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Prime-, Stress- and Cue-Induced Reinstatement of Extinguished Drug-Reinforced Responding in Rats: Cocaine as the Prototypical Drug of Abuse

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Abstract

This unit describes the testing of rats in prime-, footshock- and cue-induced reinstatement procedures. Evaluating rats in these procedures enables the assessment of treatments on behavior thought to model drug relapse precipitated by re-contact with an abused drug (prime-induced), induced by stress (footshock-induced), or by stimuli previously associated with drug administration (cue-induced). For instance, levels of reinstatement under the effects of test compound administration could be compared to levels under vehicle administration to help identify potential treatments for drug relapse, or reinstatement levels of different rat strains could be compared to identify potential genetic determinants of perseverative drug-seeking behavior. Cocaine is used as a prototypical drug of abuse, and relapse to its use serves as the model in this unit, but other self-administered drugs could readily be substituted with little modification to the procedures.

Keywords

drug relapse; drug abuse; cocaine

INTRODUCTION

Relapse in drug abusers can be precipitated by limited re-exposure to the abused drug itself (referred to as a “slip” or a “lapse”), exposure to environmental stimuli previously associated with drug use, or by stress. Preclinically, renewed drug seeking precipitated by these events can be examined with reinstatement procedures. Rats prepared with indwelling venous catheters will readily learn to press levers reinforced with intravenous infusions of several abused stimulants, depressants, opioids and glutamatergic agents, as well as nicotine (for review, see O’Connor et al., 2011). If, after having learned this response, lever pressing is no longer reinforced with drug infusion for several experimental sessions (i.e., they experience experimental extinction), responding will decrease to low rates. Reinstatement of extinguished lever pressing previously reinforced by drug infusion can then be precipitated by an experimenter-administered injection of the previously self-administered drug (i.e., administering a drug prime), by re-presenting cues (e.g., tones and lights) that had previously accompanied drug infusion, or by introduction of a stressor (e.g., footshock). Once reinstatement procedures are reliably established, they can be used to identify compound leads for treating drug relapse (such as potential medications that attenuate reinstatement) (e.g., Beardsley et al., 2005; Beardsley et al., 2010a; Beardsley et al., 2010b), or to identify liability conditions underlying perseverative drug-seeking behavior (such as possible genetic factors evidenced by strain-dependent differences in levels of reinstatement) (e.g., Dela Pena et al., 2011; Meyer et al., 2010; Miguens et al., 2011).

This unit is organized into three basic protocols and five support protocols. Basic Protocol 1 describes methods for establishing drug self-administration in the rat, extinguishing responding, and testing with prime-induced reinstatement procedures for modeling a drug “slip” or “lapse” that precipitates relapse. Basic Protocols 2 and 3 provide similar procedures for testing cue-induced reinstatement for modeling drug-paired stimulus-induced relapse, and footshock-induced reinstatement for modeling stress-induced relapse, respectively. For all basic protocols, cocaine is used as a prototypical drug of abuse. Support Protocol 1 describes the preparation of a medicated catheter locking solution used to help maintain catheter patency between self-administration sessions. Support Protocol 2 details the construction of a catheter flushing syringe assembly and sealing cap. Support Protocol 3 describes the construction of a catheter introducer used to assist in the insertion of the catheter into the jugular vein during the surgical preparation. Support protocol 4 details the construction of polyurethane indwelling venous catheters, and Support Protocol 5 describes their surgical implantation in rats. Detailed descriptions of constructing silicone intravenous catheters and their implantation in rats has been previously provided (Thomsen and Caine, 2005).

STRATEGIC PLANNING

Reinstatement protocols involve a surgical phase and three major experimental phases. During the surgical phase, rats are prepared with indwelling venous catheters. The three major experimental phases include a self-administration training phase, a period of experimental extinction, and the reinstatement test phase. Detailed protocols for making and implanting surgical catheters, as well as the initial establishment of drug self-administration have been described in this journal previously by Thomsen and Caine (Thomsen and Caine, 2005). In this, the “Strategic Planning” section, and in “Support Protocol’s 1 through 5, we provide details of catheter construction, catheter implantation and self-administration training where our procedures either meaningfully differ from the Thomsen and Caine protocol, or where peculiar to reinstatement testing. The present protocol is not aimed to reproduce the content of the Thomsen and Caine protocol. Where additional detail, or alternate approaches are needed of catheter construction, their implantation and the establishment of drug self-administration, the reader is referred to the very detailed descriptions provided by Thomsen and Caine whose protocol description is focused on these techniques.

Animals

Rat strain is an important factor when conducting reinstatement studies. The choice of strain may be driven by factors such as laboratory convention or desire to match experimental conditions from prior studies. In the absence of a strong preference for a particular strain several common rat strains used for self-administration studies may be employed. The three mostly commonly used are the Sprague-Dawley, Wistar and Long-Evans strains. Sprague-Dawley and Wistar rats are both outbred albino strains. The Long-Evans is an outbred hooded rat strain. Long-Evans rats are exclusively employed for the reinstatement studies described in this protocol. Other researchers have published reports using both Sprague-Dawley as well as Wistar rats in all three types of reinstatement procedures. Anecdotal data suggest that Sprague-Dawley rats produce less robust footshock-induced reinstatement than do Long-Evans animals. In addition, as a pigmented animal, Long-Evans rats have somewhat more acute eyesight than the albino strains that may facilitate studies such as cue-induced reinstatement that employ light cues during training and tests. A potential negative aspect of Long-Evans rats over Sprague-Dawley animals is their somewhat higher level of aggressiveness that may be problematic for technicians unfamiliar with proper rodent handling techniques.

Equipment

Operant chambers—At a minimum, operant test chambers must be equipped with: two (preferably retractable) response levers, a stimulus light above each lever, a house light and an adjustable output Sonalert or similar sound-producing device. If footshock stress reinstatement studies are anticipated, an appropriately electrically isolated floor grid with the necessary wiring harness connection is also necessary. The operant chambers must also be isolated from one another which is most commonly accomplished by placement of each chamber in a ventilated, sound-attenuating chamber of some type. Numerous manufacturers produce suitable operant chambers, some of which will provide all the necessary components as a “self-administration” package.

Operant system interfacing, power supply and control software—Software and interfacing are required to link the operant chambers and associated devices to a control computer. The software specifies the operant session conditions, chamber component function and data recording. Although operant chambers from one manufacture can often be used with interfacing and software from another vendor, it is generally advisable for investigators unfamiliar with electrical wiring and circuitry to purchase the operant chambers, interfacing and software from the same vendor to avoid potential incompatibilities. Common vendors include: Med Associates, Inc., Coulbourn Instruments and Lafayette Instrument Company, among others.

Infusion pump—The most common infusion delivery pump for rodent self-administration is the motor driven syringe pump. The complexity and price of syringe pumps varies widely. Some pumps have only a single speed motor drive, whereas others are more complex allowing the plunger motor speed to be controlled by the operant control software. For reinstatement studies described in this protocol, the requirements of a pump are fairly modest, and a suitable single speed pump delivering a 0.2 ml infusion over 10 s is acceptable. Pumps with greater flexibility may be needed if drugs, other than cocaine, are delivered, and that have different solubility and volume per delivery requirements. The pump must also be compatible with a 20 ml or larger disposable syringe. It is important to note that syringe pumps not purchased from the vendor which produces the interface system must be compatible with the 28v DC control signal output of most operant interface systems. Another common type of drug delivery pump more often used in primate self-administration studies is the peristaltic pump. Peristaltic pumps of an appropriate size can also be used for rodent self-administration and reinstatement studies although a suitable relay switching device will likely be required as these pumps do not generally have an integrated 28v control input.

Tethers, fluid swivels and swivel balance arm assembly—Each operant chamber must be equipped with a suitable catheter connection tether, fluid swivel and balance arm assembly. This assembly allows the rodent’s catheter to be connected to the drug infusion system in such a way that the animal has complete freedom of mobility within the operant test chambers. Swivel balance arms are generally purchased from the operant chamber vendor. Tethers and fluid swivels can both be manufactured in house but it is preferable to purchase these items preassembled. Model RS-P1 plastic fluid swivels, which are both durable and cost effective, can be obtained from Lomir Biomedical (Malone, NY). Model C313CS fluid connection assemblies that combine an inner polyethylene fluid tube, an outer steel spring tubing protector and a screw-down catheter connection can be purchased from PlasticsOne (Roanoke, VA).

BASIC PROTOCOL 1: TRAIN AND TEST RATS IN COCAINE-INDUCED REINSTATEMENT PROCEDURES

This protocol describes a procedure in which experimenter-administered cocaine reinstates extinguished lever pressing previously reinforced with cocaine infusion in rats. This procedure is otherwise referred to as a “prime-induced reinstatement procedure”. This effect is considered analogous to a cocaine “slip” cascading into extended relapse to cocaine seeking. Following the establishment of this procedure, tests can be conducted to determine if interventions affect cocaine-primed induced reinstatement. For example, drug pretreatments that reduce the magnitude of cocaine-reinstated lever pressing could be considered to show promise as potential medications for preventing relapse in cocaine abusers, at least in so far when relapse is typically precipitated by re-contact with cocaine.

Materials

Twelve naïve adult male Long-Evans hooded rats per dose condition instrumented with chronic indwelling jugular catheters at least 5 days prior to start of study (see Support Protocol 5 for details of catheterization surgery)

Standard laboratory rodent diet

Twelve operant conditioning chambers enclosed within sound attenuating cubicles. Chambers should be equipped with two retractable levers, two stimulus lights, house light, Sonalert®, liquid swivel/balance arm and drug infusion syringe pump

Control and interface system consisting of Windows computer, interface, control software and 28v DC power supply (Med-associates or comparable)

Twelve single channel plastic fluid swivels (RS-P1, Lomir Biomedical, Malone, NY)

Twelve catheter connection assemblies with metal spring covering (C313CS, PlasticsOne, Roanoke, VA)

One 100 ft roll of 0.040 in. (1.02 mm) internal diameter Tygon® microbore polyethylene tubing

Scale with 1 g accuracy and at least 500 g capacity for weighting rats

Analytical balance with 1 mg accuracy for weighing drugs

One box of 20 ml Luer-lock sterile disposable plastic syringes

Five boxes sterile 18 gauge disposable hypodermic needles

Four boxes of 1 ml sterile disposable plastic syringes with 26 gauge needles

One box of 3 ml sterile disposable plastic syringes with 25 gauge needles

Twenty individually packaged and sterilized blunt 25 gauge disposable hypodermic needles

One box 5.25 × 10 inch self-sealing sterilization pouches

One box 3.5 × 5 inch self-sealing sterilization pouches

One box 25 mm diameter, 0.2um membrane sterile syringe filters

Ten 30 ml sterilized serum vials and stoppers and aluminum crimp seals

One serum bottle capper

Pharmaceutical grade cocaine HCl

Ten 500 ml bags of 0.9% sterile physiological saline

One 30 ml vial of 1000 unit/ml heparin solution

One 10 ml vial of 100 mg/ml ketamine HCl

Ten vials of Timentin® antibiotic powder (GlaxoSmithKline, Research Triangle Park, NC)

Prepare a cocaine stock solution

- 1 A cocaine HCl stock solution can be prepared to facilitate the daily preparation of the self-administration solution bags. To prepare the cocaine stock solution, weigh an appropriate amount of cocaine HCl using an analytical balance. Dissolve the cocaine powder in 0.9% saline and draw into a 20 ml disposable syringe. Attach a 0.2 µm sterile syringe filter and sterile 18 gauge needle to the syringe and filter the cocaine solution into a prepared and sterilized 30 ml serum vial. Label the vial with contents, concentration, date prepared and preparer's name. Preparation of the cocaine stock solution at a concentration of 100 mg/ml is generally convenient. Store the vial refrigerated between uses.

Prepare a cocaine self-administration solution bag

- 2 A self-administration solution must be prepared. It is prepared by calculating the required unit injection dosage of cocaine and introducing sufficient cocaine stock solution into a bag of 0.9% sterile saline to achieve the appropriate concentration to deliver a 0.5 mg/kg/infusion unit cocaine dose. Heparin is also introduced to the saline bag in a quantity sufficient to achieve a 5 units/ml concentration. Factors that must be included in these calculations are saline bag volume, cocaine stock solution concentration, mean rat weight, volume per infusion and desired unit cocaine dose. For a more detailed discussion of solution preparation see the specific self-administration protocol published in a prior edition of the journal (Thomsen and Caine, 2005)

Prepare the operant test chamber

- 3 Prior to the beginning of self-administration training, prepare the operant chambers for self-administration training. Begin by inserting a liquid swivel into the round gimbal of the balance arm assembly.
- 4 Connect one end of the roll of Tygon® microbore tubing to the stainless steel hypodermic tubing protruding from the upper end of the swivel. Unroll sufficient Tygon® tubing to reach the chamber's infusion pump and cut tubing from roll.
- 5 Connect a blunt 25 gauge needle to the end of the tubing terminating at the infusion pump.
- 6 Connect a catheter connection tether assembly to the lower side of the liquid swivel.
- 7 Adjust the brass counterweight at the end of the balance arm opposite the swivel such that the weight of the swivel and fluid infusion tether is slightly less than the opposing down force of the counterweight.

