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Effects of a histone deacetylase 3 inhibitor on extinction and reinstatement of cocaine self-administration in rats

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Abstract

Rationale: A challenge in treating substance use disorder is that successful treatment often does not persist, resulting in relapse and continued drug seeking. One approach to persistently weaken drug-seeking behaviors is to pair exposure to drug-associated cues or behaviors with delivery of a compound that may strengthen the inhibition of the association between drug cues and behavior.

Objectives: We evaluated whether a selective histone deacetylase 3 (HDAC3) inhibitor could promote extinction and weaken contextual control of operant drug-seeking after intravenous cocaine self-administration.

Methods: Male Long Evans rats received a systemic injection of the HDAC3 inhibitor RGFP966 either before or immediately after the first extinction session. Persistence of extinction was tested over subsequent extinction sessions, as well as tests of reinstatement that included cue-induced reinstatement, contextual renewal, and cocaine-primed reinstatement. Additional extinction sessions occurred between each reinstatement test. We also evaluated effects of RGFP966 on performance and motivation during stable fixed ratio operant responding for cocaine and during a progressive ratio of reinforcement.

Results: RGFP966 administered before the first extinction session led to significantly less responding during subsequent extinction and reinstatement tests compared to vehicle-injected rats. Follow-up studies found that these effects were not likely due to a performance deficit or a change in motivation to self-administer cocaine, as injections of RGFP966 had no effect on stable responding during a fixed or progressive ratio schedule. In addition, RGFP966 administered just after the first extinction session had no effect during early extinction and reinstatement tests, but weakened long-term responding during later extinction sessions.

Conclusions: These results suggest that a systemic injection of a selective HDAC3 inhibitor can enhance extinction and suppress reinstatement after cocaine self-administration. The finding that behavioral and pharmacological manipulations can be combined to decrease drug-seeking provides further potential for treatment by epigenetic modulation.

Keywords

histone acetylation; epigenetics; intravenous cocaine; extinction; Long Evans rats

Introduction

Substance use disorder is a chronic, often relapsing disease that leads to a loss of behavioral inhibition and overuse of addictive drugs. In an attempt to better understand and counteract this disorder, preclinical approaches have focused on behavioral treatments such as extinction to weaken drug-seeking behaviors. The severing of relations among cues, responses, and drugs in rodents during extinction models aspects of exposure therapy in humans (Kiefer and Dinter 2013; Millan et al. 2011; Morrison and Ressler 2014). A problem in human treatment is that relapse often occurs outside of the therapeutic context or when cues previously associated with drug use are encountered. In rodents, drug-seeking behavior returns when the context is changed after extinction or when cues associated with drug intake are encountered (Bossert et al. 2013; Bouton and Todd, 2014; Fuchs et al. 1998; Farrell et al. 2018; Hitchcock et al. 2014; Hitchcock and Lattal, 2018; Shaham et al. 2003). The challenge for treatments is therefore to develop tools that may promote extinction and weaken relapse.

Recent attention has focused on epigenetic mechanisms that modulate interactions between DNA, histones, and other aspects of chromatin structure (Hitchcock and Lattal, 2014). Many studies have now documented enhancements in memory caused by administration of drugs that inhibit histone deacetylases (HDACs), which prevent the removal of acetyl groups from specific amino acid sites of histones(e.g., Dagnas et al. 2015; Foley et al. 2014). This inhibition correlates with increased histone acetylation, which corresponds to increased gene expression, protein synthesis, synaptic plasticity, and associative learning (Arrar et al. 2013; Blank et al. 2014; Bousiges et al. 2013; Guan et al. 2009; Kouzarides 2007; Lopez-Atalaya et al. 2013; Penney and Tsai 2014; Pizzimenti and Lattal 2015; Rai et al. 2010; Rosen et al. 2004; Rosenberg et al. 2014; Sarkar et al. 2011; Vecsey et al. 2007; Wang et al. 2015).

Most of what is known about pharmacological modulation of HDACs during extinction comes from the use of nonspecific HDAC inhibitors (e.g., trichostatin A, sodium butyrate, vorinostat, and valproic acid) in Pavlovian conditioning approaches. These HDAC inhibitors enhance extinction after fear or place preference conditioning (e.g., Bredy et al. 2007; Gaglio et al. 2014; Lattal et al. 2007; Malvaez et al. 2010; Raybuck et al. 2013; reviewed in Whittle and Singewald 2014; Singewald et al. 2015), but little is known about specific HDACs in extinction (Bowers et al. 2015; Malvaez et al. 2013). HDAC3 is a Class 1 HDAC highly expressed in the brain (Broide et al. 2007) and with distinct connections to other complexes and HDACs that are associated with learning. HDAC3 is therefore hypothesized to be a critical negative regulator of learning (McQuown et al. 2011). As a result, long-term inhibition of HDAC3 increases histone acetylation (e.g., on histone 4 (H4) at lysine site 8 (K8)) and acquisition of cocaine conditioned place preference (Rogge et al. 2013) in mice. In addition, systemic and short-term application of RGFP966, a novel and selective HDAC3 inhibitor enhances histone acetylation (at H3K14 and H4K8) and extinction learning in mice, resulting in decreased drug-primed reinstatement after extinction of cocaine-induced conditioned place preference (CPP; Malvaez et al. 2013). Although increases in learning and corresponding decreases in drug-seeking have been shown in Pavlovian and operant learning (Malvaez et al. 2010; Castino et al. 2015; Romieu et al. 2008), no studies to date have

determined the effects of targeting just one HDAC with extinction after drug self-administration.

The goal of the following experiments was to investigate whether HDAC3 inhibition promotes extinction of operant responding for cocaine. We first evaluated the persistent effects of a single systemic injection of the HDAC3 inhibitor, RGFP966, during extinction and several forms of reinstatement. We then assessed the effects of RGFP966 on asymptotic and progressive ratio responding for cocaine. Finally, we further evaluated the effects of a single post-extinction session administration of RGFP966 on long-term extinction. We hypothesized that RGFP966 would create a persistent decrease in drug-seeking if administered before or immediately after extinction learning.

METHODS

Animals.

Male Long-Evans rats (n = 28, Charles River Laboratories, Wilmington, NC) weighing 275–300 grams and aged 2–3 months were pair housed and allowed to habituate to the vivarium for one week after arrival. Rats had ad libitum access to food and water before behavioral training and then 4–5 pellets/day of rat chow (equivalent to 20–25g) to limit weight gain and potential complications with catheters and attached backpacks. Rats were maintained on a 12-hour light-dark cycle (lights on at 6 am and off at 6 pm), with behavioral sessions (3 hours in duration) conducted in their light cycle, between 8 am and 4 pm for 1–3 months (5–7 days/week). These procedures were approved by the OHSU IACUC, and were in accordance with the ethical guidelines set by NIH, Principles of Laboratory Animal Care (NIH Publication No. 86–23, revised 1985).

Intravenous catheter surgery.

