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Stress-Induced Relapse to Cocaine Seeking in the Rat: Contributions of Central Nervous System Corticotropin-Releasing Factor and Noradrenaline

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A Thesis in the Department of Psychology

Presented in Partial Fulfillment of the Requirements

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ABSTRACT

Stress-Induced Relapse to Cocaine Seeking in the Rat: Contribution of Central Nervous System Corticotropin-Releasing Factor and Noradrenaline

Suzanne Erb

Concordia University, 2000

The primary objective of the present thesis was to characterize the role of two neurobiological systems, corticotropin-releasing factor (CRF) and noradrenaline (NE), in stress- and cocaine-induced relapse to cocaine seeking, using an animal model of relapse. Rats were allowed to self-administer cocaine (0.5 or 1.0 mg/kg/infusion, i.v.) for 3 hours daily for 10-14 days and were then put on an extinction schedule during which lever pressing was no longer reinforced. Tests for reinstatement were given after intermittent electric footshock (10 or 15 min; 0.5-0.8 mA) and after priming injections of saline and cocaine (20 mg/kg, i.p.).

In a first series of experiments, footshock reinstated cocaine seeking in both intact animals and in animals given corticosterone replacement, but not in adrenalectomized animals. Intracerebroventricular injections of the CRF-receptor antagonist, D-Phe CRF_{12-41}, blocked footshock-induced reinstatement in both intact animals and animals given corticosterone replacement. Reinstatement by priming injections of cocaine was only minimally attenuated by adrenalectomy and by pretreatment with D- Phe CRF_{12-41}. Additionally, systemic injections of the non-peptide CRF-receptor antagonist, CP-154,526, blocked the footshock-induced reinstatement of cocaine seeking. Collectively,

these results demonstrate that CRF acting directly in the brain and independent of the hypothalamic-pituitary-adrenal axis mediates the stress- but not cocaine-induced reinstatement of cocaine seeking. In an attempt to localize where in the brain CRF acts to initiate the behaviors involved in relapse, injections of D- Phe CRF_{12-41} were made into the bed nucleus of the stria terminalis (BNST) and the amygdala (AMG), two sites that contain CRF receptors and that have been implicated in behavioral and physiological effects of stress. Injections into the BNST, but not the AMG, blocked the footshock-induced reinstatement of cocaine seeking and injections of CRF into the BNST, but not the AMG, were sufficient to induce reinstatement. These results suggest that the BNST is an import site of action for CRF in mediating the effects of footshock on relapse.

In a second series of experiments, an important role for NE in stress-induced relapse was demonstrated. Systemic injections of three alpha-2 adrenergic receptor agonists (clonidine, lofexidine, and gunabenz), which act to block NE cell firing and release, blocked footshock-induced relapse but were without effect on relapse induced by priming injections of cocaine. Together with results of the first series of experiments, these data suggest that an interaction between CRF and NE systems, possibly within the extended AMG, underlies the footshock-induced reinstatement of cocaine seeking. Additionally, the findings argue in favor of a view that different neurobiological systems underlie footshock- and drug-induced relapse to drug seeking and that, therefore, different treatment interventions may be required under different circumstances.

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Erb, S., Shaham, Y., & Stewart, J. (1998). The role of corticotropin-releasing factor and corticosterone in stress- and cocaine-induced relapse to cocaine seeking in rats. *Journal of Neuroscience, 18*, 5529-5536.

Erb, S. & Stewart, J. (1999). A role for the bed nucleus of the stria terminalis, but not the amygdala, in the effects of corticotropin-releasing factor on stress-induced reinstatement of cocaine seeking. *Journal of Neuroscience, 19*, RC35, 1-6.

Shaham, Y., Erb, S., Leung, S., Buczek, Y., & Stewart, J. (1998). CP-154,526, a selective, non peptide antagonist of the corticotropin-releasing factor type 1 receptor attenuates stress-induced relapse to drug seeking in cocaine- and heroin-trained rats. *Psychopharmacology, 137*, 184-190.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACTH.....adrenocorticotropin hormone
AMG.....amygdala
ADX.....adrenalectomized
BNST.....bed nucleus of stria terminalis
CeA.....central nucleus of amygdala
CORT.....corticosterone
CORT/PW....corticosterone replacement in pellet and drinking water
CORT/P.....corticosterone replacement in pellet
CP-154,526..... butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo [2,3-d] pyrimidin-4-yl]-ethyl-amine
CRF.....corticotropin-releasing factor
CS.....conditioned stimulus
D1.....dopamine type 1 receptor
D2.....dopamine type 2 receptor
DA.....dopamine
DBH.....dopamine beta-hydroxylase
GAD.....glutamic acid decarboxylase
GABA.....gamma aminobutyric acid
HPA.....hypothalamic-pituitary-adrenal
I_1.....imidazoline type-1 receptor
ICV.....intracerebroventricular
IP.....intraperitoneal
IR.....immunoreactive
IV.....intravenous
LH.....lateral hypothalamus
LC.....locus coeruleus
MH.....medial hypothalamus
Nacc.....nucleus accumbens
NE.....noradrenaline
Pb.....parabrachial nucleus
PVN.....paraventricular nucleus of hypothalamus
RF.....retrorubral field (A8)
SC.....subcutaneous
SHAM.....sham adrenalectomized
US.....unconditioned stimulus
VTA.....ventral tegmental area (A10)

CHAPTER 1

A GENERAL INTRODUCTION

Drug addiction can be conceptualized of as a highly recurrent pattern of drug taking, drug abstinence, and relapse to drug taking. Although the concept of relapse is relevant to a consideration of the mechanisms that maintain drug taking over days, it is the relapse to drug taking that occurs after prolonged periods of abstinence, when drug in the body cannot directly influence desire or motivation for more drug, that remains the most difficult challenge for treatment. Relatively little is known about the specific environmental factors that lead to relapse or about the neurobiological changes that may leave individuals vulnerable to relapse and, as such, relapse remains the primary problem for treatment (McKay, Rutherford, Alterman, Cacciola, & Kaplan, 1995; Stitzer & Cox, 1996).

A major focus of drug addiction research over the past 30 years has been the related roles of tolerance, physical dependence, and withdrawal in addictive behavior. This focus can be attributed, at least in part, to the attention given to the long-term consequences of opioid use. Physical dependence develops after long-term exposure to opioids, as assessed by the symptoms of withdrawal that occur upon discontinuation of drug use. As a result, investigators have hypothesized that it is the avoidance of the aversive physiological and affective consequences of withdrawal (for example, metabolic changes, irritability, and depression) that maintains drug taking and that motivates relapse to drug seeking following a period of abstinence (Siegel, 1979; Siegel, Hinson, Krank, & McCully, 1982; Solomon, 1977; Wikler & Pescor, 1967).

Although a number of withdrawal-avoidance hypotheses of relapse have been advanced, attempts to find a direct and important role for withdrawal in the relapse to

drug taking have been unsuccessful (Stewart, 1992; Stewart, de Wit, & Eikelboom, 1984). In the case of psychostimulant drug abuse, a severe physical withdrawal syndrome characteristic of opioids does not occur when drug taking is terminated. Although cocaine addicts, for example, do experience a withdrawal sometimes characterized by severe anxiety and anhedonic dysphoria, they tend to be able to withstand these symptoms until confronted by reminders of drug-taking and the euphoria induced by drug (Gawin, 1991). Even in the case of opioid abuse, a case in which development of physical dependence clearly occurs, a link between withdrawal and relapse has not been established. For example, the concordance between withdrawal symptoms and subjective reports of craving in opiate addicts is not reliable (Childress, McLellen, Ehrman, & O'Brien, 1986b; Tiffany, 1990). In animal studies as well, attempts to show a relationship between physical symptoms of withdrawal and drug seeking or drug taking of opioids have been unsuccessful. In rats trained to self-administer heroin, for example, a non-contingent injection of the training drug after a period of abstinence induces the reinstatement of drug seeking (e.g., Shaham & Stewart, 1995); however, an injection of naltrexone or naloxone, opioid-receptor antagonists that induce acute withdrawal symptoms in the presence of an opioid drug, is not effective in reinstating heroin seeking, either when given alone (Shaham & Stewart, 1996; Stewart & Wise, 1992) or when given after heroin (Shaham, Rajabi, & Stewart, 1996) or morphine (Shaham & Stewart, 1995).

An alternative to the withdrawal-avoidance view of relapse is one that emphasizes the importance of "drug-like effects" in relapse. Robinson and Berridge (1993), for example, have proposed that the neurobiology of craving and relapse involves drug-

induced changes in neuronal systems that undergo sensitization, resulting in the attribution of *incentive salience*, or attractiveness, to stimuli associated with drug taking. Related to this view is the earlier "proponent process" view (Stewart et al., 1984; Stewart & Wise, 1992) that individuals persist in drug taking or relapse to drug taking because of the pleasant, or even euphoric, state that the drug induces, not because of the unpleasant state that it alleviates (Stewart & de Wit, 1987; Stewart et al., 1984; Stewart & Wise, 1992). In other words, stimuli that serve as reminders of a previously self-administered drug can evoke "drug-like" states that motivate drug seeking and continued drug taking. Strong support for this view comes from studies conducted in laboratory animals and in humans in which a small dose of the previously self-administered drug, or a small dose of a pharmacologically similar drug, induces relapse to drug seeking in rats and monkeys (e.g., Carroll & Comer, 1996; De Vries, Schoffelmeer, Binnekade, Mulder, & Vanderschuren, 1998; de Wit & Stewart, 1981; Gerber & Stretch, 1975; Stretch & Gerber, 1973) and craving for drug in humans (e.g., Jaffe, Cascell, Kumor, & Sherer, 1989; Preston, Sullivan, Strain, & Bigelow, 1992).

EXPOSURE TO DRUG AND RELAPSE TO DRUG SEEKING

One of the basic tenets of the organization *Alcoholics Anonymous*, that consumption of a single alcoholic beverage will greatly increase the probability of a full relapse (Anonymous, 1955), supports the idea that a small amount of drug can prime desire for more drug. In animal studies, the relapse or *reinstatement* of drug seeking can be defined, operationally, as an increase in the number of occurrences of the previously reinforced behavior (e.g., number of lever presses on the previously-reinforced lever) in

response to the presentation of a drug after a period of extinction. Stretch and Gerber (1973) and Gerber and Stretch (1975) provided some of the earliest evidence that a non-contingent priming injection of a drug is able to reinstate previously-reinforced drug taking in animals. These investigators trained squirrel monkeys to self-administer amphetamine. Once drug self-administration had stabilized, the animals were given several days of extinction sessions in which responding for drug resulted in infusions of saline rather than of drug. Following extinction, the monkeys were given non-contingent intravenous (IV) priming injections of amphetamine before the start of subsequent self-administration sessions; throughout these self-administration test sessions, responding continued to result in saline infusions. In both studies, it was found that a non-contingent priming injection of the training drug reinstated drug seeking.

Since these initial reports of relapse to drug seeking in monkeys, the field has developed considerably. Using various procedures, reinstatement of responding for drug by a priming injection of the same drug has been demonstrated in rats trained to self-administer morphine (Davis & Smith, 1976), heroin (e.g., de Wit & Stewart, 1983; Shaham, Rodaros, & Stewart, 1994; Shaham & Stewart, 1995; Shaham & Stewart, 1996), cocaine (e.g., Comer, Lac, Curtis, & Carroll, 1993; de Wit & Stewart, 1981; Erb, Shaham, & Stewart, 1996; Wise, Murray, & Bozarth, 1990; Worley, Valdez, & Schenk, 1994), (Lê et al., 1999; Lê et al., 1998), and nicotine (Chiamulera, Borgo, Falchetto, Valerio, & Tessari, 1996; Shaham, Adamson, Grocki, & Corrigall, 1997a). Furthermore, cross-over priming effects have been demonstrated in which drugs with similar pharmacological properties to the self-administered drug serve as effective stimuli for the reinstatement of drug seeking. For example, in monkeys trained to self-administer

cocaine, priming injections of amphetamine (Gerber & Stretch, 1975; Slikker, Brocco, & Killam, 1984), morphine (Slikker et al., 1984), and codeine (Slikker et al., 1984) have all been shown to be effective in reinstating cocaine seeking. In general, drugs from the same pharmacological class as the self-administered drug, or those affecting similar neurotransmitter systems, appear to function most effectively in priming drug seeking (for additional examples see Carroll & Comer, 1996; Chiamulera et al., 1996; De Vries et al., 1998; de Wit & Stewart, 1981; Shaham et al., 1997a; Stewart et al., 1984; Stewart & Vezina, 1988; Wise et al., 1990; Worley et al., 1994).

Findings from cross-over priming studies with psychostimulant and opioid drugs have led to the interpretation that activation of midbrain dopamine (DA) systems, and possibly sensitization within these systems, mediates reinstatement by both classes of drugs. For example, amphetamine injected into the nucleus accumbens has been found to reinstate heroin seeking (Stewart & Vezina, 1988) and injections of morphine into the ventral tegmental area (VTA) have been found to reinstate cocaine seeking (Stewart et al., 1984); both types of priming injections mimic the effects of the self-administered drugs on the midbrain DA system, pointing to the possibility that reinstatement by stimulant and opioid drugs is related to their ability to cause sensitization within that system (Stewart et al., 1984; Stewart & Vezina, 1988). More recently, De Vries and associates (1998) have shown that in rats trained to self-administer heroin or cocaine, locomotor sensitization is observed to small doses of the same drugs that induce reinstatement, again suggesting a role for sensitization in drug seeking (see also De Vries, Schoffelmeer, Binnekade, & Vanderschuren, 1999). Finally, Tran-Nguyen et al (1998) have demonstrated time-dependent increases in the reinstatement of cocaine seeking

induced by priming injections of cocaine that correlate with cocaine-induced extracellular levels of DA in the amygdala (AMG), a structure implicated in drug reward (Caine, Heinricks, Coffin, & Koob, 1995; McGregor & D.C.S., 1993), conditioned reinforcement (Cador, Robbins, & Everitt, 1989; Everitt, Cador, & Robbins, 1989) and cue-induced reinstatement of cocaine seeking (Meil & See, 1997); rats tested for reinstatement after one month withdrawal from cocaine self-administration exhibited a significantly higher level of cocaine-induced responding than did animals tested after one day or one week of withdrawal (but see Erb et al., 1996) and these behavioral differences were accompanied by corresponding changes in extracellular DA in the AMG. These findings are consistent with the idea that sensitization of DA neurotransmission may play a role in drug seeking.

