Novel Cues Reinstate Cocaine-Seeking Behavior and Induce Fos Protein as Effectively as Conditioned Cues

by

Ryan Bastle

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Graduate Supervisory Committee:

Janet Neisewander
Michael Foster Olive
Federico Sanabria

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ABSTRACT

The capability of cocaine-associated stimuli in eliciting craving in human addicts, even after extended periods of abstinence, is modeled in animals using cue reinstatement of extinguished cocaine-seeking behavior. This study aimed to examine brain activation in response to cocaine cues in this model apart from activation produced by test novelty using a novel cue control. Rats trained to self-administer cocaine paired with either an oscillating light or tone cue underwent daily extinction training and were then tested for reinstatement of extinguished cocaine-seeking behavior elicited by response-contingent presentations of either their assigned cocaine-paired cue or the alternate, novel cue. Additional controls received saline infusions and cue presentations yoked to a cocaine-trained rat. Brains were harvested for Fos immunohistochemistry immediately after the 90-min reinstatement test. Surprisingly, conditioned and novel cues both reinstated responding to a similar degree; however magnitude of reinstatement did vary by cue modality with the greatest reinstatement to the light cues. In most brain regions, Fos expression was enhanced in rats with a history of cocaine training regardless of cue type with the exception of the Cg1 region of the anterior cingulate cortex, which was sensitive to test cue modality. Also Fos expression within the dorsomedial caudate-putamen was correlated with responding in the novel, but not conditioned, cue groups. In subsequent experiments, we observed a similar pattern of reinstatement in rats trained and tested for sucrose-seeking behavior, whereas rats trained and tested with the cues only reinstated to a novel light and tone, but not a familiar cue. The results suggest that novel cues reinstate

responding to a similar extent as conditioned cues regardless of whether animals have a history of operant-delivered drug or a natural reinforcer. Furthermore, similar brain circuits as those involved in cocaine-seeking behavior are activated by novel cues, suggesting converging processes exist to drive conditioned and novel reinforcement seeking.

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Chapter 1

INTRODUCTION

Since the early 1990s, the drug addiction field has given much attention to uncovering the mechanisms of craving and relapse using the extinction/reinstatement animal model. This procedure allows researchers to examine individual factors known to elicit drug-seeking behavior, thereby elucidating potential interventions aimed at attenuating drug craving. The face and construct validity of the model, particularly with cue reinstatement, is demonstrated by the corresponding increase of craving reported by human addicts when presented with cocaine-related stimuli (Childress et al. 1988; Ehrman et al. 1992; O'Brien et al. 1990) and the return of drug-seeking behavior in animals when presented with cues previously paired with cocaine reinforcement (Markou et al. 1993; Shaham et al. 2003) as both of these measures are thought to reflect incentive motivational effects of the cues acquired through classical conditioning (Stewart 1983; Stewart et al. 1984). Further parallels are that neuroimaging studies in human addicts and examination of immediate early gene (IEG) expression in animals reveal the same brain regions activated by cocaine-paired cues (Childress et al. 1999; Grant et al. 1996; Kufahl et al. 2009; Wang et al. 1999; Zavala et al. 2008), further supporting construct validity of the model. In addition, compounds found to decrease self-reports of craving in humans have been shown to attenuate cue-elicited drug-seeking behavior in animals (Burmeister et al. 2003; Fuchs et al. 1998; Yahyavi-Firouz-Abadi and See 2009),

demonstrating predictive validity and overall utility of the cue reinstatement model.

Neural mapping with Fos protein expression is an established method for identifying brain circuits associated with cocaine-induced conditioning effects (Brown et al. 1992; Crawford et al. 1995; Neisewander et al. 2000). Fos is a product of the IEG *c-fos*, an inducible transcription factor important for the initiation of many signal transduction pathways (Curran and Morgan 1995). When an animal undergoes physiological and/or pharmacological manipulations, *c-fos* is transiently induced in relevant brain areas, with the resulting Fos protein expression yielding a histological marker of stimulus-induced brain activity (Chaudhuri 1997; Harlan and Garcia 1998). In response to cocaine or cocaine-paired cues, Fos is expressed in brain regions commonly associated with reward processing, memory, and drug abuse (Ciccocioppo et al. 2001; Hotsenpiller and Wolf 2002; Neisewander et al. 2000). More recently, we found that presentation of response-contingent cocaine-paired cues during reinstatement testing induced a widespread pattern of Fos expression throughout the brain, including several prefrontal cortical, limbic, and striatal subregions (Kufahl et al. 2009). Although the association between cue presentation and brain activation appears to be reliant on previous drug-stimulus pairings (Guo et al. 2008; Miller and Marshall 2004; 2005), little attention has been given to the contribution of novelty on test day. While cue-elicited reinstatement of cocaine-seeking behavior is known to depend on the sensory characteristics of the cues (See et al. 1999), the propensity for animals to be aroused or motivated by environmental or procedural novelty could

also contribute strongly to both behavior and Fos expression (Bardo 2002; Bardo et al. 1996; Piazza et al. 1989).

The purpose of this study was to investigate the specificity of both the reinstatement of cocaine-seeking behavior and its associated Fos expression. In Experiment 1, rats were trained to self-administer cocaine that was paired with presentation of either an oscillating light or tone, followed by extinction training where lever presses had no consequences. Half of the rats were then tested for reinstatement of cocaine-seeking behavior with conditioned cues and half were tested with a novel cue (i.e. a cue not present during training). We hypothesized that novel cues would not reinstate cocaine-seeking behavior, but would contribute to cue-induced Fos expression in some brain regions. To control for non-motivational contributions of cue exposure to the Fos response, control rats were given saline infusions paired with cues that were yoked to reinforcement delivery in the cocaine-trained rats. In Experiment 2, we examined the specificity of the findings for drug seeking by examining rats trained with sucrose or cue only reinforcement.

Chapter 2

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 225-250 g were housed individually in a temperature-controlled colony room with a 12- (Experiment 1) or a 14-h (Experiment 2/3) reversed light/dark cycle. Animal care and housing conditions were consistent with the Guide for Care and Use of Laboratory Animals (National Research Council, 1996). Surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee at Arizona State University. Rats were acclimated to handling for 7 days and weighed 250-325 g prior to surgery or training.

Surgery

Rats were administered an analgesic (buprenorphine, 0.05 mg/kg, s.c.) prior to induction of isoflurane anesthesia (2-3%; Abbott Laboratories, North Chicago, IL) vaporized in oxygen and delivered through a plastic nose cone. Catheters were constructed from Silastic tubing (10 cm length, 0.012 in inner diameter, 0.025 in outer diameter, Dow Corning, Midland, MI) connected to a 22-gauge nonferrous metal cannula encased within a plastic screw connector (Plastics One, Roanoke, VA). A miniature ball of aquarium sealant was affixed 2.7 cm from the free end of the catheter. A burrow was made subcutaneously from an incision on the neck to an incision across the skull, and the catheter was pulled through the burrow. A small incision was made in the jugular vein, where the catheter was inserted and secured with sutures on both sides of the ball. The

cannula end of the catheter was then anchored to the skull using dental acrylic cement and four small anchor screws. The head and neck incisions were sutured and treated with a topical antibiotic and the rats were administered an anti-inflammatory (meloxicam; 1 mg/kg, s.c.). A flexible obturator made from Tygon tubing was fitted over the cannula to protect the catheter. Patency of the catheters was maintained throughout the experiment by daily flushing with 0.1 ml timentin (66.67 mg/mL; bioWORLD, Dublin, OH) in saline solution containing 70 units/mL heparin sodium. Catheter patency was tested periodically with 0.8 g methohexital sodium (Brevital, Sigma), a dose that produces rapid loss of muscle tone only when administered i.v. Following surgery, rats were left to recover for 7 days in their home cages and were handled and weighed daily.

Apparatus

Training and testing were conducted in Plexiglas operant conditioning chambers (20 × 28 × 20 cm) equipped with a food pellet dispenser and a food well located between two levers mounted on the front panel (Med Associates, St. Albans, VT). A cue light was mounted above one lever, a tone generator (500 Hz, 10 dB above background noise) was mounted on the side wall and a house light was mounted on the rear wall opposite the levers. The lever below the cue light and nearest to the tone generator was designated as the active lever. Each conditioning chamber was housed within its own ventilated, sound-attenuating cabinet. An infusion pump containing a 10-ml syringe was located outside of the cabinet. Tygon tubing connected to the syringe was attached to a liquid swivel (Instech, Plymouth Meeting, PA) suspended above the operant conditioning

chamber. The outlet of the swivel was fastened to the catheter via Tygon tubing that ran through a metal spring leash (Plastics One). The leash fastened onto the plastic screw of the cannula that was anchored on the animal's head.

