Sex Differences in Orexin Activation Patterns of Fear-Cue Induced Inhibition of Eating in Rats

Author: Jordan A. Newmark

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HP 299 Senior Arts & Sciences Honors Program Thesis

Jordan Newmark
Boston College
Advisor: Dr. Gorica Petrovich

Abstract

Inhibition of eating is an adaptive biological response to fear, however this response can become maladaptive when chronic, such as in eating disorders. We sought to understand the neurobiological basis for this phenomenon in which environmental cues override physiological cues to influence the behavioral control of feeding. To examine these mechanisms, we utilized an animal model for fear-cue induced inhibition of eating. After learning to associate a tone with foot-shocks, rats exhibited robust inhibition of eating when presented with the tone alone despite acute food deprivation. When examining sex differences between male and female rats, we observed that female rats that had received tone-shock pairings showed inhibition of eating across all test days compared to control females, whereas male rats that had received tone-shock pairings exhibited inhibition of eating the first test day compared to male controls; however, this inhibition was extinguished subsequent test days. In order to examine the neurobiological basis of these sex differences, we looked at activation of neurons that produce orexin (ORX) during the final test day. ORX is a neuropeptide that stimulates eating and is also involved in arousal, depending on the area it is expressed within the lateral hypothalamus (LHA). Data analysis revealed that female rats exhibited greater recruitment of ORX neurons in the LHA than male rats, and that no difference in ORX activation existed between control and experimental groups of either sex. Since ORX activation in the LHA typically results in increased eating, these results were unexpected but indicative that ORX does play a role in sex differences in fear-cue induced inhibition of eating and should be further investigated to provide more conclusive results that could possibly contribute information on the neurobiological origins of eating disorders.

Introduction

The behavioral implications for the role of fear in eating disorders such as anorexia nervosa have long been studied to better understand the etiology of the disorder, but only recently have researchers looked into its neurobiological effects. The DSM-IV focuses not only on the eating behavior patterns to characterize anorexia nervosa (AN) or bulimia nervosa (BN), but also on an intense fear of gaining weight and an obsession with food (Kaye, 2008). Fear has been shown to inhibit food intake as an adaptive mechanism to prepare an organism to respond to a threat, but such behavior can become maladaptive when applied to chronic stress (Cannon, 1915). Since AN and BN affect an overwhelmingly greater percentage of women over men (Kaye, 2008), this study seeks to explore the behavioral and neurobiological differences in responses to fear cue-induced inhibition of eating in male and female rats. Built upon a Pavlovian-conditioning paradigm in which rats were repeatedly subjected to an aversive, mild electric foot shock (unconditioned stimulus, US) preceded by a tone (conditioned stimulus, CS), this study observed the rats' fear-related behavioral responses (conditioned responses, CRs) as well as activation of eating and arousal-related orexin (ORX) neurons in the hypothalamus when they were later exposed to the CS.

The aversive Pavlovian conditioning is used to determine fear's affects on modulation of feeding. In previous studies using this behavioral paradigm, food-deprived rats displayed a robust decrease in food intake during tests following presentations of the tone (CS) that previously predicted a fearful, aversive cue, a foot-shock (US) ([Petrovich et al., 2009] and [Petrovich and Lougee, 2011]). The acquisition of the fear response (freezing, cessation of eating) requires only

minimal exposure with only four foot-shock presentations. It is a powerful stimulus capable of inhibiting eating even in food-deprived rats inclined to exhibit robust feeding under normal conditions.

Within this behavioral paradigm, all rats received appetitive training sessions in which food-deprived rats were exposed to food pellets and tested for food consumption. Male and female rats were divided into experimental and control groups for aversive conditioning in which both groups were exposed to the same number of tones (CSs) that immediately preceded foot-shocks in the experimental group, but were not followed by foot-shocks in the control group. The appetitive training sessions and control condition served as a basis for determining the extent to which the fear-cue (CS) inhibited eating in the experimental group for male and female rats.

In this study we examined the differences in fear-cue inhibition of feeding for males and females behaviorally and on a neuronal activation level as well. After testing, the rats' brains were stained and analyzed for c*fos* induction within ORX neurons in the hypothalamus. Melanin concentrating hormone (MCH) and ORX are two neuropeptides activated by neuropeptide Y (NPY) in separate but spatially overlapping areas of the lateral hypothalamus, perifornical region and zona incerta in the rodent and human brain ([Elmquist et al., 1999] and [Woods, 2005]). While MCH neurons are activated in response to physiological hunger, the dual nature of ORX neurons involved in arousal and stimulation of eating indicate they are activated in response to metabolic state (Elmquist et al., 1999). ORX neurons projecting to a variety of brain areas (medial septal area, medial preoptic area, substantia innominata, and locus coeruleus) have been shown via cFos immunoreactivity to be activated in response to various stressors (Berridge et al., 2010). ORX has been shown to produce a stress-like activation of catecholamines (Berridge et al., 2010). ORX fibers are in close proximity to corticotropin-releasing factor (CRF) neurons in the paraventricular nucleus of the hypothalamus (PVN) and the central nucleus of the amygdala (CeA) and have been shown to increase CRF release in the PVN as well as plasma levels of corticosterone and adrenocorticotropin hormone (ACTH), (Berridge et al, 2010). These findings indicate that ORX activates stress response systems and induces stress-like behaviors. Since ORX neurons are found near the CeA, the stress-response activation hypothesis as one function of ORX coincides with evidence that the control of eating by an aversive cue depends on the CeA (Petrovich et al., 2009), while enhancement of eating by an appetitive cue is dependent on the basolateral amygdala (Holland et al., 2002). Additionally, there is evidence of a functional separation of ORX neurons, with ORX neurons in the dorsomedial/perifornical regions of the hypothalamus related to arousal and stress, and lateral hypothalamus (LH) ORX neurons involved in appetitive and reward-seeking behavior ([Berridge et al, 2010] and [Harris and Aston-Jones, 2006]).

ORX activation induces either arousal or stress-related behavior or appetitive/reward-seeking behavior dependent upon the brain area in which the neurons are located, but these behaviors can also differ with gender. Female rats in proestrus and diestrus show rapider expression of CRF mRNA in the PVN and both rapider and higher secretion of plasma ACTH and corticosterone than do males when subjected to a foot-shock (Iwasaki-Sekino et al., 2009). In addition, there is a significant increase in plasma ACTH and corticosterone and CRF mRNA expression in females while experiencing psychological stress, while none of these levels increase in males, indicating that hypothalamic pituitary adrenal (HPA) axis response to stress depends on gender

and the type of stress (Iwasaki-Sekino et al., 2009). These findings correlate with the fact that estrogen stimulates the transcription of the human CRF gene and the rat CRF gene in the amygdala and mRNA expression in the CeA and PVN (Iwasaki-Sekino et al., 2009).

