Fear-cue Induced Inhibition of Feeding: Activation of the Central Nucleus of the Amygdala

Author: John K. Young

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Jack Young

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Introduction

Metabolic needs often drive feeding behavior in all organisms. Although the motivation to maintain homeostasis via nourishment is a strong force, it can be challenged or even overcome by other interfering motivations caused by environmental factors and as a result of learning (Petrovich et al., 2009). Stressful stimuli, for example, have been shown to hinder or even inhibit feeding despite acute states of food deprivation (Petrovich and Lougee, 2011). In the following study, the underlying neural mechanisms regarding fear-cue induced inhibition of feeding will be explored. Specifically, the mediatory role of the central nucleus of the amygdala (CEA) in modulating feeding behavior will be examined utilizing a rodent model. Differences between female and male CEA activation will also be analyzed to identify possible gender specific neural activation associated with fear-induced inhibition of feeding.

Role of the CEA in Conditioned Aversive Learning

It is vital to investigate the roles of different nuclei within the amygdala in order to better understand the function of the each individually. In a study regarding different types of fear-conditioned behavior, Killcross et al. (1997) examined the effects of lesions in not only the CEA but also in the adjacent structure of the basolateral amygdala (BLA). Here, it was found that both the BLA and the CEA are involved in conditioned stimulus (CS) – unconditioned stimulus (US)

association. Interestingly, Killcross found a disassociation between the two nuclei by showing that lesions in one or the other resulted in behavioral differences in conditioned inhibition of a learned operant response. In Killcross' paradigm, rats were first conditioned to lever-press for food. Then, the rodents received lesions of the BLA, the CEA, the BLA and CEA, or sham lesions. After surgery, the rats were then trained to associate a tone (CS+) with a footshock on one lever (US), and a tone (CS-) with no footshock on another lever. Results showed that after multiple tests, rats with BLA lesions properly inhibit lever pressing whereas CEA lesioned rats showed an impaired ability to suppress responses to the CS+ lever (Killcross et al., 1997), implicating the CEA as necessary for conditioned aversive learning.

Although it is known that both the BLA and CEA are involved in fear-cue avoidance behaviors, Killcross displays that these two nuclei can operate independently of one another and may subserve different aspects of fear avoidance behavior. The CEA seems to be involved in conditioned responses elicited by aversive CSs (Killcross et al., 1997). This finding sheds light on the role of the CEA and its direct association with behavioral responses to aversive stimuli. According to Blanchard & Blanchard (1969), in rodents the response to fear or aversive stimuli is often measured in terms of freezing duration. A study by Ciocchi et al. (2010) further connects the CEA with this behavioral response to aversive stimuli. In this study, a virus expressing channelrhodopsin-2 (ChR2) in neurons was bilaterally injected into the CEA of experimental rats. When these ChR2-expressing CEA neurons were activated via the presences of blue light, experimental rats showed significantly more freezing behavior compared to controls (Ciocchi et al., 2010). It is

clear that neurons within the CEA mediate the freezing response in one way or another. Now that the link between the CEA and the mediation of fear responses has been discussed, the CEA's role in the interaction of these fear responses with feeding behavior must be addressed.

Previous experiments have shown that the regulation of feeding behavior in the presence of an aversive stimulus is critically dependent upon the CEA, but not the BLA. Rats were conditioned to associate a tone (CS) with a footshock (US). During later testing, when the tone was presented without the shock, BLA-lesioned rats significantly inhibited feeding compared to controls whereas CEA-lesioned rats did not exhibit an inhibition of food consumption (Petrovich et al., 2009). Furthermore, both BLA-lesioned and CEA-lesioned rats revealed a reduction in freezing behavior compared to sham-lesioned rats (Petrovich et al., 2009). Thus, it can be inferred that the CS-induced inhibition of feeding behavior and the CS-induced freezing behavior activates discrete amygdalar networks. The neural connection from the BLA to the CEA is necessary for the expression of one defensive response (i.e. freezing), but CEA activation alone is crucial for the regulation of fear-cue inhibition of feeding behavior. It is evident that the CEA is involved in discrete neural networks that influencing both cue-induced freezing and the inhibition of food consumption.

Role of the CEA in Regulation of Feeding Behavior

Although most research of the CEA is typically associated with its integral role in aversive learning, the CEA has roles in modulating appetitive behaviors, including feeding behavior. One region the CEA may interact with to modulate

feeding behavior is the lateral hypothalamus (LHA). Especially, a possible interaction of the CEA with orexin (ORX) expressing neurons and melanin-concentrating hormone (MCH) expressing neurons in the LHA will be discussed.

ORX expressing neurons are almost exclusively localized in the LHA (Swanson et al., 2005). It has been shown that stimulation of these neurons lead to an increase in food consumption. Sakurai et al. (1998) revealed that intracerebroventricular (ICV) injections of orexin into the lateral ventricles increased food consumption in rats relative to controls that received an ICV injection of a vehicle. Furthermore, Sakurai and colleagues found that prepro-ORX mRNA expression is significantly upregulated in rats that were fasted for 48 hours compared to controls that were not fasted (Sakuai et al., 1998). These two findings implicate ORX in food consumption behavior. The first part of the previous study reveals that the release of ORX perpetuates food consumption. The second aspect shows that when food intake is low, the production of ORX is upregulated in order to increase the drive to consume. Clearly ORX, expressed in the LHA, modulates consumption.

MCH expressing neurons, like ORX expressing neurons, are also expressed in the LHA (Swanson et al., 2005) and are involved in the regulation of food consumption. In an experiment conducted by Clegg et al. in 2002, experimental rodents were given ICV injections of MCH into the third ventricle whereas controls were given ICV injection of a vehicle. Experimental rats revealed increases in food consumption in comparison to controls. This effect also increased with increasing dosages of MCH (Clegg et al., 2002). To further examine the role of MCH in feeding

behavior, a different study looked at the effects of an ICV injection of an MCH-1 receptor agonist. Findings indicate that the rats that received ICV injections of the MCH-1 receptor agonist increased food consumption compared to those who received the vehicle. Here, it was also found that consumption increased proportionally with the dosage of the administered MCH-1 receptor agonist (Shearman et al., 2003). This provides strong evidence that MCH release, similarly to ORX, modulates food consumption.