Note that this procedure does not detail the steps necessary to sterilize the fluid infusion pathway. The method used to accomplish this task is dependent upon available resources. Some of the plastic components may not be compatible with autoclave temperatures. In particular, the

manufacturer discourages autoclaving of the fluid swivels. One method which avoids high temperatures is to ethylene oxide sterilize all of the individual components before assembly. A second method is to treat the fluid path with a chemical sterilant after the components are assembled. However, if this latter method is used care must be taken to ensure that all traces of sterilant, which may have the potential to leach into the polyethylene tubing, are removed from the fluid path prior to connecting a rat to the system. Careful adherence to the manufacturer's recommendations for post-sterilization washing is highly advised.

- 8 Attach a blunt 25 gauge needle to a 20 ml disposable syringe and fill with the 0.5 mg/kg/infusion cocaine self-administration solution. Fit the syringe onto infusion pump and activate the pump (electronically or manually) to flush the infusion lines, seat the pump plunger and expel all air from the infusion pathway.

Train rats to self-administer cocaine

- 9 Transport the rats from the vivarium to the testing room at least 20 min prior to the start of testing, weigh the rat and record its weight.
- 10 Check the fill level of all syringes on the infusion pumps; check the proper operation of the infusion pumps, retractable levers, stimulus lights and Sonalert®.
- 11 Operate the pumps and flush the infusion lines to clear all air from the infusion pathways.

It is extremely important that there is little if any air in the infusion pathway. Therefore it is advisable to verify that each infusion set is flushed completely by visualizing the presence of drips from the end of each fluid connection tether as the pumps are running.
- 12 Load the appropriate operant conditioning software program.
- 13 Take each individual rat from its transport cage and remove the polyethylene catheter sealing cap from the catheter pedestal. Connect a flush syringe assembly (Support Protocol 3) filled with saline to hypodermic tubing extending from the top of the pedestal.
- 14 Flush at least 0.1 ml of saline through the rat's catheter.

Take note of the ease of flushing, the presence or absence of fluid welling around the catheter post where it exits the animal and the behavior of the rat. Saline should flush through the catheter with little resistance. Difficult flushing, fluid seeping from around the catheter post exit point and/or signs of discomfort such as vocalization or flinching during flushing may be signs the catheter is inpatient. If these occur, consider testing catheter patency (for details of catheter patency testing see Thomsen and Caine, 2005). Sometimes applying additional pressure to the flush the syringe can clear a minor catheter obstruction. Little is lost if this effort is unsuccessful or the catheter fails as the rat would require recatheterization in any case. However, a catheter patency test is also strongly recommended following such action if more than minimal additional force is required to clear the catheter.

- 15** Connect the catheter pedestal to the catheter connection/infusion tether. Ensure that the connection is tight or the rat may become disconnected and chew the end of the tether.
- 16** Start the 120 min self-administration training session.
- Self-administration training sessions during which 0.5 mg/kg/infusion cocaine is available occur for 2 h each day. At the start of cocaine self-administration sessions the levers are extended and the house light is illuminated. During training, each press of the right-side lever results in a cocaine infusion (i.e., infusions are arranged according to a Fixed Ratio 1 (FR1) reinforcement schedule). At the start of an infusion, the house light is extinguished, the Sonalert® is sounded, and the cue lights above each lever flash at 3 Hz. The Sonalert® and cue lights remain activated for 6 s. Six seconds following the onset of the infusion the house light is re-illuminated, and the opportunity to self-administer cocaine is again made available.
- 17** Flush the catheter with 0.1 ml of catheter lock solution (see Support Protocol 1) and replace the polyethylene cap at the conclusion of the self-administration training session.
- 18** Return the rat to the vivarium and feed 15 g of rodent chow or that necessary to maintain the rat at 320 g.
- Generally for a 320 g Long-Evans rat approximately 15 g of food per day is sufficient to maintain a stable weight but this should be adjusted on a daily basis as necessary.
- 19** Record data and examine for acquisition of self-administration, or if acquisition has previously occurred, determine whether the rat meets necessary criteria for entering into the extinction phase of the study.
- The definitions of acquisition of self-administration as well as sufficient stability of behavior and duration of access prior to beginning extinction are dependent upon the goals of the experiment. For studies that assess the effectiveness of a pretreatment drug on attenuating reinstatement, we have employed three criteria all of which must be met prior to advancing to the extinction phase of the study. Specifically: 1) at least 12 self-administration sessions have occurred; 2) at least 15 cocaine infusions have occurred in each of the last four sessions; and 3) at least 125 lifetime cocaine infusions have been obtained.
- 20** If a rat meets the acquisition criteria, proceed to extinction described below, else continue once daily self-administration training as described in steps above.
- For rats that do not spontaneously (~3–5 sessions) acquire self-administration, it is possible that increasing contact with the active lever by tactics such as “baiting” the lever with a small amount of peanut butter or other preferred food may be effective. Note that some rats may never acquire cocaine-reinforced lever pressing behavior even when levers are baited. The use of baiting, as well as the number of training sessions one chooses to allow before removing a rat from the study, should be consistent with the investigator’s goals as well as the perceived importance of these factors. This decision is based on the practicality of chamber utilization as well as the hypothesis that

refractory subjects are behaviorally unique from the idealized average rat and may therefore skew subsequent data even if they eventually acquire the self-administration operant.

Extinction training and reinstatement testing

- 21 Transport the rats from the vivarium to the testing room at least 20 min prior to the start of testing, weigh the rat and record its weight.
- 22 Check the proper operation of the retractable levers, stimulus lights and Sonalert®.
- 23 Load the appropriate operant conditioning software program.

The operant conditioning program used for extinction training and drug prime-induced reinstatement testing is identical to that used for self-administration training except that drug infusions do not occur as a result of responding on the previously active lever. All other contingencies remain as during self-administration including the 6 s flashing cue light and Sonalert® activation. An alternate condition for extinction would involve delivering saline infusion, cocaine's vehicle, following lever pressing. The advantage of discontinuing drug infusions following responding during extinction, as opposed to delivering saline, is that loss of catheter patency no longer results in the elimination of the rat from further testing. Some laboratories choose to infuse vehicle following each active-lever response to mimic all drug-associated stimuli. We have found this is unnecessary and can markedly affect data acquisition because of subject elimination due to catheter inpatency.
- 24 Connect the catheter pedestal to the catheter connection/infusion tether. Ensure that the connection is tight or the rat may become disconnected and chew the end of the tether.
- 25 Start the 120 min extinction training session.
- 26 Remove the rat from the self-administration chamber at the conclusion of the self-administration training session.
- 27 Return the rat to the vivarium and feed sufficient rodent chow to maintain a 320 g body weight.
- 28 Record the data including number of responses on the previously-active and inactive levers.
- 29 Repeat the steps above until 12 sessions of extinction have occurred. Consider a rat eligible to proceed with reinstatement testing if the mean number of responses occurring during the last three extinction sessions is the first three extinction sessions. Exclude rats from further testing if it hasn't met this criterion (because its behavior has shown little responsiveness to the extinction contingencies).

In most subjects, extinction responding will generally be high during the first and second extinction training sessions and then decline across sessions reaching a floor level that is not meaningfully decreased by additional extinction training sessions beyond the initial 12. Rats that do not meet the extinction criterion may still "reinstate", that is, increase their responding to levels greater than that occurring on the

final (12th) session of extinction. The retention of subjects that do not meet the extinction criterion after 12 days is at the discretion of the investigator.

- 30 Ten min prior to the start of the reinstatement test session administer a 17 mg/kg i.p. cocaine injection.
- 31 Place the rats in the operant chamber and execute the 2-h reinstatement test session.

Conditions during the 2-h reinstatement test session are identical to those during the prior extinction test sessions.

Analyze data and perform statistics

- 32 Tabulate the numbers of active-lever presses (i.e. presses on the right-side lever, the presses of which were previously-reinforced with cocaine) for each rat for the last session of self-administration, the last session of extinction, and for the test day. These data will be used to determine if all groups were trained to a similar level of self-administration, a similar level of extinction, and whether there were differences in the treatment groups relative to the vehicle group on the test day, respectively. Additionally, active lever presses of the vehicle group on the test day are also compared to its active lever presses during the immediately preceding (i.e., final) extinction session to determine whether the conditions used were adequate for engendering effective prime-induced reinstatement. Enter all data into a statistical software package that is able to conduct t-test, one-way ANOVA, and Dunnett's Multiple Comparison Tests, assuming you can conclude the data conform to the assumptions of those tests (normal distribution and homogeneity of variance). Substitute nonparametric tests such as the Wilcoxon matched pairs signed rank test, Kruskal-Wallis, and Dunn's Multiple Comparison Test for the previously identified parametric tests, respectively, if the data violate those assumptions. Biologically focused software such as GraphPad Prism 5 for the Macintosh or PC (GraphPad Software, Inc., San Diego, CA) is able to conduct all of these parametric and nonparametric tests.
- 33 Tabulate the numbers of inactive lever presses (i.e., presses on the left-side lever, the presses of which did not have scheduled consequences) for each rat emitted during the test session. These data are analyzed to determine whether the test compound non-specifically increases or decreases behavior in general, and if found to do so, are used to qualify the specificity of effects of test compounds on active lever pressing.
- 34 Compare active-lever presses of the vehicle group emitted during the test session with those emitted during the immediately preceding (i.e., final) extinction session using a paired, one-tailed t-test. If active-lever presses during the test session significantly exceed (typically understood to be $P < 0.05$) those during the immediately preceding extinction session, then proceed to the next step and further analysis of the data. If active-lever presses do not significantly exceed those during the immediately preceding extinction session, then you cannot conclude the conditions effectively resulted in reinstatement of responding, and further analysis of the data is not justified.
- 35 Compare active-lever presses of all groups emitted during the last self-administration session with each other using a one-way ANOVA. If the F value is not statistically significant (typically understood to be $P > 0.05$), then proceed

with analysis of the data from the final session of extinction as described below. If the F value is statistically significant (typically understood to be $P < 0.05$) then it cannot be concluded that the various groups were trained to similar levels of cocaine self-administration and useful interpretations of the test data are doubtful.

- 36 Compare active-lever presses of all groups emitted during the last extinction session with each other using a one-way ANOVA. If the F value is not statistically significant (typically understood to be $P > 0.05$), then proceed with analysis of the data from the test session as described below. If the F value is statistically significant (typically understood to be $P < 0.05$) then it cannot be concluded that the various groups were extinguished to similar levels of extinction and useful interpretations of the test data are doubtful.
- 37 Compare active-lever presses of all groups emitted during the test session with each other using a one-way ANOVA. If significant effects are found, conduct Dunnett's Multiple Comparison Tests comparing each test group with the vehicle control group to identify effective treatment effects.

Because decisions to proceed with the analysis are made in a step-wise fashion, first determining whether there was effective reinstatement by the vehicle group, then determining whether all groups were trained to a similar level of self-administration, then determining whether all groups were extinguished to a similar level, before the analyses of test session data are performed, a repeated measures ANOVA, in which data from all phases of the experiment are simultaneously included in the analysis, is not conducted. If there is only one treatment group, in addition to the vehicle control group, then t-tests (or non-parametric equivalents) are substituted for the ANOVAs.

SUPPORT PROTOCOL 1: PREPARATION OF CATHETER LOCK/ ANTIBIOTIC SOLUTION

A specially prepared solution combining the anticoagulant actions of heparin with the viscosity of glycerol and the antibiotic/bacteriostatic actions of the antibiotic Timentin (ticarcillin disodium and clavulanate potassium) is used to fill the jugular catheters between operant test sessions. Use of this solution significantly extends the longevity of the catheter and is highly recommended. The procedure below details preparation of this solution. Note that other antibiotic/bacteriostatic drugs suitable for intravenous administration such as ampicillin/sulbactam may be substituted provided that the amount of pre-flush is properly adjusted to achieve an appropriate dosage.

Materials

- 3.1 gram bottle Timentin powder (ticarcillin disodium and clavulanate potassium)
- 10 ml bottle heparin sodium (1000 units/ml concentration)
- Glycerol USP (Fisher Scientific or similar)
- 50 ml sterilized serum vial, butyl rubber stopper and foil retainer in sterilization peel pack
- 25 mm diameter - 0.2 μ m pore size sterile syringe filter
- 60 ml Luer lock sterile disposable syringe
- 20 ml sterile disposable syringes

Three 10 ml sterile disposable syringes
 Three 18 gauge sterile disposable needles
 Four 22 gauge sterile disposable needles
 100 ml beaker
 Stir plate and stir bar (optional)

Preparation of 40 ml of pre-flush solution

Bulk preparation of the solution necessary to reconstitute the Timentin antibiotic (i.e. pre-flush) is easier and less expensive than preparing the solution each time new catheter lock/antibiotic solution is required. The protocol below produces 40 ml of pre-flush solution that is sufficient to reconstitute four, 3.1 gram bottles of Timentin.

