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Incubation of cue-induced reinstatement of cocaine, but not sucrose, seeking in C57BL/6J mice

Alexandria L. Nugent, Ph.D.^{1,*}, Ethan M. Anderson, Ph.D.^{1,*}, Erin B. Larson, Ph.D.¹, and David W. Self, Ph.D.^{1,#}

¹University of Texas Southwestern Medical Center, Department of Psychiatry

Abstract

Prior studies have shown that drug-seeking behaviors increase, rather than dissipate, over weeks to months after withdrawal from drug self-administration. This phenomenon - termed incubation - suggests that drug-craving responses elicited by conditioned environmental or discrete cues may intensify over prolonged abstinence. While most of this work is conducted in rats with intravenous drug self-administration models, there is less evidence for incubation in mice that have greater utility for molecular genetic analysis and perturbation. We tested whether incubation of cocaine-seeking behavior is evident in C57BL/6J mice following 3 weeks (5 days/week) of cocaine self-administration in 2 h self-administration sessions. We compared cocaine-seeking (drug-paired lever) responses 1, 7, or 28 days after withdrawal from cocaine self-administration, and over similar times following sucrose pellet self-administration. We found that the initial re-exposure to the self-administration test chambers elicited increased reward-seeking behavior in both sucrose and cocaine self-administering mice, with maximal responses found at 7 days compared to 1 or 28 days after self-administration with either reinforcer. However, following extinction training, reinstatement of cocaine seeking reinforced by response-contingent presentation of reward-associated cues (tone/light) was significantly higher after 28 days compared to 1 or 7 days following cocaine self-administration. In contrast, cue-induced reinstatement of sucrose-paired lever pressing did not increase over this time frame, demonstrating a drug-specific incubation effect not seen with a natural reward. Thus, C57BL/6J mice display incubation of cue-induced reinstatement of cocaine seeking similar to findings with rats, but only show a transient incubation of context-induced cocaine seeking.

Keywords

incubation; cocaine; self-administration; drug-seeking; cue-induced reinstatement; extinction; sucrose; sucrose-seeking; mouse; craving

[#]Corresponding author: David Self, Ph.D., UT Southwestern Medical Center, Department of Psychiatry, 5323 Harry Hines Blvd., Dallas, TX 75390-9070, 214-648-1237, Fax: 214-648-4182, David.Self@UTSouthwestern.edu.

^{*}Denotes co-first authorship

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Introduction

Cocaine craving can be elicited by exposure to conditioned environmental cues associated with prior drug use, and drug craving has been shown to be highly predictive of the propensity for relapse after a period of abstinence¹. Drug-associated cues also elicit marked electrophysiological responses in human cocaine abusers that intensify after prolonged periods of abstinence². In rats, a similar time-dependent intensification of cocaine-seeking behavior, often referred to as incubation, is observed following chronic intravenous cocaine self-administration³⁻¹³. These rat studies often employ extinction/reinstatement procedures where cocaine seeking is measured by non-reinforced responding at a drug-paired lever after initial return to the self-administration test chambers. Following extinction of the lever-press behavior, exposing animals to the discrete injection cues associated with prior cocaine self-administration also is used to test the propensity for relapse, or reinstatement of cocaine seeking. Using these models, cocaine seeking elicited by both initial return to the self-administration context and cue-induced reinstatement of cocaine seeking progressively increases, rather than dissipates, over weeks to months after withdrawal from self-administration^{8,14,15} very similarly to the increases in cue reactivity recently described in humans cocaine abusers². Given this time-dependent exacerbation in cue responsiveness, a delineation of the neurobiological mechanisms underlying the effect may lead to more effective treatments for cocaine addiction, especially over long-term abstinence.