Previous studies implicate ORX in increased stress response in females based on estrogen's stimulation of ACTH and corticosterone levels and CRF mRNA expression in the hypothalamus and amygdala. Correlates of ORX activation and CRF, ACTH and corticosterone levels further suggest orexin's role in anxiety and the stress response. Through immunoreceptive staining of cFos-activated ORX neurons in the hypothalamus, the current study seeks to determine whether orexin is related to sex differences in behavioral responses to fear, especially on feeding.

Materials and Methods

Adapted from: Sex differences in fear-induced feeding cessation: Prolonged effect in female rats Petrovich and Lougee, 2011

Subjects

Sixteen male and sixteen female experimentally naïve Long-Evans rats (Charles River Laboratories; Portage, MI) were used in the experiment. The rats arrived to the laboratory with body weights ranging from 226-250g for both males and females. Rats were individually caged, maintained on a 12 hour light/dark cycle, and given *ad libitum* access to food and water, unless otherwise noted. Male and female rats were housed in separate housing rooms. Rats were allotted one week for acclimation to the vivarium and to handling before the initiation of behavioral training. Body weights and vaginal smears were obtained every weekday, the latter performed to ascertain that females were cycling normally. All animal 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.

Vaginal Smears

After the initial week of acclimation and handling, female rats were examined by a vaginal lavage procedure each weekday to determine estrous cycle stage. For each female rat, a small sample of water was flushed into and extracted from the vagina and placed on a glass slide. Cell types were examined under a microscope to determine the stage in the estrous cycle and ensure that female rats were cycling normally. Due to a small sample size, the estrous stage was not used as a variable in the analysis, but it did determine that none of the female rats needed to be removed from the experiment due to abnormal estrous cycling as a result of extreme stress.

Apparatus

Training and testing were conducted in a set of eight behavioral chambers (30 x 28 x 30 cm; Coulbourn Instruments, Allentown, PA) located in a behavioral testing room separate and different from the colony housing rooms. Each chamber was encased by a cubicle (79 x 53 x 53 cm; Coulbourn Instruments, Allentown, PA) consisting of monolithic rigid foam walls that isolate the chambers from ambient sound and light. A ventilation fan, placed on the back of each isolation cubicle, served to provide masking noise at 55 dB. Video cameras attached to a recording system (Coulbourn Instruments, Allentown, PA) were situated on the back of the isolation cubicle to record behavior during training and testing.

Olfactory, visual, and tactile features in the chambers were altered to provide two distinct environments: Context A and Context B, respectively. All appetitive sessions and tests were conducted in Context A, whereas aversive training sessions were conducted in Context B.

For Context A, each chamber consisted of aluminum top and sides, a transparent Plexiglas back and front, and a black Plexiglas panel placed over the grid floor to prevent rats from seeing or feeling the grids. Each chamber contained a recessed food cup (3.2 x 4.2 cm) and a "house light" (4 W light) illuminated the chamber. To create a distinct olfactory environment, 1% Acetic Acid (Fisher Scientific, Hanover Park, Illinois) was wiped over the inside of the chambers prior to placing the animal inside.

For Context B, each chamber contained two black Plexiglas sheets positioned to conceal the aluminum sides and angled to create a tent-like enclosure, while the grid floors remained uncovered. The "house light" was turned off during aversive sessions and instead the room lights served as illumination for the chambers. To create an olfactory environment distinct from Context A, 5% ammonium hydroxide (v/v, 28'30% stock, Acros Organics; Somerville, NJ) was wiped over the inside of the chambers prior to placing the animal inside for training.

Behavioral Training Procedure

The training protocol consisted of 9 sessions, of which 6 were appetitive sessions conducted in Context A (S1, S2, S4, S6, S8, S9) and 3 were aversive sessions conducted in Context B (S3, S5, S7). Prior to each training session, rats were transported from the housing room to the behavioral testing room in home cages placed on a cart. One day before the commencement of the behavioral protocol, rats were given about 1 g of the food pellets used in training and testing (formula 5TUL, 45 mg, TestDiet, Richmond, IN) in their home cages to acquaint them with the pellets.

Rats were deprived of food (lab chow) 22 hours prior to each appetitive session, though water remained available in the home cages *ad libitum*. During each session, rats were placed in Context A of the behavioral chambers with 7 g of TestDiet food pellets in the recessed food cup, and were allowed to eat for 10 minutes. Immediately after 10 minutes, rats were removed from the chambers, placed in the home cages and transported back to their respective housing rooms. Food remaining in the food cup was removed, weighed and recorded.

Between training sessions, rats were allowed *ad libitium* access to food (lab chow) and water, ensuring a time lapse of at least 24 hours between consecutive food-deprivations or before the start of aversive sessions.

During aversive training sessions, the half of the male and female rats in the Conditioned groups received tone-shock pairings, while the other half of the rats in the Control groups received tone presentations in the absence of shocks. For at least 24 hours prior to each aversive training session, rats were permitted *ad libitum* access to food (lab chow) and water. Each of the three aversive training sessions was 10 minutes long, conducted on separate days at least 24 hours after the last appetitive training session. In the first aversive session (S3), rats were merely placed in the chamber for 10 minutes to habituate them to the new training Context B. In the following two aversive training sessions (S5, S7), rats in the Conditioned groups were subjected

to 2 tone (75 dB; 2 kHz, 60 s) presentations at 3 minutes and 8.5 minutes for S5 and 1 minute and 8 minutes for S7; each tone was immediately followed by a 1mA electric foot-shock lasting 1 second (Precision Adjustable Shocker, Coulbourn Instruments, Allentown, PA). Rats in the Control groups were subjected to identical presentations of the tone with no shocks.

Food Consumption Tests

Rats were tested for fear-cue inhibition of eating in three tests conducted on different days. Prior to each test, rats were food-deprived for 22 hours, with *ad libitum* access to water. Prior to each testing session, rats were transported from the housing room to the behavioral testing room in home cages placed on a cart. For each test, rats were placed in Context A of the behavioral chambers with 7 g of TestDiet food pellets in the food cup and allowed to consume food for 10 minutes. During this time, the tone CS (75 dB; 2 kHz, 60 s) that was presented in the aversive training sessions sounded four times, starting at 1, 3, 5, and 7 minutes. After the test, rats were removed from the behavioral chambers, placed in the home cages and transported back to their respective housing rooms. Food remaining in the food cup was removed, weighed and recorded. Due to a fire alarm during testing, 14 rats (of about an equal number from each group) were removed from the study.

