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ORIGINAL INVESTIGATION

Extinction of Conditioned Responses to Methamphetamine-Associated Stimuli in Healthy Humans

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Abstract
Rationale Contextual stimuli present during drug experiences become associated with the drug through Pavlovian conditioning and are thought to sustain drug-seeking behavior. Thus, extinction of conditioned responses is an important target for treatment. To date, acquisition and extinction to drug-paired cues have been studied in animal models or drug-dependent individuals, but rarely in non-drug users.
Objective We have recently developed a procedure to study acquisition of conditioned responses after single doses of methamphetamine (MA) in healthy volunteers. Here, we examined extinction of these responses and their persistence after conditioning.
Methods Healthy adults (18–35 years; $N=20$) received two pairings of audio-visual stimuli with MA (20 mg oral) or placebo. Responses to stimuli were assessed before and after conditioning, using three tasks: behavioral preference, attentional bias, and subjective "liking."
Results Subjects exhibited behavioral preference for the drug-paired stimuli at the first post-conditioning test, but this declined rapidly on subsequent extinction tests. They also exhibited a bias to initially look towards the drug-paired stimuli at the first post-test session, but not thereafter. Subjects who experienced more positive subjective drug effects during conditioning exhibited a smaller decline in preference during the extinction phase. Further, longer inter-session intervals during the extinction phase were associated with less extinction of the behavioral preference measure.
Conclusions Conditioned responses after two pairings with MA extinguish quickly, and are influenced by both subjective drug effects and the extinction interval. Characterizing and refining this conditioning procedure will aid in understanding the acquisition and extinction processes of drug-related conditioned responses in humans.

Keywords Extinction · Pavlovian conditioning · Human · Methamphetamine · Cue · Preference · Attention bias · Associative learning

Introduction

Conditioned drug cues are widely believed to facilitate and reinstate drug seeking, and therefore extinction of these conditioned responses is considered a key target for drug abuse treatment. In substance users, drug-associated cues are thought to initiate drug seeking, maintain continued consumption, and predict relapse (Powell et al. 2010) by eliciting conditioned responses that include drug craving (Ferguson and Shiffman 2009; Drummond 2001; Bedi et al. 2011), physiological arousal (Ehrman et al. 1992), heightened emotional reactivity (Geier et al. 2000; Drobes and Tiffany 1997), and increased attention towards cues (i.e., "attentional bias;" Waters et al. 2009; Robinson and Berridge 1993). Despite a large preclinical literature on both acquisition and extinction of conditioned drug effects in laboratory animals (de Wit and Stewart 1981; See 2002), few studies have examined the acquisition and extinction of conditioned drug cue responses under controlled conditions in humans (Winkler et al. 2011; Flaten and Blumenthal 1999; Martin-Soelch et al. 2007).

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There is an extensive literature investigating responses to drug-related cues in drug-dependent individuals, using generic cues such as pictures of drugs and drug use paraphernalia (Volkow et al. 2006; Sinha 2009; O'Brien et al. 1992; Sell et al. 2000; MacKillop and Lisman 2008; Collins and Brandon 2002; Kamboj et al. 2011; LaRowe et al. 2007; Price et al. 2010). In addition, a small handful of studies have examined acquisition of novel associations between previously neutral cues and drugs of abuse, including nicotine (Mucha et al. 1998; Lazev et al. 1999) and cocaine (Foltin and Haney 2000) in drug-dependent individuals, and one study has reported the extinction of newly conditioned drug cue responses in daily nicotine users (Thewissen et al. 2006). However, it is difficult to study basic processes of conditioning in established drug users because of their complex and often unknown drug use histories, making it impossible to control for both exposures to the drugs and the cues associated with them, as well as other memories associated with the drug-taking event (Kilts et al. 2001). To avoid these issues, we have developed a paradigm to study drug cue associations de novo in healthy young adults (Mayo et al. 2013; Mayo and de Wit 2015). In the procedure, participants receive two pairings of a neutral audio-visual stimulus (nature image and corresponding sounds) with a single dose of methamphetamine (MA), and two pairings of a different stimulus with placebo (PBO). After these pairings, participants showed cue-elicited conditioned responses, including increases in behavioral preference (Mayo et al. 2013; Mayo and de Wit 2015) and attentional bias (Mayo and de Wit 2015) for the MA-paired stimuli, as compared to the PBO-paired stimuli. Mayo and de Wit (2015) also found that subjective drug "liking" during the acquisition phase was correlated with greater attentional bias towards the drug-paired cue. Importantly, these studies indicated that after only two pairings, healthy, non-dependent participants exhibited conditioned drug cue reactivity, consistent with findings using laboratory animals (see Shaham et al. 2003 and See 2005 for reviews).

A major target for substance abuse treatment is the extinction of drug-paired conditioned responses (Conklin and Tiffany 2002) through repeated presentation of the drug cue in the absence of the drug. Because the efficacy of cue exposure therapies have been limited (Havermans and Jansen 2003), the goal of the present study was to further develop our laboratory conditioning procedure by investigating the extinction phase of conditioning. As in the earlier studies, participants underwent four conditioning sessions (two MA, two PBO) each with distinctive stimuli. We studied their responses during repeated extinction test sessions (without drug administration) to assess the durability of the conditioned response. We hypothesized that the MA-paired cue would elicit conditioned responses, such as increased behavioral preference and greater attentional bias compared to the PBO-paired cue, and that these responses would attenuate with repeated, unpaired exposures to the MA-paired cue. We also expected that subjective drug effects, especially drug "liking" would be positively correlated with the strength of the conditioned responses, as indicated by more resistance to extinction (Annau and Kamin 1961; Rutten et al. 2011). Finally, on an exploratory basis, we also examined other parameters (e.g., inter-session intervals) that might predict the rate of extinction.

Materials and methods

Design

This study used a within-subjects design consisting of three phases: (i) a pre-test assessing responses to the cues before conditioning, (ii) four conditioning trials with methamphetamine (MA, 20 mg oral) or placebo paired with distinctive stimuli (counterbalanced across participants), and (iii) post-conditioning extinction tests. The primary outcome measures were three indices of conditioning of the MA-paired stimuli, behavioral preference, attentional bias, and "liking" of the stimuli. The primary goal was to examine the persistence of these conditioned responses during extinction.

Participants

Healthy volunteers ($N=20$) aged 18–35 were recruited from the university and surrounding community (Table 1). Participants underwent an in-person screening interview consisting of a psychiatric evaluation, electrocardiogram, physical examination, and reports of current and lifetime non-medical drug use history. Participants were eligible regardless of whether they had previously used stimulants or other drugs. Inclusion criteria were BMI of 19–26 kg/m, a high school education, fluency in English, resting blood pressure less than 140/90 mmHg, resting heart rate less than 90 bpm, and consumption of fewer than four standard caffeinated or alcoholic beverages a day. Exclusion criteria included current medications (except hormonal birth control for women), current or past year substance dependence, history of cardiovascular illness, abnormal EKG (as determined by a medical doctor), current or last 5-year diagnosis of attention deficit hyperactivity disorder, current or past year major Axis I DSM-IV-TR disorder (American Psychiatric 2000), mood disorders, or psychotic symptoms within the past year. Shift workers, pregnant women, and nursing mothers were also excluded. Women who were not on hormonal birth control completed all conditioning sessions during the follicular phase of their menstrual cycle (White et al. 2002). This study was approved by the University of Chicago Biological Science Division Institutional Review Board.

