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Appetitive associative learning recruits a distinct network with cortical, striatal, and hypothalamic regions

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Abstract

The amygdala, prefrontal cortex, striatum and other connected forebrain areas are important for reward-associated learning and subsequent behaviors. How these structurally and functionally dissociable regions are recruited during initial learning, however, is unclear. Recently, we showed amygdalar nuclei were differentially recruited across different stages of cue-food associations in a Pavlovian conditioning paradigm. Here, we systematically examined Fos induction in the forebrain, including areas associated with the amygdala, during early (day 1) and late (day 10) training sessions of cue-food conditioning. During training, rats in the conditioned group received tone-food pairings, while controls received presentations of the tone alone in the conditioning chamber followed by food delivery in their home cage. We found that a small subset of telencephalic and hypothalamic regions were differentially recruited during the early and late stages of training, suggesting evidence of learning induced plasticity. Initial tone-food pairings recruited solely the amygdala, while late tone-food pairings came to induce Fos in distinct areas within the medial and lateral prefrontal cortex, the dorsal striatum, and the hypothalamus (lateral hypothalamus and paraventricular nucleus). Furthermore, within the perifornical lateral hypothalamus, tone-food pairings selectively recruited neurons that produce the orexigenic neuropeptide orexin/hypocretin. These data show a functional map of the forebrain areas recruited by appetitive associative learning and dependent on experience. These selectively activated regions include interconnected prefrontal, striatal, and hypothalamic regions that form a discrete but distributed network that is well placed to simultaneously inform cortical (cognitive) processing and behavioral (motivational) control during cue-food learning.

Keywords

appetitive conditioning; Fos; prefrontal cortex; lateral hypothalamus; orexin; dorsal striatum

Appetitive associative learning is important in the control of motivated behaviors essential for survival, including feeding. Through associative learning, neutral cues from the

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environment can become signals for food and gain the ability to powerfully control feeding behavior. Associative food cues can alter multifaceted aspects of feeding behavior. Learned cues prepare the animal physiologically to ingest the food (i.e., salivation and insulin changes), increase approach and action toward the food, and increase consumption of the food even in the absence of hunger (Pavlov, 1927; Estes, 1946; Woods et al., 1976; Weingarten, 1983).

Within the laboratory the most often used form of appetitive associative learning is Pavlovian conditioning (Pavlov, 1927), where an initially neutral signal from the environment such as a tone (conditioned stimulus, CS) is repeatedly paired with reward delivery (unconditioned stimulus, US). Through this training the CS acquires the ability to act as a food signal and subsequently influence the motivation for food seeking and consumption. This conditioning forms the basis of many paradigms examining facets of appetitive learning, reward, and ingestive behavior, including cue-potentiated feeding, Pavlovian-instrumental transfer (PIT), and other forms of incentive learning, (for review see Holland and Petrovich, 2005). Even though this basic associative learning plays a critical role in guiding motivated appetitive behaviors, the neural substrates underlying the CS-food learning are largely unknown. Identifying the learning network and critical changes during CS-food association is important in revealing the neural mechanisms and plasticity underlying this form of learning and its numerous behavioral sequelae.

We recently began to study the neural substrates underlying Pavlovian appetitive conditioning, focusing on the amygdala, and using induction of the immediate early gene *c-fos* protein (Fos) as a marker of activation. We compared amygdalar recruitment during early and late training sessions. Rats received identical tone-food training sessions for either one or ten days, which therefore allowed us to directly assess differences due to the amount of learning (see Figure 1A). We found that distinct basolateral and central amygdalar nuclei were differentially activated across training (Cole et al., 2013), suggesting evidence of learning induced plasticity. These amygdalar nuclei operate through dissociable circuitries that could convey information about the CS-food association to a distributed network, and ultimately mediate different aspects of learning and the subsequent control of behavior. How that connectational network is functionally recruited during learning is unknown. Therefore, here we extended this earlier analysis and examined 27 additional telencephalic and hypothalamic regions. These regions included both areas connected with the critical amygdalar nuclei previously identified, and areas important for learning and ingestive behavior. The aim of this study was to generate a functional map of forebrain areas underlying appetitive associative learning.

2 EXPERIMENTAL PROCEDURES

2.1 Animals

Forty-seven experimentally naïve, male Long-Evans rats (276-300g) obtained from Charles River Laboratories (Portage, MI) were used. The animals were individually housed with *ad libitum* access to food and water except when otherwise noted. The colony room was maintained at 21°C on a 12 hour light/dark cycle (lights on 06:00) and all behavioral testing was conducted during the light phase of the cycle. Rats were given one week to acclimate to

the colony room during which time they were handled and weighed daily. The housing and testing procedures were in accordance with the National Institute of Health *Guidelines for Care and Use of Laboratory Animals* and approved by the Boston College Institutional Animal Care and Use Committee.

2.2 Apparatus and Behavioral Procedures

Behavioral training was described in detail previously (Cole et al., 2013), and was conducted in a set of behavioral chambers (Coulbourn Instruments, Allentown, PA, USA) located in a room different than the housing room. Each chamber was located in a sound- and light-attenuating cubicle that was equipped with a ventilation fan (55 dB) and video camera attached to a recording system in an adjacent room to record each training session (Coulbourn Instruments, Allentown, PA). The CS was a 10s 75dB, 2kHz tone, and the US consisted of two food pellets (formula 5TUL, 45mg; Test Diets, Richmond, IN, USA) delivered to the food-cup of each chamber, unless otherwise noted.

Rats were gradually reduced to 85% of their *ad libitum* weight, and remained food-restricted throughout behavioral training. All animals initially received two days of habituation to the behavioral chambers for 32 minutes each. Following the second habituation session all animals received 1g of the food US in their home cage to familiarize them with the pellets.

Experimental design is shown in Figure 1A and described next. Behavioral training sessions were 32 minutes long and consisted of eight presentations of a tone CS followed immediately by delivery of the food US into the food-cup (Paired group). The inter-trial intervals between CS presentations in each training session were random (range 110–326 seconds) and varied across days. Control groups received identical sessions in the behavioral chambers, but with eight presentations of the CS only, followed after a random interval ranging from 30–270 minutes by US (16 pellets) delivery in their home cage. One of the control groups was sacrificed after the session in the chamber (Tone group; thus they did not receive the US on the final day), and the other after food consumption (Food group). For the groups that received US delivery in the home cage during training (Tone and Food), the 16 pellets were delivered at once onto the bedding. All rats in all groups consumed all the pellets given.

To examine Fos induction early in training half of the animals were perfused and the brains collected after a single training session (early training). Animals in the conditioning group (early Paired: n=8) and one of the control groups (early Tone: n=8) were perfused 120 minutes following the beginning of the training session. The second control group (early Food: n=8) was perfused 105 minutes following US presentation in the home cage to coincide perfusion time with the middle of the training session for the Paired and Tone groups.

