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Series of Intermittent Heroin Injections Enhances Acquisition of Operant Responding for Cues Paired with Natural Rewards

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SERIES OF INTERMITTENT HEROIN INJECTIONS ENHANCES ACQUISITION OF OPERANT RESPONDING FOR CUES PAIRED WITH NATURAL REWARDS

by

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Abstract

SERIES OF INTERMITTENT HEROIN INJECTIONS ENHANCES ACQUISITION OF OPERANT RESPONDING FOR CUES PAIRED WITH NATURAL REWARDS

by

Jennifer Morrison

Advisor: Professor Nancy S. Hemmes

Repeated-intermittent heroin use has been implicated in altering learning processes. Ranaldi et al. (2009) and Morrison et al. (2011) demonstrated that repeated-intermittent heroin administration leads to an enhancement of conditioned reinforcement by a food-paired light stimulus; however, the mechanism governing this effect is still largely unknown. The aims of the present study were to examine modifications in Pavlovian and operant associations for cues paired with natural rewards after a series of intermittent heroin injections. The study consisted of three phases: (1) **Pavlovian Conditioning Phase** (4 days)- in which three groups of rats had a light stimulus paired with food, and one group had unpaired food and light presentations, (2) **Repeated Intermittent Heroin Injections (Behavioral Sensitization Test)** (9 days)- rats in each group were injected daily with either saline or heroin and tested for behavioral sensitization, (3) **Associative Learning Test Phase** (99 days)- rats were assigned to one of four conditions based on conditioning history (light paired or unpaired with food) and type of operant consequence for lever pressing (contingent or non-contingent). In this phase, rats were exposed to two test conditions (15 days each), two spontaneous recovery conditions (10 days each), and an additional two test conditions, one with no heroin (7 days) and the other with an additional heroin injection seven days prior (7 days). There was a 7-day break in between each

experimental condition. The first test condition measured conditioned reinforcement of operant responding. The light stimulus from the Conditioning Phase was presented contingent or non-contingent upon lever pressing, depending on group assignment, in the absence of primary reeinforcement. The second test condition was extinction of operant responding in which lever pressing in all groups resulted in no programmed consequence (light). The third condition was identical to the first test condition, except that animals received an additional injection of heroin or saline prior to testing. The results show that after repeated-intermittent heroin administration rats that received light-food pairings and a contingent presentation of a light stimulus demonstrated greater lever pressing for a stimulus paired with food (active lever) compared to saline controls and all other experimental conditions. These findings are consistent with the conclusion that chronic heroin administration leads to an enhancement of conditioned reinforcement, an effect that is primarily mediated by operant contingency learning.

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always told you one day, I was going to make you proud, and I hope that the day has finally come. I promise to always keep making you proud.

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Introduction

Several behavioral theories state that addictive behaviors are established and maintained by either Pavlovian conditioning (Siegel 1975; Solomon and Corbit 1974; Robinson and Berridge 1993), operant conditioning (Koob and LeMoal 1997), or both (Domjan 2005). It has been recognized that intermittent drug exposure can modify previously learned Pavlovian and/or operant associations (Robbins and Everitt 1999; Everitt and Robbins 2005; Wyvell and Berridge 2001; Ranaldi et al. 2009; Saddoris, Stamatakis, & Careli 2011; Saddoris and Carelli 2014). This dissertation examined the effects of a series of intermittent heroin injections on behavior controlled by Pavlovian and operant conditioning (i.e., conditioned reinforcement). Acquisition and maintenance of operant responding for a stimulus paired with a natural reward (food) was examined across several experimental conditions in which Pavlovian and operant contingencies were manipulated. While some studies have investigated the effect of a drug on Pavlovian and operant responding controlled by a natural reward, relatively few have studied drug effects on conditioned reinforcement. Conditioned reinforcers were established by neutral stimulus/drug pairings and by neutral stimulus/natural reward pairings. In the present study, the effects of drug on behavior controlled by conditioned reinforcers was measured using an acquisition of novel response paradigm.

In the following paragraphs, the acquisition of novel response paradigm is discussed and explained as an ideal procedure to measure the effects of drug on conditioned reinforcement. However, studies that have used the acquisition of novel response procedure did not implement control procedures needed to ascertain what is responsible for an increase in responding for a conditioned stimulus (CS) paired with the unconditioned stimulus (US) (either natural or drug

reward) (DiCiano and Everitt 2004; Davis and Smith 1975; Morrison et al. 2011; Ranaldi et al. 2009). The purpose of the present study was to have appropriate control conditions in place when measuring the effects of repeated intermittent heroin administration on responding for a food-paired conditioned reinforcer.

In the present study, the effects of heroin on conditioned reinforcement were studied under an acquisition of novel response procedure (See Mackintosh 1974 for a review). In the first phase, a Pavlovian pairing procedure was presented in which a neutral stimulus (light) was paired with a primary reward (food). After treatment with drug or saline in a novel environment (and testing for behavioral sensitization -- an increase in locomotor activity after repeated drug injection in an open field chamber), an operant conditioning phase was introduced in which the light stimulus was presented contingent on lever pressing for some groups of animals. In the present study, conditioned reinforcement was tested by comparing responding for a response-contingent presentation of the stimulus that was paired with the primary reward (food) to responding on the lever that produced no consequence. Conditioned reinforcement is inferred when responding is higher on the lever that results in the presentation of a food-paired light stimulus compared to the lever that results in no consequence.

Prior studies measuring the effects of drug on conditioned reinforcement using the acquisition of novel response procedure did not include control procedures to rule out alternatives to interpretation in terms of conditioned reinforcement. If an experimental design is inadequate to rule out alternate explanations of conditioned reinforcement it cannot successfully measure the effects of drug on conditioned reinforcement. In the present study, one alternate explanation that was tested is that stimulus presentation per se can lead to an increase in operant responding, regardless of the programmed relation between a CS (the putative conditioned

reinforcer) and the US, or between operant responding and stimulus presentation. To rule out this possibility for the operant relation, one group of animals received response-contingent stimulus presentations during testing while another group received non-contingent stimulus presentations (using a yoked schedule). To draw the conclusion that mere stimulus presentation is insufficient to increase operant lever pressing, the group of animals that receives the response-contingent light stimulus must respond at higher levels compared to the yoked non-contingent group. If this observation is confirmed, the procedure can be used to assess the effects of drug on conditioned reinforcement.

To test the assertion that behavior control by the CS in the present study is independent of a Pavlovian relation between the stimulus and primary reinforcement, one group of animals received paired CS-US presentations and another group received unpaired CS-US presentations prior to testing the acquisition of a novel response. To demonstrate that Pavlovian conditioning is necessary for a stimulus to function as a conditioned reinforcer, the group of animals that received the CS and US pairings would have to show an increase in responding to the stimulus that was paired with the primary reward compared to the group that had unpaired CS-US pairings. If this is shown, then it can be inferred that drug effects under this paradigm are dependent on Pavlovian conditioning.

Using the acquisition of a novel response paradigm to measure the effects of drug on conditioned reinforcement enables certain assertions about operant responding that could not be made using other operant conditioning paradigms (e.g., self-administration, cue-induced reinstatement, and conditioned place preference). In other paradigms that measure the effects of drug on conditioned reinforcement, during initial training phases, the Pavlovian CS-US relation is presented simultaneously with the operant relation (i.e., response/outcome). An advantage of

the acquisition of novel response paradigm is that the Pavlovian CS-US relation is formed prior to exposure to operant manipulanda (e.g., levers). If the Pavlovian and operant contingencies are introduced independently of one another, assertions can be made that responding on the active lever occurs because the stimulus that was paired with the US is now functioning as a conditioned reinforcer, assuming that the appropriate control procedures are used. If the organism learns the Pavlovian relation at the same time the operant relation (e.g.self-administration paradigm), one cannot be certain that the responding on the active lever (lever with response-contingent stimulus) that occurs during the operant conditioning phase is attributable to conditioned reinforcement, or to some other behavioral phenomenon. If these learning processes are not manipulated independently of one another, the following inferences cannot be drawn: a) differential effects of drug on Pavlovian vs. operant conditioning demonstrated by differences in acquisition of a novel response; b) whether changes in operant behavior in the drug condition are attributable to the drug, the drug-paired cue, or stimulus presentation per se; and c) attribution of maintenance of behavior change to Pavlovian and/or operant conditioning. The present study used procedures to ensure that these conclusions can be made by manipulating Pavlovian and operant contingencies independent of one another. These procedures are discussed below.

The protocol used in the present study was based on Morrison et al. (2011). Morrison et al. used an acquisition of novel response paradigm to examine the effects of repeated heroin administration on the acquisition of operant responding for a stimulus paired with natural reward, and resistance to extinction when the stimulus was presented 30 days after drug administration. In Morrison et al., the effects of repeated heroin administration were tested by manipulating the number of days between drug administration and testing of conditioned reinforcement to

examine if conditioned reinforcement was long-lasting and resistant to extinction. In the first phase, all animals received a stimulus (light) paired with food on 1/3 of the light presentations. In the second phase, animals were either given nine days of saline or heroin injections and tested for behavioral sensitization effects. In the last phase, subjects received acquisition of novel response testing either two days or 30 days after repeated heroin administration. In this phase, the presentation of light was contingent on lever pressing to examine the expression of conditioned reinforcement (lever-press followed by light) as a function of the number of days following heroin administration (either 2 or 30 days). Animals that received repeated-intermittent heroin demonstrated an increase in operant responding compared to saline controls in both the 2- and 30-day groups. These findings were interpreted as heroin enhancing the effectiveness of a stimulus paired with a natural reward, otherwise known as conditioned reinforcement. One major limitation of Morrison et al. is that the proper control conditions to ascertain if a stimulus functions as a true conditioned reinforcer were not used; therefore, alternate explanations of the increase seen in operant responding for the stimulus paired with food in Morrison et al.'s study were available.

Since Morrison et al., as well as many other studies that measured effects of drug on responding for conditioned reinforcement did not rule out alternate explanations of conditioned reinforcement, we conducted the present study to include appropriate control conditions when testing for the effects of heroin on operant responding for conditioned reinforcers. In the present study, manipulation of the Pavlovian CS-US (light/food) contingency and manipulation of the operant behavior/consequence) contingency between lever pressing and the previously trained conditioned stimulus (CS) were conducted in the same experimental paradigm. The Pavlovian contingency was manipulated by presenting a paired versus an unpaired relation between the CS

and US (light and food respectively). The function of this experimental manipulation was to determine the effects of paired CS-US presentations in comparison to unpaired CS-US presentations on the establishment of conditioned reinforcement—a manipulation that is rarely executed in studies measuring conditioned reinforcement of either food- or drug-paired cues.

To examine the effects of heroin on Pavlovian and operant learning, the Pavlovian relation between a CS (light) and US (food) was manipulated during the first phase of the study. The operant contingency between lever pressing and stimulus presentation (light that was previously paired with food) was also manipulated. Under the paired-contingent light condition (PCL), animals that were exposed to a paired CS-US relation (signified by *P* in the condition label) in the Pavlovian conditioning phase, were subsequently exposed to a contingent relation between lever pressing and presentation of the light stimulus (signified by *CL* in the condition label). Under the paired-non-contingent light condition (PNCL), animals that were exposed to a paired CS-US relationship, received yoked non-contingent light presentations (NCL) during operant training. Under the Paired-no light condition (PNL), animals exposed to a paired CS-US relation received no light stimulus presentations during the operant conditioning phase. The manipulation of the Pavlovian and operant contingency permitted us to determine if a series of heroin injections following Pavlovian conditioning enhanced contingency-based operant learning, as opposed to the alternative that heroin injections merely increased the overall level of behavior. This manipulation was critical; however, most studies that have attempted to measure the enhancement of conditioned reinforcement from repeated-intermittent administration of drug have failed to rule out this alternate explanation (Morrison et al. 2011; Ranaldi et al. 2009; Wyvell and Berridge 2001; Taylor and Jentsch 2001).

In the present study, animals were exposed to the following order of conditions: Pavlovian conditioning (Phase 1), repeated heroin injections and testing for behavioral sensitization (Phase 2), and operant conditioning sessions (Phases 3-8). In the Pavlovian conditioning phase, three groups of animals were exposed to a contingency in which the light and food were paired (PCL, PNCL, and PNL), and another group (UCL) was exposed to explicitly unpaired schedules of food and light presentations. Following Pavlovian conditioning, half of the animals in each group were exposed to repeated injections of heroin for nine days and the other half of the animals were given saline injections, followed by a test in an open-field activity chamber to assess behavioral sensitization effects.

In the operant contingency testing phases (Phases 3-8), the contingency between lever pressing and food-paired light stimulus presentation was manipulated under conditions of Pavlovian extinction (CSs were presented but no US), except for the paired no-light condition. In Phase 3, groups of previously treated heroin or saline animals received either response-contingent presentations of a stimulus previously paired with food (paired, contingent light, PCL), non-contingent yoked presentations of a food-paired light stimulus (paired, non-contingent light, PNCL), response-contingent presentations of a stimulus that was not previously paired with food (unpaired, contingent light, UCL), or no light presentation after operant responding (paired, no light, PNL). Phase 3 was designed to measure if repeated-intermittent heroin affected acquisition of responding for a food-paired conditioned reinforcer.

Following Phase 3, several additional tests of operant conditioning were conducted. In Phase 4, the same experimental conditions as Phase 3 were used, although a 7-day break preceded this phase to examine spontaneous recovery after the Pavlovian extinction programmed in Phase 3. In Phase 5, operant extinction was programmed for all experimental conditions

(PCL, PNCL, UCL, PNL); no stimulus presentations occurred. After a 7-day break, Phase 6 was initiated in which the same conditions as Phase 5 were present, to test spontaneous recovery of operant responding. A reinstatement test was used during Phase 7 to examine if operant responding would resume to similar levels as in Phases 3 or 4, if the conditioned reinforcer was presented again. In this phase, the same conditions were programmed as occurred during Phase 3 to determine if animals that were exposed to repeated-intermittent heroin would show reinstatement for a conditioned reinforcer. Prior to the last condition (Phase 8), animals were given one more injection of either saline or heroin seven days prior to testing. The purpose of Phase 8 (same conditions as in Phase 3) was to examine if one challenge injection of heroin would increase operant responding for a food-paired conditioned reinforcer compared to saline-treated animals in the PCL conditions.

The major aim of this study was to examine the effects of repeated heroin administration on the expression of Pavlovian (CS-US) associations and operant learning. It was expected that in comparison to saline-treated control animals, animals treated with repeated heroin injections would show higher levels of responding when a Pavlovian conditioned stimulus was presented response-contingently, implying greater behavioral effectiveness of the paired cue. Behavioral effectiveness was assessed by measures of acquisition of operant responding and resistance to extinction. It was hypothesized that heroin-treated PCL rats would show higher levels of operant responding for a food-paired light stimulus compared to saline controls. Another prediction was that saline and heroin PCL rats would exhibit greater levels of operant responding compared to their respective control conditions (PNCL, UCL, and PNL). The magnitude of difference between the PCL heroin group and its respective controls would also be more pronounced than the magnitude of difference between the PCL saline group and its respective controls in Phase 3.