Table 1 Participant demographics and current and lifetime drug use ($N = 20$)

	Percent (N) or mean (SEM)
Gender	
Male/Female	14/6
Race % (N)	
Caucasian	55 (11)
African-American	15 (3)
Asian	20 (4)
Other	10 (2)
Age	19.95 (0.42)
Education	13.70 (0.22)
BMI	23.00 (0.49)
Current drug use	
Servings of caffeine per day	0.83 (0.20)
Cigarettes per week	4.20 (2.00)
Alcohol beverages per week	6.86 (1.59)
Marijuana uses in last 30 days	4.70 (1.57)
Lifetime drug use % of sample (N)	
Marijuana	95.0 (19)
Opiates	25.0 (5)
Stimulants	50.0 (10)
Hallucinogens	45.0 (9)
MDMA	40.0 (8)
Sedatives	25.0 (5)

Drug

Methamphetamine (MA; 20 mg; Desoxyn, Lundbeck) was used because of its reliable subjective effects and quick onset (Martin et al. 1971; Cook et al. 1992). MA tablets were crushed and mixed into 10 ml of equal parts OraSweet and OraPlus syrup (Paddock Laboratories, Minneapolis, MN). The placebo (PBO) consisted of 5 ml of OraSweet and OraPlus alone.

Session procedures

Orientation session During an initial orientation visit, study procedures were explained to participants and informed consent was obtained. Participants practiced the study tasks and questionnaires. To minimize expectancy effects, participants were told that they could be given a placebo, stimulant, sedative, or alcohol. Participants were asked to abstain from recreational drug use for 48 h before any session (7 days for marijuana). They were allowed to consume their normal amounts of caffeine and nicotine before all sessions. Compliance was assessed at each session using breathalyzers (measuring blood-alcohol levels; Alco-SensorIII, Intoximeters, St. Louis, MO), urine drug tests (ToxCup, Branan Medical Corporation, Irvine, CA), and pregnancy tests for women (AimStickPBD, hCG professional, Craig Medical Distribution, Vista, CA). Participants were informed that testing positive for alcohol, drugs, or pregnancy would result in their exclusion from the study. No one tested positive for drug use or pregnancy.

Pre-conditioning session (session 1) In this 1-h session, participants completed three tasks that assessed responses to the to-be-conditioned visual/auditory stimuli (as in Mayo and de Wit 2015). These tasks were (1) a forced choice task measuring behavioral preference, (2) a modified visual-probe task used to track eye gazes via electrooculography (EOG), and (3) a visual analog scale (VAS) that assessed subjective "liking" of the two stimuli. Each stimulus consisted of a visual image of either an ocean or mountain scene, accompanied with appropriate sounds (waves or birds chirping, respectively). The stimuli were presented with E-Prime 2.0 (PST, Pittsburgh, PA), as the background image on the computer screen. Smaller images of the specific tasks were superimposed on the background image.

Conditioning sessions (sessions 2–5) The four conditioning sessions were conducted from 0900 to 1300 hours, 2–10 days apart. Sessions were conducted in comfortably furnished rooms that contained a television, VHS player, and computer. When not performing experimental tasks, participants were allowed to watch selected movies, read, or relax. At each session, participants first completed drug screening and pregnancy tests, pre-drug mood ratings (Profile of Mood States (POMS), Addiction Research Center Inventory (ARCI), and Drug Effects Questionnaire (DEQ)), and physiological measures (blood pressure (BP) and heart rate (HR)). The mood ratings and physiological measures were taken periodically throughout the session (15 min before and 15, 30, 70, 115, and 210 min after drug administration). At 0930 hours, MA or PBO was administered orally under double blind conditions. Subjects received MA and PBO on alternating sessions, and with the order (MA or PBO first) counterbalanced between subjects. Thirty minutes after MA/PBO administration (timed to coincide with peak drug effects, see Mayo and de Wit 2015), participants performed four simple computer tasks, the Balloon Analog Risk Taking (BART) task (Lejuez et al. 2007), a simple Reaction Time (RT) task (Leth-Steensen et al. 2000), the Go/No-go (GNG) task (adapted from Braver et al. 2001), and the Gluck task (Sheynin et al. 2013). These tasks served to maintain subjects' attention on the computer screen, where the conditioning stimuli were presented as the background. One compound stimulus (ocean or mountain image/sound) was consistently present when subjects received MA, while the other was present during PBO sessions. Cue assignments were randomized and drug orders counterbalanced between subjects. The tasks occupied about 1/3 of the screen, with the appropriate conditioning stimulus (ocean or

mountain) clearly visible in the background. The "distractor tasks" were not used as outcome measures. Participants were allowed to leave at 1300 hours if they no longer felt drug effects and their BP/HR measurements had returned to within 20 % of baseline values.

The four tasks presented during stimulus viewing lasted about 38 min, and they were presented in a counterbalanced order using E-Prime 2.0 software. The BART is a measure of risk-taking behavior in which subjects inflate virtual balloons to earn points. The RT task is a simple reaction time task in which participants press a button as quickly as possible when a stimulus appears on the screen. The Go/No-go task is a measure of impulsive action in which subjects respond when given a "go" signal, but inhibit responses when a "no-go" signal is given. The Gluck is a probabilistic classification task in which participants win or lose points as they learn to classify arbitrary stimuli. Subjects received extra money for performing the tasks.

Post-conditioning and extinction sessions (sessions 6–8) Participants attended three 1-h sessions, conducted at the same time of day as session 1, 2–10 days after the last conditioning session. Subjects completed the same three tasks they completed during the pre-test, assessing their responses to the conditioning stimuli: forced choice, visual probe, and VAS. During each of the three post-conditioning visits, subjects completed the tasks twice, in succession. Thus, we obtained two extinction assessments within each session, during the three extinction sessions.

Outcome measures

Behavioral preference (forced choice task) A forced choice task assessed behavioral preference for the two cues. Participants viewed composite images of the background scenes with distractor task images superimposed (e.g., ocean + distractor task 1; mountain + distractor task 1), first individually and then in pairs. In each trial, two composite pictures were first presented individually for 3 s (with audio corresponding to the presented background image), and then the two images were presented side by side (no audio) for the preference test. Participants indicated their preference by pressing the corresponding mouse button (left or right). A total of 28 pairs of background (two types) plus task images (four types) were presented using a full-factorial design and order presentation was randomized.