Experiment 1: Reinstatement and Fos expression elicited by conditioned versus novel cues

Self-administration training

Following recovery from surgery, self-administration (SA) training was conducted during daily 2-h sessions where rats were trained to press the active lever to receive cocaine reinforcement (0.75 mg/kg/0.1 ml, i.v.). Upon completing a schedule of reinforcement, either the blinking cue light or the pulsed tone were activated, followed one second later by activation of the infusion pump for six seconds. Following infusion, the cue light or tone was inactivated. After a 20-s timeout period, responses on the active lever were accumulated toward the next reinforcement schedule. Note that rats receiving the tone versus light cues were trained in separate rooms so that the tone would be completely novel to animals that were trained with the light cue.

For the first 5 days of training, all animals began on a fixed ratio (FR) 1 schedule of reinforcement with the capability to progress to a variable ratio (VR) 2, VR3, and finally VR5 schedule. After ending the session on a VR5 schedule for two consecutive days, animals then began the remaining sessions on a VR5 schedule. In this experiment, all animals were on a VR5 schedule exclusively for at least the last 5 days of self administration, which was considered our acquisition criteria. All animals were restricted to 16 g/day of food to facilitate

acquisition of self-administration (Carroll et al. 1981) and remained food-restricted until they ended on a VR5 schedule for three consecutive sessions. Animals were then given food *ad libitum* for the rest of the experiment. The total number of training days ranged from 13 to 21, depending on the rate of acquisition.

Extinction training

Extinction training began the day after SA training was completed. Rats were exposed to the SA environment for a 90-min session each day across 14-15 consecutive days. During these sessions, lever presses had no scheduled consequences; the rats were connected to the swivel but no infusions or light/tone cues were delivered. Responses on the active and inactive lever were recorded. Since the rats were exposed to the SA environment but not the discrete cocaine-paired cues, the incentive motivational effects of these stimuli presumably remained intact. At the end of extinction training, all rats exhibited a decrease in response rates on the active lever to less than 20 responses during a session or to 20% of the peak response rate that occurred during extinction training. Responding during the terminal extinction session was used as a baseline for statistical comparison to responding on the test day.

Test for reinstatement of cocaine-seeking behavior

Cocaine-seeking behavior was operationally defined as responses on the active lever in the absence of cocaine reinforcement. On the day after the final extinction session, rats were placed into their conditioning chambers for 90 min, during which responses on the active and inactive levers were recorded. The test

session length was chosen for optimal expression of stimulus-induced Fos protein expression (Moratalla et al. 1993).

The cocaine-trained rats were assigned to either a Novel or a CS test cue condition, with $n = 17$ in each group. For rats in the CS test cue condition, the cocaine-paired stimulus-complex (i.e. blinking cue light or pulsed tone and pump motor) was delivered on a FR1 schedule of reinforcement. For rats in the Novel test cue condition, the stimulus was a novel cue (i.e. the blinking cue light for rats trained with the tone, and the oscillating tone for rats trained with the cue light), also delivered on a FR1 schedule. Saline-yoked rats were exposed to the same cues as their cocaine-trained partners, but responses on the active lever from these rats had no consequences.

Tissue preparation

Immediately following reinstatement testing, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.). Their circulatory system was perfused with 200 ml ice-cold saline followed by 250 ml ice-cold paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Brains were removed and post-fixed in paraformaldehyde for 24 h, cryoprotected by submersion in 15% sucrose for 24 h and then submerged in 30% sucrose for at least 24 h. The brains were then sectioned using a sliding microtome (Microm International, Walldorf, Germany) connected to a freezing stage (Physitemp, Clifton, NJ). Serial coronal 40 μm sections were collected, separated by 120 μm, centered at anatomical locations corresponding to bregma +3.2, +1.6, -2.56, and -5.6 mm (Paxinos and Watson 1998). The tissue sections were then frozen and

stored at 4º C in a cryoprotectant solution comprised of 0.1 M PB (pH 7.2), 30% ethylene glycol, and 25% glycerol.

Fos protein immunohistochemistry

Free floating tissue sections were washed in 0.1 M PB (pH 7.2, nine times for 10 min each), incubated for 30 min in 1% H_2O_2 diluted 3:100 in 0.1 M PB, incubated for 30 min in 3% Normal Goat Serum (NGS; Vector Laboratories, Burlingame, CA), and then incubated for 72 h at 4ºC with rabbit polyclonal anti-Fos serum (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:5,000 in 0.1 M PB containing 0.1% bovine serum albumin (Fisher Scientific, Fail Lawn, NJ), 0.2% Triton X-100 (Fisher Scientific), and 2 % NGS. Following incubation, the sections were washed in 0.1 M PB (three times for 10 min each) and then incubated for 1 h at room temperature in biotinylated goat anti-rabbit IgG antibody (Vector Laboratories), diluted 1:500 in 0.1 M PB containing 0.1% bovine serum albumin, 0.2% Triton X-100, and 2 % NGS. The tissue was then washed in 0.1 M PB (three times for 10 min each) and then incubated for 90 min in Extravidin Peroxidase (Sigma-Aldrich, St. Louis, MO), diluted 1:1000 in 0.1 M PB containing 0.1% bovine serum albumin, 0.2% Triton X-100, and 2 % NGS. The tissue was washed in 0.1 M PB (nine times for 10 min each) and then in solution containing 0.02% 3,3'-diaminobenzidine (DAB; Sigma-Aldrich) for 20 min. The sections were then incubated in glucose oxidase (Sigma-Aldrich) for 10 min. The DAB reaction was terminated by rinsing the tissue six times for 10 min in 0.1 M PB. All of the washes and incubations described above were performed on an orbital shaker (Cole-Parmer, Vernon Hills, IL) operating at 90 rpm. The

sections were mounted onto gelatin chromium-coated slides, air-dried, dehydrated and protected with a coverslip for light-microscopic inspection.

Fos immunoreactivity analysis

Fos immunoreactivity was examined using a Nikon Eclipse E600 (Nikon Instruments, Melville, NY) microscope set at 20× magnification and counted by an observer blind to treatment conditions using the ImageJ software package (Version 1.45, National Institutes of Health, USA). The anatomical locations and boundaries of each region were determined using a rat brain atlas (Paxinos and Watson 1998) and are illustrated in Figure 1A. Sections taken at +3.2 mm from bregma contained the Cg1 region of the anterior cingulate cortex (Cg1). Sections taken at +1.6 mm from bregma contained the Cg2 region of the anterior cingulate cortex (Cg2), dorsomedial caudate/putamen (dmCPu), dorsolateral caudate/putamen (dlCPu), nucleus accumbens shell (NAcS), and nucleus accumbens core (NAcC). Sections taken at -2.56 mm from bregma included the basolateral amygdala (BlA). Sections taken at -5.6 mm from bregma included the ventral tegmental area (VTA). The sections were taken such that the rostral-caudal extent of each region of interest was sampled (340 μm). Fos immunoreactivity was counted and identified by black oval-shaped nuclei (Figs. 1C-D). Each region of interest was analyzed using both hemispheres from three tissue sections from each animal. The area of each sample measure was 0.26 mm^2 and the counts from all the sample areas from a given region were averaged and scaled to provide a mean number of Fos-positive cells per mm^2.

Experiments 2 & 3: Reinstatement after training with sucrose or cue reinforcement only

Rats in Experiment 2 received response-contingent cue presentations (either the light or tone) paired with delivery of a sucrose pellet (45 mg, Bio-Serv, Frenchtown, NJ, USA) whereas rats in Experiment 3 received response-contingent cue presentations with no other reinforcer during training. The latter served to examine the reinforcing effects of the cues themselves. The procedures used in these experiments were identical to those used in Experiment 1 with the following exceptions: 1) rats in Experiment 2 received approximately 30 sucrose pellets in their home cage prior to sucrose reinforcement training in order to familiarize them with the pellets, 2) no surgery was performed on the animals from either experiment, 3) the training, extinction, and test sessions in both experiments were only 30 min in duration to avoid satiation found with longer training sessions (Bizo et al. 1998) and to attain a comparable number of cue presentations per session as Experiment 1.

