

# Alpha-2 Adrenergic Receptor Agonists Block Stress-Induced Reinstatement of Cocaine Seeking

Suzanne Erb, Ph.D., Paul K. Hitchcott, D. Phil., Heshmat Rajabi, B.Sc., Devin Mueller, M.A., Yavin Shaham, Ph.D., and Jane Stewart, Ph.D.

*The alpha-2 adrenergic receptor agonists, clonidine, lofexidine and guanabenz, blocked stress- but not cocaine-induced reinstatement of cocaine seeking at doses that suppressed footshock-induced release of noradrenaline in prefrontal cortex and amygdala. Rats were trained to self-administer cocaine (0.5 mg/kg/infusion, i.v; 10–12 days) and, after a drug-free period (7–13 days), were returned to the self-administration chambers for daily extinction and reinstatement test sessions. Both intermittent footshock (15 min, 0.6 mA) and cocaine priming (20 mg/kg, i.p.) reinstated extinguished drug seeking. Pretreatment with either*

*clonidine (20, or 40 µg/kg, i.p.) or lofexidine (50, 100, 150, or 200 µg/kg, i.p.) attenuated footshock- but not cocaine-induced reinstatement of cocaine seeking. Guanabenz (640 µg/kg, i.p.), an alpha-2 agonist with low affinity for imidazoline type-1 receptors, also attenuated footshock- but not cocaine-induced reinstatement of cocaine seeking. The results point to an important role for NE systems in the effects of footshock on relapse to cocaine seeking.*

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“Priming” injections of an abused drug can induce craving in abstaining addicts (e.g. Jaffe 1985) and reinstate drug-seeking in monkeys and rats with a history of drug self-administration (e.g., Stretch and Gerber 1973; de Wit and Stewart 1981). Another factor thought to be important to relapse in humans is stress (Kreek and Koob 1998). In a recent laboratory study, Sinha and colleagues (1999) found that induction of psychological

stress provoked craving for drug in cocaine addicts. Using an animal model of stress-induced relapse, we and others have found in rats that exposure to brief intermittent footshock stress reliably reinstates heroin seeking (Shaham and Stewart 1995), cocaine seeking (Erb et al. 1996; Ahmed and Koob 1997; Mantsch and Goeders 1999), nicotine seeking (Buczek et al. 1999), and alcohol seeking (Lê et al. 1998).

In a search for neurotransmitter systems involved in stress-induced relapse, and for pharmacological manipulations that might prevent it, we explored in the present set of experiments the role of the noradrenergic (NE) system, comprised of a dorsal stream of neurons originating in the locus-coeruleus and a ventral stream of neurons originating in the lateral tegmental nuclei (Aston-Jones et al. 1995; Moore and Bloom 1979). Although the stress-related functions of the ventral projections have been only partially characterized (Cole and Robbins 1987; Hansen et al. 1980), NE projections from the LC have been directly implicated in a number

From the Center for Studies in Behavioral Neurobiology (SE, PKH, HR, DM, JS), Department of Psychology, Concordia University, Montreal, Quebec, Canada; Behavioral Neuroscience Department (YS), IRP/NIDA, Baltimore, MD.

Address correspondence to: Dr. Jane Stewart, Center for Studies in Behavioral Neurobiology, Department of Psychology, 1455 de Maisonneuve Blvd. W., Montreal, Quebec, Canada H3G 1M8 Tel: 514-848-2193, Fax: 514-848-2817, E-mail: Stewart@CSBN.concordia.ca.

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of stress-related responses. For example, electrical stimulation of the LC has been found to induce anxiety and to result in activation of the autonomic nervous system, whereas local injections of the *alpha*-2 adrenergic receptor agonist clonidine, which acts to inhibit NE cell firing (Aghajanian and VanderMaelen 1982) and release (Carter 1997), has been found to decrease physiological and behavioral responses to stressors (Bremner et al. 1996; Redmond and Huang 1979).

We have recently reported that systemic or intracerebroventricular (i.c.v.) injections of clonidine block footshock-induced reinstatement of heroin seeking (Shaham et al. 2000), a finding that suggests an important role for brain NE in the mediation of stress-induced relapse. In previous studies, we have shown that another neurotransmitter system, corticotropin-releasing factor (CRF), also plays an important role in mediating the footshock-induced reinstatement of both cocaine (Erb et al. 1998; Erb and Stewart 1999) and heroin seeking (Shaham et al. 1997) via its actions at extrahypothalamic brain sites. Interestingly, the CRF and NE systems interact in the brain in the modulation of stress responses (e.g., Li et al. 1998; Shimizu et al. 1994; Smagin et al. 1997; Valentino et al. 1991; Valentino and Wehby 1988), raising the possibility that an interaction between these systems underlies stress-induced relapse to drug seeking.

The present set of experiments had four main objectives. First, we assessed whether the effect of clonidine on stress-induced reinstatement previously observed in heroin-trained rats would generalize to rats with a history of cocaine self-administration. Second, we tested whether another *alpha*-2 adrenergic receptor agonist, lofexidine, would be equally effective. Third, because clonidine is known to bind at high affinity to the imidazoline type-1 ( $I_1$ ) receptor, we studied the effect of an *alpha*-2 adrenergic receptor agonist with low affinity for the  $I_1$  receptor, guanabenz (Piletz et al. 1994), on stress-induced reinstatement. Finally, we studied the effect of clonidine, lofexidine, and guanabenz on reinstatement of cocaine seeking induced by a priming injection of cocaine.

Before conducting the behavioral experiments, we assessed the ability of the three *alpha*-2 adrenergic receptor agonists to suppress footshock-induced release of NE in two terminal regions, the prefrontal cortex (PFC) and amygdala (AMG). The PFC is a major terminal region of the dorsal NE system arising from the locus coeruleus, whereas the AMG receives input from both the dorsal and ventral systems.

## MATERIALS AND METHODS

### Experiment 1: Effects of Clonidine, Lofexidine and Guanabenz on Footshock-Induced NE Release

**Subjects.** The subjects were male Long-Evans rats (Charles River, Quebec) weighing 300–350 g at the be-

ginning of the experiment. Throughout the experiment, animals were housed in a humidity- and temperature-controlled colony room on a reversed light-dark schedule (lights on 1730–0530 hours) and were given free access to standard laboratory rat chow and water. The experimental procedures followed the CCAC guidelines and were approved by the Animal Care Committee, Concordia University.

**Surgery.** Before surgery, rats were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and were injected with atropine sulfate (0.6 mg/ml; 0.2 ml/rat, SC) and antibiotic (Penlong, Rogar/STB Inc.; 0.1 ml/rat, i.m.). Guide cannulae (20 gauge; Plastics One) were implanted for subsequent insertion of dialysis probes; one was placed in PFC and one in AMG in opposite hemispheres. The animals used to study the effects of guanabenz received a single guide cannula aimed at PFC. The hemisphere into which the respective guide cannulae were implanted was counterbalanced across treatments. The stereotaxic coordinates used (relative to bregma and skull surface) were as follows: AMG, AP  $-3.0$  mm, ML  $\pm 4.5$  mm, DV  $-6.7$  mm; PFC, AP  $+ 3.2$  mm, ML  $\pm 0.5$  mm, DV  $-1.5$  mm (Paxinos and Watson 1997). Upon insertion, the shaft of the dialysis probe extended 1 mm from the guide cannulae. Implanted guide cannulae were secured to the skull with stainless steel screws and dental cement. Guide cannulae were closed with screw-in obturators. All animals were returned to the colony room for a recovery period of no less than 1 week prior to testing.

