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Characterizing conditioned reactivity to sequential alcohol-predictive cues in well-trained rats

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ABSTRACT

Implicit learning about antecedent stimuli and the unconditional stimulus (US) properties of alcohol may facilitate the progressive loss of control over drinking. To model this learning, Cofresí et al. (2017) developed a procedure in which a discrete, visual conditional stimulus (houselight illumination; CS) predicted the availability of a retractable sipper that rats could lick to receive unsweetened alcohol [*Alcoholism: Clinical and Experimental Research*, *41*, 608–617]. Here we investigated the possibility that houselight illumination, sipper presentation, and oral alcohol receipt might each exert control over alcohol seeking and drinking. We also determined the relationship between ingested dose and blood alcohol concentration, in order to validate the idea that the US is a post-ingestive action of alcohol. Finally, we tested a major prediction from the conditioning account of problematic drinking [Tomie, A., & Sharma, N. (2013). *Current Drug Abuse Reviews*, *6*, 201–219], which is that once learned, responses elicited by a CS will promote drinking. We found that despite having constrained opportunities to drink alcohol during the conditioning procedure, ingested doses produced discriminable blood concentrations that supported cue conditioning. Based on our analysis of the dynamics of cue reactivity in well-trained rats, we found that houselight illumination triggered conditioned approach, sipper presentation evoked licking behavior, and alcohol receipt promoted drinking. Reactivity to these cues, which varied in terms of their temporal proximity to the alcohol US, persisted despite progressive intoxication or satiety. Additionally, rats with the greatest conditioned reactivity to the most distal alcohol cue were also the fastest to initiate drinking and drank the most. Our findings indicate that the post-ingestive effects of alcohol may condition multiple cues simultaneously in adult rats, and these multiple cues help to trigger alcohol seeking and drinking. Moreover, identification and characterization of these cues should be helpful for designing interventions that attenuate the power of these cues over behavior.

Introduction

Problematic drinking may result from implicit learning about conditional stimuli and the unconditional stimulus (US) properties of alcohol. The more well-defined the set of conditional stimuli for the alcohol US, the greater the ability of these stimuli to elicit conditioned responses that can promote drinking (Tomie & Sharma, 2013). In support of this theory, the stimulus features of an individual's preferred alcoholic beverage (sight, smell, taste, glassware) have been shown to elicit changes in attention (Das, Lawn, & Kamboj, 2015; Field, Mogg, & Bradley, 2005; Field, Mogg, Zetteler, & Bradley, 2004; Townshend & Duka, 2001), craving (Fox, Bergquist, Hong, & Sinha, 2007; Kaplan et al., 1985; Li et al., 2015; McCusker & Brown, 1990; Monti et al., 1987; Pomerleau, Fertig, Baker, & Cooney, 1983; Witteman et al., 2015), body temperature (Newlin, 1985, 1986), heart rate (Glautier, Drummond, & Remington, 1992; Kaplan et al., 1985; Payne et al., 1992; Sinha et al., 2009; Staiger & White, 1991; Stormark, Laberg, Bjerland, Nordby, & Hugdahl, 1995), salivation (Monti et al., 1987; Pomerleau et al., 1983), and skin conductance (Glautier et al., 1992; Kaplan et al., 1985; Laberg, Hugdahl, Stormark, Nordby, & Aas, 1992).

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In order to model this implicit learning, we developed a task in which a discrete, visual conditional stimulus (CS; houselight illumination) predicted the availability of a retractable sipper that rats could lick to receive unsweetened alcohol (Cofresí et al., 2017). Our experimental set-up differs considerably from appetitive Pavlovian conditioning procedures in which i) a CS predicts the delivery of a fixed quantity (e.g., grain or sucrose pellets) or volume (e.g., liquid sucrose or alcohol) of an appetitive US that is delivered into an omnipresent magazine (e.g., food cup, fluid port), and ii) subjects are free to ingest the appetitive US at any point during the session. In our task, the CS predicts time-limited access to the magazine (sipper). Consequently, receipt of the alcohol solution is contingent upon timely interaction with the sipper, and the amount of alcohol ingested in each conditioning trial depends on this consummatory behavior. This task effectively separates 'appetitive' alcohol-seeking conditioned responses that are triggered by the CS – conditioned approach and orientation toward the sipper – from 'consummatory' licking responses. Conceptually, each conditioning trial models the stimulus sequence that is inherent in consuming a sip of alcohol, where individuals are exposed to the sight and smell of alcohol before making contact with the drinking receptacle. Blocks of conditioning trials (sessions) model the repetition of that stimulus sequence across a drinking episode, which precedes the slow-onset pharmacological effects of ingested alcohol.

Despite extensive extinction training in which the CS is presented without alcohol, the alcohol seeking and drinking sequence that is conditioned using our procedure exhibits spontaneous recovery and reinstatement (Cofresí et al., 2017). We were able to significantly reduce this relapse-like return of responding by conducting daily extinction training after an isolated CS trial (Cofresí et al., 2017), which is believed to trigger memory-updating processes that persistently alter the original CS-alcohol association (Auber, Tedesco, Jones, Monfils, & Chiamulera, 2013; Monfils, Cowansage, Klann, & LeDoux, 2009). However, we were unable to prevent response return altogether, suggesting that some alcohol cue memories remained intact. This outcome led us to consider the possibility that other stimuli in our behavioral paradigm may have also become conditioned to the pharmacological effects of ingested alcohol. In our task, houselight onset is followed 10 s later by insertion of the sipper into the conditioning chamber. Rats have access to the sipper for 10 s, after which it is retracted and the houselight turned off. Sipper presentation is accompanied by an auditory stimulus that is generated by the sipper/bottle motor assembly, creating a compound visual and auditory stimulus that signals alcohol availability and could serve as a predictor of alcohol's post-ingestive effects. Finally, licking the sipper provides access to the taste and smell of alcohol. These orosensory stimuli are likely to also act as predictors of the pharmacological consequences of alcohol ingestion.