The validity of drug-induced reinstatement of drug seeking in laboratory animals as a model of relapse is supported by parallel studies conducted in human subjects. Priming injections of an abused drug are associated with increases on measures such as desire for drug, craving for drug, and willingness to work for more drug in cocaine addicts (Breiter et al., 1997; Jaffe et al., 1989; Preston et al., 1992), alcoholics (Bigelow, Griffiths, & Liebson, 1977; Hodgson, Rankin, & Stockwell, 1979; Ludwig & Wikler, 1974), heroin addicts (Meyer & M, 1979), and nicotine addicts (Choronck, Stitzer, Gross, & Leischow, 1992). For example, Jaffe et al (1989) reported that 15 minutes after receiving an IV injection of cocaine, as compared to a placebo, subjects demonstrated a state of *needing* the drug as indicated by increased ratings in response to the question, "how much craving for the drug do you have right now?" and *desire* for the drug as demonstrated by increased ratings in response to the question, "what is the most you would pay for the drug?"; over a subsequent two-hour period, subjects' ratings in

response to these same questions declined to pre-injection levels. In a more recent study, subjects reported peak ratings of cocaine-induced rush and high within five minutes of an IV infusion of cocaine and increased ratings of craving in the first 15 minutes following the infusion; subjective ratings of rush and high correlated closely with increases in fMRI (functional magnetic resonance imaging) signal in putative brain reward circuitry, including the nucleus accumbens, basal forebrain, and VTA, and subjective ratings of craving correlated closely with increases in fMRI signal in the nucleus accumbens (Breiter et al., 1997).

EXPOSURE TO STRESS AND RELAPSE TO DRUG SEEKING

Clearly, events other than re-exposure to drugs can provoke relapse to drug seeking; after a period of abstinence, drug seeking is antecedent to drug taking. In human laboratory studies, it has been shown that exposure to environmental stimuli associated with drug taking can elicit strong craving for drug in abstinent drug users (Childress, Ehrman, McLellan, & O'Brien, 1987; Childress, Ehrman, Rohsenow, Robbins, & O'Brien, 1992; Childress et al., 1993; Childress, Mclellan, & O'Brien, 1986a; Childress et al., 1986b; O'Brien, Ehrman, & Ternes, 1986; O'Brien, Childress, Mclellan, & Ehrman, 1992). Likewise, exposure to drug-related conditioned stimuli has been shown to reinstate drug seeking in rats trained to self-administer cocaine (de Wit & Stewart, 1981; Meil & See, 1997) and morphine (Davis & Smith, 1976), albeit not as effectively as re-exposure to drug itself. Studies such as these raise the possibility that exposure to environmental events contributes to relapse.

One such event that has long been implicated in relapse to drug use in humans is exposure to stress (Kreek & Koob, 1998; Shiffman & Wills, 1985). Recently, the relationship between stress and relapse in humans was studied for the first time in an experimental setting; Sinha and colleagues (1999) reported stress-induced craving for cocaine in addicts using imagery induction procedures to induce psychological stress.

The concept of stress can be defined, in a general way, as an event that produces profound physical or psychological change in an organism by disrupting the organism's normal steady state (Akil & Morano, 1995). These changes are manifest physiologically in a variety of central and autonomic responses (see Sapolsky, 1992). Acute physiological stress responses include noradrenaline (NE) release, which is associated with increased heart rate, blood pressure, and respiration, and activation of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release, and subsequent elevation in plasma blood levels of glucocorticoids. Chronic exposure to stress can induce the suppression of immune responses and can interfere in reproductive systems and the hormonal regulation of growth and development. Physiological changes, both acute and chronic, can be expressed psychologically in the form of, for example, depression, anxiety, and attention deficits. Other factors such as genetic constitution and behavioral 'coping' strategies may influence the interaction between exposure to stress and stress responses.

In the past decade, the effects of acute stress (e.g., intermittent footshock, tail pinch, immobilization, social competition, exposure to novelty) on drug taking and drug seeking behaviors in animals have been the focus of considerable interest. Until relatively recently, however, this relationship had been studied exclusively during the

initiation and maintenance phases. For example, stressors such as footshock, observing another animal being exposed to footshock, tail pinch, and social defeat stress have been found to potentiate the initiation of amphetamine and cocaine self-administration (Goeders & Guerin, 1994; Haney, Maccari, LeMoal, Simon, & Piazza, 1995; Piazza, Deminere, Le Moal, & Simon, 1990; Ramsey & Van Ree, 1993). In addition, social defeat stress has been found to increase the rate of responding on a fixed-ratio schedule for IV cocaine self-administration during the maintenance phase (Miczek & Mutschler, 1996) and footshock stress has been found to increase the breakpoint value for animals self-administering heroin on a progressive ratio schedule of reinforcement (Shaham & Stewart, 1994).

Only in the past several years have the effects of stress on relapse to drug seeking been studied in animals. In an initial study, Shaham and Stewart (Shaham & Stewart, 1995) showed that following 7 to 10 days of extinction in rats that had been trained to self-administer heroin, 10 minutes of exposure to brief intermittent electric footshocks effectively reinstated drug seeking; footshock was still effective in inducing reinstatement after an additional 4- to 6-week drug-free period. In subsequent experiments, the effect of footshock on the reinstatement of heroin seeking was replicated (e.g., Shaham et al., 1996; Shaham & Stewart, 1996) and extended to animals with a history of cocaine (Ahmed & Koob, 1997; Erb et al., 1996; Mantsch & Goeders, 1999), alcohol (Lê et al., 1999; Lê et al., 1998), and nicotine (Buczek, Le, Stewart, & Shaham, 1999) self-administration.

Studies aimed at investigating the relationship between stress and drug taking have tended, in large part, to emphasize common neural pathways underlying the effects

of stress and drugs. Substantial evidence exists to suggest that sensitization occurs in response to acute stress in much the same way as it occurs in response to self-administered drugs, including cocaine, amphetamine, and heroin. In particular, changes in mesocorticolimbic DA transmission appear to underlie both stress-induced and psychostimulant- and opioid-induced sensitization; enduring behavioral sensitization and cross-sensitization between psychostimulants, opioid drugs, and stressors is directly related to augmented DA release in the nucleus accumbens and striatum (see reviews by Kalivas & Stewart, 1991; Sorg & Kalivas, 1993).

In studies examining the relationship between stress and relapse to drug seeking, the initial findings that exposure to intermittent footshock stress reinstated heroin and cocaine seeking as effectively as did priming injections of the self-administered drugs led to the hypothesis that footshock stress induces reinstatement by mimicking the drug effects (Shaham & Stewart, 1995). This hypothesis was soon challenged, however, by the finding that injections of D1- or D2-like DA receptor antagonists, that effectively blocked the reinstatement of heroin seeking induced by a priming injection of the drug, had no effect on the reinstatement of heroin seeking by footshock (Shaham & Stewart, 1996). These findings provided an early indication that the neural pathways mediating footshock- and drug-induced reinstatement are not identical. In subsequent studies, including those reported in this dissertation, the idea that dissociable neurobiological mechanisms mediate footshock- and drug-induced relapse to drug seeking has been substantiated.

THE PRESENT EXPERIMENTS

The experiments presented in this dissertation were conducted within the context of a larger research program aimed primarily at identifying and characterizing the neural pathways involved in stress-induced relapse to drug seeking. To date, studies have been carried out in rats with histories of heroin, cocaine, ethanol, and nicotine self-administration. All of the experiments described here, however, were carried out in rats with a history of cocaine self-administration. Thus, brief consideration of the physiological and psychological effects of cocaine is warranted.

Acute and long-term effects of cocaine

Cocaine is among the most powerful reinforcers known and is considered to be one of the major drugs of abuse. It is a psychostimulant drug that acts principally at NE, serotonin, and DA synapses. The drug inhibits the reuptake of released neurotransmitter, thereby interfering in the critical processes responsible for neurotransmitter inactivation (Wise & Bozarth, 1987; Wise et al., 1995). Although cocaine affects a number of neurotransmitter systems, the reinforcing effects of the drug have been attributed primarily to its effects on DA neurotransmission of the mesocorticolimbic system and, in particular, the pathway originating in the VTA and terminating in the nucleus accumbens (e.g., Wise, 1982b; Wise, 1989; Wise & Colle, 1984; Wise et al., 1995).

In humans, initial exposure to cocaine is typically associated with an elevated subjective sense of well-being, increases in alertness and self-confidence, and decreases in anxiety (Gawin, 1991; Gawin & Ellinwood, 1988; Kumor, Sherer, Gomez, Cone, &

Jaffe, 1989). In the laboratory, monkeys and rats will rapidly learn to press a lever for IV infusions of cocaine; under a continuous schedule of reinforcement, in which each lever press results in an infusion of cocaine, animals will readily adopt a pattern of continuous and steady-state drug taking (Roberts, 1992; Wise & Bozarth, 1987).

The transition from initial to chronic cocaine use is associated with a variety of psychological and behavioral changes, including the sensitization of its reinforcing effects in rats (Kalivas & Duffy, 1993a; Kalivas & Duffy, 1993b; Pettit, Pan, Parsons, & Justice, 1990), and possibly in humans (Holman, 1994), and the emergence of aversive drug effects such as anxiety, both in laboratory animals (Ettenberg & Geist, 1991; Yang, Gorman, Dunn, & Goeders, 1992) and in humans (Anthony, Tien, & Petronis, 1989; Gawin, 1991; Gawin & Ellinwood, 1988). In humans, increased frequency and regularity of cocaine administration is associated with a transition from occasional intermittent use to high-dose bingeing. During binges, all thoughts tend to be focused on the euphoria induced by the drug. The user often withdraws socially, and emergent negative effects of the drug, such as loss of sleep, lack of nourishment, anxiety, and withdrawal from family and friends go unrecognized. In animal studies, rats and monkeys have been reported to exhibit continuous cocaine self-administration (Gerber & Wise, 1989; Roberts, 1992). When given unlimited access to cocaine, rats will in fact self-administer the drug to the point of death. Under these conditions of free access to the drug, rats appear to die not directly from an overdose, but rather from sleep deprivation and weight loss (Bozarth & Wise, 1985).

Objectives of the present experiments

The present experiments were designed with three primary objectives in mind. The first was to assess the roles of CRF, corticosterone, and NE in the reinstatement of cocaine seeking induced by exposure to brief intermittent footshock and by priming injections of cocaine. The second, related objective was to determine the extent to which footshock- and cocaine-induced reinstatement of cocaine seeking can be dissociated pharmacologically. The third objective was to begin to map, functionally, the neural circuitry involved in stress-induced relapse to drug seeking.

GENERAL MATERIALS AND METHODS

Subjects

Subjects were male Long-Evans rats, supplied by Charles River Laboratories (Quebec, Canada). Animals were given free access to food and water throughout the experiments, unless otherwise specified, and were maintained on a reverse light-dark schedule. Other specifications regarding housing conditions are indicated in the individual experiments.

Surgery: IV catheterization

Animals were prepared with IV silastic catheters (Dow Corning, Midland, MI) in the right jugular vein under sodium pentobarbital anesthesia (65 mg/kg, IP; MTC

Pharmaceutical, Cambridge, Ontario, Canada). Just before surgery, animals were given atropine sulfate (0.6 mg/ml; 0.3 ml/animal; MTC Pharmaceutical) and penicillin B (300,00 IU; 0.2 ml/animal; Wyeth-Ayerst, Montreal, Quebec, Canada). Animals were implanted with one of two types of catheters. One consisted of a 12 cm length of silastic tubing (inner diameter 0.51 mm, outer diameter 0.94 mm) with two silicone bulbs placed 3 and 4 cm from one end of the tubing. The second type of catheter consisted of a 3-cm length of silastic tubing (inner diameter 0.30 mm, outer diameter 0.64 mm) connected with heat shrink tubing to a 9-cm length of larger-diameter silastic tubing (inner diameter 0.51 mm, outer diameter 0.94 mm). In both cases, the 3-cm length of tubing was inserted into the vein and silk sutures were tied around the heat shrink tubing to secure the catheter to the vein. The catheter was then passed subcutaneously (SC) to the top of the skull where it exited into a connector (modified 22 gauge cannula: Plastics One, Roanoke, VA) mounted to the skull with jeweler's screws and dental cement. A plastic blocker was placed over the opening of the connector during the recovery period. Following the recovery period, catheters were flushed daily with 0.1 to 0.2 ml of a saline-heparin solution (15 or 30 U/ml heparin; ICN Biochemicals, Montreal, Quebec, Canada).

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, Conn.) 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- or 20-second period in a volume of 65 or 130 µl, respectively. 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 10 or 15 minutes according to a variable time schedule at a mean interval of 40 seconds (10-70 second range). Each shock (0.6 –0.8 mA) was 0.5 seconds in duration.

