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POST-RETRIEVAL EXTINCTION ATTENUATES ALCOHOL CUE REACTIVITY IN RATS

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Abstract

BACKGROUND—Conditioned responses to alcohol-associated cues can hinder recovery from alcohol use disorder (AUD). Cue exposure (extinction) therapy (CET) can reduce reactivity to alcohol cues, but its efficacy is limited by phenomena such as spontaneous recovery and reinstatement that can cause a return of conditioned responding after extinction. Using a preclinical model of alcohol cue reactivity in rats, we evaluated whether the efficacy of alcohol CET could be improved by conducting CET during the memory reconsolidation window after retrieval of a cue-alcohol association.

METHODS—Rats were provided with intermittent access to unsweetened alcohol. Rats were then trained to predict alcohol access based on a visual cue. Next, rats were treated with either standard extinction (n=14) or post-retrieval extinction (n=13). Rats were then tested for long-term memory of extinction and susceptibility to spontaneous recovery and reinstatement.

RESULTS—Despite equivalent extinction, rats treated with post-retrieval extinction exhibited reduced spontaneous recovery and reinstatement relative to rats treated with standard extinction.

CONCLUSIONS—Post-retrieval CET shows promise for persistently attenuating the risk to relapse posed by alcohol cues in individuals with AUD.

Keywords

alcohol cue; retrieval; reconsolidation; relapse; exposure therapy

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INTRODUCTION

Drinking episodes involve taking multiple drinks of an alcoholic beverage in relatively close succession. Repetition of such episodes allows the sight, smell, and taste of alcoholic beverages, as well as the contexts in which these sensory stimuli occur, to become conditioned as cues for alcohol availability, ingestion, and intoxication. Consequently, these conditioned cues alone can increase the urge to drink (craving) and produce physiological responses (Niaura et al., 1988). Importantly, reactivity to alcohol cues has been linked to a higher risk of relapse (Monti et al., 1993; Drummond & Glautier, 1994; Rohsenow et al., 1994; Papachristou et al., 2014).

Systematic exposure to alcohol-associated cues without subsequent alcohol ingestion (cue exposure therapy, CET) may help reduce the risk for relapse posed by these cues in daily life (Monti & Rohsenow, 1999; Rankin et al., 1983). When combined with other cognitive and behavioral techniques, CET may also effectively reduce relapse to drinking (Monti et al., 1993; Rohsenow et al., 2001). As stand-alone therapy, cue exposure has also been found to reduce drinking levels self-reported by heavy drinkers (Drummond & Glautier, 1994), and to be as efficacious as standard cognitive-behavioral treatment in reducing relapse to drinking in individuals with AUD (Loeber et al., 2006). Cue exposure therapy also increased perceived ability to control cravings and resist the urge to drink (Loeber et al., 2006).

Despite promise as a treatment tool, CET may leave individuals susceptible to the return of conditioned reactions with stress, exposure to non-extinguished cues and contexts as well as the passage of time, which may in part explain eventual relapse to problematic drinking. One reason for this enduring reactivity may be that standard CET protocols generally facilitate the formation of new inhibitory associations between the cues and their predicted event (Bouton, 2004). These new inhibitory memories must then compete with the excitatory memories acquired over the individual's drinking history for control over behavior.

A potential way to reduce the persistence of reactivity to conditioned alcohol cues may be by updating existing long-term memories with response-inhibiting information, which can be achieved by conducting CET during a period of memory instability—the post-retrieval, reconsolidation window. Convergent evidence suggests that giving cue exposure during the reconsolidation window (post-retrieval extinction) can ameliorate the problem of enduring reactivity to cues after treatment (Kredlow et al., 2016). Post-retrieval extinction was first shown to persistently attenuate the return of cued fear in rats (Monfils et al., 2009). This manipulation was then adapted to persistently attenuate the return of fear responses in humans (Schiller et al., 2010). Since then, post-retrieval extinction has been applied to attenuate conditioned responses in rats after appetitive conditioning with grain or sucrose pellets (Olshavsky et al., 2013; Flavell et al., 2011; Flavell & Lee, 2013), as well as morphine or cocaine (Ma et al., 2012; Xue et al., 2012; Sartor & Aston-Jones. 2013).

Using a newly developed alcohol cue conditioning paradigm in outbred male rats, we set out to establish whether post-retrieval extinction could persistently attenuate alcohol cue reactivity. If so, then a simple procedural modification might improve treatment outcomes following cue exposure therapy for AUD.

METHODS & MATERIALS

Subjects

Adult, male Long-Evans rats from Harlan (now Envigo; Indianapolis) (n=37) were used. Rats weighed 250-275 g upon arrival and were singly housed (22±2 °C; 12hr light cycle; procedures conducted during light phase) in homecages containing bedding. Unrestricted chow and tap water were available throughout. Rats received 1 week to acclimate, during which time they were weighed daily.

Behavioral Methods

Apparatus—Phases 2 thru 4 took place in Med Associates, Inc. (Fairfax, VT) conditioning chambers (interior dimensions: 30.5 cm L × 24.1 cm W × 29.2 cm H) housed in sound-attenuating cubicles. Each cubicle was equipped with a digital video camera (KT&C USA, Fairfield, NJ) and an exhaust fan. Each MedPC-controlled chamber was equipped with a houselight and retractable bottle assembly. The houselight was installed facing downward as the top center panel of the right chamber wall. The retractable bottle assembly was placed on the right panel of the front wall such that the hole through which the metal sipper inserted into the chamber was approximately 8.5 cm above the grid floor. The sipper hole was approximately 8.5 cm to the right and 16 cm lower than the houselight.

Experiment Phase 1: Alcohol Drinking in the Homecage

After acclimation to the facility, the rats (approximately 300 g) received unsweetened ethanol solution in the homecage (15% ethanol in tap water; v/v; 15E) for 24 hr sessions on an intermittent schedule (MWF) across 5 weeks (Figure 1 Panel A) (Sparks et al., 2014). Placement of 15E and water bottles on the cage was alternated (L/R) across sessions. Rats were weighed before every session. Bottles were weighed before and after every session, with a control cage used to correct for evaporation and spillage. Two water bottles were provided on “off” days. In order to minimize attrition, rats failing to drink doses > 0.5 g/kg on session 1 and 2 were provided with 5% ethanol in tap water (v/v; 5E) on subsequent sessions until a dose ≥ 1 g/kg was achieved for 4 sessions in a row. Next, 10% ethanol in tap water (v/v; 10E) was provided until the same criterion was met. If so, 15E was provided again unless fewer than 4 sessions remained, in which case the rat was kept on 10E. Rats drinking enough 10E or 15E to achieve ≥ 1 g/kg on average across the last 3 sessions were retained for conditioning (32 out 37).