Behavioral Observations

Freezing behavior is a species-typical defense response indicative of fear, characterized by an immobile crouching posture lacking all movement except for those motions required for breathing (Fanselow, 1984). In order to determine the extent of the fear response, freezing was assessed for each rat during the video-taped food consumption tests. A metronome set at 1 beat/1.25 seconds enabled observations to be made every 1.25 seconds during the full 60 second period of each CS. The observers were "blind" as to the training group and sex of each animal observed. The 4 minutes that constitute the sum of all CS presentations represent the "total time" in the text. The percentage of this time that rats spent freezing was calculated.

Perfusions and Tissue Slicing

The rats were sacrificed 90 minutes after the commencement of the final testing session. Each rat was briefly anesthetized with exposure to isoflurane in a closed container, then euthanized by rapid anesthesia with an intraperitoneal injection of tribromoethanol (375 mg/kg). After ensuring that the rats were fully anesthetized, rats were transcardially perfused with a 0.9% isotonic saline solution followed by 400 mL of 4% paraformaldehyde in 0.01 M borate buffer solution. Following the perfusion of paraformaldehyde, rats' heads were severed and the brains extracted and stored in a 12% sucrose solution for 18-24 hours at 4°C. The brains were then blocked, rapidly frozen in hexanes cooled in dry ice, and stored at -80°C.

When ready for processing, the frozen brains were mounted upon a microtome stage with frozen KPBS solution and the tissue preserved at steady cold temperature with dry ice. The brains were sliced into 30 μm coronal segments using a Leica SM200R sliding microtome and placed into either a 0.02 M potassium phosphate-buffered saline (KPBS) or cryoprotectant (0.025 M sodium phosphate buffer with 30% ethylene glycol and 20% glycerol) solution. The tissue from each brain was collected into four series: one tray to be labeled for Nissl bodies, one tray to be double-labeled for c*fos* induction of orexin neurons, and two trays stored for future analysis. The tissue placed into the three first trays were stored in KPBS solution at 4°C, while the tissue placed into

one tray with cryoprotectant solution was stored at -20°C. Since every fourth slice was placed in the same tray, this slicing procedure allows for adjacent brain slices to be labeled with different stains for comparison purposes.

Histological Procedures

Sliced tissue to be processed for ORX/cFos double-labeling was placed in blocking solution (KPBS containing 0.3% Triton X-100 and 2% normal horse serum) to block nonspecific antibody binding, then in blocking solution containing 1:2000 anti-orexin-A primary antibody raised in goat and stored at 4°C to allow the primary antibody to bind. After 72 hours, the tissue was treated with anti-goat secondary antibody raised in horse, rinsed, bound with avidin-biotin complex (ABC) to amplify the diaminobenzidine (DAB) staining, then promptly stained with DAB. This allowed for the visualization of ORX neurons. This process was repeated, with the exception that the tissue was placed in 1:5000 anti-cFos primary antibody raised in rabbit, normal goat serum was used, and secondary antibody was anti-rabbit secondary antibody raised in goat. Additionally, the DAB staining also included nickel to allow a visual dissociation between ORX-positive and cFos-positive staining.

The presence of cFos protein is correlated with neuronal activity (Sagar et al., 1988). After treatment with antibodies, the DAB that attaches to and labels the antibodies allows for visualization of the cFos protein within each neuron. The DAB-stained tissue labeled for cFos specifies which neurons displayed high activity prior to perfusion. When tissue is double-labeled for ORX neurons and c*fos* induction, any cells labeled as ORX neurons that also display the labeled cFos protein can be determined to have been active prior to perfusion.

After being stained for c*fos* induction of ORX neurons, each brain tissue slice was mounted in anatomical order onto microscope slides. After drying at room temperature for at least one day and further drying overnight in a 45°C oven, the ORX/cFos double-labeled tissue was dehydrated in a series of washes in ethanol of increasing concentration. After extracting the fat from the tissue in a series of washes in xylenes, the slides were coverslipped with DPX to preserve the tissue.

Sliced tissue to be processed for Nissl bodies was first mounted on gelatin-subbed microscope slides and then dehydrated in a series of washes in ethanol of increasing concentration. After extracting the fat from the tissue in a series of washes in xylenes, the tissue was then rehydrated, and stained with thionin. Following stain differentiation under the microscope to determine if the Nissl-stained cells could be observed as distinct from the neural fibers, the tissue was dehydrated and washed in xylenes prior to coverslipping to preserve the tissue on the slides. The thionin interacts with nucleic acids, staining DNA and RNA in nuclei and RNA in cytoplasm, the latter of which is useful in differentiating cells from the surrounding material (Simmons and Swanson, 1993).

Tissue Analysis

Once all Nissl and DAB-stained tissue was prepared and coverslipped, the Nissl stain was used to identify specific brain regions based on Swanson's rat brain atlas (Swanson, 2004), to which the corresponding slice of ORX/cFos double-labeled could be compared. Nissl-stained tissue

served as an anatomical guide to localize the brain regions of interest and identify labeled neurons of stained tissue to reveal the extent of activation of specific brain regions.

Nissl-stained tissue was aligned with its corresponding ORX/cFos double-labeled slice to find the anatomical levels 28 and 29 (Swanson, 2004) of interest for viewing ORX neurons. An Olympus BX51 microscope with DP-72 camera was used to take 20X magnified bilateral digital images of the double-labeled tissue at the best available representation of anatomical levels 28 and 29. Three different locations within the LHA were imaged using imaging software DP2-BSW: lateral to the fornix (lateral), medial to the fornix (medial), and directly above the fornix (perifornical) (see Fig. 1). One image of the lateral LHA and one image of the medial and perifornical LHA were taken at each level.