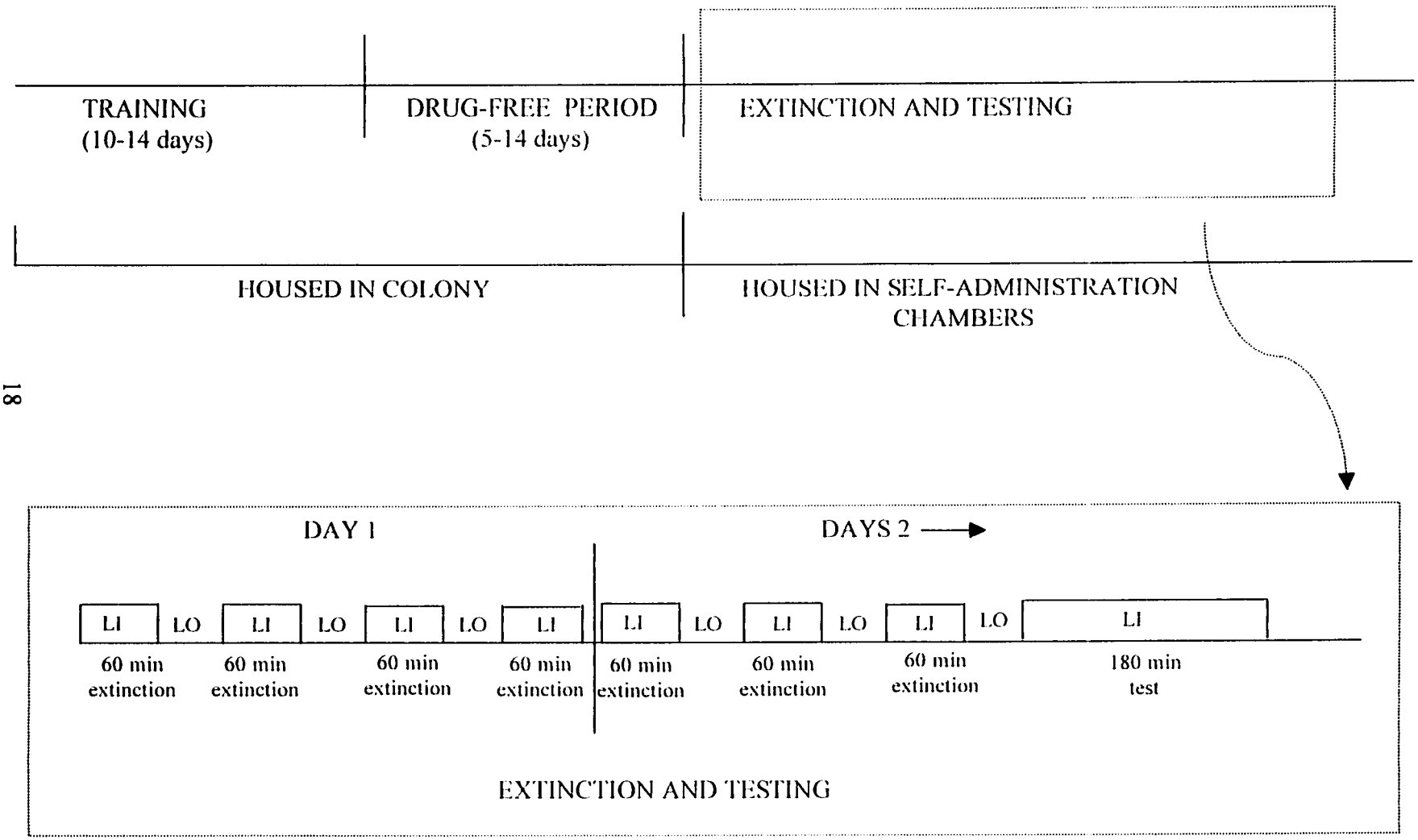
Procedures

Depending on the experiment, one of two reinstatement test procedures was used: a "complete between-days" procedure or a "within-days extinction and testing" procedure. The general procedures are described below; the type of procedure used in each of the experiments is indicated for that experiment.

Complete between-days procedure

A schematic representation of the procedure is presented in Figure 1. Following recovery from surgery, animals were transported to the self-administration chambers where they were housed 24 hours per day for the duration of the experiment. Experiments were conducted in three phases: self-administration training, extinction, and testing for reinstatement. Each day, just before lights were turned off (0900 hr), rats were weighed and the catheters were flushed with heparin. During the training and testing

Figure 1 The "complete between-days" reinstatement procedure. TRAINING: animals are given one 180 minute self-administration session per day; EXTINCTION: animals are given one or two 180 minute extinction sessions per day until a baseline criterion level of responding is reached; TESTING: animals are given one 180 minute test session per day until testing is complete.


TRAINING
(10-14 days)
DRUG-FREE PERIOD
(5-14 days)
EXTINCTION AND TESTING
HOUSED IN COLONY
HOUSED IN SELF-ADMINISTRATION CHAMBERS
DAY 1
DAYS 2
LI
LO
60 min extinction
180 min test
EXTINCTION AND TESTING

phases, animals were allowed to self-administer during one 3-hour session each day (7 days per week), three to four hours after lights off (i.e., 1200 to 1300 hr). During extinction, animals received one or two 3-hour sessions per day; when more than one session was given in a day, the sessions were separated by two hours.

Training. Rats were trained to self-administer cocaine HCl (0.5 or 1.0 mg/kg/infusion, IV) on a fixed-ratio-1 schedule of reinforcement. Animals trained to self-administer 0.5 mg/kg cocaine were allowed a maximum of 50 infusions per session; animals trained to self-administer 1.0 mg/kg cocaine were allowed a maximum of 25 infusions per session. At the beginning of each session, the retractable lever was introduced into the cage. After 10 seconds a red houselight was illuminated and the light just above the active lever was lit for 30 seconds. The house light remained illuminated throughout the session. Responses on the active lever resulted in activation of the infusion pump and illumination of the light above the lever for the drug delivery period. Additional lever presses during this period were recorded, but did not result in reactivation of the pump. Rats were given between 10 and 14 days of training; training conditions continued until stable responding (20% or less variation from day to day) was maintained over at least eight consecutive days.

Extinction. During extinction, all of the conditions present during training were maintained except that lever presses did not result in cocaine infusions. Thus, each session began with introduction of the lever, illumination of the red house light, and illumination of the white stimulus light; responses on the active lever resulted in activation of the infusion pump and illumination of the light above the lever for the

infusion period. These extinction conditions remained in place until rats reached a baseline criterion level of responding of 10 or fewer responses in a 3-hour session. At this point, a few additional extinction sessions were given before which saline injections, of the type to be given in the tests for reinstatement, were administered until animals habituated to the injection procedures and did not exceed 10 responses (saline infusions + time-out responses) in a 3-hour session. Animals were given a minimum of five days of extinction sessions before testing for reinstatement.

Tests for reinstatement. Animals were given one 3-hour test session each day. During testing, extinction conditions were maintained, such that lever presses did not result in drug infusions. All animals were typically given three tests for reinstatement: baseline (saline or no injection), cocaine, and footshock. In the saline baseline condition, animals were given a priming injection (1.0 ml/kg, IP) five minutes before insertion of the lever in the chamber; occasionally, in cases in which a footshock test but no cocaine test was given, a no injection baseline test was used. In the cocaine condition, animals were given a priming injection of 20 mg/kg cocaine (1.0 ml/kg, IP) five minutes before insertion of the lever into the chamber. In the footshock condition, animals were exposed to 10 or 15 minutes of intermittent footshock stress (0.5-0.8 mA; 0.5 seconds on; mean off period of 40 seconds) immediately before lever insertion. The dose for priming injections of cocaine was chosen on the basis of a pilot study and on the basis of a previous study in which it was found to reinstate drug seeking reliably in rats trained to self-administer cocaine (Worley et al., 1994). At this dose, reinstatement of responding does not occur immediately after the injection but it can be shown that responding occurs primarily in the first hour, with some responding in the second hour (see, for example, Fig. 3, *bottom*).

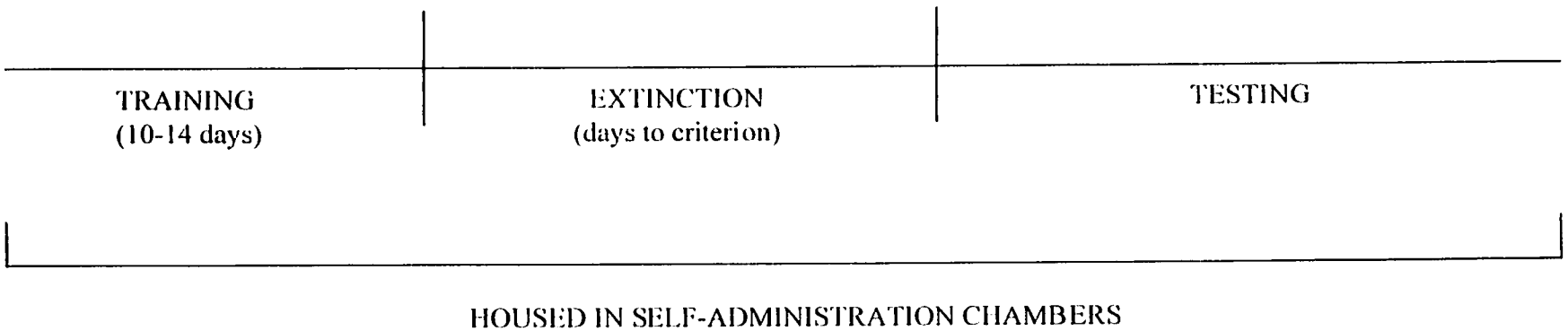
The footshock parameters are the same as those used in a previous study of relapse to cocaine seeking from this laboratory (Erb et al., 1996). All rats received the saline test first; the order of the cocaine and footshock tests was counterbalanced over consecutive days.

Within-day extinction and testing procedure

A schematic representation of the procedure is presented in Figure 2. Experiments were run in three phases: self-administration training, drug-free period, and extinction and testing for reinstatement. In contrast to the conditions of the *complete between-days* procedure, animals were housed in the colony room during training and were transported to the self-administration chambers each day for their 3-hour session. The training phase was followed by a 5- to 14-day drug-free period, during which the animals were left undisturbed in the colony room. Subsequently, animals were returned to the self-administration chambers where they were housed 24 hours per day for the duration of extinction and testing. During this final phase of the experiment, animals were given extinction sessions each day followed by a test for reinstatement; thus, extinction and testing occured *within days*.

Training. Rats were trained to self-administer cocaine under identical conditions to those described above for the complete between-days procedure. As mentioned, however, animals were housed in the colony room and were transported to the self-administration chambers only for the duration of daily sessions. Two squads of animals were run each day. Half of the animals were brought to the chambers for their self-administration session in the morning, approximately three hours after lights off, and half of the animals

Figure 2 The "within-days extinction and testing" reinstatement procedure. TRAINING: animals are given one 180 minute self-administration session per day: DRUG-FREE PERIOD: animals are left undisturbed in the colony room (home cages): EXTINCTION AND TESTING: on Day 1, animals are given four 60 minute extinction sessions with intervening periods, of variable duration, in which the lever is withdrawn. On Day 2 to the end of testing, animals are given 60 minute extinction sessions, until a baseline criterion level of responding is reached, followed by a 180 minute test session. Test manipulations (e.g., injections, exposure to footshock) are given during the intervening period preceding the test session.
LI = lever in chamber
LO = lever out of chamber


TRAINING
(10-14 days)
EXTINCTION
(days to criterion)
TESTING
HOUSED IN SELF-ADMINISTRATION CHAMBERS

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 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 extinction and testing, all animals received extinction sessions in the morning followed by a test session that typically occurred in the afternoon.

Extinction and Testing. . As mentioned, during extinction and testing, animals were housed 24 hours per day in the self-administration chambers until testing was complete. 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 first, for as many days as was necessary to complete testing, and the other group of animals was tested next. In some cases, this meant that the second group of animals had a longer drug-free period than the first group. In other cases, the experiment was arranged so that the second group of animals began training later than the first group; in this case, the duration of the drug-free period was equated for both groups. As in the *complete between-days* procedure, 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.

On Day 1 of extinction and testing, all animals were given four 1-hour self-administration sessions with intervening periods in which the active lever was withdrawn.

On subsequent days, animals were given 1-hour self-administration sessions with intervening periods until all animals responded 15 or fewer times in one hour on the active lever; this criterion was typically reached within two to three sessions. When all animals reached the baseline criterion for that day, a test for reinstatement was given. The duration of the intervening periods was kept constant throughout extinction and testing and corresponded to the time required to complete test manipulations (e.g., pretreatment injections, priming injections, exposure to footshock). At test, all animals were given the baseline (saline or no injection), cocaine and footshock tests for reinstatement described above.

CHAPTER 2

A ROLE FOR CORTICOTROPIN-RELEASING FACTOR IN STRESS-, BUT NOT COCAINE-, INDUCED RELAPSE TO COCAINE SEEKING

In an initial search for neurobiological systems involved in stress- and cocaine-induced relapse to cocaine seeking, a possible role for the hormones CRF and corticosterone was considered. CRF and corticosterone are integral components of the stress response and both also have been strongly implicated in a number of the behavioral and physiological effects of cocaine (see below). It would seem reasonable, therefore, to suspect that CRF and corticosterone may be involved in stress-induced relapse to cocaine seeking and, furthermore, that they may be involved in relapse induced by cocaine itself.

