Behavioral/Systems/Cognitive

Associatively Learned Representations of Taste Outcomes Activate Taste-Encoding Neural Ensembles in Gustatory Cortex

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Through learning processes, cues associated with emotionally salient reinforcing outcomes can come to act as substitutes for the reinforcer itself. According to one account of this phenomenon, the predictive cue associatively elicits a representation of the expected outcome by reactivating cells responsible for encoding features of the primary reinforcer. We tested this hypothesis by examining the role of neural ensembles in gustatory cortex (GC) during receipt of gustatory stimuli (sucrose and water) and cues associated with those stimuli using the immediate early genes (IEGs) *Arc* and *Homer1a*. Because these plasticity-related IEGs are expressed in the neuronal nucleus 5 and 30 min, respectively, after salient events, we examined how individual neurons encoded these stimuli in two separate behavioral epochs. In experiment 1, we showed that tasting identical sucrose solutions, but not tasteless water, in the two epochs increased both IEG activity and the degree of overlap between neural ensembles in GC. In experiment 2, odor cues associated with sucrose, but not water, evoked potentiation of IEG activity in GC similar to sucrose itself. Surprisingly, lesions of the basolateral amygdala had minimal effects on associative encoding in GC. Finally, these associatively driven representations of sucrose appeared to be outcome specific, as neural ensembles that were activated by the sucrose-associated cue were also activated by sucrose itself. This degree of overlap between associative and primary taste activity at the ensemble level suggests that GC neurons encode important information about anticipated outcomes. Such representations may provide outcome-specific information for guiding goal-directed behavior.

Introduction

Animals learn and remember information about foods they encounter. Predictive cues such as odors can be used to select food, while flavors and postingestive features of foods can guide future selection and consumption. Much research has considered the role of gustatory cortex (GC) in acquiring and retaining such information. GC, a region of the insula receiving dense ascending fibers from the gustatory thalamus, has been shown to be driven by tastants, particularly in the dysgranular layer (Kosar et al., 1986). In addition to this sensory role, GC is also important for acquiring new information about tastes on the basis of experience (Kiefer et al., 1984). Recent work has shown that such learning requires NMDA-dependent plasticity (Escobar et al., 1998) and protein synthesis (Bermúdez-Rattoni et al., 2004; Elkobi et al., 2008) in GC.

Although encoding properties of GC neurons in response to tastants have been examined (Katz et al., 2001; Lemon and Katz, 2007), less is known about GC representation of tastes in response to nongustatory cues that predict those tastes. Imaging studies in rodents using immediate early genes (Dardou et al., 2006, 2007; Kerfoot et al., 2007) and in humans using functional magnetic resonance imaging (Veldhuizen et al., 2007; Small et al., 2008) show increased activity in GC in the presence of taste-predictive cues. Similarly, GC function appears necessary for performance thought to be mediated by such associatively activated representations. For example, after selectively sating rats with one tastant, rats with GC lesions are impaired in their ability to spontaneously reduce pressing a lever that previously produced that food (Balleine and Dickinson, 2000).

These findings suggest that GC is involved in forming or using associative representations of taste outcomes. Parallel findings indicate that the basolateral amygdala (BLA) also participates in rapid encoding of cue-outcome associations (Allen et al., 1991; Hatfield et al., 1996; Balleine et al., 2003; Pickens et al., 2003). As such, connections between BLA and GC may be important for encoding features of representations in GC (Barot et al., 2008; Grossman et al., 2008; Fontanini et al., 2009).

Here we tracked how individual neurons in GC were recruited to encode such information using two immediate early genes (IEGs) *Arc/Arg3.1* (*Arc*) and *Homer1a* (*H1a*) (Link et al., 1995; Lyford et al., 1995; Brakeman et al., 1997). These IEGs are NMDA-dependent markers for plasticity and show differential temporal expression patterns in activated neurons (Guzowski et al., 2001). We exploited this temporal difference to examine patterns of neural activity across two different behavioral epochs, allowing us to map ensemble activity for both tastes (experi-

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ment 1) and taste-associated cues (experiment 2). We found that sucrose increased IEG expression in GC compared with tasteless water, and that repeated exposure to identical solutions activated overlapping populations of neurons. Furthermore, taste-associated cues increased IEG expression in GC, even in the absence of tastants, and neurons activated by sucrose-associated cues were reactivated by sucrose alone. BLA lesions failed to disrupt the content of these associative ensembles in GC, suggesting that limbic input is not necessary for GC associative taste representations.

Materials and Methods

Subjects and apparatus

Thirty-one Long–Evans rats (Charles River Laboratories) weighing between 350 and 425 g at the time of experiment served as subjects. Three of the rats used in experiment 1 had previously received exposure to saccharine in different test chambers. Rats were housed individually on a 12:12 light/dark schedule and had *ad libitum* access to standard rat chow and water until the beginning of behavioral testing procedures. All procedures were conducted in accordance with Johns Hopkins University and NIH guidelines for animal research.

Behavioral procedures

All training procedures took place in the rats' home cages, which were standard plastic tubs (19 × 10 × 9 inches), topped with a steel wire lid where chow and water were normally presented. Before testing, all food and normal cage water was removed. When fluids were presented in each session, a standard glass bottle with a rubber stopper and steel sipper tub was placed on the cage top so that the tube protruded into the box. Rats were placed on a schedule of restricted access to water 1 week before training during which they received 15 min of access to water per day at ~4:00 P.M. Rats had unrestricted access to chow during the pretraining phase.

The solutions used during testing were either reverse-osmosis-filtered (RO) water or a 10% (w/v) sucrose solution in RO water. Fluids were presented in glass bottles affixed with a steel sipper tube. The sipper tube for the test presentations was similar to the normal cage water bottle, but differed in that it used a ball bearing in the tube, while the normal cage bottle used a narrow-gauge opening at the tip.

To reduce neophobia to the sucrose solution, 2 d before testing, rats were presented with the sucrose on their home cages for 2 min during the normal water presentation time in the pretraining phase. Bottles were weighed before and after the sucrose exposure to ensure that each rat experienced the solution. Rats that failed to consume any sucrose had the bottle placed back on the home cage for an additional 2 min. At the end of pretraining, rats were allowed to drink normal cage water for another 13 min to meet their water requirements for the day. During pretraining and also during the test phase of experiment 1, rats were presented with solutions in the colony room.

In experiment 2, odorants were presented along with solutions. Odors used in experiment 2 were biologically neutral and easily discriminated. The odors, hexenol B gamma extra and isoamyl butyrate (International Flavors and Fragrances) were diluted to 1.25% (v/v) in propylene glycol. In this experiment, rats were moved to one of two different rooms for testing. Both rooms were approximately the same size, and were dimly illuminated with ambient red light. To minimize the risk of the different odors commingling, a consistent odor was used in each room during all testing.

The experimental design follows from the temporal features of IEG expression of *Arc* and *H1a*. Specifically, salient neural activity that drives plasticity will result in *H1a* mRNA being expressed in the cell nucleus 25–30 min after the event, while *Arc* mRNA can be detected 5–8 min after such an event. Because *Arc* and *H1a* have been shown to be coexpressed after exposure to salient events (Vazdarjanova et al., 2002), the temporal differences in nuclear expression of these IEGs can be used to explore how the same cell responds to two different events. To do this, the experiments used two 5 min behavioral epochs separated by 20 min, followed by killing. As a result, events 25–30 min before killing (epoch 1) will drive IEG expression that will be seen as nuclear *H1a*, while events 5 min before killing (epoch 2) will be seen as nuclear *Arc* expression. Thus, each cell will either show *H1a* only (activity only in the first epoch), *Arc* only (activity only in the second epoch), both *H1a* and *Arc* (activity in both epochs) or no IEG activity. Single IEG expression (either *Arc* or *H1a*) reflects selectivity for one of the two tastants, while double labeling demonstrates that the cell treated the two tastants similarly.