To examine how neuronal activation patterns changed as a result of learning, the other half of the animals received ten days of training (late training). Late training animals (late Paired: n=7, late Tone: n=8, late Food: n=8) were perfused at the same time points following training/stimulus presentation as early training animals. Thus, both early and late training

groups received identical training sessions, the only difference was how many sessions they received.

The control groups in this study (groups Tone and Food) were designed for Fos imaging, and as such aid interpretation of differential Fos induction. A potential alternative control group would be an unpaired group, which would receive explicitly unpaired presentations of the tone and food in the training context. However, such training would result in the animals learning a context-food association (rather than a cue-food association) and so it would not allow for differential detection of learning-driven activation, which was the main purpose of our study. Instead, the inclusion of the Tone and Food controls used here allowed identification of a critical subsystem that was selectively recruited during tone-food learning, and allows numerous alternate interpretations of the results to be ruled out (see Discussion).

2.3 Histological Procedures

Rats were anaesthetized by an intraperitoneal injection of tribromoethanol (375 mg/kg body weight) and transcardially perfused with 0.9% saline followed by ice cold 4% paraformaldehyde in 0.1M borate buffer (pH 9.4). The brains were stored for 20-24 hours at 4°C in the fixative with 12% sucrose and then rapidly frozen in hexanes cooled with dry ice and stored at -80°C. Frozen brains were cut into 30µm coronal slices using a sliding microtome (Leica SM200R) and collected into four serially adjacent sets. One series of sections was used to identify Fos with standard immunohistochemistry as described previously (Cole et al., 2013). A second series of sections were mounted from potassium phosphate-buffered saline (KPBS) onto chrome alum/gelatin-coated slides and stained with thionin (Simmons and Swanson, 1993) for identification of nuclear borders as defined in the rat brain atlas of Swanson (2004). The remaining two tissue series were collected into trays containing a cryoprotectant solution (0.025 M sodium phosphate buffer with 30% ethylene glycol and 20% glycerol) and stored at -20 °C until processing. One tissue series (n=47) underwent double immunohistochemical processing for combined detection of Fos and orexin. Free-floating sections were rinsed three times in KPBS, incubated for one hour at room temperature in a blocking solution of KPBS containing 2% normal horse serum (NHS) (S-2000; Vector Laboratories, Burlingame, CA, USA), 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), and 10% non-fat milk, and then incubated with anti-orexin-A antibody raised in goat (1:2000, SC-8070; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) in the blocking solution for 72 hours at 4°C with gentle agitation. Sections were then rinsed with KPBS, 2% NHS, and 10% non-fat milk, incubated with biotinylated secondary antibody against goat (1:500, BA-9500; Vector Laboratories) in the blocking solution, rinsed in KPBS, incubated in avidin biotin complex (ABC, PK-6100; Vector Laboratories), rinsed in KPBS, and recycled through secondary and ABC solutions with KPBS rinses in between such that the total time in each incubation was 75 minutes. Sections were recycled in this manner to further minimize non-specific labeling. Neurons immunoreactive (IR) for orexin/hypocretin (de Lecea et al., 2008; Sakurai et al., 2008) were visualized as brown after a two-minute incubation in 3, 3'-diaminobenzidine (SK-4100; Vector Laboratories). Brain sections were then rinsed three times in KPBS, and processed again in a similar manner but using normal goat serum (NGS) (S-1000; Vector Laboratories), anti-*c-fos* antibody raised in rabbit

(1:5000, SC-52; Santa Cruz Biotechnology Inc) and biotinylated secondary antibody against rabbit (1:500, BA-1000; Vector Laboratories). Nuclei IR for Fos were visualized as black after a two-minute incubation in nickel-intensified 3, 3'-diaminobenzidine (SK-4100; Vector Laboratories). Sections were then rinsed, mounted on SuperFrost slides (Fisher Scientific, Pittsburgh, PA, USA), dried at 40°C, dehydrated through graded alcohols, cleared in xylenes, and coverslipped with DPX Mountant (Electron Microscopy Services, Hatfield, PA, USA).

2.4 Image Acquisition and Analysis

2.4.1 Single-labeled Fos—To select areas for analysis, a well-trained observer unaware of experimental condition examined Fos immunoreactivity throughout the forebrain in representative brains from each group. Areas with moderate or high levels of Fos expression were chosen for quantitative analysis, except if the pattern appeared identical across groups (e.g., lateral habenula). In addition, the ARH was analyzed even though labeling was light because of its well-known role in the control of feeding behavior. This process selected 27 distinct areas, which included areas well known for their role in ingestive behavior and learning. The analysis followed parcellation and nomenclature as defined in the Swanson atlas (2004), except for the striatum, lateral hypothalamic area and paraventricular thalamic nucleus, and these exceptions are depicted in Figure 2 and described in detail below. Our analysis initially focused on the lateral hypothalamus because of prior work demonstrating its importance to appetitive learning related phenomena (e.g., Destrade and Jaffard, 1978; Petrovich et al., 2002), and involved extensive, detailed analysis throughout its rostral-caudal extent. However, the patterns of activation did not differ across the levels analyzed, which was also the case for other cell groups, and thus subsequent analyses of regions were conducted from tissue sampled bilaterally at a mid rostro-caudal level.

Images of the Fos-stained and adjacent thionin-stained sections were acquired (10X magnification) with an Olympus DP72 camera and DP2-BSW software (Olympus America Inc, Center Valley, PA, USA). Using Image J software (NIH) the images were stacked and transformed to 8-bit grayscale. After the cell group of interest was identified, either nuclear borders were drawn or a rectangular template was placed on the thionin-stained section, and automated counting was performed on the adjacent Fos-stained section in the area where the border/template was placed. The threshold for counting was set manually, and was determined from an area on each section with no specific labeling (background). Automated counting was then performed consistently across sections and brains using the same criteria. The criteria were determined before the start of analysis and accuracy was confirmed by comparing the automated counts with manual counts by an observer unaware of the experimental condition. Images were acquired bilaterally for each cell group analyzed and left and right counts were summed for each rat, then averaged for each group resulting in a mean total of Fos-labeled neurons.

2.4.1.1 Cortex: Seven regions were analyzed within the medial and lateral prefrontal cortex, and the hippocampal formation. Within the medial prefrontal cortex these regions were the dorsal part of the anterior cingulate area (ACAd), the prelimbic area (PL), and the

infralimbic area (ILA) (all Level 9, +2.80 mm from Bregma; all subsequent measurements refer to mm from Bregma).

Within the lateral prefrontal cortex, we analyzed the dorsal part of the agranular insular area (AI_d) (Level 10, +2.15 mm) and the posterior part of the agranular insular area (AI_p) (Level 22, -0.83 mm). The lateral part of the orbital area (OR_{Bl}) (Level 7, +3.60 mm) was too large to fit within a single image, and thus the sampling area was formed using a rectangular template created with Image J software centered under the claustrum (shown in Figure 2). Within the hippocampal formation, to sample from the ventral part of CA1 (Level 39, -6.06 mm) and the ventral part of the subiculum (SUB_v) (Level 39, -6.06 mm) a rectangular template created with Image J software was centered over the border of these two structures (shown in Figure 2).

