

# Accepted Manuscript

Alcohol-associated antecedent stimuli elicit alcohol seeking in non-dependent rats and may activate the insula

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PII: S0741-8329(18)30002-8

DOI: [10.1016/j.alcohol.2018.08.004](https://doi.org/10.1016/j.alcohol.2018.08.004)

Reference: ALC 6838

To appear in: *Alcohol*

Received Date: 4 January 2018

Revised Date: 22 July 2018

Accepted Date: 10 August 2018

Please cite this article as: Cofresí R.U., Grote D.J., Viet Thanh Le E., Monfils M.-H., Chaudhri N., Gonzales R.A. & Lee H.J., Alcohol-associated antecedent stimuli elicit alcohol seeking in non-dependent rats and may activate the insula, *Alcohol* (2018), doi: 10.1016/j.alcohol.2018.08.004.

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1 **Full title:** ALCOHOL-ASSOCIATED ANTECEDENT STIMULI ELICIT ALCOHOL SEEKING IN  
2 NON-DEPENDENT RATS AND MAY ACTIVATE THE INSULA

3 **Short Title:** CUE-ALCOHOL ASSOCIATIVE MEMORY IN THE RAT

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19 **Source of Support:** NIH NIAAA R37AA11852 (RAG), NIH NIAAA T32AA007471 (RUC), NIHM  
20 R01MH091147 (MHM), CIHR MOP-137030 (NC), University of Texas Undergraduate Research  
21 Fellowship (DJG)

22

**1 ABSTRACT**

2 Alcohol self-administration produces brain and behavior adaptations that facilitate a progressive  
3 loss of control over drinking and contribute to relapse. One possible adaptation is the ability of  
4 antecedent environmental stimuli that are consistently paired with alcohol to trigger alcohol  
5 seeking behaviors. We previously modeled this adaptation in rats using a Pavlovian conditioning  
6 procedure in which illumination of a houselight preceded the presentation of a sipper tube that  
7 produced unsweetened alcohol when licked. However, in our previous work we did not  
8 demonstrate whether this adaptation represented a consequence of repeated exposure to  
9 alcohol or the houselight, or whether it was the consequence of associative learning and  
10 memory. Thus, in the present study, we tested the associative basis of alcohol seeking in  
11 response to houselight illumination in our task using adult male rats that were not food or water  
12 deprived and were not dependent on alcohol. Separate groups of rats received houselight  
13 illumination that was explicitly paired or unpaired with presentation of the retractable sipper that  
14 provided access to unsweetened alcohol. Our primary dependent variable was appetitive  
15 alcohol-directed behavior: the frequency of movement toward and interaction with the hole in  
16 the wall of the chamber through which the sipper was presented during the period of houselight  
17 illumination trial before each sipper presentation. However, we also analyzed consummatory  
18 sipper licking behavior and blood ethanol concentration in the same rats. Finally, we explored  
19 the brain basis of cue-elicited alcohol seeking using c-Fos immunohistochemistry. Our findings  
20 confirmed the associative basis of cue-elicited alcohol seeking in our paradigm and mapped  
21 these onto the insular cortex, suggesting a role for this brain region in early stages of brain and  
22 behavior adaptation to regular alcohol use.

23 **Keywords:** cues; alcohol seeking; Pavlovian conditioning; low dose alcohol; Fos

24

## 1 INTRODUCTION

2 Classical conditioning allows antecedent sensory stimuli to become associated with alcohol  
3 availability, ingestion, and/or pharmacological effects. Conditioned behavioral and physiological  
4 reactivity to these alcohol-predictive cues contributes to the risk for relapse to problem drinking.  
5 We recently developed a model of alcohol cue reactivity in rats (Cofresí et al., 2017; Cofresí et  
6 al., 2018). Here, we verify whether reactivity to the alcohol cue in our model stems from  
7 associative learning and whether memory for that cue-alcohol association maps onto brain  
8 regions that are implicated in alcohol addiction.

9 In our paradigm, conditioning trials involve illumination of a houselight (the cue) and brief  
10 availability of a retractable sipper from which rats can lick out unsweetened alcohol. Houselight  
11 illumination appears to gain the ability to elicit anticipatory approach to the sipper. If this  
12 response reflects a learned association with alcohol availability, then it should only be acquired  
13 when illumination is explicitly paired with alcohol availability, in a classic “Paired” conditional-  
14 unconditional stimulus (CS-US) arrangement. However, if the same alcohol-seeking response is  
15 acquired when alcohol availability is explicitly “unpaired” with illumination by presenting the  
16 sipper only during the interval between illuminations (“Unpaired” CS/US arrangement), then it  
17 may stem from a non-associative learning process (e.g., sensitization). The associative basis of  
18 cue-alcohol associations has been shown previously in studies that used Paired and Unpaired  
19 groups (Srey et al., 2015); however, in that task alcohol delivery occurred immediately after  
20 presentation of the CS and the CS was a retractable lever.

21 Another reason for comparing a Paired and Unpaired group in our paradigm is that since  
22 alcohol delivery occurs via a sipper tube, the presentation of this sipper tube may itself be  
23 learned as a cue, and acquire the ability to initiate drinking bouts. However, sipper presentation  
24 is always experienced during houselight illumination. As such, what looks like a response to

1 another distinct cue (sipper presentation) may actually represent facilitation of consummatory  
2 sipper licking behavior by the houselight cue-alcohol association. If so, then once the houselight  
3 cue-alcohol association is formed, rats in the “Paired” cue group should initiate drinking bouts  
4 faster than rats in the “Unpaired” cue group.

5 Cue-related behavioral reactivity in other rodent models of alcohol cue reactivity has been  
6 shown to reflect associative learning. However, we cannot assume that cue-related reactivity in  
7 our procedure also reflects associative learning, as our procedure differs substantially from  
8 others. In the conditioned place avoidance/preference procedure, which has taught us that  
9 alcohol-associated cues can acquire both appetitive and aversive properties in rodents, the cue  
10 predicts experimenter-administered alcohol (e.g., Bormann & Cunningham, 1988; Bozarth,  
11 1990; Ciccocioppo, Panocka, Froldi, et al., 1999; Cunningham, 1979, 1981; Fidler, Bakner, &  
12 Cunningham, 2004; Nentwig, Myers, & Grisel, 2017; Torres, Walker, Beas, & O’Dell, 2014). In  
13 procedures where the rodent self-administers alcohol, cues typically acquire appetitive  
14 properties such as the ability to invigorate instrumental responding (Corbit & Janak, 2007;  
15 Glasner, Overmier, & Balleine, 2005; Krank, O’Neill, Squarey, et al., 2008; Milton, Schramm,  
16 Wawrzynski, et al., 2012), the ability to elicit sign-tracking conditioned responses (e.g., Krank,  
17 2003; Krank, O’Neill, Squarey, et al., 2008; Srey et al., 2015; Villaruel et al., 2016), and the  
18 ability to act as conditioned positive reinforcers for the acquisition of new instrumental  
19 responses (e.g., Srey et al., 2015; Schramm, Everitt, & Milton, 2016). However, in some cases,  
20 even cues paired with self-administered alcohol can acquire aversive properties (e.g., Stewart &  
21 Grupp, 1986). In most of the self-administration models, the cue predicts delivery of a fixed  
22 amount of alcohol, which is available for ingestion even when the cue is not present (e.g., Krank  
23 et al., 2008; Remedios et al., 2014). In many, access to food and/or water in the homecage is  
24 restricted or alcohol is sweetened to motivate rodents to drink alcohol in the conditioning  
25 chamber (e.g., Krank, 2003; Tomie et al., 2002; Millan et al., 2013). In our procedure, rats have

1 free-access to food and water in the homecage and alcohol is never sweetened. Moreover, rats  
2 are free to drink as much alcohol they want during conditioning trials. Importantly, our procedure  
3 also allows us to measure drinking behavior (consummatory sipper licking) directly for each  
4 conditioning trial.

5 Many therapies for alcohol use disorder (e.g., naltrexone, cue exposure, counterconditioning)  
6 are predicated on the idea that maladaptive alcohol-associative memories (implicit or not) drive  
7 relapse. Knowing whether alcohol-related behavior stems from associative or non-associative  
8 memory is important because different approaches (behavioral and pharmacological) are  
9 needed to target associative and non-associative aspects of behavior. Ideally, we would know  
10 how (associative v. non-associative), as well as where alcohol-related cue memories are  
11 encoded in the brain. Consequently, in the present study, using the same rats in which we  
12 characterized behavior and blood ethanol, we evaluated expression of the immediate-early  
13 gene product c-Fos as an index of cellular activity in brain regions that may be involved in  
14 maintaining and/or expressing alcohol-related cue memories, with a focus on those regions  
15 implicated in cue-induced relapse to alcohol-seeking (Koob & Volkow, 2010).

## 16 **METHODS & MATERIALS**

### 17 **Subjects**

18 Adult male Long-Evans rats were used (Envigo; Indianapolis, IN, USA). Upon arrival, rats  
19 weighed 250-275 g. All were singly housed in a temperature- and humidity-controlled room  
20 ( $22\pm 2$  °C; 12hr light cycle). The homecage contained Sani-Chips® bedding and a Bio-Serv  
21 Gummy Bone (polyurethane; 5 cm L x 2.5 cm W). Metal wire cage-tops were used. Standard  
22 chow pellets were loaded into a large cup inside the cage. Tap water was provided via gravity-  
23 fed sipper inserted at approximately 45° from the cage top. Chow and water were replenished  
24 daily. Bedding was replaced weekly. All procedures took place during the light phase of the

1 light/dark cycle. The colony room was adjacent to the procedure rooms. Ethanol (v/v) solutions  
2 were prepared every 3 days from 95% ethyl alcohol (ACS/USP grade, Pharmco-AAPER,  
3 Brookfield, CT, USA) and tap water. Solutions were kept and served at room temperature (20  
4 °C). All procedures were approved by the Institutional Animal Care and Use Committee at the  
5 University of Texas at Austin, and conducted in accordance with NIH guidelines.