It has been shown that both ORX neurons and MCH neurons are not only important in the modulation of feeding behavior and food intake, but they are also both exclusively expressed in LHA. Therefore, it is essential to understand the connection between the CEA and the LHA when examining the role of the CEA in modulating food consumption. First, a study conducted by Reppucci & Petrovich (2012) used injections of a retrograde tracer into the dorsal LHA (dLHA), the area known to contain both ORX and MCH expressing neurons. Here, Reppucci & Petrovich found that retrograde tracer injections into the dLHA labeled cell bodies in the CEA (Reppucci & Petrovich, 2012), revealing that the CEA projects to the dLHA. Another study used biotinylated dextranamine injections into the CEA for anterograde tracing. By also immunostaining for MCH or ORX neurons in the LHA, the researchers could use anterograde tracing to determine if the CEA projections synapsed on MCH or ORX neurons within the LHA. The results revealed a large number of neural connections between the CEA and MCH neurons as well as ORX neurons in the LHA (Nakamura et al., 2009). From these two studies, the connection from the CEA to LHA ORX and MCH neurons is apparent. In summary, there is a

well-established projection from CEA neurons to the MCH and ORX neurons that modulate feeding in the LHA.

The neural connectivity described above is not, however, the only way the CEA is involved in the regulation of food intake. It is important to consider alternate mechanisms for the regulation of food intake. An example of this is the effects of endogenous opioids on the CEA. It has been shown that μ-opioid agonists stimulate food intake in food deprived rats (Gosnell et al., 1986). Additionally, Sun et al. (2012) revealed that an injection of DAMGO, a μ-opioid receptor agonists, directly into the CEA increased the consumption of sucrose. Also, immunohistochemical double labeling of neurons within the CEA revealed high numbers of neurons expressing both Fos and the μ-opioid receptor. Clearly the CEA is involved in a complex network that regulates feeding behaviors.

Sex Differences in Conditioned Aversive Learning and Feeding Behavior

A study conducted by Petrovich and Lougee (2011) focused on sex differences in fear-induced feeding behavior. Results revealed that both male and female conditioned groups similarly expressed conditioned responses (CRs; increased freezing behavior and inhibited food consumption) in response to an aversive CS (tone) that was previously associated with an aversive US (footshock). Across testing days, sex differences in the extinction of the CRs was observed. The extinction of the freezing behavior occurred at a similar rate between conditioned male and conditioned female groups; however the extinction of the inhibition of food consumption occurred at a much slower rate in females than in male rats (Petrovich & Lougee, 2011). The presence of this sex difference in the extinction of

the inhibition of feeding provides a basis for further examination of the neural circuitry and especially CEA activation, underlying these differential expressions of behavior.

Hypothesis

As seen in Petrovich & Loungee (2011), sex differences in the extinction of particular behaviors induced by learned aversive cues are apparent. In this study, these differences are seen in an inhibition of food consumption but not in freezing behavior in response to a conditioned aversive cue. Additionally, the CEA has been shown to be necessary for the expression of both conditioned inhibition of feeding and conditioned freezing behavior (Petrovich et al., 2009). Thus, in the current experiment, CEA activation will be analyzed in order to shed light on how this region may be influencing these CS-driven behaviors and their extinction. Because this analysis will focus on the third behavioral test day, extinction of some of these behaviors will be evident. We have previously seen that the extinction of inhibition of consumption and the extinction of freezing behaviors in conditioned male rats occurs during the first test day, therefore I hypothesize that there will be no difference in CEA activation between conditioned males and control males on test day 3. Because females have been shown to still exhibit inhibited consumption on test day 3, I also hypothesize that there may be a difference in CEA activation between conditioned females and control females. Lastly, due to sex differences in consumption irrespective of condition, I hypothesize that CEA activation will differ between sexes.

Methods

Subjects

A total of thirty-two Long-Evans rats of both male and female sex (sixteen male and sixteen female) were used to conduct this experiment. Each rat was individually caged, maintained on a 12 hour light/dark cycle, and received standard laboratory chow and water *ad libitum* (unless otherwise specified). Male and female rats were separated into different housing rooms. Upon arrival, the rats were left in the colony room for 24 hours prior to handling. The rats were given 2 weeks prior to experimentation to experience colony life. All rats were weighed every weekday and the females were given vaginal smears 6 or 7 days a week in order to determine if the females were cycling normally. All housing and testing procedures were in compliance with the National Institute of Health Guidelines for Care and Use of Laboratory Animals, and approved by the Boston College Animal Care and Use Committee.

Behavioral Training Procedure

The rats were randomly assigned to a group (8 experimental, 8 control for both sexes). Behavioral training occurred in 9 sessions, 6 of which were appetitive (S1, S2, S4, S6, S8, S9) and 3 of which were aversive (S3, S5, S7). Water was available *ad libitum* throughout both session types.

The appetitive and aversive training were conducted in different behavioral chambers. The appetitive training took place in a Coulbourn Behavioral Box, located within an isolation cubicle. Plexiglas flooring was placed on the bottom of the behavioral box and was sprayed with 1% acetic acid. Both doors of the isolation

cubicle were closed. Both the house light and video camera (to record the training sessions) were turned on. A recessed food cup in the behavioral box contained 7g of Test Diet food pellets. Prior to each appetitive session, the rats were food deprived for 22 hours. For each appetitive training sessions, rats were placed into the behavioral box and allowed to eat for 10 minutes. After the 10 minutes, rats were immediately removed from the behavioral box, placed in their home cages, and returned to their respective housing room. The remaining food was collected and weighed.

