

Effect of yohimbine on reinstatement of operant responding in rats is dependent on cue contingency but not food reward history

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ABSTRACT

Yohimbine is an alpha-2 adrenoceptor antagonist that has been used in numerous studies as a pharmacological stressor in rodents, monkeys and humans. Recently, yohimbine has become the most common stress manipulation in studies on reinstatement of drug and food seeking. However, the wide range of conditions under which yohimbine promotes reward seeking is significantly greater than that of stressors like intermittent footshock. Here, we addressed two fundamental questions regarding yohimbine's effect on reinstatement of reward seeking: (1) whether the drug's effect on operant responding is dependent on previous reward history or cue contingency, and (2) whether yohimbine is aversive or rewarding under conditions typically used in reinstatement studies. We also used *in vivo* microdialysis to determine yohimbine's effect on dopamine levels in nucleus accumbens (NAc) and medial prefrontal cortex (mPFC). We found that the magnitude of yohimbine-induced (0.5, 1.0, 2.0 mg/kg) operant responding during the reinstatement tests was critically dependent on the contingency between lever pressing and discrete tone-light cue delivery but not the previous history with food reward during training. We also found that yohimbine (2 mg/kg) did not cause conditioned place aversion. Finally, we found that yohimbine modestly increased dopamine levels in mPFC but not NAc. Results suggest that yohimbine's effects on operant responding in reinstatement studies are likely independent of the history of contingent self-administration of food or drug rewards and may not be related to the commonly assumed stress-like effects of yohimbine.

Keywords: Conditioned place preference/aversion, dopamine, reinstatement, stress, sensory seeking, yohimbine.

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INTRODUCTION

Yohimbine is an alpha-2 adrenoceptor antagonist that increases brain noradrenaline cell firing and release (Aghajanian & VanderMaelen 1982; Abercrombie, Keller & Zigmond 1988). In psychiatric research, yohimbine is the most commonly used pharmacological stressor because it induces anxiety- and stress-like responses in humans, monkeys, dogs and rodents (Bremner *et al.* 1996a,b). Based on this literature, and the finding that alpha-2 adrenoceptor agonists (which decrease noradrenaline cell firing and release) block intermittent footshock stress-induced reinstatement of drug seeking (Erb *et al.* 2000; Shaham, Erb & Stewart 2000a), a decade ago, three laboratories determined yohimbine's

effect on reinstatement of drug seeking (Lee *et al.* 2004; Shepard *et al.* 2004; Le *et al.* 2005). Lee *et al.* found that yohimbine reinstates cocaine seeking in monkeys, an effect associated with increased plasma levels of the stress hormone cortisol and species-typical stress-related behaviors. Shepard *et al.* and Le *et al.* found that yohimbine reliably reinstates methamphetamine and alcohol seeking in rats, mimicking the effect of intermittent footshock stress on reinstatement.

Subsequent studies provided additional evidence to support the notion that, like intermittent footshock, yohimbine induces a stress-like state that leads to reinstatement of drug seeking. Both intermittent footshock- and yohimbine-induced reinstatement are critically dependent on activation of extrahypothalamic

corticotropin-releasing factor (CRF; Hansson *et al.* 2006; Marinelli *et al.* 2007; Shalev, Erb & Shaham 2010; Le *et al.* 2013). Both intermittent footshock and yohimbine increase resistance to extinction of drug seeking when administered prior to the daily extinction sessions (Highfield *et al.* 2000; Kupferschmidt, Tribe & Erb 2009). Both intermittent footshock and yohimbine increase levels of the stress hormone corticosterone; yohimbine also induces stress-like responses in the rat social interaction test that are reversed by a CRF1 receptor antagonist (Shaham *et al.* 1997; Ghitza *et al.* 2006; Marinelli *et al.* 2007). Based on these findings, and the findings that the effect of yohimbine on reinstatement is more robust and less variable than that of intermittent footshock (Shepard *et al.* 2004; Le *et al.* 2005), yohimbine has recently become the most commonly used stressor in studies on reinstatement of drug or food seeking (See & Waters 2010; Calu *et al.* 2014; Mantsch *et al.* 2014).

However, with the increasing use of yohimbine in reinstatement studies, evidence emerges for notable behavioral and neurobiological differences between the effects of intermittent footshock and yohimbine on reward seeking. Unlike footshock (Ahmed & Koob 1997; Mantsch & Goeders 1999), yohimbine potently reinstates food seeking and also increases alcohol and food self-administration (Le *et al.* 2005; Marinelli *et al.* 2007; Cifani *et al.* 2012; Ayanwuyi *et al.* 2013; Noori, Helinski & Spanagel 2014). Unlike intermittent footshock, yohimbine does not induce stress-related 22 kHz ultrasonic distress vocalizations (Mahler *et al.* 2013). Unlike intermittent footshock (Shaham *et al.* 2000b), yohimbine's effect on reinstatement of food and cocaine seeking is not blocked by the alpha-2 adrenoceptor agonist clonidine (Brown *et al.* 2009; Nair *et al.* 2009). These behavioral and neuropharmacological differences between yohimbine and intermittent footshock raise the possibility that yohimbine's effect on reinstatement of reward seeking might not be due to its ability to induce a stress-like state that motivates drug or food seeking.