To induce general anesthesia for surgery, a mixture of Ketamine (66 mg/kg) and Xylazine (AnaSed, Lloyd, Shenandoah, IA USA 1.33 mg/kg) was given to rats by intraperitoneal (IP) injection at 10% body weight (0.5ml/500g). Anesthesia was maintained by inhalation of 1–2% isoflurane gas for the remainder of the surgery.

Once rats were immobile and unconscious each rat was placed on a sterile pad for surgery and a small patch of hair was shaved for a small incision at the catheter insertion and exit sites. Catheters were pre-made out of Silastic laboratory tubing (Dow Corning, Midland, MI USA, cut to 10 cm, 0.55 mm I.D. x 0.94 mm O.D.), filled with filtered (0.22um) saline, and inserted into the jugular vein. One end of the catheter was secured into the right jugular vein, and the other end was threaded around the shoulder and out the exit incision between the shoulder blades. Two internal and three external Sofsilk sutures (Covidien, Minneapolis, MN USA, coviden3–0, wax coated braided silk) were added to the front and one external suture was added to the back of the rat at each incision point to secure the catheter in place and decrease the possibility of infection. Each catheter was briefly checked for patency by drawing blood back into the catheter and gently flushing it with filtered saline before attaching the catheter to an external backpack (Instech, Plymouth meeting, PA USA, Cat.No. CIH95AB), worn indefinitely by the rat. A steel cannula with a screw connector (Plastics

One, Roanke, VA USA, 22GA, 5mm above and below pedestal) was placed inside the backpack to attach the jugular catheter tubing to the backpack and to the tether attachment within the behavioral self-administration chamber for infusions.

Rats were given a subcutaneous (SC) injection of an anti-inflammatory drug (carprofen-Rimadyl, Pfizer, New York NY USA, 5mg/ml) to decrease swelling and associated pain, an intravenous infusions of an antibiotic (Timentin, GlaxoSmithKline, Research Triangle Park, NC USA, 238mg/ml) in filtered saline to decrease the potential for post-operative infection, and an anticoagulant (heparin, West-Ward, 100U/ml) in filtered saline to increase the patency of the catheterization. They were placed in a clean cage (singly housed), with two nestlets, and pellet food after surgery. After waking with no signs of distress, each rat was returned to the vivarium. During recovery (5–7 days after surgery) rats were monitored for any signs of pain or weight loss and given carprofen if warranted. To maintain and verify catheter patency over time, catheters were flushed with heparin, Timentin, and Brevitol intravenously. Heparin was administered before and after each self-administration session and during non-testing days (10–100U/ml, respectively). Timentin was given after each self-administration session and during non-testing days.

Drugs.

For intravenous cocaine (COC) self-administration, (−)-cocaine hydrochloride (Sigma-Aldrich CASRN 53–21-4) was dissolved in 0.9% physiologic saline (4mg/ml) and filtered (0.22um Millipore Disposable Filtration System) to make a solution of 0.2g COC/50ml saline. Syringes (BD sterile Luer-Lok Tip, Franklin Lakes, NJ, 10 ml) were attached to MedPC pumps and filled with this solution daily. Each infusion occurred after completion of the active lever schedule pumped into the attached tether and rat catheter over 5 seconds and dispensed 88.7ul of the cocaine solution (dose of 0.89mg/kg with a rat weight of 400g). For COC reinstatement, COC was dissolved in saline (10mg/ml) and injected (IP) at a dose of 10mg/kg (1ml/kg).

RGFP966 (selective HDAC3 inhibitor; provided by Repligen Corporation) was dissolved in dimethylsulfoxide (DMSO, <10% final volume), and diluted with a vehicle (VEH) solution of hydroxypropyl-β-cyclodextrin and sodium acetate (provided by Repligen, pH 5.4). RGFP966 was administered (2ml/kg vol, SC), at a dose of 10 mg/kg (Bieszczad et al., 2015; Bowers et al., 2015; Malvaez et al., 2013) after two days of habituation to vehicle injections. This compound has specific effects on HDAC3 and this dose peaks in brain concentration 30–60 min after injection, persisting in the brain for at least 2 hr (Bowers et al., 2015; Malvaez et al., 2013).

Apparatus.

Behavioral experiments were conducted in 12 MedPC modular test chambers (30.5 cm x 24.1 cm x 29.2 cm), equipped with two response levers (one of each was retractable, consistent in all chambers), two cue lights (2.5 cm white lens above each lever), one pellet receptacle between the two levers, a small hooded house light (28V DC) above the pellet receptacle, and a small opening at the top of the chamber for drug infusion equipment (swivel, tether, syringe, and pump). Each chamber side panel (left and right), grid floor, and

waste pan was made of stainless steel. The top, back, and front chamber walls were made of clear Plexiglas. Each chamber was enclosed in a sound-attenuating cubicle, equipped with a fan on one side of the chamber to circulate air and provide ambient background noise during all behavioral sessions (28V DC). On the other side of the chamber a movable metal arm connected the tether tubing to the internal rat chamber and external pump system, allowing the rat to move freely within the chamber when connected to the syringe tether.

To create two separate contexts (hereafter referred to as Contexts 1 and 2), the floor, visual cues, and spatial location of 6 out of 12 of the chambers were altered, so that half were of one type and the other half were of another type. For the tactile cue, a grid floor with either 19 large (4.8 mm) diameter bars spaced 15.6 mm apart or 26 smaller (3.2 mm) diameter bars spaced 8.0 mm apart were used for each context (MedPC, Cat.No. ENV-005 and ENV-005A-T, respectively, Crombag et al. 2002; Crombag and Shaham 2002). For the visual cues, one of two contexts had a clear Plexiglas back wall so that the exterior shell remained visible to the rat. In contrast, the back wall of the other context consisted of a 21.6 cm x 28.0 cm sheet with black and white stripes and stars. Each context was located in a different location (vertically and horizontally) within the testing room (i.e., Context 1 was 152.4 cm vertical x 91.4 cm horizontal from Context 2). The context assignment (training in Context 1 vs. Context 2) was counterbalanced between drug treatment groups (i.e., VEH vs. RGFP966 administration) so that half of the rats in each treatment group were assigned to one context, while the other half of rats were assigned to the other context.

Self-administration Protocol.

Acquisition and Maintenance—Rats were moved from the vivarium to the experimental room, their catheters were flushed with heparin (10U), and they were weighed and then placed in a pre-assigned operant chamber. Immediately after, the MedPC program was started, the house light turned on, and rats were infused with a short duration and small volume priming injection of cocaine (2s infusion). All acquisition sessions were 120-min long and were conducted in Context A (counterbalanced between Contexts 1 and 2). Once in the self-administration chamber, 1 active lever press (fixed ratio 1 (FR1) schedule) illuminated the cue light above each active lever for 5 s, activated the syringe pump, and infused the cocaine solution into the catheter for 5 s. After each active lever press and subsequent cocaine infusion, there was a 20 s timeout period, during which active lever presses had no scheduled consequence. There were no scheduled consequences for inactive lever presses. The cocaine dose, infusion rate, and timeout period remained constant in all self-administration sessions.