2.4.1.2 Striatum: Six regions were analyzed within the dorsal and ventral striatum. Within the dorsal striatum we analyzed three regions of the caudoputamen (CP). Within the anterior CP (Level 18, -0.11 mm) we sampled from its medial and lateral parts (separated by a vertical line through the center of the CP, shown in Figure 2), and these areas we refer to as the dorsomedial striatum (DMS) and the dorsolateral striatum (DLS), respectively. The third sample was from the posterior CP (Level 28, -2.45 mm). We used a rectangular template to sample from an area we refer to as the posterior dorsal striatum (pDS) (see Figure 2).

In the ventral striatum we analyzed three regions within the nucleus accumbens (ACB) (Level 13, +1.20 mm). The core region of the ACB (ACB_c) was formed by a line extending from the lateral ventricle around the anterior commissure at a distance approximately equal to half the width of the anterior commissure. The dorsal portion of the shell (ACB_{shD}) was defined ventrally by a horizontal line extending from the dorsal edge of the anterior commissure, while the ventral portion of the shell (ACB_{shV}) was bordered laterally by a vertical line extending from the lateral edge of the anterior commissure (see Figure 2).

2.4.1.3 Thalamus: Within the thalamus we analyzed the paraventricular nucleus, where we sampled from its anterior and posterior halves, areas that we refer here to as anterior (PVT_a) (Level 26, -1.78 mm) and posterior (PVT_p) (Level 30, -3.25 mm) respectively. The PVT is a large nucleus, and we assigned these divisions for separate analyses because there are connectional differences between the anterior and posterior parts (see Li and Kirouac, 2012).

2.4.1.4 Hypothalamus: Eleven hypothalamic cell groups located within the tuberal and anterior regions were examined. Within the lateral hypothalamic area, 21 distinct cell groups were identified in the most recent parcellation of this structure (Swanson, 2004). However, in order to be able to sample reliably from the same regions across different brains, we defined three larger areas for analysis: perifornical, lateral, and ventral (Figure 2). The perifornical area (LHA_{pf}) (Levels 27-30, -2.00 to -3.25 mm) we defined here contained the juxtadorsomedial and supraforinal nuclei (Swanson, 2004; Hahn and Swanson, 2010, 2012). The lateral area (LHA_l) (Levels 27-30, -2.00 to -3.25 mm) included the region lateral to the LHA_{pf}, and was bordered dorsally by a line that matches the dorsal end of the third ventricle and ventrally by the ventral edge of the fornix. The ventral area (LHA_v) (Levels 27-30, -2.00 to -3.25 mm) was defined as the area bordered medially by the lateral

edge of the ventromedial hypothalamic nucleus, dorsally by the ventral edge of the fornix, and laterally by the edge of the tissue. In addition to the LHA, we analyzed the arcuate hypothalamic nucleus (ARH) (Levels 27-30, -2.00 to -3.25 mm), the dorsomedial hypothalamic nucleus (DMH) (Levels 28-30, -2.45 to -3.25 mm), and six divisions of the paraventricular hypothalamic nucleus (PVH): the anterior parvicellular part (PVHap) (Level 24, -1.33 mm), dorsal parvicellular part (PVHdp) (Level 26, -1.78 mm), the dorsal zone of the medial parvicellular part (PVHmpd) (Level 26, -1.78 mm), ventral zone of the medial parvicellular part (PVHmpv) (Level 26, -1.78 mm), lateral zone of the posterior magnocellular part (PVHpml) (Level 26, -1.78 mm) and the periventricular part (PVHpv) (Level 26, -1.78 mm).

2.4.2 Double-labeled Orexin + Fos—To determine the distribution of Fos induction in orexin neurons, images of the tissue were taken through mid rostro-caudal LHA (Levels 28 and 29, -2.45 and -2.85 mm), where the highest density of orexin neurons are located (Swanson et al., 2005). Images were acquired using the 20x objective on the microscope equipment described above. Two images were taken bilaterally for each level. The first image encompassed the area directly above and medial to the fornix (LHApf), and the second sampled from the area lateral to the fornix (LHAf). Neurons were counted from color images on a screen manually by an observer unaware of the experimental condition of the images. Single-labeled orexin positive neurons were identified by distinct cytoplasmic staining, and double-labeled neurons (orexin + Fos) had both cytoplasmic-orexin and nuclear-Fos labeling. An orexin-containing neuron was counted as positive only if both the cell body and the nucleus were clearly visible in the image. Double-labeled neurons were counted as positive if the Fos-labeling intensity was clearly above the background and contained within the nucleus of an orexin positive cell. Bilateral counts were summed across levels for each rat for each region. The percentage of the total orexin-positive neurons that were also labeled with Fos (double-labeled orexin and Fos) was calculated for each sampling region.

2.5 Behavioral Observations

Observations of animals' behavior were made from recorded training sessions by trained observers unaware of group allocation. The primary measure of conditioning was expression of food-cup behavior. This behavior was defined as nose pokes into the recessed food-cup, or standing in front of and directly facing the food-cup. Behavior was scored every 1.25 seconds during each 10-second CS and pre-CS periods (i.e., eight observations during each trial). The pre-CS period was the 10 seconds immediately preceding each CS presentation. The total number of food-cup observations during CSs and pre-CSs for each session were summed and that number was then converted to a percentage of time rats expressed food cup during the total observation period. There were eight trials in each session. Therefore in each training session, 100% of CS food-cup behavior would reflect 64 total observations of food-cup during CS presentations. The same is true for pre-CS responding, that is 100% would reflect 64 total observations during pre-CSs of that session.

2.6 Statistical Analysis

Counts of single-labeled Fos-positive or orexin-positive neurons were analyzed with one-way ANOVAs, and when appropriate followed by Fisher's least significant difference *post hoc* comparisons. The total number and percentage of double-labeled neurons were analyzed by means of a planned orthogonal contrast testing procedure. Correlation of Fos expression between regions with significant group effects was calculated using the Pearson product-moment correlation coefficient (r). Data from the following were not collected due to tissue damage: one early training Tone (ARH), one early training Food (CA1 and SUBv), and one late training Tone (LHApf, LHA1, and LHA v). The statistical packages PSY (Bird, 2004) and SPSS (v.21) were used for all analyses and type I error rate was controlled at 0.05 unless otherwise noted.

3 RESULTS

3.1 Behavior

As shown in Figure 1B, we found evidence of appetitive learning only in the Paired group during the early training session (day 1). The Paired group spent significantly more time at the food-cup (conditioned response) during the final two CSs compared to the first two CSs ($F(1, 7) = 13, p < 0.01$), and significantly more time at the food-cup than Tone and Food groups ($F(1, 21) = 12.589, p < 0.01$). The Paired group did not show an increase in pre-CS responding (immediately prior to the tone presentation) ($p > 0.05$) confirming that the increase in food-cup behavior was specific to the CS (Figure 1C).