**Microdialysis.** Microdialysis was conducted in four hexagonal testing chambers ( $42 \times 39 \times 33.5$  cm) built from Plexiglas with wooden ceilings and stainless steel rod floors. Dark curtains were drawn around each chamber and lighting was provided on a reversed cycle by overhead light bulbs (15 W). The dialysis probe consisted of a 1.8 mm (AMG) or 3.5 mm (PFC) length of semipermeable dialysis membrane (Spectra/Por; 240  $\mu$ m o.d., 13000 M.W. cutoff), closed at one end and attached at the other to a 19 mm length of 26 gauge stainless-steel tubing. A 40- to 50-cm length of PE-20 tubing connected the other end of the stainless steel shaft to an infusion swivel stationed above the testing chamber that was in turn connected via PE-20 tubing to a variable-speed infusion pump. A small diameter, fused-silica tube extended internally through the probe, with one end resting 0.5 mm from the tip of the probe and the other end exiting the PE tubing 35 cm below the infusion swivel. The external length of PE-20 tubing was protected from damage by steel spring casings. The probes were designed so that the entire length of semipermeable membrane extended below the guide cannula tip.

Separate squads of animals were used to examine the effects of each of the drugs tested. Within each squad, each animal was randomly assigned to the treatment

condition. Animals that received vehicle injections were run in each squad, but were combined into a single group for statistical analysis. Final group sizes (PFC/AMG) were as follows: vehicle ( $n = 15/17$ ), clonidine 20  $\mu\text{g}/\text{kg}$  ( $n = 7/6$ ), clonidine 40  $\mu\text{g}/\text{kg}$  ( $n = 9/5$ ), lofexidine 75  $\mu\text{g}/\text{kg}$  ( $n=6/6$ ), lofexidine 150  $\mu\text{g}/\text{kg}$  ( $n = 5/6$ ), guanabenz 640 $\mu\text{g}/\text{kg}$  ( $n = 4$ , PFC). With the exception of the animals receiving guanabenz, all subjects were dialyzed twice, once in the PFC and once in the AMG with an interval of 3–4 weeks between tests.

The probes were inserted the day before the beginning of microdialysis testing. To prevent occlusion, artificial CSF (145 mM Na<sup>+</sup>, 2.7 mM K<sup>+</sup>, 1.22 mM Ca<sup>2+</sup>, 1.0 mM Mg<sup>2+</sup>, 150 mM Cl<sup>-</sup>, 0.2 mM ascorbate, 2 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4  $\pm$  0.1) was perfused overnight at a rate of 0.06  $\mu\text{l}/\text{min}$ . Dialysate sampling and activity monitoring began the next morning. The dialysate flow rate was increased to 0.6  $\mu\text{l}/\text{min}$ , and baseline dialysate samples ( $\sim 12 \mu\text{l}/\text{sample}$ ) were collected every 20 min. Samples were collected until a stable baseline, defined as a minimum of three consecutive samples in which dialysate NE levels varied by  $\pm 10\%$ , was achieved. Following the establishment of stable baseline NE levels, all animals received an i.p. injection (1 ml/kg) of the appropriate drug or vehicle which was given 40 min before a 10 min period of footshock. Shocks were delivered on a variable time schedule at a mean interval of 40 seconds (10–70 seconds range); each shock (0.6 mA) was 0.5 seconds in duration. Samples were collected for a further 120 minutes (6 samples). Samples were immediately analyzed using one of two similar high-performance liquid chromatography systems with electrochemical detection (HPLC-EC). The samples (10  $\mu\text{l}$ ) were loaded onto reverse-phase columns (15  $\times$  0.46 cm; HAISIL C18, 5  $\mu\text{m}$ ; Sci. Products & Equipment, Concord, Ontario) through manual injection ports (Reodyne 7125; 20  $\mu\text{l}$  loop); reduction and oxidation currents for NE, dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) were measured with dual-channel ESA coulometric detectors (Coulchem 5100, with a model 5021 conditioning cell and a model 5011 analytical cell, Sci. Products & Equipment). The currents for NE were measured independent of those for DOPAC and HVA using separate channels of the Coulchem detectors. The mobile phases (sodium acetate 36 mM, SOS 3.1 mM, EDTA 100  $\mu\text{M}$ , 5% acetonitrile, adjusted to pH 3.7 using glacial acetic acid) were circulated through each closed system at a flow rate of 1.4 ml/min by Waters 510 HPLC pumps. The peaks obtained for NE, DOPAC and HVA were integrated and quantified by EZChrom Chromatography Data System (Sci. Products & Equipment). Dialysate samples from individual rats always were analyzed with the same HPLC-EC system, and the assignment of animals to each system was counterbalanced across all treatment groups. Food was removed

from the chambers before sampling, but a water drinking tube was available. At the completion of testing, confirmation of correct probe placement was determined by examination of 30  $\mu\text{m}$  sections cut through the sites of probe placement stained with Cresyl violet.

## Experiment 2: Effects of Clonidine on Footshock- and Cocaine-Induced Reinstatement

**Subjects.** The subjects were 25 male Long-Evans rats (Charles River, Quebec) weighing 350–425 g at the start of the experiment. The animals were maintained as described in Experiment 1.

**Surgery.** Animals were prepared for surgery as described in Experiment 1. An intravenous catheter (Dow Corning) was implanted in the right jugular vein. A 3-cm length of silastic tubing was inserted into the vein (inner diameter 0.30 mm, outer diameter 0.64 mm) and was connected with heat shrink tubing to a 9-cm length of silastic tubing (inner diameter 0.51 mm, outer diameter 0.94 mm) that was passed subcutaneously to the top of the skull. The catheter was secured to the vein with silk sutures and exited into a connector (a modified 22 gauge cannula; Plastics One, Roanoke, VA) that was mounted to the skull with jeweler's screws and dental cement; a plastic cap was placed over the opening of the cannula. Animals were allowed 1–2 weeks to recover from surgery.

**Apparatus.** The self-administration chambers used in the experiments were equipped with a retractable lever (Med Associates, St Albans, VT) and a non-retractable "dummy" lever. Both levers were located 9 cm above the floor. An infusion pump (Razel Scientific Instruments, Stamford, CT) was activated by responses on the retractable, or "active," lever. Responses on the dummy lever were recorded but did not result in activation of the pump. Drug solution was delivered over a 10-s period in a volume of 65  $\mu\text{l}$ . Throughout the infusion period, a white stimulus light just above the active lever was illuminated and additional responses during this time were recorded, but did not result in reactivation of the pump. Each self-administration chamber was fitted to deliver constant-current, intermittent, inescapable, electric footshock through a scrambler to the grid floor (Grason-Stadler Generator #E1064GS or Med Associates). The footshock was delivered for 15 min according to a variable time schedule at a mean interval of 40 seconds (10–70 seconds range). Each shock (0.6 mA) was 0.5 seconds in duration. This intensity of footshocks has been found using our apparatus to be the minimal required to induce reliable reinstatement.