Rats completed 1 week of FR1 self-administration with 10 infusions/120 min session for 2 days before moving to an FR5 schedule (Bongiovanni and See 2008, Fuchs et al. 2007), and then completed 2 weeks of FR5 with 10 infusions/120 min session for 10 days (and on the last 3 days) before moving to an extinction schedule. Once rats maintained stable and high levels of FR5 self-administration for at least 10 days, inactive and active lever presses were averaged over the last 5 maintenance days of FR5 self-administration to create two balanced treatment groups prior to extinction.

Extinction—Unless otherwise noted, all extinction sessions, in which lever presses had no programmed consequences, were 2 hr in duration. Rats received RGFP966 or VEH 20-min prior to or immediately after the first extinction session in different experiments. Subsequent extinction sessions occurred until active lever responding had consistently decreased (25 active lever presses/session for 2 days, with no significant difference between treatment groups).

Reinstatement—Reinstatement testing consisted of three sub-phases: 1) *AAB Contextual Renewal (Context-induced reinstatement; CTX-R):* Rats were placed into a novel context (Context B; counterbalanced between Contexts 1 and 2) for one nonreinforced 2-hr session (no cocaine or cue lights), followed by at least two extinction sessions to return responding to low levels. *2) Cue-induced reinstatement (CUE-R)*: Rats were placed original drug-taking context (Context A) and reintroduced to the conditioned reinforcer (5 s cue-light presentation) with each active lever press in a 2 hr session. This was followed by at least two extinction sessions. 3) *Cocaine-primed reinstatement (COC-R):* Rats were administered a priming injection of cocaine (10mg/kg, 1ml/kg) prior to placement in their original drug-taking context (Context A). Responding was not reinforced with cocaine or the cue during this 2 hr session.

This order of testing was chosen so that the least amount of expected responses would be tested first, based on pilot data and previous studies on contextual renewal (Crombag et al., 2002; reviewed in Crombag & Shaham, 2002; Fuchs et al., 2005), and the highest would be tested last (cocaine-primed). Similar methods have been used by others (Berglind et al. 2007; Castino et al. 2015; Fuchs et al. 1998, 2005; Venniro et al. 2016).

Progressive Ratio—In the progressive ratio experiment, rats were trained initially on FR1 and FR5 schedules as described above, followed by two FR10 training sessions. Rats then began the progressive ratio (PR) schedule in a 180-min session for 12 sessions prior to VEH and RGFP966 treatment. During the PR schedule, an increasing number of lever presses was required to deliver each cocaine infusion (e.g., first infusion required 2 active lever presses, second infusion required 4 active lever presses, third infusion required 8 active lever presses, etc.) using a standard response ratio equation (Richardson and Roberts, 1996; with *j = 0.32*). This ratio was chosen to escalate the required active lever presses fast enough to reach a break point (when rats fail to receive a drug infusion within a 1-hour period; Richardson and Roberts, 1996) within a 3-hour session. The cocaine dose and the presentation of the cue light above the active lever were as in the FR training periods.

Data Collection and Statistical Analysis.

Data were collected with MED-PC power, control, and interface equipment, MED-PC IV control and data collection software, and MP2XL data transfer utility software. One and two-way analysis of variance (ANOVA) and within-subject comparisons with repeated measure ANOVA, followed by post-hoc tests (Bonferroni corrected if not denoted and when applicable, with a p value set at 0.05) and t-tests were used to determine statistical reliability.

RESULTS

Effects of RGFP966 delivered before extinction on extinction and reinstatement

Acquisition and Maintenance.—Over the initial days of self-administration, rats received cocaine infusions on an FR1 and then FR5 schedule. Rats were assigned to groups that would receive RGFP966 (n=10) or vehicle (n=11) that were matched in terminal FR5 performance; mean active lever presses during the final five 2-hr sessions of maintenance: RGFP966 = 107 (SEM = 8), Vehicle = 105 (SEM = 5). A 2 (drug) x 2 (lever) ANOVA revealed a reliable main effect of lever (F (1, 19) = 334.01, p < 0.001), but no effect of subsequent drug treatment and no interaction (ps > 0.5) prior to extinction.

Initial Extinction.—On Extinction Session 1 (E1) rats received a VEH or RGFP966 injection 20-min before the session. To determine the time course of the initial drug effects, data were compared at each 15-min interval of extinction Sessions 1–3. Figure 1A shows responding in 15-min blocks during the first three 2-hr extinction sessions. During E1, both groups extinguished active lever pressing during the session. There was no main effect or an interaction with drug group during E1 (Fs < 1.22, ps > 0.28) but there were reliable main effects of lever (F(1, 19)=31.54, p<0.001) and time block (F(7,133)=19.09, p<0.001), as well as a reliable lever x block interaction (F(7,133)=13.4, p<0.001).

A within-session analysis of Extinction Sessions 2 (E2) and 3 (E3) found that in both sessions, rats treated with Vehicle responded higher in the first 15 min of each session, whereas RGFP966-treated rats showed consistently low levels of responding throughout the 2-hr session. A drug x lever x bin ANOVA on responding in 15-min blocks during E2 and E3 revealed reliable main effects of lever (Fs(1,19)>19.5, ps<0.001) and time block (Fs(7,133)>10.3, ps<0.001), as well as interactions between lever x block (Fs(7,133)>3.0, ps<0.007), block x drug (Fs(7,133)>3.5, ps<0.002), lever x drug (Fs(1,19)>11.0, ps<0.005), and drug x block x lever (Fs(7,133)>3.1, ps<0.005). In addition, there was a reliable main effect of drug in E2 (F(1,19)=9.25, p<.008) but not in E3 (F(1,19)=3.6,p=0.072). Follow-up tests of the reliable 3-way interactions in E2 and E3 revealed a reliable difference between drug groups during the first 15 min of each session in responding on the previously active lever (E2: t(19)=2.6, p<0.02; E3: t(19)=3.7, p<0.002).

Mean responding on the previously active lever during the 2-hr Extinction Sessions 4–8 is shown in Figure 1B (E4-E8). Rats that received vehicle prior to E1 continued to show ordinally higher levels of responding during E4–6, but this difference was reliable only in E4 (p<0.014), with borderline trends in E5 (p=0.10) and E6 (p=0.067). There were no differences between the groups during E7 and E8, suggesting that the two groups responded at equally low levels prior to reinstatement testing.

Contextual Renewal Test.—Responding during the contextual renewal test is shown in Figure 1B (CTX). Between E8 and the renewal test, there was a reliable main effect of session (F (1, 16) = 16.862, p = 0.001), lever (F (1, 16) =9.379, p = 0.007), and a session x drug group interaction (F (1, 16) = 17.428 p = 0.015). Follow-up tests determined that no reliable main effects or interactions occurred during E8 but a reliable main effect of drug (F (1,16) = 8.204, p = 0.011) and lever (F (1, 16) = 7.313, p = 0.016) occurred during the

contextual renewal test, with no lever x drug interaction (p = 0.2). A session x drug interaction occurred for the active lever responses from E8 to the CTX test (F (1, 16) = 4.993, p = 0.040), but not for the inactive lever responses (p = 0.303).