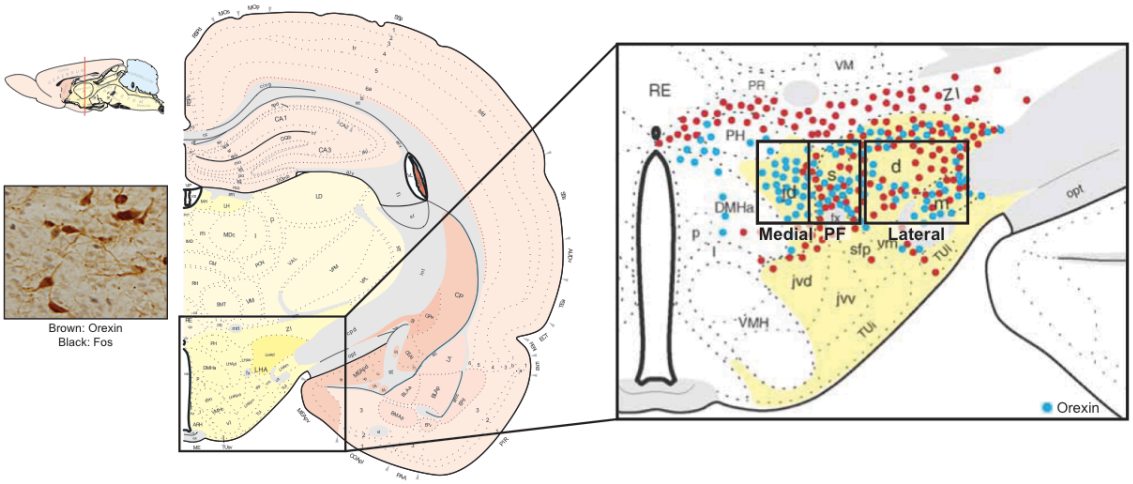

Fig. 1. A saggital (top left) and coronal (center) view of anatomical levels 28 and 29 (Swanson, 2004) of the rat brain. An enlarged view of the hypothalamus (right) outlines the lateral, medial, and perifornical regions of the lateral hypothalamic area and depicts the location of orexin neurons (blue) and melanin-concentrating hormone neurons (red) in each of these regions. A photograph of orexin neurons and cFos-activated orexin neurons in the LHA viewed through a microscope is shown on the left.

A researcher "blind" to the training group each rat brain belonged to counted the number of ORX and cFos-labeled ORX neurons in each region for levels 28 and 29 of each brain. The percentage of ORX neurons that were activated was calculated for each region across both levels according to the formula: (number of cFos/ORX double-labeled neurons divided by number of ORX neurons) x 100. The total percentage of ORX neurons activated across the three regions combined was also calculated using the above formula.

Statistical Analysis

Two-way ANOVAs with sex and condition as factors were used to analyze consumption during appetitive training sessions. Repeated measures ANOVAs with sex and condition as between-subjects factors were used for test consumption and freezing behavior across the three test sessions. Hypothalamic activation data were analyzed using a two-way ANOVA with sex and condition as factors. ANOVAs were followed by within-sex independent samples t-Tests when applicable. For all tests, $p < 0.05$ was considered significant.

Results

Rats were trained in a behavioral protocol in which alternating appetitive and aversive sessions were conducted in behavioral chambers of explicitly different contexts A and B respectively (see Materials and Methods for details). Appetitive sessions granted food-deprived rats free access to TD food pellets, while aversive sessions presented the male and female rats in the conditioned groups with tone-shock pairings and the male and female rats in the control groups with equal numbers of tone presentations without shocks.

Behavioral Training

Rats of both sexes in both the conditioned and control groups ate a considerable amount of food during appetitive training sessions. Males consumed more food overall than females, which is in concordance with their higher body weight. Males weighed the same as females upon arrival to the facility, but gained weight at a much faster rate so that they weighed more than females by the start of training. Rats of the same sex in the conditioned and control groups maintained similar body weights throughout the experiment. For the majority of the appetitive sessions, same sex members of the conditioned and control groups consumed similar amounts of pellets during training, indicating that the foot-shocks received during aversive training did not elicit change in food consumption or body weight between these groups. However, conditioned rats displayed some decrease in food consumed for appetitive training days 5 and 6 but not for the first four days, indicating conditioned rats were exhibiting influences of the aversive training only after all aversive sessions had been performed. Statistical analysis supported these observations.

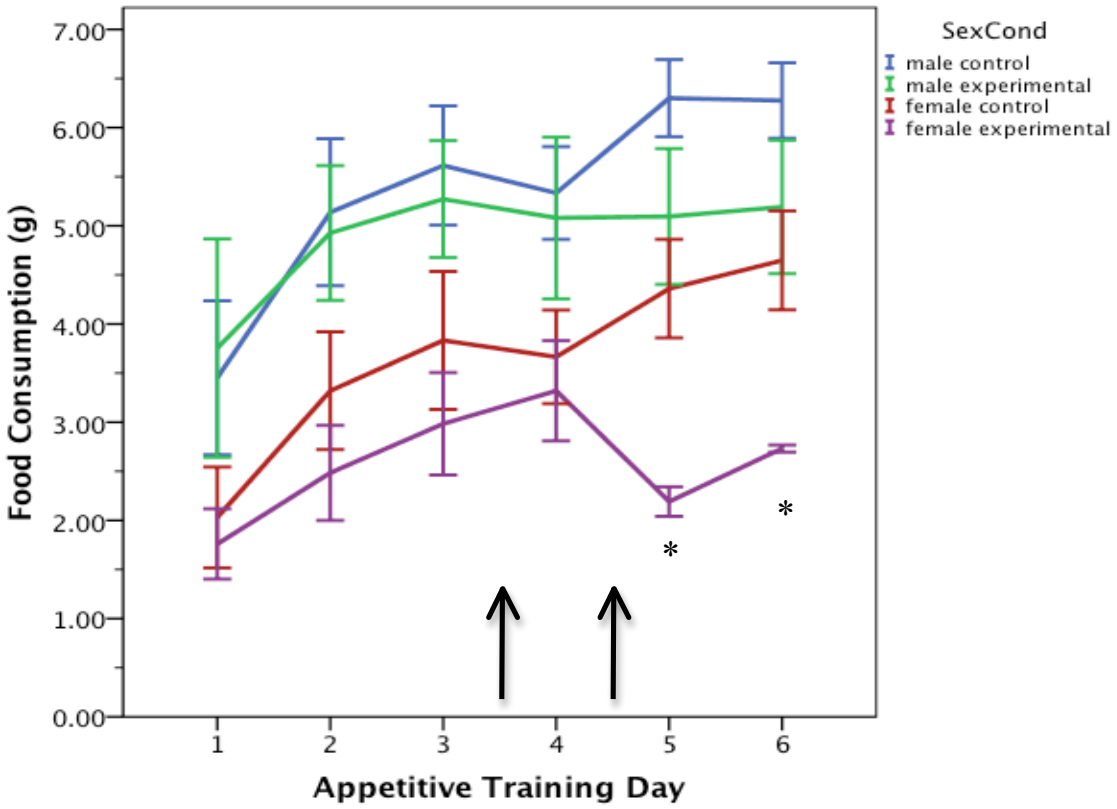

Fig. 2. Food consumption (mean ± SEM) during appetitive training sessions conducted in Context A. Asterisks indicate a significant effect of condition for female rats on those training days. Arrows indicate aversive sessions during which rats in the conditioned groups received small foot-shocks paired with a tone in Context B while rats in the control groups received tones but no shocks. These sessions were conducted between appetitive training sessions 3 and 4, and 4 and 5.