The primary outcome measure was choice preference for the drug-paired image, which was calculated as the number of trials on which subjects chose the drug-paired background on trials when both backgrounds were present on-screen, minus the number of choices for the PBO-paired backgrounds. This left a total of 16 comparisons of interest. Individual choice behavior was then used to calculate the change in preference for the drug-paired cue across sessions. Preferences for task images were also assessed by analyzing choices between tasks with the same background to rule out preference biases between tasks before and after conditioning.

Attentional bias (modified visual-probe task) Bias in attention towards the two conditioned stimuli was examined using a modified visual-probe task in combination with EOG (Wardle et al. 2012; Mayo and de Wit 2015). The task consisted of 40 trials, each beginning with the presentation of a white fixation cross (1000 ms duration) followed by the simultaneous presentation of the two study cues on the left and right side of the screen (2000 ms). After cue offset, a small brown rectangle appeared behind one of the images with either a white circle or square visual probe. Participants were told to identify the shape (circle or square) as quickly as possible by pressing one of two keyboard keys. After a response (or 10-s timeout without response), a variable inter-trial interval began (750–1250 ms) followed by the next trial. Within each trial, cue type/location (ocean/mountain, left/right), probe type (square/circle), and probe location (left/right) was counterbalanced across trials and each combination was presented randomly. EOG recordings were obtained by first cleaning and exfoliating the skin, followed by placement of 4 mm Ag/AgC1 electrode pairs (filled with electrolyte gel) 1.5 cm from the outer canthus of each eye. For the ground electrode, an 8 mm Ag/AgC1 electrode was attached to the forehead. Impedance values were measured using a Checktrode (Model 1089 MK III, UFI, Morro Bay, CA) and electrodes with impedance values above 20 kΩ were reapplied. EOG signals were amplified, digitized, and sampled at 1000 Hz using an EOG100C amplifier (Biopac MP150 system) and AcqKnowledge software (Biopac, Goleta, CA). Trained raters excluded trials using the following criteria: (1) eye gazes were not centrally fixated prior to the trial, (2) initial eye gazes were <100 ms after cue onset (indicating anticipatory eye movements), and (3) noise obscured direction of eye movements.

Two primary outcome measures from this task were quantified using EOG-based eye tracking: (1) first-gaze proportion, which was calculated as the direction of first gaze for each trial (towards the ocean or mountain image) when the cues appeared, as a fraction of total valid gazes, and (2) mean gaze time spent looking at each cue.

Self-reported liking (VAS) A VAS scale was used to assess subjective, self-reported "liking" for each cue, during the pre-test and twice during each post-test session. In this task, the compound stimuli (background image and sound) were presented individually two times each for 5 s. Participants rated how much they "liked" each image on a scale of 0 ("dislike very much") to 100 ("like very much") by moving a vertical bar on a

horizontal line to indicate their rating. The two ratings of each stimulus were averaged for each post-test session.

Subjective drug effect measures

Subjective drug effects were assessed during conditioning sessions using three measures. The Drug Effects Questionnaire (DEQ; Johanson and Uhlenhuth 1980) measures subjective drug effects and consists of five questions which asked participants to rate drug effects in terms of "like," "feel," "dislike," feeling "high," and "want more" of the drug on a 100-mm visual analog scale. The Profile of Mood States (POMS; McNair et al. 1971) measures subjective mood effects and is a 72-adjective list that asked participants to rate how much an adjective applies to them on a 5-point Likert scale, from 0 ("not at all") to 4 ("extremely"). The primary outcome measures are eight clusters of items (i.e., Anxiety, Depression, Anger, Vigor, Fatigue, Confusion, Friendliness, And Elation). From these eight clusters, two composite scales of positive mood and arousal were calculated. The Addiction Research Center Inventory (ARCI; Chait et al. 1985; Martin et al. 1971) was used to assess general subjective drug effects and is a 52-item true/false scale with two subscales of interest, arousal (A) and euphoria (MBG).

Cardiovascular drug effect measures

HR and BP were monitored during the conditioning sessions at six regular time points (TPs) during the 4-h sessions. Mean arterial pressure (MAP) was calculated using the formula: [systolic BP + 2 × diastolic BP]/3.

Statistical analysis

Subjective and physiological drug effects experienced during the conditioning sessions were analyzed using a repeated measures analysis of variance (rmANOVA) with time (baseline, five TPs after drug administration) and condition (MA, PBO) as within-subject factors. Differences at individual TPs were evaluated using post hoc pairwise comparisons with the Bonferroni correction. Similar rmANOVA tests with time and session (first vs second) were used to assess differences between the two MA sessions and the two PBO sessions. Peak change scores (PCS) were calculated using the maximum change score compared to the baseline TP 1 over the course of the six TPs during conditioning sessions, and were analyzed using a group by treatment by session rmANOVA. Correlations between subjective drug effects and outcome measures were explored by comparing PCS from baseline for MA and PBO sessions (average of two MA sessions, two PBO sessions).

The primary outcome measures were change in response to conditioned cues from before to after conditioning, as well as changes within- and between-post-test sessions for the outcome measures of behavioral preference, attentional bias, and subjective liking (VAS ratings). A paired *t* test was used to analyze changes in behavioral preference (number of MA-paired cue choices before vs after conditioning, both within- and between-post-test sessions). Subjective "liking" and attention (initial and sustained) measures were analyzed using a rmANOVA with phase (pre- and post-conditioning) and cue (MA- and PBO-paired) as within-subject factors. Violation of sphericity was tested using Mauchly's procedure and violations were corrected using the Greenhouse-Geisser adjustment.

Results

Subject characteristics

The demographic and drug use characteristics of the participants are summarized in Table 1. Most participants were male, Caucasian, and about 20 years of age, with 13 years of education. Most of the participants had used marijuana and some other drugs in their lifetime. Half reported any lifetime use of stimulant drugs but none reported prior use of methamphetamine (not shown).

Subjective and cardiovascular effects of methamphetamine

Methamphetamine (MA; 20 mg, oral) produced robust subjective effects (mood, ratings of drug effects) and physiological changes (HR, blood pressure), consistent with prior studies (Mayo et al. 2013; Mayo and de Wit 2015) (Table 2). Effects of MA did not differ from the first to the second administration for any subjective and physiological measures. Therefore, we averaged the two MA and two PBO sessions for all further comparisons. All post hoc analyses used the Bonferroni correction.

Cardiovascular measures MA increased HR and mean arterial blood pressure (MAP) from TPs 2 or 3 until the end of the session (see Supplemental Fig. 1) ($N = 20$). MA increased HR over time compared to PBO [rmANOVA treatment × time interaction, $F_{(2.82,\ 53.64)} = 15.44$, $p < 0.001$, corrected for sphericity]. MA also increased MAP over time compared to PBO [rmANOVA treatment × time interaction, $F_{(5,\ 95)} = 13.13$, $p < 0.001$]. Analysis of peak change scores (PCS) between treatments using paired *t* tests yielded similar findings (Table 2).