For late training animals, Paired animals showed greater CS-food cup behavior overall across all training sessions than Tone and Food groups ($F(1, 20) = 208.869, p < 0.0001$). Furthermore, only the Paired group showed a significant increase in CS responding across days of training ($F(1, 20) = 7.963, p < 0.05$) (see Figure 1D). Importantly, this increase in food-cup behavior in Paired animals was a specific learned response to the conditioned cue (CS), because there was no such increase during pre-CS across days ($p > 0.05$) (Figure 1D).

During the final session (day 10), only animals in the Paired group expressed robust appetitive conditioning which was specific to the CS. There was overall more food-cup behavior shown during the CSs than the pre-CS periods ($F(1, 20) = 72.459, p < 0.0001$) which was greater in Paired animals ($F(1, 20) = 120.009, p < 0.0001$) (Figures 1E-F). The level of CS-responding seen in Paired animals was comparable to prior studies employing similar training procedures (e.g., Hatfield et al., 1996).

3.2 Fos Induction

The patterns of Fos expression differed dramatically from early to late training within a number of forebrain structures. Following the early training session only three forebrain regions showed significant differences in Fos expression across groups (see Table 1 for all brain structures). None of the areas showed differential expression between Paired and Tone groups. Nevertheless, there was more Fos in the Paired and Tone groups compared to the Food group in the ACBc (p values < 0.05), LHApf (p values < 0.01), and LHA1 (p values < 0.01) (Figure 3; Table 1).

Fos induction patterns following the late training session differed from the patterns following the early session in two ways. First, we observed that after the late session a number of cortical, striatal, and hypothalamic regions had increased Fos selectively in the Paired group. Second, the activation profile we observed following the early training session (Paired and Tone greater than Food) changed. After the late session, the LHApf showed specific Paired induction, the LHA1 maintained the same pattern, and there were no longer group differences within the ACBc. Nevertheless, we found additional forebrain regions that showed this pattern (Paired and Tone greater than Food) (Table 2).

Three prefrontal cortical regions had significantly greater Fos expression in the Paired group compared with Tone and Food groups, which did not differ (Figure 4A). These regions were the PL (p values < 0.001), ILA (p values < 0.001), and AId (p values < 0.05). Figure 4 shows representative Fos induction in the PL (4B) and AId (4C) for all late training groups. In contrast, another set of cortical regions had more Fos in the Paired and Tone groups compared to the Food group (Figure 4A). These regions were the ACAd (p values < 0.05), ORBl (p values < 0.05), and CA1 (p values < 0.001).

The other regions that expressed significantly more Fos in the Paired group were in the dorsal striatum and the hypothalamus. Specifically, these cell groups were the DMS (Figure 5), PVHpml, and LHApf (Figure 6A). There was significantly more Fos in the DMS of the Paired group compared to Tone and Food groups, which did not differ (p values < 0.01). This pattern was also true for the PVHpml (p values < 0.001). Figure 6B shows representative Fos induction in the PVHpml. Interestingly, the LHApf showed a unique profile of Fos induction, where the Paired group had overall greater Fos than Tone and Food groups, but the Tone group also had more Fos compared to the Food group (p values < 0.01). This region was also unique in that it was the only area with selective Fos induction late in training that also had significant group differences early in training, where Paired and Tone groups expressed more Fos than the Food group. This difference from early to late training appears to be due to less activation in the Tone group during late training, while the Paired group remained high. Finally, the only other hypothalamic region that showed group differences in Fos activation was the LHA_v, with greater Fos in Paired and Tone groups compared to the Food group (p values < 0.01). Many areas examined showed no significant group differences in Fos expression (see Tables 1 and 2); Figure 7 shows representative Fos induction in one such region, the PVTp.

To explore the functional relationship between the brain regions that showed Paired-specific Fos induction, we examined the correlations between these structures. Of particular interest were the correlations between the medial prefrontal cortex areas (PL and ILA) with the lateral hypothalamus (LHApf). This is because prior work has demonstrated the importance of these pathways in the regulation of feeding (e.g., Petrovich et al., 2005; Mena et al., 2013). Given that our results comprise novel evidence implicating the DMS in tone-food learning, we also examined the correlations between the DMS and other regions that showed Paired-specific Fos induction. Results of statistical analyses using Pearson's correlation coefficient are displayed in Table 3. We found that Fos in the PL was significantly correlated with the LHApf ($r = .62$), and also with the ILA ($r = .89$), AId ($r = .54$), DMS ($r = .75$), and PVHpml ($r = .42$). The pattern was identical for the ILA, which also showed

significant correlation with the LHApf ($r = .65$), and with the AId ($r = .59$), DMS ($r = .75$), and PVHpml ($r = .57$). Additionally, we found significant correlations between the DMS, and all other regions examined except the PVHpml (see Table 3). These results, therefore, identify a functional network that emerges by late tone-food training.

3.3 Fos Induction in Orexin Neurons

We used double-label immunohistochemistry to examine whether Fos induction in the LHA was in orexin neurons. During early training there were no differences in double-labeled (Fos and orexin) neurons in either the LHA or LHApf across groups (p values > 0.05). There were no differences in the total number of orexin neurons identified in the LHA for either early or late training (p values > 0.05). Figure 8A shows representative photomicrographs of the LHApf for all groups following late training. During late training we observed significantly more double-labeled neurons in the LHApf of the Paired group compared with Tone and Food groups ($p < 0.05$), whereas Tone and Food groups did not differ from each other ($p < 0.05$) (Figure 8B). We found the same group differences when analyses were performed using the percentages of orexin neurons expressing Fos (Figure 8C). The Paired group had a greater percentage of double-labeled neurons than Tone and Food groups ($p > 0.05$), which did not differ from each other ($p < 0.05$).

4 DISCUSSION

Here, we examined plasticity in the recruitment of forebrain areas across early and late stages of appetitive associative learning. To accomplish this, we assessed activation profiles of forebrain areas (measured with Fos induction) during tone-food learning, and compared the pattern of recruitment following the first training session (early training) to that following the tenth training session (late training). We analyzed the functional activation of 27 cell groups that included areas important in associative learning and ingestive behavior.

We found evidence of network plasticity in that the patterns of Fos induction in several regions changed significantly from early to late training. There was selective recruitment of distinct forebrain regions in the Paired group following the late training session, but not following the early training session. We observed significantly elevated Fos induction in the Paired group within the PL, ILA, AId, DMS, PVHpml, and LHApf. Within the lateral hypothalamus we further found that the late tone-food session recruited orexin neurons in the LHApf. That area had significantly more double-labeled orexin and Fos positive neurons in the Paired group compared to controls.