The present study characterized appetitive and consummatory behavior in relation to potential cues in our oral alcohol-conditioning task. Specifically, in well-trained rats we examined the dynamics of i) conditioned alcohol-directed approach elicited by the houselight, ii) sipper contact elicited by sipper presentation, and iii) within-trial lick rate, which would have been influenced by the smell and/or taste of alcohol. Additionally, we were interested in determining the relationship between ingested dose and blood alcohol concentration in our task – something that is infrequently done in appetitive Pavlovian conditioning procedures with alcohol. Determining that alcohol is detectable in blood supports the idea that the US in our conditioning task is alcohol's post-ingestive pharmacology. In relation to this idea, we were also interested in testing a major prediction from the conditioning account of problematic drinking (Tomie & Sharma, 2013), which is that once learned, responses elicited by a CS will promote drinking.

Methods and materials

Subjects

Subjects were adult, male Long-Evans rats (Envigo; Indianapolis, Indiana) weighing 250–275 g upon arrival. All were singly housed in a temperature and humidity controlled room (22 ± 2°; 12-h light cycle). Access to chow and tap water were unrestricted in the homecage, which contained Sani-Chips® bedding and a Bio-Serv Gummy Bone (polyurethane; 5 cm L × 2.5 cm W). All procedures were conducted during the light phase of the light/dark cycle.

Apparatus

Conditioning took place in Med Associates, Inc. (Fairfax, Vermont) chambers housed within sound-attenuating cubicles that were equipped with digital video cameras and exhaust fans. The houselight and retractable bottle assembly were installed on the same chamber wall. For a detailed description, see Cofresí et al. (2017).

Behavioral methods

Drinking unsweetened ethanol in the homecage

A week after arrival, rats received 5 weeks of intermittent access to ethanol using a two-bottle choice procedure. This phase was conducted in order to acclimate rats to the taste and pharmacological effects of alcohol. Briefly, rats had 24-h access to unsweetened ethanol solution (15% ethanol in tap water; v/v; 15E) or tap water starting 4–6 h into the light phase on Monday, Wednesday, and Friday. On all other days, two water bottles were available. Ethanol and water bottle placement on the cage was alternated across sessions. Gravity-fed, metal sipper tubes were used. Rats that failed to drink ≥0.5 g/kg in week 1 were offered 5% ethanol v/v in tap water (5E) in week 2 and then 10% ethanol v/v in tap water (10E) over weeks 3–5 (for details, see Cofresí et al., 2017). Rats drinking less than 1 g/kg/session on average across week 5 were not retained for conditioning.

Cue conditioning with unsweetened ethanol

Rats received 12 sessions of cue conditioning. Briefly, sessions occurred across consecutive days and consisted of eight conditioning trials on a variable intertrial interval (ITI) with mean 280 s, minimum 160 s, and maximum 360 s. Upon initiating the Med-PC program, there was a 5-min delay period to allow rats to acclimate to the conditioning chambers, after which the exhaust fans were activated to signal session onset and the first ITI was selected. The final (9th) ITI was selected after trial 8, and the exhaust fan was turned off at the end of this ITI to signal the end of the session. In each conditioning trial, the houselight was illuminated for 20 s and the bottle assembly activated such that a metal sipper was inserted into the chamber 10 s after houselight onset and retracted upon houselight offset. The bottle assembly immediately produced a noise when it was activated and took up to 0.5 s to complete each operation (movement of the sipper tip toward or away from the plane of the wall). The sipper was attached to a bottle filled with either 10E or 15E, depending on which solution the rat was drinking at the end of the homecage drinking phase, and contained a ball bearing to prevent spillage upon insertion and retraction. Rats were given a single habituation session the day before the first conditioning session, during which the houselight and bottle assembly motor were activated on the same schedule described above, but neither sipper nor drinking solution were presented. The day immediately after conditioning session 12, rats were given 12 trials with a dry sipper. Ambient ethanol odor was absent. The

variable ITI was the same as described above except ~60 min elapsed between test trials 1 and 2 for seven rats (Cofresí et al., 2017). There were no notable differences in trial-by-trial behavior between those seven and the remaining 23 rats. Only test trials 1–8 were considered here for ease of comparison to trial-by-trial data from conditioning session 12.

Blood ethanol concentration following cue conditioning

Blood sampling took place after rats had been re-conditioned, such that their response and ingested dose levels were stable within 15% of levels across conditioning sessions 10–12. On blood sampling day, as on any day, rats had unlimited access to chow and water in the homecage until they were transferred into conditioning chambers for a conditioning session. Rats were removed from the chamber immediately after the 8th sipper presentation and anesthetized with isoflurane gas. Blood was then collected from either the saphenous vein or the trunk after decapitation.