- 1 Using the 20 ml syringe with an attached 18 gauge needle draw 20 ml of saline from the bag or bottle and empty contents of syringe into the beaker
- 2 Using a 10 ml syringe with attached 22 gauge needle draw 10 ml of heparin from vial and empty contents of syringe into the beaker.
- 3 Using a 10 ml syringe (no needle) draw 10 ml of glycerol from bottle and empty the contents of syringe into beaker.
- 4 Stir solution vigorously until the glycerol has fully incorporated into the solution.
- 5 Using the 60 ml syringe with an attached 18 gauge needle draw contents of beaker into the syringe.
- 6 Remove the needle from the 60 ml syringe and attach the 0.2 um syringe filter to the syringe.
- 7 Taking care to not contaminate the filter or needle, attach a 18 gauge needle to the end of the syringe.
- 8 Without removing the serum bottle from the sterilization peel pack, fit the rubber stopper onto the vial top, then remove the vial from the peel pack and attach the stopper retaining foil (if desired).
- 9 Insert the tip of the needle, filter and syringe assembly into the vial and filter the contents into the sterilized vial.

Insertion of a second small vent needle into the stopper will prevent over pressuring the serum vial as it fills with liquid. Also, although considerable time and moderate pressure on the syringe plunger is required to filter the solution, avoid excessive pressure on the syringe during this step as there is the potential of rupturing the filter membrane.

Final preparation of the catheter lock/antibiotic solution

- 10 Draw 10 ml of sterile filtered pre-flush into a 10 ml syringe with attached 22 gauge needle.
- 11 Add contents of syringe into 3.1 gram bottle of Timentin powder and shake vigorously until powder is completely dissolved.

It is important to note that once the catheter lock/Timentin solution is reconstituted it has a maximum refrigerated storage life of 72 hours

after which it must be discarded. Old lock solution can be distinguished by a pronounced yellow hue. If a sufficiently large cohort of animals is being tested, using the contents within 72 hours is likely. However, if few animals are tested it is possible to, using sterile vials and technique, aliquot the 3.1 grams of Timentin powder into smaller amounts and adjust the total volume of pre-flush added accordingly to minimize waste.

SUPPORT PROTOCOL 2: CONSTRUCTION OF A CATHETER INTRODUCER

A catheter introducer greatly aids in the insertion of the jugular catheter into the vein. It is easily and quickly prepared and can be cleaned and reused virtually indefinitely (see Figure 1).

Materials

- 1 ml disposable tuberculin syringe without needle
- 3/8" or longer 27 gauge disposable needle
- Rotary tool (Dremel® or similar) with mandrel and abrasive cut-off wheel
- Safety glasses

Preparation of catheter introducer

1. Don safety glasses and remove protective cap from needle.
2. Using rotary tool set at medium speed cut bevel from needle, leaving 5 mm of needle tubing remaining above plastic hub
3. Grind tip of needle flat and using the flat side of the abrasive disk.
4. Remove burrs from blunted needle by rotating needle between thumb and forefinger while in contact with the flat side of the abrasive disk.
5. Be careful to only remove the burrs and not to resharpen the needle. The goal is to make the needle blunt on the tip but smooth to the touch around the sides.
6. Wash the needle to remove traces of metal dust.
7. Place blunt needle on 1 ml tuberculin syringe and bend the needle at the hub to a 120 degree angle relative to the long axis of the syringe.

SUPPORT PROTOCOL 3: CONSTRUCTION OF A FLUSH SYRINGE ASSEMBLY AND CATHETER CAP

A flush syringe assembly is necessary both during the catheter surgery as well as subsequently during pre and post self-administration catheter maintenance. A catheter cap fashioned from Tygon® tubing is used to seal the end of the catheter between self-administration sessions (see Figure 2).

Materials

- 1 ml disposable tuberculin syringe without needle
- 25 gauge disposable needle
- Rotary tool (Dremel or similar) with mandrel and abrasive cut-off wheel
- 5.5 cm length of 0.040 inch ID Tygon® tubing

Self-sealing sterilization pouch

Safety glasses

Preparation of flush syringe assembly and catheter cap

1. Don safety glasses and remove protective cap from needle.
2. Using rotary tool set at medium speed cut bevel from needle. Grind tip of needle flat and using the flat side of the abrasive disk.
3. Remove burrs from blunted needle by rotating needle between thumb and forefinger while in contact with the flat side of the abrasive disk.

Be careful to only remove the burrs not sharpen the needle. The goal is to make the needle blunt on the tip but smooth to the touch around the sides.
4. Wash the needle to remove traces of metal dust.
5. Place the blunt needle on the 1 ml tuberculin syringe.
6. Heat end of 5.5 cm section of Tygon® tubing using open flame source until molten.
7. Pinch end of molten tubing closed with gloved fingers or surgical instrument.
8. Cut 0.5 cm from the sealed end of the Tygon® tubing to form the catheter cap.
9. Attach the remaining 5 cm of Tygon® tubing to the end of the blunt 25 gauge needle which forms the completed flush syringe assembly. (See illustration 2 for example of flush syringe assembly and catheter cap)
10. Seal the flush syringe assembly and catheter cap into the sterilization pouch and steam autoclave.

SUPPORT PROTOCOL 4: CONSTRUCTION OF A TAPERED POLYURETHANE CHRONIC INTRAVENOUS CATHETER FOR RATS

A previous protocol described the process to make silicone (silastic) catheters for rats (Thomsen and Caine, 2005). This supplementary protocol describes the construction of biocompatible tapered polyurethane catheters used in the reinstatement protocol (see Figures 3 and 4). Polyurethane tubing has advantages over silicone in that it is smoother than silicone and has been reported to be less prone to surface deposits such as proteins which may occlude the catheter and reduce its longevity. Polyurethane material is also more burst resistant than silicone. The ability to tolerate higher fluid pressures allows some difficult mechanical blockages, such as clots formed by backflow of blood, to be successfully cleared during the pre-session flushing process. Tapered catheters are also somewhat more easily inserted in smaller jugular veins during the implantation surgery due to their smaller diameter and greater stiffness.

The disadvantages of polyurethane material over silicone are the increased complexity of construction and the requirement of sterilization by a non-heat method such as ethylene oxide exposure. The catheter as described is appropriate for rats weighing between 300–350 grams. Catheters for larger or smaller rats may be produced in a similar manner with appropriate dimensional compensations. If preferred silicone catheters can, however, be used in the reinstatement protocol.

Materials

3.5 [‘diameter’? unit of measurement?] French polyurethane tubing (Access Technologies, Skokie, IL)

Hotplate (numerous suppliers)
 Sesame oil (Acros Organic/Fisher Scientific)
 Thermometer capable of reading to 200°C or higher
 Safety razor blade
 20 or 50 ml beaker
 Dumont #5/45 degree forceps (Fine Science Tools, Foster City, CA)
 Fragrance free liquid hand dishwashing detergent
 100 ml or larger wide mouth sealable container
 Three 20 ml syringes
 25 gauge blunt end needle
 Small cutting board
 Thin cyanoacrylate glue (Super Glue® or similar)
 Wooden handled cotton swab
 Catheter length gauge card (see construction below)

Preparing the catheter length gauge card

Although not required, a catheter gauge card is faster and easier to use than a ruler when producing large numbers of catheters. If multiple technicians are producing catheters it also increases overall uniformity of the product.

- 1 On a suitably sized sheet of cardstock draw a thick 18 cm straight line horizontally in the middle of the sheet.
- 2 Make vertical marks on the line at 0 mm, 26mm, 30mm and 180 mm.
- 3 Label the four marks in order from left to right: Tip, Cuff 1, Cuff 2, Button

Preparing the catheter

- 4 Fill beaker 2/3 full of sesame oil and heat to 165°C.
 If oil is overheated, begins smoking and becomes discolored, discard and begin again. Oil may be cooled, covered and reused repeatedly until discolored to make additional batches of catheters.
- 5 Cut 18 cm length of polyurethane tubing from roll.
 It is normal for the tubing to retain some curl from the roll.
- 6 Dangle tubing vertically over beaker with left hand while holding the Dumont forceps in the right hand. Immerse 2 cm of the distal end of the tubing into the hot sesame oil for 5 s. If oil is sufficiently hot the immersed tubing will curl, thicken and contract to half its original length.
If tubing does not react immediately to immersion, the oil is too cold. Increase oil temperature 10° C and test catheter material again. Use the minimum temperature necessary to soften the tubing as excessive heat may melt rather than soften the tubing.
- 7 After tubing contracts and curls, quickly remove tubing from oil, grasp the distal tip of heated tubing with Dumont forceps held in right hand. Over a period of 5 s

smoothly pull straight down, elongating and tapering the 2 cm section of tubing that was immersed to a length of 4–5 cm.

The goal is to form a gradual even taper over the entire heated length. Stretching the tubing should initially require no effort with tension noted only when stretching is completed. This step must be completed within approximately 10 s or the tubing will cool and sufficient stretching will not be possible.

- 8 Hold the tubing in place for at least 5 s to allow it to cool and set.

Forming the taper is a trial and error learning process requiring considerable practice. Expect an initially high rate of unacceptably tapered catheters. As such, when learning it is advisable to not pre-cut the catheter tubing to rough length. Instead, leave the tubing on the roll while forming the taper. Unacceptable attempts can simply be cut from the end of the roll and minimal tubing will be wasted.

Several common problems may be encountered during the tapering process. The first is excessive taper such that the distal end of the tubing is too narrow even after trimming described below. This is generally undesirable as it limits fluid flow although it can make insertion into the jugular vein easier when catheterizing smaller adolescent animals. Other problems include too little taper caused by insufficient stretching or an uneven taper with bulging at the junction between heated and unheated sections. Another common problem is excessive curling of the tapered section after having cooled. Some curling is common but excessive curling makes the catheter difficult to manipulate and insert during the implantation surgery.

- 9 Using the razor blade, remove the distal end of the catheter that was flattened by being grasped by the Dumont forceps during the stretching process.

- 10 Thoroughly wash the sesame oil from the exterior of the tubing by placing as many tapered tubing sections as desired into a sealed container half filled with warm water and a drop of dishwashing detergent. Shake the container vigorously for 30 s and drain. Repeat the exterior washing 3 times to ensure all oil is removed.

Removal of exterior oil from catheters could also be accomplished using a sonic cleaner and soapy water.

- 11 Fill a 20 ml syringe with warm soapy water made as in step 10 above. Place a blunt 25 gauge needle on end of syringe and connect proximal (full size) end of cut tubing to blunt needle. Flush tubing with entire contents of syringe. Repeat flushing with an additional 20 ml of soapy water.
- 12 Fill a clean 20 ml syringe with distilled water and flush tubing with entire contents to remove soap solution from interior of tubing.
- 13 Thoroughly rinse exterior of tubing with distilled water to remove all traces of soap residue.
- 14 Using a clean 20 ml syringe filled with air blow all remaining water from tubing. The tubing must be completely free of water to be properly sterilized. If the interior is not completely dry after the first air syringe repeat as necessary.
- 15 Place catheter on paper towel. Cover with second paper towel and allow to dry overnight.

- 16 Using the razor blade, cut two, 2 mm sections of polyurethane tubing from the roll for each catheter to be completed. These sections will form the securing cuffs of the catheter (see Figure 4).
- 17 Place a 2 mm section of tubing (the cuff) on the bent ends of the #5/45 Dumont forceps and dilate the cuff to increase its diameter enough to thread the tapered end of the tubing through the resulting aperture. Using the forceps or fingernails slide the cuff up the tubing until it reaches a point a 5 mm beyond the start of the tapered section.

Expanding the cuff can be somewhat difficult. If the tubing cannot be sufficiently dilated, a small metal spatula can be inserted between the blades of the forceps and twisted to aid in expanding the cuff. Cuffs expanded too aggressively can break. If this occurs, repeat with another 2 mm section of tubing using less expansion force.
- 18 Repeat the stretching and threading process in step 17 above with a second cuff. Advance the cuff to a point just beyond the start of the tapered section with approximately 1–2 mm between the first and second cuffs (Figure 3).
- 19 Place the catheter on the catheter gauge card lining up the two cuffs with the marks labeled cuff 1 and cuff 2. Using the razor blade cut the distal, small end of tapered section of the tubing square across the bottom at the line marked “tip”. Cut the proximal, full diameter end of the catheter at the line marked “button” (A catheter gauge card and completed catheter with catheter connection post attached as it would be when implanted in a rat are shown in Figure 4).
- 20 (Optional) Place a small amount of cyanoacrylate glue on the handle end of the wooden swab and dab a small amount of glue on each cuff where it meets the catheter tubing making sure to use the minimal amount of glue possible. When dried the glue should be virtually undetectable

This step is not required nor advisable if both cuffs are tight on the catheter tubing and do not slide unless considerable force is applied.
- 21 Sterilize catheters in accordance with requirements of your institution’s Institutional Animal Care and Use Committee (IACUC). Polyurethane material is not compatible with steam or dry heat sterilization. Best results are obtained by ethylene oxide sterilization of catheters placed in sterilization pouch peel packs.

SUPPORT PROTOCOL 5: SELF-ADMINISTRATION CATHETER IMPLANTATION IN THE RAT

A previous protocol described a method for implantation of a chronic indwelling jugular catheter in rats (Thomsen and Cane, 2005). That protocol is suitable for the implantation of jugular catheters required for reinstatement studies. Due to deviations in catheter construction and implantation techniques, an alternative catheter implantation protocol optimized for polyurethane catheters and commercially-obtained catheter connection pedestals is provided. It is worth noting that small deviations in surgical techniques, ordering of protocol steps and use of alternative instruments better suited to an individual surgeon are not problematic provided an acceptable outcome is achieved.