## 6 **Behavioral Methods**

### 7 **Paired and Unpaired Cue-Ethanol Conditioning**

8 All conditioning took place in chambers housed in cubicles equipped with a digital video camera  
9 (for detailed description of the apparatus, see Cofresí et al., 2017, 2018). Rats were acclimated  
10 to the conditioning chambers and stimuli 48 hr after familiarization with unsweetened ethanol in  
11 the homecage (see **Supplemental Information**). Rats were first familiarized with a retractable  
12 sipper in the conditioning chamber by giving a single session in which the sipper was presented  
13 for 35 min, during which time rats were free to lick out unsweetened ethanol. The next day, rats  
14 were habituated to houselight illumination in the chamber by giving a single session in which the  
15 houselight was illuminated 8 times (described below). During this session, the bottle assembly  
16 was never activated and ethanol was absent from the room.

17 Next, 2 groups matched on ingested doses across the homecage familiarization phase were  
18 created. One group of rats was assigned to our usual conditioning procedure: houselight  
19 illumination was explicitly paired with presentations of the alcohol sipper (creating group  
20 "Paired"). For the other group, the houselight stimulus was explicitly unpaired with presentations  
21 of the alcohol sipper (creating group "Unpaired").

22 Rats received 1 conditioning session per day. Each conditioning session was preceded by a 5  
23 min wait period. Session start was signaled to the rat by turning on the cubicle exhaust fan. At  
24 session start, the pre-trial interval (preTI) was selected randomly without replacement from a list

1 of possible intervals (160 s, 240 s, 250 s, 320 s, 350 s, 360 s). Upon conclusion of the 1<sup>st</sup> trial,  
2 an inter-trial interval (ITI) was selected randomly without replacement from the same list above.  
3 Each session consisted of 8 trials and each trial was 20 s long. After the 8<sup>th</sup> trial, the post-trial  
4 interval (postTI) was selected randomly without replacement from the same list above. The  
5 session ended once the postTI had elapsed. This was signaled by turning off the cubicle  
6 exhaust fan. On average, total session duration was 45 min (8 trials + 7 ITI + 1 preTI + 1  
7 postTI). Sessions were given across consecutive days and at approximately the same time  
8 every day by the same experimenter.

9 During each trial, the houselight was illuminated for 20 s. In group Unpaired, there was no  
10 consequent event. However, in group Paired, the retractable bottle assembly was activated 10 s  
11 into the illumination to present the metal sipper such that alcohol availability and illumination co-  
12 terminated. The sipper insertion hole was 8.5 cm rightward and 16 cm downward from the  
13 houselight fixture (installed on the same wall) For group Unpaired, sipper presentations instead  
14 occurred mid-ITI, beginning in ITI 2 and ending in the postTI. Houselight illumination onset,  
15 offset, and intervals were yoked between groups. Retractable bottle assembly activations were  
16 yoked within groups. On average, time between the 1<sup>st</sup> and 8<sup>th</sup> sipper presentation was 35 min.

17 Licking the sipper produced the ethanol solution each rat was drinking at the end of homecage  
18 pre-exposure. The ethanol solution was either 10 or 15% ethanol (vol/vol tap water; 10E or  
19 15E). Of the 17 rats for which data are presented in the main text, 6 drank 10E. These 6 were  
20 evenly split between groups Paired and Unpaired. The sipper contained a ball bearing to  
21 prevent fluid spill upon insertion and retraction. When inserted, the tip was typically in the plane  
22 of the wall. In other words, the sipper did not protrude far into the chamber. At most, 500 ms  
23 elapsed between activation of the bottle assembly and availability of the sipper.

#### 24 **Brain Collection Day**



1 On brain collection day, rats in 2 groups (Paired Run, Unpaired Run) were weighed and  
2 transferred into the conditioning chambers for a conditioning session. Rats in 2 other groups  
3 (Paired Not Run, Unpaired Not Run) were not handled and stayed in the homecage. Groups  
4 were matched for ingested doses across all conditioning sessions. Rats in the Not Run  
5 condition had 15 conditioning sessions total prior to brain collection. Due to technical limitations,  
6 rats in the Run condition had either 16 (n=2/group) or 18 (n=2/group) conditioning sessions total  
7 prior to brain collection. Houselight-elicited behavioral reactivity on brain collection day was not  
8 different between rats in the Run condition with 16 v. 18 sessions, so their data were pooled.

9 All brains were collected 90 min from the beginning of the conditioning session given to the Run  
10 groups. All rats were anesthetized with isoflurane gas and administered euthanasia III solution  
11 (Med-Pharmex, Inc., Ponomo, CA, USA). Following euthanasia, tissues were fixed by perfusing  
12 phosphate buffered saline (pH 7.2) (PBS) then 4% para-formaldehyde w/v in PBS (fixative  
13 solution). Brains were kept in 20% sucrose m/v in fixative solution overnight. Brains were flash-  
14 frozen on dry ice the next day and stored at -80°C until processing for Fos  
15 immunohistochemistry.

## 16 **Behavioral Measurements**

17 Trials from conditioning sessions 6, 9, and 12 as well as the sessions given on brain collection  
18 day and the day before brain collection were sampled for appetitive behavioral state from digital  
19 video recordings by making instantaneous observations every 1.25 s starting 5 s before  
20 houselight onset as in (Cofresí et al., 2017, 2018; Lee et al., 2005) such that there were 4  
21 observations per trial phase. Trial phase or bin “-1” refers the 5 s period before houselight onset  
22 and trial phase or bins 1-4 refer to 5 s periods across the houselight illumination. At each  
23 observation, the mutually exclusive rating options were “sipper site approach” (approaching,  
24 attending to, or exploring the sipper insertion hole, including sniffing, gnawing, and clawing at

1 the hole; **Figure 1 A**), “orienting” (rearing: both forepaws off the floor, supported by hindlimbs,  
2 facing any direction; **Figure 1 B**) or “other” (e.g., grooming, resting). Consummatory drinking  
3 behavior (sipper licking; **Figure 1 C**) was recorded automatically using a contact lickometer  
4 circuit. Behavioral observations were made by highly trained judges (intra- and inter-rater  
5 agreement  $\geq 95\%$ ). However, since within-trial events differed dramatically, it was impossible to  
6 blind raters to “Paired” v. “Unpaired” status, thus bias was possible. In order to corroborate  
7 “sipper site approach” observations, after the 12<sup>th</sup> conditioning session, we customized the  
8 metal panel on which the retractable bottle assembly was mounted. The portion of the panel  
9 bearing the sipper hole was cut, trimmed, and seated in a polylacticacid plastic frame (7.11 cm  
10 L, 0.32 cm W, 3.71 cm H) in order to electrically isolate it from the rest of the conditioning  
11 chamber. The exposed metal surface (6.91 cm L, 0.32 cm W, 3.66 cm H) was then wired into a  
12 second contact lickometer circuit, allowing automated recording of forepaw contact with the area  
13 around the sipper hole. In the session where both were collected, device-based sipper site  
14 contact (data not shown) exhibited the same within-session patterns as scored sipper site  
15 approach.

16 The total dose of ethanol ingested in each conditioning session was also monitored for each rat.  
17 Drinking solution intake was measured as the mass difference in bottle weight pre- and post-  
18 session after correcting for spillage. The grams of solution ingested were then converted to g  
19 ethanol and ingested dose was expressed as g/kg body weight for each rat. For every  
20 conditioning session, a weighboat underneath the retractable sipper tube/bottle assembly  
21 collected spillage such that solution intake values were corrected at the level of each rat.

## 22 **Blood ethanol analysis**

23 To determine the relationship between ingested doses and blood ethanol levels, we took blood  
24 samples after the 8<sup>th</sup> sipper presentation in a session. Blood was sampled on different days for

1 different rats (session 13 or 14) in order to measure blood ethanol concentration at  
2 approximately the same point along the blood ethanol concentration timecourse given logistical  
3 constraints (i.e., only 1 experimenter for 3 groups of 5-6 rats undergoing a yoked conditioning  
4 session at a specific time of day). As on any day, rats had unlimited chow and water in the  
5 homecage until they were transferred into conditioning chambers for a conditioning session.  
6 Rats were removed from the chamber immediately after the 8<sup>th</sup> sipper presentation and  
7 anesthetized with isoflurane gas. Blood was then collected from the lateral saphenous vein (3  
8 replicates per rat). Ethanol concentration (mg/dL) in 10  $\mu$ L whole blood mixed with 90  $\mu$ L  
9 saturated saline was determined using gas chromatography with flame ionization detection (GC-  
10 FID) as in (Carrillo et al., 2008).

#### 11 **Fos Immunohistochemistry**

12 Brain sectioning, tissue processing, and c-Fos immunostaining was performed as in (Lee et al.,  
13 2005). For details, see **Supplemental Information**.

#### 14 **Statistical Analysis & Data Visualization**

15 Behavior data were analyzed using mixed factorial analysis of variance (ANOVA). The threshold  
16 for statistical significance was  $p < 0.05$ . Significant effects in the omnibus ANOVAs were followed  
17 up as appropriate (e.g., ANOVA F-tests to decompose interactions of 2 or more factors, T-tests  
18 to decompose the main effect of a factor). Bonferroni correction was applied at every follow-up  
19 stage to prevent false discovery (i.e., the threshold p-value used to evaluate significance in a  
20 batch of tests was divided by the number of tests in the batch; e.g., a corrected threshold p-  
21 value of 0.017 would be used to evaluate the 3 F-tests for the simple 2-way interaction effect of  
22 two factors at each level of a 3-level third factor and a corrected threshold p-value of 0.006  
23 would be used to evaluate a batch of 8 pairwise t-tests).

1 Ingested dose, ethanol bottle preference ratio, and blood ethanol concentration data were  
2 analyzed using ANOVA and simple linear regression. The threshold for statistical significance  
3 was  $p < 0.05$ . Bonferroni corrected follow-ups were conducted as described above.