It was also hypothesized that heroin-treated PCL rats would show more resistance to extinction when the conditioned reinforcer was presented (Phases 3, 4, 7, and 8) during extinction conditions as compared to all other experimental conditions. These results would demonstrate that a series of intermittent heroin injections results in enhanced maintenance of operant responding by conditioned reinforcement. Lastly, we predicted that heroin-treated rats would show a behavioral sensitization effect which was demonstrated by a progressive increase in ambulatory movements (two consecutive beam breaks over repeated heroin administration) compared to saline controls.

This study extends the existing studies of effects of chronic drug administration on conditioned reinforcement by: 1) replicating Morrison et al. (2011) and extending that study by: a) manipulating Pavlovian CS-US contingency and b) manipulating the operant contingency between the response and previously exposed stimulus; 2) using control groups to rule out alternatives to conditioned reinforcement in accounting for the results of Morrison et al. (2011); and 3) examining the effects of repeated-intermittent heroin on long-term maintenance of responding in extinction. To our knowledge, these various measures have not been examined in one empirical study. It is critical that these extensions to the conditioned reinforcement literature are examined to increase our understanding of how repeated intermittent drug use alters previous and future learning for different types of rewards (i.e., natural, conditioned, drug).

Method

The protocols used in the present experiment were in accord with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Queens College Animal Care and Use Committee.

Subjects

The subjects were 64 male Long Evans rats which were between 90-120 days old from the start of the experiment. The subjects were facility-bred from males and females obtained from Charles River Laboratories (Raleigh, NC) with weights ranging from 280-410 g at the start of the study. Animals were randomly assigned to one of the eight conditions (drug by contingency) in each of the eight squads (groups of animals run together in groups of 8-one rat per experimental chamber). Each rat was kept on a 12:12 h light: dark cycle with the dark phase starting at 6 AM. All rats were tested during the active (dark) phase. Each rat had free access to water except when in experimental chambers. All rats were placed on a food-restricted diet, in which their weights were maintained at 85% of their free-feeding weight for the duration of the experiment in order to maintain a consistent motivational state across all phases of the experiment. In conventional Pavlovian and operant preparations, food restriction has been shown to affect level of responding (Cabeza de Vaca and Carr 1998; Carr 2002; Carr 2007). In our lab, it has been empirically demonstrated that food restriction was not required to demonstrate locomotor sensitization after repeated opiate injections (Ranaldi et al. 2000; Ranaldi et al. 2009; Morrison et al. 2011).

Apparatus

Locomotor Activity Chambers. Six locomotor activity chambers consisted of a sound-attenuating ventilated box. Each chamber measured 43x43x43 cm and was equipped with 16 photo-emitters positioned evenly along the length of the chamber 6 cm above the floor, each of which was directly across a photocell on the opposite side of the chamber. These photocells detected both ambulatory and stereotypical movements of the rat. Locomotor activity sessions

were run for 30-min sessions and data were collected in 5-min bins, separately for two different dependent measures: ambulatory locomotor counts and stereotypical locomotor movements.

Operant Conditioning Chambers. Operant conditioning sessions were conducted in eight operant conditioning chambers measuring 30x21x18 cm. Each chamber consisted of an aluminum top and two aluminum sides. The front side which served as the door was made of transparent plastic, as was the back wall; the floor consisted of aluminum rods. Each chamber was equipped with two levers, two white stimulus lights (6 w), and a food trough, all on the right wall. Each lever was positioned 2.5 cm from the edge of the wall and extended 2 cm into the chamber. Each white light stimulus was positioned 3 cm above a lever. The food trough measured 5x5 cm and was centered between the two levers at a height of 3 cm from the floor. Pressing one lever produced no consequence while pressing the other lever turned on the white stimulus light above that lever for 3s for some animals (contingent group), for other animals when the lever associated with the light was pressed in the matched pairs box, a signal was sent for the light to be illuminated (non-contingent), and for the last condition, lever presses were measured without any consequence. The lever associated with the light stimulus was on the right side for half of the animals, and on the left side for the other half of the animals. Each operant chamber was housed in a ventilated, sound-attenuating box.

Materials

Drug. Heroin (a gift from the National Institute on Drug Abuse, Bethesda, MD) was dissolved in 0.9 % saline to achieve a dose of 2 mg/kg and injected in volumes of 1 ml/kg (2 mg of drug to 1 ml) of saline.

Food. All rats were given once-daily access to rat chow (lab diet) within 30 to 120 min following each experimental session, according to their 85% free-feeding weight. The food was

left on the top of the cage until it was consumed, usually within the hour of placing the food on top of the cage. Bioserv (sugar pellets) food was used in the Pavlovian conditioning phase in the operant chambers. This food was not presented under any other condition.

Procedure

Most of the rats were exposed to a procedure consisting of eight experimental phases (see Method below for exceptions where some rats were not exposed to all phases). The eight experimental phases are summarized in Table 1, with further detail in the text below.

Training Phases

Phase 1: Pavlovian Conditioning (4 sessions). In the Pavlovian conditioning phase, animals were placed in the operant chambers for four consecutive daily 60-min sessions. The levers were removed prior to the sessions. During each session, 81 presentations of the 3-s light stimulus were programmed according to a random time 45-s schedule. Rats were either assigned to the paired or unpaired condition (see Table 2). Under the *paired condition*, a randomly selected one-third of the light presentations (27 in total) were paired with the delivery of two 45-mg food pellets immediately after light offset. Therefore, the interval between US presentations was approximately 135s. Under the *not paired* condition, 27 presentations of food were randomly programmed, independently of the schedule of light presentations. The mean interval between food presentations was equal to that under the paired condition. After completion of this phase, there was a three-day rest period. Prior to the next phase, subjects in both the paired and unpaired conditions were evenly divided between heroin or saline (vehicle control) conditions through random assignment.

Phase 2: Drug Treatment Phase (12 sessions). In Phase 2, animals were given either saline or heroin injections to examine if repeated heroin administration resulted in an increase in

locomotor activity compared to saline controls. In this phase, all animals were exposed to intraperitoneal (IP) injections of saline or heroin prior to 12 daily 30-min sessions. Animals were randomly assigned to experimental chambers using a table of random numbers. During the first three sessions (habituation), all animals were treated with saline. During the remaining nine sessions, half of the animals were treated with heroin (2 mg/kg) and half with saline. After receiving the injection, the animal was immediately placed in one of the six locomotor activity chambers to measure locomotor movements (ambulatory and stereotypy) for a 30-min session. Med-Associates software was programmed to record the number of beam breaks (ultra-violet light signal between two photo-transmitters opposite of one another in the chambers) registered as ambulatory locomotor counts or consecutive movements in 5 min intervals on the same beam registered as stereotypical movements. Prior to running each session, each locomotor activity chamber was tested to ensure that the beams were recording ambulatory and stereotypical movements correctly. All rats began the Associative Learning Test Phase three days following the last injection.

Associative Learning Test Phases (Phases 3-8)

The associative learning test phase included six phases (Phases 3-8) in which rats were placed in the operant chambers with the left and right operant levers inserted for consecutive daily 30-min sessions. During Phases 3-7 no saline or drug injections were given. Prior to Phase 8, one single injection of either saline or heroin (consistent with previous assignment in Phase 2) was given seven days prior to the first session in Phase 8. The procedural description of Phases 3-8 are presented below.

Phase 3: Acquisition of Novel Responding (15 sessions). In Phase 3, animals that were subjected to paired or unpaired presentations of light/food and were exposed to one of three

operant contingencies: a) contingent light, b) non-contingent light, or c) no light to determine which combination of Pavlovian and operant contingencies resulted in acquisition of a novel response. By comparing levels of operant responding under the three levels of contingency, the effects of heroin on conditioned reinforcement could be examined using rates of responding for a food-paired conditioned reinforcer. Based on what was previously learned during the Pavlovian conditioning phase (Phase 1), it was assumed that the light would function as a neutral stimulus for animals exposed to unpaired food/light presentations, and a conditioned reinforcer for animals exposed to paired food/light presentations.

In Phase 3, animals were divided into one of eight groups depending on operant contingency (contingent light [two groups- one with paired Pavlovian CS-US presentations and the other with unpaired CS-US presentations], non-contingent light, or no light) and presence vs. absence of IP drug administration (saline vs. heroin-treated animals). Selection of the active lever was conducted by randomly assigning half of the rats in each one of the eight experimental conditions to receive the light stimulus on the left side of the chamber; the opposite side of the chamber was regarded as the inactive lever. For animals that did not receive a food-paired light stimulus contingent upon lever pressing (Paired no-light groups), the active and inactive levers were assigned prior to testing and half of the saline and heroin-treated animals had the active levers assigned to the left-side of the chamber, with the other half having the active lever assigned to the right-side of the chamber.

The eight experimental groups in Phase 3 are listed in Table 2. In each of the experimental conditions explained below (paired contingent, unpaired contingent, paired non-contingent, and paired no light) there was a saline and heroin-treated group. One condition of animals was exposed to a contingency between pressing one of the two levers (the *active lever*)

and presentation of the same light stimulus that had been paired (*paired-contingent light, PCL)* with food during the conditioning phase; pressing the other (*inactive)* lever had no programed consequence. Another condition of animals were exposed to the same contingency as the PCL group, however CS-US presentations were unpaired during the Pavlovian conditioning phase (*unpaired-contingent light, UCL)*. A third condition of animals, the *paired-non-contingent light group (PNCL),* received presentations of the light that were yoked to the schedule of light presentation of the paired-contingent light subjects, and responding on either lever had no programmed consequence. Yoking was implemented by first matching an animal in the PCL condition to an animal in the PNCL condition. Every time the animal in the PCL condition pressed the active lever, a signal was sent to the next corresponding chamber where the PNCL animal was and a light stimulus was presented, Lastly, for the *paired-no light group (PNL),*no light stimulus was presented, and responding on either lever had no programmed consequence. After 15 sessions, all animals received a seven-day break in which they were placed in their respective home chamber and only handled when being weighed, and were fed according to 85% of their free-feeding weight.

Phase 4: Spontaneous Recovery (Pavlovian Responding) (10 sessions). In Phase 4, differential effects of drug on spontaneous recovery after Pavlovian extinction was studied across experimental conditions. Animals were placed into the operant chambers after the seven-day break for 10 sessions and received the same conditions under the same conditions as Phase 3. After ten sessions, all animals received a seven-day break in their respective home cages.

Phase 5: Resistance to Operant Extinction (15 sessions). In Phase 5, the light was no longer presented upon lever pressing, regardless of animals' prior testing history (PCL, PNCL, PNL and UCL) for 15 sessions. Therefore, the contingency between lever pressing and light

presentations was altered for all groups except for the PNL saline and heroin groups which never received lights. After 15 sessions, all animals were given a seven-day break in their respective home cages.

Phase 6: Spontaneous Recovery (Operant Responding) (10 sessions). In Phase 6, to examine spontaneous recovery of operant responding, after a seven-day break animals were placed back into the operant chambers for 10 sessions and received the same experimental conditions as Phase 5. After 10 days of testing, all animals received a seven-day break in their respective home cages.

Phase 7: Reinstatement Test (7 sessions). To investigate the continuing strength of the light as a conditioned reinforcer, we reintroduced the conditioned reinforcement of operant responding procedure under Pavlovian Extinction (as in Phase 3) for seven sessions in Phase 7. This phase was designed to examine if, after a prolonged operant extinction phase, presentation of the conditioned reinforcer would reinstate conditioned operant responding as a function of drug exposure and prior conditioning history. Animals in several squads were not exposed to Phase 7 because the decision to include this phase to assess reinstatement effects without an additional drug injection was made after the experiment started. Therefore the *n* was less than 8 (PCL S=4, PCL H= 4, PNCL S=4, PNCL H = 4, PNL S= 6, PNL H =6) in each experimental condition in Phase 7.

Challenge Test 1 (1 session). A single session in which all animals were given a challenge injection (either saline or heroin) was also conducted. *Challenge Test 1* was conducted after the last session of Phase 7 (Conditioned Reinforcement/Pavlovian Extinction) and was preceded by injection of whatever substance they received during Phase 2 (either saline or

heroin). Animals were then placed in the locomotor chamber for one session of ambulatory testing. A 7-day break separated this phase and Phase 8.

Phase 8: Reinstatement Test after Challenge Injection (7 sessions). In Phase 8, the conditioned reinforcement of operant responding procedure used in Phases 3 and 7 was reintroduced for an additional 7 sessions. This phase was used to investigate the difference in the reinstatement of operant responding for a conditioned reinforcer after exposure to an additional heroin or saline injection, as compared to reinstatement without an additional drug injection (Phase 7). All experimental groups were included in this phase (PCL, PNCL, UCL, and PNL).

Challenge Test 2. (1 session) *Challenge Test 2* was conducted after the last session of Phase 8 where all rats (even previously assigned saline rats) were given a challenge injection of heroin. Locomotor ambulatory movements were recorded in the same manner as explained above.

Data Analysis and α Level

An α level of .05 was used for all statistical tests. Several factorial analyses of variance (ANOVA) were used to examine the effects of chronic drug administration on the acquisition and maintenance of Pavlovian and operant conditioning. The data were further parsed by using Tukey post-hoc analyses to determine differences among groups that were observed in the primary analyses.

An important point to note is that for Phase 7 was that there was not an *n* of 8 in several of the experimental conditions. For the UCL saline and heroin-treated animals, $n = 1$ in each condition; therefore, these data were were not included in any analyses and figures for Phase 7. Two additional subjects from the PCL saline and two subjects from the PCL heroin conditions were removed from the analyses (all experimental phases) in accord with a previously

determined standard of removing outliers where the number of responses made on the active or inactive lever was +/- 2.5 standard deviations above or below the mean of that experimental condition.

Results

Effects of Heroin on Conditioned Reinforcement

The major aim of this study was to examine the effects of intermittent heroin injections on operant responding for a conditioned reinforcer. The following paragraphs describe analyses that compare responding in saline versus heroin-treated rats in each experimental phase, 3-8, across experimental conditions (PCL, PNCL, UCL, and PNL). The following behavioral phenomena were studied: acquisition of a novel response (Phase 3); spontaneous recovery of Pavlovian responding (Phase 4); resistance to operant extinction (Phase 5); spontaneous recovery of operant responding (Phase 6); reinstatement of operant responding without additional drug injections (Phase 7); and reinstatement of operant responding after receiving an additional injection of drug or saline (Phase 8). Phases 7 and 8 were omitted from omnibus 3 and 4-way ANOVAs due to the low *n* in several experimental conditions in Phase 7 (see Method for further explanation).

Overall Analyses

Figures 1 and 2 depict active and inactive lever pressing, respectively, across experimental phases (3-8) for all experimental conditions. The apparent effects of drug and training condition across experimental phases are described in the following paragraph, separately for active versus inactive lever pressing. Statistical analyses are presented in the subsequent paragraphs of this section.

As shown in Figure 1, when a response-contingent stimulus was presented in Phases 3 and 4, higher levels of *active lever pressing* were evident in saline and heroin-treated animals that received both a Pavlovian paired CS and an operant contingency (the PCL condition) compared to those in the other experimental conditions. In Phase 3, there was a marked difference between saline and heroin-treated PCL animals, with heroin-treated animals demonstrating more operant responding for a food-paired conditioned reinforcer compared to saline controls. In Phase 4, this difference subsided. During Phases 5 and 6 when the response-contingent stimulus was removed in an operant extinction test, no group differences were apparent. When the operant contingency was re-introduced during Phase 7, PCL saline and heroin-treated rats' active lever pressing appeared higher than PNL and PNCL saline and heroin-treated animals. During Phase 8 where heroin-treated animals received an additional heroin injection seven days prior to testing, it appears that PCL heroin-treated animals responded similarly to saline-treated controls which received saline seven days prior to testing. As shown in Figure 2, the effects observed for active lever pressing were not present for inactive lever pressing across experimental phases (3-8) for all experimental conditions.