**Drugs.** Cocaine HCl was obtained from BDH Chemicals (Toronto, Canada) and was dissolved in physiological saline. Clonidine HCl was purchased from Sigma

(St Louis, MO) and was dissolved in physiological saline and injected i.p. (0, 20, and 40  $\mu$ g/kg).

**Procedure.** *Phase 1: Training.* Rats were trained to self-administer cocaine HCl (0.5 mg/kg/infusion, i.v.) on a fixed-ratio-1 schedule of reinforcement during one daily 3-h self-administration session. Each day, half of the animals were brought to the operant chambers for their self-administration session in the morning, approximately three hours after lights off, and half of the animals were brought to the chambers in the afternoon, approximately seven hours after lights off. The group of animals assigned to the morning and afternoon sessions alternated daily so that by the end of training all animals had received an equal number of self-administration sessions at both times. It was important that all animals had similar experience self-administering early and late in the day since during Phase 2 all animals received extinction sessions in the morning followed by a test session that typically occurred in the afternoon. At the beginning of each session, a red house light was illuminated for 10 seconds before introduction of the retractable lever into the cage. The light just above the active lever was lit for the initial 30 seconds following presentation of the lever. The house light remained illuminated throughout the session. As indicated previously, responses on the active lever resulted in activation of the infusion pump and illumination of the light above the lever for the 10 seconds of drug delivery. Additional lever presses during the drug-delivery period were recorded but did not result in reactivation of the pump. Training conditions were in place for 10 to 12 days. At the end of training, animals were left undisturbed in the colony room for 7 to 13 days. Subsequently, extinction and testing took place. In this experiment and in Experiment 3B, groups of animals were assigned to different doses of the test drugs. All animals in each group were then subjected to three test conditions; saline, cocaine and footshock.

*Phase 2: Extinction and testing.* During extinction and testing, which occurred over four consecutive days, animals were housed 24 hours per day in the self-administration chambers. Food and water were freely available to animals, except during daily extinction and test sessions. Animals were brought to the chambers in the evening preceding the first day of extinction and testing. Since two separate groups of rats were run each day during the training phase, one group of animals was tested in the first four days of Phase 2 and the other group of animals was tested in the next four days. During extinction and testing, all of the conditions that were present during training were maintained except that lever presses did not result in cocaine infusions.

In this and all subsequent reinstatement experiments, animals were given several daily 1-h extinction sessions separated by intervening periods in which the

lever was withdrawn. On day 1, animals were given four extinction sessions; on days 2–4, animals were given two to three extinction sessions, sufficient to establish a baseline level of responding of 15 or fewer responses in one hour, followed by a 180 min test session. The duration of the intervening periods was kept constant for each experiment and corresponded to the delay required for drug absorption between pretreatment injections and the start of the test. In the present experiment, the duration of intervening periods was 40 min.

At test, separate groups of animals were pretreated with either 0, 20, or 40  $\mu$ g/kg, i.p., clonidine before each of three tests for reinstatement (saline, cocaine, and footshock), given on consecutive days and in a counter-balanced order. Clonidine was injected 40 min before the lever was inserted. For the saline and cocaine priming tests, animals were given a non-contingent, i.p., injection of saline or cocaine (20 mg/kg) 5 min before lever insertion. For the footshock tests, animals were exposed to the 15-min brief intermittent footshock stress immediately before insertion of the lever. Test sessions were 3-h in duration. The dose of cocaine and footshock parameters were chosen on the basis of previous work from this laboratory (Erb et al. 1996; Erb et al. 1998; Shaham et al. 1998). The doses of clonidine were chosen on the basis of reinstatement experiments we have conducted with heroin-trained rats (Shaham et al. 2000).

### Experiment 3A: Effects of Lofexidine on High Rates of Responding for Sucrose

Because the pharmacological profile for lofexidine has not been as well characterized as that for clonidine, an experiment was conducted to establish whether, and at what doses, lofexidine induces performance deficits. The doses in the tests for reinstatement were chosen on the basis of results obtained in this experiment.

**Subjects.** The subjects were 8 male Long-Evans rats (Charles River, Quebec) weighing 400–550 g at the beginning of the experiment. Rats were previously used in an experiment aimed at studying footshock-induced reinstatement of sucrose seeking (Buczek et al. 1999). Animals were maintained under similar conditions to those described in Experiment 1.

**Apparatus.** Each self-administration chamber was equipped with two stationary levers, symmetrically centered on one side panel, 5 cm above the floor. Responses on one lever, the “active” lever, activated a pump (Razel Sci. Stamford, CT). Responses on the other lever, the “dummy” lever, were recorded but were without consequence. Activation of the pump resulted in a 20-second delivery of 0.18 ml sucrose solution to a liquid drop receptacle located between the two levers. Throughout the sucrose delivery period, a white stimulus light just above the active lever was illuminated and

additional responses during this time were recorded but did not result in reactivation of the pump.

**Drugs.** Lofexidine HCl was generously supplied by Keith Davies, Britannia Pharmaceuticals Ltd., UK. The drug was dissolved in physiological saline and injected i.p. (0, 80, 120, 160 or 200  $\mu\text{g}/\text{kg}$ ).

**Procedure.** Animals that had previously learned to self-administer sucrose in a different study (Buczek et al. 1999) were subsequently given five sessions over five consecutive days in which they self-administered a 30% sucrose solution on an FR-1 schedule. In subsequent daily sessions, animals were injected with either vehicle (0  $\mu\text{g}/\text{kg}$ ) or lofexidine (80, 120, 160 or 200  $\mu\text{g}/\text{kg}$ , i.p.) 60 min before the start of the self-administration session. All animals received all doses of lofexidine in a counterbalanced order; the highest dose of lofexidine was tested twice. Each session in which animals were treated with lofexidine was followed by a saline pretreatment session to minimize the possibility of carry-over effects of the drug.

### Experiment 3B: Effects of Lofexidine on Footshock- and Cocaine-Induced Reinstatement

**Subjects.** The subjects were 49 male Long-Evans rats (34 from Charles River, Quebec; 15 from Harlan Sprague Dawley, USA) weighing 350–400 g at the beginning of the experiment. The animals were maintained as described in Experiment 1. There were no obvious differences in responding between animals from the two suppliers in any phase of the experiment.

**Procedure.** *Phase 1: Training.* Animals were trained to self-administer cocaine under the conditions described in Experiment 2.

*Phase 2: Extinction and testing.* In this experiment the intervening periods, as described above, were 60 min in duration. At test, separate groups of animals were pretreated with either 0, 50, 100, 150, or 200  $\mu\text{g}/\text{kg}$ , i.p., lofexidine before each of three tests for reinstatement (saline, cocaine, and footshock stress), given on consecutive days and in a counterbalanced order (see Experiment 2). Lofexidine was injected 60 min before the insertion of the lever. Test sessions were 3-h in duration. The doses of lofexidine were chosen on the basis of the results obtained in Experiment 3A.