4 Fos expression data were analyzed by running ANOVA within structures considering the  
5 between-subject factor of group (Paired v. Unpaired), the within-subject factor of brain region  
6 (e.g., in the Accumbens ANOVA, shell v. core), and their interaction. Known Fos expression  
7 differences between structures (e.g., dorsal striatum v. basolateral amygdalar complex) and  
8 missing data precluded ANOVA considering all sampled structures. Separate ANOVA were  
9 done for the “Run” and “Not Run” conditions because our primary interest was differential  
10 induction of Fos in groups Paired and Unpaired. The threshold for statistical significance was  
11  $p < 0.05$ . Bonferroni corrected follow-ups were conducted as described above.

12 All analysis was done in R version 3.41 (R Core Team, 2017) using the car package (Fox &  
13 Weisberg, 2011). Data were plotted in R using the ggplot2 package (Wickham, 2009) and  
14 finalized in Inkscape version 0.92.2 (Inkscape Team, 2017).

## 15 **RESULTS**

### 16 Acquisition of alcohol drinking and behavioral reactivity

17 A total 35 rats were obtained for this study. Of those screened, 29 ingested doses  $\geq 1$  g/kg/24hr  
18 on average over the last week of homecage drinking, and were retained for conditioning. Of  
19 those conditioned, 17 ingested doses  $\geq 0.30$  g/kg/session on average across the last 3 sessions  
20 (“Learners”), which is the dose threshold for conditioning effects of ingested ethanol that we  
21 observed in our previous study (Cofresí et al., 2018). The other 12 rats ingested doses that  
22 were consistently below that threshold (“NonLearners”). In **Supplemental Information**, we  
23 provide the following: ingested doses and ethanol bottle preference ratio across homecage  
24 sessions for both Learners and NonLearners, ingested doses across conditioning sessions for

1 NonLearners, scored sipper site approach and orienting to the light across conditioning  
2 sessions for both Learners and NonLearners, sipper licking (latency and intensity) across  
3 conditioning sessions for Learners and NonLearners, and trial-by-trial scored orienting for  
4 Learners. In the main text, we present trial-by-trial scored sipper site approach and sipper  
5 licking data for Learners in conditioning sessions 6, 9, and 12 as well as the conditioning  
6 sessions given on brain collection day and the day before. We also present blood ethanol  
7 concentrations obtained after a conditioning session alongside ingested dose and estimated  
8 blood ethanol concentrations across conditioning sessions.

#### 9 Blood ethanol concentrations and drinking across conditioning sessions

10 Both groups of rats (Paired and Unpaired) ingested similar doses across conditioning. Doses  
11 increased across sessions 1-9 and were relatively stable thereafter ( $F_{14,210}=32.17$ ,  $p<0.001$ ;  
12 **Figure 2 A**). ANOVA detected a significant interaction of session x group (group x session  
13 interaction:  $F_{14,210}=2.47$ ,  $p<0.01$ ), but follow-up revealed that while groups differed at session 1  
14 and 8, these differences were not statistically significant after Bonferroni correction. Additionally,  
15 groups ingested similar doses on brain collection day ( $n=4$ /group, group main effect:  $F_{1,6}<0.5$ ,  
16 NS). Collapsing group, rats ( $n=17$ ) drank (mean  $\pm$  sem)  $0.48 \pm 0.03$  g/kg per session across  
17 session 10-15. On brain collection day, collapsing group, rats ( $n=8$ ) drank (mean  $\pm$  sem)  $0.58 \pm$   
18  $0.04$  g/kg.

19 Blood samples were taken after the 8<sup>th</sup> sipper presentation in session 13 or 14 for ethanol  
20 analysis (only one session per rat). Body weights ranged from 409 to 505 g. Time between the  
21 1<sup>st</sup> and 8<sup>th</sup> sipper presentation in the session was 32-37 min (median = 34 min). Sampling time  
22 ranged from 8 to 12 min after the 8<sup>th</sup> sipper presentation. Blood ethanol concentration (BEC)  
23 ranged from 0 to 43 mg/dL with mean  $\pm$  sem of  $9 \pm 3$  mg/dL ( $n=17$ ). Total ingested dose across  
24 the 8 sipper presentations ranged from 0.17 to 0.94 g/kg with mean  $\pm$  sem. of  $0.45 \pm 0.05$  g/kg

1 (n=17). BEC at 8-12 min after the 8<sup>th</sup> sipper presentation was significantly correlated with the  
2 total ingested dose across the 8 sipper presentations (Pearson's  $r=+0.80$ ,  $t_{15}=5.14$ ,  $p<0.001$ )  
3 (**Figure 2 B**). The dose-BEC relationship was independent of group ( $F<1$ , NS).

4 We noticed that many rats had non-detectable or zero BEC 8-12 min after the 8<sup>th</sup> sipper  
5 presentation on the sampled day. Specifically, 5 rats in each group had non-detectable or zero  
6 BEC (in group Paired, we counted 1 rat with BEC 1.85 mg/dL as "zero" to be conservative).  
7 Whether BEC was zero or non-zero was independent of group ( $X^2<1$ , NS). The mean  $\pm$  sem  
8 across the 4 rats in group Paired with detectable BEC was  $22 \pm 8$  mg/dL, and the mean  $\pm$  sem  
9 across the 3 rats in group Unpaired with detectable BEC was  $20 \pm 2$  mg/dL. These group-mean  
10 BEC did not differ statistically ( $t_5<1$ , NS).

11 To get a better sense for possible levels of ethanol exposure across conditioning and  
12 differences between groups in exposure levels, we applied the equation for the regression line  
13 in **Figure 2 B** to estimate BEC after the 8<sup>th</sup> sipper presentation per session for each rat based  
14 on ingested dose for each session. In both groups Paired and Unpaired, estimated BEC  
15 increased across sessions 1-4 to non-zero levels, continued to increase across sessions 5-9,  
16 and then remained relatively stable ( $F_{14, 210}=13.43$ ,  $p<0.001$ ; group main effect and group x  
17 session interaction:  $F<1.5$ , NS; **Figure 2 C**). Additionally, there was no group difference in  
18 estimated BEC on brain collection day (n=4/group; group main effect:  $F<1$ , NS). Across session  
19 10-15, collapsing group, rats (n=17) could be expected to have BEC (mean  $\pm$  sem) of  $10 \pm 1$   
20 mg/dL after the 8<sup>th</sup> sipper presentation. On brain collection day, collapsing group, rats (n=8)  
21 could be expected to have BEC (mean  $\pm$  sem) of  $16 \pm 1$  mg/dL after the 8<sup>th</sup> sipper presentation.  
22 Thus, the potential level of ethanol exposure was modest, but likely non-zero, and similar  
23 between groups.

24 Reactivity to the houselight depends on the relationship between the houselight and ethanol

1 In our paradigm, houselight illumination elicits anticipatory approach and contact with hole in the  
2 wall of the conditioning chamber where the alcohol sipper is presented. To characterize this  
3 form of reactivity in our paradigm, we analyzed ethanol-directed approach and contact (i.e.,  
4 scored sipper site approach) before and during the first half of houselight illumination  
5 (specifically, trial phases -1, 1, and 2) on a per trial basis for 9 well-trained rats in group Paired  
6 and 8 well-trained rats in group Unpaired across conditioning sessions 6, 9, and 12, as well as  
7 the session before brain collection day (**Figure 3 A-D**). The data from brain collection day  
8 (**Figure 3 E**) were also analyzed, but these were treated separately because only a subset of  
9 rats (n=4 per group) received a conditioning session on brain collection day.

10 In brief, we found that houselight illumination was able to elicit ethanol (sipper)-seeking behavior  
11 only in group Paired. The vigor of this response exhibited within-session decay as early as  
12 session 9, and this within-session pattern appeared to be present in subsequent sessions.  
13 Statistical results are presented below.

14 In the analysis considering data from session 6, 9, 12, and the day before collection, we saw  
15 that the overall level of sipper site approach was greater in group Paired than Unpaired (main  
16 effect:  $F_{1,15}=26.85$ ,  $p<0.002$ ). Additionally, the pattern of approach across trials varied across  
17 sessions (trial x session interaction:  $F_{21,315}=1.92$ ,  $p<0.01$ ), and this variation was similar within  
18 groups (group x trial x session interaction:  $F<1$ , NS). Specifically, trial-by-trial variation did not  
19 emerge until session 9 (simple trial main effect within all session except 6:  $F_{7,112}\geq 5.51$ ,  $p<0.001$ ).  
20 In all sessions except 6, the level of sipper site approach was greater across trials 1-4 than 5-8  
21 ( $t_{16}\geq 2.70$ ,  $p<0.01$ ). On average across sessions, however, within-session trial-by-trial variation  
22 depended on group (group x trial interaction:  $F_{7,105}=20.71$ ,  $p<0.001$ ). Specifically, only group  
23 Paired exhibited trial-by-trial changes in approach level (simple trial main effect:  $F_{7,56}=32.33$ ,  
24  $p<0.001$ ). Sipper site approach varied across trial phases within trials differently depending on  
25 trial (trial phase x trial interaction:  $F_{14,210}=6.13$ ,  $p<0.001$ ), and this pattern of variation depended

1 on group (group x trial phase x trial interaction:  $F_{14, 210}=4.64$ ,  $p<0.001$ ), but not session (trial  
2 phase x trial x session and group x trial phase x trial x session interaction:  $F<1.5$ , NS). Sipper  
3 site approach varied within trials differently across trials only in group Paired (simple trial phase  
4 x trial interaction:  $F_{14, 112}=7.69$ ,  $p<0.001$ ). Moreover, only sipper site approach during houselight  
5 illumination exhibited variation across trials in group Paired (simple trial main effect within bin 1  
6 and 2:  $F_{7, 56}\geq 5.58$ ,  $p<0.001$ ). Specifically, sipper site approach during houselight illumination (bin  
7 1 and 2) was greater across trials 1-4 than 5-8 (collapsing session,  $t_8\geq 4.26$ ,  $p<0.01$ ).

