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ORIGINAL ARTICLE

Neural substrates of fear-induced hypophagia in male and female rats

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Abstract

Cessation of eating under fear is an adaptive response that aids survival by prioritizing the expression of defensive behaviors over feeding behavior. However, this response can become maladaptive when persistent. Thus, accurate mediation of the competition between fear and feeding is important in health and disease; yet, the underlying neural substrates are largely unknown. The current study identified brain regions that were recruited when a fear cue inhibited feeding in male and female rats. We used a previously established behavioral paradigm to elicit hypophagia with a conditioned cue for footshocks, and Fos imaging to map activation patterns during this behavior. We found that distinct patterns of recruitment were associated with feeding and fear expression, and that these patterns were similar in males and females except within the medial prefrontal cortex (mPFC). In both sexes, food consumption was associated with activation of cell groups in the central amygdalar nucleus, hypothalamus, and dorsal vagal complex, and exposure to food cues was associated with activation of the anterior basolateral amygdalar nucleus. In contrast, fear expression was associated with activation of the lateral and posterior basomedial amygdalar nuclei. Interestingly, selective recruitment of the mPFC in females, but not in males, was associated with both feeding and freezing behavior, suggesting sex differences in the neuronal processing underlying the competition between feeding and fear. This study provided the first evidence of the neural network mediating fear-induced hypophagia, and important functional activation maps for future interrogation of the underlying neural substrates.

Keywords Amygdala · Prefrontal cortex · Hypothalamus · Fear · Feeding · Anorexia

Abbreviations

ACAd	Anterior cingulate area, dorsal part
ANOVA	Analysis of variance
BLA	Basolateral amygdalar nucleus
BLAa	Basolateral amygdalar nucleus, anterior part
BLAp	Basolateral amygdalar nucleus, posterior part
BMA	Basomedial amygdalar nucleus
BMAa	Basomedial amygdalar nucleus, anterior part
BMAp	Basomedial amygdalar nucleus, posterior part
CEA	Central amygdalar nucleus
CEAc	Central amygdalar nucleus, capsular part
CEAl	Central amygdalar nucleus, lateral part
CEAm	Central amygdalar nucleus, medial part
CS	Conditioned stimulus
DMX	Dorsal motor nucleus vagus nerve
ILA	Infralimbic area
LA	Lateral amygdalar nucleus
LHA	Lateral hypothalamic area
mPFC	Medial prefrontal cortex
NTSm	Nucleus of the solitary tract, medial part
PL	Prelimbic area
PVH	Paraventricular hypothalamic nucleus
PVHdp	Paraventricular hypothalamic nucleus, dorsal parvicellular part
PVHmpd	Paraventricular hypothalamic nucleus, medial parvicellular part, dorsal zone
PVHmpv	Paraventricular hypothalamic nucleus, medial parvicellular part, ventral zone
PVHpml	Paraventricular hypothalamic nucleus, posterior magnocellular part, lateral
PVHpv	Paraventricular hypothalamic nucleus, periventricular part
PVT	Paraventricular thalamic nucleus
SO	Supraoptic nucleus, proper
US	Unconditioned stimulus

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Introduction

Cessation of eating under fear is an adaptive physiological response (Cannon 1915). This response can be triggered by either innate or learned cues, and aids survival by shifting attention to the fear-provoking cue and prioritizing the expression of defensive over other ongoing behaviors. This behavioral response can be modeled in the laboratory setting, where through Pavlovian conditioning, initially neutral cues can come to signal a fearful event and inhibit eating even in hungry subjects. Importantly, this effect of conditioned fear on feeding is a robust response that can be elicited by both simple, discrete cues (Petrovich and Lougee 2011; Petrovich et al. 2009), as well as complex, contextual stimuli (Reppucci et al. 2013) associated with past aversive events. Halting eating to attend to danger is adaptive (e.g., ceasing to eat to flee an imminent threat); however, when persistent this response could become maladaptive and contribute to the onset or maintenance of disease states (e.g., anorexia). Despite the clear importance of this behavioral response in health and disease, the neural substrates mediating fear-induced hypophagia are largely unknown. The current study used Fos imaging to determine the key neural systems recruited when a fear cue inhibits feeding despite physiological hunger in male and females rats.

Few studies have compared behavior and brain activation patterns of intact males and females (Cahill 2006; Zucker and Beery 2010), even though it is a necessary step in characterizing differences between the sexes (McCarthy et al. 2012). Here, we examined both sexes because there are sex differences in this preparation (Petrovich and Lougee 2011) that are potentially relevant to higher reported rates of anxiety and eating disorders in females compared to males (Becker et al. 2007; McCarthy et al. 2012; McLean and Anderson 2009).

We used a recently developed behavioral procedure (Petrovich and Lougee 2011) and replicated the finding that a fear cue can powerfully inhibit feeding in hungry male and female rats. In that protocol, rats in the experimental group are trained to associate a tone (conditioned stimulus, CS) with electric footshocks (unconditioned stimulus, US) in a distinct context (aversive context), and to consume food in another context (appetitive context). Rats in the control group receive only tones without any shocks. Then at test, the tone presentations halt consumption in the appetitive context in the experimental compared to the control group (Petrovich and Lougee 2011). This preparation is well suited for brain analysis because during the test the effects of the tone (CS) are isolated from the footshock (US), and the confounding effects of the aversive training context are eliminated. To specifically identify the activation patterns of brain regions mediating fear-induced hypophagia and dissociate these from patterns due to fear or feeding expression, we added two additional groups. These groups underwent training and testing identical to that of the experimental and control groups, except they did not have access to food when in the appetitive context.

Prior work with lesions demonstrated that the central nucleus of the amygdala (CEA) is critical for fear-induced hypophagia (Petrovich et al. 2009) and an amygdala–prefrontal–lateral hypothalamic circuitry is critical for the control of feeding by appetitive learned cues (for review see Petrovich 2013). Thus, we hypothesized a similar network may be mediating the control of feeding by aversive learned cues. We mapped Fos induction in these areas and other regions important for feeding or fear, including the dorsal vagal complex, during the expression of fear-induced hypophagia, and assessed whether the patterns of activation were similar between males and females.

Materials and methods

Subjects

Sixty-four experimentally naïve adult male and female Long-Evans rats (225–250 g at arrival; Charles River Laboratories; Portage, MI) were individually caged and maintained on a 12-h light/dark cycle (lights on at 6:00). Male and female rats were housed in separate colony rooms. Following arrival, rats were acclimated to the colony rooms for at least 1 week and handled daily prior to behavioral training. Rats had ad libitum access to water and standard laboratory chow (Prolab, RMH 3000, 5P00; LabDiet, St. Louis, MO; 3.2 kcal/g; 26% protein, 14% fat, 60% carbohydrate), except as otherwise noted. Body weights (for all subjects) and vaginal smears (for female subjects, to verify estrous cycling) were obtained six days a week. All housing and testing procedures were in compliance with the *National Institute of Health Guidelines for Care and Use of Laboratory Animals*, and approved by the Boston College Animal Care and Use Committee.

Apparatus

Training and testing were conducted in a set of eight 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, a transparent Plexiglas back and front, a grid floor, as well as a recessed food cup (3.2 × 4.2 cm) on one wall. Each chamber was enclosed in a sound and light attenuating isolation cubicle (79 × 53 × 53 cm; Coulbourn Instruments)

composed of monolithic rigid foam walls. A ventilation fan, located on the back wall of each isolation cubicle, provided masking noise (55 dB). Video cameras, located on the back wall of each isolation cubicle, recorded behavior during training and test, and were controlled by a software program (Coulbourn Instruments). Rats were given ~ 1 g of the training food pellets (45 mg, 5TUL; Test Diets, Richmond, Indiana; 3.4 kcal/g; 20% protein, 13% fat, 67% carbohydrate) in their home cage one day prior to the behavioral training procedure.

Behavioral training procedure

The training procedure was adapted from Petrovich and Lougee (2011) and consisted of nine sessions conducted on separate days (Table 1): six appetitive sessions (S1, S2, S4, S6, S8, S9), one habituation session to a new context (S3), and two aversive sessions (S5, S7). The appetitive and aversive training sessions took place in two distinct contexts (Context A and B, respectively), which were created by altering the visual, tactile and olfactory properties of the behavioral chambers. Context A consisted of a Plexiglas insert over the grid flooring, an illuminated house light, closed isolation chamber doors, a glass dish (107 × 87 × 70 mm), and the odor of 1% glacial acetic acid (A38-500; Fisher Scientific, Fair Lawn, NJ). Context B consisted of open isolation chamber doors, a tee-pee shaped Plexiglas insert that obscured the aluminum sides, and the odor of 5% ammonium hydroxide (205840010; Acros Organics, Fair Lawn, NJ).

Appetitive sessions Rats were acutely food deprived (~ 22 h) prior to each appetitive session. For each session, all rats were placed inside the behavioral chamber for 10 min, and half were allowed to consume food pellets located in the glass dish while the other half of the rats did not have access to food pellets (No-Food groups). At the end of each session rats were removed from the chambers, placed in their home cages, and returned to their colony rooms. Any remaining food was weighed and the amount consumed calculated. Rats in the No-Food groups received food pellets in their home cages 30–60 min following each session, and the amount given was matched to that eaten by rats of the same sex and experimental condition. Rats were returned to ad libitum access to chow for at least 24 h between consecutive food deprivations.

Aversive sessions Aversive training occurred under non-food-deprived conditions, and each session was 10 min long. The first session in Context B was habituation to the new context and no tones or shocks were administered. During each of the remaining two sessions, half of the male and female rats (experimental and no-food experimental groups) received two presentations of a tone (75 dB, 2 kHz, 60 s) each terminated with a mild electric footshock (1 mA, 1 s; Precision Adjustable Shocker, Coulbourn Instruments; variable inter-trial interval of 4 min ± 50%), while the other half of the rats (control and no-food control groups) received tone presentations but no shocks.

Food consumption test

Rats were acutely food deprived (~ 22 h) prior to test. Testing occurred in Context A, the context used during appetitive sessions. During the test, rats were placed in the behavioral chamber for 10 min and the tone (CS; conditioned stimulus) was presented four times (at minutes 1, 3, 5, and 7); no footshocks were administered. Rats in the experimental and control groups had access to food in the glass dish during the test (any remaining food was weighed and the amount consumed calculated), while the no-food experimental and no-food control groups did not have access to food. Rats were immediately returned to the colony room, and left undisturbed without access to food until sacrificed, which was 90 min from the start of the test session to measure test-specific Fos induction (Morgan and Curran 1991).

Behavioral measures

Food consumption was monitored during all training sessions and at test (for groups with access to food) and data were analyzed as the actual amount consumed (g) and as the percentage of the amounts consumed by the control group of the same sex (see Petrovich and Lougee 2011; Reppucci et al. 2013). The percentage was calculated by dividing the consumption for each subject by the mean consumption of the control group for the same sex and then multiplying the

Table 1 Experimental groups

Group	Appetitive training (sessions 1, 2, 4, 6, 8, 9)	Aversive training (sessions 5 and 7)	Test
Experimental	Access to food	Tone-shock	Access to food; tones
Control	Access to food	Tone-only	Access to food; tones
No-food experimental	–	Tone-shock	Tones
No-food control	–	Tone-only	Tones

Table indicates the stimuli presented during each training and test session; no stimuli were presented during Session 3 (habituation to the context used for aversive sessions)

result by 100; this calculation preserved the existing variance in consumption for all groups.

Additionally, freezing behavior during the test was analyzed for all subjects from recordings. Freezing behavior is a species-typical defense response that is characterized by the cessation of all movement except that required for breathing, and is considered an index of fear (Blanchard and Blanchard 1969; Bouton and Bolles 1980; Fanselow 1984). Observers were "blind" with respect to the sex and training conditions of the rats observed. The observers recorded the behavior of each rat every 1.25 s, paced by a metronome, for the 60 s prior to the first tone presentation (pre-CS period), and during each of the four tone presentations (CS period). The total number of freezing observations during each of these sampling periods was summed, and then the percentage of time spent freezing during each sampling period was calculated.

Histological procedures

Rats were briefly anesthetized with isoflurane (5%; Baxter Healthcare Corporation, Deerfield, IL), and then deeply anesthetized with an intraperitoneal injection of tribromoethanol (375 mg/kg; Sigma-Aldrich, St. Louis, MO). Rats were then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M borate buffer. Brains were extracted and post-fixed overnight in a solution of 12% sucrose dissolved in the perfusion liquid, then rapidly frozen in hexanes cooled in dry ice and stored at − 80 °C. Brains were sliced in 30-µm sections using a sliding microtome and collected into four adjacent series.