Table 1. Behavioral design for each experiment

Condition	Training	Test	
		Epoch 1	Epoch 2
Experiment 1			
SAME		Sucrose	Sucrose
DIFFERENT		Sucrose	Water
WATER		Water	Water
Experiment 2			
O1 versus O2	O1 → Sucrose (4 times) O2 → Water (4 times) No cue → Water (8 times)	O1 → Water	O2 → Water
O1 versus Sucrose	O1 → Sucrose (4 times) O2 → Water (4 times) No cue → Water (8 times)	O1 → Water	Sucrose

For behavioral testing, in experiment 1, rats were presented with either sucrose or water on their home cages in one of two time-limited epochs (Table 1). In the first epoch, rats received a presentation of a fluid for 1 min on the home cage. Twenty-four minutes later, rats received another 1 min presentation of a fluid. Five minutes after the last presentation, rats were killed. In this experiment, each rat was assigned to one of three different general conditions. In the first condition, rats received the same outcome in both epochs (SAME), either 10% sucrose ($n = 7$) or 5 Froot Loops (one per minute) ($n = 1$). In the DIFFERENT condition, rats received 10% sucrose in one epoch and plain water in the other epoch. The order of sucrose presentation was counterbalanced; 2 rats received 10% sucrose in the first epoch and water in the second epoch, and 3 rats received the solutions in the opposite order. In the final WATER control group, rats ($n = 4$) drank plain water in both epochs.

Experiment 2 was designed to assess the extent to which taste-associated cues were able to drive IEG expression in GC in the absence of the tastants themselves, and further, whether such activity was similar to that seen during primary gustation. The summary of these designs are seen in Table 1. All rats were trained to drink during two different epochs and in different contexts. Rats were run in two sessions per day. Each session was 5 min long, separated by a 20 min intersession interval. During each session, the bottle of the fluid being presented was placed on the cage 1 min following the start of the epoch. At the stopper end of the spout, an odor saturated (0.5 ml) disc of filter paper acted as a mechanism to deliver the odor cue. Scented odor cues were used for one epoch per day that used an odor-outcome pairing, but not for the control epoch in which water was presented alone. The odorant was at a concentration such that the maximum intensity was achieved when the rat was drinking from the spout, though some diffusion of odorants was likely present at a significantly lower concentration elsewhere in the testing bin. Rats drank the fluid in three 1 min bouts, each bout separated by 30 s during which the bottles were removed from the cage. Because of our concern over diffusion of odors, different rooms were used as the contexts for these sessions to minimize contamination between odors; all testing with one odor was done in one context and testing with a different odor in the other context. Many provisions, however, were taken to make the contexts as similar as possible otherwise. Both rooms were ~10 feet × 10 feet and were dimly illuminated with red lamps. Lamps were located under the tables on which the rats were placed to ensure diffuse light and to reduce the impact of visual contextual stimuli. Half the rats started in one context and the other half in the other context.

The rats underwent 8 d of training. During this phase, rats drank from standard bottles with a steel sipper tube placed on the home cage. In each of the contexts were exposed to one of two odors (either hexenol B gamma extra (context A) or isoamyl butyrate (context B), placed on the 1 inch diameter piece of filter paper. For each animal, one odor was

always paired with a bottle containing 10% sucrose solution (O1), and the other odor was always paired with normal tap water (O2). However, the identity of the specific odor/flavor pairings was counterbalanced among the rats.

Each day, rats received an odor/outcome pairing in one context (e.g., O1) and unscented plain water in the second context. This was done because it was expected that residual levels of odorants specific to the context would be ambiently present in each room even in the absence of explicit presentations. We addressed this by allowing rats to drink unscented water in both contexts to minimize effects of any such lingering odors on contextual encoding and thus enhance associability of the explicit odor cue with its paired outcome. Thus, across all 8 d of training, for each day rats received one context with odor (either O1 or O2, depending on the day) and the other context with odorless water, for a total of four O1 presentations, four O2 presentations, and eight unscented water-only presentations (four per context; Table 1). This training protocol also acclimated rats to the timing of the two-epoch protocol used on the test day.

On the day of test, rats were run on the same schedule as during training, but were assigned to one of two conditions. In the first condition, rats ($n = 5$) that had been trained to learn the associations between two odors, only one of which was paired with a flavored sucrose solution, were tested with exposure to each of those odors in different epochs. Before killing, rats were given exposure to the sucrose-paired odor (O1) presented with water instead of sucrose in one epoch, while the water-associated odor (O2) was still paired with water presentation in the other epoch. The order of O1 and O2 presentation was counterbalanced by subject during the tests.

It is known that GC and BLA share reciprocal connections, and as such, it was of interest to know whether BLA lesions modulated associative taste coding in GC. Existing evidence suggests that rats with BLA lesions fail to show behavioral evidence for specific associative representations (Hatfield et al., 1996; Balleine et al., 2003; Pickens et al., 2003). However, there is little evidence to suggest that BLA lesions affect taste perception at all (Pickens et al., 2003). As such, we predicted that lesions of BLA would also allow us to determine how BLA lesions affect GC coding of real (primary) taste representations. Thus a second group of animals in the O1 versus O2 condition ($n = 4$) were given BLA lesions and run with the same training and test as control subjects.

In the second condition, rats ($n = 5$) received the sucrose-paired O1 odor presented with water in one epoch, and actual 10% sucrose alone (i.e., no odor) in the other epoch during the test. The sucrose solution was given in the opposite context as the O1/sucrose context to minimize the effect of any associative contextual cues. This condition, therefore, assessed the degree of coexpression between "real" sucrose and the associatively activated sucrose representation. In this condition, order of O1 and sucrose presentation was counterbalanced by subject.

Surgical procedures

The effects of neurotoxic lesions of the BLA were assessed in experiment 2 in the O1 versus O2 condition to determine whether the occurrence of odor activated taste representations in GC depended on the integrity of BLA. Those rats ($n = 4$) were anesthetized with isoflurane gas mixed with oxygen, and mounted in a standard stereotaxic apparatus (David Kopf, Tijunga, CA). A midline incision was made and the scalp and fascia were retracted. The skull was leveled by bringing bregma and lambda to within 0.1 mm on the dorsoventral (DV) axis. Holes were drilled in the skull at −2.8 mm anteroposterior (AP) and ±5.1 mm mediolateral (ML) relative to bregma. Injections of the neurotoxin NMDA (20 mg/ml in 0.1 M PBS) were made using a narrow-tapered glass pipette attached to a Picospritzer (General Valve). Coordinates were taken at the skull surface at bregma, then the pipette was moved to −2.8 mm AP and ±5.1 mm ML relative to bregma. At both bilateral injection sites, the pipette was then slowly lowered to −8.7 mm DV from the skull surface at bregma and allowed to rest for 5 min. Neurotoxin (0.15 μl) was injected at rate of 0.025 μl/min over the course of 6 min. The pipette was then allowed to remain in place for another 5 min to allow the solution to diffuse and be absorbed into the surrounding neurons. The pipette was then raised to −8.3 mm DV and allowed to rest another 3 min before injecting 0.07 μl at a rate of 0.025 μl/min. Following another 5 min resting period, the pipette was slowly raised out of the brain. The scalp was then sutured shut with silk sutures and the wound was treated with topical antibiotics. Rats were allowed to recover for 30 min under warming lamps, after which they were returned to the colony room.

Histological procedures

Exactly 1 min after the end of the second testing epoch, rats were placed in a plastic tub with a tight-fitting lid saturated with isoflurane vapors for 1.25 min, and then rapidly decapitated with a guillotine. Brains were removed, notched on the rat's left side, and blocked rostrally just caudal to the rostral pole of the prefrontal cortex and caudally through the middle of the cerebellum. The brains were then lowered into supercooled (approximately −80°C) isopentane cooled externally by a dry ice and ethanol slurry and allowed to freeze for 5 min in the isopentane before being removed and wrapped in foil. Frozen brains were stored in a −80°C freezer until sectioning on a cryostat.

On the cryostat, the brain was warmed to −20°C then fixed to the platform with Tissue Tek OCT (Miles). Sections taken were either 16 μm (experiment 1) or 20 μm (experiment 2) which were immediately mounted onto slides. Mounted slides were then sealed in boxes and subsequently stored in a −80°C freezer until used for processing.

For rats that had received lesions of BLA, a separate series of sections was allowed to dry at 37°C. Sections were first fixed for 2 h in 0.1 M PB-buffered 10% formaldehyde. For staining, slides were defatted in a 1:1 mixture of ethanol and chloroform for 2 h, then were gradually rehydrated in decreasing concentrations of alcohol. Sections were stained with a 0.2% thionin stain, then gradually dehydrated. Finally, tissues were bathed in xylenes and coverslipped using DPX. Lesion validity was assessed by visual inspection with a microscope. Morphological changes such as tissue shrinkage, holes and gliosis were taken as evidence of tissue damage.