8 In the analysis of data from brain collection day, we also saw that the overall level of sipper site  
9 approach was greater in group Paired than Unpaired (main effect:  $F_{1, 5}=12.38$ ,  $p<0.05$ ). We also  
10 saw that the pattern of sipper site approach across trial phases within trials varied across trials  
11 differently between groups (group x trial phase x trial interaction:  $F_{14, 70}=3.68$ ,  $p<0.001$ ).

12 Specifically, trial and trial phase-dependent variation in sipper site approach level was only  
13 detected in group Paired (simple trial phase x trial interaction:  $F_{14, 28}=3.18$ ,  $p<0.01$ ). However,  
14 after Bonferroni correction, the simple main effect trial was not significant within any of the trial  
15 phases, and the simple main effect of trial phase was significant only within trial 3 ( $F_{2, 6}=30.18$ ,  
16  $p<0.01$ ).

17 Ethanol-directed reactivity to ethanol sipper presentation does not depend on relationship  
18 between ethanol sipper presentation and houselight illumination

19 Ethanol sipper presentation involves auditory and visual stimuli, so it is conceivable that sipper  
20 presentation could condition initiation of consummatory sipper licking (drinking). To characterize  
21 reactivity to sipper presentation, we analyzed the latency to start licking the sipper (Paired group  
22  $n=9$ ; Unpaired group  $n=7$  out of 8 due to data loss) in well-trained rats across conditioning  
23 sessions 6, 9, and 12, as well as the session before brain collection day (**Figure 4 A-D**). The  
24 data from brain collection day (**Figure 4 E**) were also analyzed, but these were treated



1 separately because only a subset of rats received a conditioning session on brain collection  
2 day.

3 In brief, we found that sipper presentation was able to elicit rapid initiation of consummatory  
4 licking (drinking) in both groups. The within-session pattern of this response also appeared to  
5 converge across sessions such that by brain collection day response vigor was subject to  
6 within-session decay in both groups. Statistical results are presented below.

7 In the analysis considering data from session 6, 9, 12, and the day before collection, we saw  
8 that the overall latency to start licking the sipper was similar between groups (group main effect:  
9  $F_{1,14}=1.03$ , NS). Additionally, latency varied by session ( $F_{3,42}=4.95$ ,  $p<0.01$ ) and by trial ( $F_{7,98}=4.84$ ,  
10  $p<0.01$ ). Although these two patterns of variation were independent of each other (trial  
11 x session interaction:  $F_{21,294}<1$ , NS), each pattern of variation was separately dependent on  
12 group (group x trial x session interaction:  $F_{21,294}<1.5$ , NS; group x trial interaction:  $F_{7,98}=4.59$ ,  
13  $p<0.01$ ; group x session interaction:  $F_{3,42}=3.26$ ,  $p<0.05$ ). Latency varied across sessions only  
14 within group Unpaired (simple session main effect:  $F_{3,24}=6.80$ ,  $p<0.01$ ). Specifically, latency  
15 decreased between session 9 and 12 ( $t_6=4.18$ ,  $p<0.01$ ). On average across sessions, latency  
16 varied by trial only within group Paired (simple trial main effect:  $F_{7,56}=11.26$ ,  $p<0.001$ ).  
17 Specifically, latency was lower across trials 1-4 than 5-8 ( $t_8=5.59$ ,  $p<0.001$ ).

18 In the analysis of data from brain collection day, we found that rats in group Paired ( $n=4$ ) and  
19 Unpaired ( $n=3$  out of 4 due to data loss) now exhibited the same trial-by-trial variation (trial main  
20 effect:  $F_{7,35}=3.20$ ,  $p<0.05$ ; group main effect and group x trial interaction:  $F<1$ , NS).

21 Drinking pattern does not depend on relationship between ethanol availability and houselight  
22 illumination

23 Although total ingested dose per session was equivalent between groups across conditioning  
24 sessions, we were curious whether the within-session pattern of consummatory sipper licking

1 (drinking) changed across sessions and whether it differed between groups. Consequently, we  
2 characterized total licks per trial across trials for 9 well-trained rats in group Paired and 7 out of  
3 8 well-trained rats in group Unpaired (due to equipment-related data loss) across conditioning  
4 sessions 6, 9, and 12, as well as the conditioning session given the day before brain collection  
5 (**Figure 5 A-D**). The data from brain collection day (**Figure 5 E**) were also analyzed, but these  
6 were treated separately because only a subset of rats received a conditioning session on brain  
7 collection day

8 In brief, the within-session pattern of consummatory sipper licking (drinking) appeared to  
9 converge across sessions such that by brain collection day both groups exhibited trial-by-trial  
10 decreases in the vigor of sipper licking.

11 In the analysis considering data from session 6, 9, 12, and the day before collection, we saw  
12 that overall licking levels were similar between groups (group main effect:  $F_{1,14}=0.21$ , NS).  
13 However, we also saw that licking varied by session ( $F_{3,42}=5.61$ ,  $p<0.01$ ) and by trial ( $F_{7,98}$ ,  
14  $p<0.001$ ). Although these two patterns of variation were independent of each other (trial  
15 x session interaction:  $F_{21,294}<1$ , NS), each pattern of variation was separately dependent on  
16 group (group x trial x session interaction:  $F_{21,294}<1.5$ , NS; group x trial interaction:  $F_{7,98}=4.17$ ,  
17  $p<0.001$ ; group x session interaction:  $F_{3,98}=3.05$ ,  $p<0.05$ ). Licking varied across sessions only in  
18 group Unpaired (simple session main effect:  $F_{3,18}=6.50$ ,  $p<0.01$ ). Specifically, licking increased  
19 from session 9 and 12 ( $t_6=3.79$ ,  $p<0.01$ ). Licking varied by trial only in group Paired (simple trial  
20 main effect:  $F_{7,56}=11.72$ ,  $p<0.001$ ). Specifically, licking was greater across trials 1-4 than 5-8  
21 ( $t_8=5.29$ ,  $p<0.001$ ).

22 In the analysis of data from brain collection day, we found that rats in group Paired ( $n=4$ ) and  
23 Unpaired ( $n=3$  out of 4 due to data loss) now exhibited the same trial-by-trial variation (trial main  
24 effect:  $F_{7,35}=3.77$ ,  $p<0.01$ ; group main effect and group x trial interaction:  $F<1$ , NS).

1 Different brain regions are responsive to Paired v. Unpaired cue-ethanol conditioning

2 To begin mapping the substrates of ethanol-associated cue memories in our models, we  
3 obtained brain tissue for all 17 rats that consistently drank  $\geq 0.30$  g/kg and evaluated expression  
4 of the immediate-early gene product c-Fos. Following immunostaining for c-Fos protein,  
5 sections were imaged and Fos+ cells counted. For each brain region, cell counts were averaged  
6 across sampling regions, atlas levels, and hemispheres to index regional activation.

7 We found no significant difference between group Paired and Unpaired (group main effect and  
8 group x sub-region interaction: NS) in either the Run or Not Run condition for mean Fos+ cell  
9 counts in the following brain regions: the medial and lateral divisions of the orbitofrontal cortex  
10 (**Figure 6 A**), the prelimbic and infralimbic divisions of the medial prefrontal cortex (**Figure 6 B**),  
11 the core and shell compartments of the nucleus accumbens (**Figure 6 C**), the medial and lateral  
12 aspects of the dorsal striatum (**Figure 6 D**), the medial and lateral divisions of the amygdalar  
13 central nucleus (**Figure 6 E**), and the substantia nigra pars compacta/ventral tegmental area  
14 complex (**Figure 6 F**).

15 However, we did find that in the Run condition, there was a significantly greater mean Fos+ cell  
16 count in group Paired across the anterior and posterior divisions of the insular cortex (group  
17 main effect:  $F_{1,6}=13.17$ ,  $p<0.02$ ; group x sub-region interaction:  $F_{1,6}<1$ , NS; **Figure 7 A**). In the  
18 Not Run condition, mean Fos+ cell counts did not differ significantly between groups Paired and  
19 Unpaired (group main effect and group x sub-region interaction:  $F_{1,7}<1$ , NS).

20 In the basolateral complex of the amygdala (**Figure 7 B**), we found significant group x sub-  
21 region interaction effects for both the Run ( $F_{2,10}=5.87$ ,  $p<0.025$ ) and Not Run condition  
22 ( $F_{2,14}=3.96$ ,  $p<0.05$ ). In the Run condition, the mean Fos+ cell count differed by region for group  
23 Paired (simple effect of region:  $F_{2,4}=8.02$ ; specifically, basal and basomedial > lateral nucleus),  
24 but not Unpaired ( $F_{2,5}=1.45$ ). However, this within-group by-region difference did not survive

1 Bonferroni correction and none of the simple effects of group within regions were significant  
2 (even before Bonferroni correction). In the Not Run condition, group Unpaired had much greater  
3 mean Fos+ cell count in the BMA (simple effect of group:  $F_{1,7}=6.61$ ); however, this too did not  
4 survive Bonferroni correction. In addition, neither simple effect of region within group was  
5 significant (even before Bonferroni correction) in the Not Run condition.

## 6 **DISCUSSION**

7 In the present study, we characterized the behavioral reactions of rats to specific alcohol-related  
8 stimuli (houelight and sipper) in an oral alcohol conditioning task to determine whether  
9 reactivity was driven by associative or non-associative memory. We also sampled blood after a  
10 conditioning session to characterize alcohol exposure level in the same rats. Finally, we used c-  
11 Fos expression as an index of cellular activation to map what brain areas might contribute to  
12 alcohol-related stimulus reactivity in the oral alcohol conditioning task.