To assess the apparent effects of heroin and experimental conditions on active and inactive lever pressing, a 2 (drug--Saline or Heroin) x 4 (condition--PCL, PNCL, UCL, PNL) x 4 (phase--3, 4, 5, and 6) x 2 (lever--active and inactive) mixed ANOVA was conducted (see Table 3 for all main effects and interactions). Data from phases 7 and 8 were excluded from this overall ANOVA because the number of subjects in those phases differed from that of the other phases. Data for Phases 7 and 8 were analyzed separately (see below). As expected, there was a significant difference among experimental conditions, F (3, 55) = 14.86 $p < .05$; phases, F (3, 165) = 66.59, $p < .05$; and levers, F (1, 55) = 35.42, $p < .05$. There was no main effect of drug F

(1, 55) = 0.003, $p > .05$. As shown in Table 3, there were significant 2-way interactions for condition x phase, F (9, 165) = 8.67, $p < .05$; drug x condition, F (3, 55) = 2.86, $p < .05$; condition x lever, F (3, 55) = 12.67, $p < .05$; and phase x lever, F (3, 165) = 28.03, $p < .05$. The drug x phase F (3, 165) = .41, $p < .05$, and drug x lever, F (1, 55) = .38, $p < .05$ interactions were not significant. There were significant 3-way interactions including a condition x phase x lever, F (9, 165) = 14.32, $p < .05$ and a drug x condition x lever interaction, F (3, 55) = 2.91, $p < .05$. The drug x phase x lever interaction, F (3, 165) = .24, $p > .05$ was not significant. The 4-way interaction was also not significant, F (9, 165) = 1.10, $p > .05$.

To parse the significant drug x condition x lever interaction, a 2-way drug x condition ANOVA was conducted at each level of lever (active and inactive) (see Table 4). The analysis for the active lever did not reveal a significant main effect of drug, F (1, 55) = .10, $p > .05$, however, as expected the main effect of condition was significant, F (3, 55) = 19.96, $p < .05$. The 2-way interaction was not significant, F (3, 55) = 1.10, $p > .05$. Tukey post-hoc tests examining condition effects revealed a significant difference between the PCL condition and all respective control groups (PNCL, UCL, and PNL) with the PCL condition pressing more on the active lever compared to other conditions, $p < .05$. The analyses for the inactive lever did not reveal any significant main effects of drug, F (1, 55) = .35, $p > .05$, or condition, F (3, 55) = .48, $p > .05$, or a significant 2-way interaction, F (3, 55) = .18, $p > .05$ (see Table 4 for F tests and Figure 2 for inactive lever pressing across phases). Overall, these results indicate that there was only a condition effect when examining active lever pressing, however, no differential drug effect among conditions.

In response to the significant condition x phase x lever interaction, a 2-way condition x phase ANOVA (Table 5) was conducted at each level of lever (active and inactive). The

analysis for the active lever revealed a significant main effect of condition, F (3, 59) = 20.26, p < .05; significant main effect of phase, F (3, 177) = 71.55, p < .05, and a significant condition x phase interaction, F (9, 177) = 16.58, p < .05. To examine differences in active lever pressing between conditions at each level of phase, separate analyses were conducted that examined group differences in Phases 3, 4, 5, and 6 (Tables 6 - 9) (note, Phases 7 and 8 were excluded from these analyses). Post-hoc Tukey analyses (Table 5) revealed that animals in the PCL condition pressed more on the active lever than animals in the PNCL condition and the PNL conditions in Phases 3, 4, 5, and 6. There was only a difference between PCL and UCL conditions in Phases 3 and 4 (see Table 5 for all statistical tests). The analyses for the inactive lever revealed a significant main effect of phase, F (3, 177) = 12.51, p < .05, however, no main effect of condition, F (3, 59) = .52, p > .05 and no 2-way condition x phase interaction, F (9, 177) = 1.08, p > .05 (see Table 5). Taken together, these findings demonstrated higher rates of active lever pressing for the PCL condition compared to control conditions (PNCL, UCL and PNL) in Phases 3 and 4.

Analyses of Individual Phases

A-priori planned comparisons were conducted to investigate the effects of drug on active and inactive lever pressing among experimental conditions for each phase. It was predicted that there would be a significant drug x group interaction in phases 3, 4, 7 and 8 because the conditioned reinforcer was presented for some conditions (PCL, PNCL, UCL), but not for PNL. It was also hypothesized that if heroin has no other effects, there should be no differences among experimental conditions when the conditioned reinforcer was not presented (Phases 5 and 6).

Acquisition of a Novel Response-Phase 3

Figure 3 represents active and inactive lever pressing in Phase 3 (Conditioned Reinforcement/Pavlovian Extinction Phase) for each experimental condition. Inspection of the figure shows higher levels of active lever pressing for those groups exposed to an operant contingency (PCL S and H, UCL S and H) compared to conditions that were not (PNCL, PNL). The aforementioned effect was further modulated by presence or absence of the Pavlovian contingency between light and food (groups PCL S and PCL H versus UCL S and UCL H). An effect of drug was apparent only when both the operant and Pavlovian contingencies were present (PCL). When animals did not have previous exposure to a Pavlovian contingency (UCL S and UCL H), an effect of drug was not apparent. In the groups that received a Pavlovian contingency but received non-contingent stimulus presentations during operant testing (PNCL S and H), there was also no effect of drug, nor was there one when animals received a Pavlovian contingency but no stimulus presentations (PNL) during operant testing.

To assess reliability of the effects described above, a 2 (drug) x 4 (condition—PCL, PNCL, UCL, PNL) x 2 (lever) ANOVA was conducted (see Table 3). As predicted, there was a main effect of condition, F (3, 55) = 14.86, $p < .05$ and a main effect of lever, F (1, 55) = 35.42, $p < .05$ and no main effect of drug, F (1, 55) = 0.03, $p > .05$. There was a significant condition x lever interaction, F (3, 55) = 12.67, $p < .05$ and drug x condition x lever interaction, F (3, 55) = 2.91, $p = .04$; however, the drug x lever interaction was not significant, F (1, 55) = .38, $p > .05$.

To parse the significant drug x condition x lever interaction in Phase 3, a 2-way drug x condition ANOVA was conducted at each level of lever (active and inactive) (see Table 6 for F tests). The analyses for the active lever did not reveal a significant main effect of drug, F (1, 55) = 0.10, $p > .05$, however, as expected the main effect of condition was significant, F (3, 55) = 19.96, $p < .05$. The 2-way drug x condition interaction was not significant, F (3, 55) = 1.10, p

>.05. Tukey post-hoc tests examining condition effects revealed a significant difference between PCL and PNCL conditions, $p < .05$; PCL and UCL conditions, $p < .05$; and PCL and PNL conditions, $p < .05$. The analyses for the inactive lever did not reveal any significant main effects of drug, condition, or a significant drug x condition interaction, $p > .05$. Overall these effects show only a difference between conditions, irrespective of drug treatment. Since there were no significant main or interactive effects involving inactive lever pressing, only data for active lever pressing were included in the between-comparison analyses reported in the following sections for Phase 3.

Comparison of operant responding in PCL saline and heroin conditions (Phase 3). An *a-priori* hypothesis was that heroin-treated rats receiving paired CS-US associations and a response-contingent light (PCL) would show a higher level of operant responding for a food-paired light stimulus compared to saline controls in Phase 3. This effect is commonly known in the conditioned reinforcement literature as the enhancement of conditioned reinforcement effect which has been previously demonstrated in our lab (Morrison et al. 2011; Ranaldi et al. 2009). The first two pairs of bars in Figure 3 show the average responding from the 15 sessions in PCL saline- and heroin-treated rats in Phase 3. A drug x lever ANOVA examining the average active and inactive lever pressing for 15 days in Phase 3 revealed a significant drug (saline vs. heroin) x lever (active vs. inactive) interaction, $F(1, 14) = 4.97$, $p = 0.04$. Post-hoc tests show that PCL heroin-treated rats pressed more on the active lever compared to saline-treated PCL controls, $p = 0.02$; however, there was no significant difference in inactive lever pressing between saline and heroin-treated animals, $p > .05$.

Comparison of operant responding in PCL and PNCL conditions (Phase 3). An additional *a-priori* hypothesis was that heroin-treated PCL rats (PCL H) would exhibit

significantly more active lever presses compared to heroin-treated rats which received non-contingent CS presentations during Phase 3 (PNCL H). A 2 (drug) x 2 (condition PCL and PNCL) ANOVA was conducted to examine whether heroin-enhanced responding depended on response-contingent presentations of the conditioned reinforcer. As predicted, the main effect of condition was significant, F (1, 25) = 47.10, $p < .05$ and the main effect of drug was not, F (1, 25) = 0.50, $p > .05$. The drug x condition interaction was significant, F (1, 25) = 3.97, $p = 0.05$. Post-hoc tests showed a significant drug effect for the PCL conditions, $p < .05$, but not the PNCL conditions, $p > .05$. This finding supports the hypothesis heroin increases lever pressing only when the operant contingency is present and does not influence behavior when the conditioned reinforcer is presented response-independently.

Comparison of operant responding in PCL and UCL conditions (Phase 3). A final point of interest for Phase 3 was whether the observed effects of heroin in the PCL condition were dependent upon the prior Pavlovian contingency between light and food. If animals that received drug administration and a prior Pavlovian contingency between light and food (PCL) demonstrated more active lever presses for a response-contingent reinforcer compared to animals that did not receive a Pavlovian contingency (UCL), it can be concluded that drug only enhances conditioned reinforcement when a Pavlovian contingency is programmed. A 2 (drug) x 2 (condition--PCL vs. UCL) ANOVA was conducted to examine whether heroin-enhanced responding depended on prior Pavlovian conditioning. As predicted, the main effect of condition was significant, F (1, 25) = 21.41, $p < .05$, and the main effect of drug was not, F (1, 25) = 1.26, $p > .05$. The drug x condition interaction was significant, F (1, 25) = 4.70, $p = 0.04$ (see Figure 3). Post-hoc tests showed a significant drug effect between the PCL groups (saline vs. heroin), $p < .05$, but not between the UCL groups (saline vs. heroin), $p > .05$. This finding supports the

hypothesis that repeated heroin injections would result in a higher rate of responding for a stimulus paired with food.

Spontaneous Recovery of Pavlovian Responding-Phase 4

Figure 4 depicts the mean number of active and inactive lever presses in Phase 4 (spontaneous recovery of Pavlovian responding). The figure shows 10-session averages separately for the active and inactive levers across experimental conditions. A 2 (drug) x 4 (condition-PCL, PNCL, UCL, PNL) x 2 (lever) ANOVA was conducted to determine if there were differences among experimental conditions after a 7-day break when subjects were tested under the same experimental conditions as in Phase 3 (see Table 7 for all statistical results). There was a significant main effect of lever, F (1, 55) = 35.80, $p < .05$ and condition, F (3, 55) = 11.52, $p < .05$. There was no main effect of drug, F (1, 55) = 0.10, $p > .05$. There was a significant lever x condition interaction, F (3, 55) = 11.91, $p < .05$, but no lever x drug interaction, F (1, 55) = 0.50, $p > .05$ or drug x condition interaction, F (3, 55) = 0.24, $p > .05$. The three-way interaction was not significant, F (3, 55) = 0.30, $p > .05$. Post hoc tests to examine the condition x lever interaction yielded significant differences in active lever pressing between PCL and PNCL conditions, PCL and UCL conditions, and PCL and PNL conditions, $p < .05$. There were no significant differences among experimental conditions on the inactive lever, $p > .05$. These findings did not support our hypothesis that there would be a drug x condition interaction whenever a conditioned reinforcer was contingent on operant responding. The lack of this drug x condition effect could be attributed to generalization of extinction decrement over repeated days of testing.

Extinction of Operant Responding-Phase 5

Figure 5 depicts the mean number of active and inactive lever presses in

Phase 5 (Extinction of operant responding). The figure shows 15-session averages separately for the active and inactive levers across experimental conditions. A 2 (drug) x 4 (condition—PCL, PNCL, UCL, PNL) x 2 (lever) ANOVA was conducted to examine the effects of repeated-intermittent heroin on extinction of operant responding, see Table 8 for all statistical tests. There was a main effect of lever, F (1, 55) = 7.85, $p < .05$ and condition, F (3, 55) = 4.33, $p < .05$; however, no main effect of drug, F (1, 55) = 0.07, $p > .05$ (see Table 8). The condition x lever, drug x lever, and drug x condition interactions were not significant, $p > .05$. The three-way interaction was also not significant, F (3, 55) = 0.30, $p > .05$. Overall, it can be concluded that animals responded more on the active lever compared to the inactive lever. For the analysis of the main effect of condition, PCL groups (saline and heroin) responded more on the active lever compared to PNCL and PNL groups (saline and heroin), $p < .05$, however not significantly more than the UCL groups (saline and heroin), $p > .05$.

Resistance to Operant Extinction-Phase 6

Figure 6 depicts the mean number of active and inactive lever presses in Phase 6 (spontaneous recovery of operant responding). The figure shows 10-session averages separately for the active and inactive levers across experimental conditions. A 2 (drug) x 4 (condition—PCL, PNCL, UCL, PNL) x 2 (lever) ANOVA was conducted to examine the effects of repeated-intermittent heroin on spontaneous recovery of operant responding, see Table 9 for all statistical results. There was a significant main effect of lever, F (1, 55) = 5.92, $p < .05$ and condition, F (3, 55) = 4.15, $p < .05$; however, no effect for drug, F (1, 55) = 0.03, $p > .05$. None of the two-way interactions (drug x lever, condition x lever and drug x condition) were significant, $p > .05$, nor was the three-way ANOVA significant, F (3, 55) = 1.34, $p > .05$. Follow-up one-way ANOVAs examining the difference among experimental conditions on the

active lever revealed a significant difference between the PCL and PNCL conditions, PCL and PNL conditions, $p < .05$, with no significant difference among any other conditions, $p > .05$.