A similar incubation of drug seeking has been shown for other abused drugs including heroin^{6,16}, alcohol¹⁷, methamphetamine¹⁸⁻²⁰, and nicotine^{21,22}, suggesting that incubation reflects a pathological process common to drug addiction in general. The incubation phenomenon also occurs with behavior motivated by natural rewards including sucrose, but the enhanced sucrose-seeking behavior fails to persist for more than a few weeks following self-administration training in rats^{6,10,23}. Thus, incubation may reflect a natural process of reward memory intensification over some discrete period that facilitates animal survival, but that drugs of abuse exacerbate and prolong this natural process leading to vulnerability to relapse long after cessation of drug use²⁴.

Studies on the incubation of cocaine-seeking behavior are extensively performed using rat models³⁻¹², but relatively few have employed mouse models²⁵⁻²⁷. Mouse models of intravenous cocaine self-administration are more difficult to perform, but allow for the use of transgenic and gene deletion models not readily accessible in rats. In this study, we tested whether the C57BL/6J mice display incubation of cocaine seeking over time in response to contextual and discrete injection cues using the extinction/reinstatement paradigm, and compared responses in cocaine-trained mice to responses in mice trained to self-administer sucrose pellets as a natural reward.

Methods

Animals

Male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine) at 6-8 weeks of age. Mice weighed on average 25 ± 0.41 g at the beginning of the experiment. The

mice were acclimated for 1 week prior to being placed in individual housing in a colony room maintained at 21°C under a 12-hour light/dark cycle (lights on at 6:00 am). Mice had free access to water and rodent chow (2016 Harlan Teklad Global Diet) except during lever-press training for 25 mg sucrose pellets when mice were food restricted for 16 h prior to each test session. All experiments were conducted during the light cycle in accordance with guidelines established by the National Institutes of Health (NIH) and the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center (UTSW).

Drugs

Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (NIDA, Research Triangle Park) and dissolved in 0.9% sterile saline.

Lever Press Training

All mice initially were food-restricted and received lever-press training for sucrose pellets in operant test chambers with levers that retract at the end of each session (Med-Associates, St. Albans, VT) on a fixed ratio 1 (FR1) reinforcement schedule in daily 1 h sessions until an acquisition criterion of 30 sucrose pellets was consumed for 3 consecutive test days. Following lever-press training, animals assigned to cocaine self-administration groups were fed *ad libitum* for at least 3 days prior to surgery. Sucrose self-administering animals were simply returned to their home cages for a week of *ad libitum* feeding prior to initiating sucrose self-administration.

IV catheter implantation surgery

Animals were anesthetized with 100 mg/kg ketamine/10 mg/kg xylazine (i.p.) prior to surgical implantation of a chronic indwelling intravenous catheter. The catheters consisted of Silastic tubing (0.02 inch i.d. 0.037 inch o.d.; Green Rubber, Woburn, MA) treated with tridodecylmethyl ammonium chloride (TDMAC) heparin (Polysciences Inc., Warrington, PA). Each catheter was secured at the jugular vein with Mersiline surgical mesh (General Medical, New Haven, CT), and passed subcutaneously to exit the back through 22-gauge cannula (Plastics One, Roanoke, VA) embedded in dental cement on a Mersiline surgical mesh base. Following surgery, animals received a prophylactic injection of penicillin (200,000IU/kg/ml, 0.2 ml, i.m.), and antibiotic ointment was applied daily to the exit wound. Catheters were flushed daily with 0.2 ml of heparinized (20 IU/ml) bacteriostatic saline containing gentamycin sulfate (0.33 mg/ml) and 5 mg/ml of enrofloxacin (Baytril®) to prevent clotting and curb infection. Catheter patency was checked weekly using methohexital sodium (5 mg/ml, 0.05ml i.v.).