Fluorescent in situ *hybridization.* Processed sections underwent processing for double label fluorescent *in situ* hybridization (FISH) for *Arc* and *H1a* mRNA. *In situ* procedures were adapted from Guzowski and Worley (2001) and Petrovich et al. (2005). *Arc* and *H1a* plasmids were linearized by separately incubating in a solution of BSA, 10× enzyme buffer and EcoR1 enzyme at 37°C for 2 h. Cutting was confirmed by running on a gel. Linearized cDNA was then purified in phenol/chloroform and precipitated in 100% alcohol. The solution was stored at −20°C overnight, after which the solution was centrifuged and the aqueous layer retained. The solution was rinsed in 70% alcohol and centrifuged three more times. The alcohol was then removed and the pellet allowed to air dry, after which TE buffer was added and the cDNA was resuspended. Finally, this cDNA was bound into a conjugate with digoxigenin-UTP (*Arc*) or fluorescein-UTP (*H1a*) and incubated at 37°C for 1.5 h. At the end of this process, the probe was purified by spinning through spin columns (Roche) for 5 min. The resulting conjugate probe was typically used that day, though in some situations the probe was kept at −20°C and was used within a week.

For the *in situ* reactions, sample tissues were allowed to warm to −20°C in a cryostat for 1 h before processing. This tissues were then fixed in fresh buffered 4% paraformaldehyde, washed in 2× SSC, then bathed in 0.5% acetic anhydride in TEA buffer. Tissues were then stabilized in a 50:50 mix of methanol and acetate, and washed again in 2× SSC. Tissues were incubated in the hybridization buffer at room temperature for 30 min in a humid chamber. Hybridization buffer was gently removed, then slides were incubated in the same hybridization buffer plus both *Arc* and *H1a* riboprobes (~10 μg/ml for each probe). Coverslips were placed over the tissue, sealed in place with DPX and then slides were incubated at 60°C overnight (~20 h). Following this incubation, slides were washed in 2× SSC at 60°C, then treated with 3 μl/ml RNase at 37°C to eliminate any nonhybridized RNAs in the tissue. Slides were washed in descending concentrations of SSC (from 4× to 0.1×) plus 0.2% w/v sodium thiosulfate. Following a rinse in Tris-buffered saline plus 2 μl/ml Tween (TBS-T), slides were moved to a humid chamber, then blocked with TSA TNB solution (PerkinElmer) at RT. Slides were then incubated with HRP-antibody conjugate anti-digoxigenin-POD (1:1000) in blocking solution at 4°C overnight. The following day, slides were washed in

TBS-T, then reacted with the fluorophor cyanine-3 (CY3) (PerkinElmer) for 30 min at RT in the humid chamber. Slides were washed in TBS-T, and then rinsed in 2% H_2O_2 to quench residual peroxidase activity. Tissues were then washed in TBS-T and removed to the humid chamber. Slides were then incubated with the antibody anti-fluorescein-POD in TSA TNB buffer (1:1000) (PerkinElmer) at 4°C overnight. The following day, slides were rinsed in TBS-T then reacted with the fluorescent marker fluorescein (PerkinElmer). Tissues were dried then coverslipped with Vectashield coverslipping media containing the nuclear Nissl-type dye 4′,6-diamidino-2-phenylindole (DAPI) to counterstain cell nuclei. Slides were kept at 4°C until imaging at confocal microscope.

Tissues were imaged using a Zeiss LSM 510 Meta confocal microscope using three lasers (488, 520 and 570 nm), each corresponding to the peak emission spectrum for DAPI (Nissl stain for cell nuclei), fluorescein (*H1a* mRNA) and CY3 (*Arc* mRNA), respectively. Data were acquired using a *z*-stack (0.5 μm thickness per section in stack), the height of which was determined by the penetration of detectable probe for each sample. For this experiment, the objective lens was set at 60× magnification for one block of animals ($n = 9$; experiment 1), 40× for another block ($n = 14$; experiment 2), and 20× for a third block of subjects ($n = 8$; experiment 1). Early sessions used the higher magnification to ensure accurate expression rates and double labeling in each cell. However, lower magnification in later replications allowed for a greater sampling of each region of interest while decreasing chances of sample bias. Typically 8 samples were taken from each animal, taking bilateral samples from four anatomical planes anterior to bregma at +0.95, +1.20, +1.45 and +1.70 mm anterior to bregma. However, where extensive tissue damage occurred due to *in situ* processing, data were not collected and the data were omitted.

Confocal fluorescent microscope imaging. Data were analyzed using LSM 510 Image Browser software (Zeiss), and cell counts were done manually. To eliminate bias, images were first viewed with only the DAPI-labeled (Nissl) cells present (e.g., turning off the filter for the CY3 and fluorescein channels). To do this, a piece of transparency was taped to the computer monitor and valid cells were circled in black. Cells were counted for analysis if the nucleus was present on at least 5 sections of the *z*-stack. Once cells were determined to be valid, the CY3 filter was turned back on to reveal only the CY3-positive markers. A second transparency was placed over the DAPI transparency and CY3-positive markers were circled in red. That sheet was removed, and the CY3 filter was turned off, while the fluorescein filter was turned on. A third transparency was overlaid on the DAPI transparency, and fluorescein-positive cells were circled in green. Analysis was done by overlaying all three transparencies. Cells which were positive for both DAPI and CY3 were considered *Arc*-positive, cells with both DAPI and fluorescein were considered *H1a*-positive, and cells with DAPI, CY3 and fluorescein were considered double-labeled for both mRNA.

Data analysis

All behavior and neural data were compiled for each subject and evaluated by repeated-measures ANOVA that treated subjects as a random effect. In some situations, significant violations of parametric assumptions demanded analysis with appropriate nonparametric analysis. Tests were computed using Statistica software (StatSoft).

Results

Experiment 1

Rats consumed substantial amounts of both normal water and the sucrose solution. Across all subjects, rats consumed an average of 9.23 ml of sucrose per epoch and 7.97 ml of water per epoch. There were no significant differences in consumption for either fluid, behavioral condition or epoch (data not shown). The rat in the SAME condition consumed all five Froot-Loop pieces during each epoch. Because we expected that consumption of identical sucrose solutions or identical cereal pellets in both epochs would induce similar rates of GC expression, data from the single Froot-Loop-consuming rat were combined with those from the sucrose-consuming rats in the SAME condition.

We then analyzed the rates of IEG expression for *Arc* and *H1a* in GC. GC was defined by anatomical boundaries determined by several factors. Several studies have shown important functional divisions in the insula in relation to the cytoarchitectonic structure of the region. For example, whereas the insular region is typically comprised of an allocortical agranular cortical region (AI), a neocortical granular cortical region (GI) and a transitional dysgranular cortical region (DI) between them, the majority of taste responsive neurons have been shown to be centered on the DI region specifically (Kosar et al., 1986; Yamamoto et al., 1989). Further, results from both lesion (Balleine and Dickinson, 2000) and recent two-photon imaging studies (Accolla et al., 2007) have demonstrated a greater importance for the more anterior portions of insula ranging from approximately +0.7 to +1.7 mm anterior to bregma for encoding taste-related information. Finally, anatomical tracing studies have shown that these same regions are reciprocally connected with limbic structures such as BLA and OFC, while more posterior sections of the insula have connections with extended amygdala structures such as central nucleus of the amygdala and bed nucleus of the stria terminalis (Allen et al., 1991). In a series of animals ($n = 4$), we confirmed these boundaries using histochemical analysis of retrograde (Fluorogold) and combined anterograde/retrograde (cholera toxin b) tracers injected into either GC or BLA (supplemental Fig. S1, available at www.jneurosci.org as supplemental material).