In the present study, we addressed two fundamental questions regarding yohimbine's effect on reinstatement of reward seeking: (1) whether yohimbine's effect on operant responding is dependent on previous reward history or cue contingency, and (2) whether yohimbine (at a dose typically used in reinstatement studies) induces an aversive state, as assessed by a conditioned place preference/aversion procedure (Tzschentke 1998). We also used *in vivo* microdialysis to determine yohimbine's effect on dopamine levels in nucleus accumbens (NAc) and medial prefrontal cortex (mPFC), brain areas important for food and drug reward (Wise 2004) and reinstatement of drug and food seeking (Bossert *et al.* 2013; Calu *et al.* 2014).

MATERIALS AND METHODS

See Supporting Information Appendix S1 for description of subjects and apparatus, drugs, training and extinction phase, reinstatement testing, *in vivo* microdialysis surgery, and microdialysis procedure.

Specific experiments

Experiment 1: effect of reward history and cue contingency on pellet priming and yohimbine-induced reinstatement of lever responding

In experiment 1, we determined the effect of previous food pellet history and cue contingency during food self-administration training on yohimbine-induced reinstatement of lever pressing after extinction. For comparison purposes, we also determined whether the different training histories affect reinstatement of lever responding induced by pellet priming, a manipulation known to selectively reinstate food seeking (de Wit 1996; Nair *et al.* 2009). We used four groups of rats ($n = 8-12$ per group) that during the training phase were exposed to the following conditions: (1) lever response-contingent pellet + cue, (2) non-contingent pellet + cue (yoked to group 1), (3) lever response-contingent cue-only and (4) non-contingent cue-only (yoked to group 3). In order to isolate the impact of lever response contingency during training on subsequent lever presses during the reinstatement tests, we installed in the self-administration chambers one retractable lever instead of both 'active' and 'inactive' levers, as we and others commonly use in reinstatement studies (Shalev, Grimm & Shaham 2002; Nair *et al.* 2009).

During the tests for pellet priming-induced reinstatement, we first gave the rats a 30 minute acclimation period in the operant chamber, after which we delivered 0 (no pellet), 1, 2 or 4 non-contingent pellets (spaced 20 seconds apart) within 1 minute after the start of the sessions. During the tests for yohimbine-induced reinstatement, we injected the rats with water (vehicle) or yohimbine (0.5, 1.0 and 2.0 mg/kg) 30 minutes before the start of the test sessions. We performed the test sessions for pellets and yohimbine during a consecutive 5-day period, with one regular extinction session between test days 2 and 3, in which we delivered no pellets or gave sham injections of water (vehicle). We included the regular extinction session in the middle of testing to ensure that baseline lever pressing is not affected by the repeated testing procedure.

Note that from a drug/food reinstatement literature terminology perspective, during testing, only the behavior of the rats in group 1 but not groups 2-4 (which did not undergo formal operant training and extinction training) can be termed reinstatement of reward seeking (Shaham *et al.* 2003). However, for the sake of consist-

ency with the reinstatement literature we refer to the different phases of the experiment as training, extinction, and reinstatement testing.

Unexpectedly, we found that during the reinstatement test yohimbine strongly increased lever pressing in both the contingent pellet + cue group and the contingent cue-only group (see Results), suggesting that some non-specific arousal or locomotor activating effects of yohimbine promote lever responding. Therefore, we also determined in subgroups of rats previously tested for reinstatement induced by pellet priming and yohimbine whether this effect of yohimbine would be mimicked by methamphetamine, a psychostimulant that increases arousal and locomotor activity (Berridge 2006). The subgroups were contingent pellet + cue: $n = 4$; contingent cue-only: $n = 4$; non-contingent cue-only: $n = 8$. One day after the last yohimbine test, we injected all 16 rats with methamphetamine (1.0 mg/kg, i.p.) 15 minutes prior to the start of the session. We compared their lever pressing after methamphetamine with their lever pressing during the last extinction session, as well as with their lever pressing during the 2.0 mg/kg yohimbine reinstatement session.

Experiment 2: effect of yohimbine on locomotor activity

In experiment 2, we directly tested whether yohimbine, at a dose that induced robust reinstatement, also increases locomotor activity. We used 15 rats from experiment 1 (water: $n = 7$; yohimbine: $n = 8$). We injected the rats with water or yohimbine (2.0 mg/kg, i.p.) 30 minutes before the locomotor activity test. In the beginning of the test, we placed the rats in the center of the chamber and allowed them to explore the apparatus for 1 hour under white light. We recorded the total distance traveled and the number of vertical movements. In experiments 2–4, we used rats that have been previously used in experiment 1 that we repeatedly tested for locomotor activity, conditioned place preference (CPP) and microdialysis dopamine levels in NAc and mPFC because we wanted to determine yohimbine's effect on these measures in rats with a history of chronic food restriction that underwent the experimental procedure of experiment 1.