Cocaine Self Administration

Animals were placed into operant test chambers for self-administration, extinction, and reinstatement testing. Each chamber was enclosed in a ventilated, sound-attenuating box and equipped with an injection pump assembly consisting of a Razel Model A injection pump (Stamford, CT) and a 10-ml glass syringe connected to Teflon® tubing which connects through a swivel mechanism to the animal's catheter exit port. Each operant chamber

contained two response levers located 2 cm off the floor; during self-administration testing, a lever-press response on the paired lever delivered an intravenous cocaine injection (0.5 mg/kg/injection) over 2.5 s in a 50 µl volume of sterile saline; lever press responses on the unpaired lever produced no programmed consequence. During the injection period, a cue light (above the lever) was illuminated for 2.5 s paired with a 2.9 kHz tone and the house light was extinguished. A 12.5 s time-out interval followed during when the house lights remained off and responding at the paired lever had no programmed consequences. The illumination of the house light signaled the end of the 15 s injection/time-out interval. Mice self-administered cocaine on a fixed ratio (FR) 1 reinforcement schedule in daily 2-h sessions, 5 days per week, for 3 weeks. Mice had no access to food or water while in the operant chambers. At the conclusion of self-administration, animals were returned to their home cages for the duration of the withdrawal period. Catheters continued to be flushed routinely during the withdrawal period.

Sucrose Self Administration

Food restricted mice received approximately 2 g of chow per day mice and were weighed daily to prevent weight loss of > 5% initial body weight. During the testing period, mice were placed in operant chambers where responding on the paired lever resulted in delivery of a sucrose pellet on a fixed ratio 1 (FR1) reinforcement schedule. Sucrose pellet delivery was paired with a similar cue light/tone cue for 2.5 s while the house light was extinguished to signal the unavailability of further sucrose pellets during the 12.5 s time-out period. At the conclusion of the time-out period, the house light was illuminated to signal the availability of further sucrose pellets. Animals were allowed to self-administer sucrose for a maximum of 60 pellets per session. Mice self-administered sucrose in daily 2-h sessions, 5 days per week, for 3 weeks. Levers retracted and lights extinguished at the end of each session or after 60 pellets were earned, but mice remained in the chamber for the 2 h session in either case. At the end of 15 days of sucrose self-administration, mice were fed *ad libitum* in their home cages for the remainder of the experiment.

Extinction and Cue-induced Reinstatement

Cocaine self administering animals were assigned to the 1, 7, or 28 day withdrawal groups with cocaine intake balanced across the 3 groups. Likewise, the sucrose self administering groups were balanced based on their latency to self-administer the first 30 sucrose pellets. Following the designated withdrawal period (1, 7, or 28 days), mice were placed back into the operant chambers under extinction conditions when lever presses resulted in neither cocaine nor sucrose pellet reinforcement nor the conditioned stimuli (light and tone). Mice remained in the operant chambers for extinction testing for 6 h. At the conclusion of the 6-h extinction period, animals were presented with 5 non-contingent cocaine- or sucrose-associated cues (light/tone for 2.5 sec) every 2 min for 10 min as a primer. Lever-pressing on the reward-paired lever produced response-contingent light/tone reward-associated cues during both the initial 10 min primer period and throughout the 1 h test.

Statistical Analysis

Responding during the first h of extinction testing and cue-induced reinstatement was analyzed by 2-way ANOVA with repeated measures on lever, or across all 6 h of extinction

with repeated measures on time. Bonferroni's tests were performed to identify differences from the 1 d WD group with significance level of $p < 0.05$.

Results

Self-administration

Groups of mice reliably self-administered cocaine starting with an initial average lever pressing of 46.7 ± 3.4 on the first day of self-administration. Over the three week period, all mice increased their lever pressing by approximately 57% to an average of 73.4 ± 2.0 presses/2 h session on the final day of self-administration. Mice were assigned into 3 withdrawal groups evenly matched for lever presses for the 15 days of training (Fig. 1a). A separate cohort of mice self-administered sucrose for 15 days and were balanced based on the number of lever presses (Fig. 1b) and the latency to achieve 30 sucrose pellets per session (Fig. 1c). Mice generally self-administered 30 pellets with a latency of ~ 16 minutes (16.3 ± 0.4 min, Fig. 1c).