Behavioral measurements

Rats were weighed before every homecage drinking or cue-conditioning session. During the homecage phase, solution intake was measured as the difference in bottle mass pre- and post-session to the nearest 0.1 g. Intake was corrected for loss due to evaporation and/or leakage by subtracting the "intake" of an empty cage subjected to identical procedures. Grams of pure ethanol ingested were calculated using corrected ethanol solution intake. During the cue-conditioning phase, intake was measured as the difference in bottle mass pre- and post-session to the nearest 0.01 g. Intake was corrected by subtracting the mass of solution leaked from the sipper in its retracted state. Leaked solution was collected by a weighboat outside the chamber and measured as the difference in weighboat mass pre- and post-session. Grams of pure ethanol ingested were calculated using corrected intake.

During the cue-conditioning phase, we also measured appetitive and consummatory behaviors. Cue-conditioning trials were sampled from digital video recordings by making instantaneous observations every 1.25 s, starting 5 s before houselight onset as in Cofresí et al. (2017). At each observation, 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, rearing, resting). Highly trained judges made these observations, with ≥95% agreement on joint ratings. Judges were blind to session/treatment parameters. For every conditioning trial in every session, "approach" behavior state ratings within each trial phase (5-s bin before houselight illumination: pre-CS; consecutive 5-s bins during illumination: CS1, CS2) were counted for every rat. Only four observations were made per trial phase, so the maximum sipper site approach frequency per trial phase on any trial of any session was 4. Consummatory sipper licking was recorded using a contact lickometer. The latency to initiate sipper licking was computed as the time to first lick following activation of the retractable bottle assembly. If no lick was registered during a trial, a maximum latency (10 s) was assigned.

Blood ethanol analysis

Ethanol concentration (mg/dL) in blood samples (10 μL mixed with 90 μL saturated saline; three replicates per rat) was determined using gas chromatography as in Carrillo et al. (2008).

Statistical analysis

Behavior patterns were characterized using within-subjects analysis of variance (ANOVA) and 2-tailed paired *t* tests. Relationships between behaviors were explored using simple linear regression. Bonferroni correction was applied as appropriate. The threshold for statistical significance was $p < 0.05$. Analyses were conducted in R version 3.3.2 (R Core Team, 2016) using the car package (Fox & Weisberg, 2011).

Ethics

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin, and conducted in accordance with NIH guidelines.

Solutions

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

Results

In total, 44 rats were obtained for the study, 37 of which were retained for conditioning based on ethanol intake in the homecage (≥1.00 g/kg per day on average across the last three sessions). To characterize cue reactivity after task acquisition, we analyzed data from 30 rats that met *a priori* minimum ingested dose criteria across experiment phases (homecage: 1.00 g/kg per day on average across the last three sessions; conditioning: 0.30 g/kg per session across the last three sessions).

Post-acquisition dynamics in reactivity to houselight illumination

We analyzed sipper site approach elicited by houselight illumination during the pre-CS, CS1, and CS2 trial phases of each trial in conditioning session 12 and the dry sipper test session (Fig. 1A and B) for all 30 rats to characterize conditioned reactivity to this visual CS, which is most distal to the post-ingestive effects of ethanol, in well-trained subjects. Within-subjects ANOVA detected a significant 3-way interaction of trial phase, trial, and session on sipper site approach ($F_{(14,406)} = 15.21$, $p < 0.001$; Fig. 1C). Follow-up ANOVA detected a significant trial phase × trial interaction in both sessions ($F_{(14,406)} = 14.54$, $p < 0.001$). In both sessions, the frequency of sipper site approach that occurred before houselight onset (pre-CS) did not vary significantly from floor across trials, but the frequency of sipper site approach that occurred during houselight illumination (CS1 and CS2) decreased significantly across trials (simple effects of trial: $F_{(7,203)} \geq 6.11$, $p < 0.001$). In both sessions, approach level during CS1 and CS2 was greater in trials 1–4 than in trials 5–8 ($t_{29} \geq 4.11$, $p < 0.001$). In both sessions, there was a consistent pattern of approach within trials (simple effects of trial phase: $F_{(2,58)} \geq 6.39$, $p < 0.004$), such that the frequency of sipper site approach was greater during CS1 than pre-CS, and greater during CS2 than CS1. In the conditioning session, pairwise comparisons of trial phases within trials were significant across trials 1–8 ($t_{29} \geq 2.27$, $p < 0.05$). In the dry sipper session, pairwise comparisons of trial phases within trials were significant in trials 1–6 and 8 ($t_{29} \geq 2.41$, $p < 0.025$), but not trial 7 ($t_{29} = 1.88$, NS). While statistically significant, it appears the 3-way interaction of trial phase, trial, and session was driven by a practically insignificant difference in within-trial pattern persistence across trials between sessions. Essentially, however, within-session houselight cue-elicited alcohol approach dynamics were identical when