Statistical Analysis

ANOVAs were performed to analyze reinforcement rates during training where training cue (light, tone) was a between-subjects factor and session was a within-subjects factor. Separate ANOVAs were used to analyze responses on the active and inactive lever during extinction, where training cue (light, tone) was a between-subjects factors and session was a within-subjects factor. Separate ANOVAs were also used to analyze responses on the active and inactive lever during reinstatement, where test cue condition (novel, CS) and test cue modality

(light, tone) were between-subjects factors and day (baseline, test) was a within-subjects factor. Separate ANOVAs, with drug history (cocaine, saline-yoked), test cue condition, and test cue modality as between-subjects factors, were used to analyze Fos protein expression for all brain regions studied. Significant interactions and main effects were followed by smaller ANOVAs, tests for simple effects, and *post hoc* tests (Tukey, Bonferroni), where appropriate. Additionally, the correlation between test-day responses on the active lever and Fos expression separated by test cue condition (novel, CS) was calculated using the Pearson product-moment correlation (r) for Experiment 1.

Chapter 3

RESULTS

All descriptive statistics are reported as mean ± standard error of the mean.

Experiment 1

Cocaine self-administration

The 16 light-trained cocaine rats received a total of 504 ± 46 cocaine infusions and the 18 tone-trained cocaine rats received a total of 413 ± 32 cocaine infusions during self-administration training. This difference in infusion rates was due to the rapid acquisition of operant responding in the light-trained rats (Fig. 2A). A significant interaction between session and training cue was found ($F_{3.7,\ 116.9} = 2.56$, $p < 0.05$), where tests of simple effects revealed that on the first four days of self-administration, the light-trained groups achieved more infusions than the tone-trained groups (ANOVAs, ps > 0.05). However, analysis of total infusions across all sessions (i.e., number of sessions ranged between 13 and 21 depending on acquisition rate) did not differ between the two training cue groups (Fig. 2B; *t*-test, $t_{32} = 1.66$, $p = 0.11$).

Extinction and reinstatement of cocaine-seeking behavior

Following cocaine self-administration, extinction training significantly reduced responding across sessions (Fig. 3A), where a main effect of session was found on the active ($F_{2.6,\ 82.4} = 33.3$, $p < 0.001$) and inactive ($F_{4.2,\ 135.6} = 3.38$, $p < 0.05$) levers and there were no group differences in response rates. *Post hoc* analyses revealed that last-day extinction responses on both levers were

significantly reduced from first-day responses (Bonferroni correction, *p*s < 0.025). No significant changes in responses on the active or inactive lever were found in the saline-yoked groups (data not shown).

To assess reinstatement of cocaine-seeking behavior, we compared responses on the active lever during testing to responses on the active lever during the last extinction session (i.e., baseline), with day as a within-subjects factor and test cue condition and test cue modality as between-subjects factors. In contrast to our predictions, no day × test cue condition interaction was found ($F_{1, 30} = 0.82$, $p = 0.37$), indicating that rats responded similarly to conditioned and novel stimuli during testing. An interaction between day and test cue modality was found ($F_{1, 30} = 10.0$, $p < 0.01$), where tests for simple effects of day revealed that rats tested with both cue modalities reinstated lever pressing compared to their extinction baseline (Fig. 3B; ANOVAs, *p*s < 0.001), but analysis of group effects on the test day ($F_{1, 32} = 10.4$, $p < 0.01$) revealed differences in the magnitude of reinstatement where rats tested with a light had higher response rates than tone-tested rats (test for simple effects; Fig. 3B). No group differences were found during baseline ($F_{1, 32} = 0.12$, $p = 0.73$).

Although no interactions were found in responses on the inactive lever, there was a main effect of test cue condition for the cocaine ($F_{1, 30} = 4.49$, $p < 0.05$) and saline-yoked ($F_{1, 12} = 6.81$, $p < 0.05$) rats, where the novel cue groups had higher response rates on the inactive lever than the CS groups, regardless of day or test cue modality (Table 1). No interactions or main effects of responding on the active lever were observed for saline-yoked groups (data not shown).

Fos Immunoreactivity

Rats in the cocaine groups exhibited significantly more Fos-immunoreactive nuclei than rats in the saline-yoked groups in all of the brain regions analyzed, with the exception of the VTA. This enhancement was evident as a significant main effect of drug history in the dmCPu, dlCPu, NAcC, NAcS, and BlA (Fig. 4; ranged from $F_{1, 45} = 7.1$ to 18.1, ps < 0.05). No other significant main effects or interactions were found in these regions, implying that Fos expression was enhanced in the cocaine-trained animals, regardless of cue conditioning or modality. In the Cg1 region of the anterior cingulate cortex, a significant main effect of drug history was also found ($F_{1, 40} = 9.45$, $p < 0.01$), as well as a significant drug history × test cue modality interaction ($F_{1, 40} = 4.32$, $p < 0.05$). Subsequent ANOVAs for the two drug conditions revealed that saline-yoked control rats tested with a light cue expressed significantly more Fos-positive nuclei than the rats tested with a tone (Fig. 5; $F_{1, 16} = 6.84$, $p < 0.05$). No such difference was found in the cocaine-trained groups ($F_{1, 32} = 1.05$, $p = 0.31$). In the Cg2 region of the anterior cingulate cortex, a significant main effect of drug history was also found (Fig. 3A; $F_{1, 40} = 9.33$, $p < 0.01$), as well as a significant drug history × test cue condition × test cue modality interaction ($F_{1, 40} = 4.32$, $p < 0.05$). Subsequent ANOVAs for the two drug conditions revealed a test cue condition × test cue modality interaction in the cocaine-exposed rats ($F_{1, 28} = 7.14$, $p < 0.05$), where *post hoc* analyses revealed a trend toward a higher number of Fos-positive nuclei in the novel light compared to the CS light group (Tukey, $p =$

0.09), which likely contributed to the interaction. No interactions or main effects were found in the Cg2 of the saline-yoked animals.

Correlations were also conducted within the cocaine groups separately for the novel versus CS test cue conditions to determine whether Fos expression in certain regions was related to the magnitude of responses on the active lever during testing. Interestingly, a positive correlation was found in the dmCPu for the novel (Fig. 6A; $r = 0.70$, $p < 0.01$), but not the CS (Fig. 6B; $r = 0.12$, $p = 0.67$) test cue condition, indicating that the higher responding on the active lever when tested with a novel cue the higher the expression of Fos in the dmCPu. No other significant correlations were found in the remaining brain regions.

Experiment 2

Sucrose Reinforcement Training

The 15 light-trained sucrose rats received a total of 382 ± 33 sucrose pellets and the 18 tone-trained sucrose rats received a total of 412 ± 22 sucrose pellets during training. A significant interaction between session and training cue was found ($F_{4.89, 154.3} = 2.38$, $p < 0.05$), where tests for simple effects of group revealed that the tone-trained rats exhibited higher reinforcement rates relative to light-trained rats during the transition from mild food restriction to *ad libitum* access, specifically on days 7 and 8 (Fig. 7; ANOVAs, $p < 0.05$). Overall, there was no group difference in the total number of pellets received across all training sessions (Fig. 7 inset; $F_{1, 31} = 2.48$, $p = 0.13$).

Extinction and reinstatement of sucrose-seeking behavior

Responses on the active lever reduced across extinction training (Fig. 8A), where a main effect of session was found ($F_{1.8, 56.4} = 92.1$, $p < 0.001$), and there were no group differences in response rates. *Post hoc* analyses revealed that last-day extinction lever presses were significantly reduced from first-day extinction lever presses (Bonferroni correction, $p < 0.05$). No significant changes were found during extinction for responding on the inactive lever (Fig. 8A).

To assess reinstatement of sucrose-seeking behavior, we compared responses on the active lever during testing to responses on the active lever during the last extinction session, with day as a within-subjects factor and test cue condition and test cue modality as between-subjects factors. Only a main effect for day was found ($F_{1, 29} = 91.5$, $p < 0.001$), indicating that test day responding increased compared to extinction baseline regardless of other factors (Fig. 8B). An interaction was found for inactive lever responding between day and test cue modality ($F_{1, 29} = 4.62$, $p < 0.05$), but tests for simple effects of day or group failed to reveal differences in responding compared to extinction baseline nor group differences during baseline or test day between test cue modalities. There was also a test cue condition × test cue modality interaction ($F_{1, 29} = 4.61$, $p < 0.05$), but *post hoc* analyses failed to reveal any group differences (Tukey, ps > 0.05). The novel light group appeared to have a higher response rate than all other groups on test day (Fig. 8B), which may have contributed to these effects.

Experiment 3

Cue reinforcement training

Across training, the 18 light cue-trained rats received a total of 65 ± 7 light presentations and the 18 tone cue-trained sucrose rats received a total of 46 ± 3 tone presentations. No interaction between session and training cue was found (Fig. 9; $F_{14, 448} = 0.94$, $p = 0.52$), but the light cue group received more cue presentations than the tone cue group overall (Fig. 9 inset; $F_{1, 32} = 5.73$, $p < 0.05$). We also examined responding on the inactive lever and observed no interaction or group differences, although there was a main effect of session ($F_{5.4, 172.5} = 3.06$, $p < 0.01$) due to nonsystematic variation across sessions regardless of training cue (data not shown).