### Experiment 4: Effects of Guanabenz on Footshock- and Cocaine-Induced Reinstatement

**Subjects.** The subjects were 18 male Long-Evans rats (Charles River, Quebec) weighing 350–400 g at the beginning of the experiment. The animals were maintained as described in Experiment 1.

**Drug.** Guanabenz was purchased from Sigma (St Louis, MO) and was dissolved in physiological saline and injected i.p. (0, and 640  $\mu\text{g}/\text{kg}$ ).

**Procedure.** *Phase 1: Training.* Animals were trained to self-administer cocaine under the conditions described in Experiment 2.

*Phase 2: Extinction and testing.* In this experiment the intervening periods, as described above, were 60 min in duration. At test, animals were pretreated with 0 or 640  $\mu\text{g}/\text{kg}$  guanabenz, i.p., before each of two tests for reinstatement (no footshock, footshock or saline, cocaine), given on consecutive days (see Experiment 2). Guanabenz was injected 60 min before the insertion of the lever. Test sessions were 3-h in duration. The dose of guanabenz was chosen on the basis of its substitutability for 40  $\mu\text{g}/\text{kg}$  clonidine in a drug discrimination procedure (Bennett and Lal 1982).

### Statistical Analyses

Microdialysis data were analyzed using a mixed-factor ANOVA for concentration of extracellular NE in 20 min dialysate samples; the between-subjects factor was dose of clonidine (0, 20, 40  $\mu\text{g}/\text{kg}$ ), lofexidine (0, 75, or 150  $\mu\text{g}/\text{kg}$ ), or guanabenz (0 or 640  $\mu\text{g}/\text{kg}$ ) and the repeated measure was time. Significant time by dose interactions were subjected to one-way ANOVAs for the factor of dose at specific time points. When appropriate, post hoc tests (Fisher's LSD,  $p < .05$ ) were conducted to compare vehicle (0  $\mu\text{g}/\text{kg}$ ) to drug conditions.

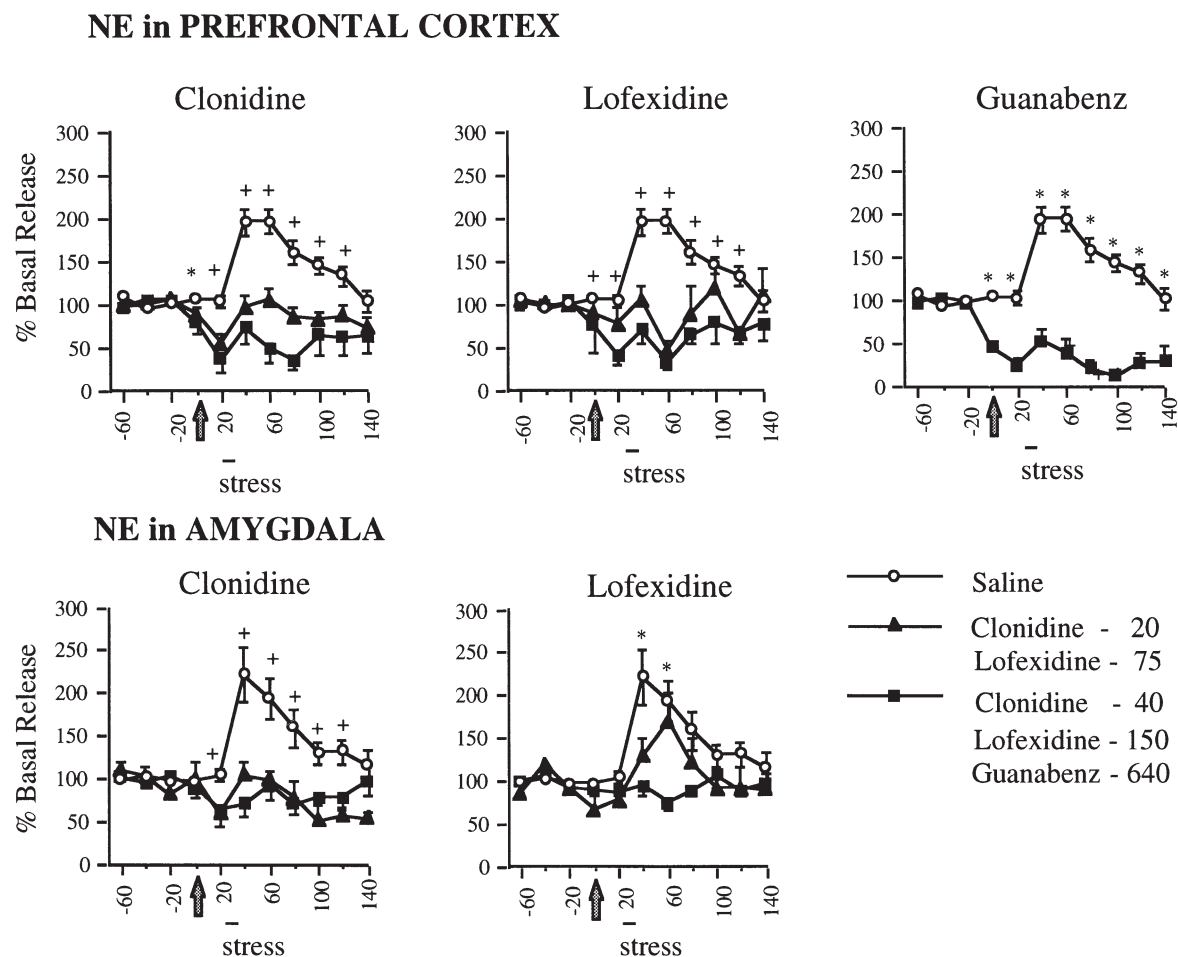
The two dependent measures in the tests for reinstatement were number of responses on the active lever (infusions + time out responses) and number of responses on the inactive lever in three hours. All behavioral data are presented as means  $\pm$  SEM. Because of considerable variability in the number of responses made in the different tests for reinstatement, the non-parametric statistics for related (Friedman and Wilcoxon) and unrelated (Kruskal-Wallis and Mann-Whitney) samples were used where appropriate.

For the sucrose study (Experiment 3A) the dependent measures were the number of responses on the active (reinforced + timeout responses) and inactive levers and the number of reinforcements obtained. Repeated measures ANOVAs were conducted with dose as the within-subject factor. When appropriate, post hoc tests (Fisher's LSD,  $p < .05$ ) were conducted to compare vehicle (0  $\mu\text{g}/\text{kg}$ ) to drug conditions.