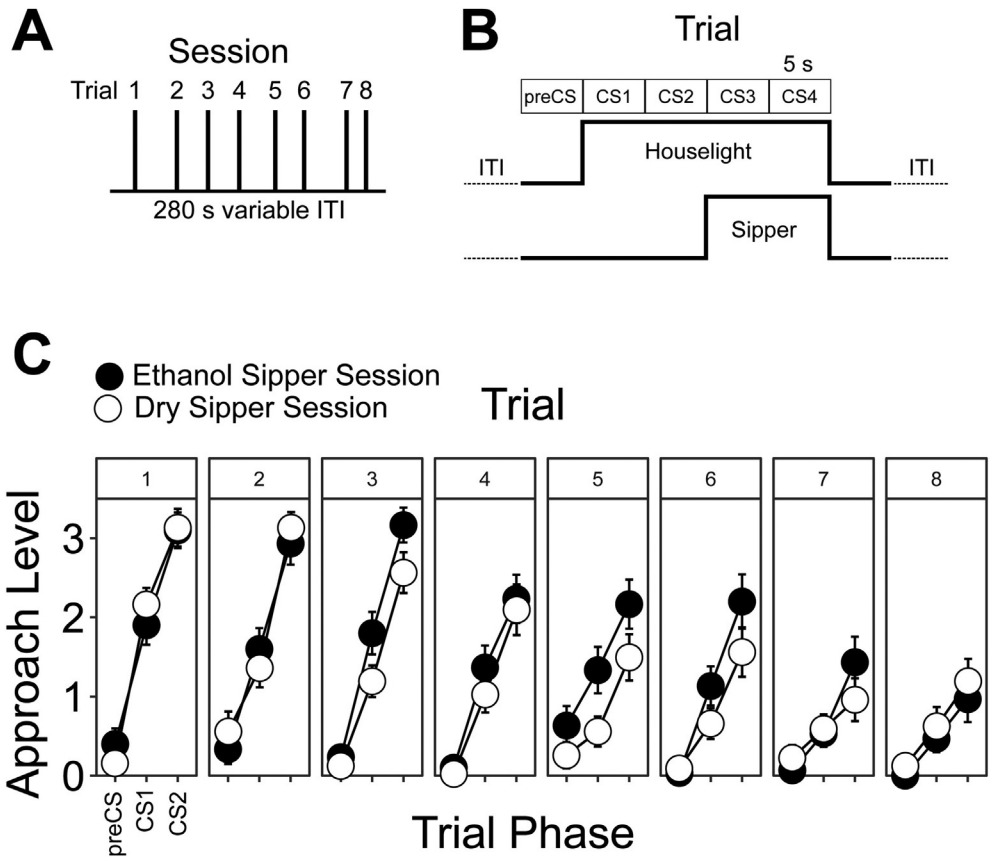

Fig. 1. Dynamics of reactivity to houselight illumination. A–B: Schematic representations of a session and trial. **C:** Mean ± S.E.M. sipper site approach level across trials phases (pre-CS, CS1, and CS2) paneled by trial for 30 adult, male Long-Evans rats. The maximum approach level per trial phase was 4 (see Behavior Measurements in main text Methods for videoscoring details). Data from conditioning session 12 are represented by black circles. Data from the dry sipper test session 24 h later are represented by white circles. Ambient ethanol odor was absent for dry sipper test trials.

tested under reinforcement (oral ethanol receipt) and non-reinforcement (dry sipper, no ethanol odor). Thus, despite some decline in conditioned reactivity across trials in each test condition, every illumination of the houselight elicited sipper site approach, and the frequency of this conditioned response increased across the period of illumination prior to sipper presentation.

Post-acquisition dynamics in reactivity to sipper presentation

We analyzed the latency to start licking the sipper per trial in conditioning session 12 and the dry sipper test session for 25 of 30 rats (five were missing lick data due to equipment malfunction) to characterize reactivity to this compound auditory-visual stimulus, which in our task is more proximal to the ethanol US than house-light illumination, in well-trained subjects. Within-subjects ANOVA detected significant main effects of trial ($F_{(7,168)} = 15.78$, $p < 0.001$) and session ($F_{(1,24)} = 12.86$, $p < 0.002$) on latency to lick (Fig. 2), but no interaction (F < 1, NS). To confirm this equivalence further, we conducted ANOVA within each session. In both sessions, the latency to start licking increased across trials (simple main effects of trial: $F_{(7,168)} = 10.86$, $p < 0.001$). Additionally, in both sessions, on average, the latency to lick was lower (namely, rats were faster to start licking) in trials 1–4 than in trials 5–8 ($t_{24} = 4.82$, $p < 0.001$). The only difference between sessions was that latency was greater across dry sipper test trials 4–6 than conditioning trials 4–6. Overall, however, within-session dynamics of sipper presentation-elicited sipper contact were identical when tested under reinforcement (oral ethanol receipt) and non-reinforcement (dry sipper, no ethanol odor). Thus, despite some decline across trials in each test condition, presentation of the sipper tended to prompt initiation of consummatory licking within the 10-sec window of opportunity.

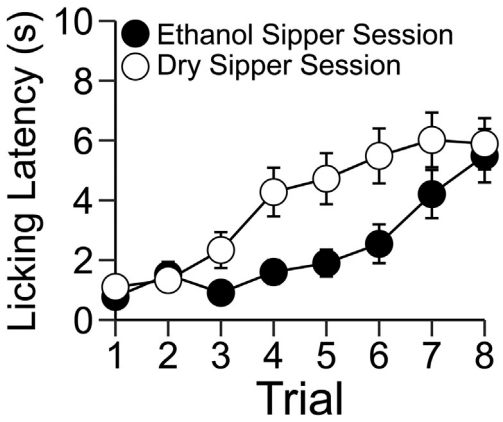

Fig. 2. Dynamics of reactivity to sipper presentation. Mean ± S.E.M. latency (sec) to start licking across trials for 25 of 30 adult, male Long-Evans rats (data were missing for 5 of 30). Maximum latency was 10 s, the total duration of sipper availability. Data from conditioning session 12 are represented by black circles. Data from the dry sipper test session 24 h later are represented by white circles. Ambient ethanol odor was absent for dry sipper test trials.