It is important to note that the increases in Fos expression we observed in the Paired group cannot be attributed to arousal associated with exposure to the tone itself, the conditioning chamber, transport, or handling, because Tone group received equal exposure to all of these stimuli. Furthermore, the observed Fos induction in Paired group is not simply due to the consumption of the food US, because the Food group consumed an identical quantity under the same food-deprived state. The amount of food given to Paired and Food animals was small, however it was sufficiently motivating to support learning as evidenced by significant behavioral change in the Paired animals. Furthermore, consumption of the food was sufficient to induce significant Fos expression (see Tables 1 and 2). Thus, the groups used in

the current study not only controlled for behavioral effects, but also were appropriately designed for Fos imaging and allowed us to identify and directly compare forebrain areas that are specifically activated by CS-food pairings across different stages of learning.

The early and late training sessions were identical. Therefore changes in activation in the late training groups suggest a re-organizing of these regions due to the learning/training experience of the animals. Despite an extensive analysis, we only detected this learning-induced change in a small subset of regions. Interestingly, the small, critical group that emerged is distributed across structural systems that include cortical, striatal, and hypothalamic areas (Figure 8).

4.1 Prefrontal Cortex

Within the medial prefrontal cortex, we found selective recruitment of the PL and ILA, but not ACAd during late tone-food training. This is especially noteworthy considering the PL and ILA have been implicated in appetitive tasks that rely upon such cue-food associations to modulate and guide feeding behavior (e.g., cue-potentiated feeding: Petrovich et al., 2007; PIT: Homayoun and Moghaddam, 2009). The increased Fos we detected in the Paired group here was not simply due to food consumption, because the Food group ate precisely the same amount of food, but expressed significantly less Fos. As such, the current findings show that the PL and ILA are recruited during tone-food learning. Therefore these structures that are important for regulating behavior in appetitive-related tasks are also important earlier when the initial cue-food associations are formed. More generally, our findings add to accumulating evidence implicating these regions in the control of feeding and appetitive-related behaviors (Corbit and Balleine, 2003; Petrovich et al., 2005; Nair et al., 2011; Mena et al., 2011, 2013; Cifani et al., 2012; Burgos-Robles et al., 2013; Calu et al., 2013; Land et al., 2014).

Within the lateral prefrontal cortex, only the AId showed elevated Fos in the Paired group. It is important to note that many studies that functionally examined the lateral prefrontal cortex in appetitive and reward behaviors targeted an area that includes at least part of both the AId and ORBI defined here. Nomenclature inconsistencies notwithstanding, these studies suggest distinct roles for these different lateral prefrontal cortical areas in reward behavior. Prior studies have demonstrated that the AId is necessary for memory of the value or magnitude of a food reward (DeCoteau et al., 1997; Kesner and Gilbert, 2007) and the value of an instrumental outcome (Balleine and Dickinson, 2000), which suggests that the AId is involved in memory related to the rewarding outcome. In contrast, the orbitofrontal cortex region is important for outcome-guided behavior and signaling outcome expectancies (see Schoenbaum et al., 2009, 2011 for review).

Anatomically, the AId is well placed to provide information on the value of the food, and associated cues, to other cortical regions important for appetitive tasks. It is reciprocally connected with the anterior basolateral amygdalar nucleus (BLAa)—the only region recruited in early training (Cole et al., 2013)—, and also sends projections to the PL as well as the posterior basolateral amygdalar nucleus (Kita and Kitai, 1990; Shi and Cassel, 1998). In accordance with this connectional pattern, here we found that the PL, ILA, and AId are all components of a functional network recruited during appetitive conditioning. Within this

network, it is likely that these regions mediate different aspects of behavior subsequent to this learning. While PL and ILA might be necessary to regulate appetitive behavior and food consumption based on motivation induced by food-associated signals, the AId might act to retrieve the value of the food outcome and guide PL and ILA and behavior accordingly. One important target for the control of feeding motivation is the LHA.

4.2 Hypothalamus

The LHA is a well-recognized, integral node of the feeding circuit and has been traditionally associated with the initiation of eating (Hetherington and Ranson, 1940; Anand and Brobek, 1951; for review see Elmquist et al., 1999). Here, we found that within the LHA, specifically the LHApf was selectively activated by tone-food pairings. Functional and anatomical studies have provided evidence linking this region to feeding and motivation (Petrovich et al., 2002, 2012; Stanley et al., 2011).

Recently, Hahn and Swanson (2010) examined the afferent and efferent connections of the suprafoaminal region of the hypothalamus, which constitutes half of the LHApf region defined in the present study. They reported a connectional pattern that would support a central role for this region in the regulation of ingestive behavior (Hahn and Swanson, 2010), and also demonstrated that it receives dense inputs from the ILA and PL. Particularly noteworthy is that we found selective activation within the PL, ILA, and LHApf, and Fos expression in these interconnected areas was highly correlated, suggesting a functional network. In accordance, a role for this prefrontal cortex to LHApf pathway in appetitive behavior is further supported by recent, converging evidence.

Previously, we showed with functional anatomical methods that a direct pathway from the mPFC to the LHA is selectively recruited by a food-associated cue that drives consumption (Petrovich et al., 2005). More recently, Mena, Selleck, and Baldo (2013) demonstrated that stimulating PL and ILA neurons through the μ -opioid system elicited feeding in sated rats, and induced Fos expression specifically in the LHApf. Moreover, they demonstrated that blockade of NMDA receptors in the LHApf significantly reduced the mPFC stimulatory effect on feeding (Mena et al., 2013). Taken together, these findings are consistent with the view that the PL and ILA could mediate feeding behavior via control over LHApf. Here, our data showed that during a late training session when a learned tone-food association was robustly expressed, the PL, ILA, and LHApf were similarly recruited, however, during the early training session none of these regions showed selective Fos expression in the Paired group. These differential patterns of activation therefore reflect a change in responding of this circuit as tone-food associations become certain. These findings suggest that during learning the PL/IL-LHApf circuitry encodes the tone-food association (the PL and ILA), and through output to the LHApf come to elicit conditioned motivation and subsequent behaviors.

The LHApf contains neurons that synthesize the neuropeptide orexin, which stimulates feeding, however orexin is also important for wakefulness and arousal as well as reward and other motivated behaviors (see Yeoh et al., 2014 for review). Prior studies have shown that orexin neurons also respond to reward and food cues (Harris et al., 2005; Petrovich et al., 2012), and that antagonizing orexin receptors decreases cue-induced responding for sucrose

and saccharin (Cason and Aston-Jones, 2013a,b). For these reasons, here we examined Fos induction in orexin neurons during tone-food learning. While there was no selective induction in the early training session, during a late training session cue-food pairings increased the induction of Fos in orexin neurons, specifically within the LHApf. This result demonstrates that orexin neurons in the LHApf become engaged during training, and may later be reactivated by learned cues acquired through this cue-food conditioning to critically regulate appetitive behaviors (e.g., during cue-potentiated feeding; Petrovich et al., 2012).