### 13 **Acquisition of houelight-elicited behavioral reactivity depends on the relationship** 14 **between alcohol availability and houelight illumination**

15 After a period of intermittent access to unsweetened alcohol in the homecage that was sufficient  
16 to promote alcohol drinking and apparent habituation of initial taste aversion (**Supplemental**  
17 **Figure 7 A-B**), rats were presented with an alcohol sipper intermittently in a different context (a  
18 conditioning chamber). Sipper presentations were accompanied by the sound of the bottle  
19 assembly motor activation. Some rats were also provided with an antecedent houelight  
20 stimulus (group Paired), while others were exposed to the same houelight stimulus explicitly  
21 unpaired with alcohol sipper presentation (group Unpaired). These conditioning stimulus  
22 arrangements were designed to test whether any resulting behavioral reactivity to houelight  
23 illumination reflected its learned association with alcohol availability.

1 In keeping with the correspondence between the two stimulus arrangements, rats in both  
2 groups learned to approach the sipper quickly upon its presentation to initiate and sustain  
3 consummatory licking across the period of alcohol availability (**Supplemental Figure 3 A-B**).  
4 However, in keeping with the key difference between the two stimulus arrangements,  
5 anticipatory approach to the site of alcohol availability was only conditioned as a reaction to  
6 houselight illumination in group Paired (**Supplemental Figure 6 A**). This is behavioral evidence  
7 that alcohol seeking behavior in response to houselight illumination in our paradigm reflects  
8 cue-alcohol associative memory, and not adaptation to repeated exposure to either the alcohol  
9 or the houselight. In previous work from one of our labs (Srey et al., 2015; Villaruel et al., 2016),  
10 we compared the learned behavioral responses of rats that received cue presentations explicitly  
11 paired and unpaired with alcohol delivery using a completely different paradigm. In that  
12 paradigm, a retractable lever was inserted into the conditioning chamber and immediately upon  
13 its retraction, unsweetened alcohol was delivered into an adjacent fluid port for consumption at  
14 any point during conditioning session. Using that paradigm, we found that alcohol seeking  
15 behavioral responses were only conditioned to the cue when the cue was explicitly paired with  
16 alcohol delivery, demonstrating the associative basis of alcohol seeking behaviors in the  
17 paradigm. Additionally, the cue only gained the ability to act as a secondary or conditional  
18 reinforcer if it had previously been explicitly paired with alcohol delivery. Together, our findings  
19 add to a growing body of preclinical work in rodent models that suggests that the potentially  
20 problematic ability of alcohol-predictive cues to elicit alcohol seeking behaviors in people may  
21 be a property that arises as a result of naturally-occurring associative learning across the  
22 alcohol use history.

23 It is worth mentioning that although the stimulus arrangement for group Unpaired was meant to  
24 eliminate the capacity of houselight onset to serve as a predictor for alcohol availability, it was  
25 possible for these rats to have learned that houselight offset predicted alcohol availability. One

1 way this latter learning could have manifested is as conditioning of the overt attentional  
2 response (orienting) (Holland, 1980; Delamater & Holland, 2008). There was some behavioral  
3 evidence for this adventitious conditioning in group Unpaired in the form of persistent, albeit low  
4 frequency, orienting toward the time of houselight offset (**Supplemental Figure 4 A**); however,  
5 we are not convinced that this behavior is related to the consequences of alcohol drinking  
6 (**Supplemental Figure 4 B**). Thus, given equivalent alcohol ingestion, the stimulus arrangement  
7 in group Paired conditioned an appetitive response whereas the stimulus arrangement in group  
8 Unpaired may have conditioned an attentional response.

#### 9 **Trial-by-trial vigor of behavioral reactivity depends on the relationship between alcohol** 10 **availability and houselight illumination**

11 After conditioning session 9, rats in group Paired exhibited a decrease in houselight illumination-  
12 elicited sipper site approach across trials (**Figure 3**), an increase in latency to initiate drinking  
13 across trials (**Figure 4**), and a decrease in overall drinking intensity or rate across trials (**Figure**  
14 **5**). Our present findings tell us that within-session patterns in group Paired are present as early  
15 as conditioning session 9 and are stable once they emerge, replicating and extending our  
16 previous finding (Cofresí et al., 2018). This within-session decrease in behavior might reflect the  
17 slow-onset of alcohol's sedative-like effects (Chuck et al., 2006; Frye & Breese, 1981). If so,  
18 then we would have predicted trial-by-trial decreases in the vigor of consummatory licking  
19 (drinking behavior) not only in group Paired, but also in group Unpaired given similar total  
20 ingested doses. This was only true on brain collection day. Alternatively, within-session  
21 decrease in behavior might reflect the progressive devaluation of alcohol reinforcer (e.g., trial-  
22 by-trial decreases in the hedonic or incentive value of oral alcohol receipt or ingestion) (Samson  
23 et al., 1998; Samson et al., 2000). Cue-elicited reinforcer-directed responses are sensitive to  
24 between-session reinforcer devaluation procedures (e.g., satiety, pairing with illness) in food  
25 and sugar cue conditioning paradigms (Holland & Rescorla, 1975; Morrison et al., 2015). If

1 within-session reinforcer devaluation were taking place in our paradigm, then we might expect  
2 the level of houselight-elicited sipper site approach in group Paired to decrease across trials.  
3 This was always the case. Tentatively, our present findings allow us to rule out alcohol-induced  
4 sedation and accept progressive within-session reinforcer devaluation as the explanation for  
5 within-session decrease in the vigor of cue-elicited alcohol seeking in group Paired. In turn, this  
6 suggests that the reason why we consistently observed within-session decreases in overall  
7 consummatory vigor in group Paired, but not group Unpaired, is that drinking behavior in group  
8 Paired is in part determined by the houselight-alcohol association. However, it is important to  
9 keep in mind that we observed neither consistently faster initiation of drinking bouts per trial nor  
10 consistently more intense drinking per trial in group Paired compared to group Unpaired.

### 11 **Alcohol drinking, blood alcohol concentrations, and the motivation for learning about** 12 **alcohol-predictive cues**

13 In the present study, as in others (Chaudhri et al., 2010; Remedios et al., 2014), we were able  
14 to observe conditioning effects of self-administered alcohol on alcohol-related behavior in rats  
15 without ever depriving them of food or fluid or sweetening the alcoholic beverage. We can  
16 therefore rule out any confounding motivation for sweet taste or hydration as the source of  
17 reinforcement. We cannot, however, rule out the possibility that the additional calories provided  
18 by metabolism of ingested alcohol played some role in alcohol-related learning. The reality is  
19 that extra calories and intoxication both contribute to the reinforcing effects of ingested alcohol.  
20 However, the within-session behavior patterns exhibited by group Paired here and in our  
21 previous study (Cofresí et al., 2018) are not observed in studies with food-deprived rats  
22 presented with cues that predict food pellet delivery where the homeostatic drive for calories  
23 likely provides the primary motivation for learning (e.g., Nasser et al., 2018).

1 Another possibility is that the post-ingestive pharmacological effects of alcohol may have  
2 motivated alcohol-related learning in our procedure. The blood alcohol results in our previous  
3 studies support this suggestion (Cofresí et al., 2018; LeCocq et al., 2018), and the blood alcohol  
4 results in the present study also provide some support (**Figure 2 B**), although the evidence is  
5 less strong. Specifically, in the present study, over half rats had zero or non-detectable blood  
6 alcohol. One explanation for this observation is that sampled blood too late after the last trial or  
7 because we only sampled blood once, which is why we then used the regression equations  
8 derived from the blood alcohol measurement data to estimate possible blood alcohol  
9 concentrations across sessions (**Figure 2 C**). However, we acknowledge that we cannot rule  
10 out the alternative interpretation, which is that the majority of rats in our present study had zero  
11 contact with alcohol's post-ingestive pharmacological effects, at least during the cue  
12 conditioning phase.

13 Additional support for the idea that cue-alcohol learning in our procedure was motivated by  
14 alcohol's post-ingestive pharmacological effects can come from the fact that in our previous  
15 study (Cofresí et al., 2018) as well as in our present study, no alcohol seeking behavior was  
16 conditioned in rats that consistently drank below 0.30 g/kg across conditioning sessions  
17 (**Supplemental Figure 2+6**). These rats may have been drinking enough to experience  
18 alcohol's peripheral but not central pharmacological effects. However, some may argue that  
19 these rats may not have experienced even alcohol's peripheral pharmacological effects. At the  
20 very least, these rats licked the sipper enough to experience alcohol as a taste stimulus  
21 (**Supplemental Figure 3D**). It is thus plausible to suggest that these rats were simply more  
22 sensitive to the aversive taste of unsweetened alcohol. These rats did not differ reliably from  
23 those that consistently drank above 0.30 g/kg across conditioning sessions in terms of initial or  
24 final alcohol preference in homepage two bottle-choice phase preceding conditioning. They did,  
25 however, exhibit lower alcohol preference and ingest lower doses on average across the



1 homecage two bottle-choice phase (**Supplemental Figure 7 C-D**), which is consistent with the  
2 idea that this group of rats may have had greater aversion to the taste of alcohol.

3 Another possibility is that the houselight cue became associated with the flavor of alcohol. This  
4 explanation hinges on the assumption that a flavor preference was first conditioned and that this  
5 then allowed the flavor of alcohol to serve as a secondary reinforcer for conditioning of an  
6 alcohol approach response to the houselight illumination (an idea first proposed by Cunningham  
7 & Niehus, 1997). This assumption and the explanation based on it are supported by the fact that  
8 rats in our paradigm drank increasingly larger doses and appeared to lose their initial aversion  
9 to the taste of unsweetened alcohol across the homecage two-bottle choice phase  
10 (**Supplemental Figure 7 A-B**). We have no blood alcohol data from the homecage phase, so  
11 we do not know the extent to which that acquired reinforcing value is a function of exposure to  
12 alcohol's post-ingestive pharmacological effects. However, the literature suggests that rats drink  
13 enough in this paradigm to experience a range of blood alcohol concentrations (see Carnicella,  
14 Ron, & Barak, 2014).