In support of these findings, we assessed total levels of IEG expression in a pilot subject after sampling tastants. For this subject, samples were taken in the DI layer of the insula from +2.0 to −0.3 mm from bregma. For this sample, IEG expression in response to sucrose exposure was greatest in the regions between +1.0 and +2.0 mm anterior to bregma, and notably diminished in the more posterior regions from +0.3 to −0.3 mm from bregma (supplemental Fig S2, available at www.jneurosci.org as supplemental material). Given this information, all subsequent analyses were restricted to regions extending from +0.95 to +1.7 mm anterior to bregma, and we referred to this circumscribed region as GC.

Samples in this experiment were taken in two separate blocks. In the initial block ($n = 9$; 6 in the SAME condition, 2 the DIFFERENT condition and 1 in WATER condition), samples were taken at 60× magnification resulting in a lower number of cells per sample. In this block, we collected an average of 346 ± 66 DAPI-labeled cells per animal. In the second block ($n = 8$; 2 in SAME, 3 in DIFFERENT, 3 in WATER), images were collected at 20× magnification, resulting in a yield of 2447 ± 117 cells per animal. No animal's tissue was imaged at both 20× and 60×. Importantly, all IEG expression analysis was done on rates of expression, which was defined as the percentage of IEG-positive nuclei (expressing *Arc*, *H1a* or both) out of the total number of DAPI-labeled cells for each sample. Thus, because analysis was done on rates rather than total numbers of observations, data were comparable across different samples and different magnification. Using these rates there were no statistical differences between IEG expression ratios in the 60× and 20× magnification groups and as such, data from these groups were combined for statistical comparisons. Representative samples of analyzed *in situ* tissue appear in Figure 1. The overall expression rates for each rat in each condition were analyzed with a repeated-measures group × test ANOVA that treated rats as a random effect.

To ensure that any effects were as a result of treatment and not to incidental differences in expression rate for *Arc* or *H1a*, we first compared the total rate of *Arc* to the total rate of *H1a*, regardless of group or stimulus order. We found no differences in the average

expression rate between Arc (20.9%) and H1a (20.3%), $t_{(16)} = 0.33$, $p = 0.75$. Thus, any differences reported here are not due to any inherent bias in expression for *Arc* compared with *H1a*. For this reason, data will be reported by treatment condition and not necessarily by order of presentation.

We first tested whether sucrose was able to drive greater IEG expression in GC than tasteless water. For these comparisons, we contrasted the total percentage of cells that expressed an IEG (*H1a*) in epoch 1 with the total percentage expressing *Arc* in epoch 2. When cells expressed both IEGs, these cells were added to both the epoch 1 and epoch 2 total count.

For both the SAME and WATER conditions, rats received identical solutions in both epochs. However, for the DIFFERENT condition, the order of the sucrose presentation was counterbalanced. For those subjects, we expected that the rate of sucrose-related IEG expression should not depend on order (or IEG). Low numbers of subjects in the DIFFERENT condition (two with sucrose first, three with water first) precluded statistical comparisons; however, numerically, solution-related IEG rates were similar (for sucrose, 27% when presented first, and 22% when presented second; for water, 12% when presented first and 13% when presented second). Thus to aid in analysis and display, we combined the IEG rates associated with sucrose and water into their respective "epochs", regardless of the order in which those solutions were presented.

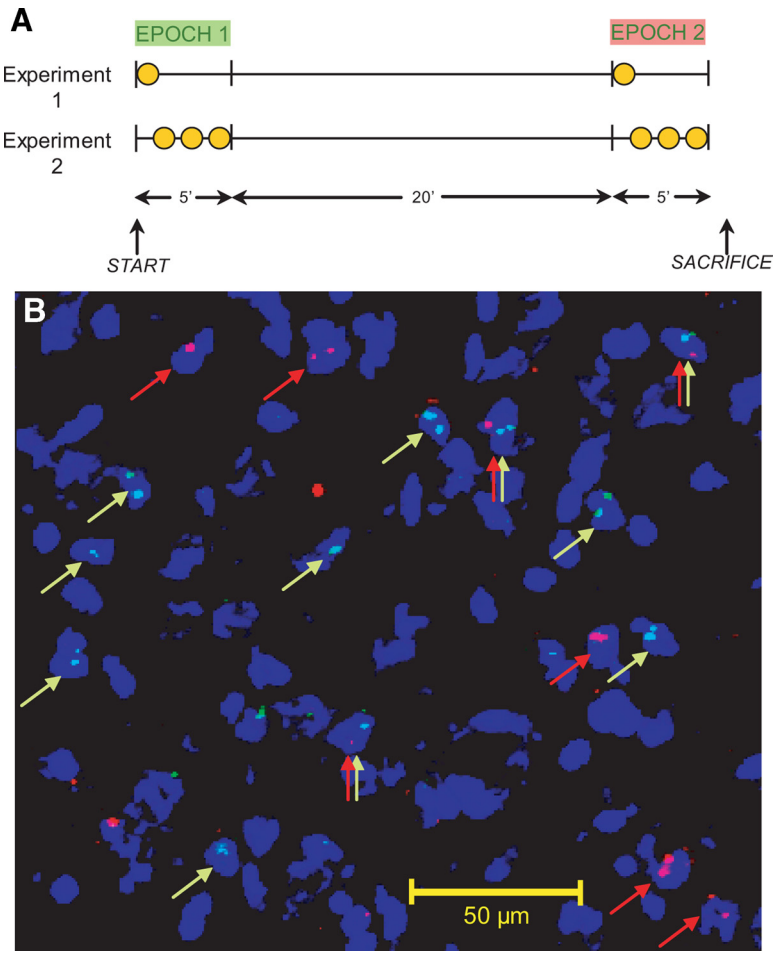

Figure 1. ***A***, Design for experiments 1 and 2. In both experiments, animals were run in two epochs, each 5 min long, separated by a 20 min interepoch interval. Orange circles are 1 min bouts of fluid exposure. Events in epoch 1 (left) that drive IEG expression will be visible as *H1a* nuclear mRNA at the time of killing (sacrifice), while events in epoch 2 (right) that drive IEG expression will be visible as nuclear *Arc* mRNA. Thus, cells active in both epochs will be double labeled for both *Arc* and *H1a*. ***B***, Sample of fluorescent *in situ* hybridized tissue. Blue regions are Nissl-stained neurons, green dots (indicated by pale arrows) show *H1a* expression, red dots (indicated by red arrows) show *Arc* expression. Cells that are double labeled for both genes are indicated by double vertical arrows. Sample image taken at 40× magnification.

In general we found that sucrose was more effective at driving IEG expression in GC than water alone (Fig. 2). A two-way ANOVA using group (SAME, DIFFERENT, WATER) and epoch revealed a significant main effect of group, $F_{(2,14)} = 4.92$, $p < 0.05$. This effect was carried by a significant linear trend where SAME showed the highest overall IEG rate, WATER the lowest and DIFFERENT at intermediate rates, $F_{(1,14)} = 9.05$, $p < 0.01$; the quadratic trend was not significant ($F < 1$). The main effect of epoch confirmed the greater role that sucrose played in increased IEG expression, as there was reliably greater IEG expression in epoch 1 (plus combined sucrose epoch in MIXED) than in epoch 2 (plus combined water epoch in MIXED), $F_{(1,14)} = 8.74$, $p < 0.05$. In both the SAME and WATER conditions, there were similar levels of IEG expression in epochs 1 and 2. Therefore, the epoch effect was primarily due to increased IEG expression in the sucrose epoch in the MIXED condition. In support of this assertion, there was a significant interaction between epoch and group, $F_{(2,14)} = 6.03$, $p < 0.02$. An a priori contrast showed that there was a significant increase in IEG expression rates in the sucrose epoch compared with the water epoch in the DIFFERENT condition, while there were no such differences between epochs in the SAME or WATER conditions, $F_{(1,14)} = 12.05$, $p < 0.005$. Thus, sucrose reliably drove greater rates of IEG expression than water both across conditions, and also within the same subjects when given both the sucrose and water stimuli.

In this analysis, it was possible that greater variability in the DIFFERENT group may have contributed to an overestimation of the variance and thus altered our interpretation. We tested, but found no evidence for outliers for any of the groups presented here, using a *z*-score of >1.96 SDs from the mean. Indeed, it was found that the two most extreme points in the sucrose epoch for the DIFFERENT condition actually decreased the effect size (Cohen's f = 0.88 with those points compared with 1.08 without those points), suggesting that our effect was robust even without the more extreme points.