Effect of withdrawal time on extinction responding

Following withdrawal time periods of either 1, 7, or 28 days in their home cages, mice returned to the test chambers under extinction conditions without response-contingent reward delivery or reward-associated discrete cues (light/tone) for 6 hours. Responding during the first h of extinction testing, and index of context-elicited cocaine-seeking behavior, was higher at the lever paired with previous cocaine injections when compared with unpaired lever responses in all groups (Fig. 2a **left**, 2-way ANOVA, effect of lever: $F_{1,29} = 74.78$, $p < 0.001$; withdrawal time $\times$ lever interaction: $F_{2,29} = 4.374$, $p = 0.022$). Cocaine-seeking responses at the drug-paired lever were significantly higher 7 days after withdrawal compared to mice tested 1 day after the last cocaine self-administration test session (Fig 2a **left**, effect of withdrawal time: $F_{2,29} = 4.097$, $p = 0.027$). Drug-paired lever responses in the 28 day withdrawal group displayed a trend ($p = 0.068$) but failed to significantly differ from the 1 day withdrawal group. This time-dependent increase in drug-paired lever responding after withdrawal from cocaine self-administration produced a significant withdrawal time $\times$ h interaction across all 6 h of extinction testing (Fig. 2a **right**: $F_{10,145} = 3.63$, $p = 0.002$). Here, responses at drug-paired lever significantly increased at both 7 and 28 days compared to 1 day withdrawal times during the first h given the added power of this analysis. However, all groups extinguished to similar low levels by the 6th h (effect of time: $F_{5,145} = 67.33$, $p < 0.001$), with mice meeting an extinction criteria of fewer than 15 lever presses per h.

Interestingly, sucrose-trained mice also showed greater initial sucrose-paired lever responding 7 days following sucrose self-administration compared to mice tested 1 day after self-administration training ended (Fig. 2b **left**, 2-way ANOVA, effect of withdrawal time: $F_{2,32} = 6.945$, $p = 0.003$). However, responding returned to the early withdrawal baseline 28 days after sucrose self-administration. Responding was substantially biased towards the sucrose-paired lever compared to the unpaired lever (effect of lever: $F_{1,32} = 60.00$, $p < 0.001$). Extinction responses over 6 h showed that mice in the 7 day withdrawal group responded at high rates at the sucrose-paired lever throughout extinction testing (Fig. 2b

right, 2-way ANOVA, effect of withdrawal time: $F_{2,32} = 5.391$, $p = 0.010$), but only mice in the 28 day withdrawal group met an extinction criteria of < 15 responses at the sucrose-paired lever by the 6th hour of extinction testing.

Effect of withdrawal time on cue-induced reinstatement of reward-seeking behavior

After 6 h of extinction training, mice were presented with 5 non-contingent light and tone cues that previously were paired with receipt of reward, and subsequent responding was reinforced by the compound cue in a 1 h reinstatement test. In cocaine-trained mice, only mice tested 28, but not 7, days after withdrawal from self-administration significantly increased responding at the drug-paired lever compared to mice tested 1 day after cocaine (Fig. 3a **left**, 2-way ANOVA, effect of withdrawal time: $F_{2,29} = 5.18$, $p = 0.012$), indicating a late forming incubation of cocaine seeking in response to discrete cues. A main effect of lever reflects responding biased at the drug-paired lever ($F_{1,29} = 12.36$, $p = 0.002$). When reinstatement responses were adjusted for baseline response rates in the 6th h of extinction testing (reinstatement responses minus extinction responses), a similar increase was observed from 1 to 28 days withdrawal from cocaine self-administration (Fig. 3a **right**, 2-way ANOVA, effect of withdrawal time: $F_{2,29} = 5.275$, $p = 0.011$), and lever pressing was again preferential for the drug-paired lever (effect of lever: $F_{1,29} = 10.74$, $p = 0.003$; lever $\times$ withdrawal time interaction: $F_{2,29} = 5.00$, $p = 0.014$). In contrast to cocaine self-administering mice, sucrose-trained mice showed similar cue-induced reinstatement of responding at the sucrose-paired lever at all 3 time points (Fig. 3b **left**) with lever pressing preferential for the paired lever (2-way ANOVA, effect of lever: $F_{1,32} = 42.29$, $p < 0.001$). The lack of incubation in cue-induced sucrose seeking remained even when responses were adjusted for differences in baseline responding during the 6th h of extinction testing (Fig. 3b **right**), and only a main effect of lever was significant (2-way ANOVA: $F_{1,32} = 20.67$, $p < 0.001$), although positive response rates indicate that discrete cues effectively increased sucrose-paired lever responding from extinction baselines.