Experiment 3: effect of yohimbine on CPP/conditioned place aversion (CPA)

We used 15 rats from experiment 2 and prior to CPP/CPA conditioning, we divided the rats into two groups. We injected one group of rats ($n = 8$) with yohimbine (2.0 mg/kg, i.p.), pairing it with one of the two end compartments, and in separate conditioning sessions injected water, pairing it with the opposite end compartment (counterbalanced). We injected the other group of rats ($n = 7$) with water throughout the experiment, and con-

fining them to each of the two end compartments during conditioning. We conducted all sessions under white light. The CPP/CPA procedure consisted of three phases: pre-exposure (1 day), conditioning (8 days), tests 1 and 2 (conducted on separate days). During the pre-exposure phase, we placed the rats in the center choice compartment and immediately opened the guillotine doors, which remained open for the duration of the pre-exposure session, allowing the rats to explore the entire apparatus for 15 min. We recorded time spent in each compartment to assess unconditioned compartment preferences.

For the conditioning phase, we confined all rats to a single compartment during a given session, which lasted for 60 minutes. We gave rats in the yohimbine-paired group yohimbine injections paired with one of the two end compartments and water with the other compartment (in a counterbalanced manner). We gave rats in the water-only group water injections paired with both end compartments in a counterbalanced order. We injected all rats with either yohimbine (2.0 mg/kg, i.p.) or water (0.5 ml/kg) 30 minutes before each conditioning session started. The daily conditioning sessions lasted for 60 minutes, during which time the center choice compartment remained closed off by the guillotine door.

We conducted the first CPP/CPA test 2 days after the last conditioning session. We placed rats in the center choice compartment and opened the guillotine doors immediately after the test started, permitting access to all three chambers. The test lasted for 15 minutes. We recorded time spent in each chamber to assess individual preference. We conducted the second CPP/CPA test 2 weeks after the first test and the procedure for this test was identical to the first test.

Experiment 4: effect of yohimbine on dopamine levels in the mPFC and NAc

We used six rats from experiments 1–3 to determine the changes in dopamine levels in the mPFC and NAc after yohimbine injections. We first obtained stable dopamine values (less than 10 percent variability) for 2–3 consecutive samples (typically after about 1–2 hours), after which we injected rats with yohimbine (2.0 mg/kg) and monitored the changes in dopamine concentrations for 90 minutes after the injection. We obtained microdialysis measurements in a single session and euthanized the rats at the end of testing.

RESULTS

Experiment 1: effect of reward history and cue contingency on pellet priming and yohimbine-induced reinstatement of lever responding

We determined the effect of food reward history and compound cue contingency during the training phase on

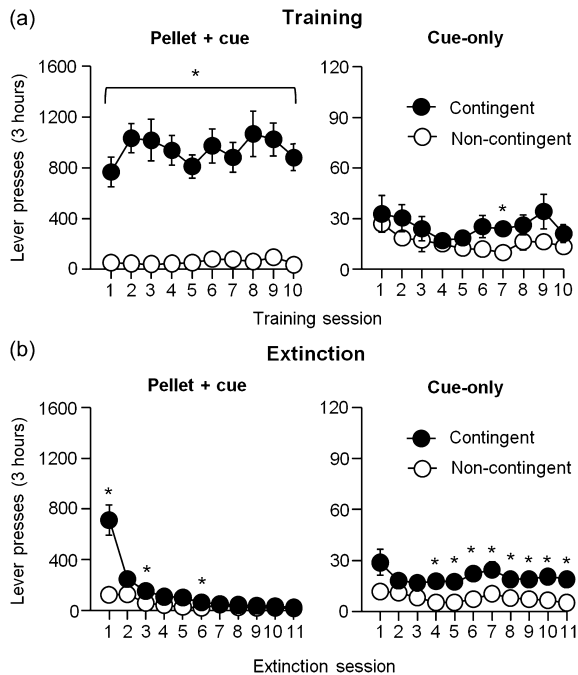


Figure 1 Lever presses during the training and extinction phases in rats trained under different food reward availability and cue contingency conditions. Left panel: response-contingent pellet + cue ($n=12$) and non-contingent pellet + cue ($n=8$, yoked condition). Right panel: response-contingent cue-only ($n=12$) and non-contingent cue-only ($n=8$). (a) Training phase. (b) Extinction phase. *Different from the respective non-contingent group within each day, $P < 0.05$. Note: the y-axis is drawn on a different scale for the pellet + cue (contingent and non-contingent) groups and the cue-only (contingent and non-contingent) groups. Data are mean \pm SEM

pellet priming and yohimbine-induced lever pressing during the reinstatement test. We used four groups of rats that during the training phase were exposed to (1) response-contingent pellet + cue, (2) non-contingent pellet + cue (yoked to group 1), (3) response-contingent cue-only and (4) non-contingent cue-only (yoked to group 3).