Subjective measures MA increased measures of stimulation and decreased fatigue, peaking at TP 4. MA increased scores on ARCI A and MBG scales [treatment × time interactions

Table 2 Mean (SEM) scores for subjective ratings and cardiovascular measures averaged across the conditioning sessions with placebo or methamphetamine

	Placebo Mean (SEM)	MA Mean (SEM)	T value
Subjective Effect			
DEQ ($N=19$)			
Feel	18.35 (4.11)	49.24 (4.60)	6.37***
Like	17.98 (4.18)	71.36 (4.56)	11.47***
Dislike	31.59 (6.91)	25.73 (5.61)	−0.87
High	10.48 (2.79)	40.62 (5.27)	6.54***
More	19.42 (5.60)	61.48 (6.40)	8.20***
POMS ($N=20$)			
Friendliness	−3.10 (0.72)	2.75 (0.86)	5.21***
Anxiety	0.08 (0.45)	1.53 (0.78)	1.92
Elation	−2.50 (0.77)	3.18 (0.84)	4.86***
Anger	0.15 (0.51)	0.33 (0.36)	1.5
Fatigue	0.18 (1.03)	−0.73 (0.78)	1.02
Depression	0.30 (0.47)	0.33 (0.54)	0.04
Confusion	0.63 (0.49)	0.13 (0.61)	0.99
Vigor	−3.48 (1.19)	4.43 (1.35)	4.04**
ARCI ($N=20$)			
A	0.15 (0.40)	3.83 (0.62)	5.12***
MBG	−0.83 (0.6)	5.55 (0.92)	6.01***
Cardiovascular ($N=20$)			
Blood pressure	−4.58 (2.09)	15.03 (2.43)	6.20***
Heart rate	−6.25 (2.71)	18.28 (3.35)	7.21***

Blood pressure is represented as mean arterial pressure

DEQ Drug Effects Questionnaire, *POMS* Profile of Mood States, *ARCI* Addiction Research Center Inventory

**$p<0.01$; ***$p<0.0001$

$F_{(5,\ 95)}=17.03$, $p<0.001$; $F_{(3.14,\ 59.65)}=20.98$, $p<0.001$; corrected for sphericity] (see Supplemental Fig. 2) at TPs 3–6 (all p's ≤0.003). For the POMS, MA increased scores on Friendliness [treatment × time interaction, $F_{(3.05,\ 54.82)}=10.73$, $p<0.001$], Elation [treatment × time interaction, $F_{(2.74,\ 49.36)}=12.63$, $p<0.001$], and Vigor [treatment × time interaction, $F_{(2.09,\ 37.58)}=13.30$, $p<0.001$] and decreased Fatigue [treatment × time interaction, $F_{(2.65,\ 47.77)}=3.20$, $p=0.037$] (see Supplemental Fig. 3) at TPs 3–6 (all p's ≤0.031). PCS analyses for both the ARCI and POMS found similar differences between MA and PBO treatments (Table 2).

For the DEQ, MA increased ratings on "feel", "like", "high", and wanting "more" drug, (see Supplemental Fig. 4) at TPs 3–6. Data from two participants were excluded because of missing values. MA effects did not differ across the two sessions so MA and PBO sessions were averaged for further analyses.

MA increased ratings of "feel", "like", feeling "high", and wanting "more" drug, compared to PBO [treatment × time interactions $F_{(3.20,\ 57.66)}=13.673$, $p<0.001$; $F_{(5,\ 90)}=22.10$, $p<0.001$; $F_{(2.32,\ 41.76)}=11.55$, $p<0.001$; $F_{(3.37,\ 60.73)}=18.84$, $p<0.001$, corrected for sphericity, except "like."], at TPs 3–6 (all p's ≤0.033). PCS analyses for all DEQ measures found similar differences between MA and PBO treatments (Table 2).

Conditioned response measures

Responses to cues were assessed before conditioning (session 1) and twice during each of the three extinction sessions (sessions 6–8). Because no differences in outcome measures were found between the within-session extinction presentations, the values from the two tests within each extinction session were averaged. All data were tested for violation of normality using the Shapiro-Wilk test. In cases where normality was violated, Wilcoxon signed-ranks tests were used instead of paired t tests. One outlier was removed from data analysis. Responses were unrelated to current and prior drug use (Table 1).

1. Behavioral preference. Behavioral preference choice scores for the MA-paired cue increased from before (session 1) to the first session after conditioning (session 6) [$t_{(18)}=2.67$, $p=0.016$] (Fig. 1, Table 3). This increase in preference was no longer evident during sessions 7 or 8 (vs session 1). Possible task bias was assessed using a one-way ANOVA, before and after conditioning, by analyzing task choices when the background cue was the same. These analyses found no systematic differences in choice behavior towards any of the tasks.
2. Attentional bias. After conditioning, there was an increase in initial gaze towards the MA-associated cue on session 6, but this was no longer evident during sessions 7 or 8 (Fig. 2). The proportion of first gaze towards the MA cue increased from before (session 1) to after (session 6) conditioning [$t_{(18)}=2.69$, $p=0.015$], but returned to near baseline levels during sessions 7 and 8 (vs session 1). Before conditioning (session 1), mean initial gaze scores were not different from 0.5 (neutral indifference point) ($p=0.971$), but after conditioning (session 6), values were significantly different from 0.5 [$t(18)=2.92$, $p=0.009$]. Mean gaze time (i.e., sustained attention) did not change after conditioning (Table 3).
3. Self-reported liking. Ratings of MA cue "liking" did not increase after conditioning. Indeed, ratings of both the MA and PBO cues declined following conditioning, an effect that persisted through sessions 7 and 8 (vs session 1) (Table 3). Wilcoxon signed-ranks tests indicated that ratings of MA and PBO cues showed significant decreases from before to after conditioning (Z's = 3.68,

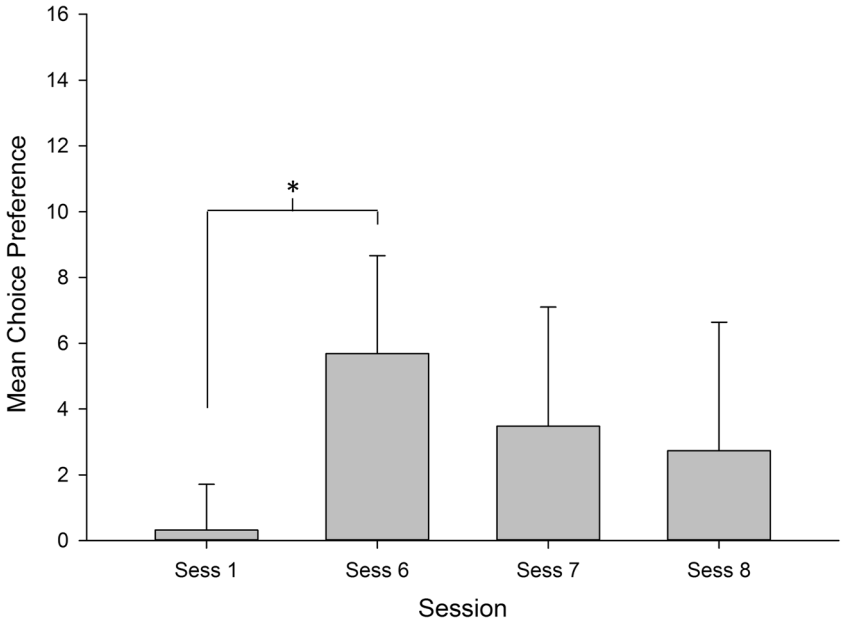

Fig. 1 Behavioral preference scores towards methamphetamine (MA)-paired stimuli before (session 1) and after conditioning at three extinction tests (sessions 6–8). Choice preference (mean ± SEM) for the MA-paired cue (MA choices minus placebo, PBO, choices) increased from before (session 1) to after conditioning at the first post-test extinction session (session 6). This increased preference attenuated over the next two extinction sessions (sessions 7 and 8), and were not significantly elevated compared to baseline pre-test values. *p values ≤0.016