The first series was stained using standard immunohistochemical procedures for visualization of Fos (as described in: Cole et al. 2013). Free-floating tissue sections were incubated in a blocking solution for 1 h at room temperature to minimize nonspecific binding. The blocking solution contained 0.02M potassium phosphate-buffered saline (KPBS), 0.3% Triton X-100 (Sigma-Aldrich), 2% normal goat serum (S-1000; Vector Laboratories, Burlingame, CA), and 10% non-fat milk (M-0841; LabScientific, Livingston, New Jersey). Then, the tissue was incubated with the primary antibody, anti-*c-fos* raised in rabbit (1:2000; SC-52; Santa Cruz Biotechnology Inc, Santa Cruz, CA, or 1:30,000; Ab-5; Calbiochem, EMD Millipore, Billerica, MA; counterbalanced across training conditions) in the blocking solution for 72 h at 4 °C. The tissue was rinsed in KPBS then incubated with the secondary antibody, biotinylated goat anti-rabbit IgG (1:500; BA-1000; Vector Laboratories) in the blocking solution for 45 min. Subsequently, the tissue was rinsed in KPBS then reacted with avidin–biotin complex (ABC solution; PK-6100; Vector Laboratories) for 45 min. To improve specific binding, this was followed by rinses in KPBS, a second 30 min incubation in the secondary antibody solution, rinses in KPBS, a second 30 min incubation in the ABC solution, and additional rinses in KPBS. To produce a color reaction, the tissue was incubated in a diaminobenzidine solution (SK-4100; Vector Laboratories) for 1–2 min with constant, manual agitation. Stained tissue was then mounted onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA) and air-dried, followed by drying in an oven at 45 °C overnight. Tissue was then dehydrated through graded alcohols, cleared in xylenes, and coverslipped with DPX (13512; Electron Microscopy Sciences, Hatfield, PA).

The second series was collected into KPBS solution, mounted onto gelatin-subbed slides, and stained with thionin (as described in: Simmons and Swanson 1993) for identification of cytoarchitectonic borders of brain structures, as defined in Swanson's rat brain atlas (Swanson 2004). The remaining series were collected into trays containing a cryoprotectant solution (0.025 M sodium phosphate buffer with 30% ethylene glycol and 20% glycerol) and stored at − 20 °C for later use. Brain perfusions, collection, slicing, and length of storage were counterbalanced across training conditions.

Image acquisition and analysis

Images of stained tissue were acquired with an Olympus BX51 light microscope at 10X and attached Olympus DP72 camera using DP2-BSW software (Olympus America Inc, Center Valley, PA). Using the ImageJ software program (NIH), borders for regions of interest were drawn onto the image of the thionin-stained tissue, and then transposed to the image of the adjacent immunohistochemically-stained tissue to allow for semi-automated counting of Fos-positive neurons as described previously (Cole et al. 2015a). In brief, images were transformed to 8-bit grayscale, a threshold was manually set to distinguish specific labeling from background, and the number of Fos-positive neurons was automatically determined based on size and circularity measures. Bilateral images were acquired and analyzed for each region of interest; counts from left and right hemispheres were summed for each rat to calculate the total number of Fos-positive neurons per region. Representative images chosen for publication were sharpened, transformed to black and white, and contrast adjusted in Adobe Photoshop CS5.5.

Identification and analysis of cell groups followed parcellation and nomenclature as defined in the Swanson atlas (2004), except for the lateral hypothalamic area (LHA; see description below). Initial analysis focused on cell groups within the amygdala, medial prefrontal cortex (mPFC), and LHA as these three regions have been shown to be critical for the control of feeding by learned cues (Petrovich 2013). Following that, well-trained observers (unaware of experimental groups) examined representative brains to determine additional areas for quantitative analysis, based on moderate to high Fos expression, whether the region had been implicated in feeding and/or fear, and known anatomical

connections to the amygdala–prefrontal–lateral hypothalamic network. In total, Fos induction was analyzed within 23 distinct cell groups.

Within the amygdala, we first completed an extensive and detailed examination of the CEA because this nucleus is critical for fear cue inhibited feeding (Petrovich et al. 2009). This analysis was conducted across the rostrocaudal extent of each part (lateral (CEAl), medial (CEAm), capsular (CEAc); Levels 24–28, − 1.33 to − 2.45 mm from bregma; all subsequent measurements refer to mm from bregma). However, the patterns of Fos induction did not differ rostrocaudally and thus we collapsed the Fos data across levels. Due to the lack of rostrocaudal differences in CEA Fos induction, and the large number of cell groups examined for multiple experimental conditions, for most of the subsequent regions we conducted analysis at a single mid-rostrocaudal level (exceptions are stated below).

The five cell groups that comprise the basolateral area of the amygdala were examined: the lateral amygdalar nucleus (LA; Level 30, − 3.25 mm), anterior part of the basolateral amygdalar nucleus (BLAa; Level 27, − 2.00 mm), posterior part of the basolateral amygdalar nucleus (BLAp; Level 30, − 3.25 mm), anterior part of the basomedial amygdalar nucleus (BMAa; Level 26, − 1.78 mm), and posterior part of the basomedial amygdalar nucleus (BMAp; Level 30, − 3.25 mm).

Within the mPFC, three regions were analyzed: the dorsal part of the anterior cingulate (ACAd), prelimbic (PL), and infralimbic (ILA) areas (all Level 9, + 2.80 mm).

Within the hypothalamus, we examined the LHA as well as the paraventricular (PVH) and supraoptic (SO) nuclei. In the LHA, three regions were analyzed (all Level 29, -2.85 mm). At this rostrocaudal level, ten distinct cell groups were identified in the most recent parcellation of the LHA (Swanson 2004); however, to consistently sample and compare areas across brains we defined three larger areas for our analysis based on their relation to the fornix. The LHA region dorsal to the fornix was divided into the dorsomedial and dorsolateral areas. For the purpose of analysis, we defined the dorsomedial area to include the juxtadorsomedial and suprafornical regions (Hahn and Swanson 2010, 2012; Swanson 2004), and the dorosolateral area to include the dorsal region and magnocellular nucleus (Swanson 2004). The third sampling area comprised all cell groups located ventral to the fornix at this level: the dorsal and ventral zones of the juxtaventromedial region, posterior zone of the suprafornical region, medial zone of the ventral region, and the intermediate and lateral parts of the tuberal nucleus (Hahn and Swanson 2015; Swanson 2004). Five cell groups within the PVH were analyzed (all Level 26, − 1.78 mm): the dorsal parvicellular part (PVHdp), dorsal zone of the medial parvicellular part (PVHmpd), ventral zone of the medial parvicellular part (PVHmpv), lateral zone of the posterior magnocellular part (PVHpml), and the periventricular part (PVHpv). Fos induction was also examined in the SO (Level 24, − 1.33 mm).

Within the thalamus, we analyzed the paraventricular thalamic nucleus (PVT). The PVT is a large nucleus and there are connectional differences between the rostral and caudal parts (Li and Kirouac 2012; Vertes and Hoover 2008) and, therefore, we analyzed samples from three different rostrocaudal levels (Level 26, − 1.78 mm; Level 28, − 2.45 mm; Level 30, − 3.25 mm); however, the patterns of activation did not differ across the levels analyzed and thus were collapsed into a single analysis.

In addition to the forebrain areas above, we also examined two cell groups within the dorsal vagal complex in the hindbrain, the medial part of the nucleus of the solitary tract (NTSm) and the ventrally adjacent dorsal motor nucleus vagus nerve (DMX). We analyzed Fos induction at two rostrocaudal levels (Level 66, − 13.28; Level 71, − 14.36); however the patterns of activation did not differ across the levels analyzed and thus were collapsed into a single analysis.

Statistical analysis

Behavioral data were analyzed using analyses of variances (ANOVAs). Two-way (sex by experimental condition) ANOVAs were used for food consumption measures, and three-way (sex by experimental condition by food access condition) ANOVAs for freezing behavior and body weights. A Pearson correlation assessed the relationship between freezing and food consumption in subjects with access to food. Due to sex differences in body weight and food consumption (see Results), Fos counts for males and females were analyzed separately using two-way (experimental condition by food access condition) ANOVAs. Post hoc independent-samples *t* tests (across experimental or food access condition) were completed to clarify reported effects, as needed.

The relationships between Fos induction patterns between brain regions were examined with Pearson correlations using the data from all groups. For this analysis, brain regions with low Fos induction across groups or where there were no main effects of experimental or food access condition were excluded (those listed in Table 4), and subregions with similar patterns of Fos induction were collapsed together (CEA = CEAl + CEAm; mPFC = ACAd + PL + ILA; LHA = dorsomedial LHA + dorsolateral LHA + ventral LHA).

The relationships between Fos induction and each of the two primary behavioral measures at test were examined with partial correlations using the data from the food access groups and collapsed across sexes (except for the mPFC where only females showed differential Fos induction across groups). This analysis was completed for brain regions that showed effects of experimental or food access condition

and examined the correlation between Fos and one behavioral measure while controlling for the effects of the other behavioral measure (i.e., Fos correlated with the amount of food consumed, controlling for freezing; Fos correlated with freezing, controlling for the amount of food consumed; Table 6). Since partial correlations examine the correlations between residuals (Brown and Hendrix 2005), plots representing these correlations (Fig. 9) were created using the standardized residuals from linear regressions against the controlling variable. For plots depicting the partial correlation between consumption and Fos induction, linear regressions against freezing were completed for both consumption and Fos induction. The resulting standardized residuals from these regressions were saved and used as the data points for scatterplots in Fig. 9 (*y* axis: standardized residual of Fos induction regressed against freezing; *x* axis: standardized residual of consumption regressed against freezing). This process was repeated for the plots depicting the partial correlation between freezing and Fos induction; linear regressions against consumption were completed for both freezing and Fos induction, and the resulting standardized residuals used as the data points for scatterplots in Fig. 9 (*y* axis: standardized residual of Fos induction regressed against consumption; *x* axis: standardized residual of freezing regressed against consumption). Verifying the data transformation, Pearson correlations between the residuals were confirmed to have *r*- and *p*-values identical to those obtained from the partial correlations using the raw data (Brown and Hendrix 2005).

Five rats were excluded from analyses due to poor health or technical malfunction during perfusion (one male experimental, one female no-food control, three female no-food experimentals; see Table 2 for final group sizes). Additionally, data for the following were not collected due to tissue damage: CEA (two male no-food controls, one female no-food control, one female no-food experimental), LA, BLAp, BMAp (two male no-food controls), BMAa (two female no-food experimentals), mPFC (one female experimental), PVT, DMX, NTSm (one female no-food experimental), PVH (four male no-food controls, four male no-food experimentals, four female no-food controls, one female no-food experimental), SO (four male no-food controls, four male no-food experimentals, four female no-food controls, two female no-food experimentals), and LHA (one female experimental, one female no-food experimental).

Graphs were generated in GraphPad Prism or SPSS, and edited in Adobe Illustrator CC. All statistics were computed using SPSS software, and in all cases $p \leq 0.05$ was considered significant.

Table 2 Body weights (mean in gram ± SEM)

Group	*n*	Prior to training	Prior to test
Male control	8	344 ± 21.0	383 ± 20.2
Male experimental	7	344 ± 17.1	386 ± 14.1
Male no-food control	8	305 ± 7.20	345 ± 9.15
Male no-food experimental	8	316 ± 6.31	352 ± 8.60
Female control	8	262 ± 7.47	275 ± 9.18
Female experimental	8	261 ± 5.91	272 ± 4.91
Female no-food control	7	245 ± 2.87	260 ± 5.01
Female no-food experimental	5	249 ± 3.82	248 ± 3.63

Results

Behavior

Training Food pellet consumption increased across appetitive training sessions for all groups that had access to food (Fig. 1). Males increased consumption across training sessions faster and thus consumed more food pellets than females by the end of training. A repeated ANOVA across training sessions confirmed a significant within-subjects main effect of session ($F_{(5,27)} = 35.8$, $p < 0.001$) and interaction of session with sex ($F_{(5,27)} = 5.87$, $p < 0.001$), as well as a significant between-subjects main effect of sex ($F_{(1,27)} = 17.0$, $p < 0.001$). Importantly, control and experimental groups of the same sex consumed similar amounts of pellets; there were no effects or interactions with experimental condition on food consumption during the training period ($p > 0.05$, all).