Extinction and test day responding for the Cue-Only condition

A significant interaction between session and training cue was found ($F_{6.0, 192.8} = 3.16$, $p < 0.01$), where tests of simple effects of group revealed that the light-trained group had higher responding on the active lever than the tone-trained group on several days throughout extinction (Fig. 10A; ANOVAs, ps < 0.05). In terms of extinction behavior, *post hoc* analyses revealed that last-day extinction lever presses were significantly reduced from first-day extinction lever presses in the light-trained group (Fig. 10A; Bonferroni correction; $p < 0.025$), but not in the tone-trained group. No significant changes were observed during extinction for responses on the inactive lever (Fig. 10A).

Surprisingly, analysis of the cue-only condition reinstatement data revealed a significant interaction between day, test cue condition, and test cue

modality ($F_{1, 30} = 4.55$, $p < 0.05$), indicating that test day behavior was under the influence of both test cue familiarity and cue modality. Tests for simple effects of day revealed that both the novel light and novel tone groups increased responding compared to extinction baseline, although in the latter case the effect was marginally significant ($p = 0.05$; Fig. 10B). Subsequent two-way ANOVAs during baseline and test day with test cue condition and test cue modality as between-subjects factors revealed no interactions or main effects during baseline, but main effects of test cue condition and test cue modality during test day ($F_{1, 30}$ = 5.09-9.22, ps < 0.05) where rats tested with a novel cue and a light cue had higher response rates than CS- and tone-tested rats, respectively (Table 2). On the inactive lever, interactions between day and both test cue condition ($F_{1, 30} = 5.81$, $p < 0.05$) and test cue modality ($F_{1, 30} = 5.55$, $p < 0.05$) were found, where tests for simple effects of day revealed that rats tested with a tone decreased responding compared to their baseline (Fig. 10B; $F_{1, 16} = 5.31$, $p < 0.05$), while rats tested with their CS+ cue exhibited only a trend toward a decrease in responding ($F_{1, 17} = 3.83$, $p = 0.07$). Tests for simple effects of group revealed no differences during baseline, but on test day rats tested with a light responded higher than tone-tested rats (Table 2; $F_{1, 32} = 8.42$, $p < 0.01$) and rats tested with a novel stimulus responded higher than CS-tested rats (Table 2; $F_{1, 32} = 4.61$, $p <$ 0.05). Again, these effects appeared to be influenced by the relatively high rate of responding on test day in the novel light group compared to all other groups (Fig. 10B).

Chapter 4

DISCUSSION

The aim of our study was to use a novel cue control group in order to parse out the contribution of experiencing novelty during cue reinstatement testing to Fos expression. On test days of typical extinction/reinstatement procedures, the animal experiences novelty in receiving a response-contingent stimulus (e.g. CS+) following a prolonged period of non-reinforced responding. Fos expression is sensitive to environmental and procedural novelty effects (Badiani et al. 1998; Handa et al. 1993; Papa et al. 1993) and we wanted to compare expression when the animal is exposed to drug-paired versus novel stimuli to further elucidate the neural circuitry specific to drug-conditioning. Surprisingly, we found that novel cues elicited similar reinstatement behavior as conditioned stimuli (Fig. 3B) and Fos analysis in the examined brain regions revealed parallel patterns of expression between the two test cue conditions. These unexpected results led us to hypothesize that cocaine self-administration may have cross-sensitized reinforcement circuits to novelty, which acts on converging reward pathways (Besheer et al. 1999; Bevins and Bardo 1999; Bevins et al. 2002). To address this question, we subsequently used a natural reinforcer, sucrose, to examine whether novel cues reinstated sucrose-seeking behavior. Non-drug reinforcers cross-sensitize behavior only under more limited circumstances than cocaine (for review, see Avena et al. 2008) and we chose parameters that are not consistent with sensitizing effects from sucrose reinforcement (Avena et al. 2005). Nevertheless, a similar outcome occurred

where all sucrose groups reinstated regardless of whether they received CS+ or novel cues (Fig. 8B), suggesting that drug-specific sensitization was not a likely explanation, and instead novel cues had similar effects on drug and non-drug reinforcement-seeking behavior.

The rewarding properties of novelty have been well documented in humans and animals (Bardo et al. 1996; Bardo et al. 1989; Besheer et al. 1999; Bevins and Bardo 1999; Bevins et al. 2002; Fagan 1970), and the degree of motivation for novelty has been a well recognized factor in predicting individual sensitivity to drug reward and reinforcement (Belin et al. 2011; Pelloux et al. 2006; Piazza et al. 1989). We therefore suggest that novel stimuli in our procedure may activate similar reinforcing processes as those involved in conditioned reinforcement-seeking behavior, which may be driving heightened responding on test day. In particular, brain regions engaged by novel cues show highly similar patterns of Fos expression as those found during CS+ re-exposure (Kufahl et al. 2009; Neisewander et al. 2000) and these regions are known to play a role in conditioning and expression of cocaine-seeking behavior (Fuchs et al. 2006; Fuchs et al. 2004; Fuchs et al. 2002; Ito et al. 2004; Kalivas and McFarland 2003; Kruzich and See 2001; McLaughlin and See 2003). Our test-day behavior of the cue-only condition further supports the reinforcing properties of response-contingent novel stimuli (Baron and Kish 1962), where the novel light and tone produced reinstatement-like behavior.

In contrast to reward, the stress-provoking effects of novel stimuli and procedures have also been examined in rodents (Kabbaj et al. 2000; Piazza et al.

1991; Rozin and Kalat 1971). Stress is known to facilitate both drug reinforcement and incentive motivation, where various stressors have the ability to reinstate drug seeking (Bossert et al. 2005; Shaham et al. 2003), but less consistently sucrose seeking (Buczek et al. 1999; Simms et al. 2011). The task of interpreting the bio-behavioral effects of novelty-induced stress is known to be complex (Beerling et al. 2011). Novel stimuli have the tendency to induce both approach and avoidance in animals, and it is the balance between these two conflicting motivations that drive subsequent behavior (Montgomery and Monkman 1955). Although exposure to discrete novel cues in our study may have induced an initial stress response, the animals appeared to approach, rather than avoid, making responses to receive novel cue presentations. This suggests that potential stress effects of the novel cues likely facilitated, rather than inhibited, the animal's motivation to seek reinforcement.

Although the stimulating effects of novel stimuli may have directed the animal to the active lever, it cannot alone account for the total motivating force behind the animal's behavior. The degree of novel cue reinstatement was robust in the cocaine and sucrose conditions, but relatively weak in the cue-only condition. Perhaps a previous history of reinforcement paired with response-contingent stimuli may have induced a strong action-outcome habit that a response-contingent novel cue re-engages during testing. Prolonged appetitive training is known to initially show goal-directed behavior and then transition to habitual responding (Belin et al. 2009; Jog et al. 1999), and contingent reinforcers likely facilitated this transition in our study, as well as initial acquisition

(Deroche-Gamonet et al. 2002). Furthermore, in a previous study a novel light/tone combination failed to produce reinstatement in animals that were not exposed to cocaine-paired cues during training (Kruzich et al. 2001), suggesting that reinforcement exposure alone during training does not produce sensitized responding to novel stimuli. Therefore, novel cues are able to reinforce responding reliably when they are presented similarly to conditioned stimuli, possibly by initiating habits that are resistant to stimulus modality changes.

While novel and conditioned cues elicited similar seeking behavior and brain activation, response magnitude and Fos effects in some cases varied depending on the stimulus modality. Several other studies have reported an impact of cue modality on responding, where a light cue was capable of eliciting greater responding than a tone cue (Baron and Kish 1962; Di Ciano and Everitt 2003; Panlilio and Schindler 1997; Reed et al. 1996; See et al. 1999). We found that a light was more effective than a tone during cocaine self-administration (Fig. 2A) and reinstatement, as well as during training sessions when only a cue served as the reinforcer (i.e. cue-only condition; Fig. 9). A light cue also induced greater Fos expression in the Cg1 of the saline-yoked control groups compared to the tone (Fig. 5). Although this might not be related to operant behavior per se, the effect may be due to attention because this region is engaged in the presence of salient stimuli (Downar et al. 2002). In contrast, the tone cue appeared to elicit slightly greater responding during sucrose training (Fig. 7). These findings are curious, but could be due to the ability of psychostimulants to amplify sensitivity to stimuli (Davis 1985; Davis et al. 1975), where the initial reinforcing and

aversive properties of response-contingent light and tone cues, respectively, may be more pronounced (Baron and Kish 1962). Regardless, our cue modality effects appear to be consistent with the sensory reinforcement literature.