Training and extinction phases

Training. We found that operant responding during the training phase was significantly higher in the response-contingent pellet + cue group than in the other three groups (Fig. 1a). The mixed ANOVA using the between-subjects factor of group and the within-subjects factor of training session showed a significant main effect of group [$F(3, 36) = 70.4, P < 0.01$], but no effect of training session ($P = 0.58$) or interaction between the two factors ($P = 0.64$).

Extinction. We found that lever presses during the extinction phase decreased over time in the response-contingent pellet + cue group, while lever presses in the

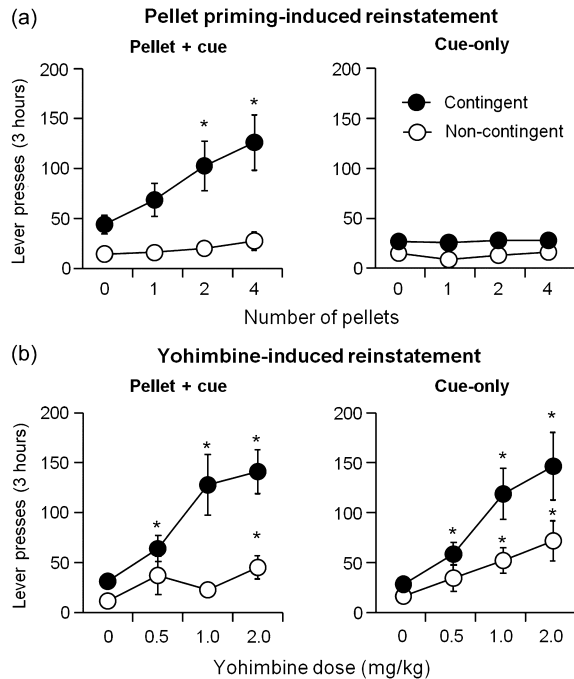


Figure 2 Effect of different reward history and cue contingency during training on pellet priming and yohimbine-induced lever pressing during reinstatement tests. Left panel: contingent pellet + cue ($n=12$) and non-contingent pellet + cue ($n=7-8$). Right panel: contingent cue-only ($n=12$) and non-contingent cue-only ($n=8$). (a) Pellet priming-induced reinstatement. (b) Yohimbine-induced reinstatement. Different from the control condition (0 pellet or water vehicle injection), * $P < 0.05$. Data are mean \pm SEM

other three groups remained relatively stable (Fig. 1b). The mixed ANOVA using the between-subjects factor of group and the within-subjects factor of extinction session showed a significant interaction between the two factors [$F(30, 360) = 19.1, P < 0.01$].

Pellet priming reinstatement test. Non-contingent delivery of pellets at the start of the test sessions increased lever responding during testing in the response-contingent pellet + cue group but not in the other groups (Fig. 2a). The mixed ANOVA using the between-subjects factor of group and the within-subjects factor of pellet number (0, 1, 2, 4 pellets) showed a significant interaction between the two factors [$F(9, 108) = 5.1, P < 0.01$]. These data demonstrate that the reinstatement effect of pellet priming is critically dependent on prior contingent pellet delivery during the training phase.

Yohimbine reinstatement test. Unlike pellet priming, during testing, yohimbine significantly increased lever presses in all groups, an effect that was substantially stronger in rats with a history of response-contingent delivery of pellet + cue or cue alone during the training phase (Fig. 2b). The mixed ANOVA using the

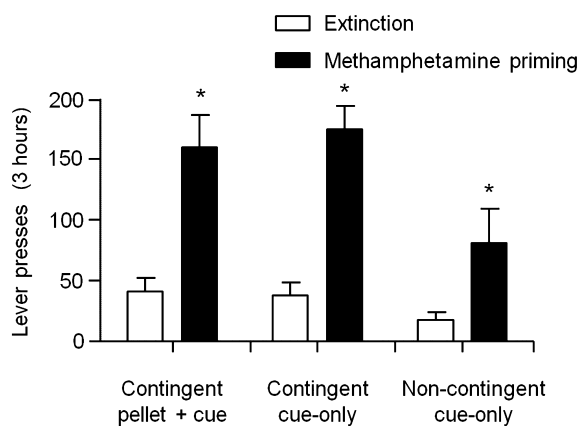
between-subjects factor of group and the within-subjects factor of yohimbine dose (0, 0.5, 1.0, 2.0 mg/kg) showed significant main effects of group [$F(3, 35) = 4.3$, $P < 0.05$] and yohimbine dose [$F(3, 105) = 19.0$, $P < 0.01$] and an approaching significant interaction between the two factors [$F(9, 105) = 1.97$, $P = 0.051$]. These data demonstrate that the effect of yohimbine on lever pressing during the yohimbine-induced reinstatement tests is independent of the history of food self-administration. Further, the different magnitude of the yohimbine-induced responding between groups suggests that the effect of the drug on lever pressing during testing is strongly potentiated when lever pressing had been previously paired with cue delivery.

