 2012). Additionally, SUBv projects to both the PL and ILA (Canteras & Swanson, 1992). The SUBv also has direct projections to the PVT (Canteras & Swanson, 1992), which

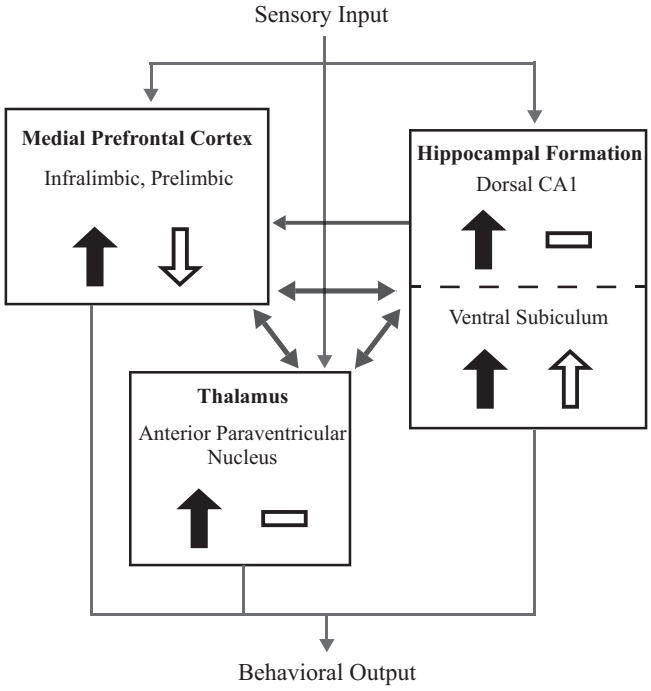

Fig. 7. Medial prefrontal cortex-hippocampal-thalamic system recruitment during context-mediated renewal of conditioned responding to food cues. Summary of major Fos induction differences in males and females are shown on a connectional diagram. Males are represented by filled arrows and females are 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.

in turn projects to the vmPFC (Hoover & 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 projections from the dorsal CA1 to the ILA, with slightly sparser projections to the PL (Hoover & Vertes, 2007). Given the well-known function of the hippocampal formation in contextual processing (Fanselow, 2000; Holland & 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 & 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, & Shors, 2010). This function depends specifically on the PL and its connections with the amygdala (Maeng & 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 et al., 2014; Khayum, de Vries, Glaudemans, Dierckx, & Doorduin, 2014; Simerly, Chang, Muramatsu, & Swanson, 1990). Estradiol can affect memory via changes in dendritic spine density in the vmPFC and the dorsal hippocampal formation (for review see Frankfurt & Luine, 2015; Inagaki, Frankfurt, & Luine, 2012; Wallace, Luine, Arellanos, & Frankfurt, 2006). Estradiol administration in the dorsal hippocampal formation increased spine density on the pyramidal vmPFC neurons (Tuscher, Luine, Frankfurt, & Frick, 2016). Another mechanism of estradiol action in renewal may be through changes in the efficacy of dopamine transmission in the PFC (Rey, Lipps, & Shansky, 2014), as D1 receptors within the ILA are critical to successful extinction (Hikind & Maroun, 2008). Lastly, estradiol was shown to change serotonergic neural transmission in the PVT in females (Krajnak, Rosewell, Duncan, & 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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