An interesting finding regarding differences in Fos expression in response to novel versus CS+ cues is that in the dmCPu, where high responding during test day was positively correlated with the degree of Fos in the novel, but not conditioned, cue groups. Although the cause of this relationship is unclear, several features and functions of the dmCPu suggest possible explanations. In terms of brain connectivity, projections from the visual/auditory cortices to the dmCPu may contribute to the relationship (Faull et al. 1986; McGeorge and Faull 1989), where a greater number of cue presentations may recruit greater input from sensory regions for novel versus familiar cues. Our reinstatement data support this claim, where cue modality had a greater effect on responding in the novel versus conditioned cue groups. In relation to cognitive-behavioral measures, the dmCPu has been implicated in behavioral flexibility and attention (for review, see Devan et al., 2011), where regional inactivation has been shown to disrupt dimensional shifting in a discrimination task and impair performance of a visually signaled time-dependent response task, respectively (Christakou et al. 2005; Ragozzino et al. 2002). Animals exhibiting low novel cue responding may lack the sensory dimensional shift and pay less attention to the stimuli, whereas the higher responding animals may show greater modality flexibility and attention to the novel stimulus. In addition, animals displaying greater novelty-seeking behavior exhibit greater *c-fos* mRNA in the dmCPu than low novelty-seekers, further

supporting our novelty-specific effects in this region (Kabbaj and Akil 2001). While the conditioned cues in our procedure may have recruited previously established habits where cognitive input is less essential, the dmCPu appears to participate in the processing and magnitude of behavioral output in response to the novel cue.

Beyond the differential contributions of the discrete cue characteristics, our procedure of testing in the original training context may also contribute to responding for novel stimuli during the test session because it is possible that any response-contingent consequence may reignite the incentive motivational value of the reinforcement context. It is well established that contextual manipulations exert control over operant responding, where reinforcement seeking and other conditioned responses are reinstated by exposure to the training context following a period of prolonged abstinence or extinction in a different context (Crombag et al. 2008; Crombag and Shaham 2002; Bouton and Peck 1989; Neisewander et al. 2000). In contrast, exposing rats to a novel context during testing (i.e. an AAB procedure) does not reinstate drug-seeking (Bossert et al. 2004; Crombag and Shaham 2002; Fuchs et al. 2005), or conditioned responses (Bouton and King 1983), highlighting the unique impact of the original training context on responding. The incentive motivational value of the context may be necessary to achieve heightened novel cue responding, perhaps through the training environment acting as discriminative stimuli or an ‘occasion setter’ (Alleweireldt et al. 2001; Holland 1992; Swartzentruber 1991). The importance of contextual

contributions to novel cue reinstatement of drug-seeking behavior will require further attention.

In order to integrate our proposed mechanisms of novel cue reinstatement, it may be worth relating our work to Robinson and Berridge's (1993) theory of incentive sensitization. According to the researchers, two dissociable processes in the form of 'liking' and 'wanting' changes over the course of addiction. While the hedonistic value of the drug-taking experience (i.e. 'liking') appears to decrease or remain stable over time, the motivation to seek out drug (i.e. 'wanting') increases and can persist even after extended periods of discontinued drug use (Lamb et al. 1991; Robinson and Berridge 1993; Robinson and Berridge 2008). The development of incentive sensitization is presumably due to the associative processes occurring over repeated drug-taking sessions, where the organism attributes incentive salience to drug-associated stimuli and this effect increases over time. In our experiment, we believe prior training with a reinforcer may have induced incentive sensitization where subsequent testing with a conditioned cue easily triggers the sensitized motivational circuits through associative learning processes. However, when a novel cue is presented during testing, the lack of direct association with previous reinforcement is superseded by other associative processes involving contextual stimuli and lever-contingent cue presentations, as well as greater modulation by the intrinsic salience of the novel stimuli, as we demonstrated with the cue-only condition. Although different, the novel and conditioned stimuli may be activating the same sensitized motivational circuit that developed during training, thus resulting in similar reinstatement effects.

Although we were unable to delineate the unique drug-conditioning contribution of stimulus presentation to Fos protein expression during reinstatement testing in our procedure, we have uncovered novel information that calls for additional thought about how reinstatement of reinforcement-seeking behavior is mediated. It appears that novelty can trigger incentive motivation and activate brain reinforcement circuits similarly to that of conditioned stimuli. We have suggested potential mechanisms that may mediate or contribute at least in part to novel cue reinstatement. These include: 1) novel stimulus-elicited activation of brain reinforcement mechanisms that in turn drive reinforcement-seeking behavior similar to CS+ stimuli; 2) response-contingent events re-engaging action-outcome processes that have become habitual; and 3) the ability of novelty or CS+ reinforcement to reinstate the incentive motivational effects of the reward-associated context. In terms of drug abuse and other addictive behaviors, the present findings suggest novel potential mechanisms by which environmental stimuli may disrupt abstinence as effectively as stimuli known to be associated with reinforcement. Further understanding of how novelty relates to craving for drugs of abuse or natural reinforcers is important for understanding relapse and for future therapeutic interventions.

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Table 1. Experiment 1 average inactive lever presses (± SEM) for cocaine and saline-yoked groups during last day extinction and reinstatement test sessions.

Group		Inactive Lever Presses / 90 min	
Drug History	Test Cue Condition	Baseline	Test Day
Cocaine	CS	3.6 ± 1.5	4.0 ± 1.4
	Novel*	13.8 ± 5.8	22.4 ± 8.7
Saline-yoked	CS	2.8 ± 1.1	2.5 ± 1.3
	Novel*	7.6 ± 2.2	5.6 ± 3.1

*Different from respective CS groups ($p < 0.05$)

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Table 2. Experiment 3 average test day response rates (± SEM) for cue-only groups.

Group			Test Day Response Rates	
Reinforcement	Test Cue Modality	Test Cue Condition	Active Lever Presses / 30 min	Inactive Lever Presses / 30 min
Cue Only	Light-tested	CS	7.0 ± 2.5*	4.1 ± 1.3*
		Novel	30.4 ± 9.6*†	10.0 ± 2.2*†
	Tone-tested	CS	4.1 ± 1.0	2.1 ± 0.5
		Novel	10.9 ± 3.3†	3.0 ± 1.1†

*Different from respective tone-tested groups ($p < 0.05$)
†Different from respective CS-tested groups ($p < 0.05$)

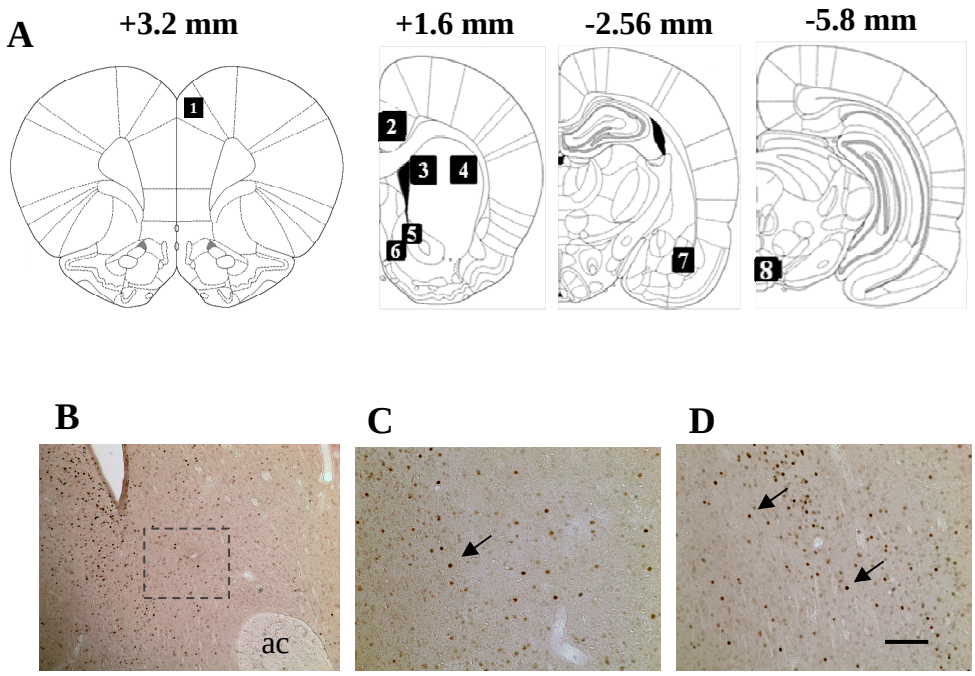

Figure 1. Schematic representations (A) illustrating the regions analyzed in brain sections taken at +3.2, +1.6, -2.56, and -5.8 mm from bregma (Paxinos and Watson, 1998), and representative photomicrographs of Fos protein expression in the NAcC with a dashed rectangle indicating sample region at 10× magnification (B), a sample of the NAcC from a saline-yoked novel tone rat (C) and cocaine novel-tone rat (D) with arrows indicating Fos-positive nuclei. The numbered regions are as follows: (1) Cg1 region of the anterior cingulate cortex (Cg1); (2) Cg2 region of the anterior cingulate cortex (Cg2); (3) dorsomedial caudate/putamen (dmCPu); (4) dorsolateral caudate/putamen (dlCPu); (5) nucleus accumbens core (NAcC); (6) nucleus accumbens shell (NAcS); (7) basolateral amygdala (BlA); (8) ventral tegmental area (VTA). All sample areas were 0.26 mm^2 and all photomicrographs were taken at 20× magnification with the scale bar is equal to 100 μm. Abbreviation: ac, anterior commissure.