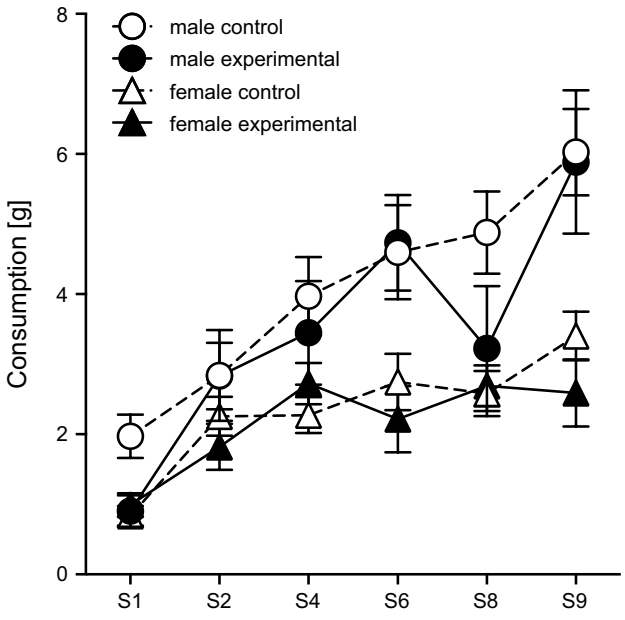

Fig. 1 Consumption during training. The amount of food pellets consumed (mean in grams ± SEM) during each appetitive training session

All subjects were the same weight at arrival; however, males gained weight much more rapidly and thus weighed significantly more than females prior to training ($F_{(1,58)}=81.4$, $p<0.001$) and prior to test ($F_{(1,58)}=164$, $p<0.001$; Table 2). There were no effects or interactions with experimental condition on body weights at either time point ($p>0.05$, all). There was an unexpected effect of food access condition, with the No-Food groups weighing less than their corresponding groups at each time point (prior to training: $F_{(1,58)}=8.85$, $p<0.01$; prior to test: $F_{(1,58)}=12.1$, $p=0.001$). However, this study was conducted in multiple replications, and closer inspection noted that in one replication, which did not include No-Food groups, rats were heavier than in the other two replications. When replication was added as an additional factor into the ANOVAs, the main effect of food access was eliminated ($p>0.05$, both) and the suspected replication effect was confirmed (prior to training: $F_{(1,58)}=87.3$, $p<0.001$; prior to test: $F_{(1,58)}=36.4$, $p<0.001$). All female rats had normal estrous cycles during training and all stages were represented at test (due to the sample size no effects of estrous cycle stage on behavioral or brain measures could be directly analyzed).

Test Subjects in the experimental groups strongly inhibited food intake compared to the control groups during the test with tone (CS) presentations ($F_{(1,30)}=23.3$, $p<0.001$). Post hoc tests confirmed that both the male ($t_{(13)}=4.43$, $p=0.001$) and female ($t_{(14)}=2.11$, $p=0.05$) experimental groups significantly inhibited food intake compared to the corresponding control groups (Fig. 2a, left). The baseline difference in food consumption observed during training was also evident at test; males consumed significantly more food pellets than females ($F_{(1,30)}=9.32$, $p<0.01$). Due to this sex difference in the amounts of food consumed there was a significant sex by experimental condition interaction effect ($F_{(1,30)}=4.80$, $p<0.05$), as the difference in consumption between the control and experimental groups was greater in males than females (3.57 vs. 1.34 g). To account for baseline sex differences in consumption, we re-calculated the amount consumed and expressed it as the percent of the mean consumption of the control group of the same sex (see "Behavioral measures" for more information). The resulting ANOVA showed that both males and females in the experimental groups inhibited food intake compared to the corresponding control groups ($F_{(1,30)}=18.6$, $p<0.001$); Fig. 2a, right; there was no main effect or interaction with sex ($p>0.05$, both).

All subjects who received tone-shock pairings during training acquired a conditioned fear response specific to the CS (Fig. 2b). There were no instances of freezing by any subject prior to the first CS presentation at test (Pre-CS: $0\pm0\%$ of time for all groups). Experimental groups showed robust freezing behavior during CS presentations, while control groups showed negligible amounts of freezing behavior ($F_{(1,51)}=77.1$, $p<0.001$; Fig. 2b). There were no effects or interactions with sex or food access condition on the expression of freezing during CS presentations ($p>0.05$, all). In

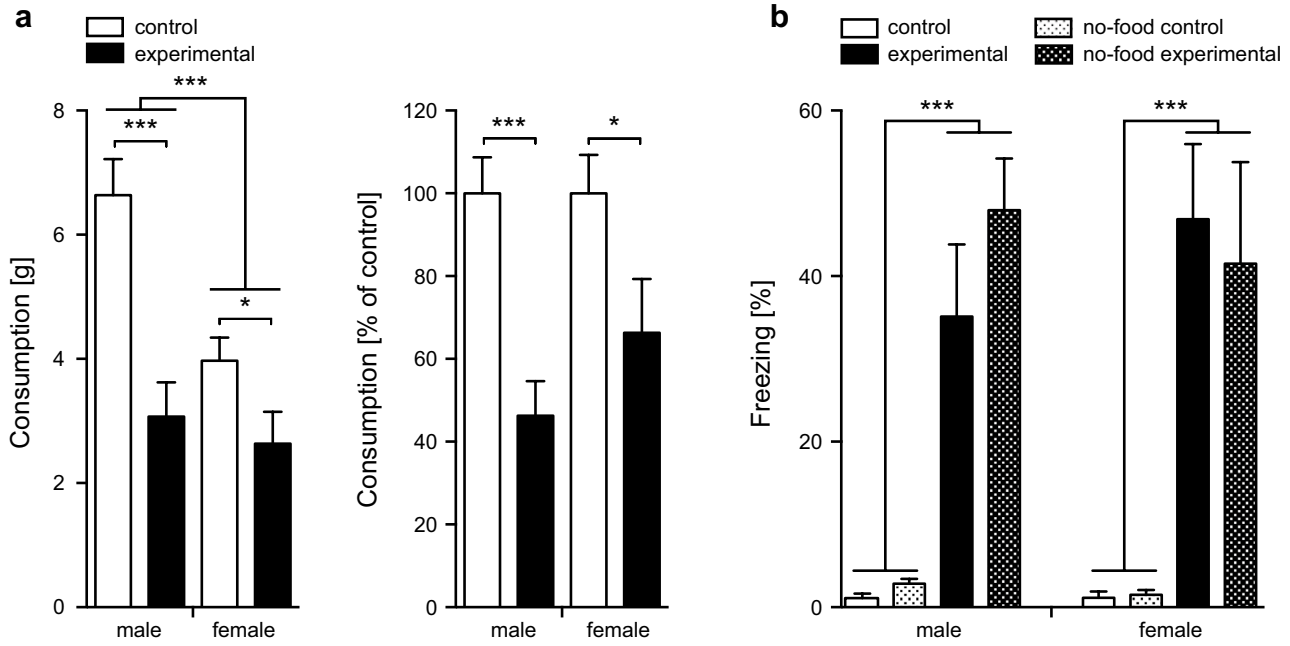

Fig. 2 Food consumption and freezing behavior during test. **a** The amount of food pellets consumed expressed in grams (left) and as the percent of the consumption of the control group of the same sex (right). **b** The percent of time subjects expressed freezing behavior during CS presentations. All data are shown as mean ± SEM; $*p=0.05$; $***p<0.001$

subjects with access to food, there was a significant negative correlation between freezing and food consumption ($r_{(32)} = -0.63$, $p < 0.001$).

Fos induction

Amygdala Within the CEA, Fos induction patterns corresponded to food consumption, specifically within the CEAl and CEAm (Fig. 3a, b). For both regions, Fos induction was significantly greater in the groups with access to food compared to the No-Food groups for males and females (see Table 3 for statistics). In males, Fos induction was significantly greater in the control compared to the experimental condition in both the CEAl and CEAm (Table 3). Post hoc tests confirmed this was due to differences between the control and experimental groups that had food access during test (CEAl: $t_{(13)} = 2.08$, $p = 0.06$; CEAm $t_{(13)} = 2.63$, $p < 0.05$), while the No-Food groups had similar Fos induction across experimental and control conditions ($p > 0.05$, both). There was no effect of experimental condition on Fos induction in

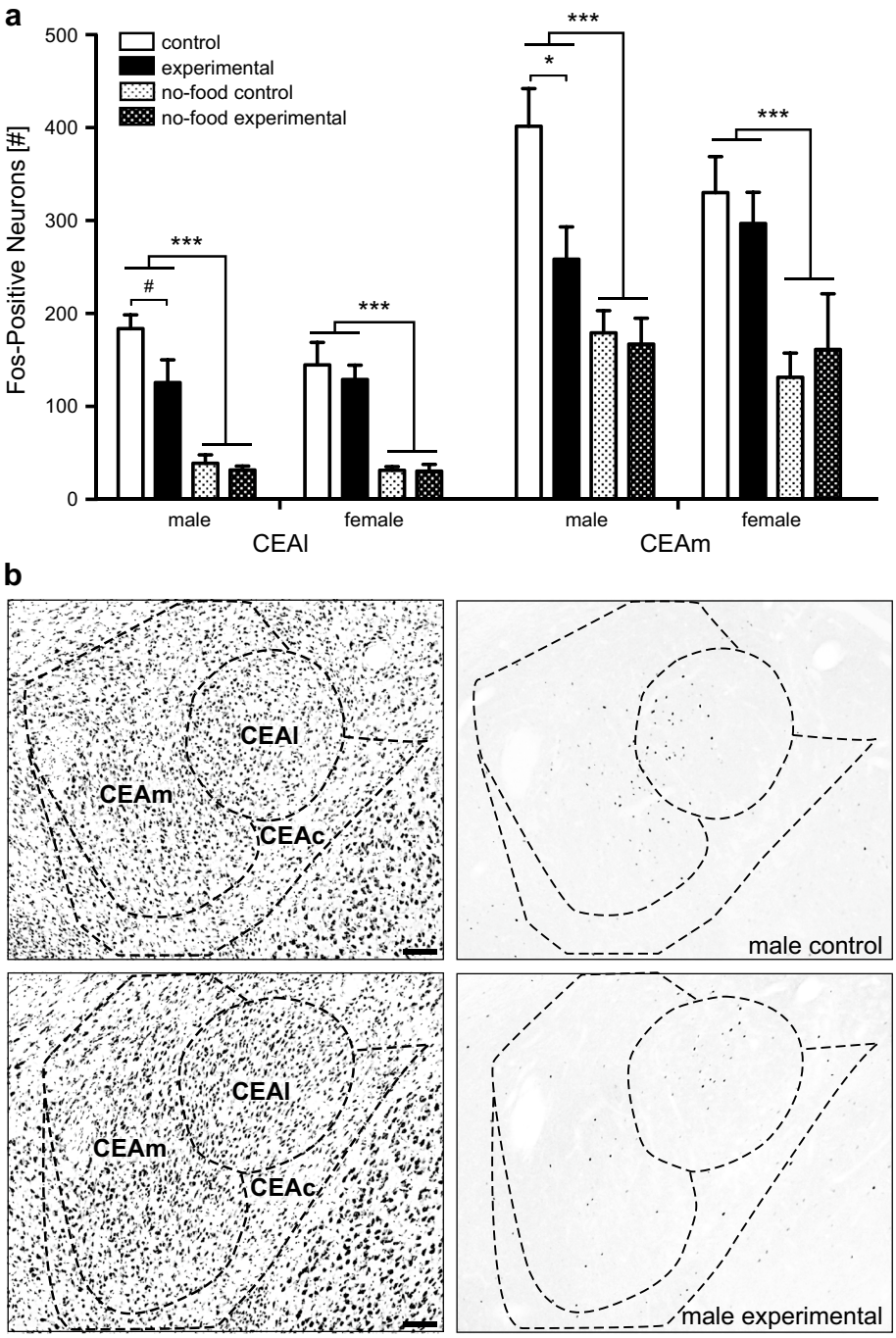

Fig. 3 Fos induction in the CEA. **a** Total number of Fos-positive neurons are shown as mean ± SEM; $^{\#}p = 0.06$, $^{*}p < 0.05$; $^{***}p < 0.001$. **b** Representative photomicrographs of the CEA showing Fos induction (right) in a distinct group of neurons within the CEAl/CEAm and adjacent thionin-stained sections (left); scale bars = 100 µm