Experiment Phase 2: Cue Conditioning

Rats were habituated to the conditioning chambers and stimuli (described above) in a single session (34-36 min total duration) conducted 48 hr after the last homecage ethanol drinking session. No bottles were loaded into the retractable bottle assembly during habituation, so there was no sipper presentation.

Following habituation, rats underwent cue conditioning across 12 consecutive days (Figure 1 Panel B). During each session, there were 8 trials using a variable intertrial interval (ITI) (Figure 1 Panel C). The ITI was selected randomly from a list (160 s, 240 s, 240 s, 250 s, 320 s, 320 s, 350 s, 360 s) until values were exhausted, at which point all values became

options again. The first trial began after a 5 min wait period and was signaled by onset of the cubicle exhaust fan. The session ended once the final ITI (selected after trial 8) had elapsed and this was signaled by exhaust fan offset. During each of these trials, the houselight was illuminated for 20 s. After 10 s, the retractable bottle assembly motor was activated to insert a metal sipper into the chamber. Licking the sipper produced ethanol. The sipper contained a ball bearing to prevent spillage upon insertion and retraction, and was attached to a bottle filled with either 10E (n=6) or 15E (n=21) depending on which solution the rat drank across the last 3 homecage ethanol drinking sessions. Different ethanol concentrations were maintained during this phase to prevent attrition in conditioning that might have arisen from switching rats that had previously received 10E to 15E. Thus, during a conditioning trial (Figure 1 Panel F), the houselight was illuminated for 20 s and the sipper was inserted 10 s after light onset such that ethanol access and light presentation co-terminated. Only rats drinking ≥ 0.3 g/kg on average across the last 3 cue conditioning sessions were retained for subsequent phases (27 out of 32).

After the final session, rats were subdivided into two groups matched on ingested doses. Assignments made on this basis were later found to have similar acquisition and final level of cue-conditioned behavioral responses. The 10E-drinking rats were split evenly between groups.

Experiment Phase 3: Cue Extinction with or without Initial Retrieval

Rats began 14 consecutive days of “retrieval-extinction” or “no retrieval-extinction” treatment 24 hr after the last cue conditioning session (Figure 1 Panel B). Each day (Figure 1 Panel D**)**, rats undergoing “retrieval-extinction” (n=13; Ret-Ext) experienced a cue memory retrieval episode followed by 1 hr in the homecage and then a cue extinction session. Rats undergoing “no retrieval-extinction” (n=14; NoRet-Ext) experienced a context exposure episode followed by 1 hr in the homecage and then a cue extinction session.

The cue memory retrieval episode consisted of an isolated trial in which the houselight was illuminated for 20 s with co-terminating sipper presentation starting 10 s after houselight onset. Importantly, there was no ethanol present in the sipper or anywhere in the cubicle (Figure 1 Panel G). This single trial was flanked by 2 variable ITIs. The first ITI was selected after a 2 min wait period and was signaled by cubicle exhaust fan onset. The episode ended when the second ITI elapsed and was signaled by exhaust fan offset. NoRet-Ext rats experienced session start and end signals (exhaust fan onset and offset, respectively), but were not given the isolated extinction trial. All rats were then returned to their homecages for 1 hr.

Subsequently, all rats were returned to the conditioning chambers for the cue extinction session. Session onset, signaled by the exhaust fan, occurred after a 2 min wait period. NoRet-Ext rats were given 12 extinction trials (as described immediately above, with variable ITI as described in Phase 2). The session ended when the final ITI (selected after trial 12) elapsed and was signaled by exhaust fan offset. Ret-Ext rats experienced session start and end signals (exhaust fan onset and offset, respectively) as well as trials 1-11, but were not given trial 12. All rats were then returned to their homecages until the next day.

These treatments involved the same amount of cue and context exposure (duration and frequency) in the absence of ethanol (i.e., under extinction conditions). The only difference was exposure to the cue plus context vs. only context 1 hr before an extinction protocol.

Experiment Phase 4: Testing

All rats underwent a long-term memory test (LTMT) to determine the persistence of extinction of cue-elicited responses 48 hr after the last extinction session. LTMT was a 4-trial cue extinction session (same variable ITIs described in Phase 2) (**Figure 1 Panel E** and **G**). Two days later, the rats underwent a test for the ability of ethanol odor to reinstate cue-elicited responses (reinstatement test: RT). RT was also a 4-trial cue extinction session except that an open vial of 10E or 15E was placed out of sight and out of reach inside the sound-attenuating cubicle to provide ethanol odor (**Figure 1 Panel E** and **H**). In both tests, response return was expected to occur on trial 1 and extinguish across trials 2-4.

Data collection

All trials were sampled for behavioral states from digital video recordings by making instantaneous observations every 1.25 s starting 5 s prior to houselight onset (Lee et al., 2005). At each observation during the 5 s before houselight onset (trial phase preCS), the 1st 5 s of houselight (trial phase CS1), and 2nd 5 s of houselight (trial phase CS2), the mutually exclusive rating options were “sipper site approach” (approaching, attending to, or exploring the sipper insertion hole, including sniffing, gnawing, and clawing at the hole) or “other” (e.g., grooming, resting, rearing). During the 1st 5 s of sipper presentation (3rd 5 s of light; trial phase CS3) and 2nd 5 s of sipper presentation (4th 5 s of light; trial phase CS4), an additional mutually exclusive rating option was possible: “sipper contact” (presumed licking: snout occluding sipper, snout angled toward and close enough to sipper plus rapid whisker movement). Observations were made by highly trained judges (≥95% agreement on joint ratings) who were blinded to treatment conditions. For every trial in every session, we counted the incidence of each behavioral state within each trial phase for every rat. Only 4 observations were made per trial phase, so the maximum behavior frequency per trial phase on any trial of any session was 4.

During Phase 1 and 2, we also monitored the dose of ethanol self-administered by each rat across sessions. Drinking solution intake was measured as the difference in bottle weight pre- and post-session after correcting for spillage. The grams of solution ingested were then converted to g ethanol and dose ingested was expressed as g/kg body weight for each rat.

Data analysis

Our main goal was to test whether Ret-Ext could reduce spontaneous recovery and reinstatement of houselight-elicited sipper site approach (trial phases CS1 and CS2) and sipper-elicited sipper contact (trial phases CS3 and CS4) relative to NoRet-Ext. Since hypotheses were directional (NoRet-Ext > Ret-Ext), planned comparisons were made using one-tailed t-tests. We also confirmed that response decay during cue extinction was comparable between treatment groups and that rats in each group were matched in histories of cue conditioning and drinking using mixed factorial ANOVA and simple effects analysis.

Post hoc comparisons were made using two-tailed t-tests. Statistical significance was met at $p<0.05$.

Ethics

All procedures were approved by our Institutional Animal Care and Use Committee and conducted in accordance with NIH guidelines.