Performing two-way ANOVAs of food consumed for each appetitive training day with sex (male or female) and training condition (tone-shock or tone-only) as factors revealed a significant main effect of sex ($F(1, 18) > 5.531$, $p < 0.05$, all) for each session. There was a significant effect of condition for appetitive training days 5 ($F(1,18) = 14.404$, $p < 0.05$) and 6 ($F(1, 18) = 12.105$, $p < 0.05$), but not for the previous four days ($p > 0.05$, all). There was no interaction of sex by condition for any training day ($p > 0.05$, all). Post-hoc within-sex independent samples t-Tests showed no effect of condition for the males on any of the appetitive training days ($p > 0.05$, all), but revealed a significant effect of condition on appetitive training days 5 ($t(1, 7) = 4.595$, $p < 0.05$) and 6 ($t(1, 7) = 4.313$, $p < 0.05$) for females; no significant effect of condition existed for the prior appetitive training days for females ($p > 0.05$, all).

Two-way ANOVA analyses of rat body weights with sex and training condition as factors showed that there was a significant main effect of sex on body weights throughout the experiment ($F(1,18) > 129.101$, $p < 0.001$, all). Male rats weighed significantly more than female rats immediately prior to training (male control: 311 ± 6 g; male conditioned 306 ± 14 g; female control: 242 ± 6 g; female conditioned: 247 ± 17 g), at the end of training (male control: 462 ± 24 g; male conditioned 439 ± 42 g; female control: 274 ± 15 g; female conditioned: 279 ± 27 g), and at the end of testing (male control: 478 ± 21 g; male conditioned 441 ± 48 g; female control: 256 ± 29 g; female conditioned: 277 ± 27 g). This analysis showed no effects of training condition on body weights ($p > 0.05$, all).

Food Consumption Tests

Following completion of all appetitive and aversive training sessions, rats were subjected to three identical food consumption tests that occurred on different days. For each of these test sessions, food-deprived rats were supplied with food pellets in the appetitive context (Context A) and presented with four tones (CS) identical to those that had been presented in the aversive context (Context B). By testing in Context A, we isolated the effects of the tone and avoided the confounding effects of the context. Presentation of the CS during the test enabled rats to recall the events previously associated with the tone in the aversive context: shock or no shock. The amounts consumed by each group on each test day are shown in Fig. 3.

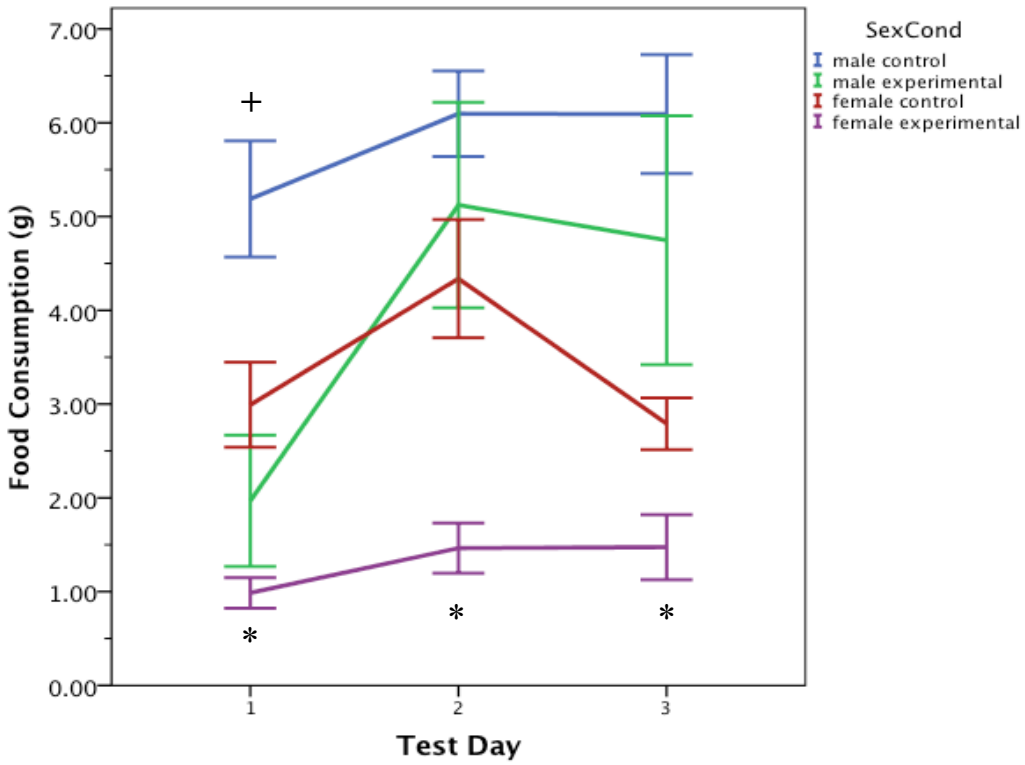

Fig. 3. Food consumption in grams (mean ± SEM) during tests with tone (CS) presentations in Context A. A plus sign indicates a significant effect of condition for male rats on that test day while asterisks indicate a significant effect of condition for female rats on that test day.

During the first test, both male and female conditioned rats demonstrated CS-driven inhibition of eating; rats that had previously received tone-shock pairings in the aversive context (Context B) consumed significantly less food than control rats of the same sex that had received only the tone in the aversive context (Test Day 1, Fig. 3). During the second and third tests, female rats in the conditioned group continued to exhibit CS-driven inhibition of eating while male rats in the conditioned group ate similar amounts to male rats in the control group. A repeated measures ANOVA comparing consumption differences across the test days with sex (male or female) and conditioning (tone-shock or shock-only training) as factors revealed a statistically significant within-subjects main effect of Test Day ($F(1, 18) = 29.619$, $p < 0.05$) and significant interactions of Test Day with both sex ($F(1, 18) = 9.810$, $p < 0.05$) and condition ($F(1, 18) = 5{,}422$, $p < 0.05$). Importantly, there was also a statistically significant three-way interaction of Test Day, sex, and conditioning ($F(1, 18) = 8.118$, $p < 0.05$), indicating that the individual groups displayed different patterns of food consumption over the test days. Additionally, there were statistically significant between-subjects main effects of both sex ($F(1, 18) = 18.544$, $p < 0.05$) and conditioning ($F(1, 18) = 11.097$, $p < 0.05$), but no interaction of sex by conditioning ($p > 0.05$). These results confirm that rats in the conditioned groups consumed more food than rats in the control groups regardless of sex, and that male rats ate more than female rats regardless of condition.