Table 3 Two-way ANOVA statistics for measures of Fos induction

Region	Sex	Experimental condition	Food access condition	Experimental condition X food access condition
Amygdala				
CEAl	Males	$\mathbf{F_{(1,28)} = 4.69, p = 0.041}$	$\mathbf{F_{(1,28)} = 62.2, p < 0.001}$	$F_{(1,28)} = 2.79, p = 0.11$
	Females	$F_{(1,25)} = 0.19, p = 0.67$	$\mathbf{F_{(1,25)} = 30.9, p < 0.001}$	$F_{(1,25)} = 0.15, p = 0.70$
CEAm	Males	$\mathbf{F_{(1,28)} = 5.27, p = 0.030}$	$\mathbf{F_{(1,28)} = 21.5, p < 0.001}$	$F_{(1,28)} = 3.73, p = 0.065$
	Females	$F_{(1,25)} = 0.002, p = 0.97$	$\mathbf{F_{(1,25)} = 17.5, p < 0.001}$	$F_{(1,25)} = 0.62, p = 0.44$
CEAc	Males	$F_{(1,28)} = 0.47, p = 0.50$	$F_{(1,28)} = 0.39, p = 0.54$	$F_{(1,28)} = 0.44, p = 0.51$
	Females	$F_{(1,25)} = 0.75, p = 0.40$	$F_{(1,25)} = 3.02, p = 0.096$	$F_{(1,25)} = 0.53, p = 0.48$
LA	Males	$\mathbf{F_{(1,28)} = 6.41, p = 0.018}$	$F_{(1,28)} = 0.29, p = 0.59$	$F_{(1,28)} = 0.68, p = 0.42$
	Females	$F_{(1,27)} = 3.27, p = 0.083$	$F_{(1,27)} = 0.000, p = 0.99$	$F_{(1,27)} = 0.60, p = 0.45$
BLAa	Males	$F_{(1,30)} = 0.22, p = 0.65$	$\mathbf{F_{(1,30)} = 5.29, p = 0.029}$	$F_{(1,30)} = 0.015, p = 0.90$
	Females	$F_{(1,27)} = 0.22, p = 0.64$	$F_{(1,27)} = 3.82, p = 0.062$	$F_{(1,27)} = 0.11, p = 0.74$
BLAp	Males	$F_{(1,28)} = 1.65, p = 0.21$	$F_{(1,28)} = 0.67, p = 0.42$	$F_{(1,28)} = 2.22, p = 0.15$
	Females	$F_{(1,27)} = 0.42, p = 0.52$	$F_{(1,27)} = 1.48, p = 0.24$	$F_{(1,27)} = 0.020, p = 0.89$
BMAa	males	$F_{(1,30)} = 0.18, p = 0.67$	$F_{(1,30)} = 0.042, p = 0.84$	$F_{(1,30)} = 0.56, p = 0.46$
	Females	$F_{(1,25)} = 0.45, p = 0.51$	$F_{(1,25)} = 0.64, p = 0.43$	$F_{(1,25)} = 2.69, p = 0.12$
BMAp	Males	$F_{(1,28)} = 3.07, p = 0.092$	$F_{(1,28)} = 0.25, p = 0.62$	$F_{(1,28)} = 1.36, p = 0.25$
	Females	$F_{(1,27)} = 3.83, p = 0.062$	$F_{(1,27)} = 0.38, p = 0.54$	$\mathbf{F_{(1,27)} = 5.01, p = 0.035}$
Cortex				
mPFC	Males	$F_{(1,27)} = 0.37, p = 0.55$	$F_{(1,27)} = 1.02, p = 0.32$	$F_{(1,27)} = 0.25, p = 0.62$
	Females	$\mathbf{F_{(1,23)} = 8.38, p = 0.008}$	$F_{(1,23)} = 0.001, p = 0.98$	$F_{(1,23)} = 0.11, p = 0.74$
Hypothalamus				
dmLHA	Males	$F_{(1,30)} = 0.56, p = 0.46$	$\mathbf{F_{(1,30)} = 5.26, p = 0.030}$	$F_{(1,30)} = 0.33, p = 0.57$
	Females	$F_{(1,25)} = 0.14, p = 0.72$	$F_{(1,25)} = 1.02, p = 0.32$	$F_{(1,25)} = 0.24, p = 0.63$
dlLHA	Males	$F_{(1,30)} = 3.14, p = 0.088$	$F_{(1,30)} = 0.018, p = 0.89$	$F_{(1,30)} = 0.89, p = 0.35$
	Females	$F_{(1,25)} = 0.007, p = 0.93$	$F_{(1,25)} = 1.93, p = 0.18$	$F_{(1,25)} = 0.31, p = 0.59$
vLHA	Males	$F_{(1,30)} = 0.38, p = 0.54$	$F_{(1,30)} = 3.76, p = 0.063$	$F_{(1,30)} = 1.20, p = 0.28$
	Females	$F_{(1,25)} = 0.41, p = 0.53$	$F_{(1,25)} = 3.41, p = 0.078$	$F_{(1,25)} = 3.92, p = 0.060$
PVHpml	Males	$\mathbf{F_{(1,22)} = 4.42, p = 0.049}$	$\mathbf{F_{(1,22)} = 16.5\ p = 0.001}$	$\mathbf{F_{(1,22)} = 4.51, p = 0.047}$
	Females	$F_{(1,22)} = 0.48, p = 0.50$	$\mathbf{F_{(1,22)} = 16.6, p = 0.001}$	$F_{(1,22)} = 0.52, p = 0.48$
PVHdp	Males	$F_{(1,22)} = 1.41, p = 0.25$	$F_{(1,22)} = 1.76, p = 0.20$	$F_{(1,22)} = 0.24, p = 0.63$
	Females	$F_{(1,22)} = 0.76, p = 0.39$	$\mathbf{F_{(1,22)} = 5.26, p = 0.033}$	$F_{(1,22)} = 0.31, p = 0.59$
PVHmpd	Males	$F_{(1,22)} = 0.28, p = 0.60$	$\mathbf{F_{(1,22)} = 15.5\ p = 0.001}$	$F_{(1,22)} = 0.21, p = 0.65$
	Females	$F_{(1,22)} = 0.015, p = 0.90$	$\mathbf{F_{(1,22)} = 5.66, p = 0.028}$	$F_{(1,22)} = 0.57, p = 0.46$
PVHmpv	Males	$F_{(1,22)} = 0.068, p = 0.80$	$\mathbf{F_{(1,22)} = 4.72, p = 0.043}$	$F_{(1,22)} = 0.50, p = 0.49$
	Females	$F_{(1,22)} = 0.26, p = 0.61$	$F_{(1,22)} = 4.34, p = 0.051$	$F_{(1,22)} = 0.54, p = 0.47$
PVHpv	Males	$F_{(1,22)} = 0.88, p = 0.36$	$F_{(1,22)} = 1.77, p = 0.20$	$F_{(1,22)} = 4.23, p = 0.054$
	Females	$F_{(1,22)} = 0.30, p = 0.59$	$F_{(1,22)} = 1.67, p = 0.20$	$F_{(1,22)} = 1.36, p = 0.26$
SO	Males	$F_{(1,22)} = 1.9, p = 0.18$	$\mathbf{F_{(1,22)} = 4.89, p = 0.040}$	$F_{(1,22)} = 2.06, p = 0.17$
	Females	$F_{(1,19)} = 0.26, p = 0.62$	$\mathbf{F_{(1,19)} = 6.14, p = 0.025}$	$F_{(1,19)} = 0.26, p = 0.62$
Thalamus				
PVT	Males	$F_{(1,30)} = 0.32, p = 0.58$	$F_{(1,30)} = 1.892, p = 0.18$	$F_{(1,30)} = 1.65, p = 0.21$
	Females	$F_{(1,26)} = 2.52, p = 0.13$	$F_{(1,26)} = 1.771, p = 0.20$	$\mathbf{F_{(1,26)} = 5.18, p = 0.032}$
Dorsal vagal complex				
NTSm	Males	$\mathbf{F_{(1,30)} = 10.0, p = 0.004}$	$\mathbf{F_{(1,30)} = 33.6, p < 0.001}$	$\mathbf{F_{(1,30)} = 10.0, p = 0.004}$
	Females	$F_{(1,26)} = 0.49, p = 0.49$	$\mathbf{F_{(1,26)} = 8.90\ p = 0.007}$	$F_{(1,26)} = 0.015, p = 0.90$
DMX	Males	$\mathbf{F_{(1,30)} = 9.19, p = 0.005}$	$\mathbf{F_{(1,30)} = 28.0, p < 0.001}$	$\mathbf{F_{(1,30)} = 9.60, p = 0.005}$
	Females	$F_{(1,26)} = 0.51, p = 0.48$	$\mathbf{F_{(1,26)} = 9.96, p = 0.004}$	$F_{(1,26)} = 0.29, p = 0.60$

Significant effects are bolded

females (Table 3). In contrast to the CEAl and CEAm, Fos induction in the CEAc was low and similar across all conditions for both sexes (Tables 3, 4).

Fos induction patterns were distinct across the five cell groups that comprise the basolateral area of the amygdala. Fos induction in the BLAa corresponded to food access condition and was significantly greater in groups with access to food compared to the No-Food groups for males, and in females this difference approached significance. For both sexes, this difference was irrespective of experimental condition (Fig. 4a; Table 3). Fos induction in the LA corresponded to experimental condition and was greater for subjects in the experimental compared to subjects in the control condition; this difference was significant for males and approached significance for females. Fos induction in the LA was not influenced by food access condition for either sex (Fig. 4b; Table 3). The BMAp had a similar pattern of Fos induction as the LA, with greater Fos induction in the experimental compared to the control condition (Fig. 4b). However, this effect was not as strong as in the LA and only approached significance in both males and females (Table 3). There was no main effect or interaction with food access condition on BMAp Fos induction in males, but in females there was a significant experimental condition by food access condition interaction (Table 3). Post hoc tests showed that in females, the control group had significantly lower Fos induction

Table 4 Number of Fos-positive neurons (mean ± SEM) in cell groups that had low Fos induction across all groups or where there were no significant main effects of experimental or food access condition for either sex

Brain region	Males				Females			
	Control	Experimental	No-food control	No-food experimental	Control	Experimental	No-food control	No-food experimental
BLAp	38.6 ± 6.70	23.6 ± 5.47	35.0 ± 2.99	36.1 ± 4.26	21.1 ± 2.44	24.4 ± 4.67	26.7 ± 2.30	28.8 ± 6.97
BMAa	66.5 ± 8.30	64.3 ± 5.01	62.8 ± 3.17	70.9 ± 8.98	51.0 ± 5.01	64.1 ± 5.52	64.9 ± 3.60	59.3 ± 6.44
CEAc	59.3 ± 11.1	45.7 ± 9.96	46.3 ± 13.3	46.1 ± 5.13	43.0 ± 5.93	53.5 ± 6.55	36.3 ± 6.94	37.3 ± 5.04
PVHdp	2.38 ± 0.84	3.00 ± 0.93	0.75 ± 0.25	2.25 ± 0.48	2.50 ± 0.53	4.00 ± 1.19	0.67 ± 0.33	1.00 ± 0.58
PVHmpd	19.6 ± 2.60	16.1 ± 4.29	4.24 ± 1.25	4.00 ± 1.87	17.3 ± 4.07	14.4 ± 4.08	3.00 ± 1.53	7.00 ± 1.00
PVHmpv	23.1 ± 4.53	17.7 ± 5.85	7.00 ± 5.12	9,50 ± 4.13	17.3 ± 3.51	16.3 ± 4.06	4.67 ± 1.20	10.3 ± 4.05
PVHpv	8.00 ± 1.39	5.86 ± 1.79	1.50 ± 0.87	7.25 ± 3.07	3.50 ± 1.09	4.25 ± 0.96	3.33 ± 1.45	1.25 ± 0.63
PVT	402 ± 57.3	324 ± 41.9	290 ± 29.3	320 ± 33.8	247 ± 32.9	266 ± 16.1	345 ± 23.8	240 ± 24.0