We observed that orexin neurons in the LHApf, but not in the LHA_l, were activated during tone-food conditioning. This is consistent with other studies that showed involvement of LHApf orexin neurons in response to food and food-associated cues (Zheng et al., 2007; Choi et al., 2010; Petrovich et al., 2012; Mena et al., 2013). Such findings are inconsistent with prior evidence that orexin neurons in the LHA_l mediate reward processing for food and drugs, while orexin neurons located more medially (LHApf and DMH) regulate stress and arousal (see Harris and Aston-Jones, 2006). Further work is needed to elucidate how these complex orexin subsystems are organized and the circuits through which they operate.

In addition to the LHA, we examined three other hypothalamic nuclei important for the maintenance of energy homeostasis and the regulation of feeding behavior, the ARH, DMH, and PVH (Schwartz et al., 2000; Swanson, 2000). Interestingly, the only hypothalamic region where we detected greater Fos expression in the Paired group, in addition to the LHApf, was part of the PVH, the PVHpml. While the specific function of the PVHpml in feeding is not known, lesions of the entire PVH lead to hyperphagia (Leibowitz et al., 1991) clearly demonstrating that this region is necessary for the regulation of feeding (Swanson, 2000). The PVHpml predominantly contains oxytocin and vasopressin neurons (Simmons and Swanson, 2009). This is particularly interesting given that manipulations of either peptide interfere with the control of feeding behavior (e.g., Pei et al., 2014; see Olszewski et al., 2010 for review), and both are important for other forms of learning (Lahoud and Maroun, 2013; see Engelmann et al., 1996 for review). Our current findings that PVHpml neurons are recruited during tone-food learning indicate a potential role for PVH oxytocin and/or vasopressin function in appetitive associative learning and memory. Clearly, more research is needed to explore the role of these systems in learning-dependent appetitive tasks.

4.3 Striatum

Interestingly, the DMS was among the few regions we identified where tone-food pairings were associated with a selective increase in Fos expression. Following the late training session we observed significantly elevated Fos induction in the DMS in the Paired group. To our knowledge this is the first study to show activation of the DMS during cue-food associative learning. Current accounts of dorsal striatal function in reward learning emphasize a role in acquiring instrumental actions for food (e.g. lever-press for sucrose) (Yin et al., 2005; Shiflett et al., 2010; Bradfield et al., 2013; Corbit et al., 2013). Consequently, the present results are exciting because they suggest that the DMS is also involved during Pavlovian cue-food associations.

The dorsal striatum is largely considered a sensorimotor area due to its primary inputs from the sensorimotor cortex. However, the DMS has a unique connectional pattern, with afferents from higher order associational areas (see Voorn et al., 2004). Notably, the DMS receives heavy innervation from the PL and lighter projections from the ILA (Berendse et al., 1992), while the amygdalar inputs are from the BLAa (Kita and Kitai, 1990; Corbit et al., 2013). These connections suggest the DMS could be an important integrative site mediating appetitive associative learning and behavior. The input from the BLAa is especially noteworthy given that we previously demonstrated that the BLAa is the only amygdalar region activated during both early and late appetitive training (Cole et al., 2013). Importantly, in the current study we found that the BLAa remains the sole forebrain area with selective Fos induction during early tone-food associations. Furthermore, the PL, ILA, and BLAa are all recruited during late tone-food training, and Fos induction in these areas correlated significantly with Fos induction in the DMS. Taken together our findings are consistent with the idea that the BLA acts as the primary processor of emotional information, which through cooperation with cortical and striatal regions results in the appropriate appetitive behavioral response (Schoenbaum et al., 1998; Piette et al., 2012; Corbit et al., 2013; Parkes and Balleine, 2013). One possibility is that the DMS activation seen here may reflect its role in encoding of an association between the cue and the unique properties of the food pellets and its current value, as suggested in a recent study by Corbit and Janak (2010).

In contrast to the dorsal striatum, the ventral striatum (and its input from the BLAa) has strongly been implicated in Pavlovian appetitive behaviors (Parkinson et al., 1999; Di Ciano et al., 2001; Parkinson et al., 2002; Ito et al., 2008; Stuber et al., 2011; Chang et al., 2012, but see Chang and Holland, 2013; McDannald et al., 2013). However, here we found no evidence of selective recruitment of any ACB subregions during either early or late training of tone-food conditioning. The absence of selective ACB recruitment here seems to be inconsistent with those prior studies implicating the ACB in appetitive conditioning and related phenomena. There are procedural and methodological differences, however, between prior research and the current study that most likely accounts for these differences. For example, in autoshaping studies the CS was the insertion of an instrumental lever that the animals can interact with (Di Ciano et al., 2001; Chang et al., 2012). Other experiments involved more complicated training with multiple stimuli (Parkinson et al., 2002), multiple levels of association formation (McDannald et al., 2013), and more complex conditioning apparatuses (Ito et al., 2008). Nevertheless, we did find greater Fos induction in Paired and Tone groups compared to the Food group during early training specifically in the ACBc, which is in agreement, at least in part, with prior work. Consistent with the current findings, in previous studies a well-trained food cue failed to selectively activate ACB neurons that project to the LHA (Petrovich et al., 2005), and disconnections of the BLA-ACB system (preventing communication between BLA and ACB) had no effect on cue-potentiated feeding (Petrovich and Holland, 2005).

4.4 Additional Regions

In addition to the regions that showed selective Fos induction in the Paired group, we also identified areas that had similarly elevated Fos induction for Paired and Tone groups

compared to the Food group. Following an early training session these regions were the ACBc, LHApf, and LHA1, while during late training these regions were the ACAd, ORBI, CA1, and LHA_v. These Fos induction patterns could be the result of a learning process (learning the tone-food association or habituating to the tone-alone presentations), or simply due to the presence of the CS. Nevertheless, it suggests that while cue-food associations may not selectively recruit these regions, they might be involved in further learning or behavioral tasks, which rely upon information regarding the cue.

4.5 Conclusion

In summary, we identified a discrete subset of forebrain regions recruited during appetitive associative learning that includes distinct, interconnected cortical, striatal, and hypothalamic regions (Figure 9). This functional network was differentially recruited across training, which suggests learning-induced plasticity. The network components are well placed to share the information about the CS-food associations simultaneously with cortical (cognitive) and behavioral (motivational) systems, and to ultimately support learning and the subsequent control of behavior.