Cue-Induced Reinstatement.—Two additional extinction sessions (E9 and E10 in Figure 1B) occurred between context- and cue-induced reinstatement (CUE in Figure 1B) to return responding to equally low levels in the two groups. A drug (RGFP966 or VEH) x session (E10 or cue-induced reinstatement) x lever (active or inactive) ANOVA revealed reliable main effects of session (F (1,16) = 58.260, p < 0.001), lever (F (1,16) = 86.408, p < 0.001), and interactions between session and drug (F (1,16) =5.672, p = 0.030), lever and drug (F (1,16) =5.457, p = 0.033), and session and lever (F (1,16) =75.006, p < 0.001). Follow-up tests determined that no main effects or interactions occurred during E10, but during cue-induced reinstatement a reliable main effect of lever (F (1,16) =88.882, p < 0.001) and an interaction of lever x drug occurred (F (1,16) =4.668, p = 0.046), with a trend of a main effect of drug (p = 0.055). One way ANOVAs for each type of lever demonstrated a significant interaction between session and drug group on active (F (1, 16) =5.007, p = 0.04) but not inactive responses (p = 0.115). As a result of cue presentation, drug-seeking increased in both drug treatment groups, as measured by increased active lever pressing, but the group given RGFP966 just prior to their first extinction session (11 days earlier) pressed the active lever reliably less than did the VEH group (CUE Figure 1B).

Cocaine-Induced Reinstatement.—Extinction occurred again for two more days (E11 and E12 in Figure 1B) before rats re-established low extinction criterion and cocaine-induced reinstatement was tested (COC in Figure 1B). Drug-seeking during cocaine-induced reinstatement increased in both drug treatment groups, as measured by increased active lever pressing during cocaine-primed reinstatement, but there was no difference in responding between rats given RGFP966 or VEH prior to their first extinction session. Reliable main effects of session (E12 vs COC: (F (1, 16) =9.131, p < 0.008), lever (F (1,160= 10.545, p < 0.005), and a session x lever interaction (F (1, 16) =7.853, p < 0.013) occurred, but no effects of drug group occurred.

In summary, one injection of RGFP966 before the first extinction session following FR5 self-administration led to a reliable and persistent reduction in drug-seeking compared to VEH treatment. This was revealed as less spontaneous recovery during the next two extinction sessions, as well as weakened AAB contextual renewal and cue-induced reinstatement, but there was no persistent effect on cocaine-primed reinstatement.

Effects of RGFP966 on lever press performance and motivation for cocaine

To determine the effects of RGFP966 on steady state responding for cocaine, we first evaluated acute effects of the compound on asymptotic responding during an FR5 schedule of reinforcement. After reaching acquisition criterion, all rats received injections of RGFP966 (2 ml/kg vol, 10 mg/kg) or VEH 20-min prior to FR5 self-administration maintenance sessions on alternating days. Figure 2A shows responding during the final sessions of maintenance (Baseline) and the sessions with RGFP966 (RGFP966) or Vehicle (VEH) treatment in Experiment 2. A drug x lever x session ANOVA revealed only a reliable

main effect of lever (F (1, 6) =74.640, p =<0.001), suggesting that RGFP966 treatment did not create a performance deficit or general motor impairment and that rats were still motivated to self-administer cocaine.

To further evaluate the effects of RGFP966 on performance and motivation for cocaine, we assessed the effects of RGFP966 in a standard progressive ratio (PR) procedure. Rats (n = 23) from the first and second experiment were regrouped and balanced for experimental history. Separate ANOVAs were completed to test if active and inactive lever responses were different based on previous drug or behavioral history and no effects were found (ps > 0.05). Once rats maintained stable and high levels of PR self-administration (after 2 weeks of cocaine SA acquisition and 3 days of consistent operant responding at the set schedule; i.e., an average of 5 infusions/PR session) responding was averaged over the last 5 days of PR self-administration to create a baseline. Following maintenance of PR self-administration, VEH or RGFP966 was administered 20-min prior to a PR self-administration session. Rats then received a final drug-free PR self-administration session.

As can be seen in Figures 2B and 2C, there were no effects of RGFP966 on responding (Figure 2B) or on number of reinforcers earned (Figure 2C) during the progressive ratio session (reliable main effect of 5-min time block on cumulative active lever presses and infusions: Fs>15, ps<0.001; no reliable main effect of drug or interaction). Results here demonstrate that neither current drug treatment (vehicle or RGFP966), nor experimental history of drug altered motivation to respond during a progressive ratio schedule of reinforcement.

Effects of RGFP966 delivered after extinction on extinction and reinstatement

Following the tests of RGFP966 on PR responding, rats remained in their home cage for 27 days. After this, the effects of RGFP966 administered immediately after the first extinction session (E1) were determined. This time in the home cage served two purposes: (1) to eliminate RGFP966 from the system (Malvaez et al. 2013), and (2) to introduce a forced abstinence period that may increase responding for cocaine (Berglind et al., 2007; Gabriele et al. 2012; Kuntz-Melcavage et al. 2009; Neisewander et al. 2000; Weiss et al. 2001). Rats were assigned to one of three subsequent treatment groups that were matched for their responding during the last PR session. These subsequent groups received different drug and extinction treatments: (1) 120-min of extinction, followed by a vehicle injection (VEH 120 n = 8), (2) 30-mins of extinction, followed immediately by a vehicle injection (VEH 30 n = 8), (3) 30-min of extinction, followed immediately by a RGFP966 injection (RGFP966 30 n = 7).

Our hypothesis was that the group that received 30-min of extinction and a vehicle injection would have the slowest rate of extinction, achieve the least amount of extinction, and have the greatest amount of reinstatement compared to the 120-min group with a vehicle injection and the 30-min group with an HDAC3 inhibitor. The two extinction durations were used to determine if the effects of the HDAC3 inhibitor could turn a weak behavioral extinction experience (30-min) into a strong behavioral extinction experience (120-min), as our lab has found with HDAC inhibitors (HDACi) and session duration effects in extinction of fear

(Stafford et al. 2012). All other extinction sessions were identical to previous sessions (120-min in duration).