Prior to each aversive training session, rats were given at least 24-hour access to lab chow *ad libitum*. This training also occurred in a Coulbourn Behavioral Box, but one of the doors of the isolation cubicle (on the right) remained opened. Double Plexiglas was placed inside the behavioral box in order to create a triangular roof, and the grid floor was exposed. The pullout trays on the bottom of the cage were sprayed with 5% ammonium hydroxide. The video camera was turned on to record the sessions. The first aversive session (S3) consisted of 10 minutes inside the box in order to habituate the rat to the aversive context. In the second and third aversive sessions (S5, S7), half of the rats (experimental/conditioned groups) were exposed to 2 tones (75db; 2khz; 60s) immediately followed by a footshock (1mA; 1s). The other half of the rats (control groups) were presented with 2 tones (75db; 2khz; 60s) but no footshock. For both groups aversive sessions lasted exactly 10 minutes.

Food Consumption Tests

In each of the 3 consumption tests, the context was identical to that of the appetitive training sessions. A tone (75db; 2khz; 60s) was introduced 4 times throughout the test. Rats were immediately removed after the 10 minutes, placed into their home cage and returned to their respective housing room. The remaining food of the 7g given was collected and weighed. Rats were allowed lab chow access *ad libitum* for at least 24 hours after each test. Due to a fire alarm during testing, 14 rats (approximately an equal number from each group) were removed from the study.

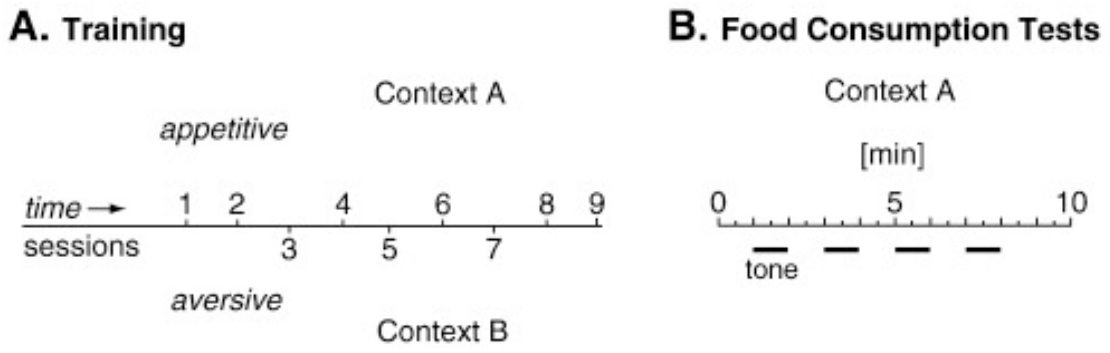


Figure 1: Experimental design showing the order of training sessions, and tone presentations during the test sessions

Vaginal Smears

Vaginal smears were obtained via lavage procedure 6 to 7 times a week. Vaginal smear cell types were identified under a microscope in order to detect estrous cycle patterns of each female rat. This procedure was used to confirm normal estrous cycling, for abnormal cycling may indicate severe stress. However, due to the small sample size, this was not used as a factor in analysis.

Tissue Collection

Rats were sacrificed 90 minutes after the start of the last (third) test. Each rat was briefly anesthetized with isoflurane, then deeply anesthetized via an intraperitoneal injection of tribromoethanol. Rats were then transcardially perfused with an isotonic saline solution followed by 400mL of 4% paraformaldehyde in a .01M borate buffer solution. After, brains were removed surgically, they were post-fixed for 24 hours in a 12% sucrose solution of the fixative, frozen in hexanes and stored at -80^{o}C. The brains were then mounted on the microtome stage via frozen KPBS solution. The microtome was set to produce 30μm slices. The tissue was sliced and collected into four series, one that was labeled for Fos presence, one was stained for Nissl bodies, and two were put into a cryoprotectant solution, stored at -20^{o}C and saved for future analysis.

Histological Procedures

The series of tissue stained for Nissl bodies was first dehydrated via a series of washes in increasing concentrations of ethyl alcohol. Then, in order to extract the fat from the tissue, it underwent a series of washes in xylenes. The tissue was then rehydrated through washes of descending concentrations of ethyl alcohol, stained with thionin, and eventually securely coverslipped. The Nissl staining was used to identify brain areas according to the Swanson atlas for the rat brain (Swanson, 2004), upon which Fos stained tissue could be compared.

Fos was then visualized on an adjacent series of tissue. The tissue was first incubated for 1 hour in a KPBS solution containing 3% Triton X-100, 2% normal goat serum, and 10% non-fat milk. The tissue was then further incubated for 72

hours in a similar KPBS solution containing anti-Fos primary antibody raised in rabbit (1:2K). After multiple rinses of KPBS solution containing 2% normal goat serum and 10% non-fat milk, the tissue was incubated in KPBS solution containing 3% Triton X-100, 2% normal goat serum, 10% non-fat milk, and secondary anti-rabbit antibody (1:500) for 45 minutes. Following rinses in KPBS, the tissue was incubated in avidin-biotin complex for 45 minutes, then again rinsed with KPBS. The tissue was then stained using a solution containing diaminobenzidine and hydrogen peroxide. Lastly, the tissue was further rinsed in a KPBS solution, mounted on slides, dehydrated with increasing concentrations of ethyl alcohol, soaked in rinses of xylenes and coverslipped.

Behavioral Data Analysis

The videotapes of each test were analyzed for freezing behavior. Freezing was defined as behavior consisting of temporarily sustained inhibition of all bodily movements except respiration. A metronome was set at 1 beat/1.25s. Thus, forty-eight beats occurred during each tone presentation. For each beat, either a yes or no for freezing behavior was recorded. The total percent of time rats expressed freezing behavior during the CS presentations at testing was then calculated.