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ABBREVIATIONS

ACAd	anterior cingulate area, dorsal part
ACB	nucleus accumbens
*ACBc	nucleus accumbens, core
*ACBshD	nucleus accumbens, shell, dorsal part
*ACBshV	nucleus accumbens, shell, ventral part
AId	agranular insular area, dorsal part
AIp	agranular insular area, posterior part
ARH	arcuate hypothalamic nucleus
BLAa	basolateral amygdalar nucleus, anterior part
CP	caudoputamen
CS	conditioned stimulus
*DLS	dorsolateral striatum
DMH	dorsomedial hypothalamic nucleus
*DMS	dorsomedial striatum
ILA	infralimbic area
IR	immunoreactive

KPBS	potassium phosphate-buffered saline
LHA	lateral hypothalamic area
*LHA_{pf}	lateral hypothalamic area, perifornical region
*LHA_l	lateral hypothalamic area, lateral region
*LHA_v	lateral hypothalamic area, ventral region
NGS	normal goat serum
ORBI	orbital area, lateral part
*pDS	posterior dorsal striatum
PIT	Pavlovian-instrumental transfer
PL	prelimbic area
PVH	paraventricular hypothalamic nucleus
PVH_{dp}	paraventricular hypothalamic nucleus, dorsal parvicellular part
PVH_{mpd}	paraventricular hypothalamic nucleus, medial parvicellular part, dorsal zone
PVH_{mpv}	paraventricular hypothalamic nucleus, medial parvicellular part, ventral zone
PVH_{pml}	paraventricular hypothalamic nucleus, posterior magnocellular part, lateral zone
PVH_{pv}	paraventricular hypothalamic nucleus, periventricular part
*PVT_a	paraventricular thalamic nucleus, anterior part
*PVT_p	paraventricular thalamic nucleus, posterior part
SUB_v	subiculum, ventral part
US	unconditioned stimulus

*Asterisks denote nomenclature that differs from Swanson (2004).

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Highlights

This study examined forebrain Fos induction across early (day 1) and late (day 10) tone-food training sessions

Late training induced Fos in a small set of interconnected prefrontal, striatal, and hypothalamic regions

Late training also induced Fos in orexin/hypocretin neurons in the perifornical area of the lateral hypothalamus

Figure 1.

A. Experimental design. CS denotes presentation of a tone. US denotes delivery of food pellets. Each training session consisted of eight presentations, with animals receiving either one day or ten days of training. **B.** Mean ($\pm$ SEM) percentage of food cup behavior across CS presentations during early training (day 1). **C.** Mean ($\pm$ SEM) CS and pre-CS responding during the first and last two CS presentations during early training. **D.** Mean ($\pm$ SEM) percentage of CS and pre-CS food cup behavior across days of training. **E.** Mean ($\pm$ SEM) percentage of food cup behavior across CS presentations during late training (day 10). **F.** Mean ($\pm$ SEM) percentage of CS and pre-CS food cup behavior during late training. ★ $p < 0.05$. Figure from Cole et al. (2013) published in *Learning & Memory*.

Figure 2.

Figure shows sampling areas (dashed lines) for analyses in the regions that did not follow parcellation and nomenclature defined in the Swanson atlas (2004). Illustrations are templates modified from the Swanson atlas (2004), and numbers in the left upper corner of each denote atlas levels. See section 2.4 for a full description of Fos counting procedure and a description of all areas analyzed.

Figure 3.
Total number of Fos positive neurons during early training (mean $\pm$ SEM). ◆ indicates $p < 0.05$ compared to the Food group.

Figure 4.

Fos induction in cortical regions. **A.** Total number of Fos positive neurons in cortical regions during late training (mean $\pm$ SEM). $\star$ indicates $p < 0.05$ compared to Tone and Food groups. $\blacklozenge$ indicates $p < 0.05$ compared to the Food group. **B.** Representative photomicrographs of Fos induction in the prelimbic area of the medial prefrontal cortex following late training (*bottom*: Scale bar = 200 μ m) and adjacent, thionin-stained sections (*top* Scale bar = 500 μ m). The box depicts the area shown in the corresponding Fos image. **C.** Representative photomicrographs of Fos induction in the dorsal part of the agranular insular area following late training (*bottom*: Scale bar = 200 μ m) and adjacent, thionin-stained sections (*top* Scale bar = 500 μ m). Each box depicts the area shown in the corresponding Fos image.

Figure 5.

Total number of Fos positive neurons in striatal regions following late training (mean $\pm$ SEM). ★ indicates $p < 0.05$ compared to Tone and Food groups.

Figure 6.

Fos induction in hypothalamic regions. **A.** Total number of Fos positive neurons in hypothalamic regions during late training (mean $\pm$ SEM). ★ indicates $p < 0.05$ compared to Tone and Food groups. ◆ indicates $p < 0.05$ compared to the Food group. **B.** Representative photomicrographs of Fos induction in the paraventricular hypothalamic nucleus following late training (*bottom*: Scale bar = 100 μ m) and adjacent thionin-stained sections (*top*: Scale bar = 200 μ m). Each box depicts the area shown in the corresponding Fos image.

Figure 7.

Representative photomicrographs of Fos induction in the posterior paraventricular thalamic nucleus following late training (*bottom*: Scale bar = 200 μ m) and adjacent thionin-stained sections (*top*: Scale bar = 500 μ m). The box depicts the area shown in the corresponding Fos image.

Figure 8.

Fos induction in orexin neurons. **A.** Low (*top*) and high (*bottom*) power representative photomicrographs of Fos induction (gray nucleus) and orexin neurons (brown cytoplasm) in the perifornical region of the lateral hypothalamic area (LHA) following late training. Scale bar = 500 μ m (*top*), 50 μ m (*bottom*). Each box depicts the area shown in the high power image. fx = fornix. **B and C.** Total number of double labeled neurons (mean $\pm$ SEM) displayed both as a total count (B) and as a percentage of orexin-positive neurons (C). ★ indicates $p < 0.05$ compared to Tone and Food groups.

Figure 9.

Schematic illustrates connectional network of forebrain areas with activation across early and late CS-food learning. Bold red text denotes selective CS-food associated Fos induction. Initial CS-food pairings (day 1) were associated with selective activation of the BLAa, while CS-food pairings during a late training session (day 10) were associated with activation of the BLAa and additional amygdalar nuclei (Cole et al., 2013), as well as the prefrontal cortical areas (PL, ILA, and AId, not shown), DMS, and the hypothalamus (LHApf and PVHpml, not shown).