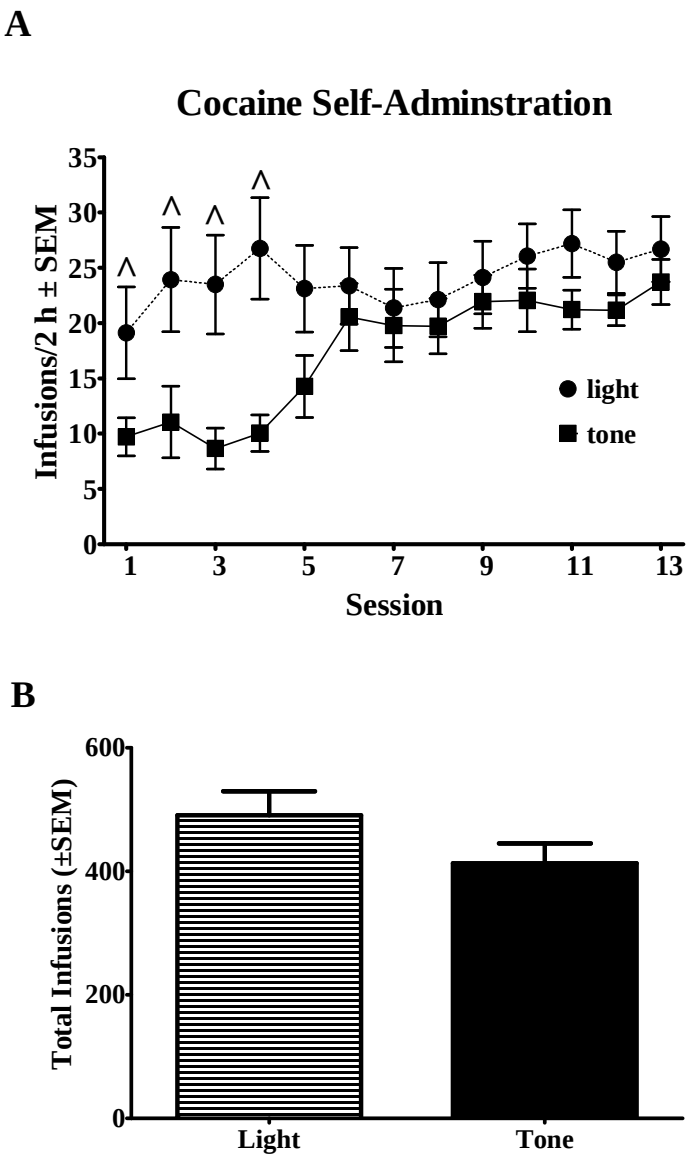

Figure 2. Daily cocaine reinforcement rates (infusions ± SEM) across sessions 1-13 (A), total number of infusions earned during self-administration (B). ^ Represents a significant difference from tone-trained group (tests of simple effects, $p < 0.05$).

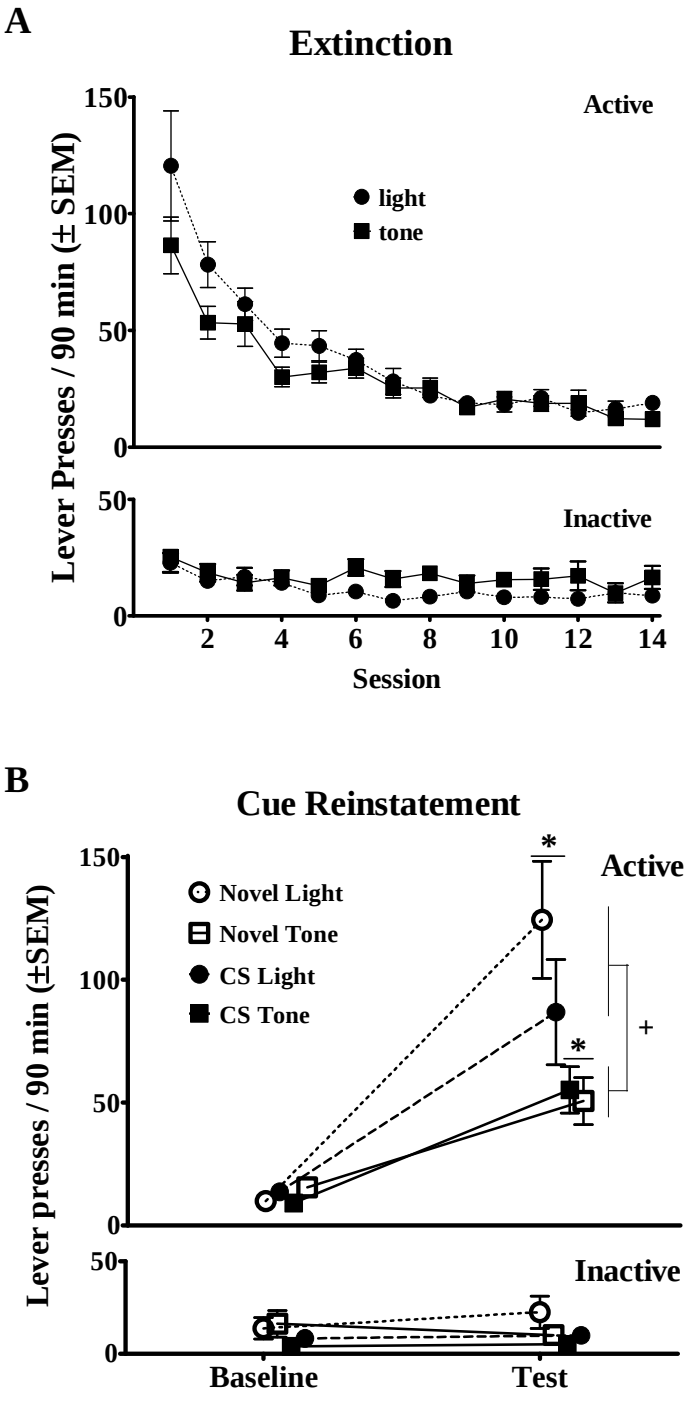

Figure 3. Active (top) and inactive (bottom) lever presses (± SEM) during extinction (A) in rats receiving light (circles) or tone (squares) cues during self-administration training. Active (i.e., cocaine-seeking behavior; top) and inactive

(bottom) lever presses/90 min ± SEM during the final extinction session (baseline) and the test day shown for groups tested with novel (open symbols) or conditioned (closed symbols) light (circles) or tone (squares) cues (B). * Represents an increase from baseline responding (ANOVA, $p < 0.05$). + Represents a difference between test cue modalities (light vs. tone; ANOVA, $p < 0.05$).

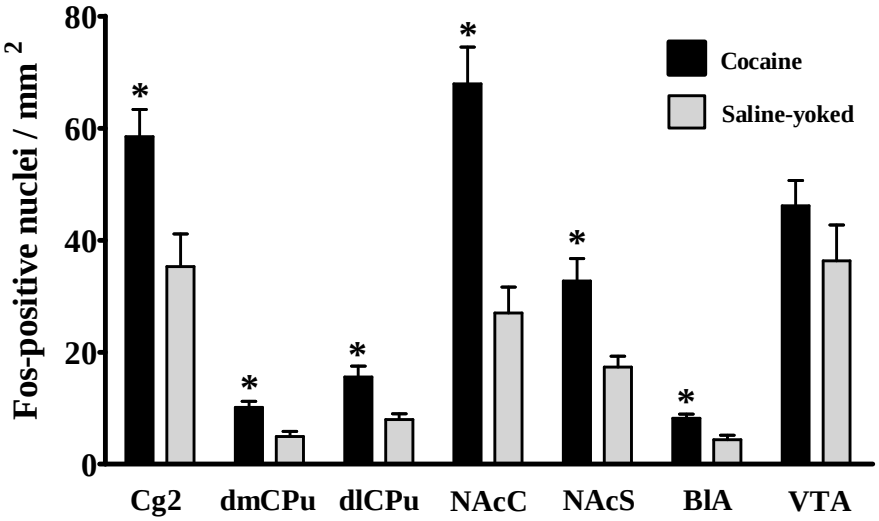

Figure 4. Number of Fos-positive nuclei/mm^2 (± SEM) in brain regions that exhibited enhanced Fos expression in rats with a history of cocaine self-administration in Experiment 1. Data is grouped by drug history. * Represents a difference from Saline-yoked groups ($p < 0.05$).