Histological Analysis

Processed tissue was imaged using the Olympus BX51 microscope and attached DP72 digital camera. Images of the tissue were taken bilaterally at level 27 (Swanson, 2004), as seen in Figure 2 and Figure 3 using Olympus' DP2-BSW imaging software. This level was chosen because it is the mid level of the lateral CEA (CEAl) and all three subdivisions of the CEA are present.

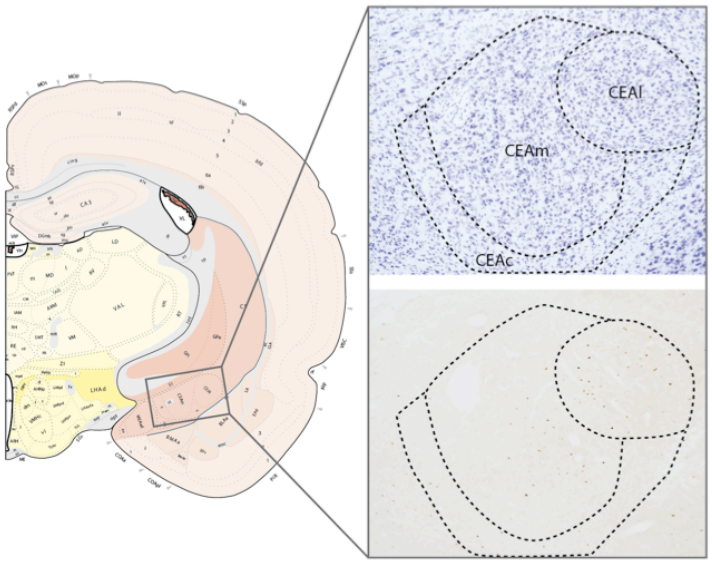


Figure 2: Level 27 of the rat brain with the CEA enlarged; coronal view. The top enlargement shows Nissl stained tissue. The bottom image shows Fos inductance.

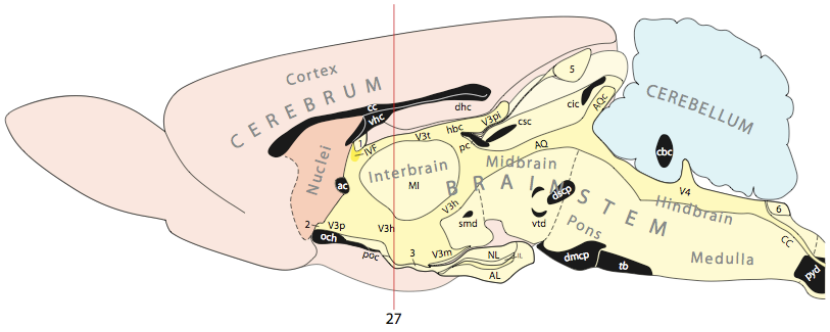


Figure 3: Level 27 of the rat brain; sagittal view

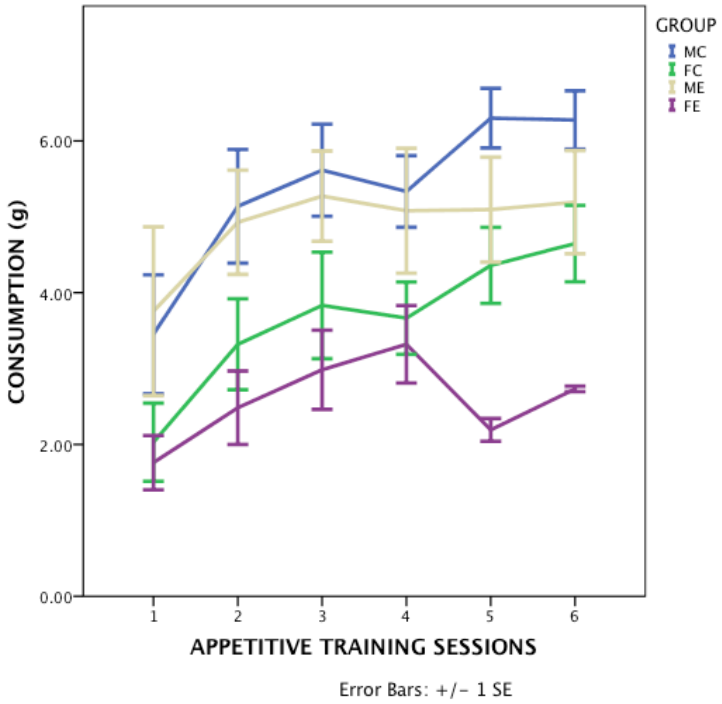
Images of the adjacent Nissl and Fos stained tissue (Nissl and Fos from the same brain) were matched such that they aligned perfectly. Using ImageJ software, the borders of the CEA and its three subdivisions, according to the Swanson Brain map, were drawn onto the Nissl-stained tissue and then superimposed onto the Fos stained tissue. The number of Fos labeled neurons within the CEA borders was then automatically counted using the ImageJ analysis program.

Results

Training Sessions

Rats were trained in the behavioral protocol described within the materials and methods section. The appetitive and aversive training sessions were conducted within two distinct environments (contexts). Food deprived rats were given *ad libitum* access to food pellets during appetitive training sessions. Half of each the male and female rats (conditioned groups) received tone-shock pairings in the aversive context, whereas the other half of the male and female rats (control groups) received the same number of tones as the conditioned groups in the aversive context, but did not received the succeeding unconditioned stimulus (shock).

As seen in Graph 1 below, all rats ate considerable amounts during the appetitive training sessions, with a net increase in consumption between appetitive session 1 and appetitive session 6. The males consistently ate more in comparison to females. Also, during the last two appetitive sessions conditioned groups (tone-shock trained rats) ate less compared to the same sex controls that did not receive a shock during training.