Table 1Early training statistical values and mean $\pm$ SEM numbers of Fos positive neurons

Brain Region	F	p	Paired	Tone	Food
<i>Cortex</i>					
ACAd	3.25	ns	256 $\pm$ 35	270 $\pm$ 33	165 $\pm$ 26
PL	2.32	ns	292 $\pm$ 27	225 $\pm$ 33	214 $\pm$ 22
ILA	2.27	ns	300 $\pm$ 28	300 $\pm$ 47	212 $\pm$ 22
ORBl	.38	ns	405 $\pm$ 56	406 $\pm$ 65	334 $\pm$ 78
Aid	.16	ns	85 $\pm$ 15	77 $\pm$ 8	77 $\pm$ 8
AIp	1.90	ns	57 $\pm$ 8	62 $\pm$ 7	43 $\pm$ 7
CA1	1.15	ns	47 $\pm$ 7	54 $\pm$ 7	40 $\pm$ 4
SUBv	.58	ns	54 $\pm$ 7	63 $\pm$ 12	50 $\pm$ 7
<i>Striatum</i>					
ACBc	4.29	.027	80 $\pm$ 14 [#]	78 $\pm$ 8 [^]	42 $\pm$ 7
ACBshD	.69	ns	111 $\pm$ 21	91 $\pm$ 18	84 $\pm$ 10
ACBshV	.05	ns	41 $\pm$ 9	44 $\pm$ 8	40 $\pm$ 10
DMS	1.71	ns	188 $\pm$ 51	12 $\pm$ 44	93 $\pm$ 8
DLS	2.13	ns	64 $\pm$ 18	33 $\pm$ 7	33 $\pm$ 9
pDS	.24	ns	73 $\pm$ 26	68 $\pm$ 9	56 $\pm$ 13
<i>Thalamus</i>					
PVTa	1.95	ns	126 $\pm$ 17	161 $\pm$ 19	121 $\pm$ 10
PVTp	1.10	ns	79 $\pm$ 14	102 $\pm$ 11	90 $\pm$ 8
<i>Hypothalamus</i>					
LHApf	15.91	.000	524 $\pm$ 42 ^{##}	643 $\pm$ 43 ^{^^}	308 $\pm$ 43
LHA1	6.89	.005	527 $\pm$ 67 ^{##}	511 $\pm$ 46 ^{^^}	301 $\pm$ 19
LHA _v	5.06	.016	375 $\pm$ 65	465 $\pm$ 47 ^{^^}	251 $\pm$ 21
ARH	3.12	ns	90 $\pm$ 16	176 $\pm$ 37	137 $\pm$ 18
DMH	2.07	ns	328 $\pm$ 18	426 $\pm$ 51	386 $\pm$ 24
PVHap	.81	ns	50 $\pm$ 7	42 $\pm$ 8	38 $\pm$ 5
PVHdp	.06	ns	6 $\pm$ 1	5 $\pm$ 2	6 $\pm$ 1
PVHmpd	2.18	ns	7 $\pm$ 2	8 $\pm$ 2	3 $\pm$ 1
PVHmpv	.52	ns	10 $\pm$ 2	12 $\pm$ 3	9 $\pm$ 1
PVHpml	.60	ns	6 $\pm$ 3	4 $\pm$ 1	4 $\pm$ 1
PVHp _v	.66	ns	7 $\pm$ 1	7 $\pm$ 2	5 $\pm$ 3

Results of one-way ANOVAs and Fisher's PLSD tests for every brain region analyzed. Fos counts are displayed as a mean total number for each area $\pm$ SEM. Significance levels for the *post hoc* comparisons refer to differences between Paired and Food

and between Tone and Food

ns denotes no significant group differences.

[#]
p < 0.05,

^{##}
p < 0.01

[^]
p < 0.05,

^{^^}
p < 0.02,

^{^^^}
p < 0.001

Table 2

Late training statistical values and mean (SEM) numbers of Fos positive neurons

Brain Region	F	p	Paired	Tone	Food
<i>Cortex</i>					
ACAd	9.16	.001	234 ± 29 ^{###}	181 ± 25 [^]	84 ± 22
PL	15.34	.000	263 ± 24 ^{*** ###}	153 ± 21	107 ± 16
ILA	19.16	.000	297 ± 28 ^{*** ###}	179 ± 12	135 ± 16
ORBl	8.14	.003	454 ± 50 ^{###}	332 ± 66 [^]	151 ± 38
Aid	4.00	.034	71 ± 5 ^{*#}	47 ± 7	46 ± 7
AIp	2.18	ns	55 ± 11	51 ± 7	33 ± 6
CA1	12.87	.000	46 ± 3 ^{###}	39 ± 4 ^{^^}	18 ± 5
SUBv	3.21	ns	54 ± 8	48 ± 7	18 ± 5
<i>Striatum</i>					
ACBc	1.58	ns	55 ± 5	37 ± 7	41 ± 9
ACBshD	.36	ns	82 ± 16	76 ± 12	63 ± 20
ACBshV	.45	ns	24 ± 5	34 ± 8	29 ± 9
DMS	6.34	.007	162 ± 34 ^{** ##}	67 ± 12	58 ± 19
DLS	2.20	ns	33 ± 8	19 ± 4	19 ± 4
pDS	2.01	ns	51 ± 8	29 ± 6	29 ± 11
<i>Thalamus</i>					
PVTa	1.82	ns	134 ± 10	115 ± 14	103 ± 9
PVTp	1.78	ns	98 ± 14	77 ± 6	70 ± 11
<i>Hypothalamus</i>					
LHApf	22.27	.000	509 ± 30 ^{** ###}	331 ± 33 ^{^^}	193 ± 37
LHA1	5.20	.016	461 ± 45 ^{##}	386 ± 60	257 ± 32
LHA _v	8.56	.002	327 ± 40 ^{###}	284 ± 74 ^{^^}	147 ± 29
ARH	1.74	ns	118 ± 25	124 ± 20	76 ± 16
DMH	1.33	ns	385 ± 53	280 ± 33	319 ± 49
PVHap	1.63	ns	36 ± 4	35 ± 2	28 ± 4
PVHdp	2.46	ns	6 ± 2	3 ± 1	3 ± 1
PVHmpd	1.19	ns	5 ± 2	5 ± 1	3 ± 1
PVHmpv	.80	ns	9 ± 2	6 ± 1	7 ± 2
PVHpml	9.85	.001	15 ± 5 ^{*** ###}	2 ± .3	2 ± .6
PVHp _v	1.52	ns	7 ± 2	6 ± 1	3 ± 1

Results of one-way ANOVAs and Fisher's PLSD tests for every brain region analyzed. Fos counts are displayed as a mean total number for each area ± SEM. Significance levels for the *post hoc* comparisons refer to differences between Paired and Tone

between Paired and Food

and between Tone and Food

ns denotes no significant group differences.

*
p < 0.05,

**
p < 0.01,

p < 0.001

p < 0.05,

p < 0.01,

p < 0.001

^
p < 0.05,

^^
p < 0.02,

^^^
p < 0.001

Table 3

Correlations in Fos expression following late training.

	AId	PL	ILA	DMS	PVHpml	LHApf
AId	1	0.536 **	0.593 **	0.452 *	0.318	0.302
PL	0.536 **	1	0.887 **	0.751 **	0.415 *	0.624 **
ILA	0.593 **	0.887 **	1	0.752 **	0.567 **	0.652 **
DMS	0.452 *	0.751 **	0.752 **	1	0.140	0.561 **
PVHpml	0.318	0.415 *	0.567 **	0.140	1	0.577 **
LHApf	0.302	0.624 **	0.652 **	0.561 **	0.577	1

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)