Reinstatement of Responding for a Conditioned Reinforcer (without additional injection) - Phase 7

Figure 7 depicts the mean number of active and inactive lever presses in Phase 7 (reintroduction of operant light reinforcement). As stated previously, there was only one subject in both the UCL saline and heroin-treated groups in Phase 7, therefore these groups were not included in the analysis. The figure shows 7-session averages separately for the active and inactive levers across experimental conditions. Figure 7 suggests that in conditions in which response-contingent conditioned reinforcement (PCL) was programmed, rats demonstrated more responding on the active lever in comparison to all control conditions (PNCL and PNL), as predicted. However, PCL saline-treated rats showed more active lever presses compared to PCL heroin-treated rats, which was not expected. A 2 (drug) x 3 (condition-PCL, PNCL, PNL) x 2 (lever) ANOVA was conducted to examine the effects of repeated-intermittent heroin on reinstatement of operant responding for a response-contingent conditioned reinforcer (refer to Table 10 for all statistical tests). As predicted, there was a significant main effect of condition, $F(2, 21) = 28.07$, $p < .05$ and a significant main effect of lever, $F(1, 21) = 40.01$, $p < .05$, however no significant main effect of drug, $F(1, 21) = .11$, $p > .05$. There was no significant condition x lever interaction, $F(2, 21) = 1.94$, $p > .05$ and no significant drug x lever interaction, $F(1, 21) = 2.04$, $p > .05$. There was a significant triple interaction, $F(2, 21) = 3.38$, $p < .05$. Post hoc analyses parsed the significant drug x condition x lever interaction with separate drug x condition ANOVAs at each level of lever (active and inactive). For the active lever analysis, there was a main effect of condition, $F(2, 21 = 35.14$, $p < .05$, no main effect of drug, $F(1, 21) = 0.77$, p

>.05, and no drug x condition interaction, F (2, 21) = 1.83, p > .05. A significant difference in active lever pressing between PCL and PNCL and PCL and PNL conditions was found with PCL saline-treated rats pressing more than PCL heroin-treated rats, p < .05. For the inactive lever analysis, no main effect of drug or condition, or a drug x condition interaction was seen, p > .05.

Reinstatement of Operant Responding after Additional Drug Injection- Phase 8

Figure 8 depicts the mean number of active and inactive lever presses in Phase 8 (effects of drug challenge under conditioned reinforcement--PCL H, PNCL H, UCL H, and PNL H groups received heroin). Each bar represents the average of 7-days of active and inactive lever presses across saline-and-heroin-treated rats. A 2 (drug) x 4 (condition--PCL, PNCL, UCL, PNL) x 2 (lever) ANOVA was conducted to examine the effects of the drug challenge under conditioned reinforcement. As predicted, there was a significant main effect of lever, F (1, 43) = 46.20, p < .05, and condition, F (3, 43) = 12.20, p < .05, however no main effect of drug, F (1, 43) = 0.74, p >.05. There was a significant condition x lever interaction, F (3, 43) = 17.95, p < .05; however no significant drug x condition or drug x lever interaction, p > .05 (see Table 11 for all tests). The three-way interaction was not significant, F (3, 43) = 0.501 p > .05. To parse the significant condition x lever interaction, differences among conditions were examined at each level of lever. For the active lever, there were significant differences between the PCL and PNCL conditions, PCL and UCL conditions, and PCL and PNL conditions, p < .05. For the comparison among experimental conditions for the inactive lever, there were no significant differences between and experimental conditions, p > .05.

Magnitude of Difference between Experimental Conditions (Active Lever, Phase 3)

Magnitude of difference in active lever pressing between PCL groups (saline and heroin) and their respective control conditions (PNCL, UCL, and PNL) was used in conjunction with absolute rates of lever pressing.

The magnitude of the difference in active lever pressing between the PCL Condition and the control conditions (PNCL, UCL, and PNL) in Phase 3 is shown in Figure 9 separately for heroin- and saline-treated animals. It was hypothesized that both heroin- and saline-treated rats in the PCL condition would exhibit significantly more active lever presses compared to their respective control conditions, and that this effect would be more pronounced in the heroin-treated rats. Figure 9 depicts the differences in mean number of responses per session (15-day average) between the PCL condition and PNCL condition (left-most pair of bars), the PCL and UCL condition (middle pair of bars), and the PCL and PNL condition (right-most pair of bars). These difference scores are presented separately for saline- and heroin-treated rats (black versus gray bars). The difference score was calculated by taking the difference of the average number of active lever presses in each control condition (PNCL S and H, UCL S and H, and PNL S and H) from the average of active lever presses in the PCL group (saline and heroin). Three separate one way ANOVAs were conducted to determine if there was a greater difference between heroin-treated rats and their respective controls compared to saline-treated animals. For the comparison between PCL and PNCL conditions, the difference scores were greater for the heroin-treated rats (M= 68.33) compared to saline-treated rats (M= 40.28), F (1, 14) = 24.41, p < .05. For the comparison between PCL and PNL conditions, heroin treated rats (M =66.31) had a greater difference score compared to saline treated rats, (M = 48.21), F (1, 14) = 21.97, p < .05. There was no effect of drug in the PCL and UCL comparison, F (1, 14) = 1.83, p > .05. These

results demonstrate a greater magnitude of difference in active lever presses between PCL heroin-treated rats and heroin-treated PNCL rats, however, no difference between heroin-treated UCL rats.

Effects of Phase Change for all Experimental Conditions

In the following paragraph, the immediate effects of each phase change were analyzed. Changes between Phase 3 to Phase 4 were studied to assess Pavlovian spontaneous recovery; differences between Phases 4 and 5 measured operant extinction; differences between Phases 5 and 6 measured the effects of a 7-day break under operant extinction; differences between Phases 6 and 7 analyzed the reintroduction of operant reinforcement; and differences between Phases 7 and 8 examined the effects of a drug challenge under conditioned reinforcement. The effects of each phase change were analyzed by comparing the frequency of responding on the active lever in session 1 of a *target phase* (defined as current phase being analyzed) divided by the average of the last 5 days in *comparison phase* (defined as the phase prior to the target phase). This measure (*percent change*) was calculated by dividing the average responding in session 1 of the target phase by the average of the last 5 days of responding in the comparison phase. A 2 (drug) x 4 (condition-PCL, PNCL, UCL, PNL) x 3 (phase comparion-3-4, 4-5, 5-6) x session (1-5) ANOVA was conducted that examined differences in percent change of responding in the comparison phase compared to the target phase. The main effect for drug F (1, 55) = 1.59, p > .05, condition F (3, 55) = .21, p > .05, phase F (2, 110) = 0.74, p > .05 and session F (4, 220) = 1.52, p > .05 were not significant. The drug x condition F (3, 55) = .94 , p > .05, drug x phase F (2, 110) = .35, p > .05, condition x phase F (6, 110) = 1.52, p > .05, drug x session F (4, 220) = 2.20, p > .05, condition x session F (12, 220) = .97, p > .05, and phase x session F (8, 440) = .76, p > .05 interactions were all not significant, p > .05. The drug x condition x phase F (6, 110)

= 1.24, $p > .05$, drug x condition x session F (12, 220) = 1.33, $p > .05$, and condition x phase x session, F (24, 440) = 1.17, $p > .05$ interactions were not significant. The overall four-way ANOVA was not significant as well, F (24, 440) = 1.17, $p > .05$. These results suggest that heroin treatment did not have any differential effects on resistance to Pavlovian and/or operant spontaneous recovery.

Behavioral Sensitization

It was hypothesized that heroin-treated rats would have more ambulatory locomotor movements (behavioral sensitization effect after nine sessions of injections) compared to saline-treated rats, and that this effect would persist until challenge test 1 (session 115) and challenge test 2 (session 123). Behavioral sensitization was defined as an increase in ambulatory motor movements as a function of the number of intraperitoneal injections. Ambulatory movements were measured by beam breaks in a locomotor activity chamber. Ambulatory movements were recorded when the animal broke two consecutive beams. Figure 10 depicts ambulatory movements during habituation (baseline; sessions 1-3), sensitization (sessions 4-12), challenge test 1 (all animals received same injection as previously), and challenge test 2 (all animals received heroin). Figure 10 shows that during sessions 1-3 (habituation) the level of locomotor activity did not differ between experimental groups, F (1, 70) = 1.95, $p > .05$ A 2 (drug-saline vs. heroin) x 4 (session- 4, 12, challenge test 1, challenge test 2) ANOVA was conducted to examine the effects of drug on locomotor activity. As predicted, there was a significant drug x session interaction, F (3, 261) = 16.60, $p = 0.01$. These findings indicate that the number of ambulatory movements was dependent on the session and what type of drug was given. A series of one-way ANOVAs was conducted to examine the differences between saline and heroin-treated animals for sessions 4, 12, challenge test 1 and challenge test 2. A one-way ANOVA

comparing ambulatory movements between saline and heroin-treated animals on Session 4 demonstrated that heroin-treated rats showed a higher level of ambulatory movements compared to saline-treated animals, F (1, 70) = 43.75, $p < .01$. During Challenge Test 1, heroin-treated animals continued to have more locomotor activity compared to saline-controls, F (1, 70) = 51.48, $p < .01$. On Challenge Test 2, animals assigned to the heroin group continued to have more locomotor activity compared to saline controls, F (1, 70) = 29.42, $p < .05$. These results suggest that heroin-treated rats were sensitized, as evidenced by a greater number of ambulatory movements, during the entire length of the experiment compared to saline-treated animals.

Discussion

The present study was a conceptual replication of other studies from our lab using an Acquisition of novel response paradigm (Morrison et al. 2011; Ranald et al. 2009) that aimed to strengthen the argument that repeated administration of heroin enhanced conditioned reinforcement. The present study was the only study to our knowledge that systematically manipulated Pavlovian and operant contingencies in the same experimental paradigm to examine the effects of a series of intermittent heroin injections on the enhancement of both of these associative learning processes. It was predicted that animals that had both Pavlovian and operant contingencies present (PCL) that were treated with heroin would differentially increase operant responding compared to saline controls only when both of these contingencies were present (Phases 3, 4, 7 and 8). The major hypothesis in the present study was supported that stated in Phase 3 when animals were acquiring the novel operant response, PCL heroin-treated rats' would demonstrate significantly greater active lever pressing compared to saline controls and all other experimental conditions (PNCL S and H, UCL S and H, and PNL S and H). It was also predicted that when the operant contingency was removed that PCL treated animals' operant

responding would be similar to control conditions (PNCL, UCL, and PNL). When the operant contingency was removed and the response-contingent stimulus was no longer presented in Phases 5 and 6, heroin-treated animals in the PCL condition showed a decrement in active lever pressing, and responding was now similar to all other heroin control groups (PNCL, UCL and PNL). Note, however, that differences observed in Phase 4 were non-significant, nonetheless, these findings may suggest that heroin selectively enhances operant responding but only when the Pavlovian and operant contingency were available (see Figure 1).

Another dependent measure used to investigate the effect of heroin on conditioned reinforcement was a difference score that examined the mean of 15-days of active lever pressing during Phase 3 comparing the level of responding in PCL (saline and heroin) groups to their respective control conditions (PNCL, UCL, and PNL). It was hypothesized that saline and heroin-treated PCL groups would have higher absolute rates of active lever pressing compared to their respective control conditions. An additional prediction was that the magnitude of difference between the PCL heroin-treated rats and their controls would be greater than the difference between PCL saline-treated rats and their controls. These hypotheses were supported in that both saline and heroin-treated PCL animals had a higher number of active lever presses compared to their respective control conditions. The hypothesis that the magnitude of difference in active lever pressing in heroin-treated PCL rats compared to heroin control groups would be greater than the magnitude of difference between saline-treated PCL animals and saline-treated PNCL, UCL, and PNL rats was partially supported (see Figure 9). PCL heroin rats had a greater difference score compared to PNCL and PNL but not the UCL heroin-treated animals. These findings suggest that the contingency between lever pressing and light presentation could have affected differential responding. Taken together, these findings suggest that heroin selectively

enhances operant responding for a food-paired conditioned reinforcer during acquisition of a novel response, but only when there is a previously learned positive contingency between the US and CS and a response-contingent presentation of the conditioned reinforcer.

The hypothesis that heroin-treated PCL rats would show more persistence in responding when the conditioned reinforcer was presented after a break (Phases 4, 7, and 8) compared to all other experimental conditions was not supported by the data. The comparison between Phases 3 and 4 did not show any significant differences between heroin and saline-treated animals in the PCL condition. Analyses for Phases 7 and 8 were not conducted owing to problems with the *n* in some of the control conditions (UCL groups specifically) in these phases since they were added after the experiment began. Generally speaking, PCL-treated rats (both saline and heroin) increased active lever pressing when the operant contingency was re-introduced (Phase 4, 7 and 8); however, there was not a significant difference between heroin and saline-treated PCL animals, as predicted.

The major aim of the present study was to replicate previous studies conducted in our lab (Morrison et al. 2011) and to determine the contingencies necessary for an increase in operant responding for a food-paired conditioned stimulus after repeated intermittent drug administration. The present study was designed to test alternatives to conditioned reinforcement as a basis for observed behavior change. These alternatives include: a) effects attributable to drug alone (PNL condition), b) mere presentation of a conditioned stimulus (PNCL), or c) response-contingent presentation of a neutral stimulus (UCL condition). The findings of the experimental manipulations used to rule out these alternate explanations are discussed below.

One alternate explanation is that the increase in operant behavior can be attributed to drug alone, and that the function is under the control of non-associative factors. This explanation was

ruled out by the PNL condition because these animals received intermittent drug injections; however, for operant testing no light stimulus was presented. If intermittent drug exposure increased general operant responding, it would be expected that animals that received heroin without a light presentation would respond similarly to animals in the PCL heroin condition. This however, was not the case; therefore, this alternate explanation can be ruled out (see Figure 3).

Another alternate explanation is that mere presentation of the conditioned stimulus results in an increase in operant responding. This alternate explanation was controlled for by having a group of animals that had a previous positive associative learning history with the CS-US, however, during testing received the food-paired light stimulus non-contingently (PNCL). If merely presenting the conditioned stimulus was the cause of the increase in operant responding demonstrated in heroin-treated rats, it would be expected that PCL and PNCL heroin-treated rats would respond similarly. This was not the case, however (see Figure 3). Generally speaking, among all heroin-treated animals, animals in the PNCL group demonstrated the lowest rates of responding on the active lever. These findings lend additional support to the conclusions that heroin only enhances operant responding when there is a contingency in place, or enhances operant contingency learning.

A final alternate explanation for acquisition of operant responding is that a response contingent presentation of a neutral stimulus (UCL groups) could result in an increase in active lever pressing. Animals that were in the UCL groups (saline and heroin) did demonstrate an increase in active lever pressing in Phase 3 compared to other controls (PNL and PNCL) (see Figure 1, panel 1). The UCL groups also showed discriminative responding on the active lever compared to the inactive lever, however, the difference between active and inactive lever

pressing was not as large compared to animals that received paired CS-US pairings during Pavlovian training (see Figure 3). Therefore, it could be inferred that receiving heroin can enhance responding for a stimulus that is presented response-contingently, but does not enhance responding for this stimulus to the degree it would if the stimulus was previously paired with an unconditioned stimulus, as was evident in the PCL condition.

A goal of the present study was to investigate long-term effects of repeated administration of drug on conditioned reinforcement and resistance to extinction. The long-term effects that were expected were that heroin-treated rats in the PCL group would demonstrate persistent responding across either 15 or 10 days when the conditioned reinforcer was presented. It was also predicted that after operant extinction, the long-term effects of heroin on enhancing responding for a conditioned reinforcer would be evident during the reinstatement of operant responding phase (Phase 7). Results did not show greater spontaneous recovery of operant responding for PCL heroin-treated animals compared to saline-treated animals and all respective control groups. For the reinstatement tests, we found that PCL rats in the heroin and saline conditions showed recovery of operant responding when training involved response-contingent presentation of the light, however, there were no differential effects of drug. We found that heroin-treated animals in the PCL group showed a slight increase in operant responding compared to saline controls, however, this difference was not statistically significant (see Figure 1). To our knowledge, no other study has attempted to measure the effects of all of these experimental manipulations in the same paradigm and studies have not attempted to specifically manipulate Pavlovian and operant contingencies (Wyvell and Berridge 2001; Ranaldi et al. 2009; Morrison et al. 2011; Galaj et al. 2013; Di Ciano and Everitt 2004; Palmatier et al. 2007; Chaudiri et al. 2007; Parkinson et al. 2005). In the studies that measured long-term effects of drugs on operant responding for a

conditioned reinforcer, few used heroin (Ranaldi et al. 2009; Morrison et al. 2011); most studies use other drugs of abuse. In these studies, changes in operant responding after repeated drug administration are typically explained by other non-associative interpretations, such as neural sensitization and anhedonia.