CRF and corticosterone are both hormones of the HPA axis. In response to an acute stressor, CRF is released into the portal vessels of the median eminance from neurosecretory cells originating in the parvocellular division of the paraventricular nucleus of the hypothalamus (PVN). This release of CRF induces release into the general circulation of adrenocorticotropin hormone (ACTH) from the pituitary gland which in turn stimulates the release of glucocorticoids from the adrenal cortex. The primary glucocorticoid in humans is cortisol and its analog in the rat is corticosterone. Glucocorticoids act at receptors in the brain, including the hippocampus, to inhibit further CRF release in the PVN, thereby serving to terminate stress-induced activation of the HPA axis through negative feedback (Herman & Cullinan, 1997; Herman, Prewitt, & Cullinan, 1996; Sapolsky, 1992). Thus, in the absence of glucocorticoid feedback, following removal of the adrenal glands or pharmacological adrenalectomy, CRF immunoreactivity in PHV perikarya and CRF peptide content in the median eminance is enhanced and this enhancement is prevented by corticosterone replacement.

In addition to serving an important hormonal function, CRF also appears to act directly in the brain, independent of the HPA axis, to mediate responses to stress. For example, intracerebroventricular (ICV) or local infusion of CRF in the locus coeruleus (LC) increases cell firing in that region and intra-coerulear infusion of a CRF-receptor antagonist reverses the effect of ICV-administered CRF (Dunn & Berridge, 1990; Valentino, Foote, & Page, 1993). Like CRF, itself, exposure to hemodynamic stress increases cell firing in the LC, an effect that is reversed by ICV or local infusion of CRF-receptor antagonists (Page & Valentino, 1994; Valentino, Page, & Curtis, 1991; Valentino & Wehby, 1988). Similarly, ICV administration of CRF has been shown to enhance the acoustic startle response, both in intact and adrenalectomized rats (Lee, Schulkin, & Davis, 1994), suggesting that the effect is likely mediated via actions of CRF directly in the brain and independent of the HPA axis.

In a series of experiments carried out with Shaham et al (1997b), it was found that ICV administration of the widely-used CRF-receptor antagonist, alpha-helical CRF_{9-41}, attenuated the reinstatement of heroin seeking induced by exposure to intermittent footshock stress. In addition, it was found that ICV administration of CRF given without footshock stress was sufficient, in itself, to induce reinstatement. Furthermore, manipulations of corticosterone, by adrenalectomy or administration of the corticosterone synthesis inhibitor metyrapone, did not affect footshock-induced reinstatement of heroin seeking. Finally, although ICV administration of the CRF-receptor antagonist mildly attenuated the reinstatement of heroin seeking induced by a priming injection of the drug, manipulations of corticosterone were without effect on heroin-induced reinstatement.

Together, these findings suggest that in the case of heroin-trained animals, the effects of CRF on relapse are independent of the HPA axis.

In the case of cocaine, however, there is considerable reason to think that the HPA axis may play an important role in relapse to cocaine seeking. Cocaine, itself, is known to cause a stress-like rise in corticosterone that is mediated by CRF secretion (Sarnyai, Biro, Penke, & Telegdy, 1992). When cocaine is administered intermittently over several days, this response to cocaine is maintained undiminished, as determined by levels of ACTH and corticosterone (Torres & Rivier, 1992). In behavioral studies, it has been found that cocaine-induced locomotion is reduced by adrenalectomy or by corticosterone synthesis inhibitors such as metyrapone (Marinelli et al., 1994; Marinelli et al., 1997a; Marinelli, Rouge-Pont, De Jesus-Oliveira, Le Moal, & Piazza, 1997b). In addition, rats with high basal levels of corticosterone are more likely to initiate cocaine and amphetamine self-administration (Goeders & Guerin, 1996a; Piazza et al., 1996b; Piazza, Maccari, Deminière, LeMoal, & Mormède, 1991), and metyrapone-induced reductions of corticosterone attenuate the intake of cocaine during maintenance of self-administration (Goeders & Guerin, 1996a; Piazza et al., 1994). Finally, there is one report that within a narrow dose range IV injections of corticosterone can reinstate cocaine seeking in intact rats (Deroche, Marinelli, Le Moal, & Piazza, 1997).

Because of their potential importance in the self-administration of cocaine, the roles of both CRF and corticosterone were studied in a series of experiments. To determine the separate roles of CRF and corticosterone on relapse induced by stress or cocaine, the effects of central blockade of CRF receptors and manipulations of

corticosterone using intact and adrenalectomized animals and animals adrenalectomized and given corticosterone replacement were assessed.

EXPERIMENT 1: EFFECTS OF THE CRF-RECEPTOR ANTAGONIST D-PHE CRF_{12-41} ON FOOTSHOCK- AND COCAINE-INDUCED RELAPSE TO COCAINE SEEKING.

This experiment was conducted to determine the effects of central administration of a CRF-receptor antagonist on stress- and cocaine-induced relapse to cocaine seeking. The CRF-receptor antagonist used was D-Phe CRF_{12-41}, a relatively new compound that is effective both behaviorally and physiologically. It has been found, for example, to reduce stress-induced defensive withdrawal (Rodriguez de Fonseca et al., 1996) and Menzaghi and colleagues (Menzaghi et al., 1994a) have found it to be five times more potent than the more commonly used peptide CRF-receptor antagonist, alpha-helical CRF_{9-41}, in reducing CRF-induced locomotor hyperactivity. An advantage of D-Phe CRF_{12-41} is that given alone it has no effects on spontaneous locomotor activity over a wide dose range. In addition, whereas alpha helical CRF_{9-41} has been shown at a high dose (25 μg, ICV) to exhibit weak agonist or stress-like effects, D-Phe CRF_{12-41} is associated with no such effects (Menzaghi et al., 1994a). D-Phe CRF_{12-41} is the antagonist of choice for making behavioral assessments of CRF function and was, for this reason, used in the present experiment.

MATERIALS AND METHODS

Subjects

The subjects were 40 male Long-Evans rats (Charles River laboratories, Canada) weighing 375-450 g at the beginning of the experiment. The animals were housed in a temperature- and humidity-controlled colony room for two to three weeks before surgery and were allowed to recover for at least two weeks before being transferred to the operant chambers where they were housed permanently for the duration of the experiment on a reverse light/dark cycle (lights on from 2100 to 0900).

Surgery

IV catheterization

Animals were prepared with IV catheters as described in the General Methods (Chapter 1).

ICV cannulation

During surgery, each rat was also implanted with a 22 gauge guide cannula (Plastics One) from which the injector extended 1 mm to end in the left lateral ventricle. Stereotaxic coordinates used for the lateral ventricle were as follows: -1.0 mm from bregma, +1.4 mm lateral from the midline, and –3.7 mm from dura (Paxinos & Watson, 1997), measured from the tip of the injector. Obdurators extending 1 mm beyond the tip of the cannula to the injection site were inserted in the cannulae. Cannula placements

were verified by giving each rat an ICV infusion of angiotensin in a volume of 50 ng/2 ml and providing access to a water bottle. Placements were considered to be accurate if a rat drank within one minute of the infusion and sustained drinking for two to three minutes (Sakai, Ma, He, & Fluharty, 1995).

Apparatus

Described in the General Methods (Chapter 1).

Drugs

Cocaine HCl was obtained from BDH Chemicals (Toronto, Canada) and was dissolved in physiological saline. D-Phe CRF_{12-41} (0.1, 0.3, and 1.0 µg/rat), generously supplied by Jean Rivier, Salk Institute, La Jolla, CA, was dissolved in physiological saline and was administered in a 1.0 µl volume.

Procedures

A *complete between-days* reinstatement procedure, described in detail in the General Methods (Chapter 1), was used in this experiment. Briefly, the experiment was conducted in three phases: self-administration training (1.0 mg/kg per infusion cocaine HCl), extinction, and testing for reinstatement. In each phase, the active lever was inserted into the chamber for one 3-hour session per day, during which time lever presses resulted in drug (training phase) or saline (extinction and test phases) infusions. In the

extinction phase of this experiment, animals reached the baseline criterion level of responding of 10 or fewer response in a 3-hour session within 5 to 14 days.

Before testing for reinstatement, animals were assigned to one of four pretreatment conditions: vehicle or 0.1 μg, 0.3 μg, or 1.0 μg of D-Phe CRF_{12-41}, delivered ICV. Injections were given 40 minutes before access to the lever during the tests for reinstatement (30 minutes before onset of footshock and 35 minutes before priming injections). The time interval between infusion and exposure to footshock was chosen on the basis of the work of Menzaghi et al (1994a). Animals in each pretreatment condition were tested on separate days after a priming injection of saline, a priming injection of cocaine, and after exposure to 10 minutes of intermittent footshock stress. See General Methods (Procedures: Chapter 1) for a detailed description of these tests.

Statistical analyses

In this and all subsequent reinstatement experiments described, the dependent measures for the tests for reinstatement were number of responses on the active and inactive levers. Separate analyses were conducted for responding on each lever. Test condition (saline, footshock, cocaine) was a within-subjects factor. The dose of D-Phe CRF_{12-41} (0, 0.1, 0.3, and 1.0 μg) was a between-subjects factor. The data are presented as mean ± SEM. Because of the very large differences in variability in the number of responses made in the different tests for reinstatement, the non-parametric statistics for non-related (Kruskal-Wallis and Mann-Whitney) and related (Wilcoxon) samples were used.

RESULTS AND DISCUSSION

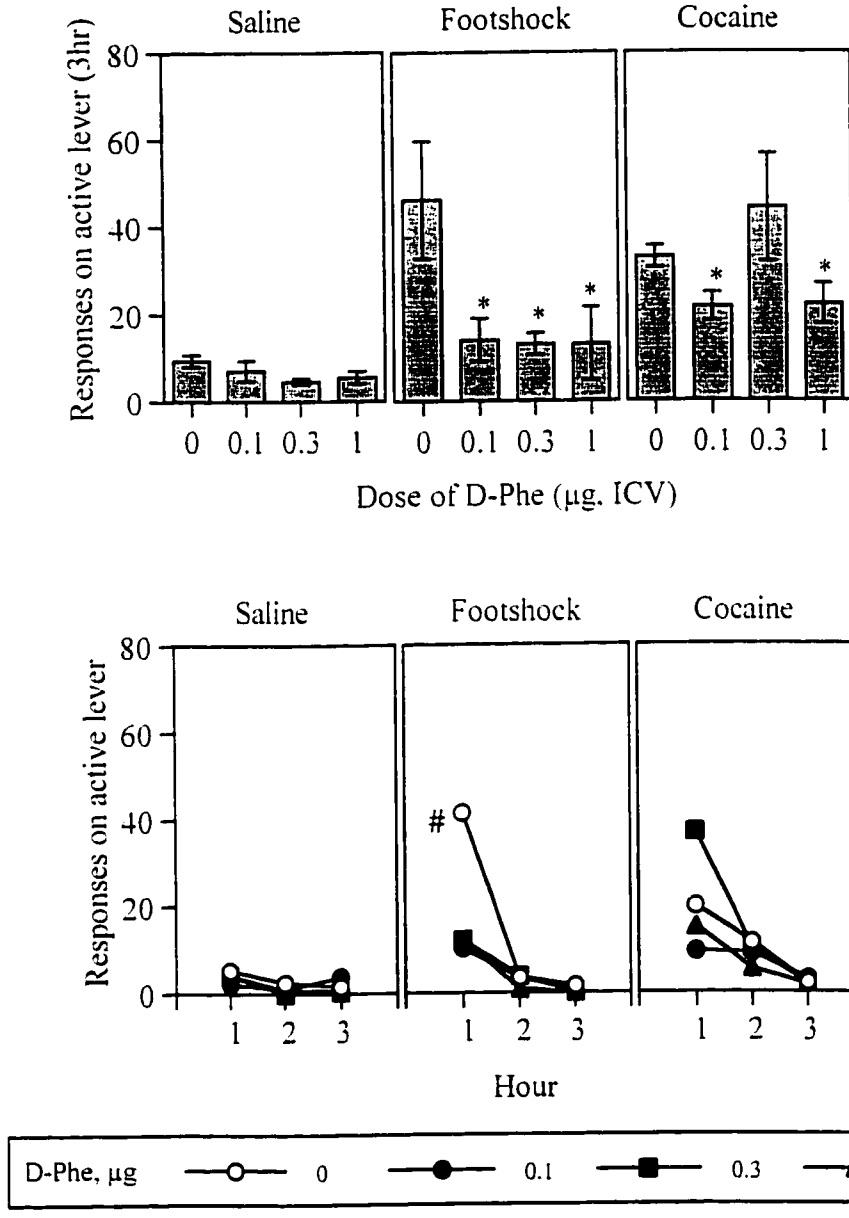
Training and extinction

During training, rats self-administered four to six infusions per hour of cocaine HCl at 1.0 mg/kg per infusion. The mean (±SEM) number of infusions made on the last two days of training were 17.9 (±1.21) and 18.18 (±1.18) infusions per 3-hour session. The mean (±SEM) number of responses (saline infusions + time-out responses) made on the active lever on the first two days of extinction was 40.95 (±6.20) and 43.77 (±6.99). By the last extinction session, all animals had reached the criterion of 10 or fewer responses.

Tests for reinstatement

Figure 3 (*top*) shows the mean number of responses (saline infusions + time-out responses) made on the active lever for each of the D-Phe CRF_{12-41}-dose groups under the three test conditions: *Saline*, *Footshock*, and *Cocaine*. Both footshock stress and priming injections of cocaine reinstated cocaine seeking. Administration of the CRF-receptor antagonist, D-Phe CRF_{12-41}, blocked the footshock effect at all doses tested [Kruskal-Wallis, ($\chi^2[3]=14.03$; $p<0.01$); Fig. 3, *Footshock*]; furthermore, the response to footshock under each of the doses did not differ from the response to saline.