Solutions

Ethanol (v/v) solutions were prepared every 3 days from 95% ethyl alcohol (ACS/USP grade, Pharmco-AAPER) and tap water. Solutions were kept and served at room temperature (20 °C).

RESULTS

Matched history of homecage alcohol drinking

Rats in groups NoRet-Ext (n=14) or Ret-Ext (n=13) drank similarly across the initial 5-week homecage alcohol pre-exposure phase (group × session and group effect: NS). As can be seen in Figure 2, ingested doses increased over sessions (session effect: $F_{14,350}=12.71$, $p<0.05$). Across the last 3 sessions, the grand mean±sem dose per session was 3.52±0.23 g/kg (n=27).

Matched history of alcohol cue conditioning

Rats in groups NoRet-Ext (n=14) and Ret-Ext (n=13) learned similarly to anticipate alcohol access based on the visual cue. Sipper site approach during trial phase preCS (5 s bin before light onset) was constant across sessions (Figure 3 Panel A) whereas approach during trial phases CS1 (1st 5 s of light) and CS2 (2nd 5 s of light) increased across sessions (**Figure 3 Panel B** and **Panel C**, respectively) (trial phase × session interaction: $F_{22,550}=2.085$, $p<0.05$). These patterns did not vary by group (group × trial phase × session, group × trial phase, group × session, and group effect: NS).

Rats in both groups also learned similarly to interact with the alcohol access device (sipper). Sipper contact during trial phases CS3 (3rd 5 s of light, 1st 5 s of sipper) and CS4 (4th 5 s of light, 2nd 5 s of sipper) increased across sessions (**Figure 3 Panel D** and **Panel E**, respectively) with contact during trial phase CS4 reaching a higher level by the end of the conditioning protocol (trial phase × session interaction: $F_{11,275}=2.19$, $p<0.05$). These patterns did not vary by group (group × trial phase × session, group × trial phase, group × session, and group effect: NS).

Finally, rats in both groups also drank similarly across conditioning (Figure 3 Panel F**)**. Ingested ethanol doses increased over sessions (session effect: $F_{11,\ 275}=47.32$, $p<0.05$), and this pattern did not vary by group (group × session and group effect: NS). Across the last 3 sessions, the grand mean±sem dose per session was 0.50±0.03 g/kg (n=27).

Equivalent extinction of conditioned responses to alcohol predictive cues

NoRet-Ext (n=14) and Ret-Ext (n=13) rats exhibited equivalent extinction of visual cue-elicited approach to the former site of alcohol access. Sipper site approach during trial phase preCS remained at floor across treatment days (Figure 4 Panel A), while approach during trial phases CS1 and CS2 decreased across treatment days (**Figure 4 Panel B** and **Panel C**, respectively) (trial phase × treatment day interaction: $F_{26, 650}$=2.83, p<0.05). These patterns did not vary by treatment group (group × trial phase × treatment day, group × trial phase, group × treatment day, and group effect: NS).

Interaction with the sipper also extinguished similarly between treatment groups. Sipper contact during trial phases CS3 and CS4 decreased across treatment days (**Figure 4 Panel D** and **Panel E**, respectively). On some treatment days, responding differed by trial phase differently for each group (group × trial phase × treatment day interaction: $F_{13, 325}$=1.79, p<0.05), but day-by-day extinction did not vary by group (group × treatment day and group effect within each trial phase: NS).

By the end of extinction (last 4 trials of E14), approach and contact (**Figure 4 Panel F** and **Panel G**, respectively) were at floor in both treatment groups (main effects of group, trial, and trial phase, all interactions: NS). This means that treatment groups not only extinguished similarly day-by-day, but also to the same final level.

Analysis of responding on the first trial of each treatment day (trial 1 for group Ret-Ext is the retrieval trial; Figure 5) further confirmed similar day-by-day extinction. Sipper site approach during trial phase preCS remained at floor, but decreased over days during trial phases CS1 and CS2 (trial phase × treatment day interaction: $F_{26, 650}$=7.96, p<0.05). This pattern did not vary by group (group × trial phase × treatment day interaction: NS). Sipper contact during trial phase CS3 and CS4 decayed differently across treatment day (trial phase × treatment day interaction: $F_{13, 325}$=2.34, p<0.05), but the patterns of decay did not vary by group (group × trial phase × treatment day interaction: NS).

Retrieval-extinction confers protection against early spontaneous recovery and alcohol odor-induced reinstatement

As expected, in the long-term memory test (LTMT) and reinstatement test (RT) response return was observed on trial 1, but rapidly extinguished across trials 2-4 (main effect of trial in each test: $F_{3, 75}$ >=3.70, p<0.05; group × trial interaction in each test: NS; data not shown).

To assess vulnerability to short-term spontaneous recovery following Ret-Ext and NoRet-Ext treatment, we first examined whether responding on LTMT trial 1 varied between groups relative to each subject's baseline response level at the end of extinction (average response level across E14 trial 9-12). Neither group exhibited spontaneous recovery of trial phase CS1 sipper site approach (Figure 6 Panel B). However, spontaneous recovery was reduced in Ret-Ext-treated rats (n=13) relative to NoRet-Ext-treated rats (n=14) for sipper site approach during trial phase CS2 (Test – Baseline for NoRet-Ext > Ret-Ext: one-tailed Welch's $t_{22.16}$=1.75, p<0.05; Figure 6 Panel C) as well as sipper contact during trial phase CS3 (Test – Baseline for NoRet-Ext > Ret-Ext: one-tailed Student's t_{25}=2.70, p<0.01; Figure 6 Panel

D) and trial phase CS4 (Test – Baseline for NoRet-Ext > Ret-Ext: one-tailed Welch's $t_{21.05}$=1.85, p<0.05; Figure 6 Panel E).

We also considered spontaneous recovery relative to an alternative end of extinction baseline that better captures the temporal context of cue presentation at test (E14 trial 1). Neither group exhibited spontaneous recovery of trial phase CS1 sipper site approach (Figure 6 Panel G). However, defined relative to this alternative baseline, spontaneous recovery was likewise reduced in group Ret-Ext relative to NoRet-Ext for sipper site approach during trial phase CS2 (Test – Baseline for NoRet-Ext > Ret-Ext: one-tailed Student's t_{25}=2.41, p<0.015; Figure 6 Panel H) as well as sipper contact during trial phase CS3 (Test – Baseline for NoRet-Ext > Ret-Ext: one-tailed Welch's $t_{19.18}$=1.77, p<0.05; Figure 6 Panel I). Both groups exhibited recovery of sipper contact during trial phase CS4 (Figure 6 Panel J), but NoRet-Ext rats made more contact than Ret-Ext rats at baseline (two-tailed Welch's $t_{14.66}$=2.58, p<0.025) and test (two-tailed Welch's $t_{20.81}$=2.13, p<0.025). Therefore, regardless of which baseline was used, our data indicate that cue memory retrieval plus extinction attenuated spontaneous recovery compared to extinction alone.