Methamphetamine reinstatement test. In a subgroup of rats (response-contingent pellet + cue: $n = 4$; response-contingent cue-only: $n = 4$; non-contingent cue-only: $n = 8$), we found that methamphetamine injections (1.0 mg/kg) increased lever presses during the reinstatement test, an effect that was more pronounced in the response-contingent cue + pellet and response-contingent cue-only groups than in the non-contingent cue-only group (Fig. 3a). The mixed ANOVA using the between-subjects factor of group and the within-subjects factor of test session (last extinction session, methamphetamine session) showed significant main effects of group [$F(2, 13) = 4.6$, $P < 0.05$] and test session [$F(1, 13) = 34.7$, $P < 0.01$], but no interaction between the two factors ($P > 0.1$). *Post hoc* differences are shown in Fig. 3, which also shows the correlation ($r = 0.48$, $P = 0.06$) between lever presses induced by yohimbine (2 mg/kg) and methamphetamine during testing. These data suggest that, like yohimbine, the effect of methamphetamine on lever pressing during testing is dependent on the cue contingency but not history of food delivery during training. However, the methamphetamine data should be interpreted with caution because we only assessed the drug's effect on lever pressing in the reinstatement test in three of the four groups of rats from experiment 1 and in a small n per condition.

Experiment 2: effect of yohimbine on locomotor activity

The goal of this experiment was to test whether the effect of yohimbine on lever presses during the reinstatement test was due to non-specific increases in locomotor activity. We found that yohimbine (2 mg/kg) had no effect on locomotor activity (Fig. 4a). The ANOVA using the between-subjects factor of yohimbine dose (0, 2 mg/kg) showed no significant effects for distance traveled ($P = 0.98$) or vertical movement counts ($P = 0.58$). These data suggest that yohimbine's effect on lever pressing

(a) Methamphetamine-induced reinstatement



(b) Correlations

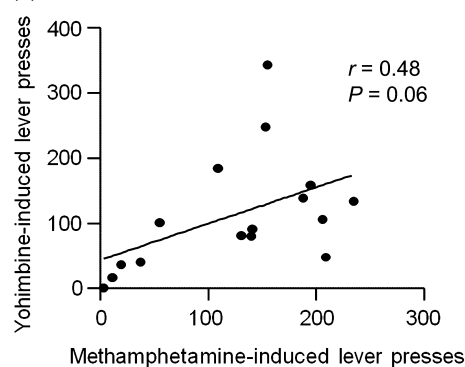


Figure 3 Effect of different reward history and cue contingency during training on methamphetamine-induced lever pressing in the reinstatement tests. (a) Methamphetamine-induced reinstatement (contingent pellet + cue: $n = 4$; contingent cue-only: $n = 4$; non-contingent cue-only: $n = 8$). *Different from the baseline extinction session, $P < 0.05$. Data are mean \pm SEM. (b) Correlation plot of lever pressing induced by methamphetamine (1 mg/kg) and yohimbine (2 mg/kg) during the reinstatement tests

during the reinstatement tests in experiment 1 is not caused by non-selective locomotor activation.

Experiment 3: effect of yohimbine on CPP/CPA

In experiment 3, we tested whether yohimbine (at a dose that strongly increases lever pressing during the reinstatement test) is rewarding or aversive, as assessed in the CPP/CPA procedure. We found that under our experimental conditions, yohimbine caused a weak CPP that diminished over time (Fig. 4b). The pre-exposure test showed that the yohimbine-paired group ($n = 8$) and water-only group ($n = 7$) had similar preference prior to conditioning. The dependent measure was a pre-exposure versus post-training change score (yohimbine paired—water paired for the yohimbine group or randomly chosen compartment 1—compartment 2 for the water group). We analyzed the change score using a

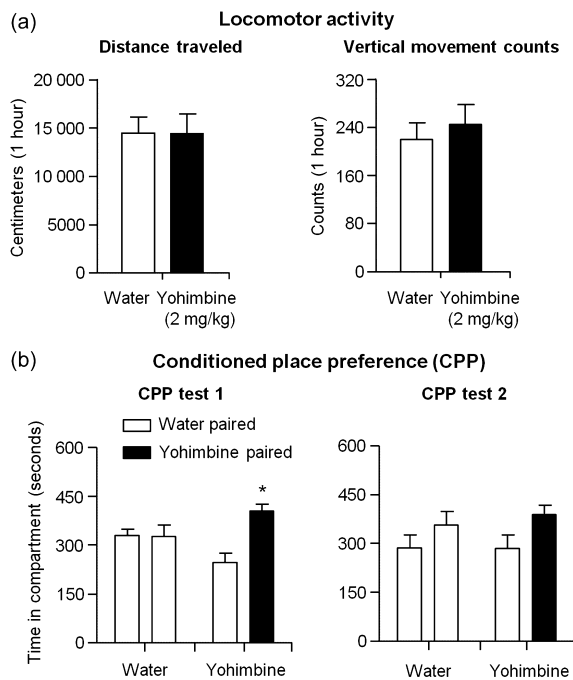


Figure 4 Effect of yohimbine on locomotor activity and conditioned place preference/aversion. (a) Locomotor activity, (b) Conditioned place preference/aversion on tests 1 and 2 conducted 2 days and 16 days after conditioned place preference training. *Different from water-paired compartment, $P < 0.05$ ($n = 7-8$ per group). Data are mean \pm SEM