One explanation of the findings of the present study demonstrating that PCL heroin-treated rats pressed more on the active lever compared to all other experimental conditions can be based on the phenomenon of neurological sensitization to heroin (Ponteri, Calo, Di Grezia, Orzi, et al. (1997); Ponteri, Monnazzi, Scontrini, Buttarelli, & Patacchioli, (2001).In previous studies conducted in our lab (Morrison et al. 2011), we only measured operant responding 30 days following drug administration. In Morrison et al., there was no way to determine if the enhancement of conditioned reinforcement resulted from behavioral sensitization effects since locomotor activity was not measured at day 30. This was the rationale of having challenge tests 1 and 2 in the present study to examine whether behavioral sensitization effects could be partially involved in operant responding for the conditioned reinforcer that was seen until Phases 7 and 8. The results from challenge tests 1 and 2 demonstrated that heroin-treated rats were still sensitized and had similar locomotor activity compared to the last session of sensitization. These findings suggest that neurological sensitization to heroin could be one of the explanations of the differences in operant responding for a food-paired conditioned reinforcer in heroin-treated vs. saline-treated animals.

Another account of the present findings could be the argument that in both human and animal literature heroin produces an anhedonic state which results in a decrease in the reinforcing effectiveness of natural rewards, such as food (Harris and Aston-Jones 2003). In the current study, if anhedonia was occurring, we would expect that heroin-treated animals in all

experimental conditions would demonstrate a decrease in operant responding for a food-paired light stimulus, however, this was not the case. An advantage to the present study is that responding for conditioned reinforcers was tested in the absence of heroin, resulting in a clean measure of the reinforcing effectiveness of other rewards in the environment, including natural rewards. Compared to the studies mentioned above, the present study measured conditioned responding for a stimulus paired with food that was presented separately from drug administration. When the drug was removed from the context where food reinforcement was being measured, the effects of heroin on food-related stimuli were observed. The present study found that when measuring operant responding for a stimulus paired with food in the absence of heroin, animals demonstrated an increase in responding for a food-paired stimulus. These differing findings may suggest that the anhedonia that is observed is possibly not a general phenomenon, but may be specific to a context that includes drug administration.

The aforementioned alternative interpretations put forth by Ponteri et al. (1997) and Harris & Aston-Jones (2003), although plausible, do not incorporate necessary control groups to rule out explanations other than conditioned reinforcement. Moreover, they fail to include long-term testing of the effects of drug on conditioned reinforcement; whereas the current study included the appropriate control groups for these alternate measures by having groups of animals receiving a yoked stimulus presentation (PNCL), unpaired stimulus presentation (UCL), and no stimulus presentation (PNL) in addition to receiving continuous-repeated testing (112 days).

Our lab is one of the few that has attempted to study the effects of intermittent heroin exposure on the enhancement of conditioned reinforcement. Morrison et al. (2011) examined the effects of repeated-intermittent heroin on protracted withdrawal (i.e., responding for a conditioned reinforcer after a 30-day abstinence period. Morrison et al. found that the

enhancement of a conditioned reinforcement effect lasted for 30-days post heroin administration. Morrison et al. was one of the few studies that have measured the long-term effects of heroin on conditioned responding; however, it lacked appropriate control groups to rule out alternate explanations of conditioned reinforcement. In Morrison et al., the only condition that was used similar to the present study was a paired-contingent light condition. The current study replicated Morrison et al.'s findings to demonstrate that heroin enhances responding for a food-paired conditioned reinforcer during the acquisition of novel response phase, with an additional advantage that instead of having 29 days of rest in the home cage, testing was conducted on consecutive days to control for any generalization decrement effects. The findings of the current study not only replicated previous studies conducted in our lab (Ranaldi et al. 2009; Morrison et al. 2011), but also extended these studies by the ability to draw conclusions about the parameters necessary for heroin to enhance responding for a food-paired conditioned reinforcer. The design used in the present study found that the only time heroin-treated animals demonstrated an increase in operant responding for a food-paired light stimulus was when the animals had a previous CS-US pairing and a response-contingent stimulus presentation. Taken together, the present study and Morrison et al. findings suggest that heroin selectively enhances operant contingency learning.

A strength of the present study was use of an acquisition of a novel response paradigm to avoid confounds of the conditioned reinforcement procedure with other possible associations between the neutral stimulus and other rewards that can be found in other paradigms such as the cue-induced reinstatement models and self-administration paradigms.. Another strength was that this study, to our knowledge, is one of the only studies that has measured repeated drug administration using continuous operant testing instead of testing at several different intervals

(e.g., 5 days, 30 days, 60 days, etc...) with long lags between these tests. The advantage of continuous testing compared to intermittent testing (see DiCiano and Everitt 2004 for an example of intermittent testing) is that additional phenomena can be examined, such as generalization decrement in extinction, without the tests being confounded by long-break periods in the home cage which is a different context and would result in more operant responding when subjects are placed back in the test chamber. A strength of using continuous testing is that the context of the test environment stays relatively unchanged under continuous, repeated testing compared to procedures that use intermittent testing after long break periods where now the test environment may constitute a novel context. Therefore, changes in operant responding can be attributed to the change in the contingency of the conditioned reinforcer, and not to novelty of the environment (i.e., generalization decrement effect, see Bouton and Bolles 1979).

Even though the present study had several methodological strengths to measure and rule out explanations other than conditioned reinforcement, there were still limitations. One limitation was that decisions regarding phase changes were made ad hoc, dependent on operant responding levels in each phase of the experiment. The justification for making ad hoc, rather than planned decisions was that there were no prior studies to inform decision making. Nonetheless, the present study provided information that informs future studies.

An additional limitation in the current study was the possibility of insufficient drug exposure prior to Phase 8. This may account for failure to observe a drug effect comparable to that seen in Phase 3. After an extensive literature search, no relevant literature was found that was comparable to the present study, therefore, we were not certain if the magnitude of this manipulation (only one drug injection) would be sufficient to elicit greater levels of operant responding compared to Phase 7 where only the conditioned reinforcer was presented. For future

studies, the number of injections prior to the reinstatement phase could be manipulated to determine if several injections are needed to have a more robust heroin reinstatement effect.

For future research, more studies need to be conducted that manipulate hierarchical associations after repeated intermittent drug administration to be able to systematically measure which specific associative learning structure is altered. To aid in examining the effects of repeated drug administration on various types of associative learning mechanisms, it would be valuable to use a devaluation paradigm. By using a devaluation paradigm, stimulus-response (S-R), response-outcome (R-O), and stimulus-outcome (S-O) learning could be teased apart further and result in stronger conclusions to be drawn about the effects of repeated-intermittent heroin on associative learning mechanisms.

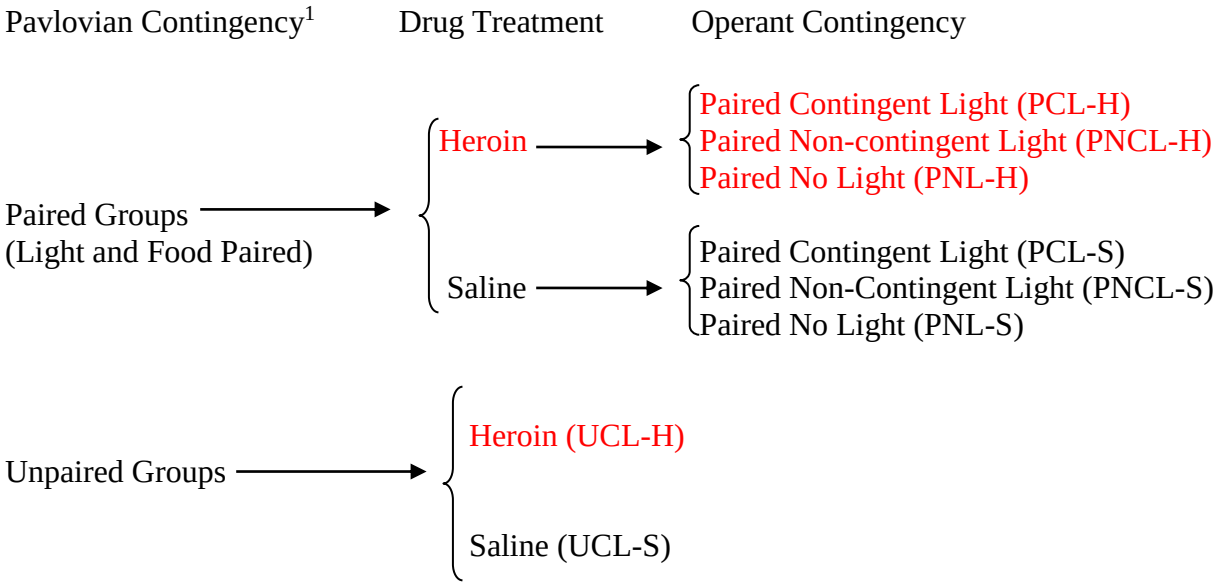
In summary, the present study found that repeated heroin administration resulted in acquisition of a novel response and enhancement of conditioned reinforcement effects that were persistent until Phase 3. The effects of heroin were evident in absolute rates of operant responding, magnitude of difference in active lever pressing between the PCL and control groups (PNCL, UCL, and PNL), and the degree of differential responding. An implication of these findings is that acute use of drugs, such as heroin, can lead to changes in associative learning mechanisms affecting learning for natural rewards. Related to applied implications, these findings could better inform clinicians about the associations that are strengthened during drug use. This may result in improved treatment outcomes as a result of a more thorough understanding of the associative structures (Pavlovian and operant) that are altered from habitual drug use.

Table 1. *Experimental Conditions*

Phase	Description	Duration/ (Session Numbers)
1) Pavlovian Conditioning *3 day break*	Rats were either given Light/food paired *or* Light/food unpaired	4 (1-4)
2) Repeated-intermittent Drug Treatment *3 day break*	***Behavioral Sensitization Test*** Rats were either assigned to receive saline or heroin IP injections and then placed in locomotor activity chambers.	12 Habituation (5-7) Sensitization (8-16)
	Phases 3-8: Associative Learning Test Phases	
3) Conditioned Reinforcement -Pavlovian Extinction *7 day break*	***Acquisition of Novel Responding*** Rats were assigned to one of four associative learning test conditions (see Table 2). No food presented	15 (16-30)
4) Conditioned Reinforcement -Pavlovian Extinction *7 day break*	**** Spontaneous Recovery (Pavlovian responding)*** Same procedure as Phase 3	10 (31-40)
5) Operant Extinction -- No CS *7 day break*	***Resistance to Operant Extinction*** Lever pressing had no programmed consequences for all groups	15 (41-55)
6) Operant Extinction --No CS *7 day break*	***Spontaneous recovery (operant responding)*** Same procedure as Phase 5	10 (56-65)
7) Conditioned reinforcement --Pavlovian Extinction ****Without Drug****	***Reinstatement Test*** Same procedure as Phase 3.	7 (66-72)
	Challenge Test 1 **7 day break**	(72)
8) Conditioned Reinforcement --Pavlovian Extinction ****With Drug****	***Reinstatement test after challenge injection*** Same procedure as Phase 3	7 (73-79)
	Challenge Test 2	(79)

Table 2.

Assignment of Rats to Pavlovian Conditioning, Drug Treatment, and Associative Learning Test Conditions



[1] Rats were randomly assigned to saline and heroin conditions, and then assigned to be in either the paired or unpaired groups. For the Paired groups, these animals were randomly assigned to either the PCL, PNCL, or PNL condition. There was only one condition for the unpaired saline and heroin-treated animals.

Table 3.
4-way ANOVA summary table comparing drug, condition, phase and lever

Variable	*df*	*F*	*p*
Drug	1	.003	> .05
Condition	3	14.86	< .05*
Drug x Condition	3	2.86	< .05*
Error	55		
Lever	1	35.42	< .05*
Lever x drug	1	.38	> .05
Lever x condition	3	12.67	<.05*
Lever x drug x condition	3	2.91	< .05*
Error	55		
Phase	3	66.59	< .05*
Phase x drug	3	.41	> .05
Phase x condition	9	8.67	< .05*
Phase x drug x condition	9	.51	> .05
Error	165		
Lever x phase	3	28.03	< .05*
Lever x phase x drug	3	.24	> .05
Lever x Phase x condition	9	14.32	< .05*
Lever x phase x condition x drug	9	1.10	> .05
Error	165		

Note: ** $p < .05$

Table 4.
Follow-up ANOVAs for Drug x Group x Lever Interaction

Variable	*df*	*F*	*p*
Active Lever			
Drug	1	.10	> .05
Condition	3	19.96	< .05*
Drug x Condition	3	1.10	> .05
Error	55		
Inactive Lever			
Drug	1	0.35	> .05
Condition	3	.48	> .05
Drug x Condition	3	.18	> .05
Error	55		

Table 5.

Follow-up ANOVAs for Condition x Phase x Lever Interaction

Variable	*df*	*F*	*p*
Active Lever			
Condition	3	20.62	< .05*
Error	59		
Phase	3	71.55	< .05*
Condition x Phase	9	16.58	< .05*
Error	177		
Post-Hoc Tukey			
Phase 3			
PCL vs. PNCL			< .05*
PCL vs. UCL			< .05*
PCL vs. PNL			< .05*
Phase 4			
PCL vs. PNCL			< .05*
PCL vs. UCL			< .05*
PCL vs. PNL			< .05*
Phase 5			< .05*
PCL vs. PNCL			> .05
PCL vs. UCL			< .05*
PCL vs. PNL			
Phase 6			< .05*
PCL vs. PNCL			> .05
PCL vs. UCL			< .05*
PCL vs. PNL			
Inactive Lever			
Condition	3	.52	> .05
Error	59		
Phase	3	12.51	<.05*
Condition x Phase	9	1.08	>.05
Error	177		

Table 6.

Follow-up ANOVAs for Drug x Group x Lever Interaction in Phase 3

Variable	*df*	*F*	*p*
Active Lever			
Drug	1	0.10	> .05
Condition	3	19.96	< .05*
Drug x Condition	3	1.10	> .05
Error	55		
Inactive Lever			
Drug	1	0.34	> .05
Condition	3	0.49	> .05
Drug x Condition	3	0.18	> .05
Error	55		

Table 7.

Follow-up ANOVAs for Drug x Condition x Lever Interaction in Phase 4

Variable	*df*	*F*	*p*
Drug	1	.08	> .05
Condition	3	11.52	< .05*
Drug x Condition	3	0.24	> .05
Error	55		
Lever	1	35.80	< .05*
Lever x Drug	1	0.50	> .05
Lever x Condition	3	11.91	< .05*
Lever x Drug x Condition	3	0.30	> .05
Lever Error	55		
Post-Hoc			
Active Lever			
PCL vs. PNCL			< .05*
PCL vs. UCL			< .05*
PCL vs. PNL			< .05*
Inactive Lever			
PCL vs. PNCL			> .05
PCL vs. UCL			> .05
PCL vs. PNL			> .05

Table 8.