Graph 1: Consumption during appetitive training sessions

Two-way ANOVAs of food pellet consumption were run for each training session using sex (male or female) and condition (experimental or control group) as factors. A significant main effect of sex was observed for each appetitive training sessions ($F(1,18) > 5.531$, $p < .05$, all). A significant main effect for condition was observed during appetitive session 5 ($F(1,18) = 14.404$, $p < .01$) and session 6 ($F(1,18) = 12.105$, $p<.01$). An interaction of sex and conditioning was never observed in any training sessions ($p > .05$, all). Pos hoc within-sex independent samples t-tests showed that conditioned females, but not conditioned males, consumed significantly less than same-sex control groups during appetitive session 5 ($t(1,7) = 4.595$, $p < .01$), and appetitive session 6 ($t(1,7) = 4.313$, $p < .01$).

Furthermore, an analysis of body weight with sex and condition as factors revealed that male rats weighed significantly more than female rats at the start of

training (F(1,18) = 123.922, p < .001; M-conditioned, 299 ± 14g, M-control, 304 ± 6g; F-conditioned 242 ± 16g, F-control 238 ± 6g) and at the start of testing (F(1,18) = 154.727, p < .001; M-conditioned, 431 ± 45g, M-control, 454 ± 23g; F-conditioned 275 ± 27g, F-control 258 ± 22g) while no effects of condition or sex by condition were observed (p > .05, all).

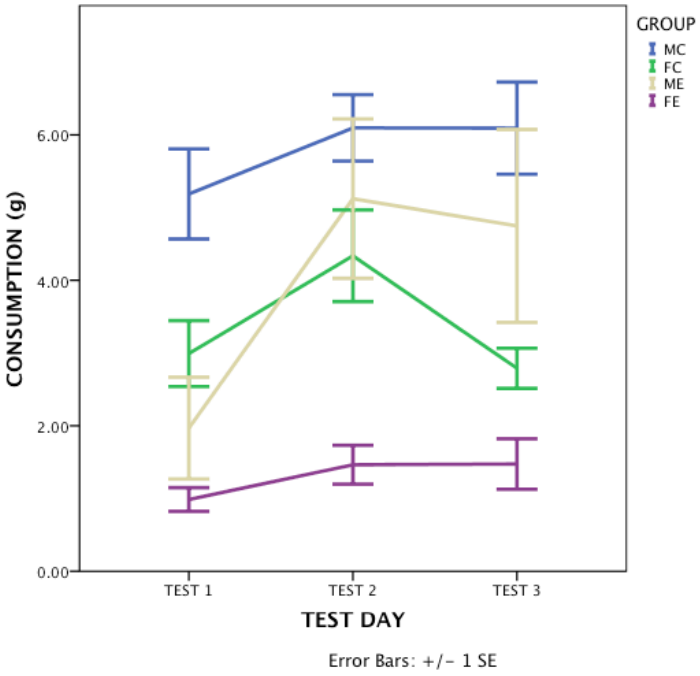
Test Sessions

After training was finished, rats were tested on three separate days. Food deprived rats were given food pellets in the appetitive context and received 4 presentations of the tone. Quantitative amounts of food consumed were observed and interpreted. Additionally, the expressions of freezing behavior during the tests was analyzed.

Food Consumption. During all tests, male rats ate more than females overall which is consistent with their consumption during training. Importantly, during the first test both male and female rats in the conditioned group ate less than the controls of the same sex. It is also important to note that although both male and female conditioned groups showed reduced food consumption compared to control groups, conditioned females inhibited food intake more drastically than did males. Between test day 1 and test day 3, conditioned males increased intake whereas conditioned females did not. A repeated (across the 3 days) two-way ANOVA was conducted. There was significant within-subjects main effect of Test Day (F(2,28) = 29.619, p < .001) as well as significant within-subjects interactions of Test Day with both sex (F(2,28) = 9.810, p = .001) and condition (F(2,28) = 5.422, p = .01), and a significant three way interaction for Test Day by sex by condition (F(2,28) = 8.118, p

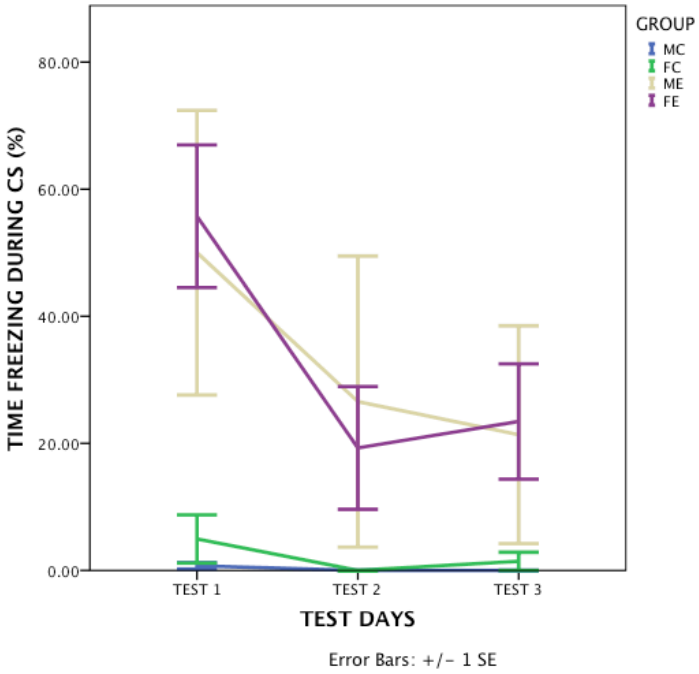
< .01). There were also significant between-subjects main effects of sex ($F(1,14)$ = 18.544, p = .001) and condition ($F(1,14)$ = 11.097, p < .01) but no interaction (p > .05).

For conditioned male rats in comparison to control males, post hoc independent samples t-Test on the first test day revealed a significant inhibition of consumption ($t(1,7)$ = 3.449, p < .05), but this inhibition was extinguished on test days two and three; on these days the two male groups ate statistically similar amounts (p > .05, both). Contrary to the behavior of the males, the conditioned female rats not only showed a significant inhibition of food consumption compared to female controls during test day one ($t(1,7)$ = 4.569, p < .01), but also maintained this inhibition of intake on test day two ($t(1,7)$ = 4.552, p < .01) and test day three ($t(1,7)$ = 2.844, p < .05).