To assess vulnerability to reinstatement following Ret-Ext and NoRet-Ext treatment, we first examined whether responding on RT trial 1 varied between groups relative to each subject's baseline response level at the end of the long-term memory test (LTMT trial 4). Reinstatement was reduced in Ret-Ext-treated rats (n=13) relative to NoRet-Ext-treated rats (n=14) for sipper site approach during trial phase CS1 (Test – Baseline for NoRet-Ext > Ret-Ext: one-tailed Student's t_{25}=1.74, p<0.05; Figure 7 Panel B) and trial phase CS2 (Test – Baseline for NoRet-Ext > Ret-Ext: one-tailed Student's t_{25}=1.56, p=0.065; Figure 7 Panel C) as well as sipper contact during trial phase CS3 (Test – Baseline for NoRet-Ext > Ret-Ext: one-tailed Student's t_{25}=3.00, p<0.005; Figure 6 Panel D), but not trial phase CS4 (Figure 7 Panel E). The marginal significance for relative reduction in reinstatement of approach during trial phase CS2 was due to 1 NoRet-Ext rat having ceiling level approach at baseline. After excluding this rat, the protective effect of Ret-Ext during trial phase CS2 met the statistical significance threshold (Test – Baseline for NoRet-Ext > Ret-Ext: one-tailed Student's t_{24}=3.01, p<0.005), in agreement with the significantly lower unadjusted level of approach during trial phase CS2 at test for group Ret-Ext than NoRet-Ext (including the rat with the problematic baseline; two-tailed Student's t_{25}=2.45, p<0.02; Figure 7 Panel C).

We also considered reinstatement relative to the temporal context-sensitive end of extinction baseline (E14 trial 1). Defined against this alternative baseline, reinstatement of sipper site approach during trial phase CS1 was small and similar between groups (Figure 7 Panel G). However, reinstatement was reduced in group Ret-Ext relative to NoRet-Ext for sipper site approach during trial phase CS2 (Test – Baseline for NoRet-Ext > Ret-Ext: one-tailed Student's t_{25}=2.44, p<0.02; Figure 7 Panel H) as well as sipper contact during trial phase CS3 (Test – Baseline for NoRet-Ext > Ret-Ext: one-tailed Student's t_{25}=1.98, p<0.05; Figure 7 Panel I). Reinstatement of trial phase CS4 sipper contact appeared to be greater in Ret-Ext rats, but this was due to significantly lower baseline sipper contact, as reported earlier (two-tailed Welch's $t_{14.66}$=2.58, p<0.025; Figure 7 Panel J). Therefore, regardless of the baseline used, our data indicate that memory retrieval plus extinction attenuated reinstatement compared to extinction alone.

DISCUSSION

Conditioned responses to alcohol-associated cues that are extinguished with standard exposure therapy readily re-emerge with the passage of time and re-exposure to non-extinguished cues. These post-treatment effects exist because the standard exposure therapy facilitates the formation of new response-inhibiting memories that must compete with existing excitatory memories for control over behavior. In the present study we were able to attenuate the return of conditioned responses to alcohol-associated cues by conducting extinction during the post-retrieval, memory reconsolidation window, an alternative approach to standard exposure therapy that may allow inhibitory learning to be incorporated into existing alcohol cue memories.

Enduring reactivity to alcohol cues in humans with AUD likely arises from the interaction of conditioning- and alcohol-induced changes in the brain that accumulate across an individual's drinking history. During the cue conditioning phase of our experiment, each trial modeled the stimulus sequence of taking a drink: olfactory and orosensory properties of alcohol preceded by specific visual stimuli. Each conditioning session (block of 8 trials) modeled the repetition of that stimulus sequence over the course of a drinking episode and as a consequence, the onset of alcohol's psychopharmacological properties, which requires alcohol to reach the brain. As such, our study only included rats that by at least the end of the conditioning phase were reliably ingesting alcohol at doses that we have previously shown to result in detectable blood alcohol concentrations (BAC) (mean±sem BAC at the end of a conditioning session was 22±7 mg/dL for mean±sem ingested dose of 0.58±0.07 g/kg, n=12) (Cofresí et al., 2015). The BACs achievable in our paradigm are within a range easily achieved by humans during drinking episodes (10-60 mg/dL or 2-13 mM) (Thombs et al., 2003; Hustad et al., 2005; Clapp et al., 2008; Dougherty et al., 2012; Clapp et al., 2009). Since humans do not drink alcohol to meet caloric or hydration needs, we neither food nor water restricted our rats—an overlooked confound in many oral self-administration paradigms. Finally, in order to attribute any conditioning effects specifically to alcohol, drinking solutions were never sweetened. We believe these choices maximize the relevance of our preclinical intervention study in rats to AUD treatment in humans.

Using only those rats with conditioned reactions to alcohol-associated cues that were ultimately reinforced by alcohol's actions on the brain, we created pseudo-randomized groups matched for drinking history (Figure 2 and Figure 3 Panel F), which led to matching on conditioned reactivity (Figure 3 Panels A-E). We then proceeded to evaluate long-term memory for extinction and susceptibility to alcohol odor-induced reinstatement following either standard- or memory retrieval-based CET. We termed the latter therapy "retrieval-extinction" treatment because it involved isolated cue presentation (memory retrieval) 1 hr before massed cue exposure all in the absence of alcohol. Rats in the standard therapy model, which we termed "no retrieval-extinction," were not presented with an isolated cue, but were exposed to the conditioning chamber as well as session start and end signals that were yoked to the retrieval-extinction group 1 hr before massed cue exposure. Similarly, the last cue presentation during massed exposure was omitted for rats undergoing retrieval-extinction, but their session did not end until the control group's session was finished. Thus,

the total amount of cue and context exposure was matched between treatments within and across the 14 consecutive treatment days.

We expected that the two treatments would similarly extinguish conditioned reactions to alcohol-associated cues (houselight illumination-elicited sipper hole approach and sipper presentation-elicited sipper contact) and thus produce similar long-term memory for extinction conditions. We hypothesized that retrieval-extinction treatment would reduce susceptibility to alcohol odor-induced reinstatement, relative to control treatment.

As expected, conditioned reactions to houselight and sipper presentation were similarly extinguished by the two treatments (Figure 4 and 5); however, when challenged with a 48 hr break in treatment, control rats exhibited spontaneous recovery of sipper site approach and sipper contact responses, whereas rats treated with retrieval-extinction did not (Figure 6). Thus, despite equivalently efficacious extinction of responding, treatments did not produce similar long-term behavioral memory: retrieval-extinction promoted better retention of response suppression.