Follow-up ANOVAs for Drug x Condition x Lever Interaction in Phase 5

Variable	*df*	*F*	*p*
Drug	1	.07	<.05
Condition	3	4.33	< .05*
Drug x Condition	3	0.87	> .05
Error	55		
Lever	1	7.85	< .05*
Lever x Drug	1	0.01	> .05
Lever x Condition	3	1.14	> .05
Lever x Drug x Condition	3	0.30	> .05
Lever Error	55		
Post-Hoc			
Active Lever			
PCL vs. PNCL			< .05*
PCL vs. UCL			> .05
PCL vs. PNL			< .05*
Inactive Lever			
PCL vs. PNCL			> .05
PCL vs. UCL			> .05
PCL vs. PNL			> .05

Table 9.

Follow-up ANOVAs for Drug x Condition x Lever Interaction in Phase 6

Variable	*df*	*F*	*p*
Drug	1	.03	> .05
Condition	3	4.15	< .05*
Drug x Condition	3	2.76	> .05
Error	55		
Lever	1	5.92	< .05*
Lever x Drug	1	0.18	> .05
Lever x Condition	3	1.94	> .05
Lever x Drug x Condition	3	1.34	> .05
Lever Error	55		
Post-Hoc			
Active Lever			
PCL vs. PNCL			< .05*
PCL vs. UCL			> .05
PCL vs. PNL			< .05*
Inactive Lever			
PCL vs. PNCL			> .05
PCL vs. UCL			> .05
PCL vs. PNL			> .05

Table 10.

Follow-up ANOVAs for Drug x Condition x Lever Interaction in Phase 7

Variable	*df*	*F*	*p*
Drug	1	0.11	> .05
Condition	2	28.07	< .05*
Drug x Condition	2	0.72	> .05
Error	20		
Lever	1	40.01	< .05*
Lever x Drug	1	2.04	> .05
Lever x Condition	2	1.94	> .05
Lever x Drug x Condition	2	3.38	< .05*
Lever Error	20		
Active Lever			
Drug	1	0.77	< .05*
Condition	2	35.14	> .05
Drug x Condition	2	1.83	> .05
Error	20		
Post-Hoc Tukey			
PCL vs. PNCL			< .05*
PCL vs. UCL			> .05
PCL vs. PNL			< .05*
Inactive Lever			
Drug	1	0.70	> .05
Group	2	1.54	> .05
Drug x Group	2	1.40	> .05
Error	20		

Note: UCL saline and heron-treated animals were excluded from this analysis as discussed in method and elsewhere throughout results.

Table 11.

Follow-up ANOVAs for Drug x Condition x Lever Interaction in Phase 8

Variable	*df*	*F*	*p*
Drug	1	0.74	> .05
Condition	3	12.20	< .05*
Drug x Condition	3	1.61	> .05
Error	42		
Lever	1	46.20	< .05*
Lever x Drug	1	0.36	> .05
Lever x Condition	3	17.95	< .05*
Lever x Drug x Condition	3	0.51	> .05
Lever Error	42		
Active Lever			
PCL vs. PNCL			< .05*
PCL vs. UCL			< .05*
PCL vs. PNL			< .05*
Inactive Lever			
PCL vs. PNCL			> .05
PCL vs. UCL			> .05
PCL vs. PNL			> .05

Figure 1.

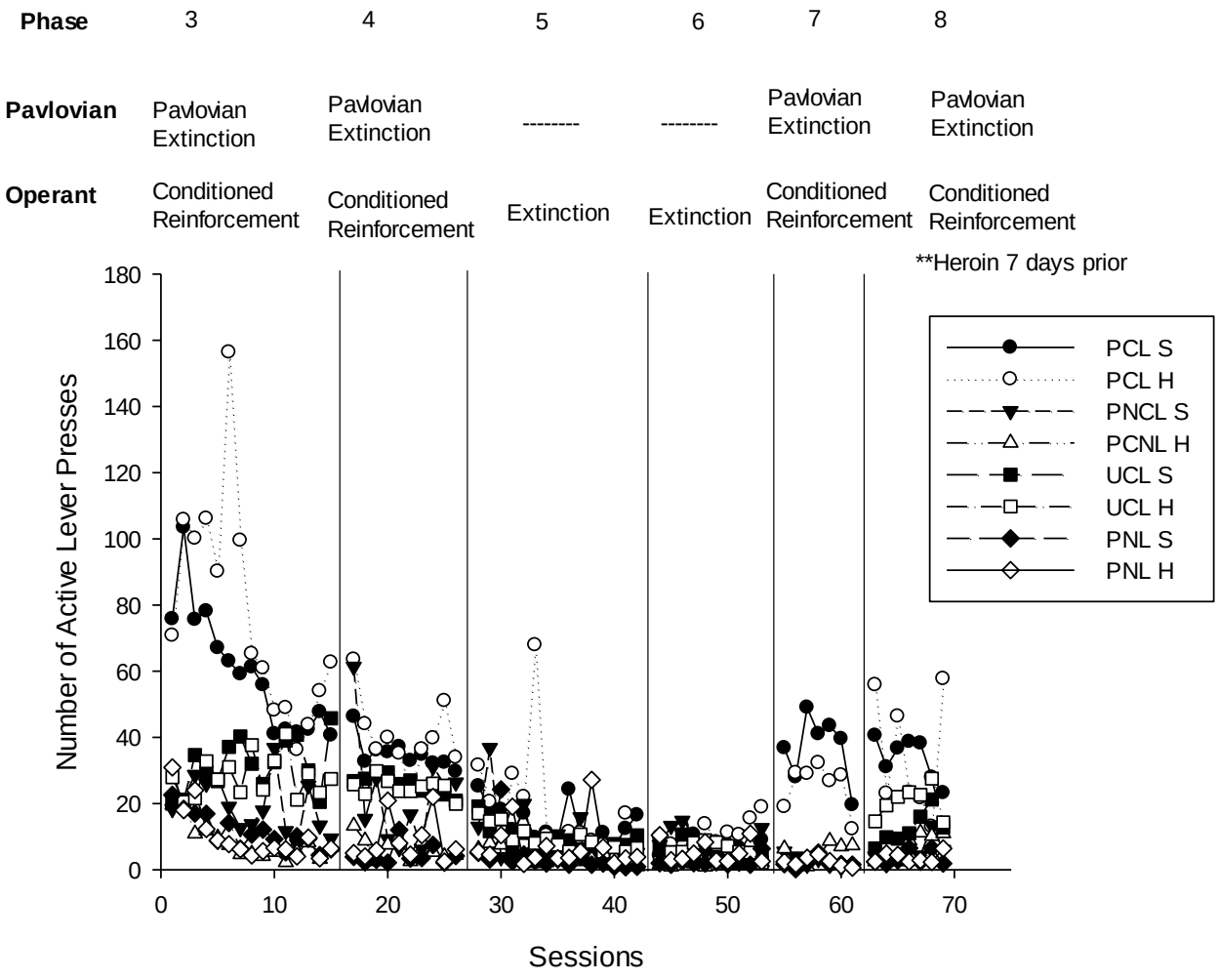


Figure 1. The mean number of active lever presses across Phases 3-8 in the PCL saline (*n*=8), PCL heroin (*n*=8), PNCL saline (*n*=8), PNCL heroin (*n*=8), UCL saline (*n*=8), UCL heroin (*n*=8), and PNL saline (*n*=8), and PNL heroin (*n*=7) groups. Each data point represents the average of active lever presses per session across saline-and-heroin-treated rats in each experimental condition.

Figure 2.

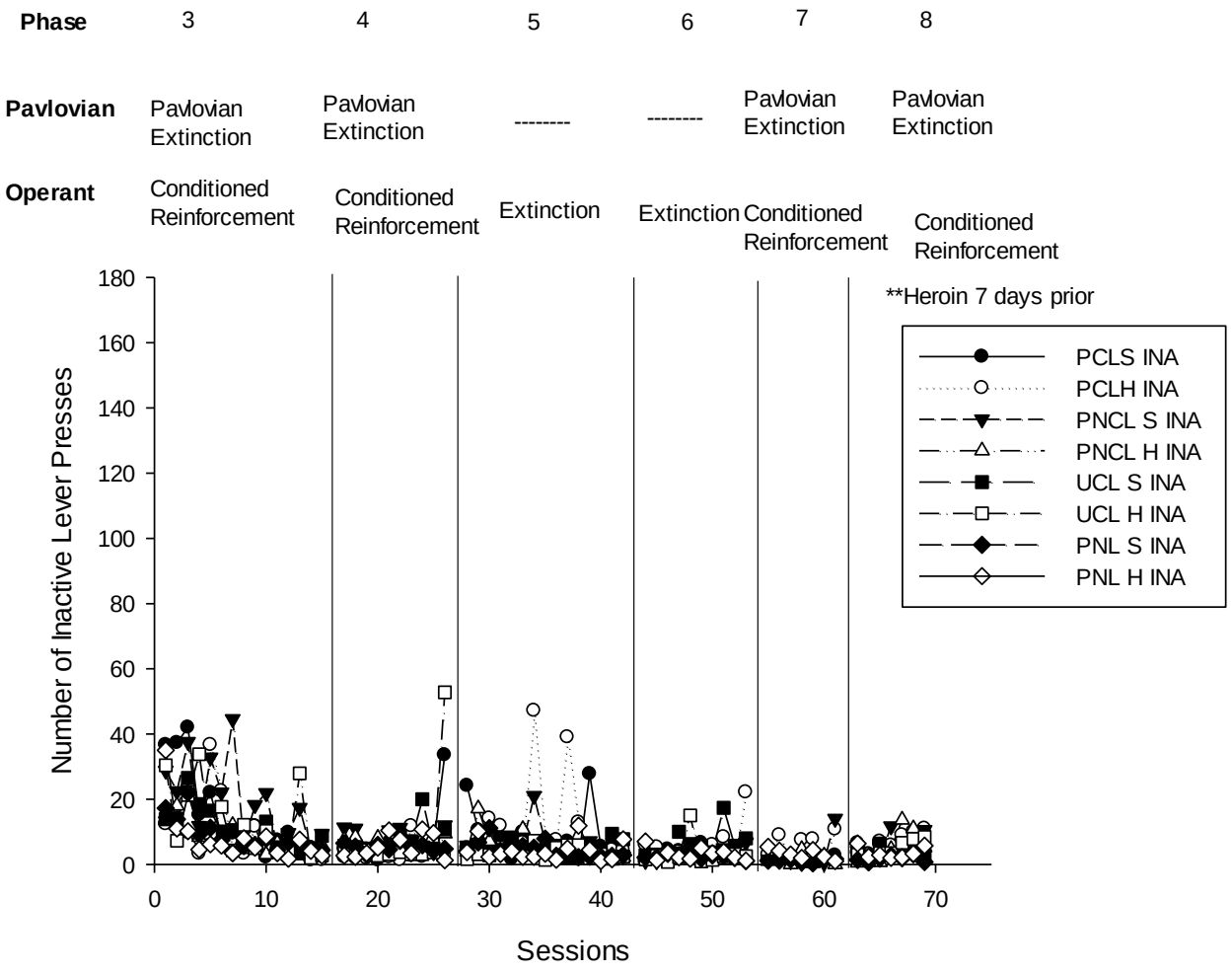


Figure 2. The mean number of inactive lever presses across Phases 3-8 in the PCL saline (*n*=8), PCL heroin (*n*=8), PNCL saline (*n*=8), PNCL heroin (*n*=8), UCL saline (*n*=8), UCL heroin (*n*=8), and PNL saline (*n*=8), and PNL heroin (*n*=7) groups. Each data point represents the average of inactive lever presses per session across saline-and-heroin-treated rats.

Figure 3.

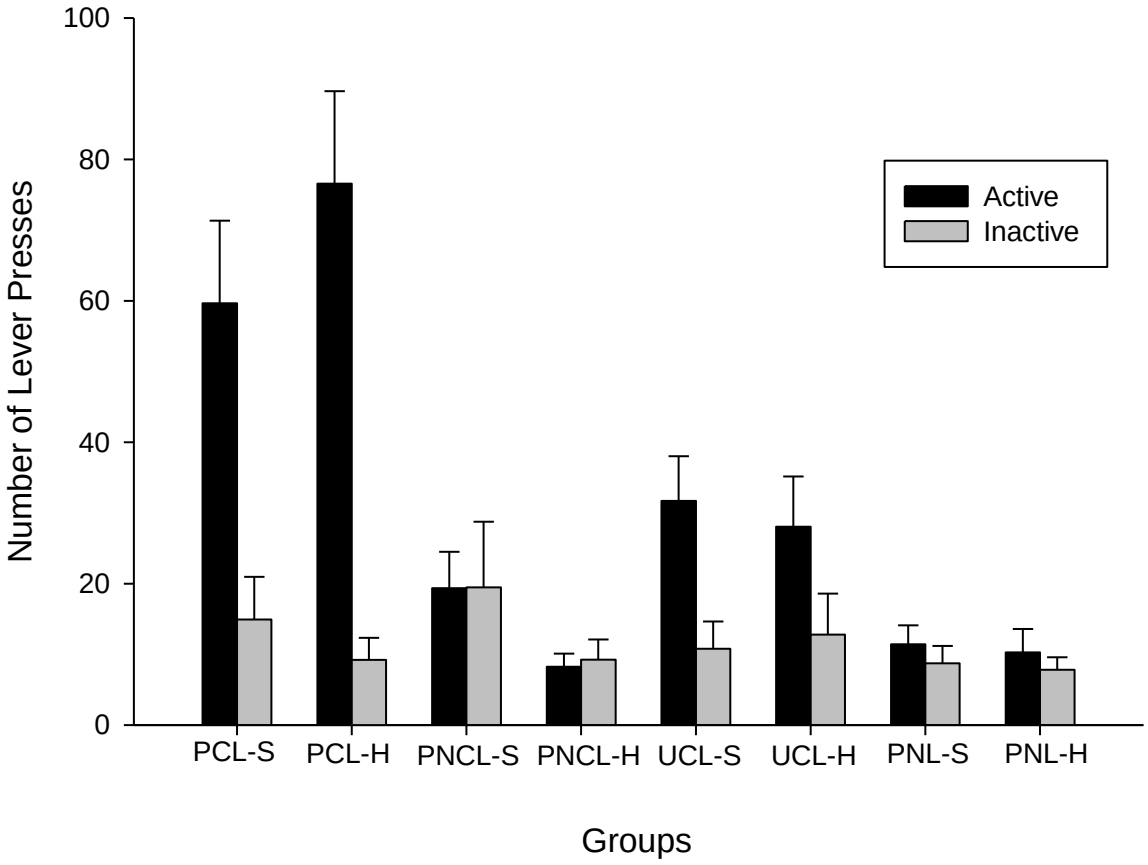


Figure 3. The mean (+ SEM) number of active and inactive lever presses in Phase 3 in the PCL saline (*n*=8), PCL heroin (*n*=8), PNCL saline (*n*=8), PNCL heroin (*n*=8), UCL saline (*n*=8), UCL heroin (*n*=8), and PNL saline (*n*=8), and PNL heroin (*n*=7) groups. Each bar represents the average of 15 days of active and inactive lever presses across saline-and heroin-treated rats.