Materials

Temperature regulated animal heating pad (e.g. Gaymar Mul•T•Pad®, Deltaphase® Isothermal Pad or similar)

Sterilized jugular catheter (see Support Protocol 4)
 Two 1 ml tuberculin syringes with needles
 500 ml bag of 0.9% sterile saline
 Sterile surgical gloves
 Steam sterilized double wrapped surgical pack consisting of:
 1 each – straight mosquito hemostats
 1 each – curved mosquito hemostats
 1 each – 1x2 teeth small Adson forceps
 1 each – fine carbide blade straight scissors
 1 each – fine Dumont forceps
 1 each – fine 1x2 teeth straight Graefe or iris forceps
 4 each - 1x1 gauze pads
 4 each - wooden handle swabs
 1 each - 12x12 inch paper surgical drape
 1 each – #2 tapered Kalt suture needle
 2 each – 10 cm length of 4–0 braided silk or nylon suture material
 4 each – 4x4 gauze pads (for manipulating non-sterile objects)
 8 each - 7.5 mm Michel suture clips (or alternate closure materials)
 1 each – 5 cm length of 0.040 inch ID Tygon® tubing
 1 each – blunt 25 gauge needle
 1 each – catheter introducer (see Support Protocol 2)
 1 each – catheter connection pedestal (#313-000BM, PlasticsOne)

Surgical procedure

1. Anesthetize the rat and prepare surgical sites (see Thomsen and Cane, 2005).
 Choice of an acceptable anesthesia protocol is at the discretion of the investigator in consultation with veterinary staff. Injection anesthesia protocols require less equipment but it is possible that the duration of anesthesia produced will be inadequate for an inexperienced surgeon to complete the entire procedure. Under these circumstances the use of vapor anesthesia supplemented by a pre-surgery opioid analgesic allows for the surgery to be conducted at a more comfortable pace.
2. Place a disposable blue underpad (a.k.a. “Chux”) on top of a suitable heating pad. Place the anesthetized and prepared rat (see Thomsen and Cane, 2005) on its stomach on the underpad with head facing the surgeon.
3. Open the outer wrapper of the surgical pack and place it in a comfortable location for the surgeon. Peel open the packaging, and without touching them, drop two 1-ml tuberculin syringes with attached 25 gauge needles, as well as the sterilized catheter, onto the sterile field formed by the interior of the outer surgical pack wrapper. Do likewise with the peel pack containing the sterilized catheter.

Proper packing of surgical instruments, sterilization protocols and maintenance of the sterile field during the surgical procedure are beyond the scope of this protocol. Consult your veterinary staff on these issues if you are unfamiliar with appropriate techniques.

4. Don mask and sterile surgical gloves and open the inner surgical pack wrapper.
5. Fill two 1 ml syringes with sterile saline using sterile technique. Remove the sharp needle from one syringe and replace it with a 25 gauge blunt needle. Attach the large diameter end of the catheter to the blunt needle, flush 0.3 ml of saline through the catheter and lay the assembly aside.
6. Perform a 3 mm incision through the skin at a point on the midline of the animals back directly between the shoulder blades using the tungsten carbide bladed fine scissors or a scalpel blade. Make a longer 2 cm incision approximately 1.5 cm lateral to the small midscapular incision.

The position of the 2 cm incision should be on the same side as the jugular vein to be catheterized. If this is the initial catheter it will be the rat's right side. If scissors are used, lifting the skin with a pair of 1x2 tooth Adson forceps facilitates the incisions.

7. Bluntly dissect a subcutaneous pocket of at least 2.5 cm diameter directly below the small midscapular incision using the fine scissors.

This may also be accomplished using straight mosquito hemostats.

8. Place a 4x4 sterile gauze pad over the incisions to protect the site and invert the rat onto its back with its head facing the surgeon.

Turning the rat onto its back without re-gloving requires either the assistance of a second person or the use of one or more additional sterile 4x4 gauze pads or sterile drapes to manipulate the animal while protecting the sterility of the surgeon's gloves.

9. Cut a 2.5 cm diameter hole in a 12 in × 12 in piece of sterile surgical drape and position the drape over the animal with the hole exposing the right side of the animal's neck.
10. Elevate the skin with the Adson 1x2 tooth forceps and with the fine scissors make a 1.5 cm rostral to caudal incision on the right side of the animal's neck approximately 7 mm to the right of midline. The most caudal point in the incision should extend to a point slightly above the clavicle.

Often, but not always, the pulsation of the jugular vein and hence the correct location of the distal end of the incision can be visualized prior to lifting the skin. The purpose of lifting the skin to perform the incision is to ensure that there is no possibility of inadvertently cutting the jugular vein.

11. Insert the closed tips of the scissors straight down into the incision and open the scissors parallel to the incision. Repeat as necessary to bluntly dissect the overlying layer of fascia and fat, exposing the jugular vein.

Do not use the scissors as a cutting instrument at any point in the vein exposure process as doing so greatly increases the possibility of accidentally cutting the jugular vein. Although use of the scissors to accomplish this step is the most expedient, inexperienced and/or very cautious surgeons may choose to dissect the overlying tissue with a combination of the Dumont and iris forceps. Care should be taken to

accomplish this procedure with a minimum of trauma as excessive dissection can result in tearing of capillaries causing bleeding that increases the difficulty of successfully visualizing the jugular vein. Locating and isolating the jugular vein is one of the greatest challenges to surgeons in training.

12. Grasp and slightly elevate the exposed portion of the jugular vein and underlying tissue with the iris forceps. Close the teeth of the forceps on the tissue under the jugular vein rather than on the jugular vein itself.
13. Insert the closed fine scissors under the elevated jugular vein and surrounding tissue perpendicular to the rostral-caudal axis of the rat. Using the closed scissors as a trocar, apply sufficient force to push the tips completely through the elevated tissue.

Be certain that the scissors tips are under the elevated jugular vein, else the possibility exists of puncturing the vein. Elevating excessive tissue along with the jugular vein and then dissecting it away is preferable to elevating too little tissue and risking a puncture. Note that other methods of separating the jugular vein from the underlying tissue are possible such as using the points of the Dumont forceps rather than the scissors to tunnel under the vein.
14. Release the grip on the vein with the iris forceps, open the scissors slightly and without removing them from under the jugular vein lay them on the surgical field. The jugular vein and a small amount of surrounding fascia will now be slightly elevated above the incision by the scissor blades.
15. Hold the Dumont forceps in one hand and the iris forceps in the other. Using both instruments gently dissect away the remaining fascia and fatty tissue from at least 1 cm of the jugular vein.
16. Wet the exposed jugular vein liberally with sterile saline.
17. Slide a 10 cm length of 4-0 braided suture under the exposed jugular vein with the aid of the Dumont forceps. Ensure that approximately 5 cm of suture is on either side of the jugular vein.

Care should be taken to avoid abrading the vein with the suture material as it is passed under the vein.
18. Tie a double-throw surgeon's knot around the exposed jugular vein to occlude its flow.

To maximize the length of accessible vein for catheter implantation, ensure that the knot is positioned as far rostrally as possible on the exposed section of jugular vein.
19. Clamp the straight mosquito hemostat on the ends of the suture and lay the hemostats on the surgical field rostral to the incision. Use the position of the hemostat to apply slight tension on the vein, which will aid in elevating the jugular vein. If necessary place the closed fine scissors under the vein to compress any interfering tissue. Re-wet vein liberally with sterile saline.
20. Insert a second 10 cm section of 4-0 braided suture under the vein. Perform the first double throw required to make another surgeon's knot but do not pull the knot tight. Instead form a loose 0.5 cm diameter loop. Position the loop as far caudally as possible on exposed section of jugular vein.

This loop will later be pulled tight to anchor the catheter firmly in the jugular vein.

21. Position the catheter with the attached flush syringe on the surgical field such that the tip is close to the incision and in the proper orientation to be easily grasped and inserted in the jugular vein.
22. Make a small nick in the top of the jugular vein between the rostral knot and caudal loop using a pair of ball-tipped Bonn artery scissors or Vannas spring scissors.

The size of the nick is critical. The optimal nick is approximately 1/3–1/2 of the vein diameter. A nick that is too small may not completely penetrate the vein wall and therefore prevent venous access by the catheter. A nick that is too large may cause the remaining jugular vein to part and retract into the underlying tissue, which is generally an unrecoverable error requiring catheterization of the opposing jugular vein. Initially erring on the side of caution is warranted as an additional deeper nick can be made more caudally on the vein if the first attempt was of insufficient depth.

23. Hold the needle of the catheter introducer parallel to the vein and insert the needle of the catheter introducer through the nick and into the vein. Once introduced, slightly angle the handle of the catheter introducer to open the nick in the vein such that a hole into which the catheter can be inserted is apparent.

Sometimes a small “flash” of blood will accompany this operation, which is an additional indication that the introducer is properly inserted in the vein.

24. Using the opposite hand, grasp the catheter approximately 5 mm from the tip with the Dumont forceps. Slide the catheter under the introducer and into the hole in the vein. Continue advancing the catheter into the vein using the forceps until inserted to the depth of the first cuff. Remove the catheter introducer.
25. Check that the catheter is properly positioned by drawing back slightly on the attached syringe plunger. Blood should flow into the catheter tubing. Push the syringe plunger forward to expel the blood back into the vein.

Depending upon the positioning of the catheter it may not be possible to pull blood back into the catheter. If no blood can be withdrawn, slowly flush 0.3–0.4 ml of saline from the syringe into the catheter and look for pooling in the incision. If no pooling of saline is noted the catheter is likely positioned correctly.

26. Grasp the catheter using the thumb and forefinger of one hand and with the aid of the Dumont forceps in the other hand to manipulate the vein, work the first cuff of the catheter completely into the vein.

A gentle back and forth twisting motion of the catheter between the thumb and forefinger is often helpful.

27. Tighten the uncompleted first throw of the surgeons knot down around the vein between the first and second cuff. Complete the second throw of the surgeon's knot, thereby anchoring the catheter into the vein. Retest that saline can still be easily infused into the vein.

Do not over tighten the knot as this may collapse the catheter. Also note that it is not always possible to introduce the first cuff into the vein. If this proves excessively difficult it is acceptable to tighten the knot around the catheter distal to the first cuff. It will be slightly less secure but additional

secondary measures in latter protocol steps should prevent the catheter from becoming dislodged.

28. Thread each suture end into the eye of the suture needle. Using the needle, pass each of the four ends of the braided suture through the fascia to the right and left of its respective knot
29. Pull the fascia over the incision to close the tissue over the catheter using the suture. Secure the opposing ends of each opposing suture with a surgeon's knots.

Steps 28 and 29 may be omitted if desired to increase the speed of the procedure but these steps provide additional catheter anchoring as well as subcutaneous tissue closure both of which are desirable.

30. Clamp the distal end of the catheter just below its attachment to the flush syringe with the curved mosquito forceps. Cut the catheter between the forceps and the syringe with fine scissors.

Clamping will prevent the backflow of blood or the introduction of air into the catheter.

31. Insert the curved mosquito forceps into the neck incision and subcutaneously tunnel the catheter around the side of the neck, exiting the larger lateral incision on the back.

Tunneling under the skin is fairly easy but some force will be required to puncture the subcutaneous tissue at the exit site.

32. Pinch close the end of the catheter with the thumb and forefinger and remove the mosquito forceps from the incision.
33. Pull the end of the catheter out the incision on the back until the loop of tubing protruding from in the incision on the neck lays flat in the neck incision.
34. Reclamp the end of the catheter with the mosquito forceps to prevent blood from backflowing into the catheter while the incision on the neck is closed.
35. Elevate and hold the skin on either side of the neck incision in opposition with the Adson forceps. Close the incision by using a mosquito hemostat to apply three or four equally spaced 7.5 mm Michel suture clips.

Method of incision closure both on the neck and later the back are entirely up to the surgeon. Reflex wound clips as well as sutures are equally effective. Although fairly unlikely, care should be taken to insure the suture clips do not clamp the catheter.

36. Reposition the animal on its stomach and redrape as necessary.
37. Insert the catheter connection post into the larger, lateral incision on the animal's back and push the post out through the small, midscapular incision. Leave the mesh and longer stainless steel needle tubing of the catheter connection post exposed.
38. Place the 5 cm section of Tygon® tubing on the end of the blunt needle attached to the flush syringe, and connect the assembly to the short section of stainless steel tubing protruding from the top of the catheter connection pedestal.
39. Flush 0.2 ml of saline through the catheter pedestal to remove any air.
40. Pinch the catheter tubing closed with one thumb and forefinger and remove the mosquito hemostat.

41. Estimate the amount of catheter tubing required to comfortably connect the catheter to the long metal needle tubing extending from the base of the connection pedestal and remove the excess catheter tubing with the fine scissors.

A careful balance between removing too much and too little excess catheter tubing is required. Removing too much tubing will place unwanted tension on the catheter as the animal moves and grows with age. Removing too little catheter tubing makes it difficult to position the excess length under the skin before final incision closure as well as risks kinking of the catheter material that will simulate a blocked catheter.