When challenged with alcohol odor, controls rats demonstrated robust reinstatement of sipper site approach and sipper contact responses. Rats treated with retrieval-extinction showed less reinstatement than controls (Figure 7), supporting our hypothesis. Furthermore, this effect was not restricted to the houselight-elicited anticipatory approach response, but rather extended to the sipper-elicited contact response. Thus, retrieval-extinction protected against reinstatement of the entire response sequence that was reinforced by alcohol ingestion during conditioning.

It has previously been shown that alcohol cue memories can undergo retrieval-induced reconsolidation in rats and that post-retrieval pharmacological interference can disrupt alcohol seeking (Barak et al., 2013; von der Goltz et al., 2009; Milton et al., 2012; Schramm et al., 2015). Our study provides the first demonstration that post-retrieval extinction, a form of behavioral interference, can also disrupt alcohol seeking. We were not, however, the first to test this possibility. A study by Millan and colleagues (2013) suggested that while in some cases, post-retrieval extinction protects against relapse to alcohol seeking, in other cases it is ineffective or exacerbates relapse risk. In that study, however, the procedure used to reactivate memory may not have induced reconsolidation. Specifically, instrumental responding during the retrieval episode was not reinforced with alcohol (i.e., it did not deliver drinking solution into the magazine) but continued to result in presentation of a discrete cue that had been paired with the drinking solution during prior operant self-administration sessions. Non-reinforcement is insufficient to trigger the reconsolidation of appetitive instrumental memories (Hernandez & Kelly, 2004; Exton-McGuiness et al., 2014) and in fact, response-contingent cue presentations in the absence of primary reinforcement can promote the formation of new inhibitory memory (Flavell & Lee, 2013). Thus, the retrieval procedure used by Millan and colleagues would not be expected to trigger reconsolidation of existing excitatory memories, but instead would be expected to initiate the consolidation of a new inhibitory memory. In this light, data from Millan and colleagues suggest that additional inhibitory learning during the consolidation of a new inhibitory memory has disparate effects on response return. Furthermore, in the study by Millan and

colleagues the motivation for alcoholic beverage seeking and drinking was critically confounded by motivation for sweet taste (malt sugars were present in the drinking solution), motivation for calories (rats were food-deprived till 1hr post-session), and motivation for water (rats were water-deprived till 1hr post-session). In contrast, rats in our study sought and drank unsweetened alcohol while neither food nor water deprived. These important parametric differences must be accounted for when comparing the present data with previously published results.

The memory reactivation procedure used in the present study was specifically designed to induce reconsolidation of the alcohol cue memory. To retrieve the memory, we presented the alcohol-predictive cue in an isolated trial. To trigger reconsolidation, we omitted the alcohol, which produces a negative prediction error (viz., we violated cue-based expectancy of subsequent alcohol access and ingestion). Negative prediction error is required for cue memory retrieval to induce a reconsolidation process (Pedreira et al., 2004; Piñeyro et al., 2013; Das et al., 2015). However, at some point during treatment, the isolated cue likely stopped producing prediction error, precluding reconsolidation-based updating of the original memory, so we cannot rule out some contribution of new response-inhibiting memory to reduced response return following our retrieval-extinction treatment.

Future studies should explore the parameters for memory reactivation to discover those that can be exploited to maximize reconsolidation-based updating of the original memory. For example, to the extent that rats learn to expect multiple presentations of the conditioned stimulus sequence—sight, smell, taste, and alcohol ingestion (non-intoxicating dose)—per day, a single stimulus sequence presentation may be enough to generate prediction error while maximally reactivating the cue memory. An internal drug stimulus-based memory reactivation procedure has also shown promise in rat models of relapse to cocaine seeking (Luo et al., 2015). Exposure to alcohol’s internal stimulus in the absence of antecedent stimuli (sight, smell, taste, and ingestion of the alcoholic beverage) naturally conditioned via routine oral self-administration may thus also be a promising strategy for reactivating alcohol-associated memories.

Our present findings suggest that there is promise in conducting CET during reconsolidation of memory for alcohol-associated cues. Additional experiments are needed to replicate and extend these findings (e.g., in rat strains selected for high alcohol drinking or preference), to determine boundary conditions (e.g., aged memories, extensively trained associations, physical dependence), and to discover any potential risks. Whether post-retrieval CET will attenuate or exacerbate other forms of response return needs to be explored (e.g., stress or alcohol prime-induced reinstatement). It would be especially important to determine if post-retrieval CET can affect, and ideally reduce rapid reacquisition of conditioned responding using our procedure. Although the post-retrieval extinction procedure in Millan et al. (2013) facilitated response reacquisition, the generalizability of this finding is unclear given the caveats discussed above.

In conclusion, we found that retrieval-extinction can attenuate the return of alcohol cue reactivity relative to standard extinction in rats. The relevance of our finding to AUD treatment is highlighted by two other recent findings. First, retrieval-extinction has been

successfully applied to persistently attenuate cue-induced craving for heroin in individuals with heroin use disorder (Xue et al., 2012). Second, it was recently demonstrated that memory retrieval with prediction error can trigger the reconsolidation of naturally acquired alcohol cue memories in hazardous heavy drinkers (Das et al., 2015). Considered alongside these results, our study suggests that a treatment approach that incorporates post-retrieval CET in individuals with AUD may help to persistently attenuate reactivity to alcohol-associated cues, thereby reducing the long-term risk that these cues pose in daily life.

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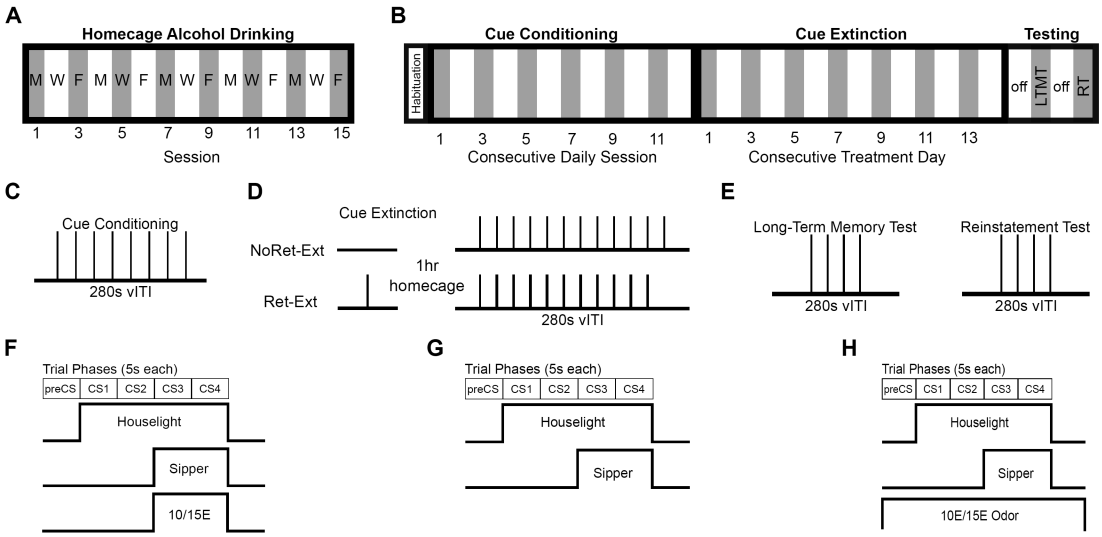