3.68, p's <0.001), and at sessions 7 (Z's = 3.72, 3.72, p's <0.001) and 8 (Z's = 3.64, 3.42 and p's ≤ 0.001).

4. Relationships among outcome measures. We examined relationships between our three outcome measures, before and after conditioning (i.e., during the acquisition phase) (N = 19). Increases in initial attentional bias towards the MA-paired cue (i.e. proportional first gazes) from sessions 1 to 6 were significantly negatively correlated with changes in sustained attentional bias (mean gaze time towards the MA-cue minus the change towards the PBO-paired cue) (Pearson's $r = -0.502$, $p = 0.028$). That is, individuals with greater changes in first gaze proportions towards the MA-paired cue showed the smallest changes in mean gaze time bias towards the drug cue, suggesting

Table 3 Outcome measure scores for methamphetamine (MA)-, or placebo (PBO)-paired stimuli across four test sessions, before (session 1) and after (sessions 6–8) conditioning

	Session 1 Mean (SEM)	Session 6 Mean (SEM)	Session 7 Mean (SEM)	Session 8 Mean (SEM)	Sig.
Outcome measure					
Behavioral preference (N = 19)	0.32 (1.40)	2.84 (1.49)	1.74 (1.81)	1.37 (1.95)	S1vs6*
Attention (N = 19)					
Initial MA first-gaze	0.50 (0.03)	0.55 (0.02)	0.52 (0.02)	0.51 (0.01)	S1vs6*
MA gaze time, in ms	607.84 (49.73)	603.17 (61.25)	600.24 (47.32)	618.66 (67.46)	
PBO gaze time, in ms	521.93 (61.30)	558.46 (61.92)	615.25 (70.35)	634.05 (80.17)	
Subjective rating (N = 19)					
MA cue	86.80 (2.7)	76.78 (3.21)	72.64 (4.06)	73.22 (4.07)	
[Median]	[89.50]	[75.50]	[69.75]	[75.25]	S1vs6***
					S1vs7***
					S1vs8***
PBO cue	85.08 (3.27)	70.24 (4.05)	69.87 (4.36)	70.01 (3.72)	S1vs6***
[Median]	[87.50]	[72.75]	[71.50]	[69.50]	S1vs7***
					S1vs8***

Behavioral preference is given by MA cue choices minus PBO cue choices in forced choice task. Initial MA first gaze is given as a proportion of first gazes towards the MA image divided by total number of valid gazes. MA and PBO gaze time are listed as average gaze time per stimulus. Subjective ratings scores are from a 100-mm VAS. For subjective rating scores, non-normal distributions necessitated the use of non-parametric tests, which compared median values across sessions

*$p \leq 0.016$; ***$p \leq 0.001$

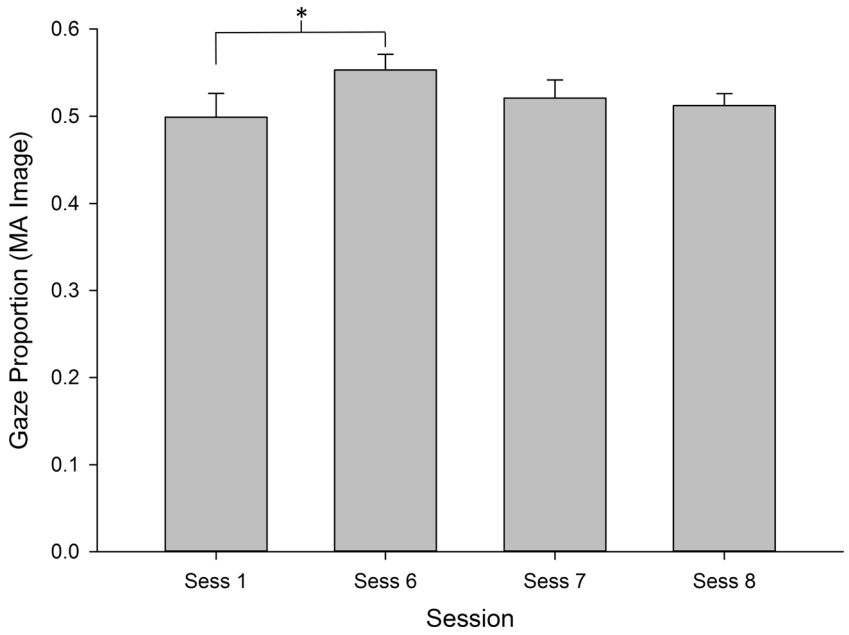

Fig. 2 Proportion of first-gazes towards the MA-paired image before (session 1) and after conditioning at three extinction tests (sessions 6–8). First-gaze proportions (mean ± SEM) for the MA-associated cue (index of initial attention) increased from pre- (session 1) to post-conditioning (first post-test extinction session, session 6). The greater proportion of MA first-gazes declined thereafter, during sessions 7 and 8, to levels not significantly different than pre-test values. *p values ≤0.016

that these attention measures are inversely related. No significant correlations were observed for the changes in behavioral choice (i.e., forced choice task).

5. Relationship between subjective drug response and conditioned responses. We examined individual differences in subjective responses to MA, using the DEQ (average PCS), in relation to the measures of conditioning. No significant relationships were identified between subjective drug effects and any of the outcome measures during the acquisition phase (i.e., sessions 1 to 6). Later at the extinction phase (i.e., sessions 6 to 7), greater feel drug scores were related to less extinction of behavior preference scores, and were significantly positively correlated with changes in choice behavior scores from sessions 6 to 7 ($r = 0.715$, $p = 0.009$) (Fig. 3). No other significant relationships were identified. Extinction analyses were conducted only using subjects who displayed conditioning effects, i.e., showed increases in outcome measures from sessions 1 to 6. One additional subject was excluded due to not following instructions on the questionnaire ($N = 12$).