Post-hoc within-sex independent samples t-Tests showed that male rats displayed a significant effect of condition on Test Day 1 ($t(1, 7) = 3.449$, $p < 0.05$), but no effect of condition on

subsequent test days ($p > 0.05$, both); female rats, however, displayed a significant effect of condition on all test days ($p < 0.05$, all). The results of the first test show that male conditioned rats ate significantly less than male control rats and female conditioned rats ate significantly less than female control rats. In contrast, female conditioned rats ate significantly less than female control rats for all test days while male conditioned rats ate similar amounts to male control rats for the last two test days.

Freezing Behavior

In addition to analyzing food consumption as a measure of fear, we analyzed the occurrence of freezing, a species-specific defensive behavior associated with fear as a conditioned response during the tests. During the first test male and female rats in the conditioned groups that had received tone-shock pairings exhibited abundant freezing behavior, while rats in the control groups exhibited no significant freezing during any of the tests (Fig. 3). None of the rats in any of the groups demonstrated freezing behavior prior to administration of the first CS tone on the first test day. The display of freezing behavior on the first test day concurs with food consumption data from the first test day, indicating male and female rats developed similar abilities to learn CS-US relationships.

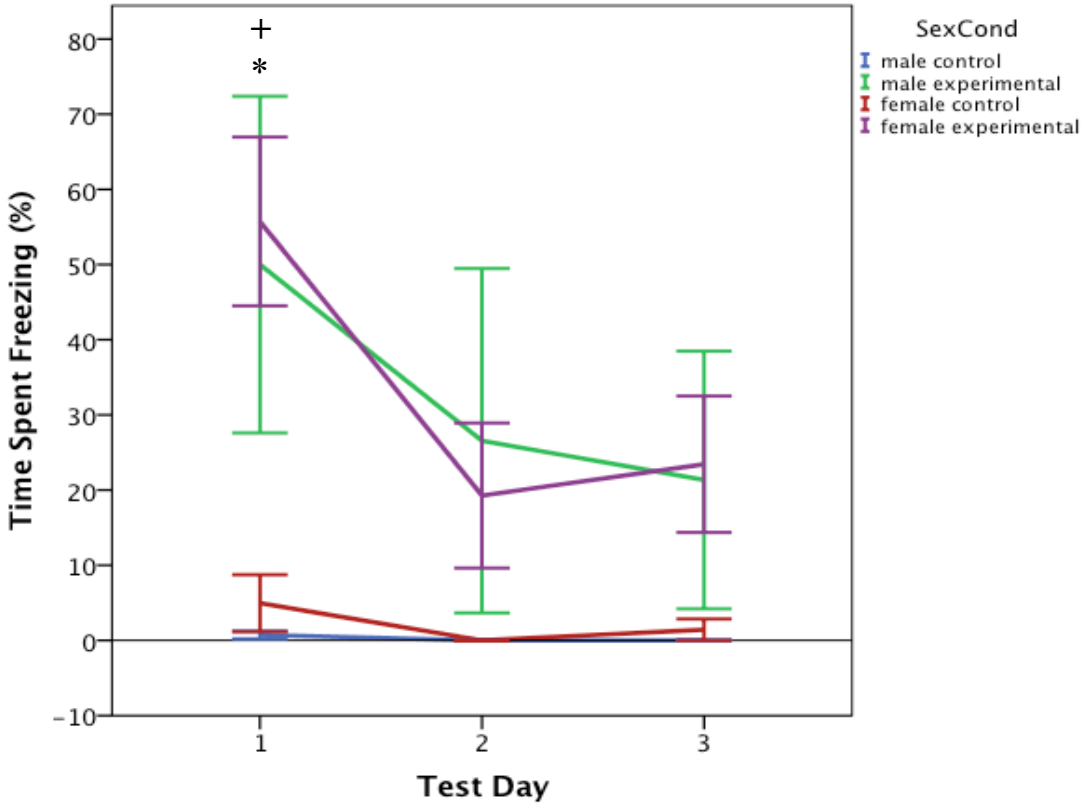

Fig. 4. Conditioned freezing behavior (mean ± SEM) during food consumption tests. Graph illustrates percentages of time spent freezing during CS presentations within each test. Observations of freezing behavior analyzed during CS periods (see behavioral observations of Materials and Methods for details). A plus sign indicates a significant effect of condition for male rats on that test day while asterisks indicate a significant effect of condition for female rats on that test day.

A repeated measures ANOVA comparing freezing behavior across the test days with sex (male or female) and conditioning (tone-shock or shock-only training) as factors revealed a statistically significant within-subjects main effect of Test Day ($F(1, 18) = 13.752$, $p < 0.05$) and a significant interaction of Test Day with condition ($F(1, 18) = 9.909$, $p < 0.05$), but not with sex or sex by conditioning ($p > 0.05$, both). The between-subjects main effect of condition ($F(1, 18) = 9.852$, $p < 0.05$), but not of sex or an interaction of sex by conditioning ($p > 0.05$, both) confirms that freezing behavior differed by Test Day and that conditioned rats froze more often than control rats regardless of sex. This analysis demonstrates changes over test days within each group on the basis of conditioning, which is consistent with extinction as shown in Fig. 4.

Post-hoc within-sex independent samples t-Tests showed that male rats displayed a significant effect of condition on Test Day 1 ($t(1, 7) = -2.503$, $p < 0.05$), but no effect of condition on following test days ($p > 0.05$, both); female rats also displayed a significant effect of condition on Test Day 1 ($t(1, 7) = -3.860$, $p < 0.05$) and no effect of condition on Test Days 2 and 3 ($p > 0.05$, both). These results determined that male and female rats in the conditioned groups displayed similar freezing patterns and froze far more than male and female controls. There were no significant differences in freezing time between male and female rats in the conditioned group nor between male and female rats in the control groups.

Orexin Activation in Lateral Hypothalamus

Subsequent to perfusion following the last testing session, rat brains were sliced, double stained for identification of cFos induction in ORX neurons, mounted, coverslipped, and examined microscopically. The anatomical location of the LHA was determined by comparison to adjacent sections that were stained with thionin. Swanson atlas levels 28 and 29 of the LHA, those with the highest concentrations of ORX neurons (Swanson et al., 2005), were examined and photographed. ORX neurons and activated cFos-labeled ORX neurons were counted in the lateral, medial, and perifornical (PF) regions of the LHA at these levels (see Fig. 1). From this data the percentage of activated ORX neurons in each LHA region of each brain was calculated. The data from the three regions was also collapsed to calculate the total percent of recruited ORX neurons in each brain.