42. Work the end of the catheter onto the stainless steel needle tubing extending from the bottom of the connection pedestal until it completely covers the stainless tubing up to the plastic post.

This fit should be quite tight. Surgeons with less finger strength may find grasping the catheter attached to the stainless steel tubing with a dry 1 in × 1 in gauze pad will make it easier to fully advance the tubing.

43. Insert the protruding lower portion of the catheter connection pedestal and catheter material into the incision. Position the center of the pedestal directly below the small midscapular incision and smooth the Dacron mesh flat against the underlying muscle tissue with the mosquito hemostats. Rotate the post if required to insure that the catheter tubing lies flat under the skin without kinking.

44. Close the larger lateral incision on the back with 3–4 equally spaced Michel suture clips, taking care to not catch the subcutaneous catheter tubing in the process.

45. Flush the catheter with 0.1 ml of catheter lock solution (see Support Protocol 1), seal the catheter post with a catheter cap (see Support Protocol 3) and recover the animal.

BASIC PROTOCOL 2: TRAIN AND TEST RATS IN COCAINE-CUE INDUCED REINSTATEMENT PROCEDURES

This protocol describes a procedure in which response-contingent presentation of stimuli (tone + stimulus light), previously associated with cocaine reinforcement, reinstates lever pressing that has been extinguished without accompanying stimuli. This procedure is otherwise referred to as a “cue-induced reinstatement procedure”. This effect is considered analogous to a drug user being exposed to stimuli which were previously associated with their drug of abuse (e.g., drug paraphernalia, a particular setting, cocaine-using peers, etc.) resulting in renewed cocaine seeking. Following the establishment of this procedure, a variety of tests could be conducted involving the determinants of cue-induced relapse. For instance, drug pretreatments that reduce the effectiveness by which cocaine-seeking can be reinstated in this way could be considered to show promise as potential medications for preventing relapse in cocaine abusers, at least in so far as when relapse is precipitated by re-contact with drug-associated stimuli.

Materials

Twelve naïve adult male Long-Evans hooded rats per dose condition instrumented with chronic indwelling jugular catheters at least 5 days prior to start of study (see Support Protocol 4 for details of catheterization surgery)

Standard laboratory rodent diet

Twelve operant conditioning chambers enclosed within sound attenuating cubicles. Chambers should be equipped with two retractable levers, two stimulus lights, house light, Sonalert®, liquid swivel/balance arm and drug infusion syringe pump

Control and interface system consisting of Windows computer, interface, control software and 28v DC power supply (Med-associates or comparable)

Twelve single channel plastic fluid swivels (RS-P1, Lomir Biomedical)

Twelve catheter connection assemblies with metal spring covering (C313CS, PlasticsOne)

One 100 ft roll of 0.040 in. (1.02 mm) internal diameter Tygon® microbore polyethylene tubing

Scale with 1 g accuracy and at least 500 g capacity for weighting rats

Analytical balance with 1 mg accuracy for weighing drugs

One box 20 ml Luer-lock sterile disposable plastic syringes

Five boxes sterile 18 gauge disposable hypodermic needles

Four boxes of 1 ml sterile disposable plastic syringes with 26 gauge needles

One box of 3 ml sterile disposable plastic syringes with 25 gauge needles

Twenty individually packaged and sterilized blunt 25 gauge disposable hypodermic needles

One box 5.25 × 10 inch self-sealing sterilization pouches

One box 3.5 × 5 inch self-sealing sterilization pouches

One box 25 mm diameter, 0.2um membrane sterile syringe filters

Ten 30 ml sterilized serum vials and stoppers and aluminum crimp seals

One serum bottle capper

Pharmaceutical grade cocaine HCl

Ten 500 ml bags of 0.9% sterile physiological saline

One 30 ml vial of 1000 unit/ml heparin solution

One 10 ml vial of 100 mg/ml ketamine HCl

Ten vials of Timentin® antibiotic powder (GlaxoSmithKline, Research Triangle Park, NC)

Prepare a cocaine stock solution

- 1 A cocaine HCl stock solution can be prepared to facilitate the daily preparation of the self-administration solution bags. To prepare the cocaine stock solution, weigh an appropriate amount of cocaine HCl using an analytical balance. Dissolve the cocaine powder in saline and draw into a 20 ml disposable syringe. Attach a 0.2 um sterile syringe filter and sterile 18 gauge needle to the syringe and filter the cocaine solution into a prepared and sterilized 30 ml serum vial. Label the vial with contents, concentration, date prepared and preparer's name. Preparation of the cocaine stock solution at a concentration of 100 mg/ml is generally convenient. Store the vial refrigerated between uses.

Prepare a cocaine self-administration solution bag

- 2 A self-administration solution must be prepared. It is prepared by calculating the required unit injection dosage of cocaine and introducing sufficient cocaine stock solution into a bag of 0.9% sterile saline to achieve the appropriate concentration to deliver a 0.5 mg/kg/infusion unit cocaine dose. Heparin is also introduced to the saline bag in a quantity sufficient to achieve a 5 units/ml concentration. Factors that must be included in these calculations are saline bag volume, cocaine stock solution concentration, mean rat weight, volume per infusion and desired unit cocaine dose. For a more detailed discussion of solution preparation see the specific self-administration protocol published in a prior edition of the journal (Thomsen and Caine, 2005)

Prepare the operant test chamber

- 3 Prior to the beginning of self-administration training, prepare the operant chambers for self-administration training. Begin by inserting a liquid swivel into the round gimbal of the balance arm assembly.
- 4 Connect one end of the roll of Tygon® microbore tubing to the stainless steel hypodermic tubing protruding from the upper end of the swivel. Unroll sufficient Tygon® tubing to reach the chamber's infusion pump and cut tubing from roll.
- 5 Connect a blunt 25 gauge needle to the end of the tubing terminating at the infusion pump.
- 6 Connect a catheter connection tether assembly to the lower side of the liquid swivel.
- 7 Adjust the brass counterweight at the end of the balance arm opposite the swivel such that the weight of the swivel and fluid infusion tether is slightly less than the opposing down force of the counterweight.

Note that this procedure does not detail the steps necessary to sterilize the fluid infusion pathway. The method used to accomplish this task is dependent upon available resources. Some of the plastic components may not be compatible with autoclave temperatures. In particular, the manufacturer discourages autoclaving of the fluid swivels. One method which avoids high temperatures is to ethylene oxide sterilize all of the individual components before assembly. A second method is to treat the fluid path with a chemical sterilant after the components are assembled. However, if this latter method is used care must be taken to insure that all traces of sterilant, which may have the potential to leach into the polyethylene tubing, are removed from the fluid path prior to connecting a rat to the system. Careful adherence to the manufacturer's recommendations for post-sterilization washing is highly advised.

- 8 Attach a blunt 25 gauge needle to a 20 ml disposable syringe and fill with the 0.5 mg/kg/infusion cocaine self-administration solution. Fit the syringe onto infusion pump and activate the pump (electronically or manually) to flush the infusion lines, seat the pump plunger and expel all air from the infusion pathway.

Train rats to self-administer cocaine

- 9 Transport the rats from the vivarium to the testing room at least 20 min prior to the start of testing, weigh the rat and record its weight.

- 10 Check the fill level of all syringes on the infusion pumps; check the proper operation of the infusion pumps, retractable levers, stimulus lights and Sonalert®.
- 11 Operate the pumps and flush the infusion lines to clear all air from the infusion pathways.

It is extremely important that there is little if any air is in the infusion pathway. Therefore it is advisable to verify that each infusion set is flushed completely by visualizing the presence of drips from the end of each fluid connection tether as the pumps are running.

- 12 Load the appropriate operant conditioning software program.
- 13 Take each individual rat from its transport cage and remove the polyethylene catheter sealing cap from the catheter pedestal. Connect a flush syringe assembly (Support Protocol 3) filled with saline hypodermic tubing extending from the top of the pedestal.
- 14 Flush at least 0.1 ml of saline through the rat's catheter using the 1 ml syringe assembly.

Take note of the ease of flushing, the presence or absence of fluid welling around the catheter post and the behavior of the rat. Saline should flush through the catheter with little resistance. Difficult flushing, fluid seeping from around the catheter post exit point and/or signs of discomfort such as vocalization or flinching during flushing may be signs the catheter is inpatient. If these occur consider testing catheter patency (for details of catheter patency testing see Thomsen and Caine, 2005). Sometimes applying additional pressure to the flush the syringe can clear a minor catheter obstruction. Little is lost if this effort is unsuccessful or the catheter fails as the rat would require recatheterization in any case. However, a catheter patency test is also strongly recommended following such action if more than minimal additional force is required to clear the catheter.

- 15 Connect the catheter pedestal to the catheter connection/infusion tether. Ensure that the connection is tight or the rat may become disconnected and chew the end of the tether.
- 16 Start the 120 min self-administration training session.

Self-administration training sessions during which 0.5 mg/kg/infusion cocaine is available occur for 2 h each day. At the start of cocaine self-administration sessions the levers are extended and the house light is illuminated. During training, each press of the right-side lever results in a cocaine infusion (i.e., infusions are arranged according to a Fixed Ratio 1 (FR1) reinforcement schedule). At the start of an infusion, the house light is extinguished, the Sonalert® is sounded, and the cue lights above each lever flash at 3 Hz. The Sonalert® and cue lights remain activated for 6 s. Six seconds following the onset of the infusion the house light is re-illuminated, and the opportunity to self-administer cocaine is again made available.

- 17 Flush the catheter with 0.1 ml of catheter lock solution (see Support Protocol 1) and replace the polyethylene cap at the conclusion of the self-administration training session.

- 18** Return the rat to the vivarium and feed 15 g of rodent chow or that necessary to maintain the rat at 320 g.

Generally for a 320 g Long-Evans rat approximately 15 g of food per day is sufficient to maintain a stable weight but this should be adjusted on a daily basis as necessary.

- 19** Record data and examine for acquisition of self-administration, or if acquisition has previously occurred, determine whether the rat meets necessary criteria for entering into the extinction phase of the study.

The definitions of acquisition of self-administration as well as sufficient stability of behavior and duration of access prior to beginning extinction are dependent upon the goals of the experiment. For studies that assess the effectiveness of a pretreatment drug on attenuating reinstatement, we have employed three criteria all of which must be met prior to advancing to the extinction phase of the study. Specifically: 1) at least 12 self-administration sessions have occurred; 2) at least 15 cocaine infusions have occurred in each of the last four sessions; and 3) at least 125 lifetime cocaine infusions have been obtained.

- 20** If the rat meets acquisition criteria, proceed to extinction described below, else continue once daily self-administration training as described in steps above.

For rats that do not spontaneously (~3–5 sessions) acquire self-administration it is possible that increasing contact with the active lever by tactics such as “baiting” the lever with a small amount of peanut butter or other preferred food may be effective. Note that some rats may never acquire cocaine-reinforced lever pressing behavior even when levers are baited. The use of baiting, as well as the number of training sessions one chooses to allow before removing a rat from the study, should be consistent with the investigator’s goals as well as the perceived importance of these factors. This decision is based on the practicality of chamber utilization as well as the hypothesis that refractory subjects are behaviorally unique from the idealized average rat and may therefore skew subsequent data even if they eventually acquire the self-administration operant.

Extinction training and reinstatement testing

- 21** Transport the rats from the vivarium to the testing room at least 20 min prior to the start of testing, weigh the rat and record it’s weight.
- 22** Check the proper operation of the retractable levers, stimulus lights and Sonalert®.
- 23** Load the appropriate operant conditioning software program.

The operant conditioning program used for extinction training differs from that used in self-administration training and reinstatement testing in that neither cocaine infusions, nor the 6 s flashing cue light and Sonalert® activation occur as a result of responding on the previously active lever. All other contingencies remain as during self-administration.

- 24 Connect the catheter pedestal to the catheter connection/infusion tether. Ensure that the connection is tight or the rat may become disconnected and chew the end of the tether.
- 25 Start the 120 min extinction training session.
- 26 Remove the rat from the self-administration chamber at the conclusion of the self-administration training session.
- 27 Return the rat to the vivarium and feed sufficient rodent chow to maintain a 320 g body weight.
- 28 Record the data including number of responses on the previously-active and inactive levers.
- 29 Repeat the steps above until 12 sessions of extinction have occurred. Consider a rat eligible to proceed with reinstatement testing if the mean number of responses occurring during the last three extinction sessions is the first three extinction sessions. Exclude rats from further testing if it hasn't met this criterion (because its behavior has shown little responsiveness to the extinction contingencies).

In most subjects extinction responding will rapidly decline to very low levels in the absence of cocaine-paired exteroceptive stimuli. Few rats should fail to reach extinction criteria, although rats that do not meet the extinction criterion may still “reinstatement”, that is, increase their responding to levels greater than that occurring on the final (12th) session of extinction. The retention of subjects that do not meet the extinction criterion after 12 days is at the discretion of the investigator.

- 30 Place the rat in the operant chamber and execute the 2-h reinstatement test session.

Conditions during the 2-h reinstatement test session are similar to those during self-administration. Specifically a 6 s flashing cue light and Sonalert® activation should be programmed to occur following each response on the lever which resulted in cocaine infusions during self-administration training. Reinstatement conditions differ from self-administration in that responding does not result in cocaine infusions. Some laboratories choose to infuse vehicle following each active-lever response during reinstatement to mimic all drug-associated stimuli. We have found this is unnecessary to elicit robust cue-induced reinstatement and can markedly affect data acquisition because of subject elimination due to catheter inpatency.