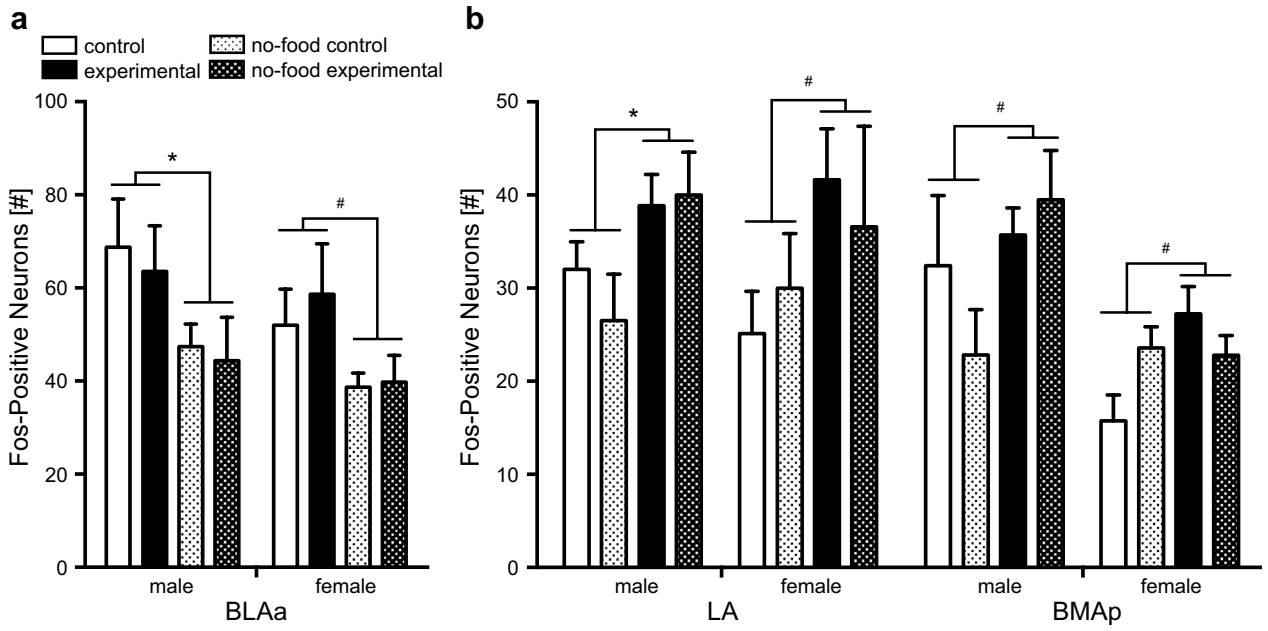

Fig. 4 Fos induction in the basolateral area of the amygdala. **a** Total number of Fos-positive neurons (mean ± SEM) in the cell group where Fos induction was greater for groups with access to food compared to the No-Food groups. **b** Total number of Fos-positive neurons (mean ± SEM) in cell groups where Fos was greater in the experimental compared to the control condition; $^{\#}p < 0.10$; $^{*}p < 0.05$

than the experimental group ($t_{(14)} = 2.85$, $p < 0.05$) and the no-food control group ($t_{(13)} = 2.14$, $p = 0.05$). Fos induction in the remaining two basolateral area cell groups (BLAp, BMAa) was similar across all conditions for both sexes (Tables 3, 4).

Medial prefrontal cortex Within the mPFC, Fos induction was much greater in the PL and ILA compared to the ACAd for both sexes (Fig. 5). In males and females, repeated (across the three mPFC regions) two-way (experimental condition by food access condition) ANOVAs confirmed a significant within-subjects main effect of region (males: $F_{(2,54)} = 106$, $p < 0.001$; females: $F_{(2,46)} = 91.0$, $p < 0.001$), and this effect did not interact with experimental or food access condition ($p > 0.05$, all). Importantly, there was a sex difference in the Fos induction patterns across experimental conditions. In females, there was a significant between-subjects effect of experimental condition; rats in the experimental groups had significantly greater Fos induction than those in the control groups (Table 3). There was no between-subjects effect of experimental condition in males, or of food access condition in either sex (Table 3).

Hypothalamus Fos induction patterns in the hypothalamic regions analyzed (LHA, PVH, SO) were related to food access. Within the LHA, there was robust Fos induction across regions in both sexes and in all conditions (Fig. 6a). In males, there was significantly greater Fos induction in the groups with access to food compared to the No-Food groups in the dorsomedial LHA, while in the dorsolateral LHA a main effect of experimental condition approached significance (Table 3). In females, there were no significant effects of food access or experimental condition on Fos induction within the dorsomedial or dorsolateral LHA (Table 3). In the ventral LHA, there was greater Fos induction in groups with access to food compared to the No-Food groups and this

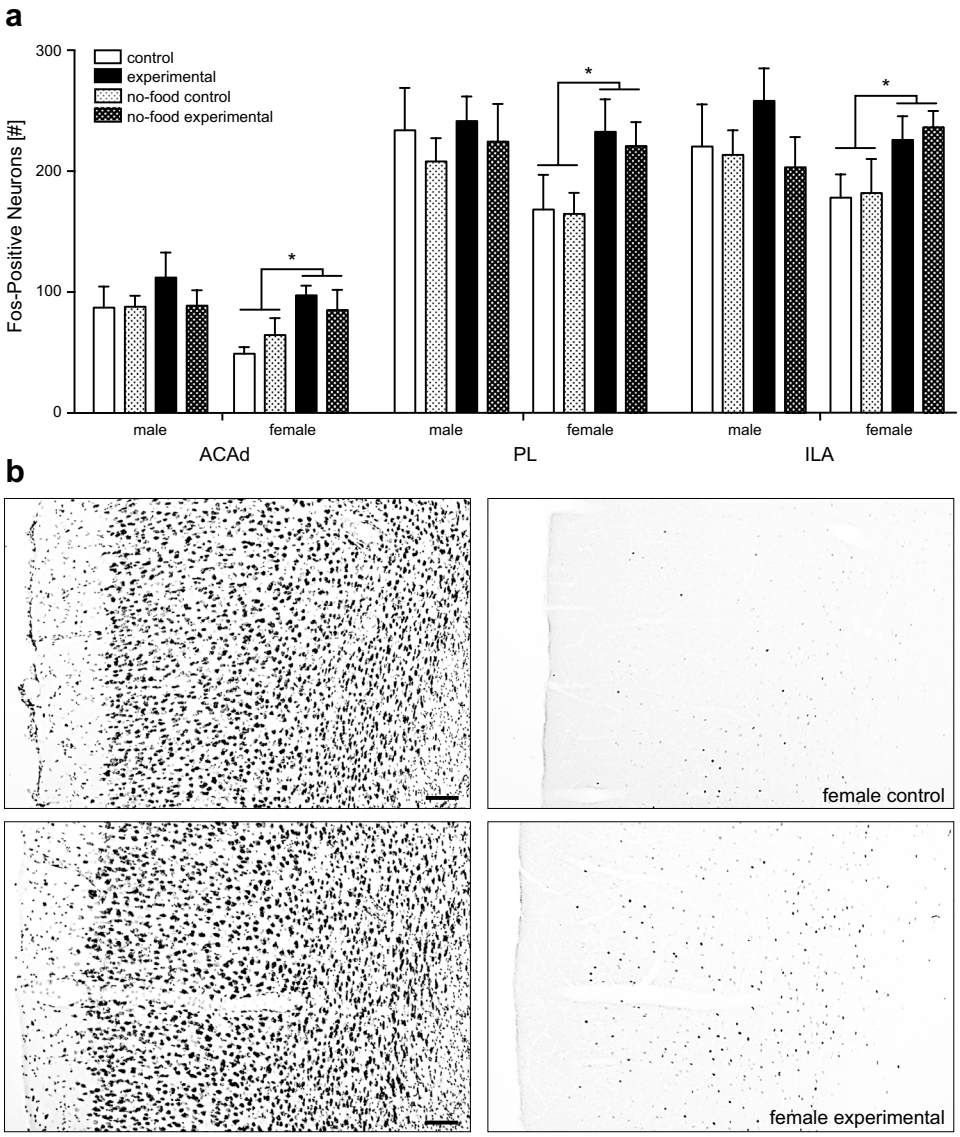

Fig. 5 Fos induction in the mPFC. **a** Total number of Fos-positive neurons within the ACAd, PL, and ILA (mean ± SEM); *$p < 0.01$ across all three areas. **b** Representative photomicrographs of PL Fos induction (right) and adjacent thionin-stained sections (left); scale bars = 100 µm

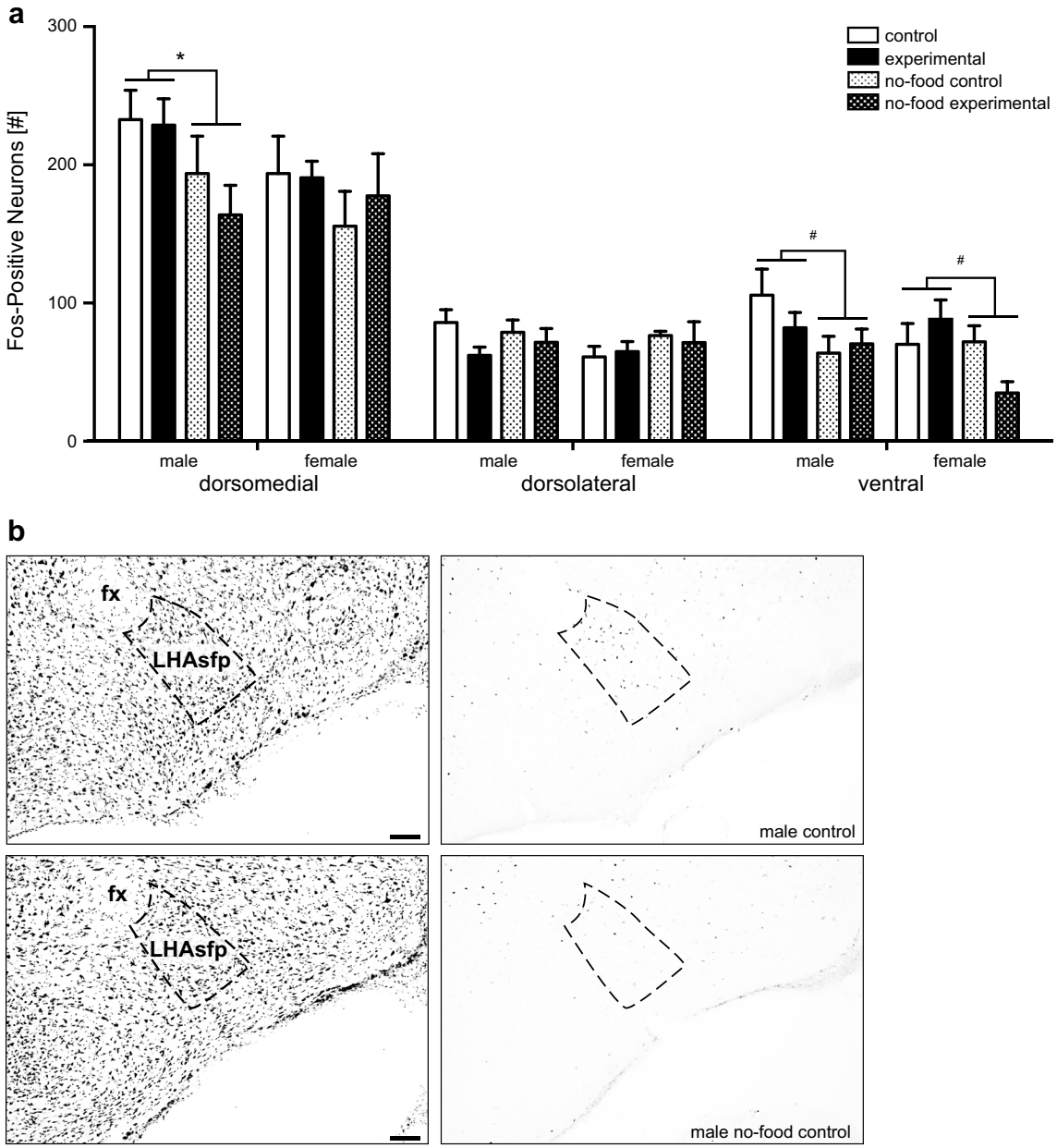

Fig. 6 Fos induction in the LHA. **a** Total number of Fos-positive neurons within each sampling region of the LHA (mean ± SEM;) $^{\#}p < 0.10$, $^{*}p < 0.05$. **b** Representative photomicrographs of the ventral LHA sampling area Fos induction (right) and adjacent thionin-stained sections (left); scale bars = 100 µm, *fx* fornix

induction was concentrated within an area that corresponds to the posterior zone of the suprafornical region (LHAsfp; Fig. 6b); however, the main effect of food access condition only approached significance in both sexes (Table 3).

