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Research report

Contextual fear cues inhibit eating in food-deprived male and female rats ☆

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ABSTRACT

Previously we have shown that food-deprived male and female rats inhibit food consumption when presented with a discrete conditioned stimulus that signals danger. Here, in a series of three experiments, we examined whether contextual conditioned stimuli can exert the same effect. Experiment 1 paired a distinct context with footshocks, to examine food intake in food-deprived rats upon re-exposure to the context. Experiment 2 used a discrimination protocol with two alternating contexts; rats were given food in one, and received footshocks in the other. This protocol allowed us to monitor food consumption during training in a context never associated with footshocks, and to evaluate consumption at test in a context that had been previously paired with footshocks. Experiments 1 and 2 compared experimental groups to controls that never received footshocks. Experiment 3 used a within-subjects design to assess the specificity of the inhibition by the contextual cues, separate from any generalized effects due solely to the prior experience of footshocks. Our results demonstrate that similar to discrete cues, contextual cues previously associated with aversive events can inhibit feeding in food-deprived animals. These findings are important for our understanding of environmental contributions to the control of food intake.

Introduction

Motivated behaviors (i.e. ingestive, defensive, reproductive, and foraging behaviors) are essential for survival of the individual and the species (Swanson, 2000). Such behaviors are controlled by both internal, physiological signals from the body, as well as external, environmental signals. In certain instances, environmental cues can override physiological or homeostatic signals. In studies of feeding behavior it has been demonstrated that learned cues from the environment can induce food intake despite satiation (Holland, Petrovich, & Gallagher, 2002; Petrovich, Ross, Gallagher, & Holland, 2007; Reppucci & Petrovich, 2012; Weingarten, 1983; Weingarten, 1984) and inhibit food intake despite acute food deprivation (Petrovich & Lougee, 2011 Petrovich, Ross, Mody, Holland, & Gallagher, 2009).

Recently, we developed a behavioral paradigm to show that a learned cue that signals danger can inhibit feeding in food-deprived male and female rats (Petrovich & Lougee, 2011 Petrovich et al., 2009). In those studies we used a discrete conditioned stimulus (CS), a tone previously paired with footshocks. Here we examined whether the training environment alone, in the absence of any discrete cues, could accomplish the same.

Context and its associated cues are important for learning. Contextual cues can act as occasion setters for discrete CS's, but they can also become conditioned stimuli themselves in the absence of discrete cues (Holland & Bouton, 1999). Context has been increasingly studied in both the role it plays in aversive conditioning processes (e.g. Urcelay & Miller, 2010; Westbrook, Iordanova, McNally, Richardson, & Harris, 2002) and its influences on feeding (e.g. Petrovich et al., 2007; Stroebele & De Castro, 2004; Todd, Winterbauer, & Bouton, 2012). Thus, here we assessed if contextual cues associated with past aversive events would modulate food intake in food-deprived rats.

Importantly, we tested males and females to determine if learned contextual cues would modulate feeding similarly in both sexes. This was notable since few studies compare behavior of intact males and females (Cahill, 2006; Zucker & Beery, 2010), even though it is a necessary step in characterizing differences between the sexes (McCarthy, Arnold, Ball, Blaustein, & De Vries, 2012). Further, women have higher reported rates of anxiety and eating disorders but remain grossly underrepresented in both basic research and in clinical trials, including in models of diseases which disproportionately affect females (McCarthy et al., 2012; Zucker & Beery, 2010).

The present study had two aims. First, we examined whether aversive contextual conditioned stimuli could inhibit feeding despite acute physiological hunger in male and female rats. Second,

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we compared behavior across the sexes to determine if there were differences in the expression of this fear-induced feeding inhibition. In Experiment 1 we used a simple aversive context conditioning protocol to test whether food-deprived rats would inhibit food consumption when given the opportunity to eat in a context previously paired with mild electric footshocks. In Experiment 2 we used a discrimination protocol with two distinct contexts. Training sessions in these two contexts occurred in an alternating order. In one context rats were provided with food, and in the other they were given footshocks. At test, rats were allowed to consume food in the context where they had received footshocks. This preparation allowed us to monitor food consumption during training in a context that was never associated with footshocks, and to evaluate consumption at test in a context that had been previously paired with footshocks.

Experiments 1 and 2 compared consumption of the experimental groups to the consumption of controls that did not receive footshocks during training. Thus, these experiments could not directly rule out a possible context generalization in inhibition of consumption due to prior experience with footshocks. Experiment 3 used a within-subjects design to assess the specificity of the inhibition by the contextual cues. In that experiment, during training rats received equal exposure to two distinct contexts, but were only given footshocks in one. Food was not provided during training in either context. Rats were then tested for consumption in the two contexts. This allowed direct comparison of novel consumption in a neutral context to novel consumption in a context that had been paired with footshocks in the same rats.

Materials and methods

Subjects

Twenty-eight male and female Long-Evans rats (Charles River Laboratories; Portage, MI) from a previous study (Petrovich & Lougee, 2011) were used in Experiment 1. Thirty-two experimentally naïve, male and female Long-Evans rats (Charles River Laboratories; Portage, MI) weighing 226–250 g at arrival were used in Experiment 2. Sixteen male and female Long-Evans rats (Charles River Laboratories; Portage, MI) from a previous study (a potentiation of feeding experiment; protocol similar to: Petrovich et al., 2007) were used in Experiment 3. For all experiments, rats were individually caged and maintained on a 12 h light/dark cycle (lights on at 6:00). All training and testing were conducted during the light phase, approximately between 10:00 and 15:00. Rats were given *ad libitum* access to standard laboratory chow (LabDiet 5P00, Prolab RMH 3000; Saint Louis, MO; 3.2 kcal/g; 26% protein, 14% fat, 60% carbohydrate) and water except as otherwise noted. Male and female rats were housed in separate colony rooms. Rats were acclimated to the colony rooms and handled daily for at least one week prior to the start of any behavioral training. Body weights for all rats, and vaginal smears of the female rats, were obtained 6 days a week for the duration of each experiment. 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 Animal Care and Use Committee.

Apparatus

For both experiments, 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 an isolation cubicle (79 × 53 × 53 cm; Coulbourn Instruments, Allentown, PA) composed of monolithic rigid foam walls, which isolate from ambient sound and light. A ventilation fan, located on the back wall of each isolation cubicle, provided masking noise (55 dB). Video cameras controlled by Digital Video Security System Digital Video Recorder software program (Coulbourn Instruments, Allentown, PA) were mounted on the back wall of each isolation cubicle to record behavior during training and testing. Rats were pre-exposed to ~1 g of the training food pellets (5TUL; Test Diets; Richmond, Indiana; 3.4 kcal/g; 20% protein, 13% fat, 67% carbohydrate) the day before the start of the behavioral training procedure. These food pellets have a similar caloric density and macronutrient energy composition to standard laboratory chow, with the exception that the carbohydrates are from starch in the chow and sucrose in the pellets.