#### 15 **The cue-alcohol associative memory formation or expression may involve the insular** 16 **cortex**

17 Overall, despite differences in study design, mean Fos+ cell counts in the present study were in  
18 line with those reported by others using rat models of oral alcohol conditioning (Dayas et al.,  
19 2007; Radwanska et al., 2008; Jupp et al., 2011). Our Fos expression findings corroborate the  
20 idea that some, but not all, brain regions involved in tests for alcohol cue-induced relapse-like  
21 behavior after extinction training may be those involved in maintaining or expressing alcohol-  
22 related cue memories before extinction training (**Figure 6 + Figure 7**) (Chaudhri et al., 2010,  
23 2013; Gass et al., 2011; Millan et al., 2015). The only brain region in which we observed  
24 noteworthy differential Fos expression between groups Paired and Unpaired was the insula

1 **(Figure 7 A)**. Activity in the insula appears to be important for maintaining cue-reward  
2 associative memory (Nasser et al., 2018). Additionally, deactivation of the insula appears to be  
3 important for the interoceptive effects of alcohol (Jaramillo et al., 2016). Since “Run” rats  
4 ingested equivalent alcohol doses and exhibited similar drinking behavior within the session on  
5 brain collection day, our findings suggest that either memory for the learned association of  
6 alcohol availability with houselight illumination in group Paired involves cells in the insula or that  
7 ingested alcohol has different effects in the insula of rats in group Paired and Unpaired.

8 Our Fos findings come with critical caveats. First, we do not know the identity of the cells  
9 activated (i.e., expressing Fos) in each brain region. Projection neurons and interneurons, and  
10 their neurochemically-defined subtypes, play important roles in communication within and  
11 between brain regions. Furthermore, Fos induction also has been observed in astrocytes  
12 (Arenander et al., 1989; Edling et al., 2007; Hermann & Rogers, 2009). Second, our study  
13 considered only 4-5 rat brains per group. Third, we lack some control groups such as alcohol  
14 and/or conditioning-naïve control groups, and groups exposed to CS without alcohol ingestion.  
15 Fourth, it is important to remember that over half the rats in the present study had zero blood  
16 alcohol when sampled. However, among rats that received a conditioning session prior to brain  
17 collection (viz., those in the “Run” condition), non-zero blood alcohol had been detected in 3 out  
18 of 4 rats in group Paired (we counted 1 rat with BEC 1.85 mg/dL as “zero” to be conservative)  
19 and 2 out of 4 rats in group Unpaired. Given these caveats, more work will be needed to  
20 illuminate the brain bases of reactivity to alcohol-related cues present in voluntary oral self-  
21 administration paradigms, including manipulation of putative substrates in order to confirm their  
22 involvement in alcohol-associative versus non-associative memory.

## 23 **Conclusion**

1 In the present study, we showed that behavioral reactivity to an antecedent visual stimulus  
2 signaling the opportunity to self-administer alcohol resulted from associative learning, rather  
3 than from non-specific effects of repeated exposure to the oral alcohol or visual stimulus. We  
4 also showed that memory for this conditioned alcohol cue reactivity may involve cells in the  
5 insular cortex. Our findings support continued investigation of the progression of brain and  
6 behavioral adaptations to chronic voluntary oral alcohol self-administration.

**1 ACKNOWLEDGEMENTS**

2 Funding was provided by NIH NIAAA R37AA11852 (RAG), NIH NIAAA T32AA007471 (RUC),  
3 NIHM R01MH091147 (MHM), CIHR MOP-137030 (NC), and a University of Texas  
4 undergraduate research fellowship (DJG). We thank Dr. Greg Loney for suggesting the idea to  
5 automate sipper site/faceplate contact measurement. We thank Jonathan Rosenzweig for his  
6 help with designing, prototyping, and fabricating the frame used to automate sipper  
7 site/faceplate contact measurement in conjunction with the Longhorn Maker Studio, the  
8 Aerospace Research Machine Shop, the Mechanical Engineering Machine Shop, and  
9 Mechanical Engineering Wood Shop. We thank Jarod Chaney and Lauren Schneider for their  
10 help with microscopy and cell counts. We thank Dr. Rheall Roquet is thanked for her help with  
11 brain collection.

12

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- 8

## 1 FIGURE CAPTIONS

2 **Figure 1. Behavioral measurements. A-B:** Digital video recordings of conditioning trials were  
3 scored for **(A)** “sipper site approach” (defined as approach and/or contact: locomotion toward  
4 the hole in the wall at which the sipper was presented or exploring the hole, including sniffing,  
5 gnawing, and clawing at the hole) or **(B)** “housetlight orienting” (defined as rearing: forepaws off  
6 the floor, supported by hindlimbs, facing any direction). **C:** A contact lickometer circuit was used  
7 to automatically record the latency and intensity of alcohol drinking bouts (sipper licking) during  
8 conditioning trials.

9 **Figure 2. Ethanol exposure across conditioning. A:** Mean  $\pm$  sem ingested ethanol doses  
10 across conditioning sessions for adult, male Long-Evans rats. Dashed horizontal line shows a  
11 *priori* study inclusion criterion ( $\geq 0.30$  g/kg across sessions 10-12). Black and white squares  
12 represent group Paired (n=9) and Unpaired (n=8), respectively. Data on brain collection day  
13 (session “BCD”) are from n=4/group. **B:** Relationship between ethanol concentrations in whole  
14 blood samples taken 8-12 min after the 8<sup>th</sup> drinking opportunity in a conditioning session and  
15 ingested ethanol doses for the same 17 rats. Regression line and 95% confidence limits shown  
16 as solid and dashed lines, respectively. **C:** Mean  $\pm$  sem estimated blood ethanol concentrations  
17 across conditioning sessions (8-12 min after the 8<sup>th</sup> drinking opportunity in the session) using  
18 ingested doses from **A** and regression equation from **B** for the same 17 rats.

19 **Figure 3. Housetlight cue-triggered ethanol sipper-seeking per trial.** Mean  $\pm$  sem level of  
20 anticipatory sipper site approach state in the 5 s before light onset (CS bin -1) and 10 s post-  
21 light onset but pre-sipper onset (CS bin 1 and 2) paneled by trial (1-8) within select conditioning  
22 sessions for adult, male Long-Evans rats. Approach data (maximum response level was 4) were  
23 derived from offline manual videoscoring.

1 **Figure 4. Latency to initiate drinking per trial.** Mean  $\pm$  sem latency (s) to start licking (viz.,  
2 initiate drinking bout) by trial within select conditioning sessions for adult, male Long-Evans rats.  
3 Latencies (maximum latency was 10 s; omissions were recorded as maximum latency) were  
4 derived from lickometer data.

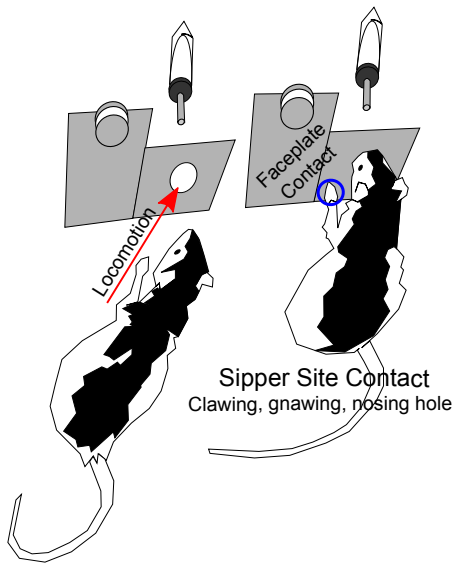
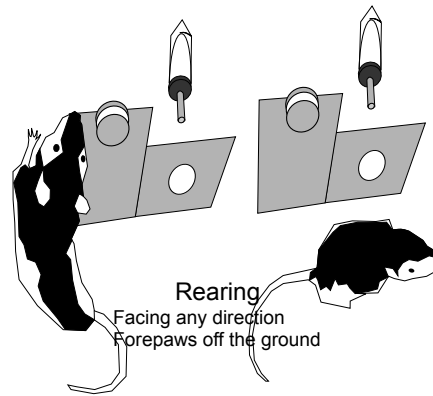
5 **Figure 5. Drinking intensity per trial.** Mean  $\pm$  sem total licks (viz., drinking bout size or  
6 intensity) by trial within select conditioning sessions for adult, male Long-Evans rats. Licking  
7 was measured directly using a lickometer.

8 **Figure 6. Brain c-Fos expression after a conditioning session or its omission in well-**  
9 **trained rats.** Group-level mean  $\pm$  sem of Fos-positive cell counts (averaged across sampling  
10 region, level, and hemisphere) across specific structures in the adult, male Long-Evans rat  
11 brain. See **Supplemental Information** for immunostaining details. Data from rats that  
12 underwent conditioning on brain collection day (“Run” condition) and rats that remained in  
13 homecage on brain collection day (“Not Run”) are shown in separate panels for each brain  
14 structure. Dark gray bars represent data from group Paired. White bars represent data from  
15 group Unpaired. Unless otherwise indicated, sample sizes were: Run Paired n=4, Run Unpaired  
16 n=4, Not Run Paired n=5, and Not Run Unpaired n=4.