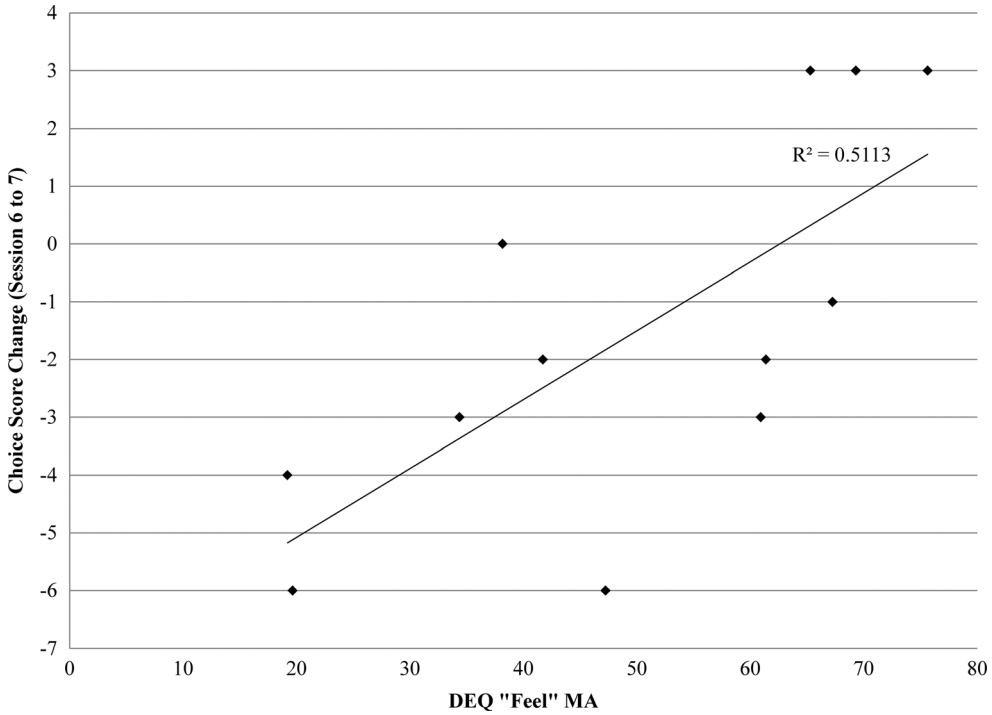

Fig. 3 Correlation between DEQ "feel" scores and changes in behavioral preference (choice scores) during the extinction phase. Average peak change scores (PCS) for the DEQ "feel" category, during the MA sessions, were significantly positively correlated with changes in forced choice behavior scores from session 6 to 7 (Pearson's $r = 0.715$, $p = 0.009$). Positive changes indicate increases in choices for the MA cue from session 6 to 7, while negative changes indicate a reduction in MA-cue choices, i.e., extinction. Note that choice behavior scores were only included for individuals that displayed a conditioned response after conditioning, e.g., increased choice behavior from session 1 to 6 ($N = 12$)

6. Relationship between inter-session interval and conditioned responses. For the acquisition phase, no systematic relationships were found between conditioned responses and mean inter-session intervals (ISIs, in days) for conditioning sessions (overall mean ± SEM, 5.62 ± 0.31). Differences in conditioned responses were also unrelated to mean session intervals between the last day of conditioning and first day of extinction (i.e., first post-test; 3.05 ± 0.30). During the extinction phase, shorter mean ISIs for the three sessions were associated with greater extinction of choice behavior (Fig. 4). Mean extinction ISIs for all extinction sessions (1.68 ± 0.20) were significantly positively correlated with choice behavior score changes from sessions 6 to 7 (Spearman's rho = 0.798, $p = 0.001$). Similar positive correlations were observed when comparing mean extinction ISIs for all extinction sessions with choice behavior score changes from sessions 6 to 8 (Spearman's rho = 0.781, $p = 0.002$) (data not shown). Additional analyses found that mean ISIs for extinction sessions 6 to 7 were positively correlated with choice behavior score changes from sessions 6 to 7 (Spearman's rho = 0.823, $p = 0.001$), and that sessions 7 to 8 ISIs were positively correlated with score changes from sessions 6 to 7 (Spearman's rho = 0.601, $p = 0.03$) (data not shown). Note that these analyses were only conducted for participants who showed acquisition of the conditioned response (i.e., increased choice behavior from sessions 1 to 6, $N = 13$).

Discussion

The purpose of this study was to extend our previous demonstration of the acquisition of de novo drug cue associations in healthy adults (Mayo et al. 2013; Mayo and de Wit 2015), to characterize the extinction of the conditioned drug cue responses over three extinction sessions. Two pairings of methamphetamine (MA) and neutral visual/auditory stimuli produced increases in behavioral preference and initial attentional bias towards the drug-associated cue, but these responses attenuated rapidly and were not observable by the second of three post-conditioning test sessions. Counter to our prediction, we did not find any relationship between subjective drug effects (e.g., drug "liking") and conditioned response magnitude for behavioral preference or attentional bias. Interestingly, greater subjective drug effects (e.g., "feel" drug) were related to smaller declines in the conditioned behavioral preference during the extinction phase. This suggests that the conditioned responses are more persistent in those who experience stronger drug effects. Finally, we found that longer inter-session intervals (ISIs) during the extinction phase were correlated with less extinction of the behavioral preference measure, suggesting that intermittent, or spaced, presentations of conditioned stimuli may contribute to persistence of drug-seeking behavior. Together, these results indicate that novel, non-drug-related stimuli can acquire conditioned properties after being presented during a drug-taking experience. Further, these conditioned drug cue responses diminish with repeated presentations of the drug-associated cues in the