Figure 3 *Intracerebroventricular D-Phe CRF_{12-41}: Top.* The effect of the CRF receptor antagonist, D-Phe CRF_{12-41}, on the mean (±SEM) number of responses (infusions + time-out responses) on the previously active lever in 3-hour tests for reinstatement after *Saline* (physiological saline, 1.0 ml/kg, IP; 5 min before the start of the session), *Footshock* (intermittent footshock, 10 min; 0.5 mA; 0.5 sec on; mean off period of 40 sec; immediately before the start of the session), and *Cocaine* (20 mg/kg, IP; 5 min before the start of the session). Groups of intact animals were tested in each reinstatement test condition under one dose of D-Phe CRF_{12-41} given intracerebroventricularly (0 µg, n=10; 0.1 µg, n=9; 0.3 µg, n=11, and 1.0 µg, n=10). *Bottom.* The effect of D-Phe CRF_{12-41} on the mean number of responses (saline infusions + time-out responses) on the previously active lever in each hour of each of the 3-hour tests for reinstatement.
* Different from the vehicle (0) µg dose of D-Phe CRF_{12-41} in the same test condition; ps<.05.
# Different from other doses of D-Phe CRF_{12-41}, Hour 1, *Footshock* condition; ps<.05.


Saline
Footshock
Cocaine
Responses on active lever (3hr)
80
60
40
20
0
0 0.1 0.3 1 0 0.1 0.3 1 0 0.1 0.3 1
Dose of D-Phe (µg, ICV)
Saline
Footshock
Cocaine
Responses on active lever
80
60
40
20
0
#
1 2 3 1 2 3 1 2 3
Hour
D-Phe, µg
0
0.1
0.3
1.0

Although the effect of dose on reinstatement by the priming injection of cocaine was statistically significant ($X^2[3]=7.65$; $p=0.05$), inspection of Figure 3, *Cocaine*, shows that reinstatement was mildly attenuated at the lowest (0.1 μg) and highest (1.0 μg) doses, but not at the intermediate dose. In all cases, the response to cocaine was significantly greater than the response to saline.

Figure 3 (*bottom*) shows the mean number of responses made on the active lever at each hour of the tests for reinstatement under the various conditions. Kruskal-Wallis tests conducted for dose (0, 0.1, 0.3, and 1.0 μg of D-Phe CRF_{12-41}) in each hour of testing revealed that the only significant dose effect was found in Hour 1 of the footshock test, when animals pretreated with vehicle (0 μg) exhibited a higher level of responding than animals pretreated with the antagonist. Responding by vehicle-pretreated animals extinguished over the second and third hours of testing.

In this Experiment, and in Experiments 2 and 3 below, responding on the inactive lever was minimal (means ranging from 0.62 to 5.31), and in no case was there a significant effect of treatment on the number of responses on this lever.

These data suggest that activation of CRF receptors may be a critical condition for footshock-, but not cocaine-, induced reinstatement of cocaine seeking. Although the findings point, therefore, to a potentially important role for CRF systems in mediating the effects of footshock on relapse, it cannot be determined on the basis of these findings whether the CRF-receptor antagonist D-Phe CRF_{12-41} has its effects by altering function of the HPA axis (that is by interfering in the rise in corticosterone that would normally accompany footshock stress) or whether it has its effects through direct actions in the

brain. Subsequent experiments were conducted, therefore, to attempt to determine how the CRF-receptor antagonist might act to interfere in the effects of footshock on relapse. Experiment 2 was conducted to assess, specifically, what contribution the HPA axis might make to the footshock-, as well as cocaine-, induced reinstatement of cocaine seeking.

EXPERIMENT 2: THE EFFECTS OF ADRENALECTOMY WITH AND WITHOUT CORTICOSTERONE REPLACEMENT ON FOOTSHOCK- AND COCAINE-INDUCED REINSTATEMENT OF COCAINE SEEKING

As discussed previously, there is considerable reason to think that the HPA axis may be important for mediating the effects of footshock and cocaine on relapse to cocaine seeking. In Experiment 2, footshock- and cocaine-induced reinstatement of cocaine seeking was assessed in animals that, following the final day of cocaine self-administration, received a sham surgery, were adrenalectomized, or were adrenalectomized and given corticosterone replacement.

MATERIALS AND METHODS

Subjects

Thirty male Long-Evans rats (375-450 g; Charles River laboratories, Canada), maintained under the conditions described in Experiment 1, served as subjects.

Surgery

Adrenalectomy

Bilateral adrenalectomies were performed rapidly under methoxyflurane anesthesia (Metofane; Jannsen Pharmaceuticals, Mississauga, Ontario, Canada) the day after the last day of training, one to two hours after the start of the light cycle. Adrenalectomized animals were given physiological saline in their drinking bottles. Animals given corticosterone replacement had a pellet containing 50 mg corticosterone (CORT) and 50 mg cholesterol implanted SC at the time of surgery (Meyer, Micco, Stephenson, Krey, & McEwen, 1979), and corticosterone 21-hemisuccinate, in a concentration of 50 μg/ml, was added to the drinking water (CORT/PW) (Marinelli et al., 1994).

Measurement of plasma corticosterone

At the end of the experiment, tail blood was collected in heparanized tubes immediately before and after exposure to 10 minutes of footshock. A radioimmunoassay for plasma corticosterone was conducted to verify that the adrenalectomies were complete. The assays were conducted with the help of Claire-Dominique Walker, Douglas Hospital Research Center, McGill University, using a kit from ICN Biochemicals (Medicorp, Montreal, Quebec, Canada) and ^{125}I-corticosterone as the tracer. The limit of detection was 0.31 μg/dl.

Procedure

A *complete between-days* reinstatement procedure, described in detail in the General Methods (Chapter 1), was used in this experiment. On the day after the final training session (in which animals self-administered 1.0 mg/kg per infusion cocaine HCl), animals were either adrenalectomized (ADX), adrenalectomized and given corticosterone replacement (CORT/PW), or given a sham surgery (SHAM). The animals were allowed 36 hours to recover from surgery before the start of the first extinction session. Extinction proceeded under conditions similar to those of Experiment 1. In the extinction phase, animals reached the baseline criterion level of responding of 10 or fewer responses (infusions + time-out responses) in a 3-hour session within 5 to 14 days. In this experiment animals received two cocaine and two footshock tests, preceded by one saline test. Cocaine and footshock tests were given on alternate days with some animals receiving a cocaine test first and others receiving a footshock test first. See the General Methods (Procedures: Chapter 1) for a detailed description of the test conditions.

Statistical analyses

Test condition (saline, footshock, cocaine) was a within-subjects factor; surgery condition (SHAM, ADX, and CORT/PW) was a between-subjects factor. As in Experiment 1, the data are presented as mean ± SEM and were analyzed using the non-parametric statistics for non-related (Kruskal-Wallis and Mann-Whitney) and related (Wilcoxon) samples.

RESULTS AND DISCUSSION

Verification of adrenalectomy and corticosterone replacement.

Plasma corticosterone levels (μg/dl) for animals in the different groups are presented in Table 1 (p. 49). Analyses were done in combination with data obtained from samples in Experiment 3 (see Experiment 3 below).

Training

The mean (±SEM) number of infusions made on the last two days of training were 20.9 (±0.96) and 20.5 (±1.08) infusions per 3-hour session. The mean (±SEM) number of responses (saline infusions + time-out responses) made on the active lever on the first two days of extinction were 40.95 (±6.20) and 43.77 (±6.99). By the last extinction session, all animals had reached the criterion of 10 or fewer responses.

Extinction

Because of the possible effects of adrenalectomy and corticosterone replacement on responding during extinction, data for the three groups (SHAM, ADX, and CORT/PW) were subjected to one-way ANOVAs for the number of days to criterion (mean ± SEM, 7.78 ±0.86; 6.73 ± 0.36; and 8.17 ± 0.4, respectively) and for the number of responses made on the first two days of extinction (SHAM, 46.11 ± 10.88 and 23.11 ± 4.17; ADX, 29.36 ± 4.63 and 35.09 ±7.68; and CORT/PW, 53.00 ± 16.78 and 28.50 ±

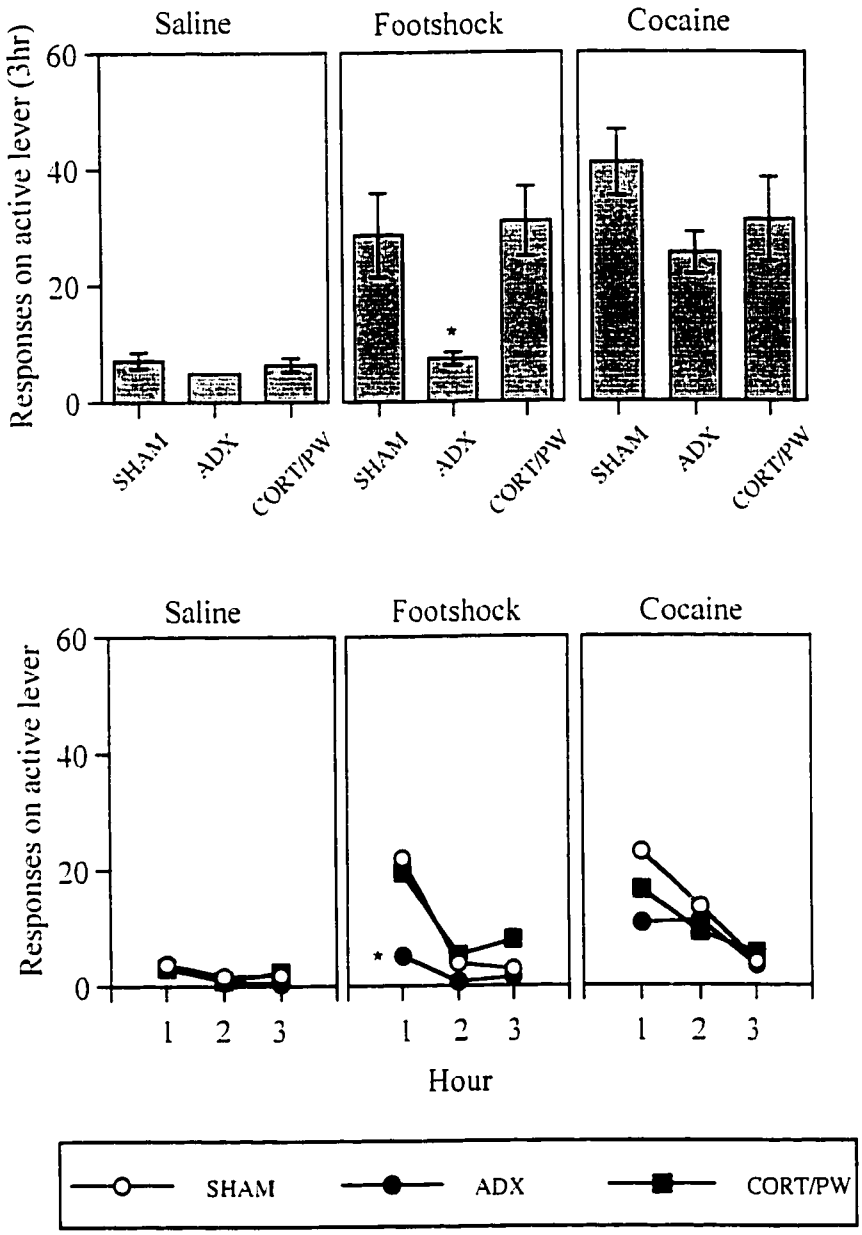
10.71). No significant differences between groups were found (X^2[2]=1.79; p=0.41). By the last extinction session, all animals had reached the criterion of 10 or fewer responses.

Tests for reinstatement

Figure 4 (*top*) shows the number of responses (mean of two tests) made on the active lever (saline infusions + time-out responses) for SHAM, ADX, and CORT/PW animals under the *Saline*, *Footshock*, and *Cocaine* test conditions. Both footshock and priming injections of cocaine reinstated cocaine seeking in SHAM and CORT/PW animals. Footshock, however, did not reinstate cocaine seeking in ADX animals. Kruskal-Wallis analyses for the factor of group (SHAM, ADX, and CORT/PW) performed for each of the test conditions, *Saline*, *Footshock*, and *Cocaine*, revealed a significant effect of group only in the *Footshock* test condition (X^2[2]=10.53; p<0.05); the effect of group was not significant in either the *Cocaine* (X^2[2]=3.67; p=0.16) or *Saline* (X^2[2]=2.08; p=0.35) test conditions.

Figure 4 (*bottom*) shows the mean number of responses made on the active lever for each group at each hour of testing and under the various test conditions. The patterns of responding over the 3-hour test sessions were very similar to those observed in Experiment 1. In the *Footshock* test condition, most of the responding was in the first hour; in the *Cocaine* test condition, responding occurred in both the first and second hours. Kruskal-Wallis analyses for the factor of group (SHAM, ADX, and CORT/PW) performed for each of the test conditions at each hour of testing revealed significant

Figure 4 *Adrenalectomy and corticosterone replacement: Top.* The effect of adrenalectomy (ADX; n=11), corticosterone replacement (CORT/PW; n=9), and sham surgery (SHAM; n=10) on the mean (±SEM) number of responses (infusions + time-out responses) on the previously active lever in the 3-hour tests for reinstatement after *Saline* (physiological saline, 1.0 ml/kg, IP; 5 min before the start of the session), *Footshock* (intermittent footshock, 10 min; 0.5 mA; 0.5 sec on; mean off period of 40 sec; immediately before the start of the session), and *Cocaine* (20 mg/kg, IP; 5 min before the start of the session). Animals in each group (SHAM, ADX, and CORT/PW) were tested under all three test conditions. *Bottom.* The effect of adrenalectomy (ADX; n=11), corticosterone replacement (CORT/PW; n=9), and sham surgery (SHAM; n=10) on the mean number of responses (saline infusion + time-out responses) on the previously active lever in each hour of the 3-hour tests for reinstatement. *Different from SHAM and CORT/PW in the *Footshock* condition: ps<.05.