Extinction.—Figure 3A shows responding during the first two sessions of extinction in 15-min time blocks. All groups responded similarly during the initial 30 min of Extinction 1 prior to injection of RGFP966 or vehicle (no reliable main effect of group: $F_{(2, 20)} = 0.615$, $p = 0.550$; reliable main effect of lever: $F_{(1, 20)} = 77.522$, $p < 0.001$). The 120-min group showed within-session extinction during the session, with main effects of lever $F_{(1, 7)} = 22.912$, $p = .002$) and 30-min time block ($F_{(1.898, 13.288)} = 27.459$, $p < 0.001$), and an interaction of lever x block ($F_{(1.280, 8.962)} = 7.968$, $p = 0.016$). During Extinction Session 2, all groups responded at similar levels, with a reliable main effect of lever ($F_{(1, 20)} = 68.954$, $p < 0.001$), time block ($F_{(1.321, 26.416)} = 50.576$, $p < 0.001$), and an interaction between block and lever ($F_{(1.378, 27.555)} = 44.161$, $p = 0.001$). There was no effect of treatment, but a near trend for an interaction between lever and treatment (Fs < 2.617, ps > 0.098) during the entire 120-min of Extinction 2.

Figure 3B shows mean responding during the 12 sessions of extinction prior to reinstatement testing. There were no effects of treatment during these sessions, with an ANOVA revealing reliable main effects of extinction session ($F_{(11,198)} = 19.93$, $p < 0.001$) and lever ($F_{(1,18)} = 115.06$, $p < 0.001$), as well as a reliable session x lever interaction ($F_{(11,198)} = 18.36$, $p < 0.001$. All other main effects and interactions were not reliable (ps > 0.122).

Contextual renewal.—Figures 3C and 3D show responding during the reinstatement sessions, including extinction that occurred before or between reinstatement tests. Groups did not differ during the final extinction session in Context A prior to the test in Context B (E12 in Figure 3D; ANOVA: main effect of lever $p < 0.001$, treatment $p = 0.910$, lever x treatment $p = 0.852$). During the Context B test (CTX in Figure 3D), all rats increased their active lever pressing during the 2-hour session with no effect of treatment. A 3 (treatment) x 2 (lever-repeated) x 2 (session-repeated) ANOVA confirmed main effects of session ($F_{(1, 18)} = 14.137$, $p = 0.001$), and lever (($F_{(1, 18)} = 32.9$, $p < 0.001$), but this interaction and all treatment effects (main and interactions) were not reliable (Fs < 1.172, ps > .293). Although RGFP966-treated rats demonstrated an ordinal decrease in responding compared to vehicle-treated rats, there was not a reliable treatment effect over the full session or in the first 30-min (ps > 0.193 for main effects and interactions with treatment).

During the extinction sessions between Context and Cue reinstatement testing (E13 to E15 in Figure 3C), there were main effects of session ($p < 0.001$) and treatment ($p = 0.044$) on active lever presses, with a near effect between the RGFP966 and the VEH 30 groups ($p = 0.066$) compared to the VEH 120 group (ps > 0.152). There was also a main effect of inactive presses over sessions ($p = 0.013$), but with no effect of treatment ($p > 0.366$). There was no interaction of session by treatment group between E13–15 for active or inactive lever presses (ps > .476), suggesting that differences in active lever presses by treatment emerged by E13 and remained similar until E15. One-way comparisons for each session determined that there was no treatment effect at E13 ($p = 0.47$), a trend at E14 ($p = 0.082$) on active

responses (as the RGFP966 group continued to extinguish to baseline levels), and no effects on E15.

Cue-induced Reinstatement.—Prior to cue-induced reinstatement testing, responding was equally low during E16 (ANOVA: 3 treatments x 2 levers-repeated, main effect of lever $p < 0.001$, treatment $p = 0.441$, lever x treatment $p = 0.984$). The next day rats were placed into the same context (Context A) where each active lever press illuminated the cue light above the lever. All rats increased their active lever pressing during the 2-hour Cue session, and although RGFP966-treated rats showed ordinally lower responding, there was no reliable main effect of treatment (Cue in Figure 3D). There were reliable main effects of session ($F (1, 18) = 29.478$, $p < 0.001$), lever (($F (1, 18) = 34.297$, $p < 0.001$), and an interaction between the two ($F (1, 18) = 29.900$, $p < 0.001$), but all treatment effects (main and interactive) were not reliable ($Fs < 0.791$, $ps > 0.469$).

Extinction after cue-induced reinstatement.—The differences in responding that existed prior to cue-induced reinstatement continued during extinction sessions after that reinstatement test (E17 to E23 in Figure 3C). An ANOVA revealed reliable main effects of session ($F(6,108)=23.2$, $p<0.001$), lever ($F(1,18)=60.9$, $p<0.001$), and treatment ($F(2,18)=3.8$, $p<0.05$), as well as interactions between lever and treatment ($F(2,18)=4.2$, $p<0.05$), session and lever ($F(6,108)=15.4$, $p<0.001$), and the three-way interaction ($F(12,108)=1.84$, $p=0.05$). Further analysis of the three-way interaction revealed a difference between VEH 30 and RGFP966 groups ($p = 0.038$) but not the VEH 120 ($ps > 0.33$) group over sessions E17–23 with both levers included. These effects occurred during the active lever (Veh 30 vs RGFP966: $p=0.029$; Veh 120 vs RGFP966: $p=.22$), but not during the inactive lever ($ps>0.81$). In the active lever there were main effects of session ($p < 0.001$) and treatment ($p = 0.031$), and a near interaction of treatment x session ($p = 0.06$) in active responses. In the inactive lever, there was an effect of session ($p = 0.001$), but no treatment effect ($p = 0.450$), or an interaction between session and treatment ($p = 0.886$). To determine which sessions and groups differed, further testing revealed this effect was not caused by the VEH 120 group ($ps > 0.215$) but by differences between the RGFP966 and VEH 30 ($p = 0.029$) groups, with trends for treatment differences after CUE-R at E17 and E18 ($ps < 0.064$), and significant treatment effects in active responses at session E19-E23 ($0.007 < ps < 0.046$), compared with one-way ANOVAs for each session).

Cocaine-induced Reinstatement.—Although responding decreased prior to cocaine-induced reinstatement testing, the seven extinction sessions after cue-induced reinstatement testing failed to bring the groups to equivalent levels of responding (ANOVA: 3 treatments x 2 levers-repeated, main effect of lever $p < 0.001$, treatment $p = 0.043$, lever x treatment $p = 0.099$). All groups increased their responses in the presence of cocaine, relative to E23 (main effect of session: $F (1, 18) = 15.427$, $p = 0.001$, lever: $F (1, 18) = 22.874$, $p < 0.001$, and interaction of session x lever effect: $F (1, 18) = 13.156$, $p = 0.002$), with no effect of prior extinction or drug treatment ($ps > 0.560$; Figure 3D). Therefore, one post-extinction (30-min session) injection of RGFP966 did not alter context-, cue-, or cocaine-induced reinstatement, in this experiment, but did result in persistent differences in responding during extinction between reinstatement sessions.