Behavioral training procedure: Experiment 1

All subjects in Experiment 1 were previous participants in a behavioral experiment that utilized tones and shocks, and half of the subjects had received tone-shock pairings while the other half of the subjects received only tones (Petrovich & Lougee, 2011). For the current experiment, groups were counterbalanced in respect to the prior experience and thus, the new groups had equal numbers of rats from the two prior conditions. All rats also had experience with the training food pellets (Petrovich & Lougee, 2011). The current experiment started ~2 weeks after the end of the prior study. Prior to behavioral training for the current study, rats received a habituation session where they were allowed to consume food pellets *ad libitum* from the food cup in a new context that was distinct from the two contexts used in the prior study. To create the context for the current study the behavioral chambers were modified in the following way: the grid floor was exposed, the doors to the isolation cubicle were closed, and a "house light" (4 W light) provided illumination. Additionally, the chambers were wiped with 5% ammonium hydroxide (205840010; Acros Organics, Fair Lawn, NJ) before placing the rat inside. For this, and every following session, rats were transported to and from the colony rooms and testing room in their home cages via a pushcart.

Experiment 1 consisted of four sessions conducted over approximately 2 weeks: a baseline food consumption test, two aversive training sessions, and a final food consumption test. For the baseline and final food consumption tests acutely food-deprived rats (20–22 h food deprivation, *ad libitum* access to water) were placed in the behavioral chambers and allowed *ad libitum* access to food pellets in the food cup for 10 min. No stimuli were presented during the food consumption tests. At the end of this time, rats were removed from the chambers and returned to their respective colony rooms. The food remaining in the food cup was then weighed, and the amount consumed was calculated. Rats were returned to *ad libitum* access to chow ~30 min after the end of each session.

The aversive training sessions were conducted under sated conditions (*ad libitum* access to chow) and were 5 min and 10 s long. For each of the aversive training sessions half of the male and female rats (experimental groups) received four mild electric footshocks (1 s, 1 mA each) administered at the 2nd, 3rd, 4th, and 5th minute; the other half of the male and female rats (control groups) received no footshocks. At the end of each session, rats were removed from the chambers and returned to their respective colony rooms.

Two identical replications of the behavioral training procedure were used in Experiment 1 ($n = 12$, $n = 16$), with equal numbers of rats in every group for each replication.

Behavioral training procedure: Experiment 2

For Experiment 2 the behavioral training procedure consisted of alternating appetitive and aversive training sessions. There were a total of 8 training sessions: 6 were appetitive sessions (S1, S2, S4, S6, S7, and S8) and 2 were aversive sessions (S3 and S5). A food consumption test was given following training completion. The appetitive and aversive sessions were conducted in two distinct contexts (A and B, respectively) that were created by altering the visual, tactile and olfactory properties of the behavioral chambers. The rats, in their home cages, were placed on a cart for transport to and from the testing room for each training session and the food consumption test. The behavioral training procedure was completed over the course of three weeks.

For Context A, each chamber had a black Plexiglas panel placed on top of the grid floor so that rats could not see or feel the grid. The "house light" was turned off, but the doors of the isolation cubicles were in an open position so that the testing room light provided illumination for the chambers. Each chamber had a circular glass dish (70 mm × 50 mm) containing food pellets. The chambers were wiped with 1% acetic acid (A38-500; Fisher Scientific, Fair Lawn, NJ) before placing the rat inside.

For Context B, each chamber had a black Plexiglas insert sheet that was positioned at an angle and hid one aluminum side. The grid floor was exposed, and the circular glass dish was present but empty. The doors of the isolation cubicle were closed, and a "house light" (4 W light) provided illumination. The chambers were wiped with 5% ammonium hydroxide (205840010; Acros Organics, Fair Lawn, NJ) before placing the rat inside.

During the six 10 min appetitive training sessions, acutely food-deprived rats (22 h food deprivation, *ad libitum* access to water) were allowed to consume food pellets *ad libitum* from the glass dish in Context A. Rats were immediately removed at the end of each appetitive training session and returned to their respective colony rooms. The food remaining in the glass dish was weighed, and amount consumed was calculated. Rats were returned to *ad libitum* access to chow ~30 min after the end of each appetitive training session, and were allowed at least 24 h of *ad libitum* access prior to subsequent deprivation, or prior to the start of an aversive training session.

During both of the 10 min aversive training sessions, sated rats (at least 24 h *ad libitum* access to chow) were placed in Context B where half of male and female rats (experimental groups) received two mild electric footshocks (1 s, 1 mA each) and the other half of the male and female rats (control groups) received no footshocks. The shocks were administered at the 7th minute and the 9th minute during the first aversive session and at the 5th and 9th minute during the second aversive session. Rats were immediately removed following each aversive training session and returned to their respective colony rooms.

Rats were acutely food deprived (22 h food deprivation, *ad libitum* access to water) prior to the food consumption test. Rats were placed in Context B (the aversively conditioned context) and were allowed to consume food pellets *ad libitum* from the glass dish for 10 min; no footshocks were administered. As with the training sessions, rats were removed from the chambers at the end of the 10 min and returned to their respective colony rooms. The food remaining in the glass dish was weighed, and the amount consumed was calculated. Rats were returned to *ad libitum* access to chow ~30 min after the end of the test.

Behavioral training procedure: Experiment 3

All subjects in Experiment 3 participated in a prior experiment in which they had extensive experience with the training food pellets. Importantly, they had no prior experience with footshocks, and no prior exposure to the training contexts used in Experiment 3. The behavioral training for the current study began ~2 weeks following the completion of the prior experiment, and training conditions were counterbalanced with respect to prior experience.

For Experiment 3 the behavioral training procedure utilized a within-subjects design in which rats were trained to discriminate between two distinct, initially novel contexts. One context remained neutral during training while rats received footshocks in the other context. The two contexts (A and B, respectively) were made distinct by altering the visual, tactile and olfactory properties of the behavioral chambers. For Context A, each chamber had a black Plexiglas panel placed on top of the grid floor so that rats could not see or feel the grid. The "house light" was turned off, but the doors of the isolation cubicles were in an open position so that the testing room light provided illumination for the chambers. For Context B, each chamber had a black Plexiglas insert sheet that was positioned at an angle and hid one aluminum side. The grid floor was exposed, the doors to the isolation cubicle were closed, and a "house light" (4 W light) provided illumination. Additionally, the chambers of Context B were wiped with 5% ammonium hydroxide (205840010; Acros Organics, Fair Lawn, NJ) before each session.