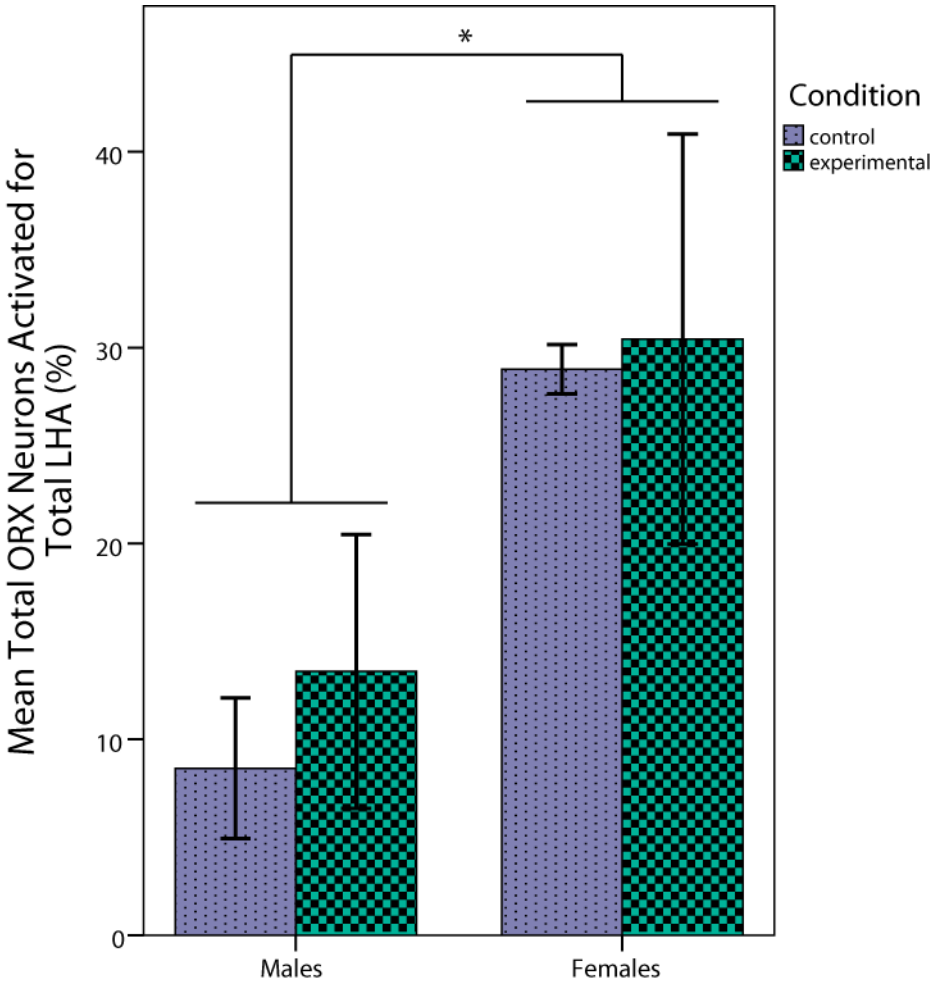

Fig. 5. Percentage of orexin neurons (mean ± SEM) activated in total combined lateral, medial, and perifornical regions for Swanson atlas levels 28 and 29 in male and female rats in control and conditioned groups. Asterisk indicates significant difference between sexes, but not condition for either sex.

A two-way ANOVA analysis of the percentage of activated ORX neurons in the lateral LHA with sex (male or female) and condition (tone-shock or shock-only training) as factors revealed a significant main effect of sex ($F(1, 18) = 6.23$, $p < 0.05$). Similar results were obtained following ANOVA analyses of the medial region of the LHA, with a significant main effect of sex ($F(1, 18) = 5.25$, $p < 0.05$), and also for the PF region of the LHA ($F(1, 18) = 7.672$, $p < 0.05$). There was neither a main effect of condition nor an interaction of sex by condition for any of the three regions ($p > 0.05$, all). Since the statistical results were similar for all three regions of the LHA, the data was collapsed into an analysis of the total LHA to reveal a significant effect of sex ($F(1, 18) = 7.10$, $p < 0.05$) and no significant effect of condition or sex by condition ($p > 0.05$). Females exhibited a greater percentage of ORX neuron activation in the LHA than males irrespective of condition (Fig. 4).

Although there was a significant difference between sexes for ORX activation, a within-sex post-hoc t-Test comparison confirmed that ORX activation in control and conditioned groups of each sex was similar for each LHA region and the total LHA measure ($p > 0.05$, all).

Discussion

This study sought to examine the neurobiological basis of female rats exhibiting prolonged fear-cue inhibition of eating compared to males that showed much faster extinction of such behavior. We used a recently developed behavioral paradigm (Petrovich et al., 2009; Petrovich & Lougee, 2011) involving acquisition of a fear response to the presentation of a tone (CS) followed by a foot shock (US). Rats were tested in an appetitive context in which presentation of the CS elicited inhibition of eating. Both male and female rats acquired conditioned fear to the CS during training similarly, as shown by both sexes exhibiting similar fear responses in the form of freezing behavior and inhibition of eating on the first day of testing with the tone presented in the appetitive context. There were no sex differences in the extinction of freezing behavior in conditioned rats. However, differences among the sexes emerged over the following two test days when male rats showed extinction of CS-induced inhibition of eating while female rats continued to express mild fear in the form of inhibition of eating for all three test days.
After analyzing these sex differences in inhibition of eating and extinction of fear, we examined the LHA for differences in activation of ORX neurons between the different sexes and conditions (experimental or control). We found that the lateral, medial, and perifornical regions of the LHA all displayed similar patterns of ORX activation when analyzed by sex and condition. We also discovered that there was no significant difference in ORX activation between the different training conditions for either sex, but that females displayed significantly greater ORX activation for both conditions than males for both conditions.

Based on the behavioral results we obtained with conditioned rats of both sexes exhibiting fear-cue inhibition of eating relative to controls, the lack of significant difference in ORX activation patterns between conditions for either sex deserves further exploration. The brains of the male and female rats that were analyzed for this experiment were from those rats perfused after Test Day 3, by which time male conditioned rats had already exhibited extinction of inhibition of eating, but females had not. Therefore, no significant difference in ORX activation was expected between male rats of the conditioned and control groups, but since a behavioral difference still existed on that day between female conditioned and control rats, some difference in ORX activation between the conditions was expected.