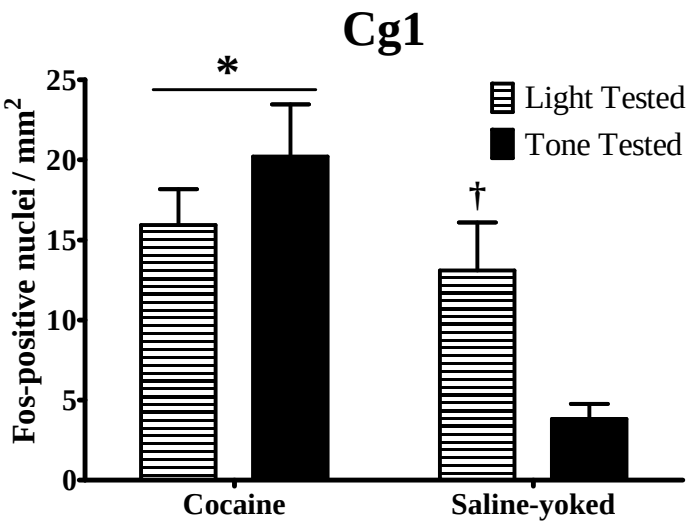

Figure 5. Fos expression in the Cg1 grouped by drug history and test cue modality. * Represents a difference from Saline-yoked groups ($p < 0.05$). † Represents a difference from the saline tone-tested group (ANOVA, $p < 0.05$).

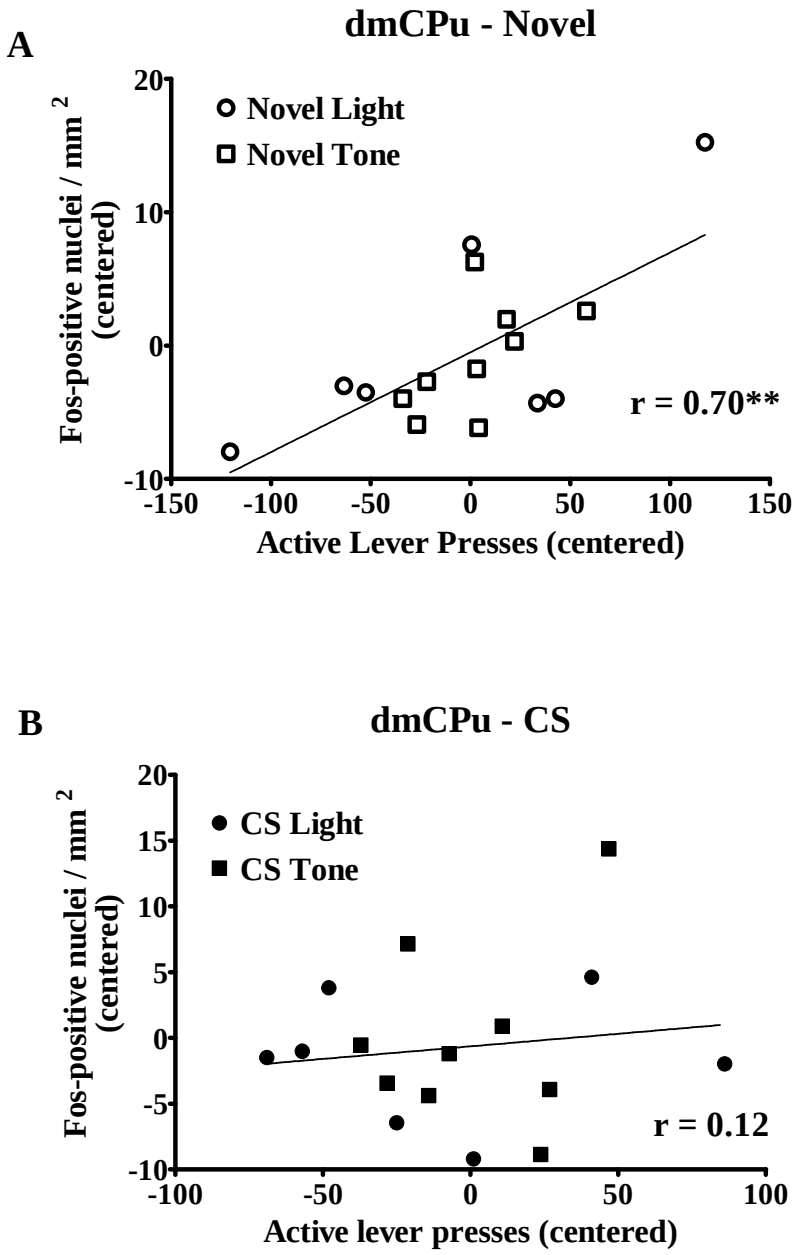

Figure 6**.** Scatter plot of dmCPu for the cocaine novel (A) and CS (B) groups with Fos expression vs. test session cocaine-seeking behavior in Experiment 1. Fos-positive nuclei/mm^2 and test session responding on the active lever were centered

to where ‘0’ represents the mean of each test cue modality in order to remove group differences. The line represents a best-fit linear relationship between the two variables. ** Represents a positive linear correlation among responding on the active lever and Fos-labeled cells ($p < 0.01$).

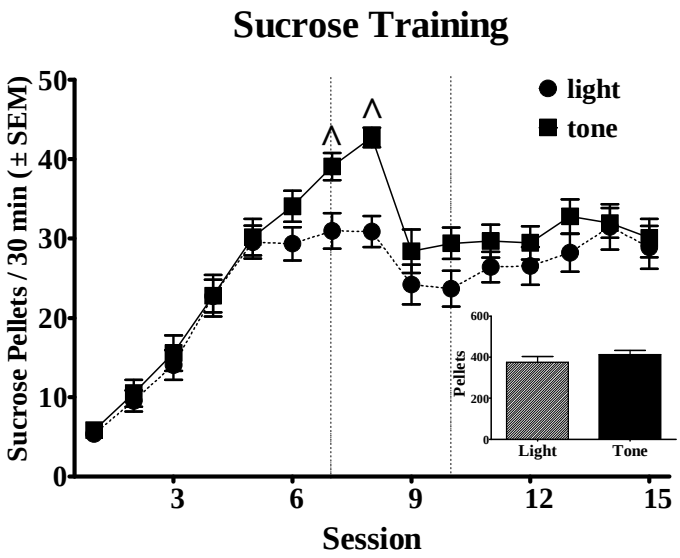

Figure 7. Daily reinforcement rates for rats trained to lever press for sucrose pellets (± SEM) that were paired with a light (circles) or tone (squares). Inset graph shows the total number of sucrose pellets attained across all training sessions. Dashed vertical lines represent the transition period from food restriction (~16-22 g chow/day) to food *ab libitium*. ^ Represents a difference from the light-trained group (tests of simple effects, $p < 0.05$).