Graph 2: Consumption across all three test sessions

Freezing Behavior. Freezing behavior was also analyzed during the food consumption tests. Both male and female rats in the conditioned groups spent a greater percent of time freezing during the conditioned stimulus when compared to control rats (control rats show almost 0% time freezing), especially during Test 1. This difference in freezing based on condition and not on sex reveals that males and females learned the CS-US association equally well.



Graph 3: Percent of time spent freezing during CS presentations across the test sessions.

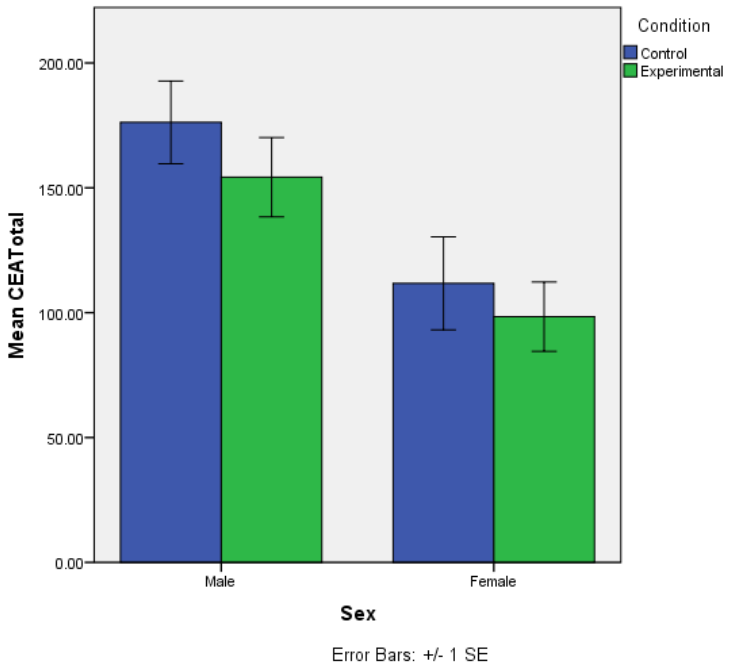
A repeated (across 3 test days) two-way ANOVA was conducted. There was significant within-subjects main effect of Test Day ($F(2,28) = 13.752$, $p < .001$) and significant within-subjects interaction of Test Day with condition ($F(2,28) = 9.909$, $p = .001$). There was also significant between-subjects effect of condition ($F(2,28) = 9.852$, $p < .01$), but no effect of sex ($p > .05$).

Specifically, post hoc within-sex independent samples t-Test showed that the conditioned males, when compared to the control males, showed significantly more freezing behavior on test day one ($t(1,7) = -2.503$, $p < .05$). Similar to the males, the females also showed significantly more freezing behavior than control females during test one ($t(1,7) = -3.860$, $p < .01$). Expressions of freezing behavior by the conditioned groups extinguished across the test days such that there was no statistical difference in this behavior during test two and test three compared to the corresponding control groups ($p > .05$).

Fos-induction of CEA neurons during Test 3. Male rats had higher Fos induction than female rats regardless of condition within the entire CEA. A two-way ANOVA of Fos induction in the CEA revealed a significant main effect of sex ($F(1,18) = 13.618$, $p < .01$), with males showing significantly greater Fos induction in the CEA than females overall during test 3. Both male and female rats of the control groups revealed higher Fos induction within the CEA compared to their same-sex control groups, although this difference is not statistically reliable for either sex ($p > .05$, both).

An analysis of Fos-induction within each of the three subdivisions of the CEA was then completed. Two-way ANOVAs of Fos induction within both the CEAl ($F(1,18) = 6.788$, $p < .05$) and medial CEA (CEAm) ($F(1,18) = 19.093$, $p = .001$) subdivisions revealed the same significant main effect of sex as seen in the whole CEA analysis, with males showing greater Fos induction than females in both cases. A main effect of condition trended toward significance in CEAl Fos expression

($F(1,18) = 3.140$, $p < .10$), with control rats showing greater Fos-induction than conditioned rats.



Graph 4: Total CEA neurons expressing Fos protein after sacrifice on test day 3.

Within the capsular CEA (CEAc), a two-way ANOVA revealed a significant main effect of condition ($F(1,18) = 9.022$, $p < .01$), with conditioned groups showing greater Fos induction than control groups. This effect of condition is likely due to sampling error and extremely small samples (control groups showing about 3 Fos-positive neurons with conditioned groups showing 7 Fos-positive neurons on average). Thus, this effect will not be considered in further discussions.

Discussion

Results of consumption during appetitive sessions reveal relatively few differences between control groups and conditioned groups within sexes. Conditioned rats and controls show similar consumption patterns within sexes until

the 5th and 6th appetitive sessions in which conditioned females show significantly less consumption compared to female controls. This difference in consumption can be attributed to the aversive sessions used in the behavioral training paradigm. As seen in Figure 1, by appetitive sessions 5 and 6, conditioned rats have already been exposed to 3 aversive sessions, the final two of which expose conditioned rats to shocks. Thus, the female conditioned group's inhibition of consumption in appetitive sessions 5 and 6 (as well as the male conditioned group's decrease in consumption that was not statistically reliable) can be attributed to a generalized effect due to prior experience with footshocks.

It is clear that total consumption by males is greater than that of females, but what is most compelling is the difference in consumption trends over the three test sessions. Conditioned males seem to disinhibit their feeding behavior inhibition and show greater food consumption after test 1. By tests 2 and 3, conditioned male rats consumed similar amounts compared to male controls. Conditioned females, on the other hand, maintain a significant inhibition of food consumption throughout tests 1, 2, and 3. This reveals a failure to extinguish the inhibition of food intake caused by aversive fear-cue conditioning in females, but not it males.