paired *t*-test (pre- versus post-conditioning) for each group. For the initial test, which occurred 2 days after the last conditioning session, the results showed a significant effect for yohimbine-paired group (pre-exposure: 14.4 ± 33.6 ; post-conditioning test 1: 157.0 ± 42.9 ; $t_7 = 2.5$, $P < 0.05$), suggesting that these rats developed modest preference to the yohimbine-paired side regardless of their initial preference. This preference was not evident 16 days after CPP/CPA training (Fig. 4b). There was no difference in place preference in the water-only group (post-conditioning test 1: $t_6 = 0.8$, $P = 0.46$; post-conditioning test 2: $t_6 = 0.0$, $P = 0.99$). Together, our results indicate that yohimbine, at a dose typically used in reinstatement studies, is not an aversive stimulus in the CPP/CPA procedure.

Experiment 4: effect of yohimbine on extracellular dopamine levels in mPFC and NAc

In a previous study, we found that mPFC dopamine plays a critical role in yohimbine-induced reinstatement of food seeking (Nair *et al.* 2011) and in experiment 1, we found that the effect of yohimbine on lever pressing during the reinstatement tests is mimicked by methamphetamine, which increases dopamine levels in mPFC (Staiti *et al.* 2011). In experiment 4, we determined whether yohimbine would increase mPFC dopamine

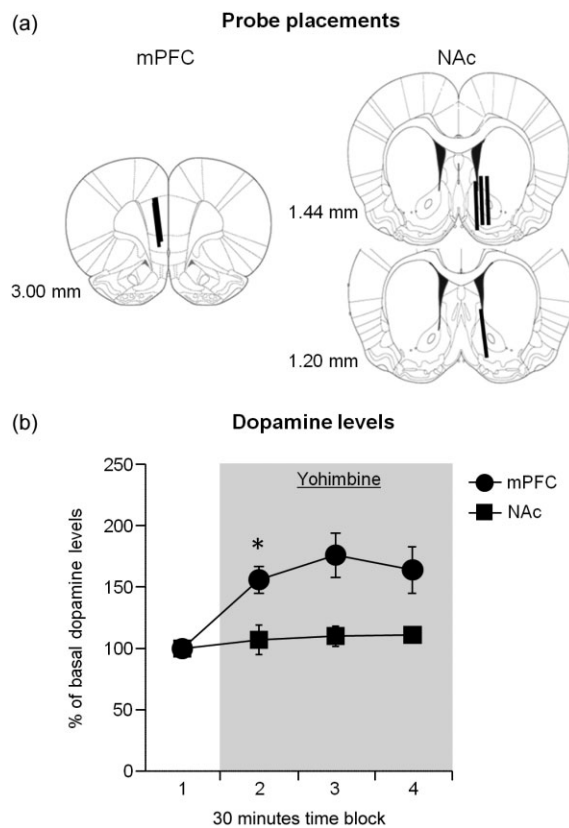


Figure 5 Effect of yohimbine on extracellular dopamine levels in NAc and mPFC. (a) Coronal sections (Paxinos & Charles 2005) showing the microdialysis probe placements. (b) Percent change from baseline dopamine levels after yohimbine (2 mg/kg) injections. Each time block represents average data in 30 minutes from samples collected every 10 minutes. *Different from baseline (block 1), $P < 0.05$. Data are mean \pm SEM

levels and also determined the drug's effect on NAc dopamine levels. We found that yohimbine (2 mg/kg) increased dopamine levels in mPFC but not NAc (Fig. 5). We analyzed the data in 30 minute time bins, using the within-subjects factor of session time (pre-treatment, yohimbine 0–30 min, yohimbine 30–60 min, yohimbine 60–90 min). The analysis showed a significant effect of session time for mPFC [$F(3, 6) = 11.0$, $P < 0.01$] but not NAc ($P = 0.61$).

DISCUSSION

We found that the effect of yohimbine on reinstatement of operant responding is critically dependent on the history of response-contingent cue delivery during training but not response-contingent food delivery. In contrast, pellet priming-induced reinstatement is critically dependent on the history of response-contingent food delivery during training but not response-contingent cue delivery. Additionally, yohimbine at a dose commonly used in

reinstatement studies of food or drug seeking did not induce an aversive state, as assessed in the CPP/CPA procedure. Furthermore, yohimbine had no effect on locomotor activity, suggesting that the drug's effect on lever pressing during reinstatement is not due to non-specific hyperactivity. Finally, in agreement with a previous study (Tanda, Bassareo & Di Chiara 1996), yohimbine increased dopamine levels in mPFC but not NAc. Our results suggest that the effect of yohimbine on operant responding in reinstatement studies in rats is independent of the history of contingent self-administration of food or drug reward and may not be related to the commonly assumed stress-like effects of yohimbine.