Saline
Footshock
Cocaine
Responses on active lever (3hr)
60
40
20
0
SHAM
ADX
CORT/PW
Responses on active lever
1
2
3
Hour
SHAM
ADX
CORT/PW

effects in both Hour 1 ($X^2[2]=7.64$, $p<0.03$) and Hour 2 ($X^2[2]=9.39$, $p<0.03$) of the *Footshock* test.

These results suggest that although a basal level of corticosterone is necessary for the footshock-induced reinstatement of cocaine seeking, a stress-induced rise in corticosterone is not necessary (i.e., footshock was effective in CORT/PW but not ADX animals). In the case of cocaine-induced reinstatement of cocaine seeking, on the other hand, the data suggest that neither basal nor stress-induced rises in corticosterone are involved in mediating the effects of priming injections of cocaine on relapse. Furthermore, the results of this experiment suggest that the effects of the CRF-receptor antagonist in Experiment 1 were likely due to the antagonist's direct actions in the brain. Experiment 3 was conducted to assess this conclusion directly.

EXPERIMENT 3: EFFECTS OF D-PHE CRF_{12-41} ON FOOTSHOCK-INDUCED REINSTATEMENT OF COCAINE SEEKING IN ADRENALECTOMIZED ANIMALS GIVEN CORTICOSTERONE REPLACEMENT

In Experiment 1 it was found that ICV administration of the CRF-receptor antagonist, D-Phe CRF_{12-41}, blocked the footshock-induced reinstatement of cocaine seeking. In Experiment 2 it was found that adrenalectomy also blocked the footshock-induced reinstatement of cocaine seeking, a result that by itself would seem to suggest an important role for the HPA axis in mediating the effects of footshock on relapse in cocaine-trained animals. When adrenalectomized animals were given back basal levels of corticosterone (i.e., group CORT/PW), however, the effect of adrenalectomy was

completely reversed. Thus, although footshock-induced reinstatement of cocaine seeking appears to be dependent on the presence of basal levels of corticosterone, a stress-induced rise in corticosterone is not necessary. On the basis of Experiments 1 and 2, together, it would seem reasonable to hypothesize that ICV administration of D-Phe CRF_{12-41} should be effective in blocking the footshock-induced reinstatement of cocaine seeking not only in intact animals but also in adrenalectomized animals given corticosterone replacement. Experiment 3 was conducted to test this hypothesis. Adrenalectomized animals given corticosterone pellets sufficient to maintain steady plasma levels of corticosterone were tested under footshock and no footshock test conditions following pretreatment with vehicle and D-Phe CRF_{12-41}.

MATERIALS AND METHODS

Subjects

Five male Long-Evans rats (375-450 g; Charles River laboratories, Canada), maintained under the conditions described in Experiment 1, served as subjects.

Surgery

Adrenalectomy

All animals received bilateral adrenalectomies and were given corticosterone replacement in the form of a pellet implanted SC (CORT/P) The adrenalectomy and hormone replacement procedures are described in Experiment 2. Following adrenalectomy, animals were given physiological saline in their drinking bottles.

Measurement of plasma corticosterone

Plasma corticosterone was collected and assayed in the same manner described in Experiment 2.

Procedure

Self-administration training (1.0 mg/kg per infusion cocaine HCl) and extinction phases were conducted as described in Experiment 2. On the day after the final training session, animals were adrenalectomized and received corticosterone replacement pellets (CORT/P). Extinction, which was conducted under conditions similar to those of Experiment 1, ranged from 6 to 10 days to criterion. In the reinstatement test phase, all animals were tested in both footshock and no footshock conditions after pretreatment with both D-Phe CRF_{12-41} (1.0 μg, ICV) and vehicle (ICV). Thus, animals received a total of four tests for reinstatement: D-Phe CRF_{12-41} + footshock; vehicle + footshock; D-Phe CRF_{12-41} + no footshock; and vehicle + no footshock. See General Methods (Procedure: Chapter 1) for a description of the footshock and no footshock test conditions. Three animals received D-Phe CRF_{12-41} pretreatment first, and two received vehicle pretreatment first; within each pretreatment condition, the no footshock test preceded the footshock test. The timing of the ICV infusion of D-Phe CRF_{12-41} was as described in Experiment 1.

Statistical analyses

Test condition (no footshock, footshock) and pretreatment condition (vehicle, D-Phe CRF_{12-41}) were within-subjects factors. Data were analyzed using the non-parametric statistics for related samples (Friedman, Wilcoxon).

RESULTS AND DISCUSSION

Verification of adrenalectomy and corticosterone replacement

The concentration (μg/dl) of corticosterone in plasma samples collected from rats before and after footshock (10 minutes; intermittent; 0.5 mA) was determined for the different groups of Experiments 2 and 3. Two animals showing significant increases in corticosterone in response to stress, indicative of incomplete removal of the adrenals, were excluded from the study. Plasma corticosterone concentrations for SHAM, ADX, and CORT/PW and CORT/P animals are shown in Table 1. The number of animals in each of the two CORT groups is reduced by one because of the loss of a single "before" or "after" sample. A two-way ANOVA for group by time (before and after stress) showed that there was a significant group effect ($F[3,29]=36.17$; $p<0.001$); SHAM animals had higher basal and stress levels of corticosterone than did all other groups. The levels in ADX animals were minimal and significantly different from those in all other groups. The significant group by time interaction ($F[3,29]=3.0$; $p<.05$) reflects the fact that only in the SHAM group was there an increase in levels in response to stress.

Table 1. Mean (±SEM) plasma corticosterone levels (μg/dl) immediately before and after 10 minutes of intermittent footshock stress

Group	Before	After
SHAM (n=10; Exp. 2)	12.12 ± 1.45	16.38 ± 1.44
ADX (n=11; Exp. 2)	0.73 ± 0.38	0.82 ± 0.51
CORT/PW (n=8; Exp. 2)	9.27 ± 1.25	8.08 ± 1.40
CORT/P (n=4; Exp. 3)	6.92 ± 2.78	6.33 ± 2.41

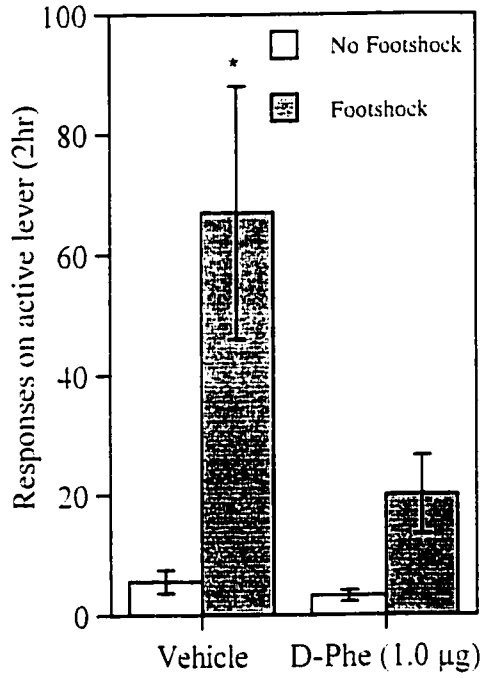
Training and extinction

The mean (±SEM) numbers of infusions made on the last two days of training was, respectively, 16.4 (±3.2) and 17.0 (±3.1) infusions in each 3-hour session. The mean (±SEM) numbers of responses (saline infusions + time-out responses) made on the active lever on the first two days of extinction was 32.80 ±5.99 and 25.40 ± 2.77. By the last extinction session, all animals had reached the criterion of 10 or fewer responses.

Tests for reinstatement

Figure 5 shows the mean number of responses made on the active lever (saline infusions + time-out responses) by CORT/P animals during *No Footshock* and *Footshock* test sessions after pretreatment with vehicle or D-Phe CRF_{12-41}. Pretreatment with D-Phe CRF_{12-41} greatly attenuated the footshock-induced reinstatement. Although in both pretreatment conditions there was an increase in responding on the active lever after footshock (Wilcoxon, $p<0.05$), the level of responding in response to footshock after vehicle was significantly greater than that after D-Phe CRF_{12-41} pretreatment (Wilcoxon, $p<0.05$). There was no difference in responding between pretreatment conditions in the absence of footshock ($p=0.22$). As in Experiments 1 and 2, the greatest number of responses after footshock occurred in the first hour of the sessions. It can be noted that the level of responding in the vehicle condition in this experiment was higher than that in similarly treated animals in Experiment 2. This difference probably reflects the fact that there were fewer animals in Experiment 3 and a higher degree of variability.

Figure 5 *Intracerebroventricular D-Phe CRF_{12-41} and corticosterone replacement:* Effect of D-Phe CRF_{12-41} in adrenalectomized animals with corticosterone replacement (CORT/P; n=5) on the mean (±SEM) number of responses on the previously active lever (infusions + time-out responses) in the 2-hour tests for reinstatement after *No Footshock* and *Footshock* (intermittent footshock, 10 min; 0.5 mA; 0.5 sec on; mean off period of 40 sec; immediately before the start of the session). All animals were tested after pretreatment with vehicle and with D-Phe CRF_{12-41} given intracerebroventricularly 40 minutes before the the *No Footshock* and *Footshock* tests. The number of responses in the *Footshock* tests was greater than that in the *No Footshock* tests with both vehicle and D-Phe CRF_{12-41} (Wilcoxon, $p<.05$).
* Different from other conditions: $ps<.05$.


100
80
60
40
20
0
Responses on active lever (2hr)
No Footshock
Footshock
*
Vehicle
D-Phe (1.0 µg)

As predicted, ICV administration of D-Phe CRF_{12-41} strongly attenuated the footshock-induced reinstatement of cocaine seeking in adrenalectomized animals given corticosterone replacement. This result offers direct support for the hypothesis that CRF-receptor antagonists act directly in the brain, independent of the HPA axis, to interfere in the reinstatement of cocaine seeking by footshock stress. Put differently, this result offers direct support for the hypothesis that activation of CRF receptors directly in the brain mediates the footshock-induced reinstatement of cocaine seeking.

Although the findings discussed so far clearly point to a neurotransmitter role for CRF in mediating the effects of footshock on relapse, an obvious question that arises concerns where in the brain the CRF-receptor antagonist, D-Phe CRF_{12-41}, acts to block footshock-induced reinstatement of drug seeking. Experiment 4 was conducted to address this question.

EXPERIMENT 4: EFFECTS OF INJECTIONS OF D-PHE CRF_{12-41} INTO THE BNST AND AMG ON THE FOOTSHOCK-INDUCED REINSTATEMENT OF COCAINE SEEKING

Two brain sites that are likely to be involved in the effects of ICV-administered D-Phe CRF_{12-41} on footshock-induced reinstatement of cocaine seeking are the bed nucleus of the stria terminalis (BNST) and the AMG. Both of these sites contain CRF receptors (Chalmers, Lovenberg, & De Souza, 1995; Potter et al., 1994) and both have been implicated in a number of physiological and behavioral responses to stress (see

Chalmers et al., 1995; Lee & Davis, 1997; Makino et al., 1994a; Makino et al., 1994b; Potter et al., 1994).

Although initially evidence pointed to the AMG as the primary site of action of CRF in emotional behaviors, Davis and colleagues (Gewirtz, McNish, & Davis, 1998; Lee & Davis, 1997) have shown dissociations between the behavioral effects of CRF in the BNST and AMG. Importantly for the experiments discussed in this chapter, they have reported a role for the BNST, and not the AMG, in CRF-induced enhancement of the acoustic startle reflex (Lee & Davis, 1997), suggesting that the actions of CRF in this region may be important in unconditioned anxiety, perhaps acting to sensitize the emotional response to startle. On the basis of these and other findings they have argued as well that the AMG may be preferentially involved in mediating conditioned emotional responses (Gewirtz et al., 1998; Walker & Davis, 1997).

In light of these arguments and because reinstatement of drug seeking can be induced by footshock stress in animals exposed to it for the first time, it seemed reasonable to hypothesize that the BNST and not the AMG would play a primary role in mediating the effects of CRF-receptor antagonists in the stress-induced relapse to cocaine seeking. Thus in Experiment 4, the effects of local BNST and AMG injections of the CRF-receptor antagonist, D-Phe CRF_{12-41}, on the footshock-induced reinstatement of cocaine seeking were assessed in intact animals. In addition, the ability of intra-BNST and intra-AMG injections of CRF, itself, to induce relapse was assessed.

MATERIALS AND METHODS

Subjects

Sixty-nine male Long-Evans rats (350-450 g; Charles River laboratories, Canada) served as subjects. Animals were housed in a colony room (described in Experiment 1) until the time of extinction and testing. Throughout the experiment, animals were maintained on a reverse light-dark cycle (lights on to 1730 to 0530 hr).

Surgery

Intracranial cannulation

At the time of IV catheterization, animals were implanted bilaterally with cannulae (22 gauge; Plastic Products) aimed 2 mm above either the BNST or the AMG. Coordinates for entry of cannulae from the skull surface (midline at bregma) were taken from the atlas of Paxinos and Watson (Paxinos & Watson, 1997). Cannulae were aimed to inject into the ventrolateral division of the BNST or the intersection of the central and basolateral nuclei of the AMG. BNST coordinates (arms positioned at 15 degrees) were: A/P: -0.6; M/L: +3.7; D/V: -4.6 mm; AMG coordinates (arms positioned at 0 degrees) were: A/P: -2.4; M/L: +4.7; D/V: -5.4 mm. Obdurators extending 2 mm beyond the tip of the cannula to the injection site were inserted in the cannulae.