Analyze data and perform statistics

- 31 Data from rats not emitting at least one press on the active lever during the reinstatement test are eliminated from the analyses because they are never exposed to the reinstating cues. For all other rats, tabulate the numbers of active-lever presses (i.e. presses on the right-side lever, the presses of which were previously-reinforced with cocaine) for each rat for the last session of self-administration, the last session of extinction, and for the test day. These data will be used to determine if all groups were trained to a similar level of self-administration, a similar level of extinction, and whether there were differences in the treatment groups relative to the vehicle group on the test day, respectively. Additionally, active lever presses of the vehicle group on the test day are also

compared to its active lever presses during the immediately preceding (i.e., final) extinction session to determine whether the conditions used were adequate for engendering effective cue-induced reinstatement. Enter all data into a statistical software package that is able to conduct t-test, one-way ANOVA, and Dunnett's Multiple Comparison Tests, assuming you can conclude the data conform to the assumptions of those tests (normal distribution and homogeneity of variance). Substitute nonparametric tests such as the Wilcoxon matched pairs signed rank test, Kruskal-Wallis, and Dunn's Multiple Comparison Test for the previously identified parametric tests, respectively, if the data violate those assumptions. Biologically focused software such as GraphPad Prism 5 for the Macintosh or PC (GraphPad Software, Inc., San Diego, CA) is able to conduct all of these parametric and nonparametric tests.

- 32 Tabulate the numbers of inactive lever presses (i.e., presses on the left-side lever, the presses of which did not have scheduled consequences) for each rat emitted during the test session. These data are analyzed to determine whether the test compound non-specifically increases or decreases behavior in general, and if found to do so, are used to qualify the specificity of effects of test compounds on active lever pressing.
- 33 Compare active-lever presses of the vehicle group emitted during the test session with those emitted during the immediately preceding (i.e., final) extinction session using a paired, one-tailed t-test. If active-lever presses during the test session significantly exceed (typically understood to be $P < 0.05$) those during the immediately preceding extinction session, then proceed to the next step and further analysis of the data. If active-lever presses do not significantly exceed those during the immediately preceding extinction session, then you cannot conclude the conditions effectively resulted in reinstatement of responding, and further analysis of the data is not justified.
- 34 Compare active-lever presses of all groups emitted during the last self-administration session with each other using a one-way ANOVA. If the F value is not statistically significant (typically understood to be $P > 0.05$), then proceed with analysis of the data from the final session of extinction as described below. If the F value is statistically significant (typically understood to be $P < 0.05$) then it cannot be concluded that the various groups were trained to similar levels of cocaine self-administration and useful interpretations of the test data are doubtful.
- 35 Compare active-lever presses of all groups emitted during the last extinction session with each other using a one-way ANOVA. If the F value is not statistically significant (typically understood to be $P > 0.05$), then proceed with analysis of the data from the test session as described below. If the F value is statistically significant (typically understood to be $P < 0.05$) then it cannot be concluded that the various groups were extinguished to similar levels of and useful interpretations of the test data are doubtful.
- 36 Compare active-lever presses of all groups emitted during the test session with each other using a one-way ANOVA. If significant effects are found, conduct Dunnett's Multiple Comparison Tests comparing each test group with the vehicle control group to identify effective treatment effects.

Because decisions to proceed with the analysis are made in a step-wise fashion, first determining whether there was effective reinstatement by the vehicle group, then determining whether all groups were trained to a similar level of self-administration, then determining whether all

groups were extinguished to a similar level, before the analyses of test session data are performed, a repeated measures ANOVA, in which data from all phases of the experiment are simultaneously included in the analysis, is not conducted. If there is only one treatment group, in addition to the vehicle control group, then t-tests (or non-parametric equivalents) are substituted for the ANOVAs.

BASIC PROTOCOL 3: TRAIN AND TEST RATS IN FOOTSHOCK-INDUCED (STRESS) REINSTATEMENT PROCEDURES

This protocol describes a procedure in which pre-session experimenter-administered intermittent inescapable footshock reinstates extinguished lever pressing previously reinforced with cocaine infusion in rats. This procedure is otherwise referred to as a “stress-induced reinstatement procedure”. This effect is considered analogous to stressful events precipitating renewed cocaine seeking in abusers of cocaine. Following the establishment of this procedure, tests can be conducted examining the determinants of stress-induced reinstatement. For example, drug pretreatments that reduce the effectiveness by which cocaine-seeking can be reinstated in this way could be considered to show promise as potential medications for preventing relapse in cocaine abusers, at least when relapse is precipitated by stressors.

Materials

Twelve naïve adult male Long-Evans hooded rats per dose condition instrumented with chronic indwelling jugular catheters at least 5 days prior to start of study (see Support Protocol 4 for details of catheterization surgery)

Standard laboratory rodent diet

Twelve operant conditioning chambers enclosed within sound attenuating cubicles. Chambers should be equipped with two retractable levers, two stimulus lights, house light, Sonalert®, liquid swivel/balance arm and drug infusion syringe pump

Twelve current regulated footshock delivery devices.

Control and interface system consisting of Windows computer, interface, control software and 28v DC power supply (Med-associates or comparable)

Twelve single channel plastic fluid swivels (RS-P1, Lomir Biomedical)

Twelve catheter connection assemblies with metal spring covering (C313CS, PlasticsOne)

One 100 ft roll of 0.040 in. (1.02 mm) internal diameter Tygon® microbore polyethylene tubing

Scale with 1 g accuracy and at least 500 g capacity for weighting rats

Analytical balance with 1 mg accuracy for weighing drugs

One box 20 ml Luer-lock sterile disposable plastic syringes

Five boxes sterile 18 gauge disposable hypodermic needles

Four boxes of 1 ml sterile disposable plastic syringes with 26 gauge needles

One box of 3 ml sterile disposable plastic syringes with 25 gauge needles

Twenty individually packaged and sterilized blunt 25 gauge disposable hypodermic needles

One box 5.25 x 10 inch self-sealing sterilization pouches
One box 3.5 x 5 inch self-sealing sterilization pouches
One box 25 mm diameter, 0.2um membrane sterile syringe filters
Ten 30 ml sterilized serum vials and stoppers and aluminum crimp seals
One serum bottle cap
Pharmaceutical grade cocaine HCl
Ten 500 ml bags of 0.9% sterile physiological saline
One 30 ml vial of 1000 unit/ml heparin solution
One 10 ml vial of 100 mg/ml ketamine HCl
Ten vials of Timentin® antibiotic powder (GlaxoSmithKline, Research Triangle Park, NC)

Prepare a cocaine stock solution

- 1 A cocaine HCl stock solution can be prepared to facilitate the daily preparation of the self-administration solution bags. To prepare the cocaine stock solution, weigh an appropriate amount of cocaine HCl using an analytical balance. Dissolve the cocaine powder in saline and draw into a 20 ml disposable syringe. Attach a 0.2 um sterile syringe filter and sterile 18 gauge needle to the syringe and filter the cocaine solution into a prepared and sterilized 30 ml serum vial. Label the vial with contents, concentration, date prepared and preparer's name. Preparation of the cocaine stock solution at a concentration of 100 mg/ml is generally convenient. Store the vial refrigerated between uses.

Prepare a cocaine self-administration solution bag

- 2 A self-administration solution must be prepared. It is prepared by calculating the required unit injection dosage of cocaine and introducing sufficient cocaine stock solution into a bag of 0.9% sterile saline to achieve the appropriate concentration to deliver a 0.5 mg/kg/infusion unit cocaine dose. Heparin is also introduced to the saline bag in a quantity sufficient to achieve a 5 units/ml concentration. Factors that must be included in these calculations are saline bag volume, cocaine stock solution concentration, mean rat weight, volume per infusion and desired unit cocaine dose. For a more detailed discussion of solution preparation see the specific self-administration protocol published in a prior edition of the journal (Thomsen and Caine, 2005)

Prepare the operant test chamber

- 3 Prior to the beginning of self-administration training, prepare the operant chambers for self-administration training. Begin by inserting a liquid swivel into the round gimbal of the balance arm assembly.
- 4 Connect one end of the roll of Tygon® microbore tubing to the stainless steel hypodermic tubing protruding from the upper end of the swivel. Unroll sufficient Tygon® tubing to reach the chamber's infusion pump and cut tubing from roll.
- 5 Connect a blunt 25 gauge needle to the end of the tubing terminating at the infusion pump.

- 6 Connect a catheter connection tether assembly to the lower side of the liquid swivel.
- 7 Adjust the brass counterweight at the end of the balance arm opposite the swivel such that the weight of the swivel and fluid infusion tether is slightly less than the opposing down force of the counterweight.

Note that this procedure does not detail the steps necessary to sterilize the fluid infusion pathway. The method used to accomplish this task is dependent upon available resources. Some of the plastic components may not be compatible with autoclave temperatures. In particular, the manufacturer discourages autoclaving of the fluid swivels. One method which avoids high temperatures is to ethylene oxide sterilize all of the individual components before assembly. A second method is to treat the fluid path with a chemical sterilant after the components are assembled. However, if this latter method is used care must be taken to ensure that all traces of sterilant, which may have the potential to leach into the polyethylene tubing, are removed from the fluid path prior to connecting a rat to the system. Careful adherence to the manufacturers recommendations for post-sterilization washing is highly advised.

- 8 Attach a blunt 25 gauge needle to a 20 ml disposable syringe and fill with the 0.5 mg/kg/infusion cocaine self-administration solution. Fit the syringe onto infusion pump and activate the pump (electronically or manually) to flush the infusion lines, seat the pump plunger and expel all air from the infusion pathway.

Train rats to self-administer cocaine

- 9 Transport the rats from the vivarium to the testing room at least 20 min prior to the start of testing, weigh the rat and record it's weight.
- 10 Check the fill level of all syringes on the infusion pumps; check the proper operation of the infusion pumps, retractable levers, stimulus lights and Sonalert®.
- 11 Operate the pumps and flush the infusion lines to clear all air from the infusion pathways.

It is extremely important that there is little if any air is in the infusion pathway. Therefore it is advisable to verify that each infusion set is flushed completely by visualizing the presence of drips from the end of each fluid connection tether as the pumps are running.

- 12 Load the appropriate operant conditioning software program.
- 13 Take each individual rat from its transport cage and remove the polyethylene catheter sealing cap from the catheter pedestal. Connect a flush syringe assembly (Support Protocol 3) filled with saline hypodermic tubing extending from the top of the pedestal.
- 14 Flush at least 0.1 ml of saline through the rat's catheter using the 1 ml syringe assembly.

Take note of the ease of flushing, the presence or absence of fluid welling around the catheter post and the behavior of the rat. Saline should flush through the catheter with little resistance. Difficult flushing, fluid seeping from around the catheter post exit point and/or

signs of discomfort such as vocalization or flinching during flushing may be signs the catheter is inpatient. If these occur, consider testing catheter patency (for details of catheter patency testing see Thomsen and Caine, 2005). Sometimes applying additional pressure to the flush the syringe can clear a minor catheter obstruction. Little is lost if this effort is unsuccessful or the catheter fails as the rat would require recatheterization in any case. However, a catheter patency test is also strongly recommended following such action if more than minimal additional force is required to clear the catheter.

- 15 Connect the catheter pedestal to the catheter connection/infusion tether. Ensure that the connection is tight or the rat may become disconnected and chew the end of the tether.
- 16 Start the 120 min self-administration training session.
 Self-administration training sessions during which 0.5 mg/kg/infusion cocaine is available occur for 2 h each day. At the start of cocaine self-administration sessions the levers are extended and the house light is illuminated. During training, each press of the right-side lever results in a cocaine infusion (i.e., infusions are arranged according to a Fixed Ratio 1 (FR1) reinforcement schedule). At the start of an infusion, the house light is extinguished, the Sonalert® is sounded, and the cue lights above each lever flash at 3 Hz. The Sonalert® and cue lights remain activated for 6 s. Six seconds following the onset of the infusion the house light is re-illuminated, and the opportunity to self-administer cocaine is again made available.
- 17 Flush the catheter with 0.1 ml of catheter lock solution (see Support Protocol 1) and replace the polyethylene cap at the conclusion of the self-administration training session.
- 18 Return the rat to the vivarium and feed 15 g of rodent chow or that necessary to maintain the rat at 320 g.
 Generally for a 320 g Long-Evans rat approximately 15 g of food per day is sufficient to maintain a stable weight but this should be adjusted on a daily basis as necessary.
- 19 Record data and examine for acquisition of self-administration, or if acquisition has previously occurred, determine whether the rat meets necessary criteria for entering into the extinction phase of the study.
 The definitions of acquisition of self-administration as well as sufficient stability of behavior and duration of access prior to beginning extinction are dependent upon the goals of the experiment. For studies that assess the effectiveness of a pretreatment drug on attenuating reinstatement, we have employed three criteria all of which must be met prior to advancing to the extinction phase of the study. Specifically: 1) at least 12 self-administration sessions have occurred; 2) at least 15 cocaine infusions have occurred in each of the last four sessions; and 3) at least 125 lifetime cocaine infusions have been obtained.
- 20 If the rat meets acquisition criteria proceed to extinction described below, else continue once daily self-administration training as described in steps above.