Figure 4.

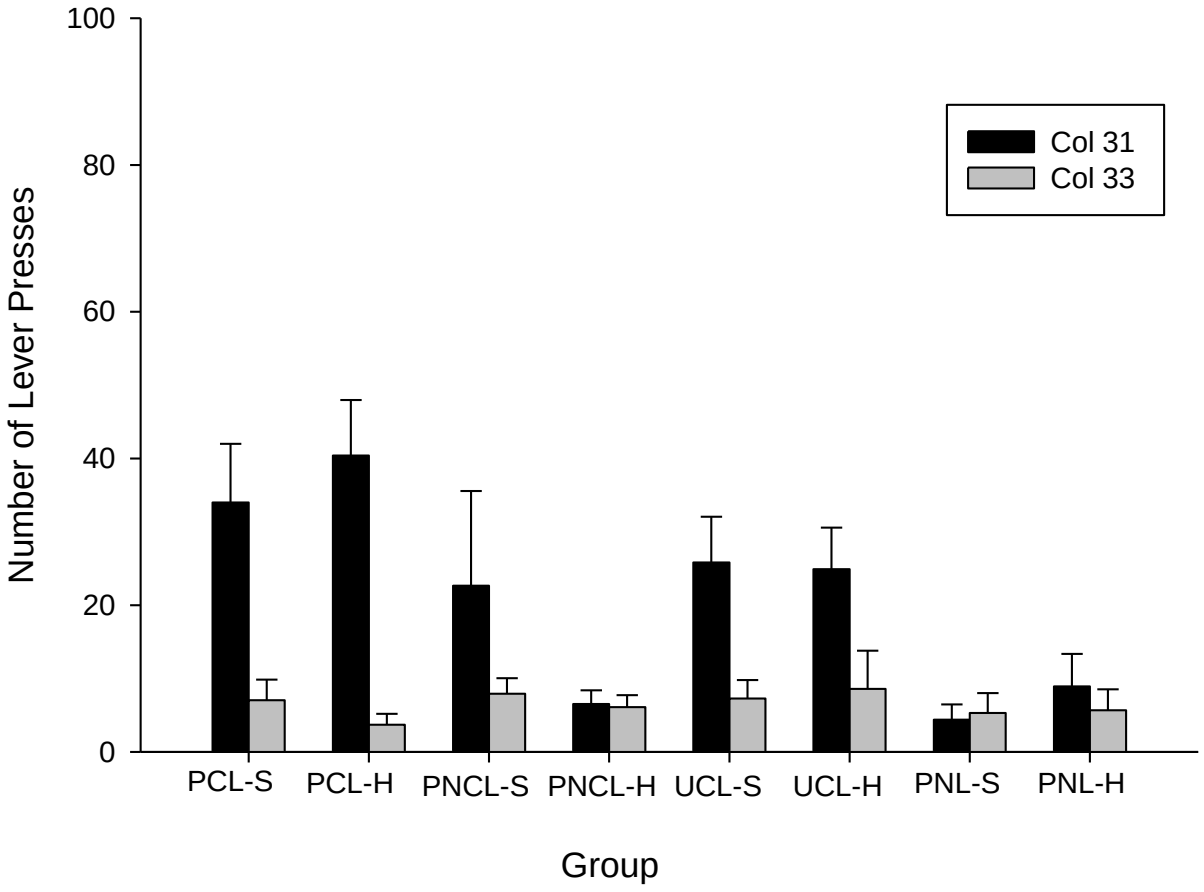


Figure 4. The mean (+ SEM) number of active and inactive lever presses in Phase 4 in the PCL saline (*n*=8), PCL heroin (*n*=8), PNCL saline (*n*=8), PNCL heroin (*n*=8), UCL saline (n=8), UCL heroin (*n*=8), and PNL saline (*n*=8), and PNL heroin (*n*=7) groups. Each bar represents the average of 10 days of active and inactive lever presses across saline-and-heroin-treated rats.

Figure 5.

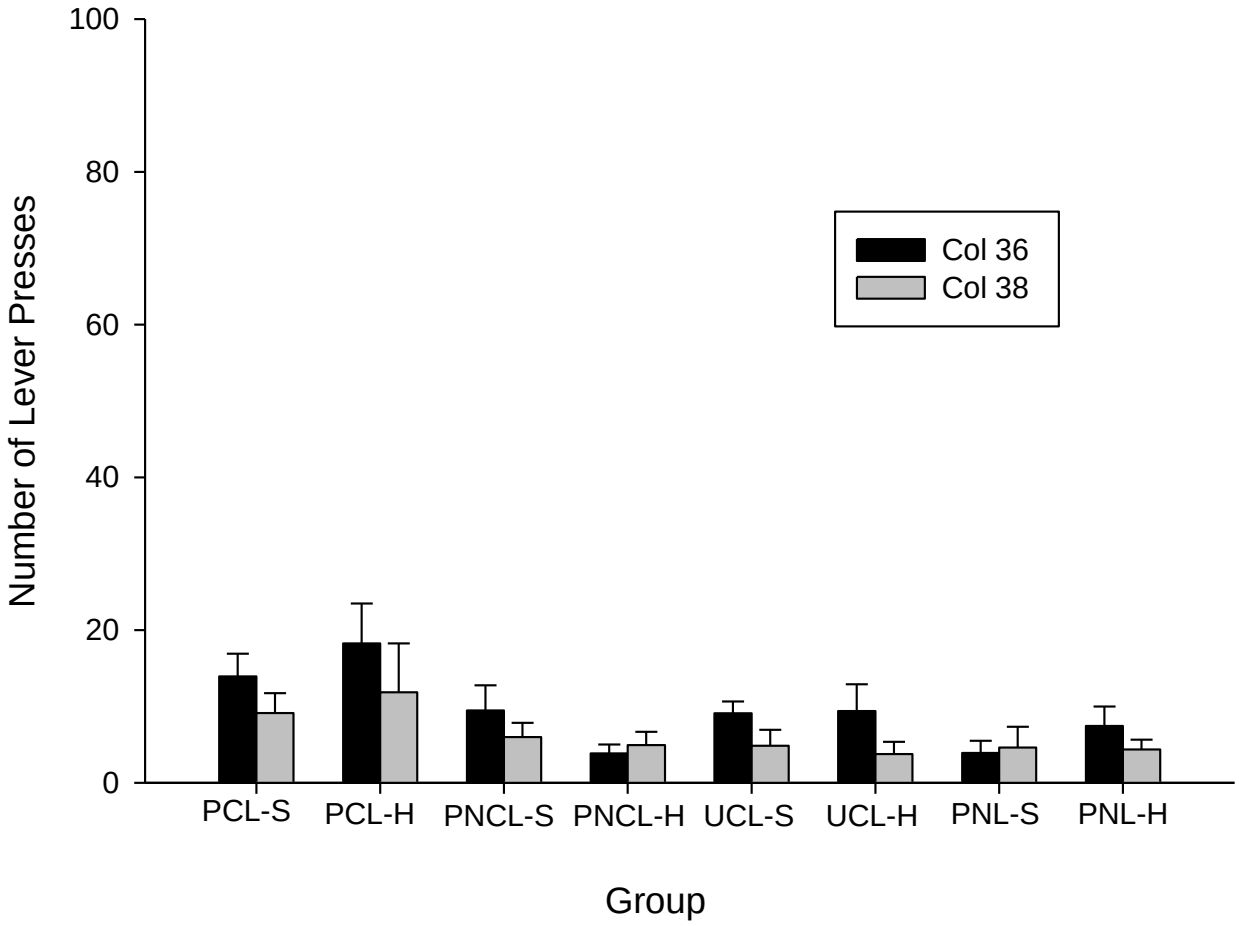


Figure 5. The mean (+ SEM) number of active and inactive lever presses in Phase 5 in the PCL saline (*n*=8), PCL heroin (*n*=8), PNCL saline (*n*=8), PNCL heroin (*n*=8), UCL saline (*n* =8), UCL heroin (*n*=8), and PNL saline (*n*=8), and PNL heroin (*n*=7) groups. Each bar represents the average of 15 days of active and inactive lever presses across saline-and-heroin-treated rats.

Figure 6.

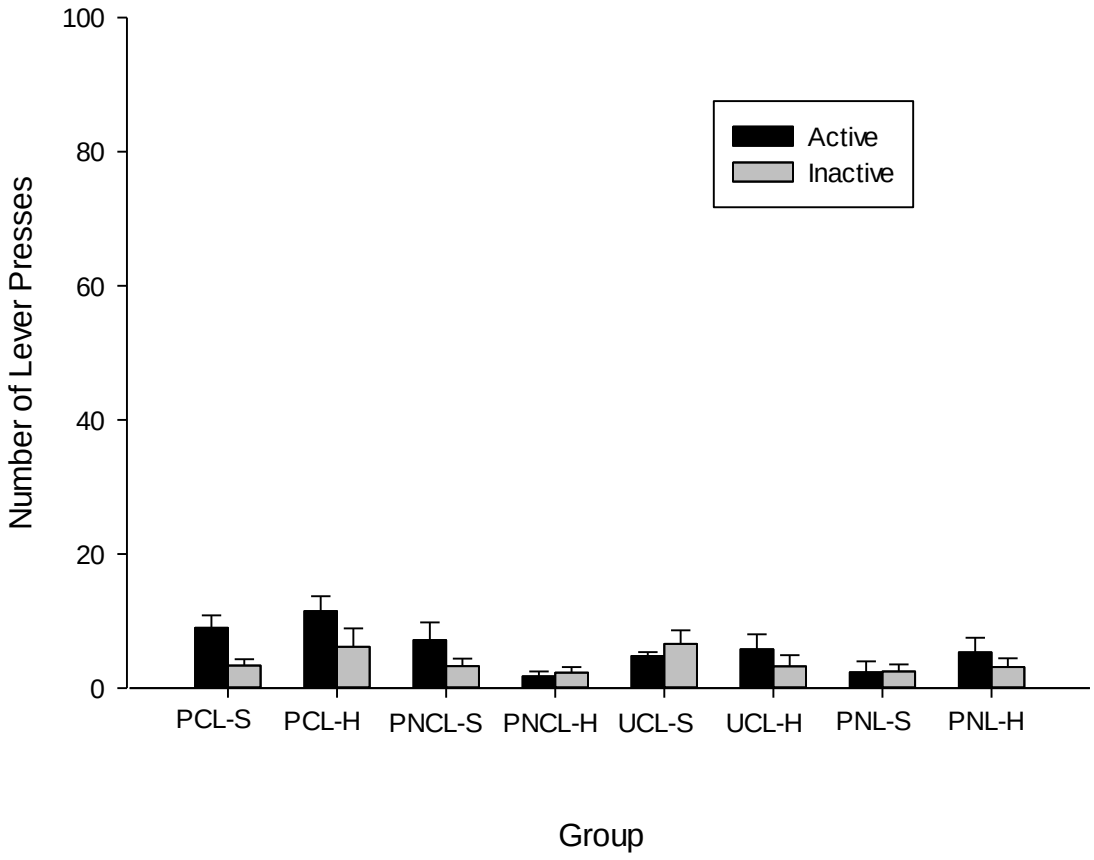


Figure 6. The mean (+ SEM) number of active and inactive lever presses in Phase 6 in the PCL saline (*n*=8), PCL heroin (*n*=8), PNCL saline (*n*=8), PNCL heroin (*n*=8), UCL saline (*n*=8), UCL heroin (*n*=8), and PNL saline (*n*=8), and PNL heroin (*n*=7) groups. Each bar represents the average of 10 days of active and inactive lever presses across saline-and-heroin-treated rats.

Figure 7.

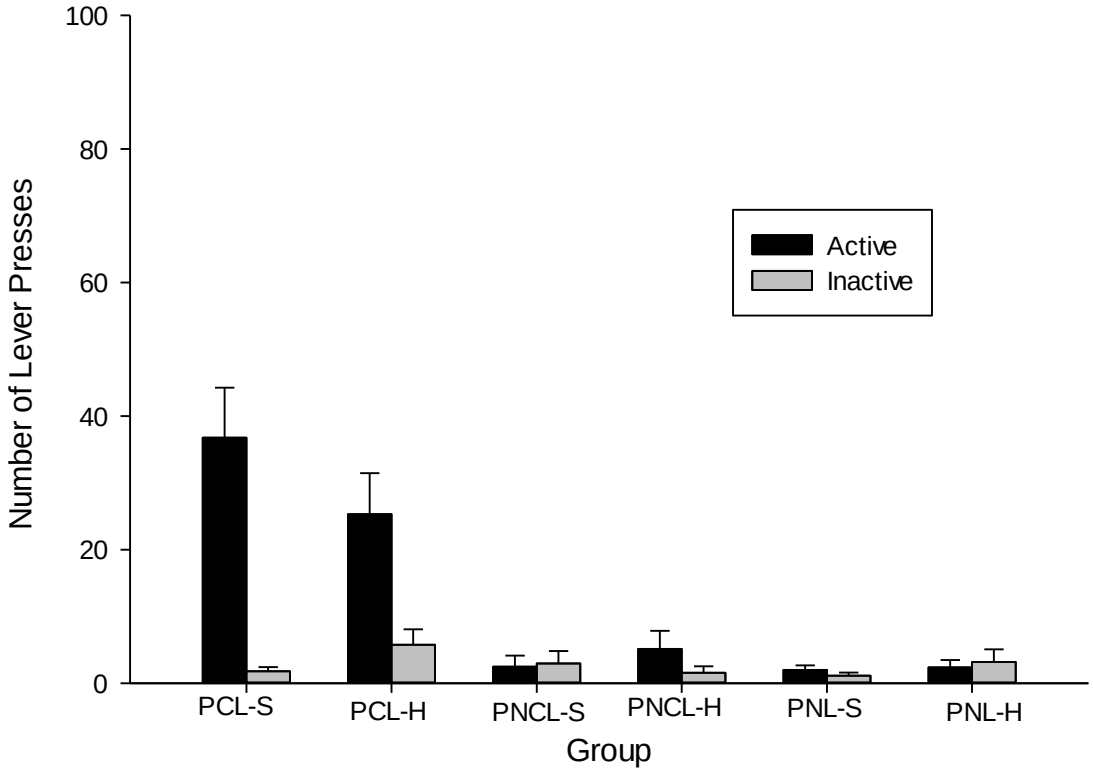


Figure 7. The mean (+ SEM) number of active and inactive lever presses in Phase 7 in the PCL saline (*n*=4), PCL heroin (*n*=4), PNCL saline (*n*=4), PNCL heroin (n=4), UCL saline (*n*=1), UCL heroin (*n*=1), and PNL saline (*n*=6), and PNL heroin (*n*=5) groups. Each bar represents the average of 7 days of active and inactive lever presses across saline-and-heroin-treated rats before receiving an additional injection of heroin or saline.

Figure 8.