The rats received four 10 min long training sessions, two in each context with order counterbalanced across subjects. For aversive training sessions rats were placed into Context B and received mild electric footshocks (1 s, 1 mA each) at the 3rd, 5th, 7th, and 9th minute mark. During the neutral training sessions rats were placed into Context A, and no shocks were given. The rats were transported via a pushcart in their home cages to and from the testing room for all training and testing sessions; they were immediately removed from the chambers at the end of each session and returned to their colony rooms. During training rats had *ad libitum* access to chow and water in their home cages.

After training completion rats were tested for food pellet consumption in each context. The consumption tests were 10 min long and were conducted on separate days in a counterbalanced order. The context order (i.e., the first test occurring in the neutral or aversive context) for the food consumption tests was counterbalanced against the final training session. Prior to each of the two tests rats were acutely deprived of food (22 h food deprivation, *ad libitum* access to water). During each test session rats were allowed to consume food pellets *ad libitum* from the food cup; no footshocks were administered during tests. The food remaining in the food cup was weighed, and the amount consumed was calculated. Rats were returned to *ad libitum* access to chow ~30 min after the end of the test. The behavioral training procedure was completed over the course of 2.5 weeks.

Vaginal smears

Female rats in all experiments were examined 6 days a week by a vaginal lavage procedure to determine the estrous cycle stage. The vaginal smears were obtained, placed on glass slides, and samples were observed under a microscope (Leica ATC 2000) to identify the estrous cycle stage. The procedure was applied to ensure that the female rats were showing normal cycling; however, due to the small sample size, the estrous cycle stage was not used as a variable in analyses.

Behavioral observations

Freezing and food cup behaviors were assessed for each rat during the final consumption tests in all three experiments. Freezing behavior is a species-typical defense response that is characterized

by the cessation of all movement except that required for breathing (Blanchard & Blanchard, 1969; Fanselow, 1984). "Food cup behavior" was qualified whenever the rat was eating the food near the food receptacle or when the rat was standing close to and facing the food receptacle (except when the rat was freezing near the receptacle). Latency to approach the food receptacle was also recorded in Experiment 3. Observers were "blind" with respect to the training condition and sex of the rats observed. The observer noted the behavior ("freezing", "food cup", or "other") of each rat every 1.25 s, paced by a metronome, during the first 10 s of each minute that the rat spent in the behavioral chamber. This allowed for representative sampling of freezing and food cup behaviors over the course of the test. The percentage of time rats exhibited freezing or food cup behavior during each of the sampled intervals was combined to calculate the percent of the total amount of time spent exhibiting freezing or food cup behavior during all the sampled intervals. Additionally, videos of aversive training sessions for all experiments were watched to confirm that footshocks were received at the intended times.

Statistical analysis

Behavioral data for Experiments 1 and 2 were analyzed using two-way (Sex × Test Group) ANOVAs and post hoc *t*-Tests in SPSS. Behavioral data for Experiment 3 were analyzed using repeated measures (Context A vs. Context B) ANOVAs with Sex as a factor and post hoc *t*-Tests in SPSS. In all cases, $p < 0.05$ was considered significant.

Results

Experiment 1

The training procedure began with a baseline consumption test to determine how much rats would eat in behavioral chambers under acutely food-deprived conditions. The baseline test was followed by two aversive training sessions that occurred while rats were sated. A final food consumption test under food-deprived conditions was then given.

Food consumption tests

There were no differences in consumption between experimental and control groups of the same sex during the baseline food consumption test (Male Control: 6.44 ± 0.31 g, Male Experimental: 6.63 ± 0.28, Female Control 2.95 ± 0.28, Female Experimental 3.74 ± 0.39). However, food consumption was significantly lower in the experimental group compared to the control group for both sexes during the final food consumption test (Fig. 1A). Two-way ANOVAs revealed a significant main effect of Sex ($F(1,24) = 98.92$, $p < 0.001$) during the baseline test, and significant main effects of Sex ($F(1,24) = 11.09$, $p < 0.01$) and Test Group ($F(1,24) = 30.68$, $p < 0.001$) during the final test. Post hoc within-sex independent samples *t*-Tests confirmed that both male ($t(1,12) = 3.75$, $p < 0.01$) and female ($t(1,12) = 5.39$, $p < 0.001$) experimental groups consumed less than the corresponding control group during the final test.

To account for baseline differences in consumption between the sexes we re-calculated the amount consumed and expressed it as the percent of the mean consumption of the control group of the same sex to determine if there were differences in the extent of inhibition of food intake between the sexes. This calculation was done by dividing the consumption for each subject, control and experimental, by the mean consumption of the same-sex control group and then multiplying the result by 100; this calculation preserved the existing variance in consumption for all groups. Here, two-way ANOVAs again showed no significant differences between groups during the baseline test ($p > 0.05$). There was a significant main effect of Test Group ($F(1,24) = 39.36$, $p < 0.001$) during the final consumption test. There was no longer a main effect of Sex for either test ($p > 0.05$, both). Thus, males and females in the experimental groups inhibited their intake a similar amount during the final consumption test (Fig. 1B).

Freezing and food cup behaviors

Both the male and female experimental groups exhibited robust freezing behavior during the final food consumption test, while neither of the control groups exhibited any instances of freezing (Fig. 1C). A two-way ANOVA on total percent of time freezing during the test confirmed a significant main effect of Test Group ($F(1,24) = 57.41$, $p < 0.001$), but no effect of Sex ($p > 0.05$). Post hoc within-sex independent samples *t*-Tests confirmed that both the male and female experimental groups froze significantly more than the control groups of the same sex ($t(1,12) > 4.80$, $p < 0.001$, both).