Discussion

The development and persistence of memory and addiction is thought to be regulated, in part, by histone posttranslational modifications (Maze et al. 2012). The experiments reported here demonstrated that a histone deacetylase 3 inhibitor can promote extinction of operant responding for cocaine. This was true when the HDAC3 inhibitor was delivered 20-min before or immediately after an extinction session, though these extinction enhancements were revealed in different ways. A pre-session injection of RGFP966 promoted extinction, measured in terms of rate of extinction across days and persistence of extinction, revealed as weakened contextual renewal and cue-induced reinstatement. The extinction-enhancing effects appear not to be due to general performance or motivational effects, because RGFP966 did not alter stable responding on an FR5 schedule or progressive ratio responding. Further, a pre-session injection of RGFP966 did not immediately cause changes in responding, either during extinction (Figure 1) or during the progressive ratio session (Figure 2), both of which lead to cessation of responding. This suggests that within-session extinction occurs normally in the presence of this compound. Post-session delivery of RGFP966 did not significantly alter reinstatement, but differences emerged during additional extinction sessions that followed reinstatement sessions, consistent with what we have observed with the persistent effects of acute stressors that occur before extinction begins (Pizzimenti et al. 2017). Together, these results suggest that extinction can be promoted by HDAC3 inhibitors and emphasize the importance of assessing potential extinction enhancements using a variety of behavioral measures (with effects on early extinction, reinstatement, and late extinction).

Our post-session extinction enhancement also occurred in animals that had an extensive history of RGFP966 treatment paired with reinforced cocaine sessions. That we still observed a persistent extinction effect is consistent with the idea that the most recent experience with the compound drives subsequent effects, independent of the history of that compound being paired with self-administration or extinction sessions. Further, the behavioral treatments in the vehicle groups (30 min vs 120 min extinction session) had very little impact on behavior, either during subsequent extinction or reinstatement testing, suggesting that RGFP966 results in changes that are greater than those added by additional exposure times in the extinction session.

Our experiments are the first to show that HDAC3 inhibition enhances extinction after cocaine self-administration. Previous work has shown that RGFP966 can promote extinction of cocaine CPP, long-term object location memory, and auditory operant discrimination (Bieszczad et al. 2015; Malvaez et al. 2013; McQuown et al. 2011b). Together, these findings, as well as others using genetic manipulations of HDAC3 (Rogge et al. 2012) suggest that HDAC3 inhibition may generally promote memory. However, this may not always occur, as RGFP966 has been shown to have no effect on extinction of cued fear (Bowers et al. 2015). More work is clearly needed to determine how systemic administration of compounds that target HDACs interact with the overlapping and distinct systems that mediate extinction in appetitive and aversive procedures.

Our findings add to a large body of evidence that HDAC inhibition in general can promote extinction or otherwise weaken drug self-administration. For example, class I HDAC inhibitors (such as sodium butyrate (NaB) and trichostatin A (TSA)) have been found to weaken drug self-administration under different circumstances (Castino et al. 2015; Jeanblanc et al. 2015; Romieu et al. 2008, 2011; Simon-O'Brien et al. 2014; reviewed in Kennedy and Harvey 2015). The effects of these compounds, however, interact with a number of factors that need to be considered when making general conclusions about HDAC inhibitors and addiction. For example, Romieu et al. (2008) found that maintenance and motivation to self-administer cocaine decrease with multiple pre-treatments of class I HDAC inhibitors (TSA and phenylbutyrate under FR1 schedule; TSA and depudecin under PR schedule) but that these effects did not occur with a higher dose of cocaine (0.75 mg/kg/ infusion; comparable to the dose used in our experiments).

Another study demonstrated that rats with a history of heavy drinking (reaching ethanol levels near 1g/kg/30min) decreased ethanol self-administration on a progressive ratio schedule when given a class I HDAC inhibitor thought to be more specific for HDAC1 (MS-275) at least two times and three hours prior to drug intake or reacquisition (Jeanblanc et al. 2015). This difference could mean that a class I HDAC inhibitor, but not a selective HDAC3 inhibitor, decreases the motivation for drugs, or that a general HDACi increased the reinforcing property of the drug, such that animals received the same reinforcing effect from less drug.

It may also be that differences between drug exposure or total infusions may lead to effects based on dependence (~10/session in our study versus > 20/session in Romieu et al. 2008). This may be similar to the way in which dependent (but not non-dependent) rats limit ethanol intake with a non-specific HDACi (Simon-O'Brien et al. 2014) or how rapid ethanol tolerance can be reversed with a non-specific HDACi (Sakharkar et al. 2012). These effects support the possibility that HDAC inhibition may lead to different behavioral and cellular outcomes due to the baseline cellular and behavioral environment during treatment (i.e., treatment after more or less dependence or tolerance, or during drug taking or extinction) and has the potential to enhance or weaken persistent behaviors associated with aspects of addiction.

In addition, previous studies used multiple consecutive injections (IV, 30 min prior to self-administration) of a non-specific HDACinhibitor (i.e., TSA or depudecin) or vehicle (saline or 10% DMSO, daily for 10 days), which may underlie the different result from our studies after a progressive ratio schedule, as we used a greater duration of time between HDAC inhibitor deliveries as compared to others (e.g., Romieu et al. 2008) who saw decreases in self-administration with decreased time between HDACi treatment. One of the benefits of targeting a specific HDAC is that non-specific HDAC inhibitors target multiple HDACs and may disrupt important protein-protein interactions that HDACs have with corepressors such that other functions besides deacetylation are impacted. Non-specific inhibitors may also lead to dynamic changes in the cellular environment, in the structure of chromatin, transcription targets, and to the life cycle of proteins that may be involved (Dokmanovic et al. 2007). For example, class I inhibitors (e.g., NaB, RGFP963, TSA, etc.) occasionally lead

to indirect peripheral and central nervous system effects that could more likely influence performance if given before behavioral testing (Andersen et al. 2013; Tran et al. 2014).

Evidence exists of non-specific HDAC inhibition enhancing Pavlovian extinction but there are fewer investigations on selective HDAC inhibition, or in translating this to operant behaviors. One study has demonstrated that extinction of fear is enhanced with a pre-extinction administration of a class I HDACi but not with RGFP966 (Bowers et al. 2015). An additional study found that pre-extinction administration of valproic acid (VPA, non-specific HDACi and GABAergic enhancer) rescued retrieval and consolidation deficits of fear extinction in learning impaired mice, yet MS-275 (non-specific HDACi thought to be HDAC1 selective) did not enhance extinction acquisition (Whittle et al. 2013).

One of few studies to investigate the effects of HDACi on operant drug extinction found that a post-extinction injection (IP) of a class I inhibitor (NaB) decreased nicotine-seeking during cued-extinction (one day after treatment), that at least six days of post-extinction treatment decreased the time it took to reach extinction criterion, and weakened reinstatement (Castino et al. 2015). This effect supports our results with post-session delivery, with faster rates of extinction criterion reached in the RGFP966-treated rats compared to vehicle-treated rats in our studies.