In the PVH, Fos induction was greatest in the PVHpml where groups with access to food had robust Fos induction compared to the No-Food groups (Fig. 7a, b). This observation was supported by a significant main effect of food access condition for both sexes in the PVHpml (Table 3). In males, there was also a significant main effect of, and interaction with, experimental condition (Table 3). Post hoc tests revealed this interaction was driven by significantly greater Fos induction in the control compared to the experimental group ($t_{(13)} = 2.96$, $p = 0.01$), while the no-food control and no-food experimental groups had similar low levels of Fos induction ($p > 0.05$). There was no main effect or interaction with experimental condition in females (Table 3). Within the remaining PVH divisions, Fos induction related to food access, as seen in the

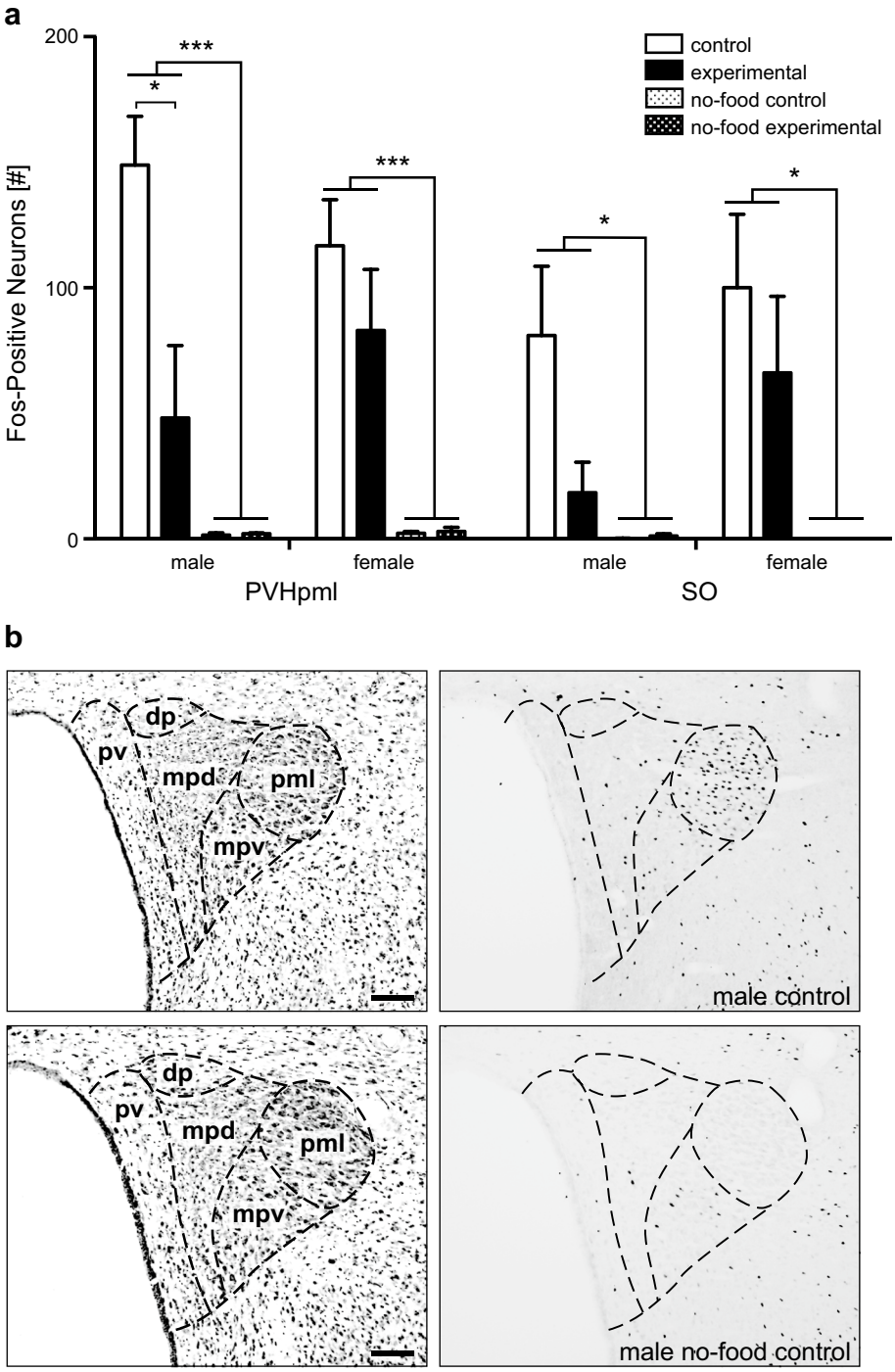

Fig. 7 Fos induction in the PVHpml and SO. **a** Total number of Fos-positive neurons (mean ± SEM); $*p < 0.05$, $***p < 0.001$. **b** Representative photomicrographs of PVH Fos induction (right) and adjacent thionin-stained sections (left); scale bars = 100 µm

PVHpml, but was markedly lower (see Table 3 for statistics and Table 4 for group Fos induction means).

Fos induction patterns in the SO were similar to the patterns in the PVHpml; there was greater Fos induction in the groups with access to food compared to the No-Food groups (Fig. 7a). There was a significant main effect of food access condition in both sexes, and no main effect of experimental condition for either sex (Table 3).

Paraventricular thalamus Within the PVT there was robust Fos induction in all groups for both sexes, and the patterns differed across conditions in females but not in males (Table 4). In males, there were no significant effects of experimental or food access condition, while in females there was a significant interaction (Table 3). Post hoc tests found that in females the no-food control group had greater Fos induction than the no-food experimental ($t_{(9)} = 2.87$,

$p < 0.05$) and control ($t_{(13)} = 2.35$, $p < 0.05$) groups. Fos induction in the female experimental group did not differ from that of the female control or female no-food experimental groups ($p > 0.05$, both).

Dorsal vagal complex Fos induction patterns in the NTSm and DMX were similar (Fig. 8). In both regions there was significantly greater Fos induction in the groups with access to food compared to the No-Food groups in males and females (Table 3). In males, there was also a significant main effect of experimental condition on Fos induction in both regions, and significant experimental by food access condition interactions (Table 3). Post hoc tests showed these interaction effects in males were driven by significantly greater Fos induction in the control compared to the experimental group (NTSm: $t_{(13)} = 3.06$, $p < 0.01$; DMX: $t_{(13)} = 2.96$, $p < 0.05$); the male No-Food groups had similar negligible amounts of Fos induction in both regions ($p > 0.05$, both). In females, there were no main effects of interactions with experimental condition on Fos induction for either region (Table 3).

Correlations of Fos induction patterns across brain regions To examine the relationship in Fos induction patterns across brain regions, Pearson correlations were completed using the data from all groups (see "Statistical Analysis" for more information). This analysis revealed three main clusters of brain regions that were significantly and positively correlated with each other (Table 5): CEA-BLAa-mPFC-LHA, CEA-PVHpml-SO-NTSm-DMX, and BLAa-LA-BMAp-mPFC. Additionally, the BLAa was significantly positively correlated with the NTSm and DMX, and the LHA was significantly positively correlated with the BMAp and PVHpml. The only significant negative correlation was between the BMAp and PVHpml.

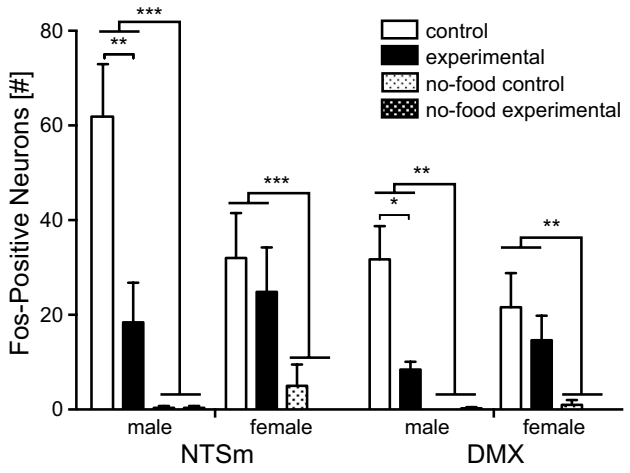

Fig. 8 Fos induction in the NTSm and DMX. Total number of Fos-positive neurons (mean ± SEM); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Correlations of Fos induction patterns and behavior at test To evaluate the contribution of each behavior on patterns of Fos induction, partial correlation analyses were completed using the data from the food access groups (see "Statistical Analysis" for more information). Fos induction in the CEAl, CEAm, dorsolateral LHA, ventral LHA, and NTSm was significantly positively correlated with food consumption, and was not correlated with freezing (Table 6; Fig. 9a, b, e, f, h). Fos induction in the LA significantly positively correlated with freezing behavior while Fos induction in the PVHpml significantly negatively correlated with freezing; in neither region was Fos induction correlated with consumption (Table 6; Fig. 9c, g). In females, mPFC Fos induction was significantly positively correlated with both consumption and freezing behavior (Table 6; Fig. 9c). Fos induction in the remaining regions analyzed (BLAa, BMAp, dorsomedial LHA, PVHdp, PVHmpd, PVHmpv, PVHpv, SO, DMX) did not significantly correlate with consumption or freezing behavior (Table 6).

Discussion

The current study provided the first evidence of the neural network underlying fear-induced hypophagia. We found distinct patterns of recruitment associated with feeding and fear expression. Typically, these patterns were similar between males and females, except for the mPFC. In both males and females, activation of the CEA, LHA, PVH, SO, NTSm, and DMX was associated with food consumption, BLAa activation with food-associated cues, and LA and BMAp activation with fear expression. There was selective recruitment of the mPFC in females only, and this was the only area where activation was significantly correlated with both feeding and freezing behavior. This sex-specific mPFC recruitment during the control of feeding by fear offers an interesting avenue for future research regarding female susceptibility in anxiety and eating disorders.

In this preparation, the inhibition of food intake was specifically driven by presentation of the fear cue at test. During training, we monitored food consumption across the appetitive training sessions and found no evidence that footshock experience during aversive sessions affected subsequent food intake during appetitive sessions (Fig. 1). This is consistent with other reports that footshock experience in one context did not affect food consumption in a different context that was associated with food (Holmes and Westbrook 2014; Reppucci et al. 2013). During the test, none of the subjects expressed freezing behavior prior to the first tone (CS) presentation, further indicating the contextual specificity of the initial learning. Importantly, at test, males and females expressed equal levels of freezing behavior during the CS, indicating similar learning and memory across

Table 5 Correlations of Fos induction between brain regions

		CEA	BLAa	LA	BMAp	mPFC	LHA	PVHpml	SO	NTSm	DMX
CEA	*r*		.58**	-0.019	0.080	.35*	.48**	.56**	.34*	.74**	.69**
	p		<0.001	0.89	0.57	0.010	<0.001	<0.001	0.031	<0.001	<0.001
	N		55	54	54	54	53	44	41	55	55
BLAa	*r*	.58**		.27*	.34**	.67**	.47**	0.16	0.17	.47**	.31*
	p	<0.001		0.042	0.002	<0.001	<0.001	0.29	0.29	<0.001	0.016
	N	55		57	57	58	57	46	43	59	59
LA	*r*	-0.019	.27*		.36**	.36**	0.18	-0.18	-0.19	0.073	0.061
	p	0.89	0.042		0.007	0.007	0.19	0.25	0.22	0.59	0.65
	N	54	57		57	56	55	44	41	57	57
BMAp	*r*	0.080	.40**	.36**		.55**	.32*	-.34*	-0.26	0.084	-0.026
	p	0.57	0.002	0.007		<0.001	0.016	0.024	0.099	0.53	0.85
	N	54	57	57		56	55	44	41	57	57
mPFC	*r*	.35*	.67**	.36**	.55**		.55**	-0.20	-0.058	0.19	0.078
	p	0.010	<0.001	0.007	<0.001		<0.001	0.19	0.72	0.16	0.56
	N	54	58	56	56		56	45	42	58	58
LHA	*r*	.48**	.47**	0.18	.32*	.55**		.31*	0.27	.43**	.41**
	p	<0.001	<0.001	0.19	0.016	<0.001		0.042	0.085	0.001	0.002
	N	53	57	55	55	56		44	43	57	57
PVHpml	*r*	.56**	0.16	-0.18	-.34*	-0.20	.31*		.73**	.56**	.61**
	p	<0.001	0.29	0.25	0.024	0.19	0.042		<0.001	<0.001	<0.001
	N	44	46	44	44	45	44		43	46	46
SO	*r*	.34*	0.17	-0.19	-0.26	-0.058	0.27	.73**		.51**	.46**
	p	0.031	0.29	0.22	0.099	0.72	0.085	<0.001		0.001	0.002
	N	41	43	41	41	42	43	43		43	43
NTSm	*r*	.74**	.47**	0.073	0.084	0.19	.43**	.56**	.51**		.86**
	p	<0.001	<0.001	0.59	0.53	0.16	0.001	<0.001	0.001		<0.001
	N	55	59	57	57	58	57	46	43		59
DMX	*r*	.69**	.31*	0.061	-0.026	0.078	.41**	.61**	.46**	.86**	
	p	<0.001	0.016	0.65	0.85	0.56	0.002	<0.001	0.002	<0.001	
	N	55	59	57	57	58	57	46	43	59	

Significant positive correlations are indicated by green squares, and significant negative correlations by red squares. *Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed)

the sexes, consistent with prior reports (Gruene et al. 2014; Maren et al. 1994; Pryce et al. 1999). Further, all subjects in the experimental condition expressed similar levels of freezing behavior, irrespective of food access. Thus, the presence of food at test did neither influence the expression of the conditioned freezing response to the fear cue, nor did potential positive contextual associations with food access and consumption from training. Last, there was a large baseline sex difference in consumption that emerged during training and was evident during testing, similar to prior observations (Petrovich and Lougee 2011; Reppucci et al. 2013). Greater food consumption by males may be due to their greater body weight which results in a larger gastric capacity (Bull and Pitts 1971), or other factors due to organizational and activational effects of sex hormones (Asarian and Geary 2013; Fukushima et al. 2015). However, despite a sex difference in absolute consumption, males and females exhibited a similar hypophagic response to the fear cue (Fig. 2a).