Additionally, we analyzed food cup behavior, the amount of time spent in close proximity to the food receptacle, during the test. As expected from the consumption data we found that rats in the control groups spent more time engaging in food cup behavior than rats in the experimental groups of the same sex (Male Control: 43.99 ± 4.74%; Male Experimental: 14.61 ± 8.29%; Female Control: 25.00 ± 5.73%; Female Experimental: 12.34 ± 5.90%). A two-way ANOVA on the total percent of time spent engaging in food cup behavior during the test confirmed a significant main effect of Test Group ($F(1,24) = 11.14$, $p < 0.01$). Post hoc within-sex independent samples *t*-Tests revealed a significant difference

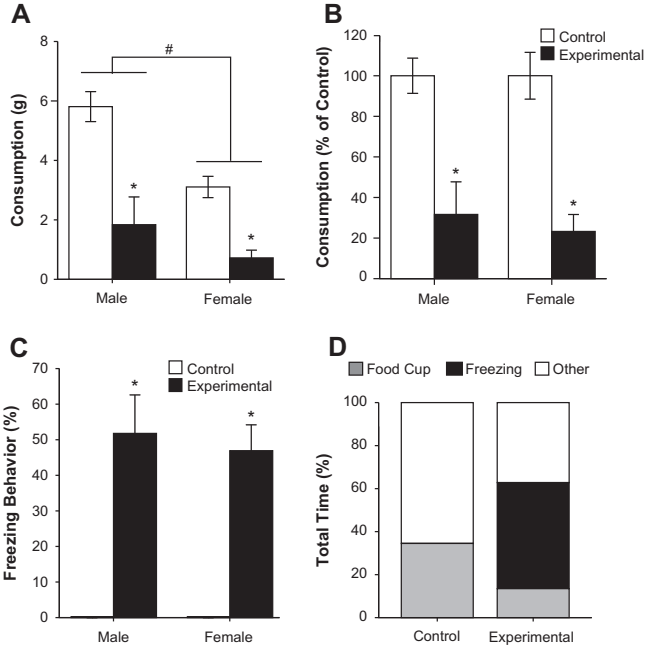

Fig. 1. Results from the final test in Experiment 1. (A) The amount of food pellets consumed (g) in the conditioned aversive context (mean ± SEM). (B) The amount of food pellets consumed expressed as the percent (mean ± SEM) of the consumption of the control group of the same sex. (C) Freezing behavior during the test; data show freezing-positive observations as the percent (mean ± SEM) of the total number of observations. (D) Representation of how rats divided their time during the final food consumption test; data show the percent of time experimental and control groups (collapsed by sex) spent expressing food cup, freezing, or other behaviors during the test. *Indicates a significant difference ($p < 0.05$) between control and experimental groups of the same sex. #Indicates a significant difference ($p < 0.05$) between males and females.

Table 1
Body weights during Experiment 1. Data is presented as mean ± SEM grams.

	Prior to training (g)	At test (g)
Male Control	470.29 ± 15.82	480.86 ± 23.68
Male Experimental	456.57 ± 20.71	460.57 ± 22.3
Female Control	265.00 ± 2.38	274.71 ± 6.88
Female Experimental	275.00 ± 5.46	274.71 ± 6.97

between the experimental and control groups in males ($t(1,12) = 3.08$, $p = 0.01$), but not in females ($p = 0.15$). Also, similar to the consumption results there was a trend towards a main effect of Sex ($p = 0.10$) and a significant difference in food cup behavior between the male and female control groups ($t(1,12) = 2.55$, $p < 0.05$).

Since there were no statistical main effects of Sex in the expression of freezing or food cup behavior, data were collapsed across the sexes to visualize how rats divided their time during the test session. As shown in Fig. 1D neither freezing nor food cup behaviors were near 'ceiling' for either condition. Importantly, experimental subjects spent ~50% of their total time freezing during the test, while the control groups spent only ~35% of their time exhibiting food cup behavior during the test. Thus, despite having ~50% of their time unallocated to freezing (i.e., they can freely move around) rats in the experimental groups did not engage in food cup behavior at the level of the control groups.

Body weights and estrous cycle

Males weighed more than females throughout Experiment 1, but same-sex experimental and control groups were similar in weight (Table 1). Two-way ANOVAs confirmed significant differences in body weight between the sexes at both baseline and test ($F(1,24) > 133.20$, $p < 0.001$, both), and no effect of Test Group ($p > 0.05$, both) at either time. Female rats of both groups were observed to be cycling normally throughout Experiment 1, and all stages of estrous were represented at test. Although not directly analyzed due to sample size, results suggest that estrous cycle stage did not affect female feeding or freezing behavior since behavioral data variability (as interpreted by the SEM) was no greater in females than in males. Thus, the experience of receiving footshocks during training did not produce changes in body weight or estrous cycling in the experimental groups compared to the control groups that did not receive any shocks.

Experiment 2

Rats were trained in a behavioral protocol with alternating appetitive and aversive sessions that were conducted in distinctive behavioral chambers (Context A and Context B, respectively; see Materials and methods for details). During appetitive sessions food-deprived rats were given free access to food pellets. During aversive training, half of the male and female rats received footshocks (experimental groups), while the other half received no shocks (control groups). Rats were then tested for food consumption in the aversive context under food-deprived conditions.

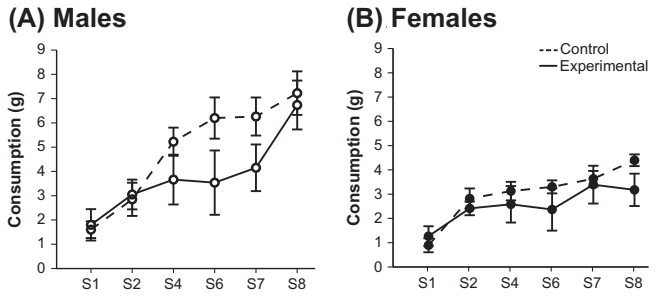

Fig. 2. Consumption during training in Experiment 2. Mean (±SEM) food pellet consumption in grams is shown for each appetitive training session.

Training

Food pellet consumption increased across the appetitive training sessions for all groups (Fig. 2). There were no differences in consumption between experimental and control groups of the same sex during training. During the appetitive sessions that occurred after sessions with shocks (S4, S6), rats in the experimental groups ate slightly less than the controls, however this difference was not statistically reliable ($p > 0.05$, all). This was not a reduction in the amount experimental groups consumed compared to their consumption on the prior appetitive training day, rather there was no additional increase as seen in the controls. Importantly, this stagnation in consumption was transient, and by the final appetitive training session there was no difference in consumption between the experimental and control groups. Two-way ANOVAs showed males and females consumed similar amounts of food pellets during the first two appetitive sessions ($p > 0.05$), but male rat consumption increased more rapidly across training and resulted in males consuming significantly more than females during the remaining four appetitive sessions ($F(1,28) > 4.90$, $p < 0.05$, all).

Food consumption test

Both male and female experimental groups strongly inhibited food pellet intake at test compared to the control group of the same sex (Fig. 3A). A two-way ANOVA revealed significant main effects of Sex ($F(1,27) = 8.64$, $p < 0.01$) and Test Group ($F(1,27) = 14.00$, $p = 0.001$). Post-hoc independent samples t-Tests confirmed that both the male ($t(1,14) = 3.08$, $p < 0.01$) and female ($t(1,13) = 2.29$, $p < 0.05$) experimental groups significantly inhibited their intake compared to the control group of the same sex.