Post-acquisition dynamics in reactivity to oral ethanol receipt

The receipt of unsweetened ethanol solution is an orosensory stimulus and it is the antecedent stimulus that is most proximal to ethanol's post-ingestive effects in our task. In order to characterize reactivity to this stimulus in well-trained subjects, we analyzed lick rate (licks per 2-sec bin within the 10-sec sipper presentation) in each trial in conditioning session 12 and the dry sipper test session for 25 of 30 rats (five were missing lick data due to equipment malfunction). Within-subjects ANOVA detected a significant 3-way interaction of bin, trial, and session on lick rate ($F_{(28,672)} = 1.65$, $p < 0.02$; Fig. 3). Follow-up ANOVA detected a significant bin × trial interaction on lick rate in the conditioning session ($F_{(28,672)} = 1.98$, $p < 0.0025$), but not in the dry sipper test session ($F_{(28,672)} = 1.01$, NS). In the conditioning session, the simple effect of bin was significant in every trial ($F_{(4,96)} \geq 5.21$, $p < 0.001$). In every trial, initial receipt of oral ethanol triggered a spike in lick rate (bin 1 vs. bin 2: $t_{24} = 2.54$, $p < 0.02$). The final lick rate in every trial was also always greater than the starting rate (bin 1 vs. bin 5: $t_{24} = 3.59$, $p < 0.002$). What varied between trials was whether lick rate was stable after its initial spike or increased with more oral ethanol receipt. In 6 of 8 trials, there was an increase in rate across or sometime between bin 2 and bin 5. In only four of eight trials was the final rate (bin 5) greater than rate after the spike due to initial oral ethanol receipt (bin 2 or bin 3). However, none of these latter comparisons was statistically significant after correction for multiple comparisons. The simple effect of trial was also significant in every bin ($F_{(7,168)} \geq 6.09$, $p < 0.001$), reflecting the overall decline in lick rate across trials in the conditioning session. Overall, lick rates in the dry sipper test session were significantly lower than in the conditioning session (collapsing bins and trials, $t_{24} = 13.35$, $p < 0.001$). In the dry sipper test session, there was a significant main effect of trial ($F_{(7,168)} = 14.67$, $p < 0.001$) driven by a drop in the average lick rate (across bins) per trial to near-floor by the second half of the dry sipper test session (trials 1–4 vs. trials 5–8: $t_{24} = 8.54$, $p < 0.001$). A statistically significant main effect of bin was also detected in the dry sipper test session ($F_{(4,96)} = 3.1$, $p < 0.02$). However, none of the pairwise comparisons between bins (collapsing across trials) was statistically significant after correcting for multiple comparisons. Thus, despite some decline across trials, initial receipt of oral ethanol solution tended to accelerate the rate of consummatory licking within every trial. The same within-trial pattern was not observed when the sipper failed to deliver fluid and ambient ethanol odor was absent.

Blood ethanol concentrations in the conditioning task

To determine whether ethanol ingested in the conditioning task produced detectable levels of ethanol in blood, we obtained blood samples from 24 rats (13 from saphenous vein, 11 from trunk). The time between the 1st and 8th sipper presentation in the session was 34–37 min, due to the variable ITI. Blood sampling time ranged from 2.5 to 12.5 min after the 8th sipper presentation. Body weights ranged from 416 to 547 g. Blood ethanol concentration (BEC) was detectable in most rats at the end of the conditioning session, and was significantly related to ingested dose (Pearson's $r = +0.73$, $t_{22} = 5.01$, $p < 0.001$; Fig. 4). The mean ± S.E.M. ingested dose was 0.59 ± 0.04 g/kg. The mean ± S.E.M. BEC was 16 ± 3 mg/dL. BEC ranged from 0 to 57 mg/dL.

Conditioned reactivity to houselight illumination promotes ethanol drinking

We used simple linear regression to test the prediction that sipper site approach elicited by the houselight promotes ethanol intake during sipper exposure after task acquisition. Specifically, we modeled drinking-related measurements as a function of reactivity

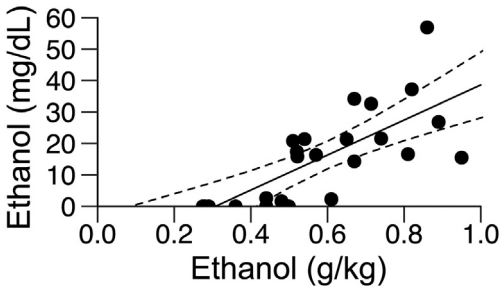

Fig. 4. Ethanol in blood after a conditioning session. Relationship between blood ethanol concentrations detected at the end of a conditioning session and ingested ethanol doses. Data were from 24 of 30 adult, male Long-Evans rats. Solid line represents the regression line. Dashed lines represent the upper and lower 95% confidence limits around the regression line.