Drugs

D-Phe CRF_{12-41} was purchased from Bachem (Torrance, California); rat/human CRF was purchased from Sigma (Oakville, Ontario). D-Phe CRF_{12-41} (10, 50, and 500

ng) and CRF (100 and 300 ng) were dissolved in 0.5 µl saline or distilled water, respectively.

Procedure

A *within-days extinction and testing* reinstatement procedure, described in detail in the General Methods (Procedures; Chapter 1), was used in this experiment. Briefly, the experiment was conducted in three phases: self-administration training (0.5 mg/kg per infusion cocaine HCl), drug-free period, and extinction and testing for reinstatement. During training and testing sessions, the active lever was inserted into the chambers for one 3-hour session per day, during which time lever presses resulted in drug infusions (training phase) or were without consequence (test phase). During extinction sessions, the active lever was inserted into the chambers for several 1-hour sessions each day, during which time lever presses were without consequence. Between the training and extinction/testing phases, animals were left undisturbed for five days in the colony room.

Tests for reinstatement following intra-BNST or -AMG injections of D-Phe CRF$_{12-41}$

On Day 1 of the extinction and testing phase, animals were given four 60-minute self-administration sessions with 60-minute intervening periods during which the active lever was withdrawn. On Days 2 and 3, when testing occurred, animals were given 60-minute self-administration sessions with 60-minute intervening periods until they made 15 or fewer responses on the active lever in 60 minutes; this criterion was reached within

two to three sessions. When all animals reached the baseline criterion for that day, a test for reinstatement was given.

Two tests for reinstatement (no footshock, footshock) were given on consecutive days and in a counterbalanced order. Animals were pretreated with D-Phe CRF_{12-41} either in the BNST (0, 10, or 50 ng/side) or in the AMG (0, 50 or 500 ng/side) 30 minutes before insertion of the active lever into the chamber. Different groups of animals were assigned to different doses of the antagonist. The doses of D-Phe CRF_{12-41} were chosen on the basis of doses used in Experiment 1.

Tests for reinstatement following intra-BNST or -AMG injections of CRF

Two additional groups of animals were trained and prepared for testing as just described. On Day 1 of this phase, animals were given four 60-minute extinction sessions with 30-minute intervening periods during which the active lever was withdrawn. On Days 2 to 4, when testing occurred, animals were given 60-minute extinction sessions with 30 minute intervening periods until all animals responded 15 or fewer times in 60 minutes on the active lever; this criterion was reached within two to three sessions. When all animals reached the baseline criterion for that day, a test for reinstatement was given.

Animals were pretreated with CRF either into the BNST (0, 100, or 300 ng) or into the AMG (0 or 300 ng/side) 15 minutes before insertion of the active lever in the self-administration chamber. No footshock was administered. All animals were tested at each dose of CRF on consecutive days and in a counterbalanced order.

Statistical Analysis

The data from the CRF-receptor antagonist groups and CRF groups with BNST and AMG cannula placements were analyzed separately. In the case of the CRF-receptor antagonist groups, where there were considerable differences in variability in the various test conditions, the data were analyzed using the non-parametric statistics for non-related (Kruskal-Wallis and Mann-Whitney) and related (Wilcoxon) samples. In the case of the CRF groups, where there were no marked differences in variability in the various test conditions, the data were analyzed using ANOVAs for repeated measures or paired-samples t-tests.

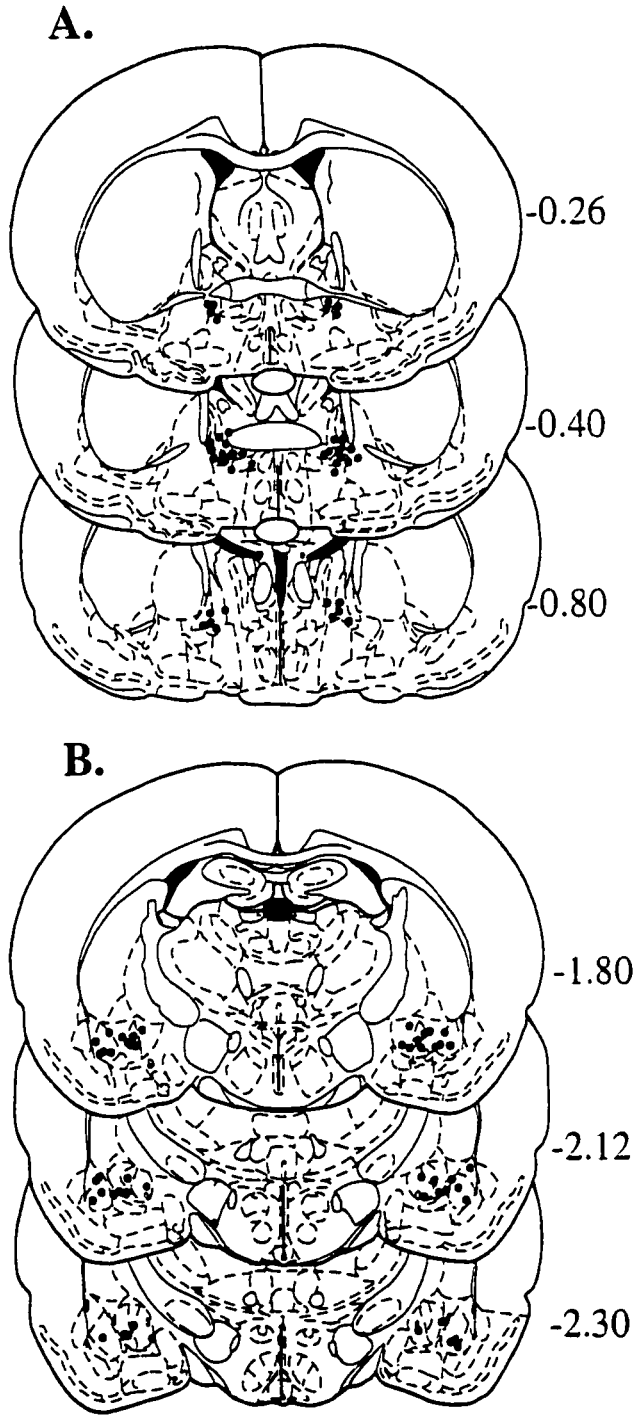
RESULTS

Intra-BNST injections of D-Phe CRF_{12-41}

Training and Extinction

The mean number of infusions of 0.5 mg/kg cocaine made in the 3-hour sessions on the last two days of training was 39.42 (±2.64) and 38.88 (±2.63), respectively. The mean (±SEM) number of responses (infusions + time out responses) made during the first three 1-hour extinction sessions on Day 1 of the extinction and testing phase was 55.58 (±5.68), 25.35 (±3.63), and 14.88 (±2.86).

Figure 6 *Top* Placement of injector tips for animals with BNST cannula implants for D-Phe CRF_{12-41} and CRF: *Bottom* Placement of injector tips for animals with amygdala cannula implants for D-Phe CRF_{12-41} and CRF. Values at right of figures represent mm from bregma. Drawings adapted from Paxinos and Watson, 1997.

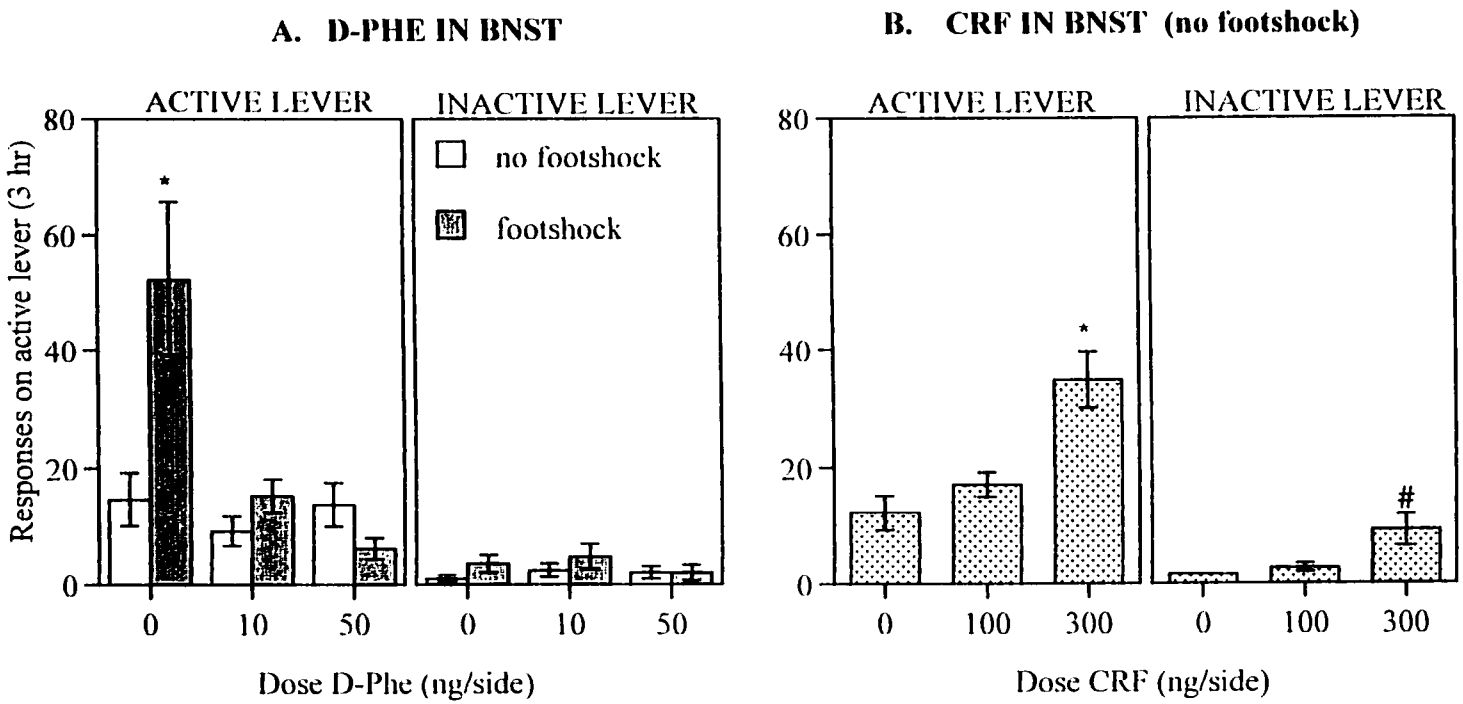

A.
-0.26
-0.40
-0.80
B.
-1.80
-2.12
-2.30

Tests for reinstatement

A total of eight animals per group was used in the analyses for the tests for reinstatement. One animal that had improperly-placed cannulae was excluded from the analyses. This animal, whose cannulae lay just posterior to the BNST, was given 50 ng D-Phe CRF_{12-41} and showed robust footshock-induced reinstatement (110 responses on the active lever in three hours). See Figure 6. (*top*) for a composite diagram showing the distribution of injector tips of animals used in the analyses with BNST cannula implants.

The number of responses made on the active and inactive levers during the 3-hour *No Footshock* and *Footshock* tests for reinstatement following pretreatment with D-Phe CRF_{12-41} is shown in Figure 7A. It can be seen that pretreatment with 10 and 50 ng D-Phe CRF_{12-41} completely blocked the footshock-induced reinstatement of cocaine seeking. A Kruskal-Wallis analysis D-Phe CRF_{12-41} conducted for responses on the active lever in the *Footshock* test condition was significant ($\chi^2[2]=13.95$, $p<.001$). Wilcoxon comparisons revealed that only animals in the vehicle condition responded more in the *Footshock* than *No Footshock* test condition (significant differences indicated by asterisks in Figure 7A, $p<.05$). Similar analyses conducted for responses on the inactive lever were not significant.

Figure 7 *Intra-BNST D-Phe CRF_{12-41}:* (A) Mean (±SEM) number of responses (infusions + timeout responses) on the inactive and previously active levers in the 3-hour tests for reinstatement after exposure to *No Footshock* or *Footshock* (intermittent footshock, 15 min; 0.8 mA; 0.5 sec on; mean off period of 40 sec; immediately before the start of the session). Separate groups of animals were tested in each reinstatement test condition under one dose of D-Phe CRF_{12-41} (0 ng, n=8; 10 ng, n=8; 50 ng, n=8). (B) Mean (±SEM) number of responses (infusions + timeout responses) on the inactive and previously active levers in 3-hour tests for reinstatement in which no footshock was given. All animals (n=10) were tested after injections of 0, 100, and 300 ng CRF.
* Different from other conditions (active lever); ps<.05.
# Different from other conditions (inactive lever); ps<.05.


A. D-PHE IN BNST
ACTIVE LEVER
INACTIVE LEVER
no footshock
footshock
Responses on active lever (3 hr)
80
60
40
20
0
*
0
10
50
0
10
50
Dose D-Phe (ng/side)
B. CRF IN BNST (no footshock)
ACTIVE LEVER
INACTIVE LEVER
80
60
40
20
0
*
#
0
100
300
0
100
300
Dose CRF (ng/side)

Intra-BNST injections of CRF

Training and extinction

The mean number of infusions of 0.5 mg/kg cocaine made in the 3-hour sessions on the last two days of training was 37.6 (±3.77) and 34.6 (±5.04), respectively. The mean (±SEM) number of responses (infusions + time out responses) made during the first three 1-hour extinction sessions on Day 1 of the extinction and testing phase was 35.8 (±9.31), 21.8 (±3.73), and 16.9 (±3.14).

Tests for reinstatement

A total of 10 animals was used in the analyses for the tests for reinstatement; no animals were excluded. See Figure 6 (*top*) for a composite diagram showing the distribution of injector tips of animals used in the analyses with BNST cannula implants.