Psychological and neuropharmacological mechanisms of the reward-independent effects of yohimbine

Yohimbine has been used in many studies on reinstatement of drug and food seeking (See & Waters 2010; Calu *et al.* 2014; Mantsch *et al.* 2014). A common assumption is that yohimbine-induced reinstatement of lever pressing after extinction is due to stress-induced reinstatement of the previously reinforced drug- or food-associated operant response. Our results challenge this assumption by demonstrating that the contingent cue-only training group had a similar dose-response curve for the effect of yohimbine on 'reinstatement' as that of the response-contingent pellet + cue group (Fig. 2b). What psychological and neuropharmacological mechanisms might account for the reward-independent effects of yohimbine?

Psychological perspective

We propose that the primary action of yohimbine in reinstatement studies is to invigorate responding for visual or auditory stimuli/cues that under normal conditions have weak or moderate rewarding effects in rodents (Stewart 1960; Deroche-Gamonet *et al.* 2002; Donny *et al.* 2003; Olsen & Winder 2009; Shin *et al.* 2010). We see evidence for weak sensory seeking in the higher lever pressing of the contingent cue-only versus non-contingent cue-only groups (Fig. 1), an effect that is potentiated by yohimbine (Fig. 2b). The findings that yohimbine strongly enhances cue-induced reinstatement of drug seeking (Feltenstein & See 2006; Banna *et al.* 2010; Feltenstein, Ghee & See 2012) support the notion that the primary effect of yohimbine in reinstatement studies is to potentiate cue responding.

Our finding that yohimbine also somewhat increased lever pressing in the yoked response-non-contingent pellet + cue or response-non-contingent cue-only training groups seems inconsistent with the idea that the primary action of yohimbine in reinstatement studies is

to enhance cue responding. However, it is challenging to make operant lever pressing a cue-free manipulation because pressing an operant lever results in a clicking noise that is itself a sensory cue; additionally, the lever-pressing action may have some sensory properties. Thus, we speculate that yohimbine-induced increases in lever pressing during testing in the yoked training groups are likely due to potentiation of the motivational effects of sensory cues that are distinct from the tone-light cue.

Other studies using delay discounting and five-choice serial reaction time tasks suggest alternative psychological effects of yohimbine, which act either to increase preservative choice of previously learned behaviors (Schwager, Haack & Taha 2014) or to increase motor impulsivity (Sun *et al.* 2010). While yohimbine's effect on lever pressing during the reinstatement tests in the present study may be due to the drug's ability to increase motor impulsivity, it is difficult to assess the impact of yohimbine on perseverative choice since across all phases of experiment 1, the response contingency on the single lever remained unchanged for the contingent cue-only and non-contingent pellet + cue and cue-only groups.

Neuropharmacological perspective

We propose that yohimbine-induced potentiation of responding to sensory cues is mediated by mPFC dopamine transmission. Several lines of evidence support this notion. First, operant responding for sensory cues in rats is increased by the indirect dopamine agonists amphetamine (Winterbauer & Balleine 2007; Keller *et al.* 2014) or methamphetamine (Fig. 3 and Gancarz *et al.* 2012), and is decreased by knockdown of D1-dopamine receptors in mice (Olsen & Winder 2009). Second, yohimbine-induced reinstatement of food seeking is decreased by systemic or dorsal mPFC injections of the D1-family receptor antagonist SCH 23390 (Nair *et al.* 2011). Third, as previously reported (Tanda *et al.* 1996) and replicated in our study, yohimbine increases mPFC dopamine release (Fig. 5).

Another possibility is that yohimbine-induced potentiation of responding to sensory cues is mediated by noradrenaline transmission, as suggested by the finding that the beta adrenoceptor antagonist, propranolol, decreases yohimbine-induced reinstatement of cocaine CPP in mice (Mantsch *et al.* 2010). However, two lines of evidence are inconsistent with this possibility. First, lesions of the ventral or dorsal noradrenergic bundles have no effect on yohimbine-induced reinstatement of alcohol seeking (Le *et al.* 2009). Additionally, yohimbine-induced reinstatement of food or cocaine seeking is not blocked by the alpha-2 adrenoceptor agonist clonidine (Brown *et al.* 2009; Nair *et al.* 2009). However, these negative results should be interpreted with caution because they may be due to higher alpha-2 receptor

occupancy by yohimbine versus clonidine within the dose range used in these studies.

Alternatively, there is evidence that yohimbine reduces dorsal raphe cell firing and serotonin release (Millan *et al.* 2000). Thus, yohimbine may induce sensory-seeking behavior by suppressing the serotonergic system. Indeed, this system has been implicated in sensory reinforcement, as inactivation of median and dorsal raphe increases visual stimulus seeking (Vollrath-Smith, Shin & Ikemoto 2012). Our previous study showing that serotonin releaser fenfluramine reduced yohimbine-induced reinstatement of food seeking (Pickens *et al.* 2012) further supports this hypothesis.