As in Experiment 1, we re-calculated the amount consumed and expressed it as the percent of the mean consumption of the same sex control group to assess if there were differences in the extent of inhibition of food intake between the sexes. Here, a two-way ANOVA confirmed a significant main effect of Test Group ($F(1,27) = 14.15$, $p = 0.001$), and no longer showed an effect of Sex ($p > 0.05$). Thus, as in Experiment 1, males and females in the experimental groups inhibited their intake a similar amount during the food consumption test (Fig. 3B).

Freezing and food cup behaviors

Both the male and female experimental groups exhibited much more freezing behavior during the final food consumption test than the control groups (Fig. 3C). A two-way ANOVA on total freezing during the test confirmed a significant main effect of Test Group ($F(1,27) = 27.14$, $p < 0.001$), and no effect of Sex ($p > 0.05$). Post hoc within-sex independent samples t-Tests confirmed that both the male and female experimental groups froze significantly more than the control groups of the same sex ($t(1,14) > 3.12$, $p < 0.01$, both).

Similar to results from Experiment 1, and in accordance with consumption data, rats in the control groups spent more time engaging in food cup behavior than rats in the experimental groups of the same sex (Male Control: 52.81 ± 7.73%; Male Experimental: 31.56 ± 10.37%; Female Control: 39.38 ± 6.50%; Female Experimental: 18.75 ± 5.72%). A two-way ANOVA on total percent of time engaging in food cup behavior during the test confirmed a significant main effect of Test Group ($F(1,27) = 6.98$, $p < 0.05$), and no effect of Sex ($p > 0.05$).

Since there were no sex differences in the expression of freezing or food cup behavior, data were collapsed across the sexes to visualize how rats divided their time during the test session (Fig. 3D).

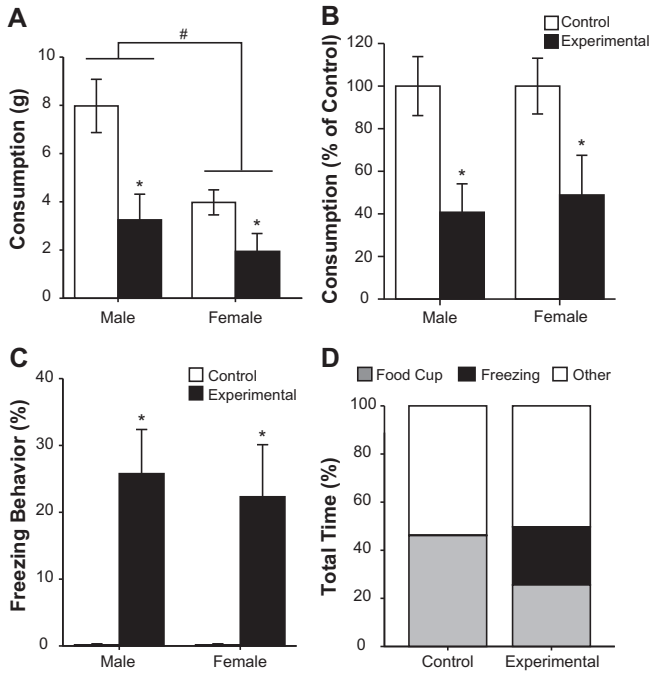

Fig. 3. Results from the final test in Experiment 2. (A) The amount of food pellets consumed (g) in the conditioned aversive context (mean ± SEM). (B) The amount of food pellets consumed expressed as the percent (mean ± SEM) of the consumption of the control group of the same sex. (C) Freezing behavior during the test; data show freezing-positive observations as the percent (mean ± SEM) of the total number of observations. (D) Representation of how rats divided their time during the food consumption test; data show the percent of time experimental and control groups (collapsed by sex) spent expressing food cup, freezing, or other behaviors during the test. *Indicates a significant difference ($p < 0.05$) between control and experimental groups of the same sex. #Indicates a significant difference ($p < 0.05$) between males and females.

Similar to the results from Experiment 1, the patterns of food cup and freezing behaviors suggest that the inhibition of feeding was not a consequence of immobilization due to freezing. Rats in the experimental groups spent only ~25% of their time freezing during the test, which left more than a sufficient amount of time to match the ~45% of the time that the rats in the control groups spent engaging in food cup behavior. Nevertheless, rats in the experimental groups did not match the amount of time spent exhibiting food cup behavior or the amount consumed by the control groups.

One female subject in the experimental group did not express any freezing behavior during the test, and this was interpreted as a failure to learn the context-shock association. Thus, the data from this subject were removed from all behavioral (freezing, food cup, and consumption) analyses at test.

Body weights and estrous cycle

Males weighed more than females throughout Experiment 2, but the weights of the experimental and control groups of the same sex were similar (Table 2). Two-way ANOVAs confirmed a significant difference in body weight between the sexes both prior to training and at test ($F(1,28) > 253.21$, $p < 0.001$, both), and no effect of Test Group ($p > 0.05$, both) at either time. Female rats of both groups were observed to be cycling normally throughout Experiment 2, and all stages of estrous were represented at test. Although not directly analyzed due to sample size, results suggest that estrous cycle stage did not affect female feeding or freezing behavior since behavioral data variability (as interpreted by the SEM) was no greater in females than in males. Thus, the experience of receiving footshocks during training did not produce changes in body weight or estrous cycling in the experimental groups compared to the control groups that did not receive any shocks.

Experiment 3

Rats were trained in a behavioral protocol with alternating neutral and aversive sessions that were conducted in distinctive behavioral chambers (Context A and Context B, respectively; see Materials and Methods for details). Rats were then tested for food consumption in each context under food-deprived conditions.

Food consumption tests

Males strongly inhibited intake in the aversive context compared to their intake in the neutral context, while females ate similar small amounts in both contexts (Fig. 4A). A repeated measures ANOVA revealed a significant within-subjects interaction of Context with Sex ($F(1,14) = 5.79$, $p < 0.05$). Post-hoc comparisons confirmed that males ate significantly less in the aversive context compared to the neutral context ($t(1,7) = 3.22$, $p < 0.05$), while females ate similar amounts in each context ($p > 0.05$). The amount consumed by females was similar to that of males in the aversive context ($p > 0.05$), and significantly less than the consumption of males in the neutral context ($t(1,14) = 4.38$, $p = 0.001$).