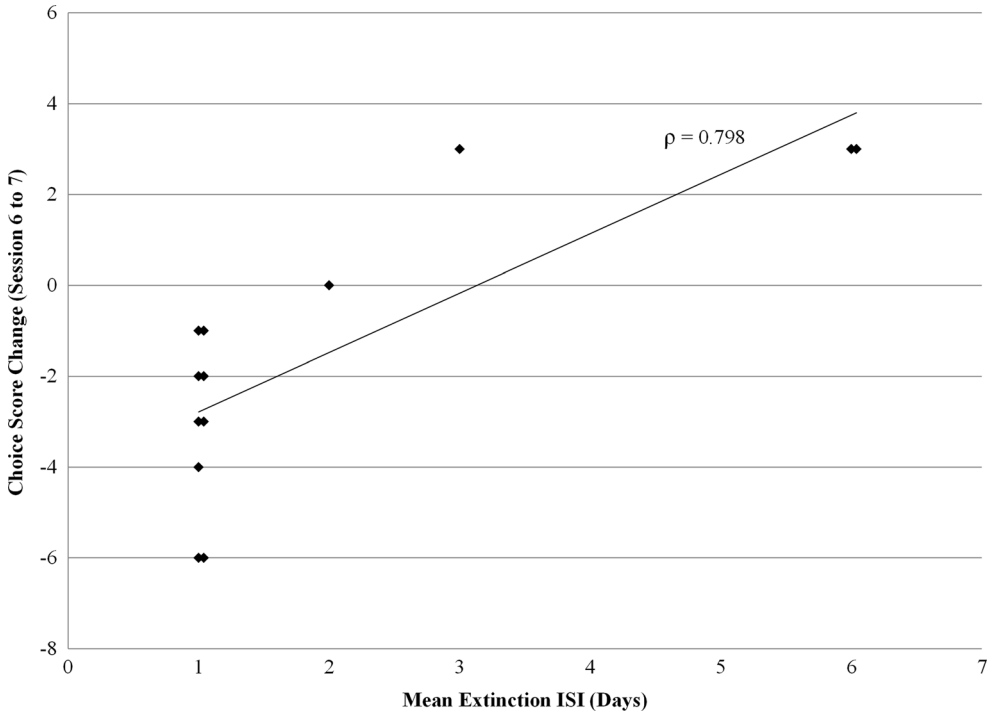

Fig. 4 Correlation between extinction inter-session interval (ISI) values and changes in behavioral preference choice scores during the extinction phase. Mean ISI values for the three extinction sessions were significantly positively correlated with changes in forced choice behavior scores from sessions 6 to 7 (Spearman's rho = 0.798, $p = 0.001$). Positive changes in choice behavior indicate greater choices for the MA-cue from sessions 6 to 7, while negative changes indicate fewer MA cue choices, i.e., extinction. Note that analysis was only conducted for individuals that showed increased choice behavior from session 1 to 6 ($N = 13$). Note that *double diamond symbols* indicate overlapping data points

absence of drug, both as a function of subjective drug response and the timing between extinction sessions.

To our knowledge, this study is the first to demonstrate apparent extinction of conditioned behavioral preference for de novo drug-paired cues, and to show extinction of attentional bias towards novel conditioned drug cues in healthy young adults. Understanding how these conditioned drug cue responses are extinguished could facilitate new avenues of treatment. Both behavioral preference (O'Brien et al. 1992; Bozarth 1987) and attentional bias (Garland et al. 2012; Field and Eastwood 2005; Waters et al. 2003a, b; see Field and Cox 2008; Robbins and Ehrman 2004 for reviews) are indices of conditioned appetitive responses that are thought to drive continued drug use. Despite their putative importance, this is the first study to measure the extinction of behavioral preference towards conditioned drug cues, and few studies have examined extinction of attentional bias. Kamboj et al. (2011) found that in heavy alcohol drinkers, conditioned attentional bias towards alcohol-related cues was attenuated after two extinction sessions, compared to only one session in our study. It is difficult to compare our results with those reported in Kamboj et al. (2011) because they studied extinction using individualized, in vivo cue exposure (e.g., handling and smelling drink of choice) in established drug users. It is similarly difficult to compare our results to the broad extinction literature because most of these studies employed naturalistic drug-related stimuli (e.g., drug paraphernalia, favorite alcoholic beverage, drug images, etc.) in drug-dependent populations, and used outcome measures (e.g., craving, Price et al. 2010) that are not easily assessed in healthy adults.

An interesting finding in this study was that shorter extinction ISIs were correlated with greater reductions of conditioned behavioral preference measure, suggesting that massed extinction trials reduced the conditioned drug cue response more effectively than spaced trials. Although these results should be viewed with caution because of the small sample ($N = 13$), they are generally consistent with animal research on conditioned fear, showing that massed extinction training using short ISIs produces greater conditioned response decrements (Cain et al. 2003; Oler and Baum 1968). In the animal literature, short ISIs (i.e., massed extinction) typically occur on the scale of minutes, while long ISIs (i.e., spaced training) proceed over intervals of hours to days (Cain et al. 2003; Li and Westbrook 2008). Therefore, the "short" and "long" ISI intervals reported here may not be analogous to massed and spaced extinction, respectively. Regardless of these definitional differences, the literature concerning massed vs spaced extinction is mixed (see Li and Westbrook 2008; Rescorla and Durlach 1987; Westbrook, et al. 1985). For example, a human study examining extinction of cue-induced drug craving in MA-dependent individuals (Price et al. 2010) found that short extinction ISIs (e.g., ≤4 days) produced slightly less extinction than longer ISI (e.g., ≥4 days). Although it is tempting to attribute these differences to the state of drug dependence of the participants, it is difficult to compare the Price et al. (2010) results to the present study because of differences in study parameters, subject sample, and outcome measures. Regardless, the presence of this relationship helps to bolster our interpretation that the observed reduction in conditioned responses is due to extinction rather than general decrements produced by processes unrelated to associative learning, e.g., habituation.

A second notable finding was that greater reports of "feeling" the drug effect during the acquisition phase were correlated with a smaller decline in behavioral preference, possibly indicating a greater resistance to extinction. Because resistance to extinction is often taken to represent the associative strength between cue and reward (Annau and Kamin 1961), the present findings suggest that those individuals who experienced more robust drug effects also developed stronger drug cue learning. To our knowledge, these findings have not been shown before in healthy adults. However, evidence from the animal literature, using conditioned place preference as a proxy for the rewarding effects of drugs of abuse, indicates that higher doses of opiates are more resistant to extinction (Rutten et al. 2011). It is not clear why the magnitude of subjective drug effects in the current study was related to resistance to extinction but not to the strength of the conditioned response. Future parametric studies, with larger samples, may reveal the relationships between magnitude of the unconditioned stimulus (the drug effect in this case) and the magnitude of the acquired conditioned response, relative to the persistence of the conditioned response during extinction conditions.

The present results can be considered relative to previous findings from our laboratory (Mayo et al. 2013; Mayo and de Wit 2015). Despite some minor methodological differences in the present study (e.g., lower compensation, different distractor tasks during conditioning, and more choices during the preference task), conditioning readily occurred to the neutral background cues, and subjects developed both behavioral preference (Mayo et al. 2013) and attentional bias towards the MA-paired stimulus (Mayo and de Wit 2015), although not with subjective evaluation of conditioned drug cues (Mayo and de Wit 2015). In this study, participants rated their liking of both the MA- and PBO-paired cues as lower after conditioning, compared to before conditioning. This decline in "liking" was observed previously both in our laboratory (Mayo et al. 2013; Mayo and de Wit 2015) and in a study with conditioning in cigarette smokers, in which cues increased emotional activity, but not "liking" of the cues (Winkler et al. 2011). One possible explanation is that the images were initially positive but repeated exposure reduced their attractiveness due to boredom or habituation. It is interesting that choice behavior and attentional bias increased with conditioning despite the decline in "liking." This suggests

simple subjective ratings of "liking" of an image may play a secondary role to more implicit conditioned responses such as attentional bias. Quantitatively, our results were very similar to, and even more robust than, the results observed in Mayo and de Wit (2015), who found that 55.5 and 64.7 % of participants exhibited conditioned responses for the choice and attentional bias measures, respectively, compared to the present findings of 68.4 % (13/19 subjects) and 78.9 % (15/19) for choice and attentional bias measures, respectively. In contrast with our study, Mayo and de Wit (2015) observed conditioned increases in sustained attentional bias (measured using mean gaze time), whereas in our study we only detected a change in initial attention (first gazes) towards the MA-paired cue. Although initial gaze and sustained attention may measure different underlying processes (see LaBerge 1995; Robbins and Ehrman 2004 for discussion), the reasons for the differential findings are unclear. The younger age of our participants (mean age 19.95 vs 24.6 years reported in Mayo and de Wit 2015) might have been a factor, as brain areas involved in regulating attention develop late in adolescence (Fuster 2002), and reward processing is also known to change during development (Fareri et al. 2008). Because adolescents are also sensitive to disturbances in attention (Yurgelun-Todd 2007), these factors make it possible that the ~5-year age difference between our studies might have contributed to the attentional bias findings.