17 **Figure 7. Brain c-Fos expression after a conditioning session or its omission in well-**  
18 **trained rats.** Group-level mean  $\pm$  sem of Fos-positive cell counts (averaged across sampling  
19 region, level, and hemisphere) in the insular cortex (A) and the basolateral amygdala (B). Data  
20 from rats that underwent conditioning on brain collection day (“Run” condition) and rats that  
21 remained in homecage on brain collection day (“Not Run”) are shown in separate panels for  
22 each brain structure. Dark gray bars represent data from group Paired. White bars represent  
23 data from group Unpaired. Sample sizes were: Run Paired n=4, Run Unpaired n=4, Not Run

- 1 Paired n=5, and Not Run Unpaired n=4. Black-filled asterisk indicates Bonferroni-corrected
- 2  $p < 0.05$ , white-filled asterisk indicates  $p < 0.05$  before correction.
- 3

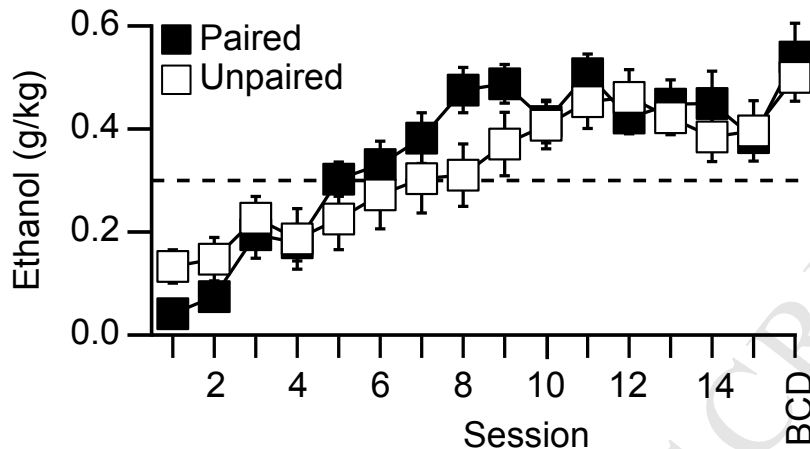
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**A. Sipper Site Approach****B. Housetlight Orienting****C. Drinking**

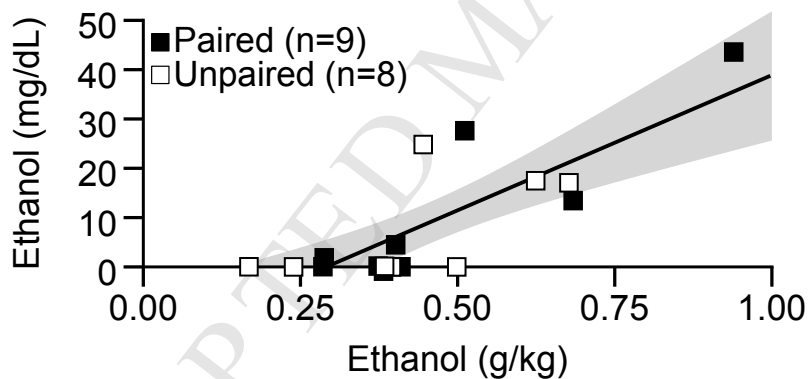
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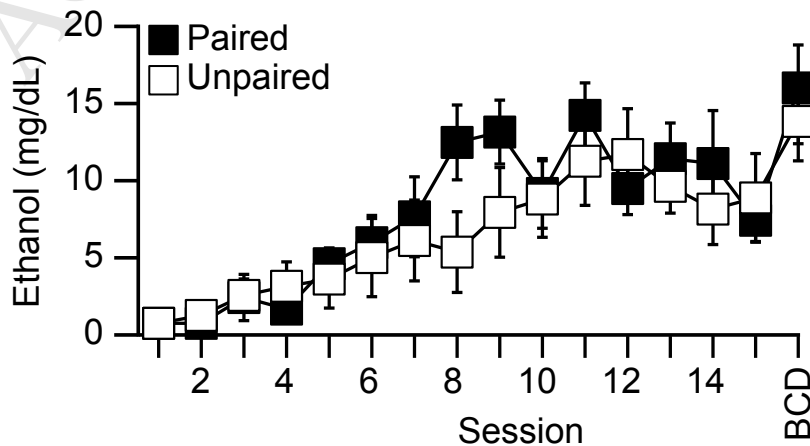
## A. Ingested Doses

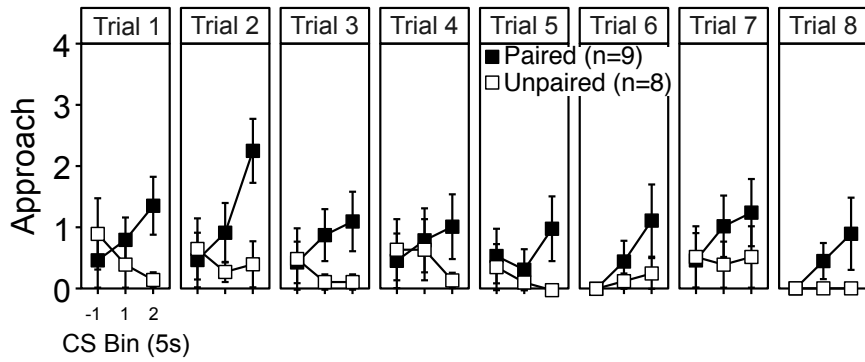


## B. Measured Blood Ethanol

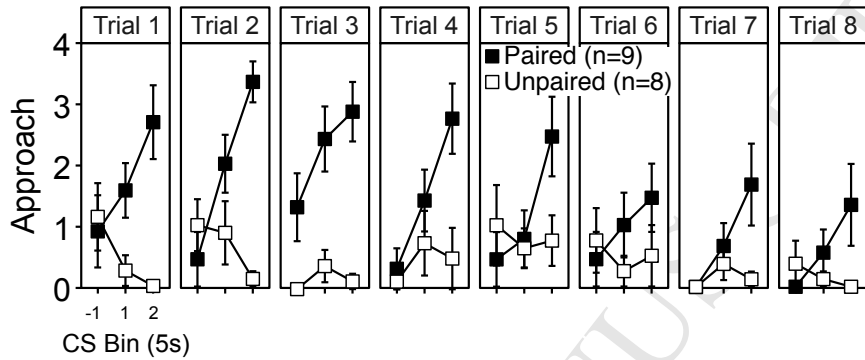


## C. Estimated Blood Ethanol

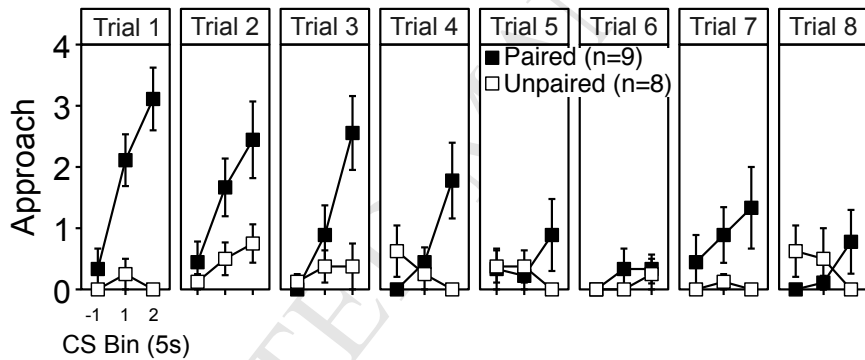




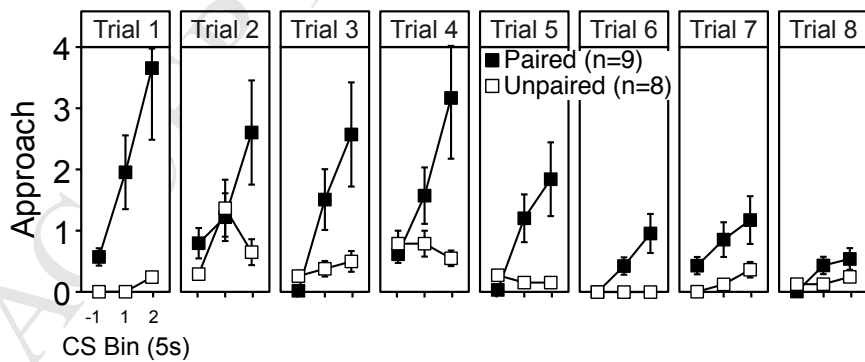
**B. Session 9**



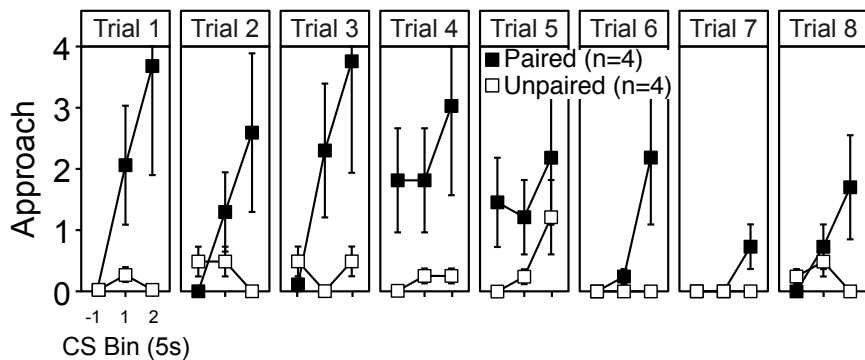
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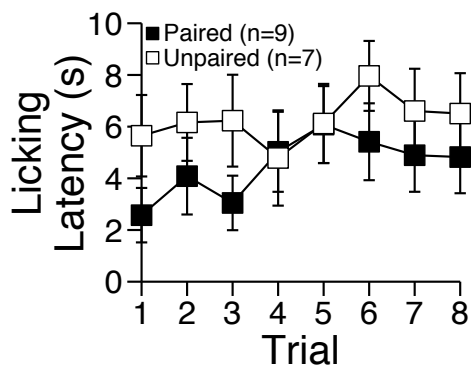