## RESULTS

### Experiment 1: Effects of Clonidine, Lofexidine and Guanabenz on Footshock-Induced NE Release

Figure 1 shows basal and footshock-induced levels of NE in PFC (top row) and/or AMG (bottom row) after injection at Time 0 with clonidine (left panel), lofexidine (center panel), or guanabenz (right panel). Mixed-factor ANOVAs revealed significant Time by Dose interac-



**Figure 1.** Microdialysis – Prefrontal cortex and amygdala: Mean ( $\pm$ SEM) NE concentrations in 20 min dialysate samples in prefrontal cortex and amygdala. Animals were injected with either clonidine (0, 20, or 40  $\mu$ g/kg, i.p.), lofexidine (0, 75, or 150  $\mu$ g/kg, i.p.), or guanabenz (0 or 640  $\mu$ g/kg, i.p.) at Time 0, and 40 min later were exposed to 10 min of intermittent foot-shock stress (0.6 mA). Significant Time by Dose interactions were obtained for the prefrontal cortex and amygdala after clonidine ( $F(16,192) = 4.40, p < .01$  and  $F(16,168) = 2.03, p < .03$ , respectively), lofexidine ( $F(16,168) = 5.09, p < .01$  and  $F(16,192) = 2.09, p < .03$ , respectively), and guanabenz ( $F(8,120) = 6.22, p < .001$ ). \* Different from high dose only,  $p < .05$ ; + different from both other doses,  $p < .05$ .

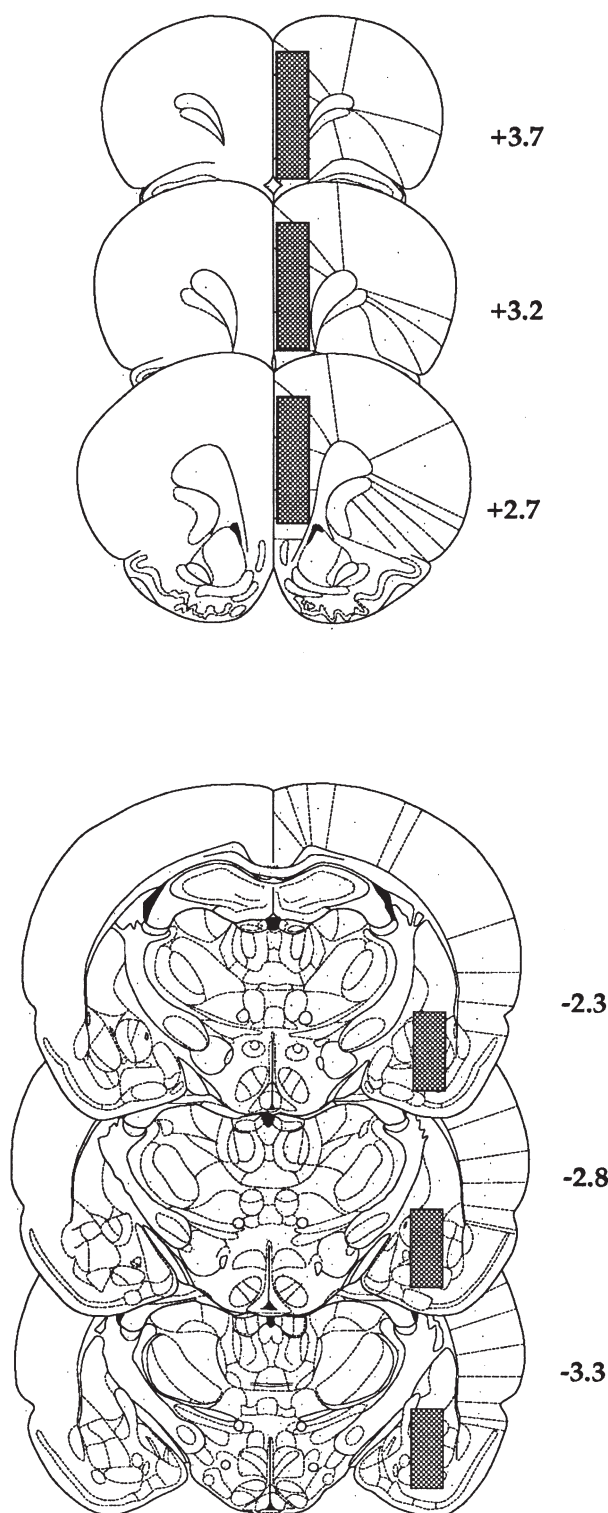
tions in both brain regions and for each of the three drugs. The statistics for each of these interactions are indicated in the figure legend. One-way ANOVAs conducted at specific time points were, when statistically significant, followed by post-hoc comparisons (Fisher's LSD). Significant differences are indicated in Figure 1. See Figure 2 for a diagram showing the areas of the PFC and AMG where the active regions of the dialysis probes were located.

### Experiment 2: Effects of Clonidine on Footshock- and Cocaine-Induced Reinstatement

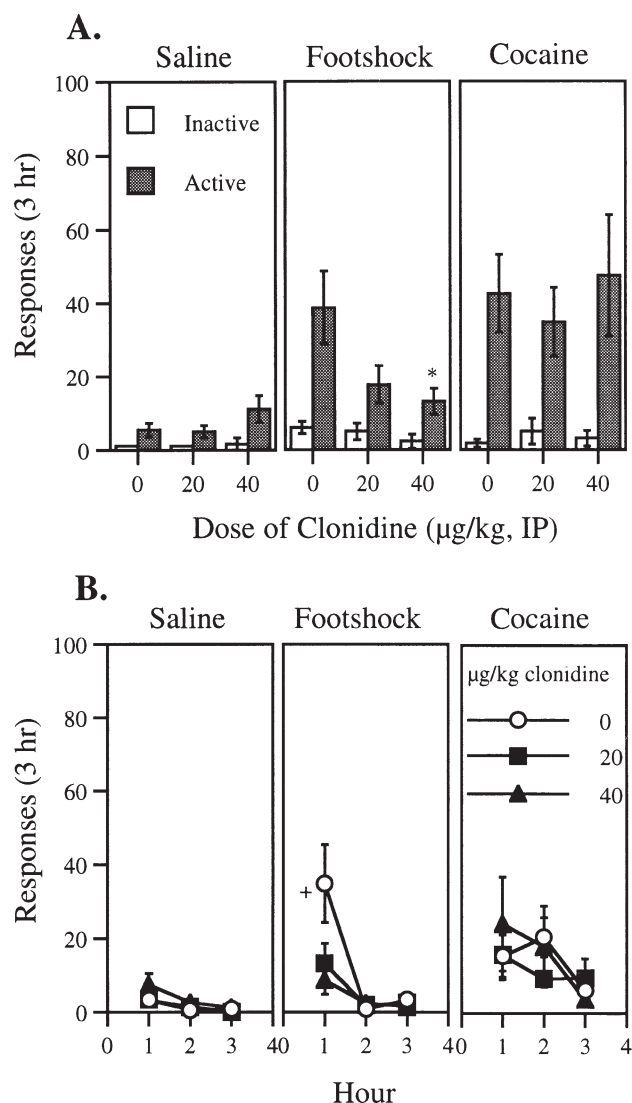
**Training and Extinction.** The mean ( $\pm$ SEM) number of infusions of 0.5 mg/kg cocaine made in the 3-h session on the last two days of training was 32.24 ( $\pm$ 2.77)

and 25.76 ( $\pm$ 2.96), respectively. The mean ( $\pm$ SEM) number of responses (infusions + time out responses) made during the first three 1-h extinction sessions on day 1 of Phase 2, was 28.92 ( $\pm$ 2.81), 13.16 ( $\pm$ 2.03), and 9.68 ( $\pm$ 2.04). There was no difference in the rate of extinction between animals tested in the first four days of Phase 2 and those tested four days later.