For rats that do not spontaneously (~3–5 sessions) acquire self-administration, it is possible that increasing contact with the active lever by tactics such as “baiting” the lever with a small amount of peanut butter or other preferred food may be effective. Note that some rats may never acquire cocaine-reinforced lever pressing behavior even when levers are baited. The use of baiting, as well as the number of training sessions one chooses to allow before removing a rat from the study, should be consistent with the investigator’s goals as well as the perceived importance of these factors. This decision is based on the practicality of chamber utilization as well as the hypothesis that refractory subjects are behaviorally unique from the idealized average rat and may therefore skew subsequent data even if they eventually acquire the self-administration operant.

Extinction training and reinstatement testing

- 21 Transport the rats from the vivarium to the testing room at least 20 min prior to the start of testing, weigh the rat and record it’s weight.
- 22 Check the proper operation of the retractable levers, stimulus lights and Sonalert®.
- 23 Load the appropriate extinction training software program.

The operant conditioning program used for extinction training prior to footshock stress-induced reinstatement testing is identical to that used for self-administration training with the two exceptions: 1) a 15-min timeout, in which the animals remain in the darkened chamber with the levers retracted, precedes the start of each extinction session 2) drug infusions do not occur as a result of responding on the previously active lever. All other contingencies remain as during self-administration including the 6 s flashing stimulus light and Sonalert® activation. An alternate condition for extinction would involve delivering saline infusion, cocaine’s vehicle, following lever pressing. The advantage of discontinuing drug infusions following responding during extinction, as opposed to delivering saline, is that loss of catheter patency no longer results in the elimination of the rat from further testing. Some laboratories choose to infuse vehicle following each active-lever response to mimic all drug-associated stimuli. We have found this is unnecessary and can markedly affect data acquisition because of subject elimination due to catheter inpatency.
- 24 Connect the catheter pedestal to the catheter connection/infusion tether. Ensure that the connection is tight or the rat may become disconnected and chew the end of the tether.
- 25 Start the 120 min extinction training session.
- 26 Remove the rat from the self-administration chamber at the conclusion of the self-administration training session.
- 27 Return the rat to the vivarium and feed sufficient rodent chow to maintain a 320 g body weight.
- 28 Record the data including number of responses on the previously-active and inactive levers.

- 29** Repeat the steps above until 12 sessions of extinction have occurred. Consider a rat eligible to proceed with reinstatement testing if the mean number of responses occurring during the last three extinction sessions is the first three extinction sessions. Exclude rats from further testing if it hasn't met this criterion (because it's behavior has shown little responsiveness to the extinction contingencies).

In most subjects extinction responding will generally be high during the first and second extinction training sessions and then decline across sessions reaching a floor level that is not meaningfully decreased by additional extinction training sessions beyond the initial 12. Rats that do not meet the extinction criterion may still "reinstatement", that is, increase their responding to levels greater than that occurring on the final (12th) session of extinction. The retention of subjects that do not meet the extinction criterion after 12 days is at the discretion of the investigator.

- 30** On the subsequent day test for footshock-stress induced reinstatement.

The footshock-stress induced reinstatement session initiates with a 15 min period of intermittent, inescapable footshock in the operant conditioning chamber. The footshock is administered at an intensity of 0.63mA for 0.5 s/shock. The inter-shock interval is randomized with a mean inter-shock interval of 40 s. Following the 15 min footshock period a 120 min reinstatement test session is conducted. During the reinstatement test sessions conditions are identical to those present during extinction training in that drug infusions do not occur as a result of responding on the previously active lever but other contingencies remain as during self-administration including the 6 s flashing cue light and Sonalert® activation.

Analyze data and perform statistics

- 31** Tabulate the numbers of active-lever presses (i.e. presses on the right-side lever, the presses of which were previously-reinforced with cocaine) for each rat for the last session of self-administration, the last session of extinction, and for the test day. These data will be used to determine if all groups were trained to a similar level of self-administration, a similar level of extinction, and whether there were differences in the treatment groups relative to the vehicle group on the test day, respectively. Additionally, active lever presses of the vehicle group on the test day are also compared to its active lever presses during the immediately preceding (i.e., final) extinction session to determine whether the conditions used were adequate for engendering effective stress-induced reinstatement. Enter all data into a statistical software package that is able to conduct t-test, one-way ANOVA, and Dunnett's Multiple Comparison Tests, assuming you can conclude the data conform to the assumptions of those tests (normal distribution and homogeneity of variance). Substitute nonparametric tests such as the Wilcoxon matched pairs signed rank test, Kruskal-Wallis, and Dunn's Multiple Comparison Test for the previously identified parametric tests, respectively, if the data violate those assumptions. Biologically focused software such as GraphPad Prism 5 for the Macintosh or PC (GraphPad Software, Inc., San Diego, CA) is able to conduct all of these parametric and nonparametric tests.

- 32 Tabulate the numbers of inactive lever presses (i.e., presses on the left-side lever, the presses of which did not have scheduled consequences) for each rat emitted during the test session. These data are analyzed to determine whether the test compound non-specifically increases or decreases behavior in general, and if found to do so, are used to qualify the specificity of effects of test compounds on active lever pressing.
- 33 Compare active-lever presses of the vehicle group emitted during the test session with those emitted during the immediately preceding (i.e., final) extinction session using a paired, one-tailed t-test. If active-lever presses during the test session significantly exceed (typically understood to be $P < 0.05$) those during the immediately preceding extinction session, then proceed to the next step and further analysis of the data. If active-lever presses do not significantly exceed those during the immediately preceding extinction session, then you cannot conclude the conditions effectively resulted in reinstatement of responding, and further analysis of the data is not justified.
- 34 Compare active-lever presses of all groups emitted during the last self-administration session with each other using a one-way ANOVA. If the F value is not statistically significant (typically understood to be $P > 0.05$), then proceed with analysis of the data from the final session of extinction as described below. If the F value is statistically significant (typically understood to be $P < 0.05$) then it cannot be concluded that the various groups were trained to similar levels of cocaine self-administration and useful interpretations of the test data are doubtful.
- 35 Compare active-lever presses of all groups emitted during the last extinction session with each other using a one-way ANOVA. If the F value is not statistically significant (typically understood to be $P > 0.05$), then proceed with analysis of the data from the test session as described below. If the F value is statistically significant (typically understood to be $P < 0.05$) then it cannot be concluded that the various groups were extinguished to similar levels of and useful interpretations of the test data are doubtful.
- 36 Compare active-lever presses of all groups emitted during the test session with each other using a one-way ANOVA. If significant effects are found, conduct Dunnett's Multiple Comparison Tests comparing each test group with the vehicle control group to identify effective treatment effects.

Because decisions to proceed with the analysis are made in a step-wise fashion, first determining whether there was effective reinstatement by the vehicle group, then determining whether all groups were trained to a similar level of self-administration, then determining whether all groups were extinguished to a similar level, before the analyses of test session data are performed, a repeated measures ANOVA, in which data from all phases of the experiment are simultaneously included in the analysis, is not conducted. If there is only one treatment group, in addition to the vehicle control group, then t-tests (or non-parametric equivalents) are substituted for the ANOVAs.

COMMENTARY

The toll of drug abuse can be measured in the billions of dollars in the United States, and incalculable in terms of human suffering. Although users can come to abstain from drug use, or be coerced into abstinence, relapse is intractable. Preclinical methods for studying drug relapse can be valuable for understanding its determinants and for identifying potential

pharmacological and behavioral leads for its treatment. Reinstatement procedures provide one approach for modeling clinical drug relapse. In this unit, “reinstatement procedures” refer to those procedures that involve the restoration or elevation of extinguished responding previously reinforced with drug administration. Reinstatement procedures have a degree of face validity with clinical drug relapse in that they involve subjects who have had a history of volitional drug use, undergo a period of drug abstinence, and who experience conditions that then precipitate renewed drug-seeking behavior. Although there is substantial face validity between reinstatement procedures and clinical relapse, it is not complete. Most researchers who employ reinstatement procedures use experimental extinction for inducing abstinence, but extinction is rarely responsible for clinical abstinence (e.g., rarely is the lack of rendering a drug upon payment the reason for extended abstinence). However, the use of experimental extinction in reinstatement procedures provides both a means of driving response rates to low levels enabling an expression of reinstatement, as well as integrally providing a concurrent monitor of response levels consequentially permitting the statistical evaluation of reinstatement. As such, experimental extinction retains strengths, and no other procedure for imposing abstinence while also insuring low response levels has emerged as a serious replacement for it. Of course, face validity by itself has little worth, and whether reinstatement procedures have predictive validity, for instance as serving as screens for potential pharmacotherapies for treating drug relapse, or construct validity, for instance as serving as a system for identifying mechanisms mediating clinical relapse, is what is important but will only be determined with additional study. Employing procedures as those described in this unit will help generate the databases for evaluating the level of predictive and construct validity they may have.

Amongst the earliest demonstrations of reinstatement of previously drug-reinforced responding include those by de Wit and Stewart in rats (de Wit and Stewart, 1981; de Wit and Stewart, 1983), and of Stretch and Gerber in squirrel monkeys (Stretch and Gerber, 1973; Stretch et al., 1971). Since those reports, there has been a dramatic expansion in the number of reinstatement reports utilizing operant procedures as did these earliest reports, with most of this expansion occurring within the last 12 years. As an example, there have been 463 reinstatement reports involving cocaine alone, with all but 38 published after the year 2000 (as measured by a PubMed search on “reinstatement AND cocaine AND self administration”, 02/02/12). Other than reinstatement procedures involving operant contingencies, there are those that use conditioned place preference procedures (for reviews see, Aguilar et al., 2009; Liu et al., 2008; Tzschentke, 2007); (for detailed methods, see Roux et al., 2003). In conditioned place preference procedures, administration of a drug of abuse is associated with one of two distinctive test chambers, and administration of its vehicle is associated with the other. Typically, there are three pairings of each type of injection, and then during a post-conditioning test session the subject is placed in a neutral zone between the two compartments, and the amount of time then spent in each compartment is then recorded. It is inferred that more time spent in the drug-paired compartment reflects the abuse properties of the drug. Subjects having been trained under conditioned place preference procedures can then have their preferences extinguished by allowing them to explore the two compartments in the absence of paired injections, and then can be tested for reinstatement by pretreating them with a drug prime or stressor, and then measuring their distribution of time spent in two compartments. If more time is spent in the previously drug-paired compartment, compared to subjects primed with vehicle or not exposed to the stressor, reinstatement of place conditioning is inferred. Two features of conditioned place preference procedures bear consideration in choosing it, as opposed to the operant procedures described in this unit. First, never in the history of the conditioned place preference subject does it demonstrate volitional drug self-administration as do drug abusers, thus markedly decreasing its face validity relative to operant reinstatement procedures. There are scores of reports documenting that the neuropharmacological and

genetic effects of self-administered versus experimenter-administered drug can differ, if not being diametrically opposite to one another (for review, see Jacobs et al., 2003). Secondly, the conditioned place preference effect has multiple interpretations when considered along side other reports utilizing similar conditioning procedures, but that have documented conditioned effects opposite to those that the drug, itself, elicits (Siegel, 1978; Siegel, 1990; Siegel et al., 2000). Other weaknesses of this procedure have been observed (Schechter and Calcagnetti, 1993). For these, and other reasons, we have focused on presenting reinstatement procedures that have utilized operant contingencies during the drug treatment phase, that is, in which drug delivery contingent upon the subject's own responding has been an integral component of the subject's history.

Critical Parameters and Troubleshooting

Major technical considerations IV: Selection of extinction period duration—

The term, “extinction”, has several meanings depending on the context. We use the term in this protocol to refer to the *process* in which delivery of drug infusion following completion of a previously reinforced response is discontinued. The objectives of the extinction period are twofold: 1) It provides a period of drug abstinence; and 2) It drives response rates down enough to permit the expression of “reinstatement”. An expression of reinstatement is inferred when response rates following a prime, reinstitution of cue-contingent responding, or presentation of footshock exceed those during the final session of extinction. Another meaning that the term, “extinction”, can sometimes have refers to a final *state* of near-zero responding, and not just to a process as we use the term. It would be incorrect to use the term in this latter sense (referring to a near-zero state of responding) after imposing just 12 sessions of extinction (as the described protocols employ) because response rates can be, and typically are, still well above zero levels. This is particularly true for the prime and footshock reinstatement procedures in which cues previously associated with drug infusion accompany responding during extinction, which, in turn, retards dissipation of responding despite the absence of drug infusion. If for some reason the experimental objectives require a near-zero response state at the end of the extinction period, expect to invest several additional weeks of extinction for this to occur during the prime and footshock reinstatement protocols (if even it occurs then). If experimental considerations preclude protracted extinction training, the use of a within-session extinction and reinstatement protocols in which reinstatement testing occurs at the end of a single long extinction session may constitute an alternative approach (Neisewander et al., 1996). The terminal level of extinction responding is less an issue during the cue protocol because extinction occurs in the absence of accompanying drug-associated cues, and consequentially, responding can diminish to near-zero levels after 12 sessions of extinction. Utilizing 12 sessions of extinction, as we do in these protocols, strikes a balance between constraining response rates to low enough levels on the 12th session of extinction permitting statistically significant elevations of responding during each of the reinstatement test conditions, while maximizing the progression of rats completing training and testing in the experimental chambers. Twelve days of abstinence for some drugs, such as heroin, also appears to maximize the magnitude of the reinstatement effect, at least those of primes (Shalev et al., 2001).