The similarities in ORX activation in the LHA for the different conditions of the female rats could possibly be explained by the different roles of ORX. ORX is involved not only in eating and reward, but also in stress, anxiety and arousal (Harris, 2006). It is possible that the control females could be activating the ORX neurons involved in feeding and reward, which is consistent with their feeding behavior during testing. Increased ORX activation in the LHA of sated rats corresponded to increased eating in response to a cue previously paired with food (Petrovich et al., 2012), so the heightened activation of ORX neurons in female control rats could be in response to increased eating in the environmental cue of testing in the appetitive context. In contrast to the females, ORX activation in the male control and experimental groups was not different; since the experimental group had fully extinguished their fear responses, reward and food-associated ORX neurons were likely equally activated across the two male groups. It has been suggested previously that ORX neurons can be activated in response to all arousing stimuli, regardless of whether they are aversive or appetitive cues (Berridge, 2010). Conditioned females that still exhibit fear-cue inhibition of eating during testing could therefore be activating ORX

neurons in the LHA in response to arousal, stress, and anxiety. Although these ORX neurons are usually associated with the dorsomedial LHA in stressful contexts while ORX neurons activated by eating are typically associated with the lateral LHA (Harris, 2006), these regions are directly adjacent to each other and it is possible there is some overlap in the different kinds of ORX neurons expressed in the two regions. This would create no statistical difference for the number of ORX neurons activated in the different LHA regions for the different training conditions, and therefore patterns of ORX activation would appear similar between the two conditions even though different types of ORX neurons are activated in experimental and control females. It is possible the training paradigm utilized was not specific enough to differentiate between the types of ORX neurons activated under each condition, and a more sensitive paradigm should be set up in future experiments to accurately determine the type of ORX neurons being activated under each condition. The addition of a "no food" control group that does not have access to food pellets during training and testing sessions may aid in dissociating ORX activation as a result of food and reward versus stress or arousal.

These findings, however unexpected, have possible implications for the manifestation of anorexia. It has been observed that individuals with AN have altered concentrations of the neurochemicals CRH, NPY, endorphin, and the adiposity signal leptin, all of which are associated with feeding behavior and energy metabolism, and correlate with the irregular feeding behavior seen in those afflicted with AN (Kaye, 2008). Despite discrepancies in these neuropeptide concentrations, individuals with AN still experience physiological hunger signals, but demonstrate an emotional control over feeding that restricts food intake (Kaye, 2008). Individuals suffering from AN have higher levels of orexin A in their blood plasma (Bronsky et al., 2010), indicating that ORX is over-expressed in these persons. Thus, it is possible that in anorexic individuals hunger signal that should activate ORX neurons to stimulate eating instead trigger fear and anxiety that inhibits eating. Therefore it can be proposed that the strong physiological hunger signals released by anorexic individuals activate ORX neurons in the LHA that induce arousal, fear, and resulting inhibition of eating seen by female conditioned rats in this experiment, rather than ORX neurons in the same or nearby LHA region that stimulate eating. Human females are afflicted by a disproportionate percentage of eating disorders compared to males (Kaye, 2008), which is consistent with the greater recruitment of ORX neurons in the LHA of female rats of both control and experimental conditions than males of either condition. It can thus be theorized that greater activation of LHA ORX neurons in females can be correlated with females' greater susceptibility to eating disorders than males.

Although a greater percentage of ORX neurons activated in the LHA of female rats is a significant finding, it is not the only factor that could have contributed to the slower extinction to fear response exhibited by conditioned females. While ORX in the LHA has been implicated in stimulation of feeding behavior, and this study's results imply inhibition of eating as well, other brain regions have been shown to be responsible for this fear response. Activation of the HPA axis via CRF release in the PVN stimulates the release of ACTH from the pituitary gland that in turn increases secretion of corticosteroids corticosterone and cortisol that produce a physical stress response (Berridge, 2010). CRF release in the amygdala has been strongly implicated with stress as well; ORX fibers lie in close proximity to both the CeA and PVN and have been shown to activate PVN neurons, as well as increasing release of catecholamines, to produce a heightened stress response (Berridge, 2010). Stressful conditions such as dehydration have been

shown to induce corticotropin-releasing hormone (CRH) mRNA in the LHA as well, resulting in anorexia (Watts et al., 1999). Since ORX neurons have been shown to activate PVN neurons to stimulate a stress response, it is possible that under stressful conditions ORX could interact with CRH to induce inhibition of eating.

A sex-related difference exists in the activation of these systems that reveals females to be more greatly affected by stress. Female rats exhibit a rapider expression of CRF mRNA in the PVN than do males, as well as a rapider and greater secretion of plasma ACTH and corticosterone in response to a foot-shock (Iwasaki-Sekino et al., 2009), indicating a heightened physiological stress response compared to male rats. Female rats also show an increased expression of CRF mRNA in the PVN, plasma ACTH levels and corticosterone levels in response to psychological stress, whereas male rats exhibit no increase in any of these substances (Iwasaki-Sekino et al., 2009). Females in general are more susceptible to stress than males, as estrogen stimulates transcription of both the human and rat CRF genes in the amygdala and PVN, initiating activation of the HPA axis (Iwasaki-Sekino et al., 2009). More specifically, fear-cue inhibition of feeding is dependent on an intact CeA (Petrovich et al., 2009), a brain region where CRH neurons and estrogen receptors are present, and may lead to the prolonged inhibition in female rats seen in current and past research (Petrovich and Lougee, 2011). ORX may interact with any of these systems to induce a stress response correlated with inhibition of eating, especially in females that exhibit greater sensitivity and heightened responses to stress.

In conclusion, we have found extended fear-cue inhibition of eating in food-deprived female rats compared to males and also greater ORX activation in the LHA of female rats than male rats. While greater induction of ORX neurons did not correspond to training condition, the dual nature of ORX is implicated in heightened activation of ORX neurons in females. Orexin's role in the appetitive reward circuitry of the brain as well as its increased activation in response to food-related cues is implied in greater ORX activation in control female rats; orexin's role in increasing fear and anxiety in response to stressful stimuli is implied in increased activation of LHA ORX neurons corresponding with prolonged inhibition of eating in conditioned females. The role of ORX in fear and anxiety and possible connotations for inhibition of eating under stressful conditions has interesting implications for its role in anorexia nervosa. As AN has a high co-morbidity with anxiety disorders and is associated with increased ORX expression (Bronsky et al., 2010), the role of ORX in AN deserves further exploration. This neuropeptide known for its dual nature in increasing both appetite and arousal may be a large contributing factor to one of the deadliest psychological disorders in existence.

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my understanding of orexin, the hypothalamus, physiological hunger signals and the brain circuitry involved as well as eating disorders such as anorexia nervosa mentioned in this paper.

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