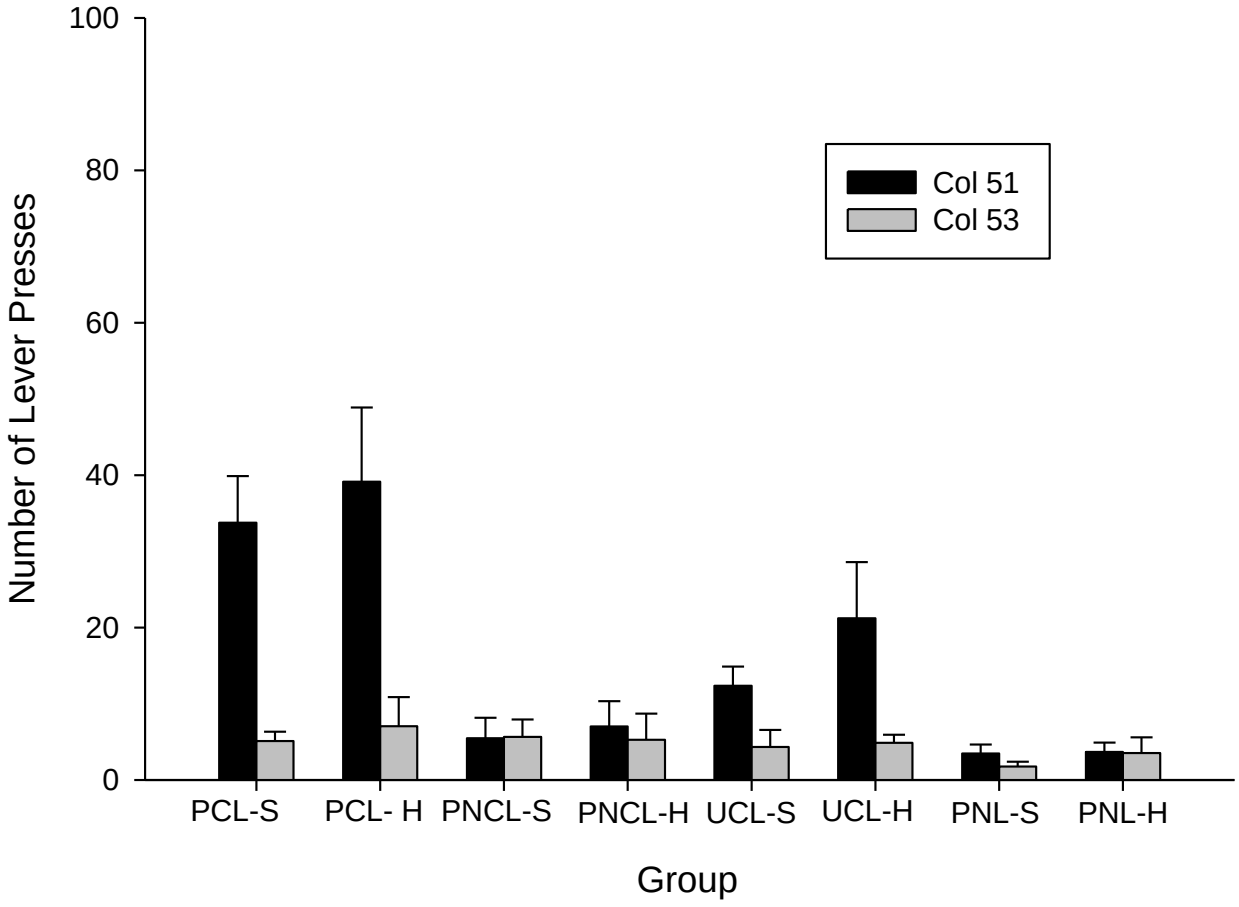


Figure 8. The mean (+ SEM) number of active and inactive lever presses in Phase 8 in the PCL saline (*n*=6), PCL heroin (*n*=6), PNCL saline (*n*=7), PNCL heroin (*n*=6), UCL saline (*n*=5), UCL heroin (*n*=5), and PNL saline (*n*=8), and PNL heroin (*n*=8) groups. Each bar represents the average of 7 days of active and inactive lever presses across saline-and-heroin-treated rats after receiving an additional injection of either heroin or saline 7 days prior.

Figure 9.

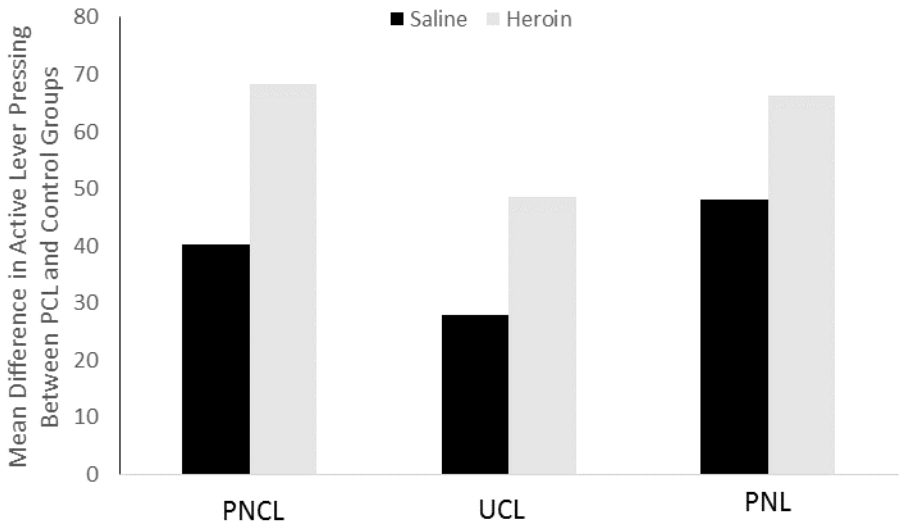


Figure 9. Mean difference in active lever presses between PCL (saline or heroin) and the three control conditions in Phase 3. Differences were calculated as a 15 day mean of PCL groups and means of control conditions (PNCL, UCL, PNL), separately for heroin and saline-treated animals.

Figure 10.

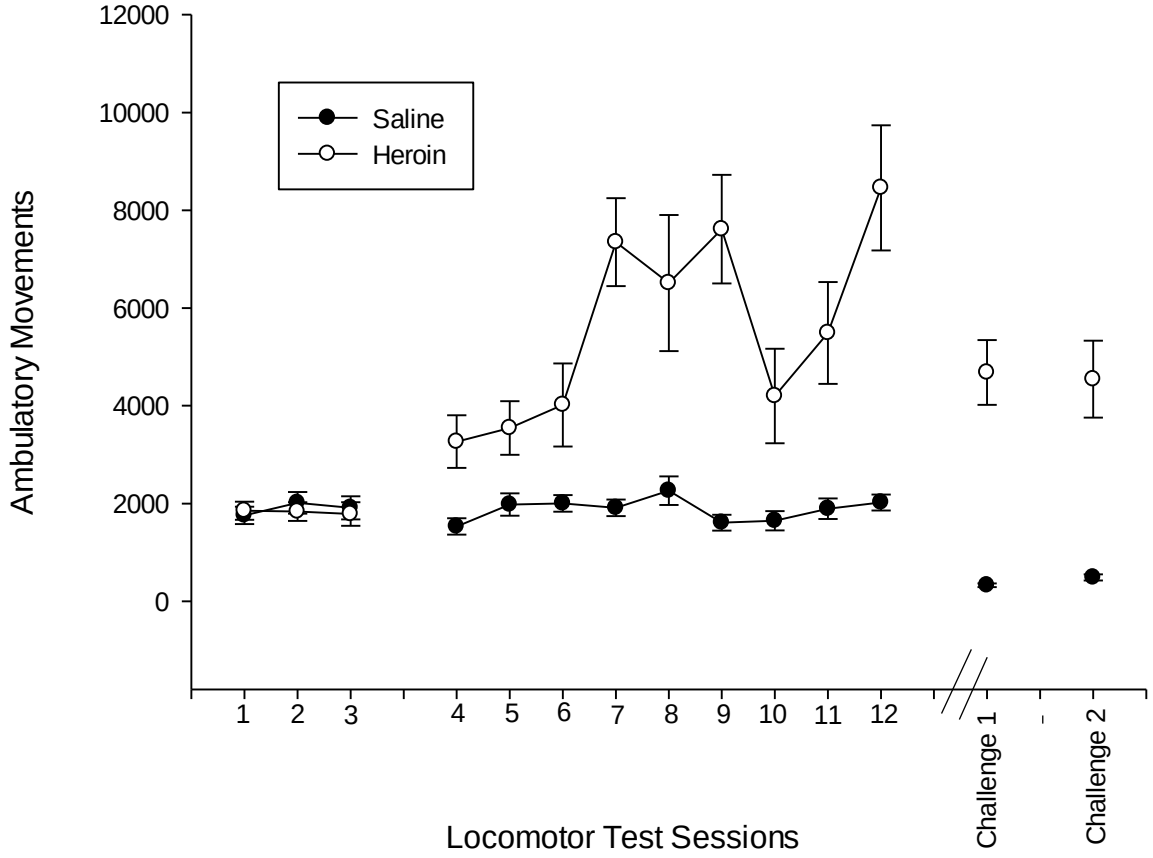


Figure 10. Mean (+ SEM) locomotor activity counts (measured as consecutive photo beam breaks during the habituation phase (session 1-3), during the treatment phase (session 4-12), Challenge test 1 where each rat received the same injection they received prior (challenge 1) and Challenge test 2 where all rats now received heroin (challenge 2). In the treatment phase, rats were treated daily with heroin (N=37) or saline (N=37) for 9 consecutive sessions.

Bibliography

Bouton, M. E. & Bolles, R. C. (1979). Role of conditioned contextual stimuli in reinstatement of Extinguished fear. *Journal of Experimental Psychology: Animal Learning and Cognition, 5,* 368-378.

Cabeza de Vaca, S. & Carr, K. D. (1998). Food restriction enhances the central rewarding effect of abused drugs. *Journal of Neuroscience, 18,* 7502-7510.

Carr, K. D. (2002). Augmentation of drug reward by chronic food restriction: behavioral evidence and underlying mechanisms. *Physiology and Behavior, 76,* 353-364.

Carr, K. D. (2007). Chronic food restriction: enhancing effects on drug reward and striatal cell signaling. *Physiological Behavior, 91,* 459-472.

Chaudhri, N., Caggiula, A. R., Donny, E.C., Booth, S., Gharib, M., Craven, L., Palmatier, M. L., Liu, X., & Syed, A. F. (2007). Self-administered and non-contingent nicotine enhance reinforced operant responding in rats: impact of nicotine dose and reinforcement schedule. *Psychopharmacology (Berl), 190,* 353-362.

Di Ciano, P., & Everitt, B. J., (2004). Conditioned reinforcing properties of stimuli paired with self-administered cocaine, heroin, or sucrose: implications for the persistence of addictive behavior. *Neuropharmacology, 47,* 202-213.

Domjan, M. (2005). Pavlovian conditioning: a functional perspective. *Annual Reviews in Psychology, 56,* 179-206.

Everitt, B. J., & Robbins, T. W. (2005). Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. *Nature Neuroscience, 8,* 1481-1489.

Galaj, E., Cruz, I., Schachar, J., Koziolck, M., & Ranaldi, R. (2013). Differential effects on natural reward processing in rats after repeated heroin. *Psychopharmacology, 229,* 125-

132.

Harris, G. C., & Aston-Jones, G. (2003). Altered motivation and learning following opiate withdrawal: evidence for prolonged dysregulation of reward processing. *Neuropsychopharmacology, 28,* 865-871.

Koob, G. F., & Le Moal, M. (1997). Drug abuse: hedonic homeostatic dysregulation. *Science, 278,* 52-58.

Mackintosh, N. J. (1974). The psychology of animal learning. *Oxford: Academic Press.*

Morrison, J., Thornton, V., & Ranaldi, R. (2011). Chronic intermittent heroin produces locomotor sensitization and long-lasting enhancement of conditioned reinforcement. *Pharmacology, Biochemistry and Behavior, 99,* 475-479.

Palmatier, M. L., Liu, X., Matteson, G. L., Donny, E. C., Caggiula, A. R., & Syed, A. F. (2007b). Conditioned reinforcement in rats established with self-administered nicotine and enhancement by noncontingent nicotine. *Psychopharmacology, 195,* 235-243.

Palmatier, M. L., Matteson, G. L., Black, J. J., Liu, X., Caggiula, A. R. , Craven, L., Donny, E. C., & Syed, A. F. (2007c). The reinforcement enhancing effects of nicotine depend on the incentive value of non-drug reinforcers and increase with repeated drug injections. *Drug and Alcohol Dependence,*

Parkinson, J.A., Roberts, C., Everitt, B. J., & Di Ciano, P. (2005). Acquisition of instrumental conditioned reinforcement is resistant to the devaluation of the unconditioned stimulus. *The Quarterly Journal of Experimental Psychology, 58B,* 19-30.

Ponteri, F. E., Calo, L., Di Grezia, R., Orzi, F., et al. (1997). Functional correlates of heroin sensitization in the rat brain. *European Journal of Pharmacology, 335,* 133-137.

Ponteri, F. E., Monnazzi, P., Scontrini, A., Buttarelli, F. R., & Patacchioli, F. R. (2001).

Behavioral sensitization to WIN55212.2 in rats pretreated with heroin. *Brain Research, 898,* 178-180.

Ranaldi, R., Egan, J., Kest, K., Fein, M., & Delamater, A. R. (2009). Repeated heroin in rats produces locomotor sensitization and enhances appetitive Pavlovian and instrumental learning involving food reward. *Pharm biochem Behav, 91,* 351-357.

Ranaldi, R., Munn, E., Neklesa, T., & Wise, R. A. (2000). Morphine and amphetamine sensitization demonstrated under moderate-and high dose NMDA receptor blockade with MK-801. *Psychopharmacology, 151,* 119-127.

Robbins, T. W., & Everitt, B. J. (1999). Drug addiction, bad habits add up. *Nature, 398,* 567-570.

Robinson, T. E., & Berridge, K. C. (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Research Reviews, 18,* 247-291.

Saddoris, M. P., & Careli, R. M. (2014). Cocaine self-administration abolishes associative neural encoding in the nucleus accumbens necessary for higher-order learning. *Biological Psychiatry, 75,* 156-164.

Saddoris, M. P., Stamatakis, A., & Carelli, R.M. (2011). Neural correlates of Pavlovian-to instrumental transfer in the nucleus accumbens shell are selectively potentiated following cocaine self-administration. *European Journal of Neuroscience, 33. Psychopharmacology, 142,* 31-40.

Siegel, S. (1975). Evidence from rats that morphine tolerance is a learned response. *Journal of Comparative & Physiological Psychology, 89,* 498-506.

Solomon, R. L., & Corbit, J. D. (1974). An opponent-process theory of motivation: I. Temporal dynamics of affect. *Psychological Review, 81,* 119-145.

Taylor, J. R., & Jentsch, J. D. (2001). Repeated intermittent administration of psychomotor stimulant drugs alters the acquisition of pavlovian approach behavior in rats: differential effects of cocaine, D-amphetamine and 3, 4-methylenedioxymethamphetamine ("Ecstasy"). *Biological Psychiatry, 50,* 137-143.

Wyvell, C. L., & Berridge, K. C. (2001). Incentive sensitization by previous amphetamine exposure: increased cue-triggered "wanting" for sucrose reward. *Journal of Neuroscience, 21,* 7831-7840.