Discussion

This study found prominent and late forming incubation of discrete cue-induced reinstatement after 28, but not 1 or 7 days, following withdrawal from cocaine self-administration in C57BL6/J mice, one of the most commonly used genetic background strains for analysis of genetic deletion or transgenic over-expression. Our findings are consistent with previous studies in rats demonstrating that discrete cue-induced reinstatement of cocaine seeking incubates over time⁸. In contrast to cue-induced reinstatement, initial re-exposure to the self-administration context during extinction testing elicited only transient increases in cocaine or sucrose seeking after a 7 day withdrawal period that declined after 28 days to levels observed 1 day after self-administration training. These extinction findings differ from studies in rats that generally find prominent and long-lasting enhancement in initial context-induced cocaine seeking after longer withdrawal periods^{3,8}. One possibility is that the incentive properties of contextual memories are longer lived in rats when compared to mice²⁸, while the incentive properties of discrete cues gain salience over time. Another possibility is that since discrete cues are more closely associated

with drug delivery, this association may survive for longer times compared to contextual cues that are less temporally associated with the receipt of individual cocaine injections⁶.

A similar transient enhancement (7 days) in the response elicited by the environmental/contextual cues associated with a natural reinforcer, sucrose was found in C57BL/6J mice. However, there was no evidence of incubation in discrete cue-induced sucrose seeking as reinstatement responses remained elevated at all 3 withdrawal times even when corrected for differences in extinction baselines. These results are somewhat similar to studies using rats where responding for sucrose-paired cues dissipate quickly over time^{6,10,16,23}, or are not observed²⁵, while drug-paired cues progressively increase over months in rats²⁴. However, some studies do show an incubation with both cocaine and sucrose in rats, although these studies examined responses at earlier times (day 15 or 21 withdrawal)^{10,11,29,30}, or do not dissociate the initial drug-seeking in extinction from the reinstatement induced by discrete-cues^{20,30,31}. As discussed earlier, this transient incubation of initial context-induced sucrose seeking may represent a natural phenomenon to promote and enhance memories essential for survival by facilitating reward-related cues over a brief time period. However, if animals can survive beyond this time, the incentive salience evoked by these memories returns to normal levels. There are many reports of conditioned responses to natural appetitive stimuli that either remain relatively constant or decay over time^{32,33}.

There are other experimental caveats that could contribute to differences between cocaine and sucrose reinstatement. For instance, although we capped the maximum number of sucrose reinforcers available to avoid satiety and approximate the number of cocaine reinforcers mice self-administer under similar conditions in 2 h sessions, mice self-administer most of the sucrose pellets within the first 20 min of the session. In contrast, mice self-administer cocaine infusions more evenly spaced over the 2 h session. Another notable difference is that the overall rates of cue-induced reinstatement in the sucrose-trained group were much higher than in cocaine-trained animals, despite no evidence for incubation of the cue response. The lack of food availability in the operant chambers may have contributed to this higher response in the 7 h extinction/reinstatement sessions. The palatability of the food pellets may also be a factor, since other studies using rats have shown incubation in animals trained with liquid sucrose solutions^{10,23,30}. To our knowledge, incubation of liquid sucrose seeking has not been shown in mice, and a lack of incubation with food pellets is consistent with the current mouse literature²⁵. Finally, it is important to note that the present findings could be unique to the C57BL/6J strain of inbred mice, and may not be representative of all other mouse strains.