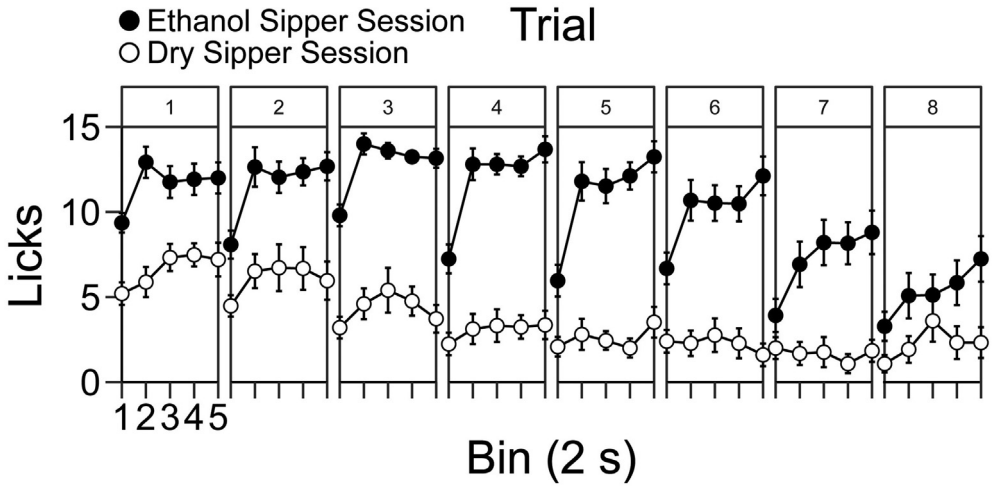

Fig. 3. Dynamics of reactivity to oral ethanol receipt. Mean ± S.E.M. licks per 2-sec bin paneled by trial for 25 of 30 adult, male Long-Evans rats (data were missing for 5 of 30). The sipper was available for 10 s total, hence 5 bins per trial. Data from conditioning session 12 are represented by black circles. Data from the dry sipper test session 24 h later are represented by white circles. Ambient ethanol odor was absent for dry sipper test trials.

to houselight illumination as indexed by sipper site approach during trial phase CS2 per trial. Data were averaged across sessions 10–12 to get the best estimates of asymptotic individual behavior and dose levels. Data from all 30 rats were available for regression of dose on conditioned approach level, but data from only 25 rats were available for regressions of licks and lick latency on conditioned approach level (data were missing for five of 30 rats due to equipment malfunction). Conditioned reactivity to the houselight was related to the latency to start licking per trial, the number of licks per trial, and the total ingested dose. Greater reactivity to houselight illumination predicted shorter latency to start licking ($t_{23} = -9.15$, $p < 0.001$; Fig. 5A), greater number of licks ($t_{23} = 6.32$, $p < 0.001$; Fig. 5B), and larger ingested doses ($t_{28} = 2.06$, $p < 0.05$; Fig. 5C). Houselight cue reactivity levels accounted for 78% of the variance in licking latencies, 63% of the variance in licks, and 13% of the variance in ingested doses.

Discussion

Here, we set out to: i) characterize reactivity to potential conditional stimuli for alcohol availability and/or alcohol's pharmacological effects in our paradigm, ii) confirm that alcohol consumed during conditioning sessions was detectable in blood, and iii) test the prediction that conditioned responses elicited by an alcohol-predictive CS can promote drinking.

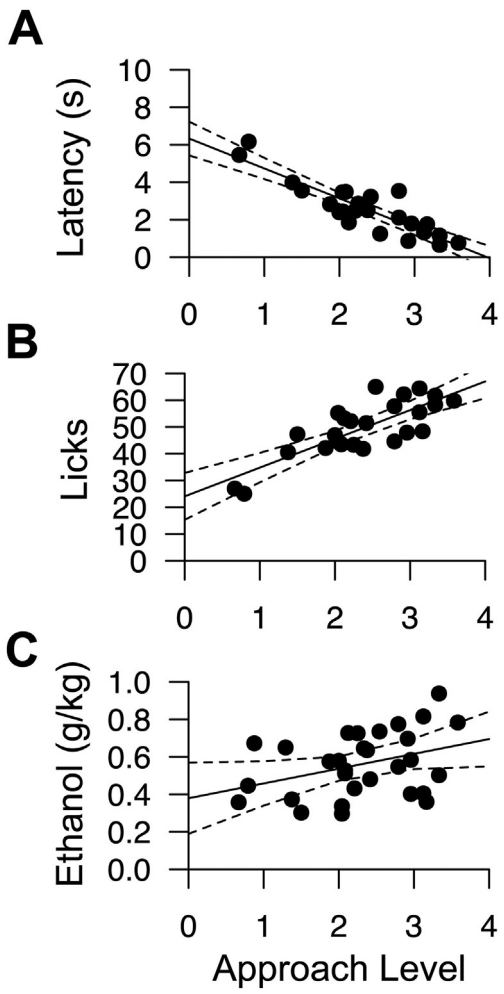

Fig. 5. Houselight cue reactivity predicts drinking. Relationships of latency to start licking per trial (**A**), total licks per trial (**B**), and ingested dose per session (**C**) to houselight-elicited sipper site approach level per trial (maximum = 4) on average across conditioning sessions 10–12. Data were from 25 to 30 adult, male Long-Evans rats (lick data were missing for 5 of 30). Solid lines in each panel represent the regression line. Dashed lines represent the upper and lower 95% confidence limits around the regression line.