Figure 1. Experiment timelines

Panel A: Singly housed, adult male Long-Evans rats were induced to drink unsweetened alcohol in the homecage using an intermittent 24hr access schedule: alcohol available on MWF for 5 weeks. Water and standard chow were always available (ad libitum). **Panel B:** Following habituation to conditioning chamber and stimuli, rats had 12 consecutive days of cue conditioning followed by 14 consecutive days of cue extinction treatment. A test of long-term memory (LTMT) for extinction was conducted 48hr after the last day of extinction. A test of reinstatement (RT) was conducted 48hr after LTMT. **Panel C:** Cue conditioning sessions consisted of 8-trial blocks. **Panel D:** Cue extinction treatment days involved 12 extinction trials in either “no retrieval-extinction” or “retrieval-extinction” arrangement. **Panel E:** LTMT and RT consisted of 4-trial blocks. Panels C-E: The same variable inter-trial interval (vITI) was used (mean=280s; sd=68s; min=160s; max=360s). **Panel F:** Conditioning trials involved 20s chamber houselight illumination with co-terminating 10s access to alcohol (10% or 15% ethanol v/v in tap water; 10E/15E) sipper starting 10s after light onset. **Panel G:** Extinction trials for both Extinction and LTMT consisted of 20s houselight illumination with co-terminating 10s access to dry sipper starting 10s after light onset. **Panel H:** For RT, extinction trials were given, but an open vial of 10E/15E (20mL) was hidden in the cubicle housing the conditioning chamber.

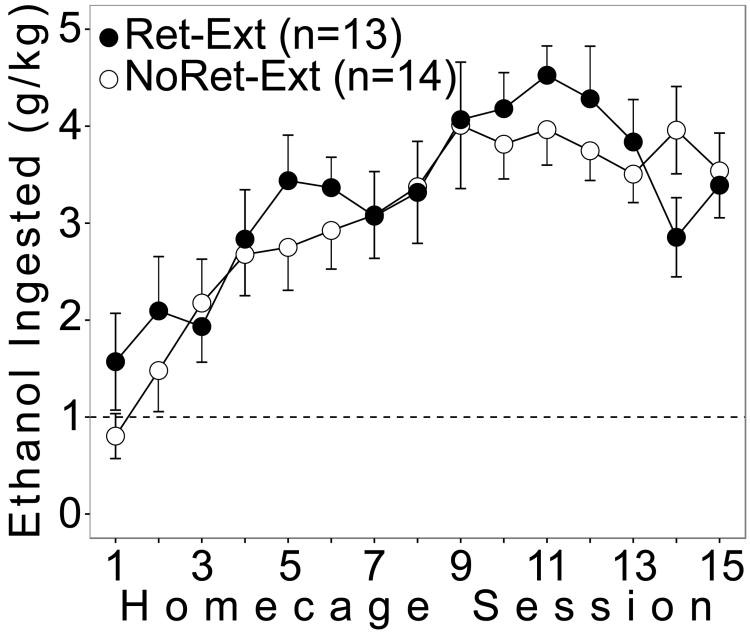

Figure 2. Treatment groups were matched on drinking in the homecage

Group mean ± SEM shown for ingested doses per day. Open circles represent group NoRet-Ext (n=14). Filled circles represent group Ret-Ext (n=13). Horizontal line indicates *a priori* study inclusion criterion: mean across last 3 days >=1.00 g/kg.

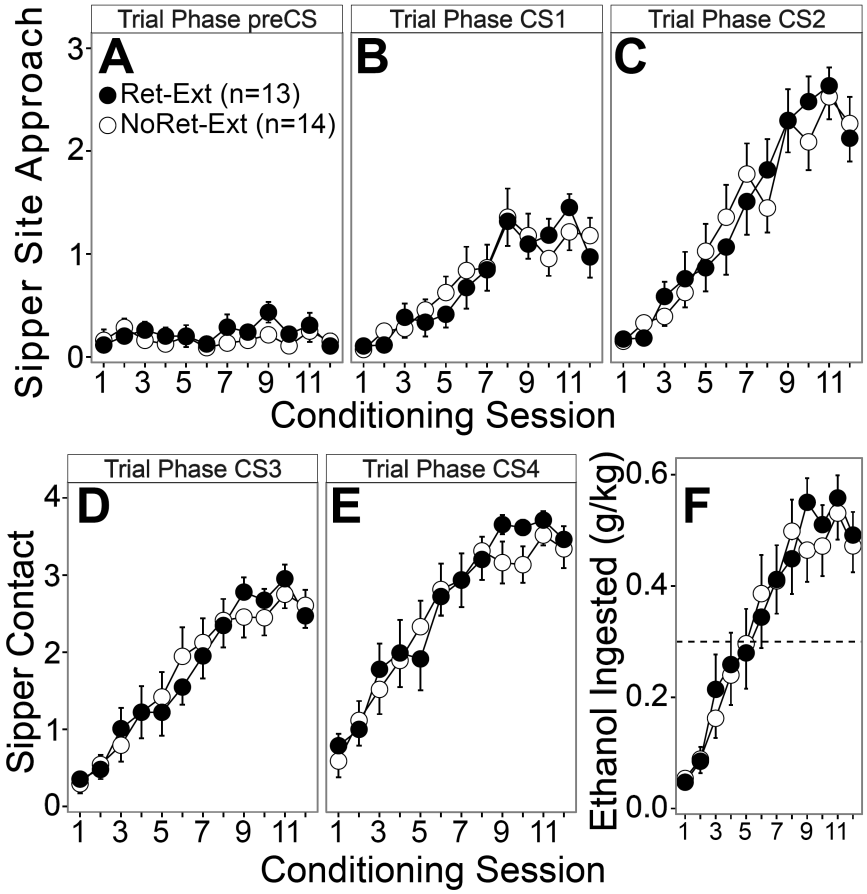

Figure 3. Treatment groups were matched on cue-conditioned response acquisition and drinking during conditioning

Panels A-C: Group mean ± SEM shown for sipper site approach level (max level = 4). Open circles represent group NoRet-Ext (n=14). Filled circles represent group Ret-Ext (n=13).