There are relatively few previous studies showing incubation of cocaine seeking in mice in response to various combinations of discrete and contextual cues. One study found incubation of cue-induced reinstatement in unspecified wild-type littermates of GluR1 knockout mice 66 days after cocaine self-administration²⁶, although this study tested the same mice for cue-induced reinstatement at different times following a single extinction session. Thus, this effect could reflect spontaneous recovery rather than incubation. Two other studies have shown incubation of cocaine seeking when mice are initially returned to the self-administration test chambers but with a combination of both contextual/environmental and response-contingent presentation of discrete injection cues during

extinction^{25,27}. Our approach to test contextual and discrete cues separately suggest that this effect may be entirely due incubation in the response to discrete cocaine injection cues (light/tone) and not contextual factors.

Given that incubation of drug seeking in rats also is found with self-administration of heroin^{6,16}, alcohol¹⁷, methamphetamine¹⁸⁻²⁰, and nicotine^{21,22}, it is likely that similar incubation could be found in mice self-administering these abused drugs. Mouse incubation models could be used to study strain-specific differences in the genetics underlying addictive disorders, or to study the effects of cell-specific genetic deletion or transgenic expression on the incubation phenomenon, similar to their pivotal use in understanding other aspects of addiction including reinstatement^{26,34,35}. Since incubation of drug craving can be observed in humans^{2,36}, non-human primates³⁷, rats³⁻¹³, and mice²⁵⁻²⁷; future studies in mouse incubation models may have high translational impact to improve treatment strategies in abstinence that to decrease relapse rates in human addicted populations.

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Highlights

- Incubation of cue-induced reinstatement of cocaine seeking in C57BL/6J mice
- Transient incubation of context-induced cocaine or sucrose seeking
- No incubation of cue-induced reinstatement of sucrose seeking

Figure 1. Cocaine or sucrose self-administration

a) The number of lever presses for self-administered cocaine infusions (0.5 mg/kg/injection) during 2 h sessions over 15 days of cocaine self-administration training for each withdrawal (WD) group: 1 day WD ($n = 9$), 7 day WD ($n = 11$) or 28 day WD ($n = 12$). The number of **b)** lever presses for self-administered sucrose pellets (60 max) and **c)** the latency to earn the first 30 sucrose pellets in 2 h sessions over 15 days of sucrose self-administration training for each WD group: 1 day WD ($n=12$), 7 day WD ($n=12$) or 28 day WD ($n=11$).

a: Cocaine **b: Sucrose**

Figure 2. Both cocaine- and sucrose-seeking behavior peaks after 7 days WD during an extinction session

a-left) Cocaine-paired and unpaired lever presses during the first hour of extinction at 1, 7, and 28 days after WD from cocaine self-administration. **a-right)** Paired (black lines) and unpaired (grey lines) lever pressing over a 6 h extinction session for all cocaine WD groups relative to lever presses on the last day of cocaine self-administration training. **b-left)** Sucrose-paired and unpaired lever presses during the first hour of extinction after at 1, 7, and 28 days after WD from sucrose self-administration. **b-right)** Sucrose-paired (black lines) and unpaired lever presses (grey lines) over a 6 hour extinction session for all sucrose self-administration WD groups relative to lever presses on the last day of sucrose self-administration. Differs from 1 day WD * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Bonferroni tests.

a: Cocaine **b: Sucrose**

Figure 3. Incubation of cue-induced reinstatement of cocaine- but not sucrose-seeking behavior after 28 days WD

a-left) Cocaine-paired and unpaired lever responding during cue-induced reinstatement immediately after 6 hours of extinction training (Fig. 2). **a-right)** Reinstatement of cocaine-paired and unpaired lever responding after subtraction of baseline extinction response rates during the 6th hour of extinction training. **b-left)** Sucrose-paired and unpaired lever responding during cue-induced reinstatement immediately after 6 hours of extinction training. **b-right)** Reinstatement of sucrose-paired and unpaired lever responding after subtraction of baseline extinction response rates during the 6th hour of extinction training. Differs from 1 day WD, ** $p < 0.01$ by Bonferroni tests