**Tests for reinstatement.** The number of responses made on the active and inactive levers during each 3-h test for reinstatement following pretreatment with clonidine is shown in Figure 3A. Figure 3B shows the number of responses on the active lever during each hour of testing. It can be seen that pretreatment with 20 or 40  $\mu$ g/kg clonidine blocked footshock-induced re-



**Figure 2.** Diagrams showing the regions (shaded areas) of the prefrontal cortex (top panel; 46 placements) and amygdala (bottom panel; 40 placements) in which the active region of the dialysis probes were found to be placed after histological analysis. The values of the right of each figure indicated mm from bregma (Paxinos and Watson 1997).



**Figure 3.** Systemic clonidine - reinstatement: **(A)** Mean ( $\pm$ SEM) number of responses (infusions + timeout responses) on the inactive and previously active levers in the three hours after an i.p. injection of saline, exposure to 15 min of intermittent footshock stress (0.6 mA), or an i.p. priming injection of cocaine (20 mg/kg). **(B)** Mean ( $\pm$ SEM) number of responses on the previously active lever in each hour after a saline injection, exposure to footshock stress, or a priming injection of cocaine. Rats were pretreated with vehicle (0) ( $n = 9$ ) or 20 ( $n = 8$ ) or 40 ( $n = 8$ ) µg/kg clonidine, i.p., 40 min before the start of the self-administration session. Lever presses were without consequence during the tests. \* Different from vehicle (0) condition, + different from other doses,  $p < .05$ .

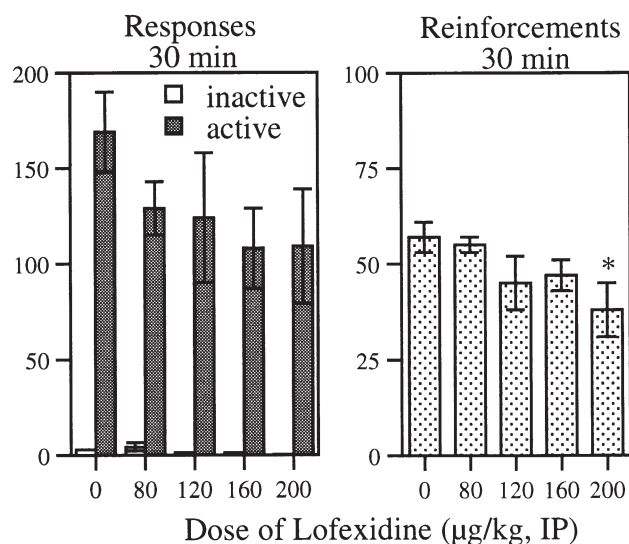
lapse to cocaine seeking, but had no effect on relapse induced by a priming injection of cocaine. Kruskal-Wallis tests conducted at each hour of testing revealed a significant effect of dose of clonidine in the footshock condition in Hour 1, when most of the responding occurred



( $X^2(2) = 6.0, p < .05$ , footshock); both doses of clonidine were effective ( $p < .05$ ). There were no significant effects of clonidine in either the saline or cocaine conditions in Hour 1; no effects were seen in any of the test conditions in Hours 2 or 3. Figure 3A shows that responding on the inactive lever was low under all pretreatment and test conditions. This was the case in all subsequent reinstatement experiments, where there were no significant effects of the number of responses on the inactive lever.

### Experiment 3A: Effects of Lofexidine on Responding for Sucrose

Figure 4 shows the total number of responses (reinforced + time out responses) on the active and inactive levers, and the number of sucrose reinforcements made, following injections of vehicle (0  $\mu\text{g/kg}$ ) or lofexidine. There was no effect of lofexidine on the number of responses on the active lever,  $F(4,28) = 1.79$ , ns. There were, however, dose effects on the number of reinforcements and number of responses on the inactive lever ( $F(4,28) = 2.80, p < .05$  and  $F(4,28) = 2.85, p < .05$ , respectively); this reflects the fact that when compared with the vehicle (0  $\text{g/kg}$ ) condition, animals obtained fewer sucrose reinforcements and made fewer responses on the inactive lever following pretreatment with the highest dose of lofexidine (200  $\mu\text{g/kg}$ ).



**Figure 4.** Systemic lofexidine – responding for sucrose: Mean ( $\pm$ SEM) number of responses on the inactive and active levers 60 min after pretreatment with 0, 80, 120, 160 and 200  $\mu\text{g/kg}$  lofexidine, i.p. ( $n = 8$ ). Responding on the active lever was reinforced with 0.18 ml of a 30% sucrose solution. \* Different from vehicle (0) condition,  $p < .05$ .

### Experiment 3B: Effects of Lofexidine on Footshock- and Cocaine-Induced Reinstatement

**Training and Extinction.** The mean ( $\pm$ SEM) number of cocaine infusions made in the 3-h session on the last two days of training was 30.07 ( $\pm 2.37$ ) and 30.91 ( $\pm 2.06$ ), respectively. On day 1 of extinction, the mean ( $\pm$ SEM) number of responses made in the first three 1-h extinction sessions (infusions + time out responses) was 31.32 ( $\pm 3.22$ ), 16.84 ( $\pm 3.29$ ), and 16.23 ( $\pm 2.35$ ). There was no difference in the rate of extinction between animals tested in the first four days and those tested in the subsequent four days of Phase 2.

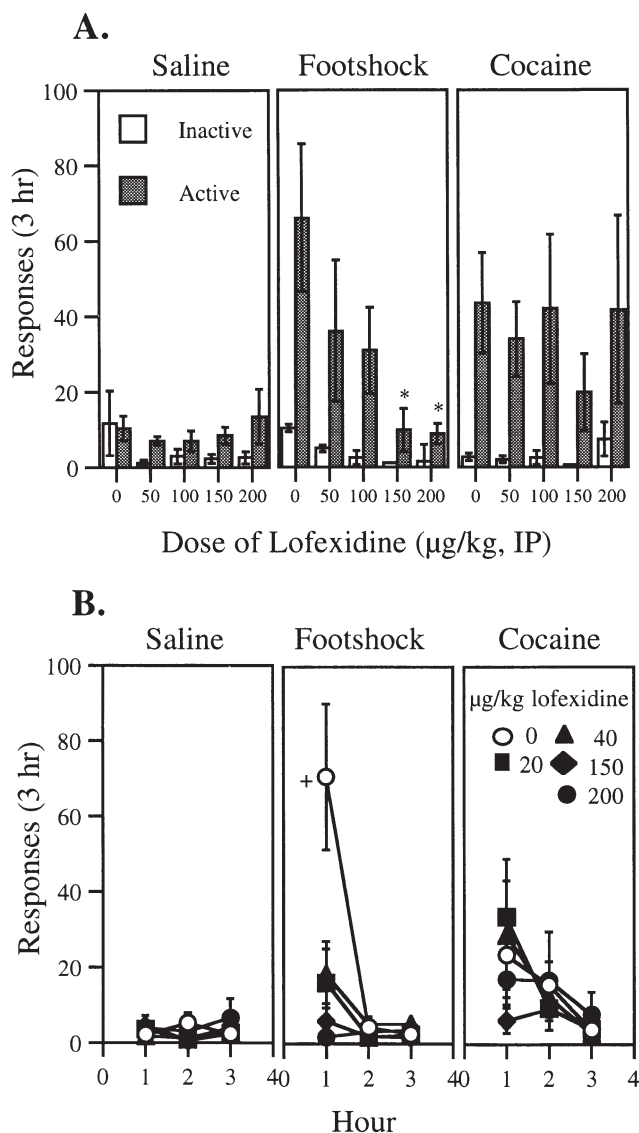
**Tests for Reinstatement.** Figure 5A shows the number of responses made on the active and inactive levers during each 3-h test for reinstatement following pretreatment with vehicle (0  $\mu\text{g/kg}$ ) or lofexidine. Figure 5B shows the number of responses on the active lever during each hour of testing. It can be seen that both footshock stress and priming injections of cocaine induced reinstatement of cocaine seeking behavior. Only the effect of footshock stress was attenuated by lofexidine. This is confirmed by the Kruskal-Wallis tests conducted for the 3-h tests for reinstatement where the only significant effect was found in the footshock condition ( $X^2(4) = 10.50, p < .05$ ). Subsequent Mann-Whitney comparisons revealed a significant difference between the 0 and 150  $\mu\text{g/kg}$  and 0 and 200  $\mu\text{g/kg}$  lofexidine doses ( $ps < .05$ ); furthermore, the response to footshock after 150 and 200  $\mu\text{g/kg}$  doses did not differ from the responses in the saline condition. Separate analyses conducted at each hour of testing revealed a significant effect, again, only in Hour 1 of testing ( $X^2(4) = 17.31, p < .01$ , footshock), when all doses of lofexidine blocked the footshock effect ( $ps < .05$ ).