Following the initial food consumption tests all rats received one set of re-training sessions (one session in each context; the order counterbalanced), and were then tested again for consumption in each context (order counterbalanced across subjects). This was done for two reasons. One was to potentially help females in case they were deficient in discriminating between the contexts. The other was to assess whether there would be a generalization across the contexts due to aversive training experience. That is, the goal was to determine whether rats would decrease consumption indiscriminately after experiencing shocks, or whether they would specifically decrease consumption in the aversive context.

The re-training sessions did not change the consumption patterns observed during the original tests. During the re-tests both the males (neutral context: 6.45 ± 0.23 g, aversive context: 4.86 ± 0.94 g) and females (neutral context: 3.84 ± 0.53 g, aversive context: 3.51 ± 0.51 g) consumed amounts similar to those observed during the original tests. Males significantly inhibited intake in the aversive compared to the neutral context ($t(1,7) = 2.39$, $p < 0.05$), while females consumed similar amounts in both contexts ($p > 0.05$). Thus, the additional training did not help the females.

Importantly, there was no evidence of cross-context generalization. A direct comparison of consumption between the tests and re-tests using repeated-measures ANOVAs for Session (Test vs. Re-Test) revealed significant main effects of both Session and Sex ($F(1,14) > 7.80$, $p < 0.05$, both) in the neutral context; subjects consumed more in the neutral context following re-training, and as expected males consumed significantly more than females ($t(1,14) = 4.78$, $p = 0.001$). Consumption in the aversive context was statistically similar between the Test and Re-Test sessions ($p > 0.05$, all).

Freezing and food cup behaviors

At test, both the male and female rats exhibited much more freezing behavior in the aversive context compared to the neutral context (Fig. 4B). A repeated measures ANOVA on total percent of time freezing during the tests confirmed a significant main effect of Context ($F(1,14) = 41.13$, $p < 0.001$), and no effect of Sex ($p > 0.05$). Post hoc within-sex paired t-Tests confirmed that both males and females froze significantly more in the aversive than in the neutral context ($t(1,7) > 4.64$, $p < 0.01$, both).

Both sexes also exhibited a greater latency to approach the food cup in the aversive context (Males: 26.50 ± 10.4 s, Females: 34.38 ± 9.46 s) compared to their latency to approach the food cup in the neutral context (Males: 6.75 ± 3.16 s, Females: 7.88 ± 3.39 s). A repeated measures ANOVA on the latency to

Table 2
Body weights during Experiment 2. Data is presented as mean ± SEM grams.

	Prior to training (g)	At test (g)
Male Control	403.00 ± 8.76	481.75 ± 12.02
Male Experimental	403.75 ± 10.60	476.38 ± 14.32
Female Control	271.25 ± 6.50	286.50 ± 6.89
Female Experimental	271.38 ± 6.65	282.75 ± 7.74

approach the food cup during the tests confirmed a significant main effect of Context ($F(1,14) = 10.87$, $p < 0.01$), and no effect of Sex ($p > 0.05$). Post hoc within-sex paired t-Tests revealed that the latency difference between the two contexts was significant for females ($t(1,7) = 2.99$, $p < 0.05$), and approached significance for the males ($p = 0.11$).

In accordance with the consumption data, male rats spent more time engaging in food cup behavior in the neutral context (38.44 ± 5.19%) than in the aversive context (15.63 ± 6.75%), and this was more than the food cup behavior expressed by female rats in either context (neutral: 16.41 ± 3.25%, aversive: 22.19 ± 6.91%). A repeated measures ANOVA on the total percent of time engaging in food cup behavior during the tests revealed a significant within-subjects interaction of Context with Sex ($F(1,14) = 8.65$, $p < 0.05$). Post hoc comparisons confirmed that males exhibited significantly more food cup behavior in the neutral compared to the aversive context ($t(1,7) = 3.69$, $p < 0.01$), and also compared to food cup behavior of females in the neutral context ($t(1,14) = 3.60$, $p < 0.01$).

As shown on Fig. 4C, rats spent less than 50% of the total time expressing freezing behavior in the aversive context and less than 50% of the total time expressing food cup behavior in the neutral context. Thus, similar to the results from Experiments 1 and 2, there was a sufficient length of time (>50%) when rats were not immobilized due to freezing behavior to match the amount of food cup behavior expressed in the neutral context. Despite this, rats did not match the amount of time spent feeding in both contexts.

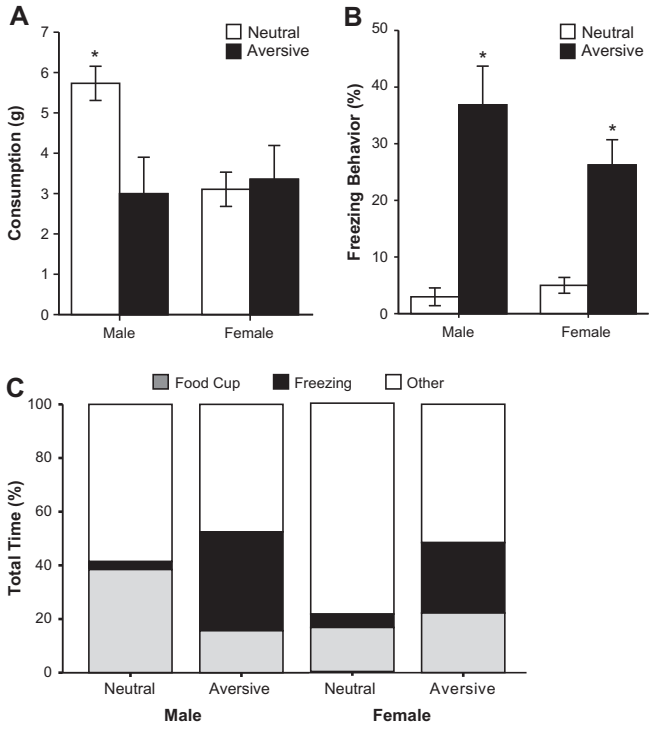

Fig. 4. Results from the tests in Experiment 3. (A) The amount of food pellets consumed (g) in the conditioned aversive and neutral contexts (mean ± SEM). (B) Freezing behavior in each of the two contexts; data show freezing-positive observations as the percent (mean ± SEM) of the total number of observations. (C) Representation of how rats divided their time during the food consumption tests; data show the percent of time males and females spent expressing freezing, food cup, and other behaviors in each of the two contexts. *Indicates a significant difference ($p < 0.05$) between control and experimental groups of the same sex.