If GC is important for encoding taste properties of stimuli, then it was expected that repeated exposure to identical tastants should recruit highly overlapping neural ensembles, while different tastants should activate separate ensembles. To assess this, we compared the rate of double labeling as a percentage of those cells expressing both *H1a* and *Arc* to all valid cells. A one-way ANOVA showed a significant main effect, $F_{(2,14)} = 6.54$, $p < 0.01$, Figure 3. This effect was due to increased rates in the SAME condition compared with the DIFFERENT (Tukey: $p < 0.05$) and WATER conditions (Tukey: $p < 0.05$), though DIFFERENT and WATER

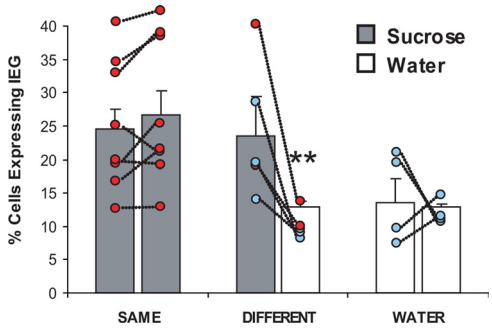

Figure 2. Experiment 1 IEG results. Rats in the SAME condition were given identical tastants in both epochs, either 10% sucrose ($n = 7$) or sweet cereal ($n = 1$), and showed similar rates of IEG expression. Similarly, rats in the WATER condition ($n = 4$) received unflavored water in both conditions and showed significantly lower rates of IEG expression. In the DIFFERENT condition, rats ($n = 5$) received sucrose in one epoch and water in the other. All rats in this condition showed greater activity in the sucrose epoch than in the water epoch, regardless of order presented. Colored circles show individual rates for each subject. Red circles indicate averages for rats that experienced sucrose in the first epoch, while blue circles are for the rats that experienced water in the first epoch. The identity of the tastant, rather than the order of sucrose presentation, drives differential IEG expression in GC. Left bars (SAME and WATER conditions) are for epoch 1, right bars for epoch 2; for the DIFFERENT condition, the left bars are the average of the sucrose presentations and the right bars are the average for the water presentations. Gray shading indicates that sucrose was presented in that epoch, white indicates water presentation. $^{**}p < 0.05$ for sucrose greater than water in DIFFERENT condition. Error bars are ±SEM between subjects.

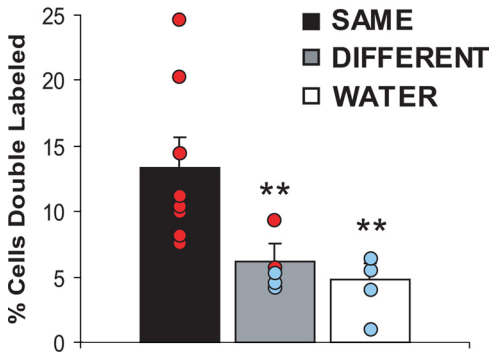

Figure 3. Experiment 1 double labeling results. Data on the left show the absolute rate of double labeling, expressed as a percentage of double labeled cells out of all cells counted. Using this metric, there were significantly more double labeled cells in the SAME group compared with both the DIFFERENT and WATER groups, although there was no difference between DIFFERENT and WATER. This suggests that similar ensembles in GC are reactivated when animals taste identical tastants, but not when they experience either different tastants or tasteless water. Individual data points for each subject are shown by the colored circles overlying each bar. Rats that received sucrose in the first epoch are indicated by red circles and rats that experienced water first are indicated by blue circles. $^{**}p < 0.005$ compared with the SAME condition.

were not different from each other ($p = 0.92$). However, it was uncertain whether low rates of double labeling in the WATER and DIFFERENT conditions were simply due to low rates of single labeling. For example, tasting water in both epochs may lead to a high degree of double labeling as a percentage of total cells activated, but the double labeling rate would be minimal because of a lower baseline of IEG activity. To test this across treatments, the rate of double labeling was normalized as the percentage of cells active in the first epoch that were subsequently reactivated in the second epoch. This epoch 1 Reactivation Rate (E1RR) was calculated as follows: E1RR = [(Cells expressing both *Arc* and *H1a*)/All cells expressing *H1a*].

E1RR that approaches 100% indicate a greater rate of overlap between ensembles, while lower numbers (near 0) show distinct

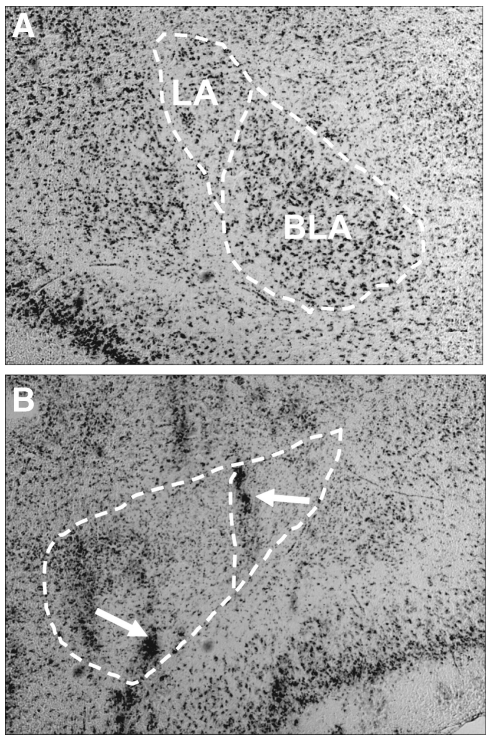

Figure 4. Histology on BLA sections for O1 versus O2 subjects. ***A***, Sham-lesioned animal shows intact (BLA) and lateral (LA) subregions of the BLA nucleus. Note large pyramidal cells in both subregions. ***B***, Neurotoxic NMDA injections damage cells in both BLA and LA. In both subregions, there is noted cell shrinkage and gliosis observable throughout the region. However, this damage did not encroach significantly into adjacent cortical or subcortical regions. White arrows indicate terminal location of needles used to inject neurotoxin.

ensembles. As with the overall rate, a significant effect of group, $F_{(2,14)} = 4.88, p < 0.03$ showed that E1RR was reliably greater in the SAME condition (E1RR = 53%) than in either the DIFFERENT (E1RR = 33%) or WATER (E1RR = 36%) conditions, $F_{(1,14)} = 9.74, p < 0.01$. Further, despite receiving the same water solution in both epochs of the WATER condition, rates of E1RR in that condition were not greater than in the DIFFERENT condition, $p > 0.5$. Thus, identical flavored tastants, but not tasteless water or the production of similar consummatory responses, are able to increase reactivation rates of GC neural ensembles across multiple exposures.

Experiment 2

Histology

In the rats that received neurotoxic lesions of BLA, tissue damage was assessed by absence of nuclear staining, gliosis and cell shrinkage (Fig. 4). In all subjects, damage was bilateral and extended rostrally to −1.8 mm and caudally −3.8 mm from bregma. Damage was focused on the basolateral and lateral nuclei of the amygdala, leaving the central nucleus largely intact (Fig. 5). Anatomical studies (Allen et al., 1991; Saddoris, 2008) indicate that this region is densely and reciprocally connected with the levels of GC that were analyzed in experiment 1. Thus these bilateral lesions effectively remove BLA input into GC. In some cases, damage often extended both ventrally into posterior piriform cortex and laterally into perirhinal cortex. However, this damage was only seen at a few levels and failed to encompass more than partial damage to those regions at any given level. Follow-up analyses failed to show any correlation between size of