Panels D-E: Group mean ± SEM shown for sipper contact level (max level = 4). Open circles represent group NoRet-Ext (n=14). Filled circles represent group Ret-Ext (n=13).

Panel F: Group mean ± SEM shown for ingested doses per session. Open circles represent group NoRet-Ext (n=14). Filled circles represent group Ret-Ext (n=13). Horizontal line indicates *a priori* study inclusion criterion: mean across last 3 sessions >= 0.30 g/kg.

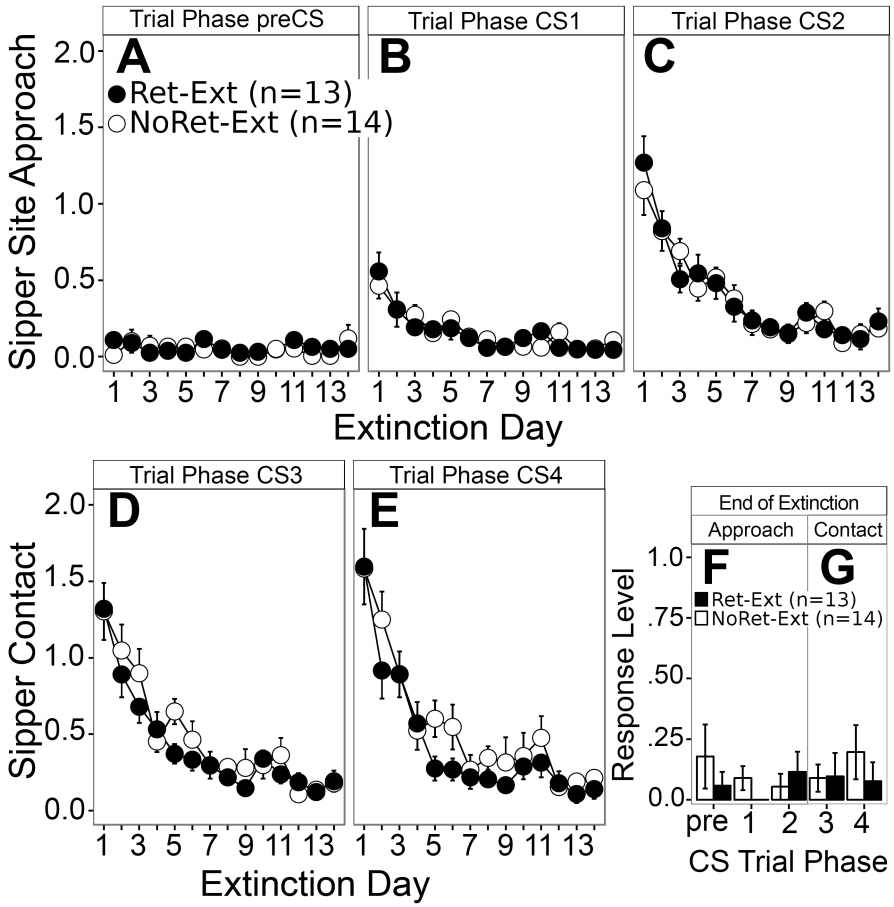

Figure 4. Isolated cue plus context or context only exposure 1 hr before massed cue exposure similarly extinguished cue-conditioned responses across treatment

Panels A-C: Group mean ± SEM shown for sipper site approach level (max level = 4). Open circles represent group NoRet-Ext (n=14). Filled circles represent group Ret-Ext (n=13).

Panels D-E: Group mean ± SEM shown for sipper contact level (max level = 4). Open circles represent group NoRet-Ext (n=14). Filled circles represent group Ret-Ext (n=13).

Panels F-G: Group mean ± SEM shown for sipper site approach and sipper contact levels (max level = 4) across the last 4 trials on the last day of treatment. Open bars represent group NoRet-Ext (n=14). Filled bars represent group Ret-Ext (n=13).

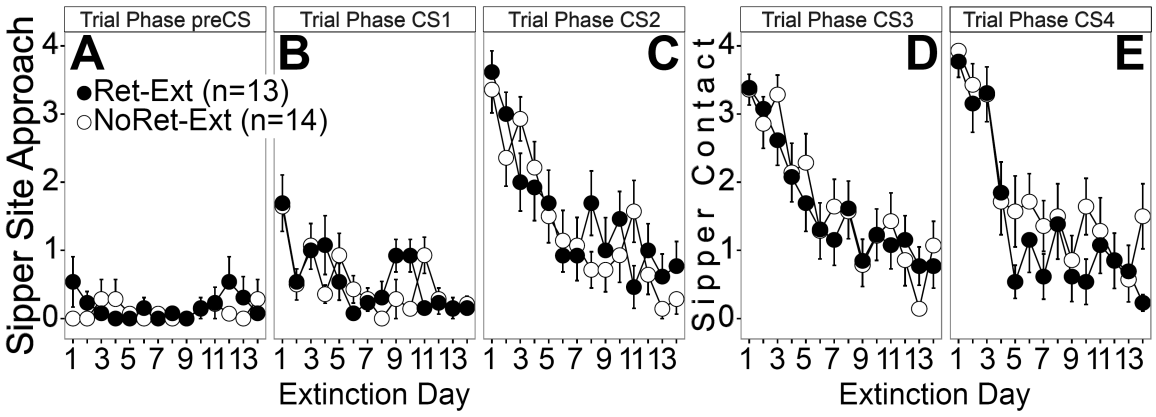

Figure 5. Similar daily recovery of cue-conditioned responses between groups across treatment

Panels A-C: Group mean ± SEM shown for sipper site approach level (max level = 4) on trial 1 across extinction days (retrieval trial for Ret-Ext group). Open circles represent group NoRet-Ext (n=14). Filled circles represent group Ret-Ext (n=13). **Panels D-E**: Group mean ± SEM shown for sipper contact level (max level = 4) on trial 1 across extinction days (trial 1 = retrieval trial for Ret-Ext group). Open circles represent group NoRet-Ext (n=14). Filled circles represent group Ret-Ext (n=13).