Supporting our findings for a role of HDAC3 in operant behavior, recent evidence demonstrated that post-session RGFP966 injections can enhance consolidation in a reward based auditory learning assay (Bieszczad et al. 2015) and the development of stimulus-response learning (Malvaez et al. 2018). This is further evidence that effects of HDAC3 (and potentially epigenetic regulation in general), directly relate to the learning event that occurs concurrently with drug treatment (McQuown and Wood, 2011) rather than altered behavior in general, or additional HDAC3 activities (e.g., TF recruitment; Liu and Bagchi, 2004; Nott et al. 2016). However, a limitation of our approach is that we used only one dose of RGFP966. Several previous studies have found that systemic administration of 10 mg/kg of RGFP966 promotes memory and plasticity (Bieszczad, et al. 2015; Malvaez et al. 2013; Phan et al. 2017). Malvaez et al. (2013) found that a lower dose (3 mg/kg) enhanced object memory relative to vehicle but this effect was smaller compared to 10 mg/kg and 30 mg/kg of RGFP966. They found similar relations with extinction of cocaine-induced CPP, where a 3 mg/kg dose did promote extinction, but at a slower rate compared to a 10 mg/kg dose. Our findings are in accordance with these other findings with the 10 mg/kg dose, but given some of the caveats with our extinction enhancements (less pronounced effects with post-session injections, for example), it will be important to fully characterize the dose-response effects of this compound in future studies. This importance is magnified by another study that failed to observe extinction enhancing effects in extinction of fear (Bowers et al. 2015) and by other studies that have found that HDAC inhibitors and other drugs can have opposite effects on behavior at higher and lower doses (Gulick & Gould, 2007; Schroeder, et al. 2007). Further, in the specific case of assessing the effects of a compound on the motivation to respond for cocaine, it will be important to evaluate the interaction between doses of the compound and doses of cocaine, both of which will change the sensitivity of progressive ratio responding (Depoortere et al. 1993).

Another limitation of our approach is that with a systemic injection, the brain regions that are implicated are unclear. Enhancements in extinction over multiple days that persists across context and cued reinstatement tests implicate not only HDAC3, but the involvement of multiple neural circuits. Other studies have found spatial memory enhancements induced by RGFP966 treatment (Malvaez et al. 2013) and focal HDAC3 deletions in the dorsal hippocampus have enhanced object location memory and cocaine CPP acquisition (McQuown et al. 2011; Rogge et al. 2013). Similarly, selective HDAC3 point mutations in the dorsal hippocampus enhanced memory retrieval of object location and memory formation during cocaine CPP extinction (Alaghband et al. 2017). It is therefore possible that context-induced reinstatement for cocaine seeking behavior and context-specific extinction may be particularity susceptible to HDAC3 modulation. Further experiments should evaluate how HDAC inhibitors work in a variety of circuits that may recruit different neurotransmitters (Whittle et al. 2016).

Our data represent the first evidence that pre-extinction administration of an HDAC3 inhibitor promotes rapid decreases of drug-seeking, promotes long-term learning early in extinction, and reduces context- and cue-induced reinstatement in operant behavior. This research confirms that HDAC3 inhibition can not only enhance Pavlovian but also operant extinction, and create a less context-dependent form of extinction that may persist across contexts and when exposed to drug-related cues. In addition, administration of the same HDAC3 inhibitor after extinction had a minimal effect on early extinction and reinstatement, but a pronounced effect on later extinction after reinstatement. These findings suggest that extinction enhancements can be revealed in different ways and they emphasize the importance of the use of multiple tests to reveal persistent extinction effects.

Finally, at a translational level, these results add to a growing body of literature suggesting that drugs that target histone acetylation coupled with behavioral experiences may be useful to pursue in the clinic. Promoting the rate of extinction may help to decrease attrition that occurs with prolonged therapies and promoting the persistence of extinction outside of the extinction context is the ultimate goal of any treatment. The difficulty is having control over what the patient experiences when HDAC inhibitors are delivered and it is likely that for this pharmacological treatment approach to be viable in the clinic, the clinician must take care to ensure that the experience of the patient in that setting is one consistent with the goal of the behavioral intervention.

Acknowledgments

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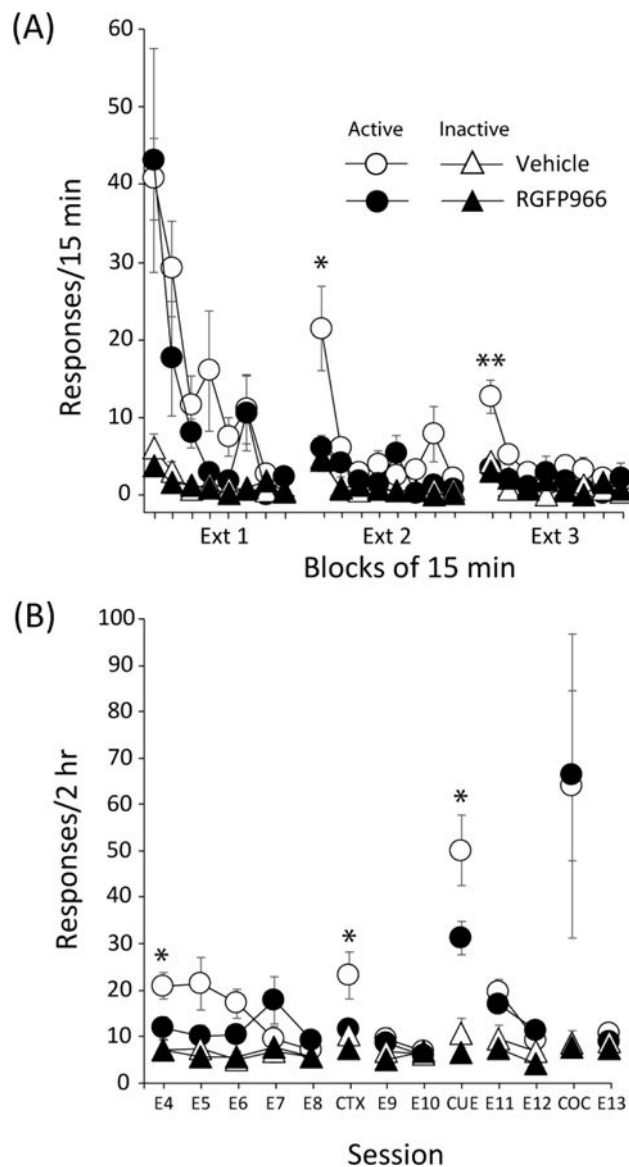
Figure 1. Effects of RGFP966 on extinction and reinstatement.
(A) Average active (reinforced, circles) and inactive (not reinforced, triangles) lever presses during the first three 2-hr extinction sessions (shown in 15-min time blocks). Rats received a single injection of Vehicle (VEH, N = 9) or RGFP966 (RGFP966, N = 9) 20 min prior to the first extinction session (Ext 1). **(B)** Responding during subsequent extinction and reinstatement sessions. Data are presented as means of the 2-hr sessions during additional extinction sessions (E4-E8, E9-E10, E11-E12, and E13), and reinstatement testing in a novel context (CTX), with the cue previously associated with cocaine (CUE), and following a priming injection of cocaine (COC). Error bars indicate the standard error of the mean (±SEM). * p<0.05, **p<0.005 (see text for statistical details).