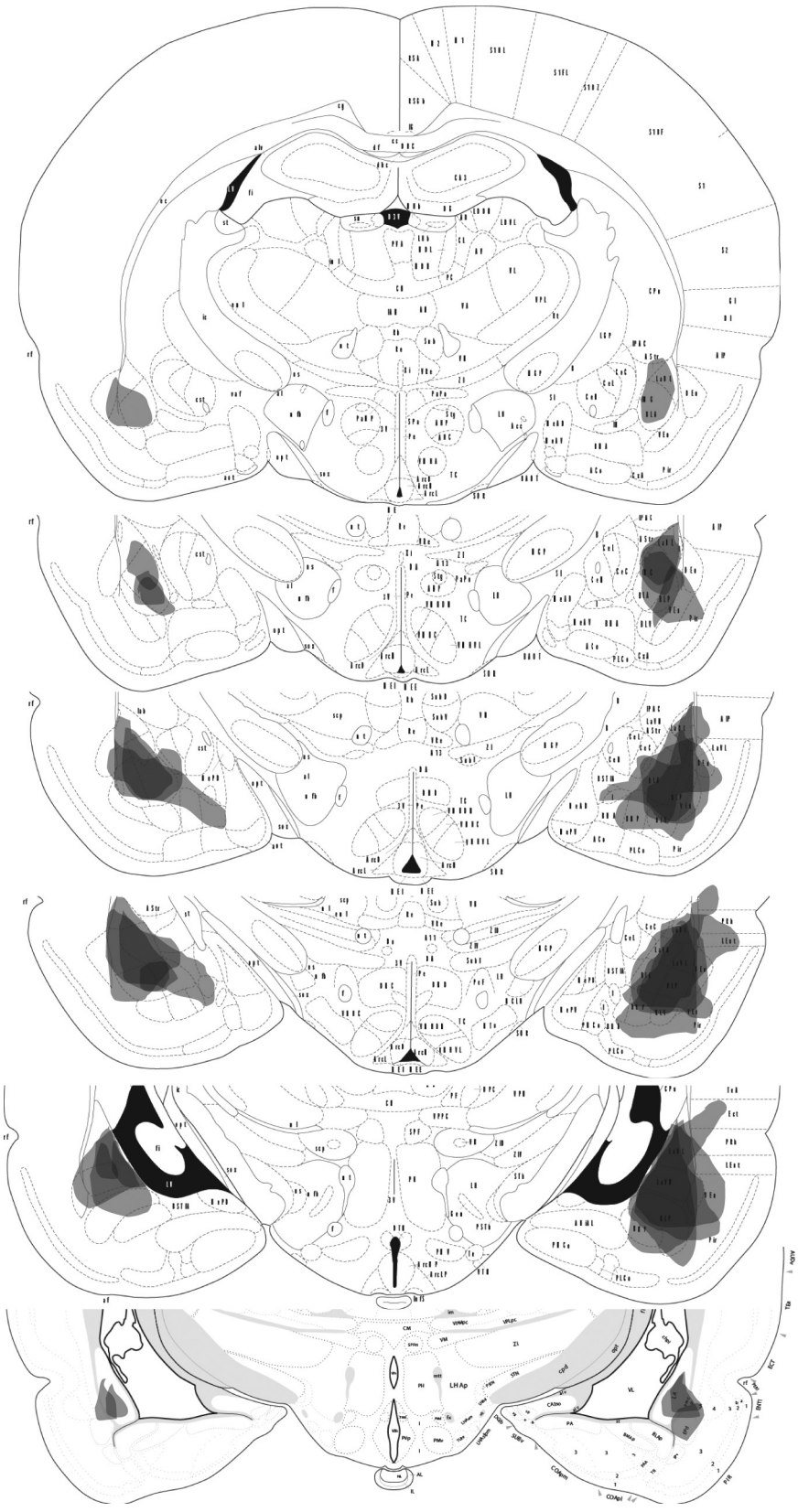

Figure 5. Composite drawings of BLA lesions. Individual lesions are in transparent light gray such that darker regions indicate regions of greater overlap between lesions. Numbers at right indicate mm from bregma; lesions extended from −2.0 to −3.8 mm posterior to bregma. Images adapted from Paxinos and Watson, 1997.

damage to these regions and IEG encoding rates in GC (data not shown).

Behavior

All rats in experiment 2 quickly overcame neophobia to the novel sucrose solutions during pretraining. In the O1 versus O2 condition, no rats in either group required multiple presentations to consume the requisite amounts of sucrose in the 2 min time limit. During subsequent training (supplemental Fig. 3, available at www.jneurosci.org as supplemental material), there was a significant difference in the rates of consumption for the different fluids, $F_{(2,24)} = 11.58$, $p < 0.02$. Rats consumed significantly less of the water when presented along with O2 than either sucrose paired with O1 or water alone, $F_{(1,12)} = 20.23$, $p < 0.001$, though rats consumed similar amounts of sucrose paired with O1 and water alone, $F_{(1,12)} = 0.54$, $p = 0.47$. However, there were no effects of training day (days 1–8), lesion (sham vs BLA) or interactions with those factors, all p values >0.2. Finally, during the test session, overall consumption varied across condition, $F_{(2,11)} = 16.82$, $p < 0.001$ (not surprisingly, rats in the O1 vs sucrose condition consumed more overall than rats in the O1 vs O2 condition, $F_{(1,11)} = 33.39$, $p < 0.001$). Notably, rats in each condition drank similar amounts of fluid in the two test epochs. Neither the main effect of epoch, $F_{(1,11)} = 0.85$, $p = 0.38$, nor the interaction of epoch and condition, $F_{(2,11)} = 0.48$, $p = 0.63$, was significant. Thus, observed differences in IEG expression rate within each condition (below) were not attributable to differences in consumption during test.

IEG expression

In the O1 versus O2 condition, we collected an average of 525 ± 86 cells in the sham-lesioned rats, and 3689 ± 207 cells in the BLA-lesioned rats. In the O1 versus sucrose condition, we collected an average of 674 ± 156 per subject. Differences in cell yields were due to differences in magnification for image capture and analysis; images for the sham-lesioned animals (both in the O1 vs O2 and O1 vs sucrose conditions) were taken at 40× magnification, while tissue from BLA-lesioned rats was imaged at 20×. In addition to similar rates found across different magnifications in experiment 1, other analyses in our lab has shown that the larger sample reduces variability across samples but does not affect the observed rates of IEG expression.

As in experiment 1, we first analyzed the total rate of IEG expression for *Arc* and

H1a to ensure that effects were due to treatment and not to incidental differences in the expression rates of *Arc* and *H1a*. A two-way ANOVA compared rates of IEG expression for *Arc* and *H1a* between the three different groups [O1 vs O2 (sham), O1 vs O2 (BLA-lesion), and O1 vs sucrose], and there were no effects of IEG type (*Arc*: 14.28%, *H1a*: 14.36%; $F_{(1,11)} = 0.005$, $p = 0.95$) nor an interaction between group and IEG type, $F_{(2,11)} = 0.55$, $p = 0.59$. Indeed, encoding rates between *Arc* and *H1a* within each group were strikingly similar [Tukey: O1 vs O2 (sham), $p = 1.0$; O1 vs O2 (BLA-lesion), $p = 0.97$; O1 vs sucrose, $p = 0.97$]. Thus, any effects seen in each group in the experiments were due to behavioral treatment, and not to differential activity between IEGs.

Because different stimuli were used during test, data from O1 versus O2 and O1 versus sucrose were considered separately. In the O1 versus O2 conditions, we found a reliable increase in the rate of IEG expression for the sucrose-paired odor (O1) compared with the water-paired odor (O2) despite the fact that identical water solutions were consumed in both test epochs, $F_{(1,7)} = 10.66$, $p < 0.02$ (Fig. 6*A*). Indeed, this effect was robust as there was greater IEG expression in the O1 epoch compared with the O2 epoch in all five sham-lesioned rats and all four BLA-lesioned rats. However, BLA lesions appeared to moderately decrease the overall rate of IEG expression in GC compared with sham-lesioned rats, $F_{(1,7)} = 5.90$, $p = 0.045$, but there was no interaction between lesion status and odor identity, $F_{(1,7)} = 1.16$, $p = 0.32$. Indeed, sham and BLA-lesioned rats showed similar differences between the O1 and O2 epoch, $t_{(7)} = 1.08$, $p = 0.32$. Thus, despite tasting the same water solution in both epochs, the presence of the sucrose-paired odor O1 potentiated IEG expression.

In a separate analysis, IEG rates in the O1 versus O2 sham group were compared in the GC and BLA of the same animals. Within subjects, GC neurons were more likely to show potentiated expression for O1 over O2, while BLA neurons showed similar rates of expression for both O1 and O2 (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Thus, there was no main effect of region (GC vs BLA) or stimulus (O1 vs O2), but there was a significant interaction, $F_{(1,3)} = 12.84$, $p < 0.05$. These findings indicate that BLA and GC play complementary roles in the encoding of tastants in associative representations.