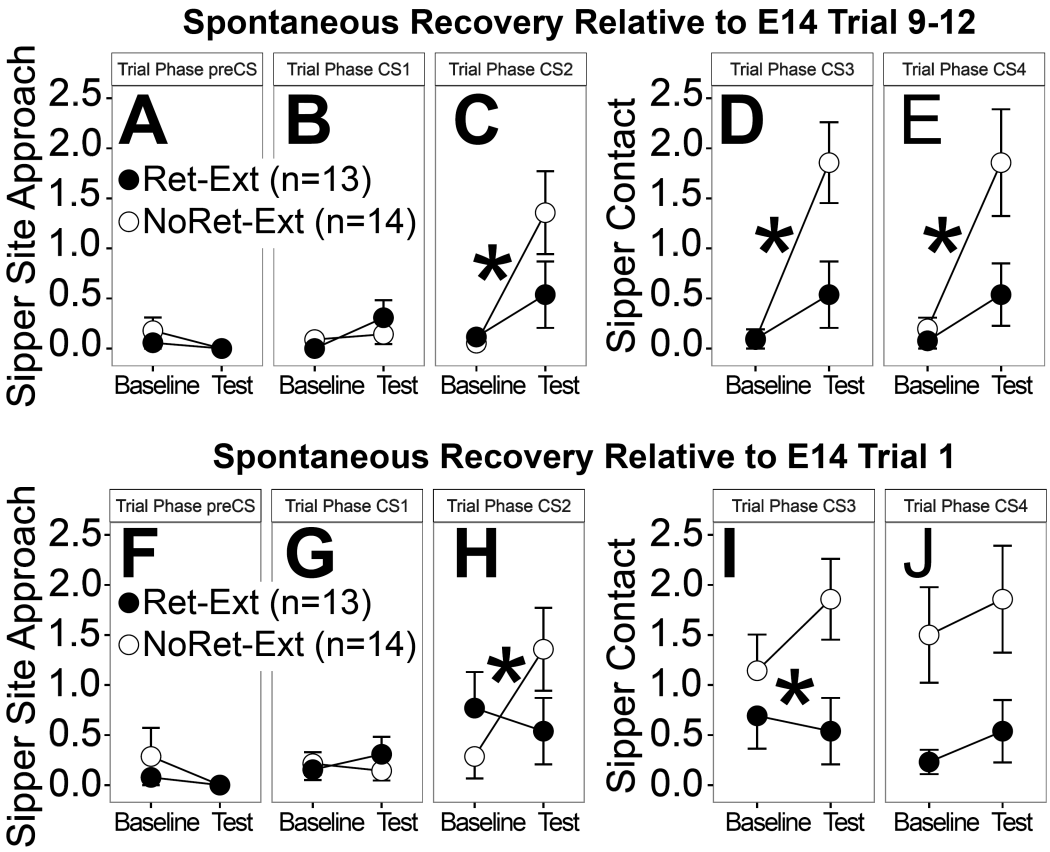

Figure 6. Retrieval-extinction treatment protects against early spontaneous recovery of cue-conditioned responses relative to two different baselines

Group mean ± SEM shown for sipper site approach and sipper contact levels (max level = 4) (**Panels A**, **B**, **C**, **F**, **G, H** and **D**, **E**, **I**, **J** respectively). Open circles represent group NoRet-Ext (n=14). Filled circles represent group Ret-Ext (n=13). **Panels A-E**: Baseline refers to average across E14 trials 9-12. **Panels F-J**: Baseline refers to E14 trial 1 (retrieval trial for Ret-Ext group). **All Panels:** Test refers to long-term memory test trial 1. Asterisk (*) signifies one-tailed $p<0.05$ for directional comparison on response level change (Test – Baseline for NoRet-Ext > Ret-Ext).

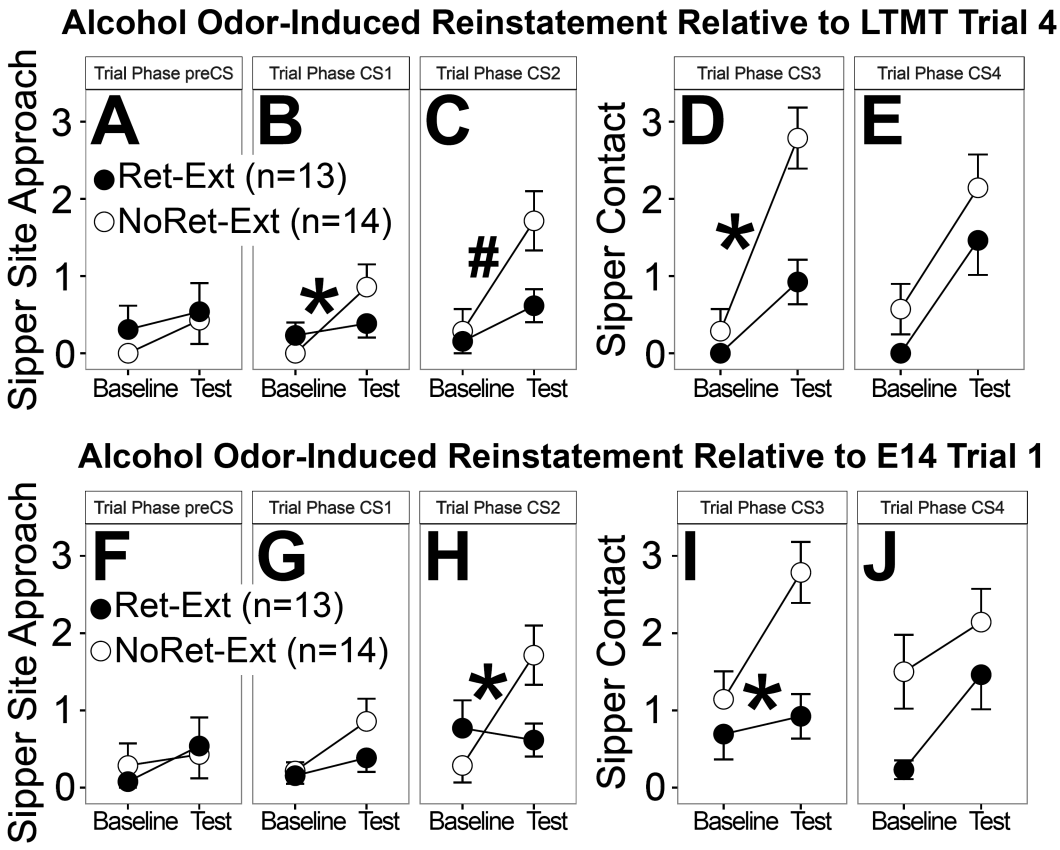

Figure 7. Retrieval-extinction treatment protects against alcohol odor-induced reinstatement of cue-conditioned responses relative to two different baselines

Group mean ± SEM shown for sipper site approach and sipper contact levels (max level = 4) (**Panels A**, **B**, **C**, **F**, **G, H** and **D**, **E**, **I**, **J** respectively). Open circles represent group NoRet-Ext (n=14). Filled circles represent group Ret-Ext (n=13). **Panels A-E**: Baseline refers to long-term memory test trial 4. **Panels F-J**: Baseline refers to E14 trial 1 (retrieval trial for Ret-Ext group). **All Panels:** Test refers to reinstatement test trial 1. Asterisk (*) and pound sign (#) signify one-tailed $p<0.05$ and $p<0.07$, respectively, for directional comparison on response level change (Test – Baseline for NoRet-Ext > Ret-Ext).