Cue conditioning with unsweetened alcohol in non-deprived rats

In the present study, non-deprived adult male rats learned to drink unsweetened alcohol from a sipper that was presented in a conditioning chamber, and learned to react to houselight illumination – an antecedent conditional stimulus (CS) for alcohol access – with approach to the site of alcohol access. It is likely that providing rats with intermittent access to unsweetened alcohol in the homecage prior to cue conditioning facilitated this learning (Supplemental Figs. 1–3). Acclimation to the taste and pharmacological effects of alcohol during this phase may have permitted subsequent conditioning with unsweetened alcohol as the appetitive US in a different context, without the need for fluid or food deprivation or sweetened alcohol solution (see also Carnicella, Ron, & Barak, 2014; Chaudhri, Sahuque, Schairer, & Janak, 2010; Cofresí et al., 2017; Remedios, Woods, Tardif, Janak, & Chaudhri, 2014).

Control of alcohol seeking and drinking by multiple alcohol-predictive cues

The primary objective of the present study was to characterize the behavioral reactions of well-trained subjects to distinct stimuli – houselight illumination, sipper presentation, and alcohol solution – that comprise conditioning trials in our paradigm. We found that houselight illumination (visual cue) elicited approach to the location of the sipper (Fig. 1), and sipper presentation (multimodal cue) elicited consummatory licking (Fig. 2). Moreover, the receipt of alcohol for oral consumption (orosensory cue) elicited increases in lick rate across the sipper presentation period (Fig. 3). Although the conditioned response sequence within trials remained stable, responding was greatest at the beginning of a session and decreased across trials within that session. This decline in overall reactivity across trials may have been due to the slow onset of alcohol's sedative-like effects (Chuck, McLaughlin, Arizzi-LaFrance, Salamone, & Correa, 2006; Frye & Breese, 1981). Alternatively, it may have been due to decreases in the momentary motivational value of alcohol as a function of consumption (namely, satiety) (Samson, Czachowski, & Slawecki, 2000; Samson, Slawecki, Sharpe, & Chappell, 1998).

In our paradigm, the explicit stimulus that was designed to acquire a conditioned response was houselight illumination, but other stimuli present in the task may also have acquired conditioned responses, specifically, alcohol sipper presentation and oral alcohol receipt. Our results show that sipper presentation elicited the initiation of consummatory licking, even in the absence of oral alcohol and ambient alcohol odor. However, it should be noted that sipper presentation occurred in the context of houselight illumination. Thus, it is possible that licking initiation was controlled by the houselight, and not a reaction to sipper presentation. To distinguish between these alternatives, one future study with well-trained subjects could probe reactivity to the sipper without antecedent houselight illumination. Another future study might compare the behavior of subjects trained to drink alcohol under houselight illumination to that of others trained to drink in its absence. Another possibility is that houselight illumination and sipper presentation were learned as a compound cue. This can be tested by probing reactivity to the constituent elemental stimuli in isolation after training or extinction of the compound stimulus. A related, important consideration is that sipper presentation itself is

a multimodal compound stimulus and such stimuli can engender stronger conditioned responses (Rescorla, 1973; See, Grimm, Kruzich, & Rustay, 1999; Weiss, 1964).

Our results also show a dramatic spike in lick rate following initial receipt of oral alcohol and a less robust spike in lick rate toward the end of drinking opportunities. No such patterns were observed when licking did not deliver oral alcohol. These findings not only confirm that oral alcohol receipt sustains consummatory licking after it is initiated, but also suggest that the initial receipt of oral alcohol may act as a cue capable of eliciting increases in drinking speed (consummatory vigor) within drinking occasions. To confirm this possibility, a future study could probe lick rate reactivity to receipt of an alternative liquid (e.g., water or quinine with and without ambient alcohol odor) after training with oral alcohol. Another future study could compare the lick rate of subjects conditioned with alcohol to that of subjects conditioned with different liquid reinforcers.

A final caveat worth mentioning is that conditioned reactivity is multiply determined. Other factors known to influence the form and dynamics of reactivity beyond those already described include the nature of the US (Jenkins & Moore, 1973), the nature of the CS (Timberlake & Grant, 1975), and the interval between them (Esmorís-Arranz, Pardo-Vázquez, & Vázquez-García, 2003; Waddell, Morris, & Bouton, 2006). The present study was not designed to disambiguate the role of specific factors. Despite all the caveats presented above, we believe our results support the idea that multiple, distinct alcohol-predictive cues influence alcohol seeking and drinking, at least in our model (and perhaps also in naturalistic drinking by humans).

The primary motivation for the present study was to gain insight into whether and, if so, how different cues elicit behavioral reactivity in our model, because in prior work using this model, we observed that conducting retrieval + extinction, an arrangement of CS-no US trials that is believed to persistently update the original CS-US association formed during conditioning, attenuated but did not completely prevent the subsequent return of responding in spontaneous recovery and relapse tests (Cofresí et al., 2017). Importantly, the retrieval cue consisted of houselight illumination and dry sipper presentation. This procedure may have reactivated and updated only memories related to the houselight and sipper cues. Memory for cues more proximal to alcohol's post-ingestive pharmacology (the putative US), such as the smell and taste of the alcohol drinking solution, may not have been reactivated. Thus, a retrieval + extinction procedure that included olfactory and orosensory alcohol cues may allow broader or more robust memory reactivation and updating.