A different pattern of results emerged in the O1 versus sucrose condition. Here, despite tasting two discriminable solutions in the two epochs (water and sucrose), IEG expression in the O1 epoch was not reliably different from that in the sucrose alone epoch ($t_{(4)} = 1.24$, $p = 0.28$, Fig. 6*B*). Indeed, these rates were numerically similar to those found in experiment 1 in which rats sampled sucrose alone (25% in experiment 1 and 20% in O1 vs sucrose). Thus, a sucrose-associated odor was able to drive IEG expression at rates statistically similar to that when rats taste sucrose alone.

Next we compared rates of double labeling for all conditions in experiment 2. As noted above, double labeling is a mechanism by which to explore the extent to which ensembles of neurons overlap in activity across epochs. Data from the O1 versus sucrose illustrates the utility of this measure. In that condition, it was possible that similar IEG expression rates driven by exposure to O1 and sucrose was carried by two different populations of neurons. For example, associative representations may reflect activity from limbic afferents, while primary gustatory responses may be driven by nonoverlapping, bottom-up sensory processes. In that case, one would expect similar rates of single labeling for both O1 and sucrose, but low rates of double labeling between those outcomes. In contrast, if associative representations of sucrose reactivate ensembles engaged in primary gustatory processing, then there should be a high degree of double labeling between O1 and sucrose ensemble activity. Thus all data were analyzed for rates of double labeling. Recall that in experiment 1, we found that rates of double labeling were similar in the DIFFERENT condition regardless of order of presentation, suggesting that different neural ensembles are recruited for the different tastants, rather than one tastant activating a subset of the neurons that encoded the other tastant. Thus, for this analysis in experiment 2, we looked at overall double labeling for all three conditions (O1 vs O2: Sham, O1 vs O2: BLA and O1 vs sucrose), regardless of whether the first epoch was O1, O2, or sucrose. We found a significant increase in the rate of double labeling in the O1 versus sucrose condition

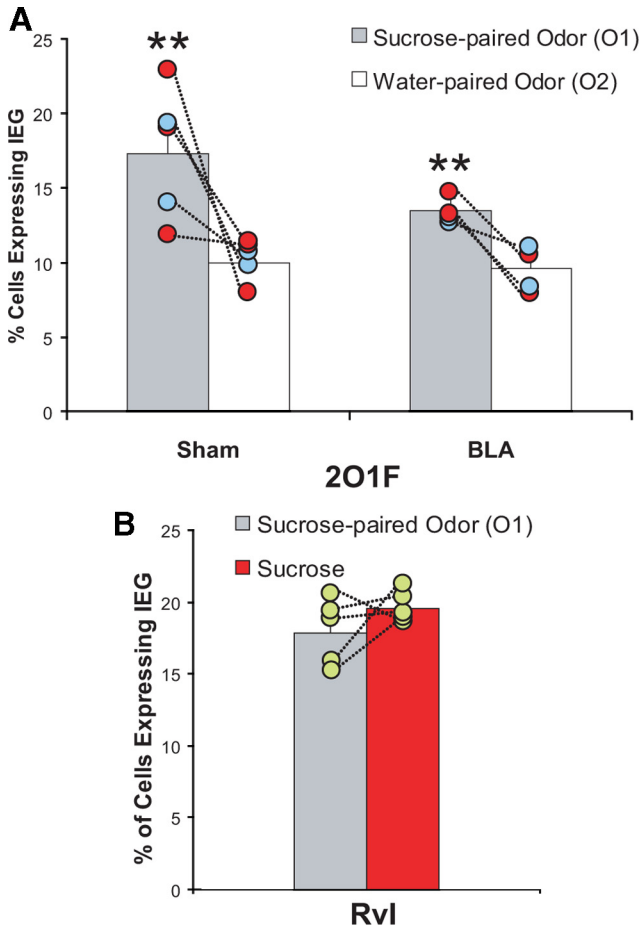

Figure 6. Experiment 2 IEG results. ***A***, In the O1 versus O2 condition, rats were trained with pairings of odor 1 (O1) with sucrose and odor 2 (O2) with water. During test, all rats received water paired with O1 in one epoch and O2 in the other epoch. Despite tasting the same water in both epoch, rats in both sham-lesioned (5 of 5) and BLA-lesioned (4 of 4) conditions showed significantly greater IEG activity in O1 epoch compared with the O2 epoch (**$p < 0.05$ greater for O1 compared with O2 IEG expression). As in experiment 1, order had no effect on IEG rates. Red circles are data from subjects that received O1 in epoch 1, and blue circles are for subjects that received O2 in epoch 1. BLA lesions had a moderate effect on overall IEG expression rates ($p < 0.05$), but did not affect the ability for O1 to potentiate IEG expression over O2. ***B***, In the O1 versus sucrose condition, rats received the same training as in O1 versus O2, but on the day of test received water paired with O1 in one epoch (same as in O1 vs O2), and unscented sucrose in the other epoch. Rats showed similarly elevated rates of IEG expression in the O1 epoch, and indeed, this activity was nearly identical to receipt of sucrose itself in the other epoch. These data suggest that in GC, a representation of a taste outcome is as effective at driving GC IEG expression as tastants themselves.

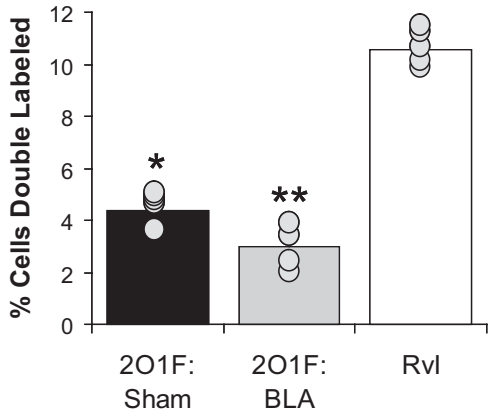

Figure 7. Experiment 2 double labeling results. In the O1 versus O2 condition, rats consumed water in both test sessions, but received O1 in one epoch and O2 in the other. Consonant with findings in experiment 1, this led to low levels of double labeling in both sham controls and BLA-lesioned subjects. Further, there was a significant decrease in the rate of double labeling between sham and BLA-lesioned subjects in the O1 versus O2 condition, $p < 0.05$. However, subsequent analysis using a normalized E1RR index showed that this was simply due to lower overall IEG expression rates in the BLA-lesioned condition. In contrast, in the O1 versus sucrose condition, rats that consumed sucrose in one epoch and water + O1 in the other showed significantly greater rates of double labeling than in either of the O1 versus O2 conditions (*$p < 0.01$ less double labeling than O1 vs sucrose; **$p < 0.01$ less double labeling than the O1 vs sucrose condition and O1 vs O2 Sham). Importantly, rats in the O1 versus sucrose condition actually consumed two different solutions—water and sucrose—across epochs. However, O1 was able to activate the same ensembles as tasting sucrose alone, as suggested by double labeling indexes that are numerically similar to that found in the SAME condition in experiment 1 (Fig. 3).

compared with the O1 versus O2 conditions ($F_{(2,11)} = 205.92$, $p < 0.0001$, Fig. 7). Tukey unequal-*n post hoc* tests revealed that such double labeling was significantly greater in O1 versus sucrose than in both the O1 versus O2 sham ($p < 0.001$) and O1 versus O2 BLA ($p < 0.001$) groups. Further, within the O1 versus O2 subjects, double labeling in the sham group was significantly greater than in the BLA-lesioned group ($p < 0.05$).

As before, we suspected that lower basal rates of IEG expression might account for this difference in total double labeling between O1 versus O2 groups. Using the E1RR index for double labeling instead of the overall rate, we again found a significant difference across the three groups, $F_{(2,11)} = 19.35$, $p < 0.0005$. This difference was due to almost exclusively to an increase in the E1RR for the O1 versus sucrose animals (E1RR = 59%) compared with either of the O1 versus O2 groups; *post hoc* comparisons show a significant increase for O1 versus sucrose over the O1 versus O2 sham (Tukey: $p < 0.001$; E1RR = 32%) and O1 versus O2 BLA-lesion (Tukey: $p < 0.005$; E1RR = 33%) groups. However, with this metric lesions of BLA did not alter the rate of double labeling compared with sham controls in the O1 versus O2 condition, Tukey: $p = 0.98$. Thus, differences in double labeling with overall rates between the two O1 versus O2 groups were due to a general decrease in overall IEG expression rather than a lesion-induced alteration in content of the GC associations. This argues that BLA lesions may modulate the magnitude of representational encoding in GC while leaving intact the content of those representations.