### Experiment 4: Effects of Guanabenz on Footshock- and Cocaine-Induced Reinstatement

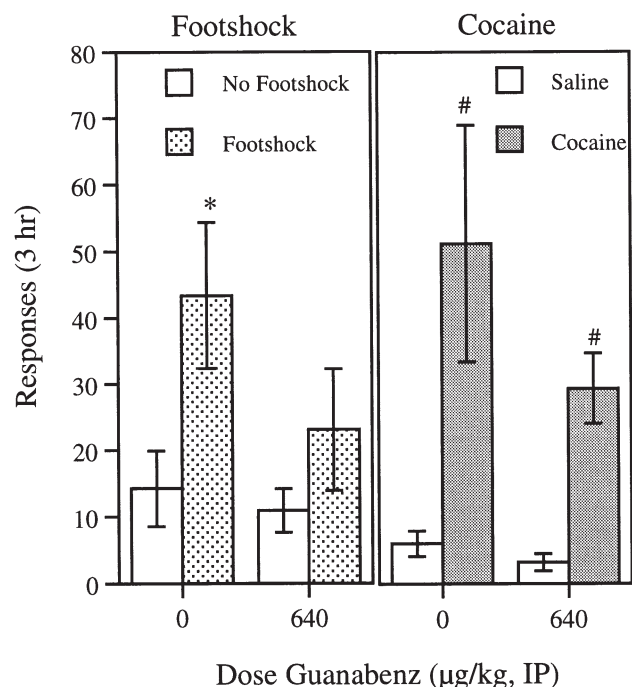
**Training.** The mean ( $\pm$ SEM) number of infusions of cocaine made in the 3-h session on the last two days of training was 36.63 ( $\pm 3.56$ ) and 40.12 ( $\pm 3.65$ ), respectively.

**Tests for Reinstatement.** Figure 6 shows the number of responses made on the active lever in tests for reinstatement after pretreatment with either 0 or 640  $\mu\text{g/kg}$  guanabenz. It can be seen that guanabenz attenuated the effect of footshock stress. A Friedman analysis on the footshock and no footshock tests revealed a significant effect of test condition ( $X^2(3) = 8.36, p < .05$ ) and subsequent comparisons using the Wilcoxon test showed that the footshock (0  $\mu\text{g/kg}$ ) condition differed from the others ( $ps < .05$ ). Inspection of Figure 6 also reveals what appears to be a reduction in responding in the cocaine test following pretreatment with guanabenz. However, a comparison of the scores on the cocaine test using the Mann-Whitney revealed no signifi-





**Figure 5.** Systemic lofexidine – reinstatement: **(A)** Mean ( $\pm$ SEM) number of responses (infusions + timeout responses) on the inactive and previously active levers in the three hours after an i.p. injection of saline, exposure to 15 min of intermittent footshock stress (0.6 mA), or an i.p. priming injection of cocaine (20 mg/kg). **(B)** Mean ( $\pm$ SEM) number of responses (infusions + timeout responses) on the previously active lever in each hour after saline injection, exposure to footshock stress, or a priming injection of cocaine. Rats were pretreated with vehicle (0) ( $n = 9$ ) or 50 ( $n = 11$ ), 100 ( $n = 11$ ), 150 ( $n = 9$ ), or 200 ( $n = 9$ ) µg/kg lofexidine, i.p., 60 min before the start of the self-administration session. Lever presses were without consequence during the tests. \* Different from vehicle (0) condition, + different from other doses,  $p < .05$ .



**Figure 6.** Systemic guanabenz – reinstatement: Mean ( $\pm$ SEM) number of responses (infusions + time out responses) on the previously active lever during 3-hour test sessions (no footshock and footshock; saline and cocaine) after pretreatment with 0 or 640 µg/kg guanabenz. \* Different from all other conditions,  $p < .05$ . # different from saline test condition,  $p < .05$ .

cant difference between the two groups (0 and 640 µg/kg;  $z = 0.73$ ,  $p = .46$ ).

## DISCUSSION

Two major findings emerge from the present series of experiments. First, systemic injections of clonidine and lofexidine attenuate the footshock-induced reinstatement of cocaine seeking, but have no effect on reinstatement induced by priming injections of cocaine. Second, the  $\alpha$ -2 adrenergic receptor agonist, guanabenz, which unlike clonidine and lofexidine has a low affinity for  $I_1$  receptors (Piletz et al. 1994), also attenuates footshock-induced reinstatement. These effects were obtained by drugs that at similar doses and under similar stress parameters to those used in the tests for reinstatement suppressed the footshock-induced increase in NE release in AMG and/or PFC in naïve animals. These findings suggest that reduction of brain NE activity may be an effective way to prevent stress-induced relapse to cocaine seeking.