Body weights and estrous cycle

Males weighed more than females at the start of training (Males: 466.38 ± 14.22 g, Females: 256.50 ± 3.26 g) and at test completion (Males: 468.86 ± 15.86 g, Females: 250.38 ± 3.81 g). Independent samples *t*-Tests confirmed a significant difference in body weight at both time points ($t(1,14) > 13.40$, $p < 0.001$, both). Female rats were observed to be cycling normally throughout Experiment 3, and all stages of estrous were represented at test. Although not directly analyzed due to sample size, results suggest that estrous cycle stage did not affect female feeding or freezing behavior since behavioral data variability (as interpreted by the SEM) was no greater in females than in males.

Discussion

Here we demonstrated in three experiments that conditioned aversive contexts can inhibit eating in food-deprived rats. Experiment 1 showed that following simple aversive context conditioning (pairing a distinct context with electric footshocks) food intake in the conditioned context was significantly inhibited in the experimental compared to the control groups. Since Experiment 1 used only one context, we were unable to definitively conclude that the effect was driven by conditioned contextual cues and not simply due to a generalized effect of prior experience with footshocks. Thus, to determine if this inhibition of food intake was specific to the conditioned context, in Experiments 2 and 3 we used context discrimination training protocols. In Experiment 2, training consisted of appetitive and aversive context training sessions which allowed us to monitor food consumption during training in a context that was never associated with footshocks. Here, we showed that regardless of experimental condition, rats of the same sex ate similar amounts in the appetitive context. At test, when subjects were re-exposed to the aversive context, rats in the experimental groups significantly inhibited food intake compared to the control groups despite being in an acute physiological hunger state. In Experiment 3, we used a within-subjects design to directly assess whether the inhibition is specific to the learned aversive contextual cues, or whether it is a general effect due to prior experience of shocks. Rats were trained to discriminate between a neutral and an aversive context and then tested for consumption in each context. Therefore, in this experiment all rats experienced shocks during training and were then tested in contexts that were novel in respect to food. As expected from Experiments 1 and 2, males showed a robust inhibition specific to the aversive context. Thus confirming that the inhibition of food intake upon re-exposure to the conditioned aversive context is due to the specific learned contextual cues and not simply due to a generalized effect of footshock experience.

In addition to monitoring food consumption during the tests, we also analyzed the expression of freezing behavior, a conditioned response often used to assess the extent of aversive conditioning (Bouton & Bolles, 1980; Fendt & Fanselow, 1999; LeDoux, 2000; Maren, 2001). Conditioned rats expressed significantly more freezing at test compared to no-shock controls in Experiments 1 and 2. Similarly, rats froze more in the aversive context compared to the neutral context in Experiment 3. This is in accordance with well-established prior work which has shown that subjects will exhibit robust freezing behavior when placed into a

context where they had previously received shocks, but will exhibit very low amounts of freezing when placed into a different context (Bolles & Collier, 1976; Fanselow, 1980; Fanselow, 1990).

In the current study, males and females expressed similar amounts of freezing behavior in all three experiments. This is in contrast to prior work which showed that females froze less than males following context-shock training (Maren, De Oca, & Fanselow, 1994). We hypothesize that differences in the number and intensity of the footshocks, as well as in the experimental design of the two studies contributed to differential results. Subjects in the current experiments received 8 or 4 footshocks of 1 mA intensity, while those in the prior study received 3 shocks of either 0.4 or 0.8 mA intensity. Indeed, in the prior study the amount of freezing increased with the intensity of the shocks, and the difference between males and females was slightly decreased with higher shock intensity (Maren et al., 1994). This is consistent with other prior work, which has shown that conditioned freezing to both discrete and contextual cues increases as shock intensity increases (Baldi, Lorenzini, & Bucherelli, 2004; Fanselow, 1980). Another important difference between the two studies is that subjects in the prior study were not pre-exposed to the training context, and received only one training session (Maren et al., 1994). It has been well-documented that pre-exposure to the context prior to training facilitates learning and later expression of conditioned contextual fear (Fanselow, 1990; Rudy, 1996; Rudy & O'Reilly, 1999; Rudy & Pugh, 1998). In the current study, subjects were habituated to the training context (Experiment 1), and had multiple training sessions (Experiments 1–3). It is likely that these procedural differences made learning the context-shock association easier in our paradigms (especially in Experiments 1 and 2), and thus males and females attained the context-shock association equally well. In agreement with the Maren et al. (1994) finding that females perform poorer in contextual learning paradigms, the experimental subject who did not exhibit any freezing in Experiment 2 was a female.

In addition to freezing behavior, in the current study we also analyzed food cup behavior. This food cup behavior measure provided an assessment of the time rats spent in close proximity to the food even if they were not eating. We then compared the total amount of time rats spent expressing each behavior during tests to examine whether the time spent immobilized prevented rats from dedicating time to feeding. This was important to address since immobilization associated with freezing prevents rats from simultaneously expressing any other behavior. Furthermore, prior work has shown that conditioned suppression of appetitive and consummatory behaviors—bar pressing for food and sucrose solution licking, respectively—correlate to the amount of freezing behavior in the presence of an aversively conditioned CS (Bouton & Bolles, 1980). Thus, we wanted to address whether behavioral response competition might be the reason for the inhibition of consumption in the current experiments.

In the experiments here we found that rats in the conditioned aversive context had a sufficient amount of time to move around and eat, but they did not. We found that the period of time conditioned rats were not immobile due to freezing was longer than the duration controls spent feeding (Experiments 1 and 2) or the duration of their own feeding behavior in a neutral context (Experiment 3). Therefore, freezing *per se* was not driving the suppression of consumption, rather freezing and feeding inhibition were both driven by the aversive contextual cues. Nevertheless, our experiments were designed to induce mid-range behaviors of interest (freezing and inhibition of feeding), and it is possible that at high levels of freezing some portion of the cessation of feeding is driven by response competition. Similarly, it is possible that at lower levels of fear, suppression of consumption may be a slightly more sensitive measure than freezing.

Previously using discrete cues, we also showed that CS-driven inhibition of feeding is not merely a consequence of immobilization due to conditioned freezing induced by the CS. Lesions of the periaqueductal gray or the basolateral nucleus of the amygdala abolished conditioned freezing to a discrete cue that signaled footshocks, but left inhibition of eating following that same cue intact (Petrovich, Ross, Holland, & Gallagher, 2006; ; Petrovich et al., 2009). Together, the current data using contextual CSs and our prior work with discrete CSs provide strong evidence that inhibition of feeding is not merely a consequence of immobilization due to conditioned freezing, rather freezing and feeding inhibition are separable behavioral responses driven by the same CS.