Since the goal of the current study was to identify the patterns of recruitment due to fear-induced hypophagia, it was important to dissociate them from Fos induction patterns due to other behaviors such as freezing or feeding. Comparisons of Fos induction patterns between the experimental groups with and without food access allowed identification

Table 6 Partial correlation statistics of Fos induction and behavior at test

Region	Consumption	Freezing
Amygdala		
CEAl	$\mathbf{r_{(28)} = 0.52, p = 0.003}$	$r_{(28)} = 0.001, p = 0.99$
CEAm	$\mathbf{r_{(28)} = 0.49, p = 0.006}$	$r_{(28)} = 0.016, p = 0.93$
LA	$r_{(28)} = 0.13, p = 0.49$	$\mathbf{r_{(28)} = 0.40, p = 0.026}$
BLAa	$r_{(28)} = 0.28, p = 0.13$	$r_{(28)} = 0.031, p = 0.87$
BMAp	$r_{(28)} = 0.32, p = 0.076$	$r_{(28)} = 0.35, p = 0.057$
Cortex		
mPFC	$\mathbf{r_{(12)} = 0.63, p = 0.016}$	$\mathbf{r_{(12)} = 0.72, p = 0.003}$
Hypothalamus		
dmLHA	$r_{(27)} = 0.25, p = 0.19$	$r_{(27)} = 0.061, p = 0.75$
dlLHA	$\mathbf{r_{(27)} = 0.39, p = 0.031}$	$r_{(27)} = 0.027, p = 0.89$
vLHA	$\mathbf{r_{(27)} = 0.38, p = 0.036}$	$r_{(27)} = 0.11, p = 0.56$
PVHpml	$r_{(28)} = 0.22, p = 0.24$	$\mathbf{r_{(28)} = -0.41, p = 0.023}$
PVHdp	$r_{(28)} = 0.086, p = 0.65$	$r_{(28)} = -0.004, p = 0.99$
PVHmpd	$r_{(28)} = -0.005, p = 0.98$	$r_{(28)} = -0.27, p = 0.14$
PVHmpv	$r_{(28)} = 0.15, p = 0.44$	$r_{(28)} = -0.20, p = 0.30$
PVHpv	$r_{(28)} = 0.14, p = 0.45$	$r_{(28)} = 0.046, p = 0.81$
SO	$r_{(26)} = 0.20, p = 0.30$	$r_{(26)} = -0.23, p = 0.24$
Dorsal vagal complex		
NTSm	$\mathbf{r_{(28)} = 0.59, p = 0.001}$	$r_{(28)} = -0.004, p = 0.99$
DMX	$r_{(28)} = 0.33, p = 0.078$	$r_{(28)} = -0.13, p = 0.50$

Significant effects are bolded

of activation due to the inhibition of feeding from activation associated with other responses elicited by the fear cue. Similarly, comparisons of Fos induction patterns between the control groups with and without food access allowed identification of activation specific to food consumption. Finally, since freezing and inhibition of feeding are both behavioral indexes of conditioned fear and were correlated in the current study, we evaluated the contribution of each to Fos induction using partial correlation analyses.

Amygdala

Fos induction in the CEA, specifically CEAl and CEAm, corresponded to food consumption during test. Fos was robustly induced in all rats with access to food compared to those in the No-Food groups, and was substantially lower in the male experimental group that strongly inhibited food intake compared to the male control group. Females with food access did not show significantly different Fos induction patterns between the experimental and control condition, very likely due to a quantitatively smaller difference in the amounts consumed between these two groups at test compared to the corresponding groups in males. There was a baseline difference in food intake between the sexes, and at test the difference in grams consumed between the male control and experimental groups was > 2.5 fold greater than the difference in grams consumed between the female control and experimental groups (3.57 vs. 1.34 g, respectively). Thus, the smaller difference in grams consumed between the two female groups, we postulate, was insufficient to produce differences in Fos expression at the group level, but at the individual level CEA Fos was associated with feeding in females, just like in males. In support, Fos induction in the CEAl and CEAm significantly correlated with the amount of food subjects (both sexes included) consumed at test, when freezing was controlled (Fig. 9a, b). Alternatively, these patterns could represent sex differences in the way males and females process food information or interact with food (Asarian and Geary 2013; Becker and Taylor 2008). There could also be sex differences in the processing of fear-related information (Cover et al. 2014; Gruene et al. 2015; Lebron-Milad and Milad 2012; Petrovich and Lougee 2011; Reppucci et al. 2013) or associated anxiety, which may have contributed to the observed sex differences in consumption and Fos induction patterns.

The pattern of CEA activation in the current study is consistent with reports of increased CEA Fos induction following ingestion driven by homeostatic need (Li et al. 2012) or learned cues (Holland and Hsu 2014), exposure to contexts previously associated with food (Schiltz et al. 2007), and of 2-deoxy-D-glucose administration (which induces hunger; Ritter and Dinh 1994). Our results are also consistent with the CEA's recently proposed role in the network underlying refeeding (Zseli et al. 2017). Although nearly exclusively GABAergic (Swanson and Petrovich 1998), CEA neurons are highly diverse (e.g., Cassell et al. 1986; Marchant et al. 2007; Wray and Hoffman 1983), and this heterogeneity is one potential mechanism by which different CEA neuronal populations could respond to stimuli that promote or inhibit food intake. Indeed, recent studies have identified two subsets of mutually exclusive CEA neurons, one marked by the expression of serotonin receptor Htr2a which promotes food intake (Douglass et al. 2017) and the other marked by the expression protein kinase C- δ which inhibits food intake (Cai et al. 2014).

In the current study, CEA Fos induction did not correlate with conditioned freezing behavior and there was no differential Fos induction between the experimental and control conditions in the No-Food groups. The CEA is well-recognized as the amygdalar output for conditioned fear (for reviews see Ehrlich et al. 2009; Ledoux 2012; Maren 2001; Pare and Duvarci 2012); however Fos induction patterns within this nucleus during conditioned fear expression have been inconsistent. Prior reports have shown increases, decreases, or no change in Fos

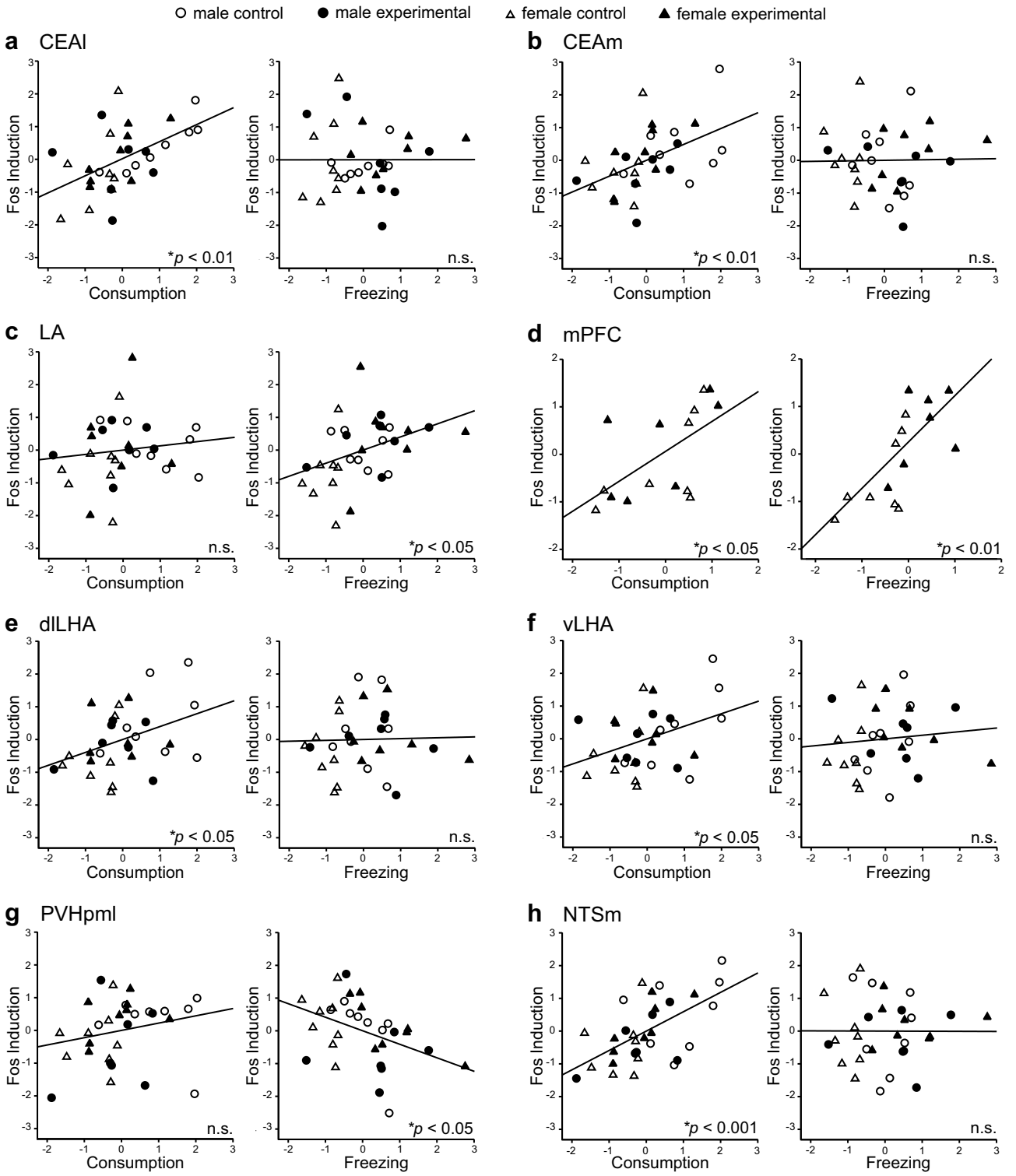

Fig. 9 Scatter plots depict the partial correlations for regions where Fos induction significantly correlated with behavior at test (see "Statistical Analysis" for information on plot creation). *n.s.* not significant

expression (Beck and Fibiger 1995; Day et al. 2008; Hall et al. 2001; Pezzone et al. 1992; Rosen et al. 1998; Scicli et al. 2004). These inconsistencies are likely due to differences in the behavioral preparations used across studies, including differences in test length and type of cue (e.g., discrete vs. contextual), and potential differences in the parts of CEA that were compared across studies.

Within the BLAa, Fos induction was greater in subjects with access to food compared to the No-Food groups, irrespective of experimental condition, and did not correlate with feeding or freezing. Thus, unlike the CEA where Fos induction corresponded with food consumption, Fos in the BLAa only corresponded to whether the group had access to food during the test. This Fos induction pattern was unique to the BLAa, which is particularly intriguing given that the BLAa was the only cell group in the forebrain that showed selective recruitment during early cue-food learning (Cole et al. 2015a, 2013). Since the subjects with food access at test were previously fed in that context during training, the observed Fos induction could be due to learned food associations with the testing context or with food, rather than consumption itself. In support of this interpretation, earlier studies did not find changes in BLA Fos induction following ingestion of a palatable meal (Park and Carr 1998), but did observe BLA Fos induction following exposure to a food-associated context (Schiltz et al. 2007).

Within the LA, Fos induction was greater in the experimental compared to the control condition, irrespective of food access. This is consistent with prior studies that showed increased Fos induction in the LA following presentation of conditioned fear stimuli (Scicli et al. 2004; Senn et al. 2014; but see Hall et al. 2001). The LA is critical for conditioned fear acquisition and expression (Campese et al. 2015; Blair et al. 2005; Jimenez and Maren 2009), and is considered the primary amygdalar input region for auditory and somatosensory information (for reviews see Ehrlich et al. 2009; Pare and Duvarci 2012; Pare et al. 2004; Sigurdsson et al. 2007). A recent study employing optogenetic techniques highlighted the importance of the convergence of these sensory inputs into the LA by showing that pairing activation of auditory input terminals in LA with an aversive event was sufficient to create a fear memory, and that reactivation of these inputs was sufficient to elicit conditioned fear behaviors (Kwon et al. 2014). This suggests an intriguing possibility that the LA Fos induction in the experimental condition in the current study may reflect reactivation of the same neurons that were recruited by the auditory (tone) and somatosensory (footshock) stimuli during the initial tone-shock conditioning.