Yohimbine and place preference/aversion

We found that yohimbine did not cause the expected CPA as previously reported by File (1986). The present results, which are in agreement with a previous report (Morales, Perez-Garcia & Alguacil 2001), stand in contrast to the stress-like effects of yohimbine that have been documented in many studies using different behavioral measures (Davis, Redmond & Baraban 1979; Pellow, Chopin & File 1985; Chopin, Pellow & File 1986; Johnston, Baldwin & File 1988; Bhattacharya, Satyan & Chakrabarti 1997; Ghitza *et al.* 2006). The reasons for the different results between our study and the Morales *et al.* study versus the S. File study are unknown, and may be due to the yohimbine dose, feeding conditions and prior history (our rats were food restricted and had prior experience with yohimbine), and the use of a biased CPP/CPA procedure in the S. File study. Overall, our results extend previous findings with cocaine, caffeine, and nicotine, demonstrating that anxiogenic agents that induce stress-like response are not invariably aversive (Brockwell, Eikelboom & Beninger 1991; File, Kenny & Cheeta 2000; Sarnyai, Shaham & Heinrichs 2001; Ettenberg 2009; Trigo, Zimmer & Maldonado 2009).

Implications of the present finding for studies on reinstatement of food and drug seeking

We found that both yohimbine and methamphetamine caused 'reinstatement' in rats that did not undergo formal contingent operant training and extinction training for food reward. These results have implications for future reinstatement studies in which investigators commonly assume that reinstatement of reward seeking after extinction is due to the prior history of food or drug self-administration. One implication is that the 'selectivity' of different pharmacological and neurobiological manipulations in reinstatement studies should be assessed using a contingent cue-only group, instead of the commonly used 'inactive' lever control condition. Unlike the inactive lever control condition, the contingent cue-only control

condition is identical to the experimental group in all aspects except for the omission of the critical variable under study (the food or drug reward). Another implication is that results from studies using psychostimulant priming or yohimbine manipulations should be interpreted with caution because they may not necessarily demonstrate evidence for 'reinstatement of reward seeking.'

An issue to consider based on the current results is whether the effect of intermittent footshock on reinstatement of food or drug seeking is also independent of the self-administration training history. We believe that this is not the case because several studies have reported that footshock-induced reinstatement is observed in rats with a history of cocaine (Ahmed & Koob 1997; Mantsch & Goeders 1999), nicotine (Buczek *et al.* 1999) or alcohol (Le *et al.* 1998) self-administration, but not food self-administration. Since footshock does not reinstate food seeking under conditions equivalent to the pellet + cue group in our study, it is unlikely that footshock would reinstate lever pressing in the cue-only condition.

Finally, our data do not rule out the contribution of stress-like effects of yohimbine on reinstatement. Indeed, there is evidence that both extrahypothalamic CRF and the bed nucleus of the stria terminalis, which play critical roles in mediating both stress responses (Davis *et al.* 2009) and footshock stress-induced reinstatement (Shaham *et al.* 2000a; Shalev *et al.* 2010), also contribute to yohimbine-induced reinstatement (Hansson *et al.* 2006; Marinelli *et al.* 2007; Shalev *et al.* 2010; Buffalari & See 2011; Le *et al.* 2013). Additionally, human studies indicate that yohimbine induces both physiological and psychological stress-like responses and increases opiate craving and intake (Stine *et al.* 2002; Greenwald, Lundahl & Steinmiller 2013), as well as alcohol and cocaine craving (Umhau *et al.* 2011; Moran-Santa Maria *et al.* 2014).

Concluding remarks

Since 2004 (Lee *et al.* 2004; Shepard *et al.* 2004), yohimbine has been used in many studies as a pharmacological stressor to study the mechanisms of stress-induced reinstatement of drug and food seeking (See & Waters 2010; Bossert *et al.* 2013; Calu *et al.* 2014). Our results challenge two commonly accepted assumptions in reinstatement studies: that yohimbine-induced reinstatement of lever pressing after extinction primarily reflects stress-induced reinstatement of responding for the previously reinforced drug or food reward and that psychostimulant priming-induced reinstatement of reward seeking is primarily controlled by the prior history response-contingent drug self-administration. Finally, our results extend previous results on the profound effect of conditioned sensory stimuli/cues on operant responding commonly assumed

to be controlled by current or past history of experience with unconditioned food or drug rewards (Caggiula et al. 2002; Bastle et al. 2012).

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Disclosure/Conflict of Interest

The authors declare that they do not have any conflicts of interest (financial or otherwise) related to the data presented in this manuscript.

Authors Contribution

YWC, YS and DJC contributed to the design of the experiments and the data analysis; YWC, KAF and SZB conducted all behavioral experiments; GT conducted the microdialysis experiments and related analysis; YWC, YS and DJC wrote the manuscript. All authors reviewed the content and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1 The effect of yohimbine on reinstatement of operant responding in rats is dependent on cue contingency but not food reward history.