In the current study, there were no sex differences in the extent of inhibition of feeding in the first two experiments, but there was a pronounced sex difference in baseline consumption. Throughout these two experiments male rats ate more than female rats, which was in accordance with the difference in body weight between the sexes (Bull & Pitts, 1971). All rats were the same weight at arrival, however males gained weight at a much faster rate, as expected given the standard growth charts available from the supplier. Importantly, regardless of the baseline difference, in Experiments 1 and 2 the experimental groups of both sexes consumed significantly less than the controls of the same sex, and when the consumption data for these two experiments was normalized against the control groups we showed that the extent of the inhibition of food intake was similar between the sexes.

Interestingly, in contrast to the first two experiments during which males and females inhibited feeding similarly, we found a sex difference in Experiment 3. In that experiment, only males showed selective inhibition of feeding in the aversive context while females indiscriminately ate small amounts of food in both contexts. The freezing data suggested that females were able to discriminate between the two contexts. Yet, to further investigate the nature of this sex difference and to confirm females' ability to discriminate between the contexts we also assessed the latency to approach the food in Experiment 3. Here we found that all subjects (male and female) exhibited increased latencies to approach the food cup in the aversive context compared to their approach latencies in the neutral context. This is in agreement with a prior study where rats received footshocks after either approaching or consuming a cookie in a distinct training context (Gustavson & Gustavson, 1982). In accordance with our current findings, rats that had received footshocks displayed a strong context-specific increase in latency to approach a cookie; there was no increase in approach latency when rats were tested in familiar (homecage) or novel contexts (Gustavson & Gustavson, 1982).

Despite the consumption sex difference in Experiment 3, we found similar freezing and latency measures in both sexes. Thus, we conclude that females learned to discriminate between the two contexts as well as males. It is therefore unclear why they ate indiscriminately across the two contexts. The most parsimonious explanation is that the indiscriminate consumption reflects suppression of eating in both contexts, due to prior experience with shocks. That is, females, unlike males, might be inhibiting feeding in that setting due to a more generalized state of fear, likely akin to anxiety, that is below the threshold for freezing. Future work is needed to further characterize these sex differences. Nevertheless, this relates to our prior finding that females, unlike males, continued to inhibit feeding after they have extinguished freezing behavior (Petrovich & Lougee, 2011). Together with the current findings this suggests a dissociation of freezing- and feeding-driven behavioral responses to the same CSs.

These results illustrate the importance of comparing behavior in intact males and females as an important step in characterizing sex differences (Cahill, 2006; McCarthy et al., 2012). Most research is conducted in males, and our findings suggest that the results

might not always translate to the opposite sex. The current study also aimed to begin to remedy the gross underrepresentation of female subjects in both basic and clinical research (Zucker & Beery, 2010), which is paradoxical given that women have higher reported rates of anxiety and other psychiatric and eating disorders (McCarthy et al., 2012). Our study is also a precursor for future functional neuroanatomical investigations into the brain mechanisms underlying fear-induced inhibition of feeding.

Our findings of conditioned inhibition of food intake also relate to prior literature on conditioned lick suppression (e.g. Nageishi & Imada, 1974; Urcelay & Miller, 2010; Witnauer & Miller, 2012). In that paradigm water-deprived rats will cease licking a water spout when presented with cues previously paired with aversive events (i.e. footshocks). The primary feature of both models is the inhibition of a consummatory behavior due to learned cues despite strong physiological drives. Although the basic learning mechanisms of these two conditioned behaviors are likely similar, the hormones, peripheral targets, neuropeptides and brain circuits underlying fluid and energy balance are vastly different (Shin, Zheng, & Berthoud, 2009; Stricker, 2004; Swanson, 2000). Thus, how shared learning mechanisms might interact within these two distinct neural systems to overcome the physiological drives for thirst and hunger is a question for future research.

Our models of fear-induced eating cessation under conditions of acute food-deprivation may be useful tools in increasing our understanding of one facet of anorexia nervosa, an exceedingly complex and multifaceted disease. Indeed, intense fear of weight gain even though patients are underweight is a key symptom and a diagnostic criterion of anorexia nervosa (American Psychiatric Association, 2000; Attia, 2010; Kaye, 2008; Klein & Walsh, 2004; Treasure, Claudino, & Zucker, 2010). Furthermore, anorexia nervosa patients often maintain low body weight through restricted eating despite the presence of physiological hunger signals (Jimerson & Wolfe, 2006). Our models suggest that fear might aid that paradoxical behavior.

Indeed, links between anorexia nervosa and fear have been proposed (Strober, 2004), and pre-meal anxiety level is inversely correlated with food intake in anorexia nervosa patients (Walsh, 2011). Although the brain mechanisms underlying anorexia nervosa are exceedingly complex and not well understood (Watts & Salter, 2004), abnormalities in regions of the fear network have been reported in anorexia nervosa patients (Giordano et al., 2001; Seeger, Braus, Ruf, Goldberger, & Schmidt, 2002; Takano et al., 2001; Uher et al., 2004), and recruitment of these regions is correlated with increased anxiety in healthy young women without a history of disorders when viewing pictures of idealized female bodies (Friederich et al., 2007). Future studies that examine how contextual fear cues override hunger signals triggered by food-deprivation to inhibit feeding would greatly add to our understanding of this complex disease.

Contextual fear cues might also play a role in relapse of this disease. Inpatient treatment of anorexia nervosa is not uncommon, especially in severe cases. Unfortunately, relapse rates following treatment are often high (Attia, 2010; Eckert, Halmi, Marchi, Grove, & Crosby, 1995; Klein & Walsh, 2004), and it has been suggested that this may be attributed to mechanisms similar to those responsible for relapse following drug abuse treatment, including the role of contextual cues (Crombag, Bossert, Koya, & Shaham, 2008; Crombag & Shaham, 2002; O'Brien et al., 1992). Given our current findings, we hypothesize that contextual cues may play a role in relapse by eliciting activation of fearful memories, which in turn may activate physiologic fear responses that lead to a cessation of food intake (Cannon, 1915). Interestingly, it has been suggested that in humans, emotional states themselves may serve as contextual cues (Bouton & Swartzentruber, 1991). Nevertheless, whether contextual fear cues contribute to relapse following treatment remains to be determined.

In conclusion, we demonstrated in three experiments that conditioned aversive contextual cues can inhibit eating under physiological conditions where rats would normally consume a large amount of food. Our findings provide novel paradigms for future behavioral and brain analyses on the role of contextual learning in food intake. These models might also enhance our understanding of maladaptive consequences of environmental influences on the control of feeding, including mechanisms by which threatening and fearful environments may contribute to the maintenance of restricted eating in anorexia nervosa.

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