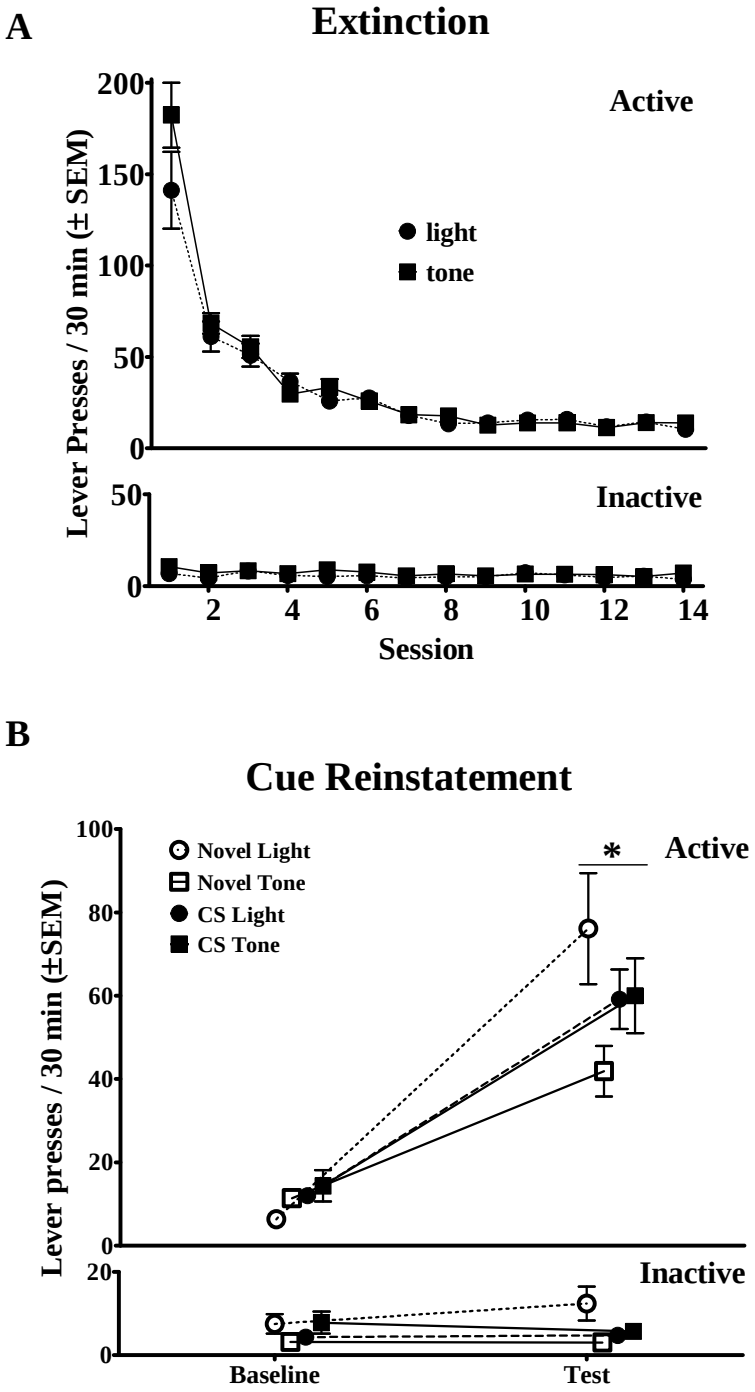

Figure 8. Active (top) and inactive (bottom) lever presses (± SEM) during extinction (A) in rats receiving light (circles) or tone (squares) cues during

sucrose training. Active (i.e., sucrose-seeking behavior; top) and inactive (bottom) lever presses/30 min ± SEM during the final extinction session (baseline) and the test day shown for groups tested with novel (open symbols) or conditioned (closed symbols) light (circles) or tone (squares) cues (B). * Represents a difference from baseline responding ($p < 0.05$).

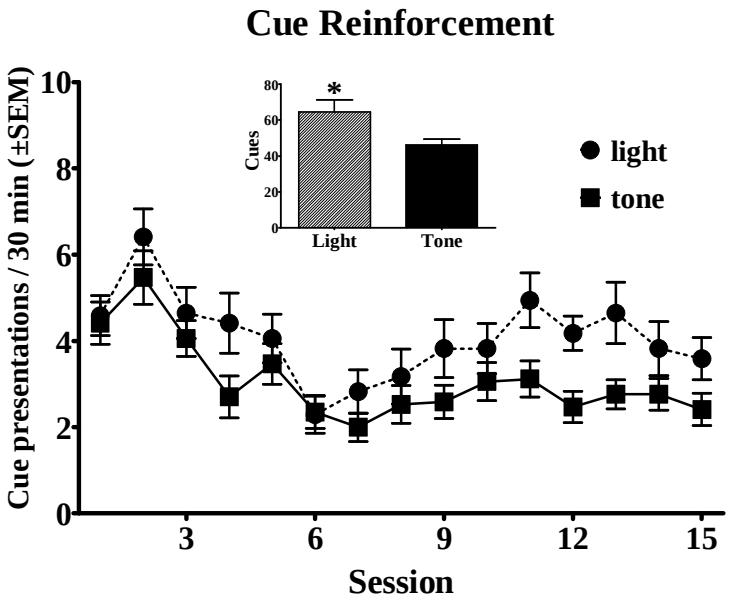

Figure 9. Daily reinforcement rates for rats trained to lever press for either a light (circles) or tone (squares) cue presentation (± SEM). Inset graph shows the total number of cue presentations earned across all training sessions. * Represents a difference from the tone-trained group in total number of cue presentations ($p < 0.05$).

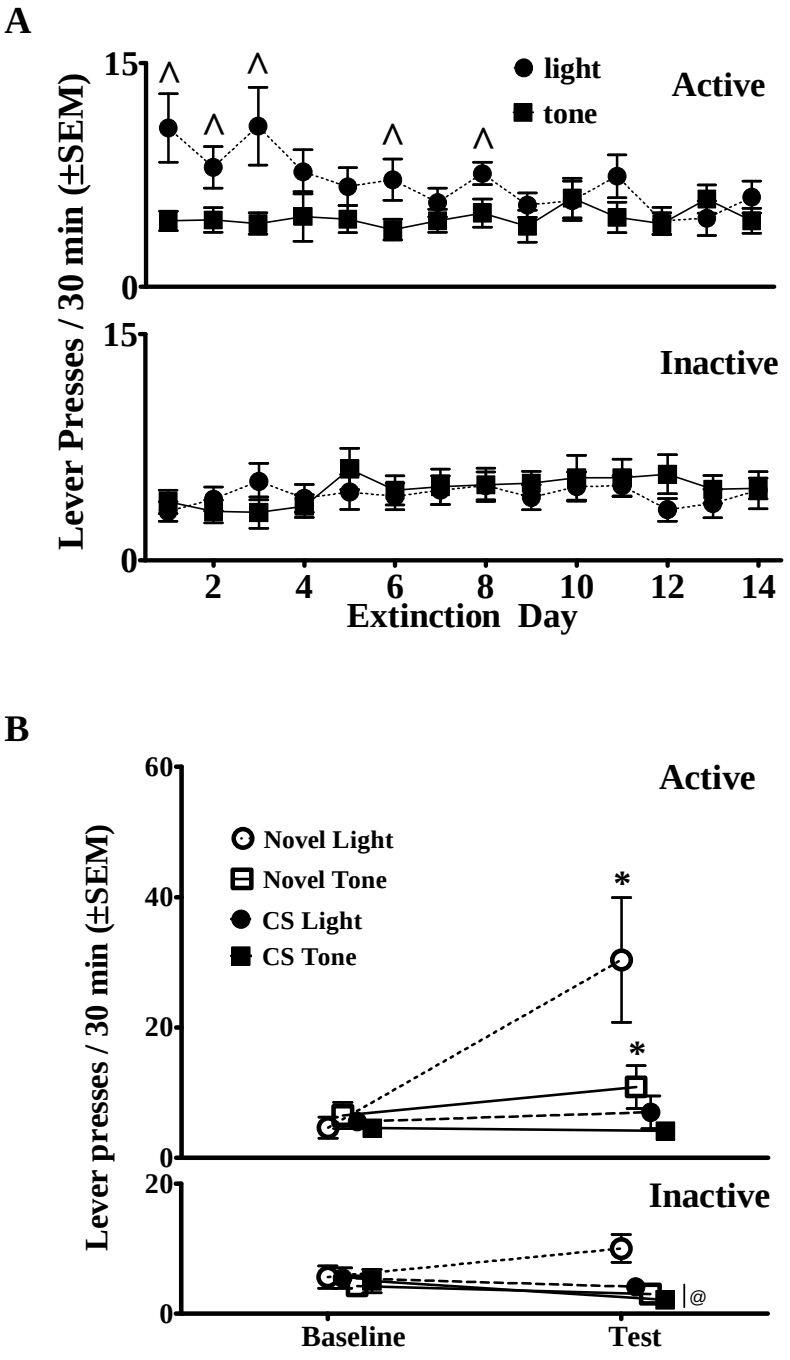

Figure 10. Active (top) and inactive (bottom) lever presses (± SEM) during extinction (A) in rats receiving light (circles) or tone (squares) cues during cue reinforcement training. Active (i.e., cue-seeking behavior; top) and inactive

(bottom) lever presses/30 min ± SEM during the final extinction session (baseline) and the test day shown for groups tested with novel (open symbols) or conditioned (closed symbols) light (circles) or tone (squares) cues. * Represents an increase from baseline responding ($p \leq 0.05$). @ Represents a decrease from baseline responding in the tone-tested groups (ANOVA, $p < 0.05$).