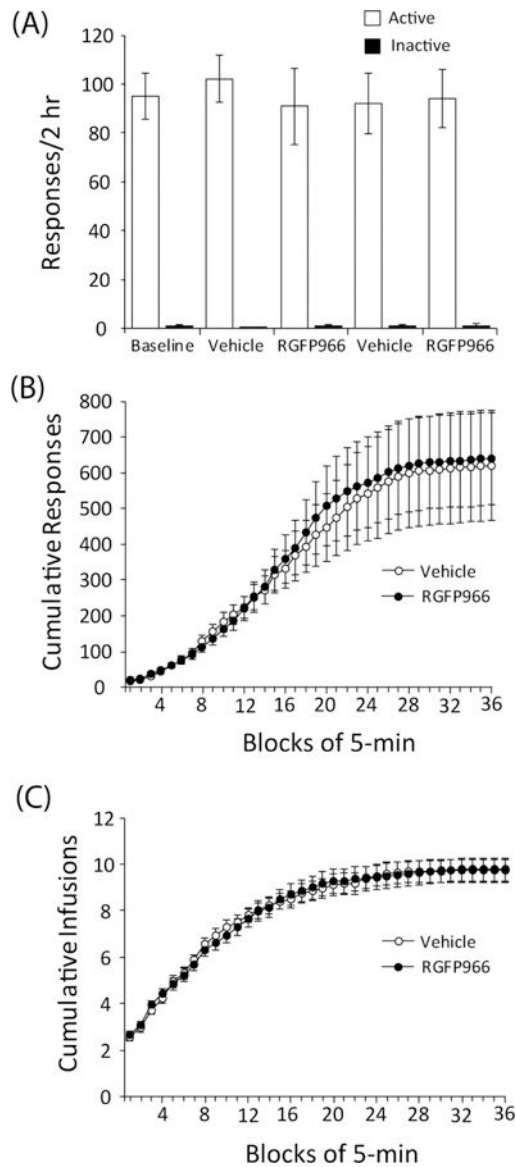**Figure 2. Acute effects of RGFP966 on asymptotic responding for cocaine on during an (A) FR5 or (B) progressive ratio schedule of reinforcement.**

(A) Average active and inactive lever presses during fixed ratio (FR5) maintenance self-administration (2-hr duration) sessions that were preceded by no injection (baseline) or injection of vehicle or RGFP966 (n=7). **(B)** Average active and inactive lever presses during 3-hr progressive ratio sessions during baseline and followed by vehicle or RGFP966 injections (n=23). Error bars indicate the standard error of the mean. The main effect of group was not reliable and did not interact with lever or time block.
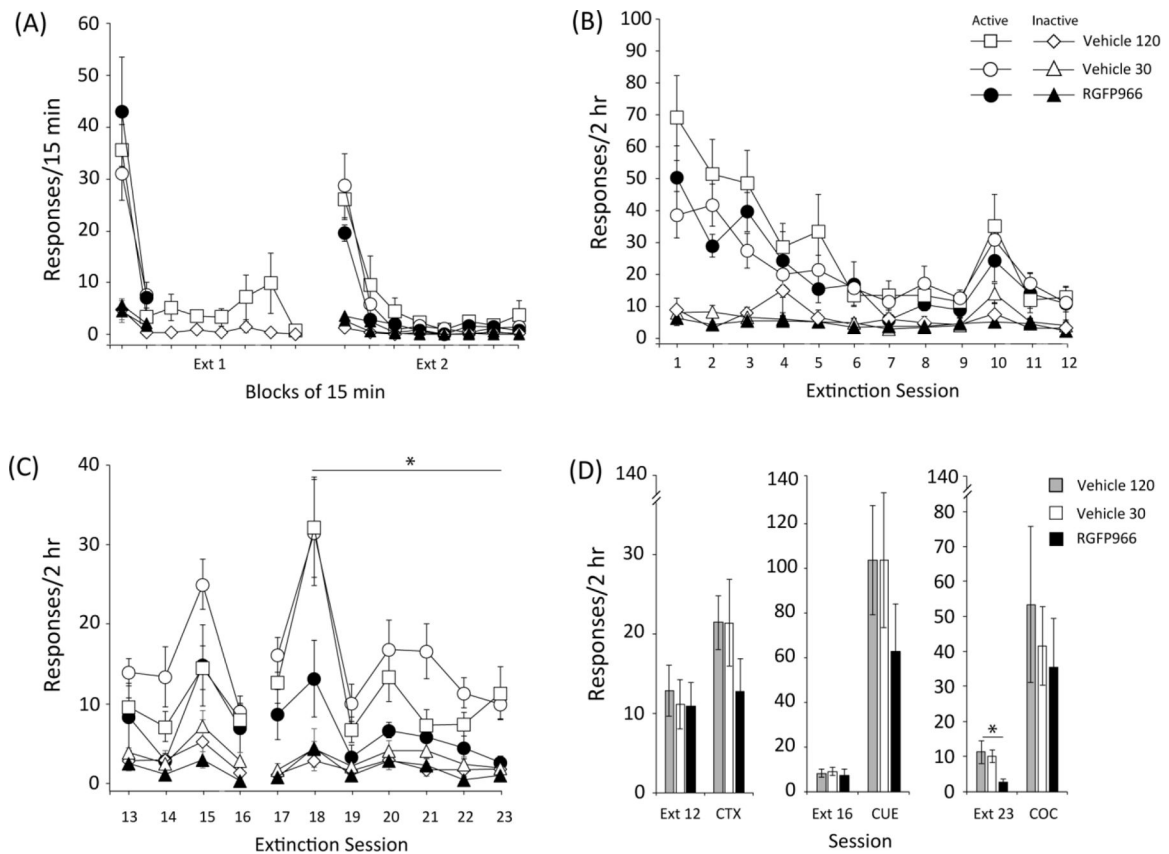
Figure 3. Effects of post-session RGFP966 on extinction and reinstatement.
(A) Average active and inactive lever presses during the first two extinction sessions (shown in 15-min time blocks). Two groups received a 30-min session duration in Extinction Session 1 followed by either injection of RGFP966 (n=7) or Vehicle (n=8). A third group received a 2-hr extinction session followed by Vehicle (n=8). All groups received a 2-hr extinction session the next day (Ext 2). Extinction across 2-hr sessions are shown for **(B)** Sessions 1–12 and **(C)** Sessions 13–23, which occurred between reinstatement sessions. **(D)** Responding during the first 12 extinction. **(C)** Responding during each extinction session prior to reinstatement and during reinstatement testing in a novel context (CTX), with the cue previously associated with cocaine (CUE), and following a priming injection of cocaine (COC). Error bars indicate the standard error of the mean (±SEM). * p<0.05 (see text for statistical details).