This conditioning procedure has both advantages and disadvantages as a model for studying extinction of drug-related responses. First, it is noteworthy that not all participants exhibited conditioned responses towards the drug-paired cue. Conditioning did not appear to be related to demographic characteristics of the participants or to their responses to the drug. It is possible that this variation across individuals reveals inter-subject variability in susceptibility to conditioning. It is also possible that some individuals would develop conditioned responses only with higher doses of a drug, or with more pairings. Alternatively, it is possible that these participants also formed associations, but that we did not detect them with our dependent measures. The existence of inter-individual variability provides a unique opportunity to explore what factors confer resistance to the rewarding properties of drugs of abuse, or conversely, which factors predispose individuals to greater drug cue reactivity, such as impulsivity (Field and Cox 2008). This will be a goal of future analyses, as we test sufficient numbers of subjects to draw conclusions.

Another interesting observation in this study is that the conditioned responses assessed with different outcome measures are not highly correlated. That is, individuals differ in the extent to which they exhibit conditioned responses, but these vary with the outcome measures. This raises several possibilities. First, some individuals may acquire conditioning more readily with different aspects of the pairings (e.g., attention to the stimuli, "liking" of the stimuli). Second, individuals may acquire the underlying association in the same way, but express this differentially, with different dependent measures. In either case, the observation that the conditioned responses are not unitary highlights the complex and multifaceted nature of conditioned drug cue responses. We speculate that implicit behavioral choice and/or attentional bias towards the drug-paired cue might contribute to drug-seeking behaviors, but the precise mechanism of these processes, and their individual contribution, remain to be explored.

The use of healthy, non-dependent participants removes the possible confound of long, unknown histories of drug use and drug conditioning. For instance, although some of the participants reported some lifetime use of stimulant drugs, none reported prior use of methamphetamine. This feature permits the use of rigorous Pavlovian conditioning procedures such as those used in animal studies. Furthermore, the procedure provides good control over the processes of acquisition and extinction, which helps with cross species comparisons, and with investigating the basic learning processes underlying conditioned drug cues. On the other hand, the conditioned responses extinguished rapidly, making it difficult to develop interventions that would slow the extinction process. Although it is notable that acquisition occurred after only two pairings, with a low dose of the drug, administered by a route that has a slow onset of effect, it would be useful to develop a procedure in which extinction responding persisted for a longer period. The extinction of naturalistic drug cues in populations with a history of drug dependence proceeds gradually, and is sometimes incomplete by the end of extinction training (O'Brien et al. 1990; Price et al. 2010; Foltin and Haney 2000; MacKillop and Lisman 2008; Collins and Brandon 2002; Kamboj et al. 2011). Thus, a laboratory procedure with robust conditioning, and more persistent cue-induced responses, might enable researchers to test novel pharmacological or behavioral treatments that might speed the extinction process. We continue to explore ways to refine this procedure, within the limits of ethical and practical constraints related to human subjects testing.

There were several limitations to this study. First, we used healthy, non-dependent adults with only moderate drug experience to avoid confounds of extended drug use history. If we develop a successful method for studying extinction, it is not certain that extinction would proceed similarly in established drug users after years of drug use. The procedure described here examines conditioned responses after only a handful of pairings of drug and stimuli, whereas experienced drug users are exposed to hundreds or thousands of pairings, so that their conditioned responses are likely to be far more robust. This aspect of the procedure may limit its clinical relevance. Yet, investigating the early acquisition of stimulus-drug associations in healthy individuals is important for at least two reasons. First, studying early acquisition of de novo-conditioned associations may help to identify individuals who condition most rapidly, and thus may be at higher risk for developing

dependence with repeated pairings. Second, this type of study with human volunteers provides an important bridge to preclinical studies with Pavlovian drug conditioning in animal models, and thus is a critical link in generalizing across species. Another limitation stems from a methodological problem with the attentional measure: Some participants failed to look at either neutral image during the pre-conditioning test, making their results unusable. By limiting our analysis to individuals who showed some attention towards the neutral stimuli at baseline, we may have biased our subject selection. A third limitation concerns the narrow conditions that are of necessity part of any laboratory model. To characterize the temporal kinetics of extinction, we repeatedly assessed the conditioned responses using the tasks with each cue presentation. It is possible that repeated exposure to the tasks influenced our measure of extinction, and so a future variation might be to expose subjects to the cues without the outcome measures. Another issue is that the simple learning paradigm modeled here may not replicate the naturalistic drug-taking situation, in terms of the environmental stimuli and the drug itself. In the natural situation, drug use occurs in complex contexts comprised of numerous multimodal cues, any of which might acquire conditioned value. Our use of a single discrete audio-visual cue might not reflect the learning between drugs and cues that operate in a typical drug use scenario. Furthermore, MA is typically abused via inhalation or intravenous routes, which produce faster peak plasma drug concentrations, more robust subjective effects, and greater abuse potential (Rawson 2013). Therefore, the present use of oral MA, with much slower absorption kinetics, did not duplicate the potential rewarding properties of the drug and thus not fully replicate the conditioning environment that occurs in naturalistic settings. Finally, although our results are consistent with conditioned extinction, future experiments should test for the presence of extinction by investigating other associated phenomena, including the re-emergence of extinguished conditioned responses after additional drug administration sessions (e.g., reinstatement).

The present results are preliminary in that they report data from a modest sample size ($N = 20$), and of these, only eight showed a clear decrease in choice behavior during extinction. However, our findings further validate the utility of this associative learning model developed by Mayo et al. (2013) as a reliable method to study the acquisition and reduction of novel conditioned drug cue responses in healthy, non-dependent adults, findings that are consistent with extinction. We have reported that a single extinction session of repeated drug cue presentations without drug abolished behavioral preference and attentional bias towards conditioned drug cues that developed after two pairings of novel cues and MA administration. The results add to the growing body of literature showing that environmental cues paired with drugs of abuse acquire appetitive properties and can be attenuated using extinction training. These findings are an additional step towards understanding the processes that reduce conditioned drug cue responses, and will hopefully yield new methods to accelerate extinction in drug-dependent populations and make the benefits of their behavioral impact last.

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Compliance with ethical Standards

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Disclosure The authors declare no conflict of interest.

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