Discussion

Previous work has shown that GC plays a role in both the perception and memory for tastants. Here we used a 2-IEG *in situ* hybridization technique to characterize coding in ensembles of GC neurons. First, a tastant (sucrose) drove greater expression of plasticity-related IEGs in GC than tasteless water. Second, when sucrose was consumed in two different test epochs, overlapping ensembles of neurons were recruited in encoding those tastants, whereas dissimilar tastants or water alone failed to reengage those ensembles to the same extent. Third, odor cues associated with sucrose provoked GC IEG expression similar to that induced by sucrose itself. Despite consuming only water in both epochs, rats in the O1 versus O2 condition in experiment 2 showed a greater percentage of neurons recruited when presented with a previously sucrose-paired odor than with a previously water-paired odor. Moreover, in rats in the O1 versus sucrose condition in experiment 2, presentation of a sucrose-paired odor and sucrose itself recruited similar ensembles of neurons. Thus, features that were coded for in a primary gustatory stimulus were also captured in associatively activated representations of that tastant. Finally, although many studies have implicated BLA in the encoding of outcome-expectant representations, BLA lesions failed to alter the content of these associative GC representations.

These findings provide evidence that IEG markers of plasticity track important aspects of both primary and associative taste encoding. This is consistent with the results of other studies, which demonstrate the importance of protein synthesis in GC for the maintenance of taste-associated learning. For example, infusions of NMDA or AMPA receptor antagonists in GC prevent the formation and maintenance of CTA (Yasoshima et al., 2000). Mirroring these observations, recent work has shown a critical role for NMDA-dependent *Arc* in neural plasticity by regulating the number of AMPA receptors at locally activated synapses (Park et al., 2008; Waung et al., 2008). Thus, the present experiments provide evidence to support the expression of these IEGs during learning about taste and taste-associated cues.

Compared with some previous experiments in which this two-gene technique was used, we found a much lower rate of coexpression across epochs given identical tastants. For example, Vazdarjanova et al. (2002) found that repeated exposure to an identical context induced E1RR that was >90% in parietal cortex and hippocampus, while repeated exposure to identical sucrose solutions or sucrose and a sucrose-related cue in the present experiment induced a much lower 50% reactivation rate. These data, however, are in line with previous electrophysiological studies which have shown that taste-responsive neurons in GC have broadly tuned response characteristics (Katz et al., 2001, 2002; Stapleton et al., 2006). Indeed, the majority of neurons recorded in GC do not appear to be selective for chemosensory taste properties. As few as 14% of cells using averaging techniques (Yasoshima and Yamamoto, 1998) and as many as 41% using more sensitive temporal analyses (Katz et al., 2001; Soares et al., 2007) selectively encoded taste information. Thus, such broad tuning as well as the integration of somatosensory and taste information inherent in the region may limit the sensitivity of IEG ensemble analysis in GC. A recent study that examined CTA learning in GC and BLA with *Arc* reported similar overall rates of overall encoding (7–10%) and double labeling (~4%) in these regions when presented with taste-illness pairings (Barot et al., 2008). Thus, our data are consistent with mapping the ensembles selective for taste information even within the limits of such a system.

GC appears to be important not only for encoding tastants, but also the associative representations between cues and the motivational value of specific tastants. Previous studies have shown that GC lesions interfere with performance on CTA learning for multiple reinforcers (Braun et al., 1981) or a selective-satiation devaluation task where rats must use such associative representations to guide outcome-specific operant responding

(Balleine and Dickinson, 2000). In these cases, GC lesions do not interfere with the ability for animals to acquire accurate learning about either cues or operant responses, but rather impair the ability to use a learned representation of outcomes to guide responding. Indeed, evidence suggests that both GC and BLA may be involved with a form of mediated learning known as taste-potentiated odor aversion (TPOA), in which illness is paired with a cue-evoked representation of food. While normal rats show a decrease in the amount of fluid consumed in the presence of the odor CS alone (Palmerino et al., 1980), lesions or inactivation of GC or BLA cause impairments in TPOA (Lasiter et al., 1985; Hatfield et al., 1992; Ferry et al., 1995; Hatfield and Gallagher, 1995) while training in this procedure in normal rats produces increased IEG activity in GC and BLA (Dardou et al., 2006, 2007). Thus, the present data support this role for GC and BLA for playing a role not only in taste perception, but also associative representations of tastes and taste memory.

Although other studies have shown increased activity in GC in the presence of taste-predictive cues, those studies have used between-subjects designs to explore differences between different treatment conditions. By using a within-subjects design in the present study, we found greater activity for the sucrose-paired cue than the water-paired cue. However, it is not clear that this potentiated IEG expression was necessarily due to taste-related processing. For example, O2 was always paired with water during training and also during test, while O1 was paired with sucrose in training but surprisingly paired with water in test. Thus, the increase may instead be capturing the novelty of the new situation compared with the well predicted nature of the O2 epoch.

Data from the O1 versus sucrose condition argue against this interpretation. Here we saw that sucrose-paired O1 was able to drive IEG expression in GC ensembles as much as sucrose itself, a solution that rats had received many times over the course of training and preexposure. Indeed, the rate of coexpression across these epochs was nearly the same as when rats had sucrose in both epochs as in experiment 1. This suggests that at the level of GC, tastants and associative representations of those tastants are encoded by the same ensembles of neurons. One objection to these findings is that sucrose is more hedonically motivating than water, even to thirsty rats. At the concentrations used, we always found a preference for the sucrose solution compared with water when the rats had a two-bottle forced choice. Thus, GC representations may simply be encoding the rewarding value of the expected outcome. Previous lesion data suggest that GC is important for assigning a motivational value to specific tastant representations (Balleine and Dickinson, 2000). However, future studies will be required to distinguish the hedonic and sensory-specific aspects of these GC associative representations.

Surprisingly, lesions of BLA failed to abolish this potentiated IEG expression in GC. Many studies have shown that BLA plays an important role in assigning value to cue-outcome associations. In many learning paradigms, electrophysiological recordings show that BLA neurons develop cue-selective firing even before animals are making accurate choices (Schoenbaum et al., 1999; Saddoris et al., 2005), while BLA lesions impair the ability of OFC neurons to develop firing patterns that reflect learned cue-outcome associations (Schoenbaum et al., 2003). This associative encoding in BLA appears to be specific to the sensory properties of the reinforcer, as lesions of BLA prevent animals from avoiding a lever paired with a devalued outcome (Balleine et al., 2003) or potentiating a specific operant response that shares an outcome with a presented Pavlovian cue (Blundell et al., 2001; Corbit and Balleine, 2005).

Those studies suggest a critical role for BLA in developing sensory-specific associative representations that can be used in guiding behavior in the absence of primary reinforcers. Nonetheless, in the present studies, GC ensembles in BLA-lesioned rats showed a similar increase in IEG expression for O1 compared with O2 as in sham-lesioned animals, suggesting that lesioned animals were able to form odor-taste associations in the absence of BLA input. Indeed, some studies have shown that rats are able to form specific cross-modal associations between neutral stimuli without an intact BLA (Blundell et al., 2003; Dwyer and Killcross, 2006), a feature that is similar to the stimulus-stimulus associative functions in adjacent perirhinal cortex (Nicholson and Freeman, 2000). BLA, then, appears to be engaged when assigning the motivation significance to associations formed in the cortex. However, the overall decreased rate of IEG expression in BLA lesioned rats might suggest that BLA plays a modulatory role in GC, diminishing but not eliminating the content of associative GC ensembles. Thus if associative outcome information in GC is indeed intact following BLA lesions, then BLA may not be essential for the encoding of outcome-specific representations in the brain. Previous findings showing such dependence may consequently reflect an interdependence of BLA with GC input (Blundell et al., 2001; Balleine et al., 2003; Corbit and Balleine, 2005), and that specifically, GC-generated representations may need to be communicated to BLA for assignment of motivational significance. We will explore this possibility in future experiments.

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