Within the BMAp, Fos induction was greater in the experimental compared to the control condition, similar to the patterns of Fos induction observed in the LA. The similarity in Fos patterns for the BMAp and LA is consistent with dense reciprocal connections between these two cell groups (Petrovich et al. 1996; Pitkänen et al. 1995; Savander et al. 1996) which are likely the reason for the significant positive correlation in Fos induction between these regions in the current study. Reports of increased BMAp Fos induction during the expression of conditioned fear have been inconsistent across studies (Beck and Fibiger 1995; Hall et al. 2001; Senn et al. 2014), and, as discussed above, this is likely due to differences between preparations or in sampling regions. However, neurons within the BMA have been shown to acquire excitatory responses to discrete CSs, and are activated during the expression of conditioned fear (Amano et al. 2011). This activation may be critical for fear expression, as post-training lesions (Anglada-Figueroa and Quirk 2005) or temporary inactivation (Amano et al. 2011) of an area which encompassed the BLA and BMA blocked the expression of conditioned freezing behavior.

Medial prefrontal cortex

We found sex-specific Fos induction patterns in the mPFC. Females in the experimental condition had significantly greater Fos induction than those in the control condition, while in contrast, all male groups showed similar amounts of Fos induction. Interestingly, Fos induction in females correlated with both food consumption and freezing during the test, suggesting that the mPFC is involved in both the appetitive (feeding) and aversive (freezing) aspects of this behavioral paradigm. These findings are in agreement with prior evidence for mPFC function in both feeding and fear associated behaviors. The mPFC is necessary for the potentiation of feeding by learned contextual cues (Petrovich et al. 2007), and exposure to a context previously associated with food induced Fos in the mPFC (Choi et al. 2010; Schiltz et al. 2007). Additionally, the dorsal mPFC (ACAd and dorsal PL) has been linked to the expression of conditioned fear, while the ventral mPFC (ventral PL and ILA) has been linked to the inhibition of conditioned fear especially during extinction (for reviews see Courtin et al. 2013; Sotres-Bayon and Quirk 2010). Thus, in the experimental condition, Fos induction in the ACAd and PL could be due to fear cue presentation and subsequent expression of conditioned fear responses, and Fos induction in the ILA could be due to inhibition of the conditioned fear response to consume food. Further, separate populations of mPFC neurons could subserve distinct feeding and fear-related functions during the food consumption tests under fear, which could be why Fos induction was high, but not different, across all conditions in males.

Importantly, the mPFC is part of the executive control network that guides on-going behaviors (Dalley et al. 2004; Euston et al. 2012; Gilmartin et al. 2014), and its sex-specific recruitment during fear-induced hypophagia suggests differential control of feeding and fear competition in males and females. In that regard, in this preparation females continue to inhibit feeding under fear for much longer than males (Petrovich and Lougee 2011; Reppucci and Petrovich 2014), and this is in general agreement with evidence that females are more risk adverse (less likely to risk footshock punishment to obtain a food reward) than males (Orsini et al. 2016).

Therefore, enhanced recruitment of the mPFC in females during this initial testing might be predictive of prolonged expression of fear-induced hypophagia in females. Intriguingly, in an fMRI study, women with anorexia nervosa rated food images as more fear inducing and had greater BOLD responses in the mPFC compared to healthy control subjects (Uher et al. 2004). Together these findings suggest that mPFC overactivation under fear may be a susceptibility factor in females, consistent with higher reported rates of eating and anxiety disorders in women (McCarthy et al. 2012; McLean and Anderson 2009).

Hypothalamus

Fos induction patterns in the LHA were associated with food access and consumption in both sexes during the test. These results are consistent with the LHA's critical role in feeding behavior (reviewed in Elmquist et al. 1999) and cue-food associative learning (Cole et al. 2015a; Sharpe et al. 2017). The LHA is important for both homeostatic and non-homeostatic feeding drives, and is anatomically positioned to integrate cognitive and hedonic environmental influences with the physiological motivation to eat (e.g., Berthoud and Münzberg 2011; Petrovich 2013, 2018; Swanson 2000, 2005). Meal anticipation (Poulin and Timofeeva 2008; Verhagen et al. 2011), ingestion of a palatable meal (Park and Carr 1998), exposure to food-associated contexts (Choi et al. 2010; Schiltz et al. 2007), cue-food associative learning (Cole et al. 2015a), and cue-induced food seeking after punishment (Campbell et al. 2017) have all been shown to increase Fos expression in the LHA. Further, lateral hypothalamic neurons expressing orexin or melanin-concentrating hormone (MCH) have been implicated in the control of feeding by learned cues (Cole et al. 2015b; Sherwood et al. 2015). In addition, recent work using opto- and pharmacogenetic techniques revealed subpopulations of GABAergic neurons (that do not co-express orexin or MCH) that when activated, increased appetitive and consummatory measures of feeding behavior (Jennings et al. 2015). Thus, LHA Fos induction in the current study could have been driven by consumption or by food cues, and may have engaged orexin and MCH circuitries, or GABAergic systems.

Similar to the LHA, Fos induction in the PVH and SO was associated with food access and consumption, and Fos induction between these two regions was positively correlated. Subjects with access to food had greater Fos induction than those without food access, and this effect was blunted in subjects who were inhibiting food intake in response to the fear cue. This is consistent with PVHpml recruitment by well-learned food cues (Cole et al. 2015a), Fos expression in SO and magnocellular PVH after feeding in food-restricted rats (Poulin and Timofeeva 2008), and more broadly with the roles of these regions in the maintenance of energy homeostasis and the regulation of feeding behavior (Schwartz et al. 2000; Swanson 2000). Interestingly, these two regions are characterized by the presence of oxytocin- and vasopressin-synthesizing neurons (Choy and Watkins 1977; Simmons and Swanson 2009; Vandesande and Dierickx 1975). In the PVH, activation of oxytocin neurons corresponded with meal termination (Olszewski et al. 2010) and activation of vasopressin neurons inhibited food intake (Pei et al. 2014). In the SO, oxytocin and vasopressin neurons responded to glucose and insulin (Song et al. 2014). Thus, PVH and SO Fos induction patterns observed in the current study could represent activation of processes that terminate feeding under fear, potentially using these satiety mechanisms.

Paraventricular thalamus

Fos induction in the PVT was robust in all conditions in both sexes, which may reflect the role of the PVT as an integrator of visceral and emotional information (Kirouac 2015) and is consistent with its involvement in both feeding- and fear-related behaviors. For example, Fos expression in the groups with access to food could have been driven by the food-associated context (Schiltz et al. 2007) or meal anticipation (Poulin and Timofeeva 2008), while Fos induction in the experimental groups could be due to the fear cue, since an intact PVT is necessary for conditioned fear expression (Li et al. 2014).

Dorsal vagal complex

Within both the NTSm and DMX Fos induction was associated with consumption, consistent with prior findings that feeding increased Fos expression in the NTS in food-restricted rats (Poulin and Timofeeva 2008). The NTSm and DMX are the primary components of a hindbrain circuit mediating the regulation of feeding behavior (Norgren 1983; Travagli et al. 2006) via the vagus and other cranial nerves relaying gustatory information (McLean and Hopkins 1982; Norgren 1983; Norgren and Smith 1988). The similarity and correlation in Fos induction patterns between these two regions is consistent with their interconnectivity (Norgren 1978; Rogers et al. 1980), and could represent activation in response to taste or the activation of neurons coordinating aspects of feeding behavior and gastrointestinal functioning.

The CEA: a critical node in the functional network that coordinates expression of fear-induced hypophagia?

The organization of the neural networks underlying motivated behaviors is conceptualized as a triple descending pathway of converging cortical, striatal, and pallidal inputs

to hypothalamic and brainstem controllers that coordinate behavioral expression (Swanson 2000, 2005). Representative nodes from nearly all levels in this organizational schema were analyzed in the current study, defining an emerging functional network that supports the expression of fear-induced hypophagia. With respect to the triple descending pathway, the mPFC and cortical (LA, BLA, BMA) and striatal (CEA) parts of the amygdala (Swanson and Petrovich 1998) send converging direct and indirect pathways to hypothalamic (LHA, PVH, and SO) and brainstem (NTSm, DMX) regions (these anatomical connections are reviewed further in Reppucci 2015; Reppucci and Petrovich 2016). The CEA, we hypothesize, is a critical node within the network that integrates appetitive and aversive sensory information from cortical (mPFC and basolateral amygdalar area) inputs to coordinate fear-induced hypophagia via the hypothalamus and brainstem. In support, we found that Fos induction in the CEA was significantly correlated with Fos induction in cortical (i.e., BLAa, mPFC), hypothalamic (i.e., LHA, PVHpml, SO), and brainstem (i.e., NTSm, DMX) regions.

Basolateral area inputs to the CEA are important for both fear and feeding behaviors. For example, functional disconnection of the LA and CEA abolishes conditioned fear expression (Campese et al. 2015; Jimenez and Maren 2009), an effect likely mediated via the BMAp (Pare and Duvarci 2012; Pitkänen et al. 1997; reviewed in Reppucci 2015). Additionally, optogenetic inhibition of basolateral area projections to the CEA impaired cue-shock fear conditioning, but promoted cue-food learning (Namburi et al. 2015). Interestingly, LA and BLA inputs to the CEA compete, as activation of a subset of BLA neurons blocked LA-stimulation-induced excitation of the CEAm (Jasnow et al. 2013). Similarly, mPFC pathways to the CEA are recruited during feeding (Zseli et al. 2017), are important for the control of conditioned fear expression (Moscarello and LeDoux 2013), and gate the effectiveness of basolateral area inputs (Quirk et al. 2003). Fos patterns in the current study, including the correlations, support a functional network where information about the fear stimulus from the LA, BMAp, and mPFC, and food-associated cues from the BLAa and mPFC could be relayed to the CEA where this integration could contribute to decreased consumption during fear (and corresponding changes in CEA Fos induction) via projections to downstream hypothalamic (LHA, PVH, SO) and brainstem (NTSm, DMX) targets. Further, the activation patterns within the mPFC suggest the mPFC–CEA circuitry within this network may be functioning in a sex-specific way during fear and feeding competition.

The hypothalamic and brainstem regions analyzed in the current study receive CEA inputs (direct or indirect), and all displayed Fos induction patterns associated with food consumption/access during the test session. The similarities in CEA and LHA Fos patterns are consistent with the reciprocal connections between these two regions (for review see Reppucci 2015; Reppucci and Petrovich 2016), and with a recent report that unilateral lesions of the LHA suppressed food cue-induced Fos expression in the CEA (Wheeler et al. 2014). Although the CEA may not directly innervate the PVH or SO, it is anatomically positioned to influence these regions through dense pathways via the bed nuclei of the stria terminalis (Dong et al. 2001; Sawchenko and Swanson 1983), and CEA stimulation results in robust PVH Fos induction (Petrov et al. 1995).

The CEA sends substantial projections to the NTSm and weaker projections to the DMX (Danielsen et al. 1989; Liubashina et al. 2000; Zhang et al. 2003), and receives reciprocal projections mainly from the NTSm (Ricardo and Koh 1978). This anatomical framework supports integration of convergent information from the CEA with gustatory and viscerosensory inputs within the NTS and DMX. Indeed, electrical stimulation of the CEA modulated the response of NTS and DMX neurons to gastrointestinal stimuli (Zhang et al. 2003). The CEA can also influence these hindbrain sites indirectly via the PVH (Petrov et al. 1995). The organization of connections between the NTS, DMX and CEA, and the correlated patterns of Fos induction reported here together suggest processing within this circuitry regulates feeding under fear.

In conclusion, we propose that a forebrain–brainstem network, which includes the CEA, is critical for fear and feeding competition. This emerging network overlaps with the previously defined amygdala–prefrontal–lateral hypothalamic network underlying the control feeding by learned appetitive cues (Cole et al. 2015a, 2013; for review see Petrovich 2013), including sex-specific mPFC engagement (Anderson and Petrovich 2017, 2018). However, there are important differences. Notably, we postulate that the CEA is a key integrator for the expression of fear-induced hypophagia. The network identified in the current study provides a novel functional map for future interrogation of the neural substrates underlying the competition between fear and feeding.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed. All proce-

dures performed in this study involving animals were in accordance with the ethical standards of the Boston College Institutional Animal Care and Use Committee.

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