Alcohol consumed during cue-conditioning sessions was pharmacologically active

Except for Tomie and colleagues (Tomie, Lewis, Curiotto, & Pohorecky, 2007; Tomie, Uveges, Burger, Patterson-Buckendahl, & Pohorecky, 2004; Tomie et al., 2006), preclinical researchers modeling the appetitive conditioning effects of human alcohol consumption in rodents do not typically verify that ingested alcohol can be detected in blood. If alcohol can be detected in the blood, then it is reaching the brain, and it can be argued that conditioned behavior in these rodent models stems from some action of alcohol on the brain, as we believe it does in humans. However, species differences and task parameters can make it so that little to no ingested alcohol reaches the brain. Thus, verifying blood alcohol is important for the relevance of findings from these rodent models to cue-triggered alcohol use in humans. Despite the limited number of drinking opportunities and the spacing of those opportunities in time in our task, alcohol was detectable in a large majority of subjects' blood at the end of conditioning sessions. Although pre-session stomach content was not controlled, blood alcohol concentration (BAC) was still strongly related to total ingested dose (Fig. 4). The 95% confidence interval around the BAC-dose regression line included zero until ~0.4 g/kg. This suggests that our *a priori* minimum ingested dose criterion for "alcohol-reinforced conditioning" (average dose ≥0.30 g/kg across the last three conditioning sessions) may be too lenient. However, without mid-session blood samples, we cannot exclude the possibility that rats drinking between 0.30 and 0.40 g/kg had detectable BAC earlier in the session. Perhaps more importantly, we do not know how BAC changes over time within the conditioning session. Thus, we cannot say whether conditioning took place on the ascending or descending limb or both limbs of the BAC-time curve. Additionally, we do not know how phases of the BAC-time curve affect expression of CS reactivity at asymptote.

However, doses ingested by rats at asymptote in our paradigm were in a range (0.30–0.95 g/kg) that produces discriminable internal states, and the average ingested dose (~0.56 g/kg) would substitute for the internal state produced by the same dose injected intraperitoneally (Macenski & Shelton, 2001). Additionally, ingested doses were similar to those that maintain operant self-administration of unsweetened alcohol by rats (Czachowski, 2005; Czachowski, Chappell, & Samson, 2001). Furthermore, we know that BACs detected in our rats were within a range that is easily achieved by humans in naturalistic drinking situations (10–60 mg/dL or 2–13 mM) (Clapp, Min, Shillington, Reed, & Ketchie Croff, 2008; Clapp et al., 2009; Dougherty et al., 2012; Hustad & Carey, 2005; Thombs, Olds, & Snyder, 2003). These data support the contention that our paradigm allows us to study conditioning processes that are ultimately reinforced by alcohol's post-ingestive pharmacology, specifically its central neuropharmacology. Although ingested alcohol doses that produce central effects also produce peripheral effects, the central effects of ingested alcohol appeared to be critical for conditioning effects of alcohol in our paradigm. We saw no evidence for cue conditioning in rats that consistently ingested enough alcohol for peripheral effects, but not enough alcohol for central effects (Supplemental Fig. 4).

Reactivity to an alcohol-predictive CS promotes alcohol intake

Based in part on prior studies showing that the US properties of alcohol can alter responding elicited by a CS that predicts sweet taste or food, Tomie and colleagues (Tomie & Sharma, 2013; Tomie, Festa, Sparta, & Pohorecky, 2003; Tomie et al., 1998) proposed a model of alcohol abuse that predicts that reactivity to an alcohol-predictive CS would promote alcohol drinking. In agreement with this prediction, we found that in well-trained rats, greater reactivity to the houselight predicted faster initiation of drinking, more drinking, and the ingestion of larger alcohol doses (Fig. 5A–C). These relationships may represent a causal stimulus-response chain or between-subject differences in biological and psychological factors that influence conditioning rates, final levels of cue reactivity, and drinking behaviors.

The link between cue reactivity and drinking in our paradigm is consistent with the finding that current and former heavy drinkers tend to show greater autonomic, behavioral, and neural reactivity to alcohol cues (Sinha et al., 2009; Sjoerds, van den Brink, Beekman, Penninx, & Veltman, 2014; Townshend & Duka, 2001; Vollstädt-Klein et al., 2010). This link is also in accordance with the finding that alcohol cue reactivity measurements can predict alcohol-use disorder relapse after treatment (Drummond & Glautier, 1994; Monti et al., 1993; Papachristou, Nederkoorn, Giesen, & Jansen, 2014; Rohsenow et al., 1994; but see; Witteman et al., 2015).

Summary

Using a rat model of alcohol cue conditioning in which alcohol's post-ingestive pharmacology arguably serves as the unconditional stimulus, we were able to measure alcohol-directed appetitive and consummatory behaviors while tracking the level of alcohol exposure within and across drinking episodes. This allowed us to describe the dynamics of behavioral reactivity potentially conditioned to distinct antecedent stimuli for alcohol's post-ingestive effects. We found that cue reactivity within drinking episodes persisted despite progressive intoxication or satiety, and predicted overall levels of drinking. Insight gained here about the incidental conditioning of multiple alcohol-predictive cues can help guide future work on ways to attenuate the control that such cues exert over alcohol seeking and drinking.

Conflicts of interest

None.

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Appendix B. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.alcohol.2017.11.034.

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