In contrast to the sex differences in extinction of the conditioned inhibition of food intake, males and females showed similar patterns of freezing behavior across testing. Both sexes of the conditioned group show a greater percentage of time spent freezing relative to the same-sex control rats throughout all of the test sessions. However, this difference was only statistically significant on test day 1 as both conditioned groups showed a decrease in percentage of time spent freezing

across test days 2 and 3. In this case, both male and female conditioned groups reveal the same extinction of freezing behavior after test day 1. Male and female conditioned groups exhibit the extinction of the freezing response to the CS, but only males exhibit the extinction of the inhibition of food consumption. This data suggests a dissociation between the CS-induced inhibition of feeding and the CS-induced freezing, and may therefore unveil the role of the CEA in mediating both fear and feeding behaviors.

It is clear that the CEA is involved in distinct neural pathways modulating both the fear behavior and feeding behavior. It is unclear, however, the exact influence the CEA has upon behavior when both fear responses and feeding behavior need to be modulated simultaneously. In this study, we looked to further understand the role of the CEA in this specified instance.

As discussed above, there is a clear behavioral difference between males and females by test day 3. The neuronal activation of the CEA on this test day may shed light on the circuitry underlying this behavioral difference. During test day 3 there was a significantly greater number of activated CEA neurons in males than in females overall. Also, there was a small difference in total activation of the CEA between conditions. This reveals information indicating the function of the CEA in influencing a fear-cue response. As previously discussed, conditioned rats extinguish freezing behavior by test session 3 regardless of sex, yet we see differences in CEA activation between sexes. Additionally, within each sex, control subjects had greater activation than experimental subjects. Thus, these results suggest that the observed CEA activation is likely due to differences in food

consumption between the groups, not due to differences in the expression of freezing behavior.

Before the role of the CEA is to be discussed, it is imperative to understand the quantification of CEA activation used in this study. *c-fos* is a gene that can be expressed in rodent neurons. The protein produced by this gene is called Fos. The expression of the *c-fos* gene is highly correlated with neuronal activation and, thus, in response to extracellular stimuli, neurons fire and concurrently begin transcribing the *c-fos* gene, eventually producing its associated Fos protein. Fos levels typically peak approximately 90 minutes after neural activation. Although the presence of Fos in a neuron simply correlates with its activation, Fos is still a strong and reliable biological marker for indicating neuronal firing (Curran & Morgan, 1994).

Various studies have been conducted that not only link activation of the CEA to fear responses, but also consider the effect of sex on both CEA activation and the conditioned fear response. Many of these studies focus on the effects of corticotropin-releasing hormone (CRH), a stress hormone that helps to increase the classic sympathetic stress response. CRH neuronal synapses are abundant in the CEA, where the chemical is used as a stress-induced neurotransmitter (Swiergiel et al., 1993), revealing a potential neurochemical substrate that mediates the importance of CEA in eliciting the behavioral fear response. In addition, sex differences are present in the levels of expression of CRH mRNA in the paraventricular nucleus of the hypothalamus (PVN) where the chemical is used as a hormone involved in the production of stress-related glucocorticoids. Additionally,

CRH mRNA expression is much higher in the CEA of female rats than in male rats (Iwasaki-Sekino et al., 2009). The PVN is not only a brain region vital to stress related behavioral responses, but it also directly influences and projects to the CEA (Gray et al., 1989). Similarly, the CEA directly projects to the PVN but to a lesser extent (Berk & Finkelstein, 1981).

CRH release causes an increase in plasma adrenocorticotropic hormone (ACTH) levels (Lee et al., 2012). It has been shown that stress induces significant increases in ACTH (Lennartsson et al., 2012). ACTH causes the release of glucocorticoids from the adrenal glands and further functions by increasing the bioavailability of cholesterol in cells of the adrenal cortex. ACTH acts by increasing the transport of cholesterol into cellular mitochondria, and by stimulating the production of enzymes that catalyze the synthesis of pregnenolone, a vital prohormone in the synthesis of progesterone (Rafnsson et al., 2005). All of these responses allow for the production of available energy and organic chemicals associated with fear and the "flight or fight" response.

The high level of CRH mRNA expression in the PVN only reveals part of its role in response to stress. The PVN contains not only CRH neurons, but also neurosecretory cells that produce vasopressin, a peptide hormone that acts synergistically with CRH in order to upregulate synthesis of ACTH. Not only do females show an increase in ACTH secretion compared to males, but they also reveal higher levels of corticosterone (a derivative of progesterone and major stress hormone), thus leading to higher, stress related hormone levels than in males (Iwasaki-Sekino et al., 2009). These high hormone levels might influence the over-

activation of the amygdala, specifically via activation of glucocorticoid receptors in the CEA in a stress induced state. Furthermore, with the knowledge that CRH induced activation of the CEA is higher in females than in males, it could be used as part of the biological explanation for why females typically exhibit a more sustained behavioral fear responses to aversive stimuli.

Further, past research has shown that the CEA has strong neural projections to the bed nuclei of the stria terminalis (BNST), a brain area associated with anxiety, via CRH neurons (Swanson & Petrovich, 1998). Although the BNST was not found to have modulatory effects upon unconditioned anxiety behaviors, the BNST did have modulatory effects on sustained fear-potentiated behavior (modeling conditioned anxiety) in rats exposed to a tone after being conditioned to a tone-shock pairing (Sink et al., 2012). This further emphasizes the role of CRH in the fear response, and also suggests separate neural pathways for conditioned versus unconditioned responses. Perhaps these pathways involve specific subsets of neurons within the CEA.