Major technical considerations VII: Selection of electric shock delivery device, its amplitude and duration—

The stress-induced reinstatement procedure described in Basic Protocol 3 uses brief, intermittent inescapable electric footshock to elicit reinstatement behavior. The tolerance requirements of acceptable footshock delivery devices (shockers) for this procedure are more stringent than devices used for other experimental goals such as providing negative reinforcement consequences. Specifically, footshock that is too intense will result in variable reinstatement behavior with some animals reinstating at very high levels while others simply freezing and emitting few if any responses. Conversely,

footshock of insufficient intensity does not result in robust reinstatement. Numerous factors can alter this intensity, the most critical of which is the resistance of the electrical circuit completed by the animal as it stands on the operant chamber's bar floor. The resistance of a circuit in a particular chamber is affected by animal itself, the shocker to floor grid cable length, component connection quality and cleanliness of the floor grid among others. Some less costly shock delivery devices do not provide compensation for these factors and therefore provide inconsistent shock intensities across subjects. This concern is mitigated by utilizing a shock delivery device with internal feedback regulating circuitry that senses and compensates for differences in circuit resistance. Several years ago our laboratory teamed with a custom design shop to construct shockers to our specifications. In addition to precisely regulating shock intensity, these shockers also include an internal fault detection readout to verify the integrity of the shocker and the shocker to floor grid connection. At the time of publication of this protocol, these devices were available for purchase from a company that is unaffiliated with our laboratory (Custom Design and Fabrications, Inc., Sandston VA). Other operant device manufactures, notably Coulbourn Instruments (Whitehall, PA), also purports to offer a feedback regulated shocker that would likely be similarly suitable, although we have not tested it ourselves.

Troubleshooting

Please refer to Table 1.

Anticipated Results

Discussions of the expectations, pitfalls and troubleshooting approaches in developing refined catheter construction and implantation techniques, and for establishing self-administration in rats, are beyond the scope of the present protocol, but are elaborated in detail in the protocol previously provided in this journal by Thomsen and Caine (Thomsen and Caine, 2005). We have provided details only of significant differences in our methodology relative to those described in this earlier protocol. Those differences, however, do not directly translate into differences of anticipated results, at least insofar as establishing and maintaining self-administration, and a detailed treatment of anticipated results during catheterization and self-administration is not provided here. However, there are varying degrees of difficulty, levels of reinstated responding, and consequential yields in proportions of successfully reinstating rats in cocaine prime-, cue-, and footshock-induced reinstatement procedures.

One should expect to get a similar proportion of rats to successfully reinstate, that is emit more responses during the reinstatement test session than during the final session of extinction, in cocaine prime- and cue-induced procedures, but more so than during footshock-induced procedures. For instance, examining the most recently tested 24 rats from cocaine prime-, cue- and footshock-induced procedures in our laboratory, 23, 23, and 20 successfully reinstated, respectively (all rats had been given pre-session administrations of vehicle). Figure 5 shows representative self-administration, extinction and reinstatement results from groups of 12 rats each tested in each of the three reinstatement procedures. Levels of lever pressing on the final day of self-administration varied little across the groups, and ranged from a mean ($\pm$ SEM) of 42.58 ($\pm$ 3.42) in the footshock group, to 46.00 (4.43) in the cocaine prime group. Levels of extinction responding are more variable, and in this sampling of rats ranged from a mean ($\pm$ SEM) of 13.17 (2.60) in the cue-tested group, to 32.25 ($\pm$ 7.84) in the prime-tested group. It should be expected that levels of extinction responding will be lowest in cue-tested rats because extinction occurs without the accompaniment of tone or light cues during extinction, unlike that of either the prime- or footshock-tested rats. Mean ($\pm$ SEM) levels of reinstated responding were highest for cocaine-primed rats at 122.70 ($\pm$ 24.57) responses, then for cue-reinstated rats at 87.50

(± 10.82) responses, and lowest for footshock-reinstated rats at 46.58 (± 11.02) responses. For illustration, a one-way ANOVA was conducted on this sample of rats and significant [$F(2,33)=5.164$; $P=0.0112$] differences were found. Bonferroni's Multiple Comparison post-hoc tests indicated that levels of reinstated responding were significantly ($P<0.05$) greater in the prime-tested rats than the footshock-tested rats ($t=3.211$). Observing the greatest levels of reinstatement in prime-tested rats, and the lowest levels in footshock-tested rats, has frequently (with some exceptions) been the observation in our laboratory.

Typically, numbers of presses of the inactive lever are low during the reinstatement test. For instance, mean ($\pm$ SEM) inactive lever presses during the reinstatement tests shown in Figure 5 ranged from 6.93 (± 2.55) during the footshock test, to 8.17 (± 2.74) during the prime test. Because of the low level of inactive presses that typically occur during the reinstatement test, their usefulness is limited in making inferences following experimental manipulations because of a "floor" effect. However, in some instances the experimental manipulation can be so powerful that significant depressions of responding can occur relative to control levels. For instance, Figure 6 shows the effects of pretreatment with the antipsychotic, haloperidol, on active (upper frame) and inactive lever presses (lower frame) on footshock-induced reinstatement (the experimental conditions were similar, but not identical to those described in this protocol). A significant reduction of footshock-induced reinstatement occurred relative to vehicle levels at the 0.3 mg/kg dose. However, a significant reduction in inactive lever presses also occurred at the 0.3 mg/kg dose that complicates interpretations of the effectiveness of haloperidol in blocking stress-induced reinstatement because the data suggest it may nonspecifically suppress behavior engendered by a variety of events at that dose.

Pharmacological Manipulation (Example 1)

Evaluating the effects of a pharmacological agent on prime-, cue- and footshock-induced reinstatement of responding previously reinforced with cocaine

Testing the effects of the kappa opioid receptor (KOR) antagonist, JDTic: Figure 3 illustrates the effects of JDTic (vehicle, 3, 10 and 30 mg/kg i.g.), a KOR antagonist, on prime-, cue- and footshock-induced reinstatement of extinguished lever pressing previously reinforced with 0.5 mg/kg/infusion cocaine. Separate groups of 12 rats each were used for each dose condition. The effects of JDTic on prime- and footshock-induced reinstatement had been previously reported (Beardsley et al., 2005) using similar data. Separate nonparametric (Kruskal Wallis) tests were conducted within each reinstatement condition on active lever presses across dosage groups for the last session of self-administration and for last session of extinction and were found to not significantly ($P<0.05$) differ indicating that the rats had been trained to similar levels of cocaine self-administration and extinction prior to reinstatement testing (data not shown). These analyses thus established that rats within each treatment group had been self-administering cocaine comparably before extinction, and that extinction had comparable effects on responding before testing, enabling their comparisons during reinstatement testing. Separate one-way t-tests were conducted on active lever presses of individual vehicle groups within each reinstatement condition to their respective final sessions of extinction to determine if cocaine primes, cue presentations and footshocks were effective in reinstating responding. In each of the three comparisons, active lever presses significantly ($P<0.05$) increased during reinstatement tests relative to their immediately preceding extinction session. Mean ($\pm$ SEM) numbers of active lever presses during the last session of extinction emitted by the vehicle treatment group for the prime-, cue- and footshock conditions were 17.67 (± 2.548), 14.17 (± 3.426) and 36.75 (± 8.890), respectively, and increased to 51.67 (± 10.96), 54.92 (± 5.578) and 64.75 (± 14.33) during reinstatement testing. These increases were statistically significant (prime: $t=3.170$, $df=11$,

$P=0.0045$; cue: $t=10.83$, $df=11$, $P<0.0001$; footshock: $t=2.314$, $df=11$, $P=0.0205$). These significant increases in active lever responding during the reinstatement conditions, relative to their respective final extinction sessions, indicates that the conditions used effectively resulted in reinstatement in the vehicle treated groups for each reinstatement condition.

When tested with JD_{Tic}, levels of footshock-induced reinstatement were reduced relative to vehicle conditions, significantly so at the 10 and 30 mg/kg conditions (Dunn's Multiple Comparison Tests, $p<0.05$). Levels of prime- and cue-induced reinstatement were unaffected by JD_{Tic}. The selectivity of effects on footshock-induced, but neither on prime- nor cue-induced reinstatement, suggests that the effects on stress-induced reinstatement were likely not attributable to non-specific suppressing effects of JD_{Tic} (or all reinstatement conditions would have been affected by JD_{Tic}). The use of multiple reinstatement tests in this way can provide one approach for deciding the likely selectivity of a potential medication for treating relapse.

Time Considerations

Please refer to Table 2.

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Figure 1. Catheter introducer components showing unmodified 1 ml syringe and 27 gauge needle above and completed catheter below.

Figure 2. Catheter flush syringe assembly with attached Tygon® tubing. Two catheter-sealing caps are shown in the upper left.

Figure 3.
Magnification of the tapered tip of a polyurethane jugular catheter.

Figure 4. Completed tapered polyurethane catheter on gauge card. Also shown is catheter connection post/button as assembled during surgery.

Representative Levels of Responding During Self-Administration, Extinction, and of Reinstatement

Figure 5.

Mean active lever presses during the final session of self-administration and of extinction, and during the cocaine prime-, cue- and footshock-induced reinstatement tests in rats that had previously self-administered cocaine. Brackets through bars indicate SEM. Intragastric injections of vehicle (saline for prime and cue tests, and 0.25% methylcellulose for footshock tests) preceded tests by 30 min. N=12 for all groups.

Figure 6.

Figure 6 (upper frame). Mean numbers of active lever presses during the cocaine footshock-induced reinstatement test session as a function of haloperidol dose. Brackets through the bars indicate $\pm$ SEM. "VEH" = results of the vehicle-treatment group. Asterisks indicate significantly different ($P < 0.05$) from vehicle. $N = 12$ for each condition.

Figure 6 (lower frame). Mean numbers of inactive lever presses during the cocaine prime-induced reinstatement test session as a function of Drug X dose. Other details as in Figure 2A.

Figure 7.

Mean active lever presses during cocaine prime-, cue- and footshock-induced reinstatement tests in rats when tested with vehicle or doses (mg/kg i.g.) of JDtic which had previously self-administered cocaine. Brackets through bars indicate SEM. Dotted lines indicate range of mean responses on the last day of extinction. N=12 for all groups.

Table 1

Troubleshooting

Problem	Possible Cause	Solution
Surgical issues, failure to acquire self-administration or attain stabilized responding	Many	See protocol by Thomsen and Caine (Thomsen and Caine, 2005).
High levels of attrition due to not attaining extinction criteria	Pumps still activated delivering infusions, unusually low levels of responding during the initial 3 sessions of extinction, training drug	Check to ensure pumps are not still infusing during extinction. Typically, substantial responding occurs during the initial sessions of extinction; if many rats show extremely low levels, it may represent a rat strain or training drug difference and criteria may need adjustment.
Low levels of prime reinstatement	Inaccurate drug solution preparation, poor, such as misplaced (not intra-peritoneal) injection	If all rats show poor prime reinstatement, suspect improperly prepared cocaine solutions, and remake. If rats showing poor reinstatement are associated with a specific technician, verify proper injection technique.
Low levels of cue reinstatement	Failure of light or tone stimuli to occur following responding	Check for proper illumination of light and sounding of tone during the reinstatement test, but also during self-administration.
Low levels of stress reinstatement	Shock intensity suboptimal, either too high or too low	Clean floor grids before each test session. Calibrate shock generators. Test to be sure shock generators <i>consistently</i> produce the intended shock level. If a substantial proportion of all tested rats fail to reinstate despite being shocked by different shock units, manipulation of shock intensity, downwards or upwards, may need to be made to accommodate the specific strain or generation of rat.

Table 2

Time Considerations

Phase	Approximate time	Considerations
Ordering and receipt of rats	1 week	Sometimes rats are unavailable at the desired weight (~275–300 g) and delays can occur in receipt.
Habituation to vivarium	minimum of 5 days	
Catheterization surgery	~25 to 35 min for an efficient surgeon, longer for a less experienced surgeon	Catheters and connection pedestals must be pre-assembled and sterilized with backups.
Post-operative recovery time	minimum of 5 days	A longer post-operative recovery time may be necessary if swelling is observed at surgical sites.
Acquisition of drug self-administration	A little as 1 session, but some rats may never acquire.	It is unusual for a rat not to spontaneously acquire self-administration within the first 5 sessions. The experimenter needs to pre-specify a maximum number of sessions allowed a rat to acquire self-administration before eliminating it from subsequent study. Allowing over 10 sessions for acquisition may result in the retention of rats responding to subsequent experimental manipulations atypically.
Stabilization of self-administration	~ 10 sessions	Catheter loss, and the consequential waiting period before recatheterization can extend this period substantially.
Extinction of lever pressing	12 days	
Reinstatement testing	1 day	