The finding that clonidine, lofexidine, and guanabenz attenuate footshock-induced but not cocaine-

induced reinstatement was not unexpected in view of our previous findings that stress- and drug-induced relapse can be dissociated pharmacologically. As mentioned, CRF receptor antagonists attenuate footshock-induced, but not drug-induced relapse (Erb et al. 1998; Shaham et al. 1997). From these data it seems logical to consider that footshock stress may have its effects on reinstatement through an interaction between the CRF and NE systems. Although the present data do not bear directly on where in the brain this interaction might take place, we have recent data that do (Erb and Stewart 1999; Shaham et al. 2000). We found first that 6-OHDA lesions of the ventral NE pathway attenuate footshock-induced reinstatement of heroin seeking, whereas injections of clonidine or ST-91 (a less diffusible charged analogue of clonidine) directly into the locus coeruleus were not effective (Shaham et al. 2000). On the basis of this work and additional studies on the site of action of CRF and its receptor antagonist, D-Phe CRF<sub>12-41</sub>, we have proposed that the effects of footshock on relapse may be mediated by the interaction between NE and CRF in the bed nucleus of the stria terminalis (BNST) and/or the central nucleus of the AMG. We have found that local injections of CRF and its receptor antagonist, D-Phe-CRF<sub>12-41</sub>, in the BNST act to induce and block stress-induced reinstatement, respectively (Erb and Stewart 1999). The ventral NE bundle provides the primary source of NE to both the BNST and central nucleus of the AMG (Aston-Jones et al. 1995; Terenzi and Ingram 1995) and both structures are known to contain CRF cell bodies (Phelix and Paull 1990; Veinante et al. 1997); furthermore, the CRF cell bodies of the central nucleus of the AMG project to the BNST (Sakanaka et al. 1986).

Whereas footshock-induced reinstatement may be mediated by the NE and CRF systems, the effects of priming injections of psychostimulant and opioid drugs on relapse to drug seeking have been shown to be mediated by the midbrain dopaminergic system (Self and Nestler 1998; Stewart 1984; Stewart and Vezina 1988). It is interesting, however, that clonidine is known to have effects on the functioning of midbrain dopaminergic neurons (Lategan et al. 1990). For example, it has been shown that systemic injections of *alpha*-2 adrenergic receptor agonists decrease dopamine release in the nucleus accumbens (Murai et al. 1998) and the PFC (Gobert et al. 1998). These effects may be due to the effect of *alpha*-2 adrenergic receptor agonists on dopamine cell firing. Grenhoff and Svensson (1989) have shown that clonidine regularizes midbrain dopamine cell firing in anesthetized rats, an effect that should reduce dopamine release from terminals (Roth et al. 1987). These changes, however, may not have been sufficient to prevent increases in extracellular dopamine in the nucleus accumbens, in the face of cocaine-induced blockade of dopamine reuptake. It is also possible that previous exposure to cocaine during the self-adminis-

tration phase of the study served to increase dopamine availability in the nucleus accumbens, thereby increasing sensitivity to a priming injection of the drug (Kaliyas and Duffy 1993).

The effects of clonidine and lofexidine observed in this study could have been mediated through their actions at I<sub>1</sub> rather than *alpha*-2 receptors. The nM affinity of clonidine for the *alpha*-2 receptor is  $28 \pm 2.6$  and for the I<sub>1</sub> receptor is  $0.99 \pm 0.43$  (Ernsberger et al. 1990); the affinity of lofexidine for these receptors is almost identical (Buccafusco et al. 1995). *Alpha*-2 and I<sub>1</sub> receptors have been shown, however, to be differentially involved in biochemical and behavioral effects of *alpha*-2 adrenergic receptor agonists (for a review see Piletz et al. 1994). For example, in drug discrimination studies, *alpha*-2 receptors appear to be responsible for a clonidine-induced cue, for which both lofexidine and guanabenz (a compound with nM affinity for *alpha*-2 receptor of  $7.2 \pm 0.6$  and for the I<sub>1</sub> receptor of  $> 1,000,000$ ) substitute dose-dependently (Bennett and Lal 1982; Jordan et al. 1993; Lal and Yaden 1985). On the other hand, I<sub>1</sub> receptors of the medulla are thought to mediate the anti-hypertensive actions of these drugs (Buccafusco et al. 1995; but see Guyenet 1997). Although not definitive in view of the fact that guanabenz was not tested independently for rate-reducing effects, it appears unlikely that the attenuation of the footshock-induced reinstatement of cocaine seeking was I<sub>1</sub>-receptor mediated; guanabenz, a drug with a low affinity for the I<sub>1</sub> receptor, attenuated footshock-induced reinstatement of cocaine-seeking at a dose that has been shown to substitute for 40  $\mu$ g/kg clonidine in a drug discrimination task (Bennett and Lal 1982).

Three issues might be seen to complicate the interpretation of the present findings. First, clonidine, lofexidine and guanabenz might affect motor performance (see Monti 1982; Stanford 1995); second, these drugs might act at peripheral rather than central receptors (Buccafusco et al. 1995); and finally, it is possible that the analgesic actions of these drugs (see Codd et al. 1995) mediate their effect on footshock-induced reinstatement. With respect to the first, it is unlikely that the effects were due to a performance deficit. In the saline and cocaine priming conditions, neither clonidine, lofexidine nor guanabenz reduced responding on the active lever. Furthermore, the doses of clonidine and lofexidine used in the tests for reinstatement were chosen because they had little or no effect on high rates of responding for sucrose. After the highest dose of clonidine tested (40  $\mu$ g/kg) in sucrose-trained animals (Shaham et al. 2000), animals responded nearly 100 times in 20 min; likewise, in Experiment 3A, animals injected with the highest dose of lofexidine (200  $\mu$ g/kg) obtained close to 40 reinforcements in 30 min and demonstrated no significant reduction in response rate.

The possibility that the effects observed in the

present study were due to the actions of the *alpha*-2 agonists at peripheral rather than central receptors also seems unlikely. First, we found in a study with heroin-trained rats that i.c.v. injections of clonidine were as effective as systemic injections in blocking the footshock-induced reinstatement (Shaham et al. 2000). Additionally, we have given systemic injections of 40 µg/kg ST-91 (the charged analogue of clonidine which does not effectively cross the blood brain barrier) to cocaine-trained animals and have found that ST-91-treated animals show a comparable footshock-induced reinstatement of responding ( $90.75 \pm 26.37$  in three hours) to vehicle-treated animals ( $115.63 \pm 24.49$  in three hours; unpublished data). It would appear, therefore, that the effects of systemic injections of clonidine on footshock-induced reinstatement of both heroin and cocaine seeking are mediated centrally.

Finally, it seems unlikely that footshock-induced reinstatement of drug seeking is mediated by analgesic effects of these drugs at the doses used. Observations made during the footshock sessions revealed that both vehicle- and drug-pretreated animals reacted similarly to the footshocks throughout the shock period. These observations are consistent with the finding that clonidine, within a similar dose range to the one used in the present study, does not alter threshold sensitivity to footshock (Soderpalm and Engel 1988).

In conclusion, the present findings may have clinical implications. First, the results presented here may provide a rationale for the use of *alpha*-2 adrenergic receptor agonists in treatment programs for the prevention of relapse to drug use. Currently, these drugs are being used with some success in the short-term treatment of opioid withdrawal (Lin et al. 1997; Warner et al. 1997). The present findings suggest that they may be effective in the treatment of cocaine users (McDougle et al. 1994) if given for a more prolonged period (see Herman and O'Brien 1997). Second, the present experiments demonstrate a similar efficacy of clonidine and lofexidine in preventing footshock-induced reinstatement of cocaine seeking. This finding is of potential clinical significance in that lofexidine has been reported in humans to be associated with fewer adverse side effects than clonidine, in particular fewer hypotensive effects (Carnwath and Hardman 1998; Kahn et al. 1997; Lin et al. 1997).

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