However, in the current study, sex differences in CEA activation are likely not a result of freezing behavior, for the conditioned groups show a similar extinction of the defensive response irrespective of sex. Thus, it may be helpful to consider the CEA's role in inhibition of feeding between sexes. In a study conducted by Kuriyama and Shibasaki (2004), male and female rats were exposed to either a stressful environment (experimental group) or a non-stressful environment. The experimental group was placed in a behavioral box surrounded by three others, each containing rats who received 2 footshocks every minute for an hour. The

experimental group could smell, hear, and see the rats being shocked. The control group was put into the same behavioral box but was surrounded by three empty behavioral boxes. The amount of food consumed by each rat was recorded. A greater degree of inhibition of feeding by the experimental group was found in female rats compared to their male counterparts. This inhibitory effect was revealed to be dependent on estradiol and corticotropin-releasing hormone (CRH) type 1 receptor (Kuriyama and Shibasaki, 2004). Increases of CRH from stress were shown correlate with an increase in the inhibition of food consumption. Increased female CEA neuronal activation, previously shown to increase with CRH stimulation, has also exhibited its inhibitory effects on food consumption in previous research (Spina et al., 1996). Although this previous research seems to contradict some of the results in our paradigm, it can be explained by the fact that we analyzed CEA Fos-induction during late extinction where the conditioned groups of both sexes were no longer expressing freezing behavior, and where conditioned males no longer inhibited food consumption.

Although CEA activation has been previously shown to increase behavioral fear responses consistently, sex differences in CEA activation are not expressed as the expected behavioral differences during test 3. Although female rats have been shown to more readily express CRH mRNA (Iwasaki-Sekino et al., 2009), a transcript of a hormone directly associated with CEA activation and the circuitry underlying the conditioned fear response, the conditioned females in the current study had lower CEA activation than conditioned males yet exhibited a similar freezing response. Despite previous studies indicating a positive correlation between CEA

activation and the inhibition of food consumption, male groups exhibit higher CEA Fos expression and a disinhibited conditioned inhibition of food consumption. With these seemingly contradictory results, we must now consider a different perspective on role of the CEA to account for behavioral differences between male and female conditioned rats.

As discussed in the introduction, the CEA has been shown to regulate feeding in multiple ways. The CEA can mediate consumption via projection from CEA neurons to the MCH and ORX neurons (Nakamura et al., 2009) that modulate feeding in the LHA (Clegg et al., 2002; Sakuai et al., 1998). It can also act as a consequence of opioid signaling modulation. For instance, it was found that rats peripherally injected with naltrexone (NTX), a μ-opioid receptor antagonist, inhibited food intake in a food-restricted paradigm. Contrarily, when NTX was injected into PVN it did not inhibit sucrose intake. These two findings suggest that opiods, particularly via binding with the μ-opiod receptor regulate food intake (Naleid et al., 2007). In the CEA, μ-opiod receptor activity has been shown to modulate feeding regulation (Beckman et al., 2008). Specifically, it was shown that administration of NTX into the CEA of rats suppresses the intake of some foods (Glass et al., 2000). Thus, this information further emphasizes the regulatory effects of the CEA upon feeding behavior.

An experiment conducted by James Pomonis and colleagues (1997) utilized naloxone, another μ-opiod receptor antagonist, to further explain the role of the CEA in the modulation of consumption. The study consisted of 5 groups of rats that were administered two chemicals, separated by 30 minutes. The five groups were

administered saline then saline, naloxone then saline, saline then NPY, saline then NPY (but were deprived food post-injection), and naloxone then NPY. All NPY administrations were injections into the PVN. Saline and naloxone were administered peripherally. The study found an increase in Fos induction in the CEA in response to both naloxone and NPY administration. Moreover, the response to NPY and naloxone was additive (Pomonis et al., 1997). The additive nature of CEA activation in this experiment implies that each chemical stimulated a different set of neurons within the CEA. This further offers evidence that the function of the CEA is more than inhibitory; it is also modulatory. The CEA seems to be involved in the modulation of feeding behavior by showing reactivity to μ-opiod antagonist (previously associated with inhibition of food intake) and NPY, a neuropeptide associated with increasing consumption.

It is evident that the role of CEA in feeding behavior is complex and not fully understood, yet this last study may provide insight into our results. CEA activation is not a 1:1 correlate with the conditioned fear response or feeding modulation. The CEA has multiple functions that are processed simultaneously, and different parts of the CEA are likely mediating different aspects. The results obtained in our study could be explained by this functional disassociation within the CEA. Perhaps the increase in CEA Fos in males compared to females can be attributed mostly to a group of neurons that are dedicated to the modulation of feeding via projections to the LHA, or through the release of endogenous opioids. Perhaps the active neurons involved in the fear response within the CEA are equally abundant between sexes, resulting in similar freezing behavior. Although the exact role of the CEA and its

different subgroups is still unclear, current research is aiding our understanding of its wide variety of complex actions and behavioral influences.

Conclusions

In this experiment, there were no behavioral differences between conditioned and control males during the third test day. Thus, it was hypothesized that there would be no difference in the CEA activation in males during that test. As expected, behavioral and neural similarities were seen between the two conditions in male rats. In contrast to males, conditioned female rats have been shown to exhibit significant inhibition across all three tests. For this reason, it was hypothesized that the CEA activation patterns would differ between the two conditions in females. However, it was found that the CEA activation was similar within all females, across the two conditions. It is important to note that the sample size in the current experiment was small and additional subjects are needed before final conclusions can be drawn. Additionally, more extensive analysis across rostro-caudal extent of the CEA will also be conducted in future studies.

Although there was not a significant difference in activation between experimental and control groups of the same sex, there was a trend for greater CEA activation in the control groups compared to the experimental groups regardless of sex. Lastly, it was hypothesized that there would be significant differences in neural activation between sexes. This effect was observed, with males showing greater CEA activation than females.

In conclusion, the overall results implicate the CEA as a mediator of feeding behavior: greater CEA activation correlated with groups that consumed more food.

The female rats low CEA activation, reduced consumption during appetitive training sessions, and inability to extinguish inhibition of consumption during the test sessions indicates that females may be showing a greater generalized response as a result of aversive experiences than males. These findings may help delineate the underlying neurological basis for increased female susceptibility to anxiety and eating disorders.

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