**D. Day Before Brain Collection**

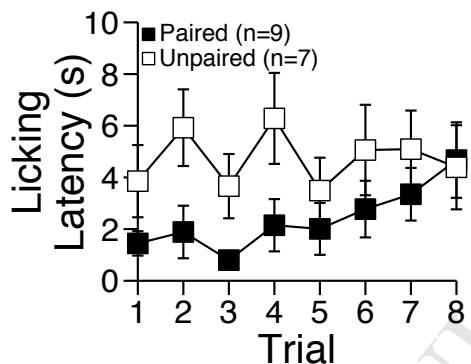


**E. Brain Collection Day**

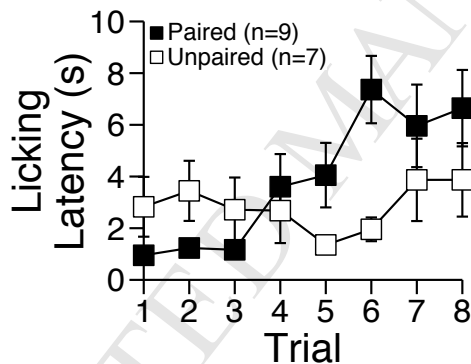




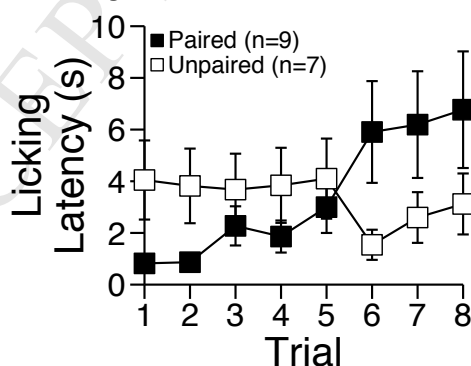
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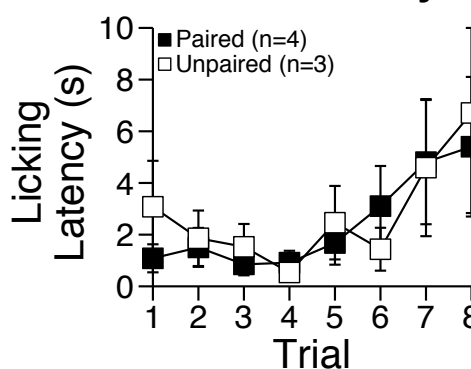
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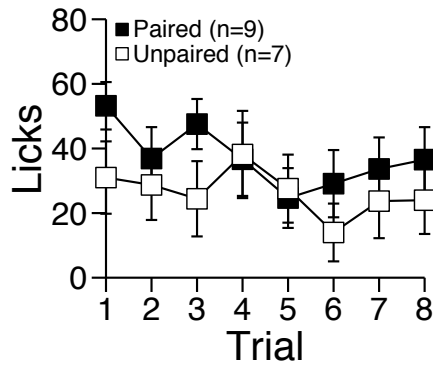
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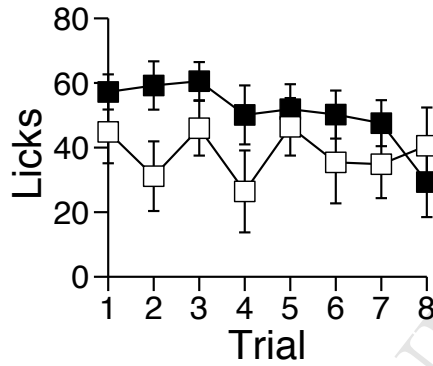
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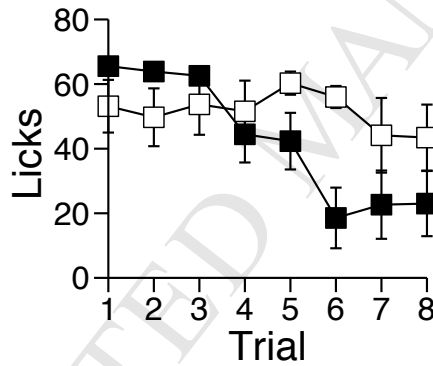
**A. Session 6**



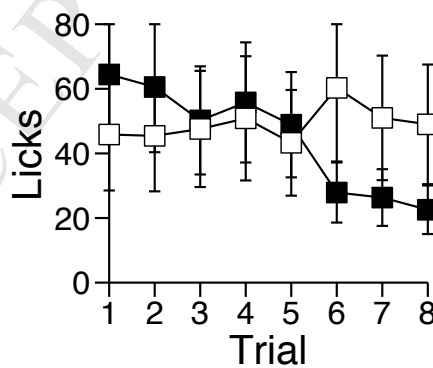
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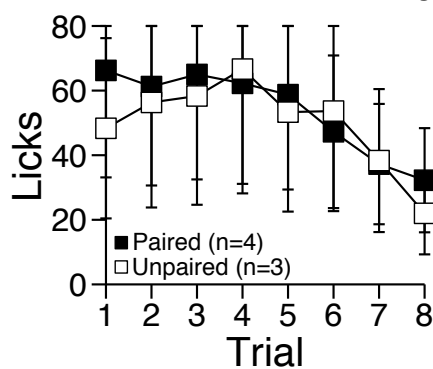
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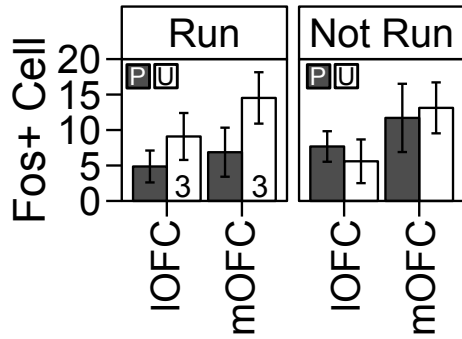
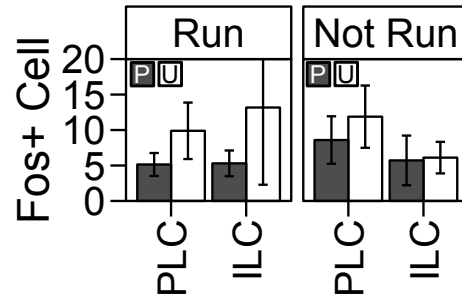
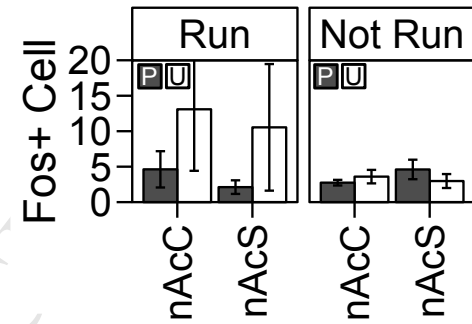
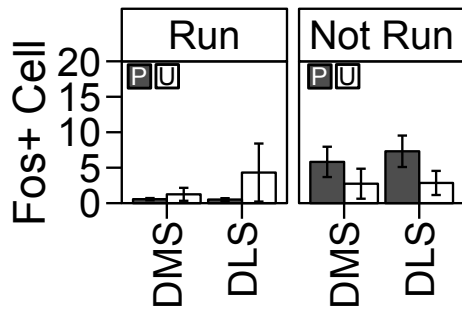
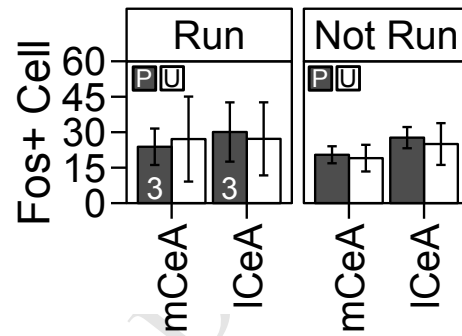
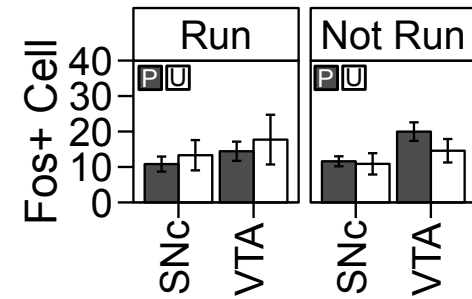


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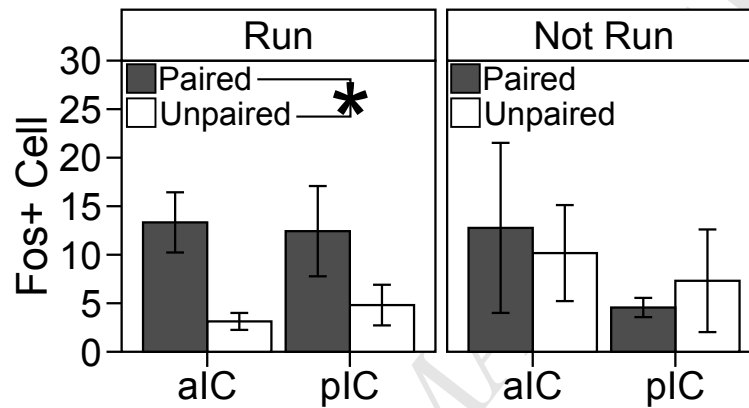


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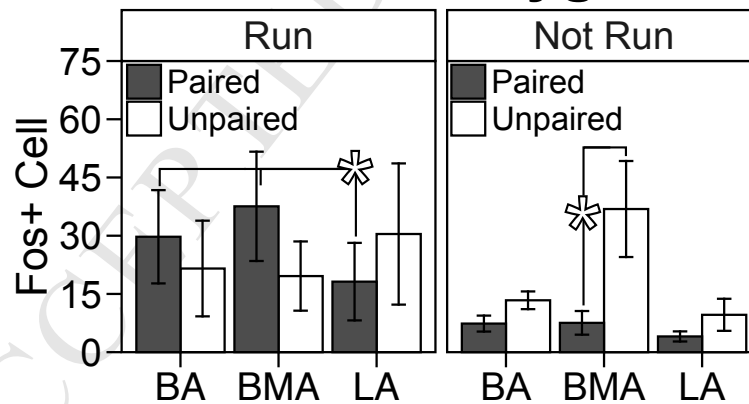


**A. OFC****B. mPFC****C. Accumbens****D. Striatum****E. CeA****F. DA Midbrain**

## A. Insular Cortex



## B. Basolateral Amygdala



## Highlights

- In rats that received light cue-alcohol sipper pairings, but not rats exposed to the light cue explicitly unpaired with the alcohol sipper, the light cue gained the ability to elicit anticipatory alcohol seeking
- In both groups of rats, the alcohol sipper gained the ability to elicit the initiation of alcohol drinking
- In rats that received light cue-alcohol sipper pairings, the vigor of alcohol seeking and drinking was subject to progressive devaluation of the alcohol reinforcer within the conditioning session
- In rats that received light cue-alcohol sipper pairings, behavioral expression or maintenance of memory for association of the light cue with alcohol access induced c-Fos expression in the insular cortex