The mean (±SEM) number of responses made on the active and inactive levers in the 3-hour tests for reinstatement following injections of CRF (0, 100 and 300 ng) is shown in Figure 7B. The 300 ng dose of CRF induced reinstatement of cocaine seeking. A repeated measures ANOVA for number of responses on the active lever revealed a significant effect of dose ($F[2,18]=18.17$, $p<.001$). A similar analysis of responding on the inactive lever was also significant ($F[2,18]=5.89$, $p<.03$). Therefore, difference scores (number of responses on the active lever minus number of responses on the inactive lever) were calculated for responding at each dose and these scores were entered into a repeated measures ANOVA. This analysis revealed a significant effect of dose

(F[2.18]=5.13, p<.03). Reliable post-hoc comparisons (Fisher's LSD, p<.05) are indicated in Figure 7B.

Intra-AMG injections of D-Phe CRF_{12-41}

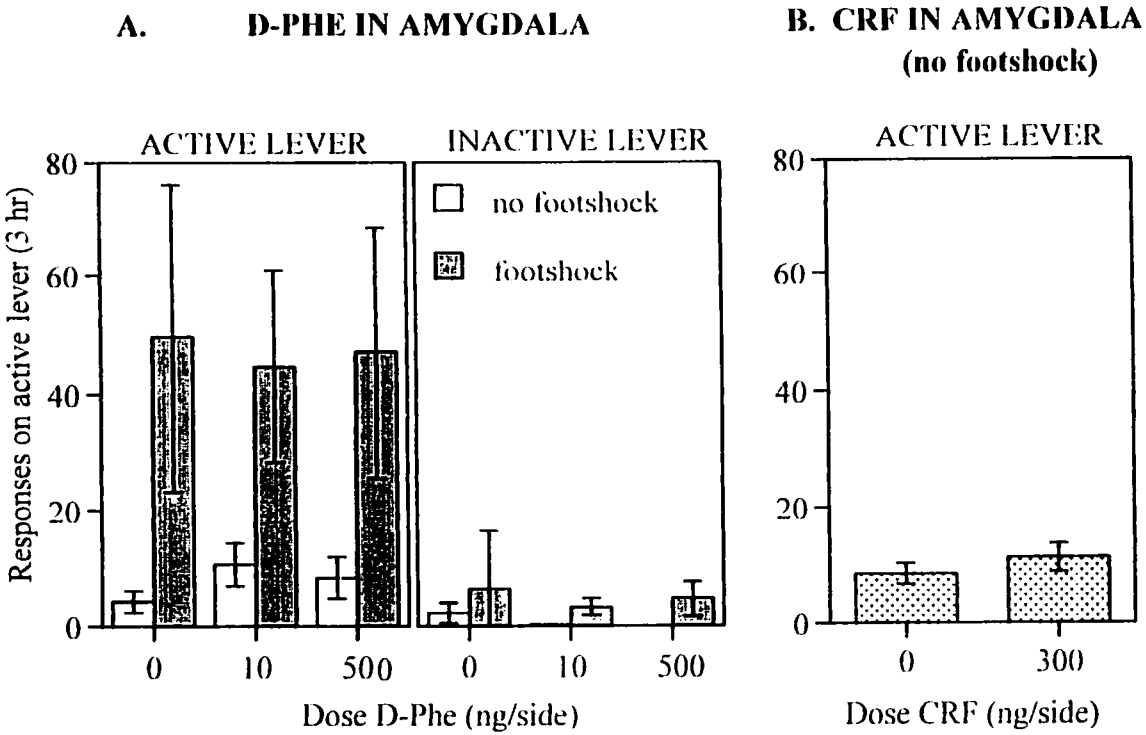
Training and extinction

The mean number of infusions of 0.5 mg/kg cocaine made in the 3-hour sessions on the last two days of training was 31.2 (±4.09) and 31.67 (±3.63), respectively. The mean (±SEM) number of responses (infusions + time out responses) made during the first three 1-hour extinction sessions on Day 1 of the extinction and testing phase was 28.8 (±5.08), 27.4 (±12.44), and 14.4 (±2.79).

Tests for reinstatement

A total of five animals per group was used in the analyses for the tests for reinstatement. Eleven animals, four from the 50 ng and seven from the 500 ng group, were excluded from the analyses because of misplacement of one of the cannulae bilaterally. (It can be noted, however, that removal of the data for these animals made no difference to the outcome, inasmuch as no effects of AMG injections were found). Because there was a relatively wide distribution of injector tips throughout the central and basolateral nuclei (Figure 6A), and because of the known differential effects of CRF on cell firing in these two regions (Rainnie, Fernhout, & Shinnick-Gallagher, 1992), separate analyses were conducted for the animals with placements in basolateral and central nuclei. There was no significant difference between the effects in the two areas

Figure 8 *Intra-amygdala D-Phe CRF_{12-41}:* (A) Mean (±SEM) number of responses (infusions + timeout responses) on the previously active and inactive levers in 3-hr tests for reinstatement after exposure to *No Footshock* or *Footshock* (intermittent footshock, 15 min; 0.8 mA; 0.5 sec on; mean off period of 40 sec; immediately before the start of the session). Separate groups of animals were tested in each reinstatement test condition under one dose of D-Phe CRF_{12-41} (0 ng, n=5; 50 ng, n=5; 500 ng, n=5). The number of responses in the *Footshock* tests was greater than that in the *No Footshock* tests with both vehicle and D-Phe CRF_{12-41} (Wilcoxon, $p<.05$). (B) Mean (±SEM) number of responses (infusions + timeout responses) on the inactive and previously active levers in 3-hour tests for reinstatement in which no footshock was given. All animals (n=10) were tested after injections of 0 and 300 ng CRF.


A. D-PHE IN AMYGDALA
ACTIVE LEVER
INACTIVE LEVER
no footshock
footshock
Responses on active lever (3 hr)
80
60
40
20
0
0
10
500
0
10
500
Dose D-Phe (ng/side)
B. CRF IN AMYGDALA
(no footshock)
ACTIVE LEVER
80
60
40
20
0
0
300
Dose CRF (ng/side)

after either the D-Phe CRF12-41 or CRF injections (see below). See Figure 6 *(bottom)* for a composite diagram showing the distribution of injector tips of animals used in the analyses with AMG cannula implants.

The number of responses made on the active and inactive levers during the 3-hour *No Footshock* and *Footshock* tests for reinstatement following pretreatment with D-Phe CRF_{12-41} is shown in Figure 8A. Neither the 50 nor 500 ng dose of D-Phe CRF_{12-41} interfered in the footshock-induced reinstatement of cocaine seeking. A Kruskal-Wallis analysis conducted for responding on the active lever in the *Footshock* test condition was not significant ($\chi^2[2]=0.31$, $p=0.86$). A Wilcoxon analysis comparing responses in the *No Footshock* and *Footshock* tests, however, was significant ($Z=3.13$, $p<.01$). Thus, regardless of dose, animals responded more under the *Footshock* than *No Footshock* test condition. Similar analyses conducted for responding on the inactive lever were not significant.

Intra-AMG injections of CRF

Training and extinction

The mean number of infusions of 0.5 mg/kg cocaine made in the 3-hour sessions on the last two days of training was 39.3 (±3.43) and 27.1 (±4.30), respectively. The mean (±SEM) number of responses (infusions + time out responses) made during the first three 1-hour extinction sessions on Day 1 of the extinction and testing phase was 56.7 (±12.06), 13.88 (±3.22), and 2.25 (±1.83).

Tests for reinstatement

A total of 10 animals was used in the analyses for the tests for reinstatement. One animal that had improperly-placed cannulae was excluded from the analyses. Another animal that was found to have extremely enlarged ventricles and an atrophied hippocampus was also excluded from the analyses. See Figure 6 *(bottom)* for a composite diagram showing the distribution of injector tips of animals used in the analyses with AMG cannula implants.

The mean (±SEM) number of responses made on the active lever in the 3-hour tests for reinstatement following injections of CRF (0 and 300 ng) is shown in Figure 8B. The same dose of CRF that reinstated cocaine-seeking when injected into the BNST had no effect when injected into the AMG. A paired samples t-test for number of responses on the active lever following 0 versus 300 ng CRF was not significant ($t[9]=0.89$; $p=.40$).

DISCUSSION

Two principle findings emerge from Experiment 4. First, intra-BNST administration of a CRF-receptor antagonist at a dose 10 times lower than that effective when given ICV abolished the footshock-induced reinstatement of cocaine seeking in rats. In contrast, intra-AMG injections of doses as high as those that were effective when given ICV were completely without effect. Thus, the BNST, but not the AMG, would appear to be an important site involved in mediating the effects of D-Phe CRF_{12-41} administered ICV. Second, local administration of CRF into the BNST, but not the

AMG, induced reinstatement of cocaine seeking, mimicking, at least in part, the effects of footshock on relapse.

Although intra-BNST injections of both the CRF-receptor antagonist and CRF were effective, and consistent with the findings of others, one must be concerned about diffusion of injected compounds to other structures, such as the septum, substantia innominata, and preoptic area, that lie close to the BNST and contain moderate to high levels of CRF receptors (Chalmers et al., 1995). It is reassuring, therefore, to note that injections of D-Phe CRF_{12-41} made through cannulae placed improperly just posterior to the BNST were ineffective in blocking the effects of footshock on relapse.

The findings obtained in Experiments 1 to 4 are clearly of theoretical importance with respect to delineating the neurobiological basis of footshock- and cocaine-induced reinstatement of cocaine seeking (the implications of the findings will be discussed in greater detail shortly). Although of theoretical significance, however, the findings are of limited applicability in that antagonists such as D-Phe CRF_{12-41} are large peptide molecules that would not be expected to cross the blood brain barrier if injected systemically and that, therefore, must be injected centrally. In this regard, there has been considerable interest recently in the development of non-peptide CRF-receptor antagonists that can be injected systemically. Experiment 5 was conducted to test the effectiveness of one recently-developed non-peptide CRF-receptor antagonist in blocking the footshock-induced reinstatement of cocaine seeking.

EXPERIMENT 5: EFFECTS OF THE NON-PEPTIDE CRF_1-RECEPTOR ANTAGONIST, CP-154-526, ON THE FOOTSHOCK-INDUCED REINSTATEMENT OF COCAINE SEEKING

CP-154,526 is a potent and behaviorally-effective non-peptide CRF-receptor antagonist that is selective for the CRF_1 receptor subtype and that can be administered systemically (Chen et al., 1997). This compound has been shown to inhibit the CRF-induced cell firing of NE neurons of the LC and to block CRF-induced ACTH release, positive evidence of its ability to penetrate the blood brain barrier. As well, the drug has been found to be behaviorally effective in blocking anxiogenic responses, as measured by the acoustic startle and elevated plus-maze procedures, and to exert antidepressant-like effects in the learned helplessness procedure (Lundkvist et al., 1996; Mansbach, Brooks, & Chen, 1997; Schulz et al., 1996). Experiment 5 was conducted to determine whether systemic injections of CP-154,526 would be effective in blocking footshock-induced reinstatement of cocaine seeking, in a manner similar to that observed following central administration of a peptide CRF-receptor antagonist.

MATERIALS AND METHODS

Subjects

Ten male Long-Evans rats (375-450 g; Charles River laboratories, Canada), maintained under similar conditions to those described in Experiment 1, served as subjects.

Surgery

Animals were prepared with IV catheters, as described in the General Methods (Chapter 1).

Drugs

CP-154,526, butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo [2,3-d] pyrimidin-4-yl]-ethyl-amine, generously supplied by Pfizer, Groton, Conn., USA, was dissolved in distilled water containing 0.1% methylcellulose (pH adjusted to 5.5-5.6). CP-154,526 (15 or 30 mg/kg) or the vehicle was injected SC at a volume of 2 ml/kg. The doses were chosen on the basis of previous reports (Mansbach et al., 1997; Schulz et al., 1996).

Procedure

A *complete between-days* reinstatement procedure, described in detail in the General Methods (Procedures; Chapter 1), was used in this experiment. Briefly, the experiment was conducted in three phases: self-administration training (1.0 mg/kg per infusion cocaine HCl), extinction, and testing for reinstatement. In each phase, the active lever was inserted into the chamber for one 3-hour session per day, during which time lever presses resulted in drug (training phase) or saline (extinction and test phases) infusions. In the extinction phase of this experiment, animals reached the baseline criterion level of responding of 10 or fewer response in a 3-hour session within 5 to 14 days.

In the reinstatement test phase, all animals received six tests for reinstatement on six consecutive days. The tests were vehicle pretreatment + no footshock, vehicle + footshock, 15 mg/kg CP-154,526 + no footshock, 15 mg/kg CP-154,526 + footshock, 30 mg/kg CP-154,526 + no footshock, and 30 mg/kg CP-154,526 + footshock. All animals received the vehicle + no footshock condition first; the other five conditions were given in a counterbalanced order (i.e., the rats were exposed to different sequences of the test conditions). Previous work has indicated very little or no habituation to the effect of footshock on reinstatement when it is given repeatedly (Shaham et al., 1997b; Shaham & Stewart, 1996); it seemed reasonable, therefore, to use a within-subjects design in the present experiment. Vehicle or CP-154,526 was injected 30 to 40 minutes before exposure to the footshock or no footshock test conditions. Details of the test conditions are described in the General Methods (Procedure: Chapter 1).

Statistical Analyses

Dose of CP-154,526 (0, 15, 30 mg/kg) and test condition (no footshock, footshock) were within-subjects factors. Data were analyzed using the non-parametric statistics for related samples (Friedman, Wilcoxon).

RESULTS AND DISCUSSION

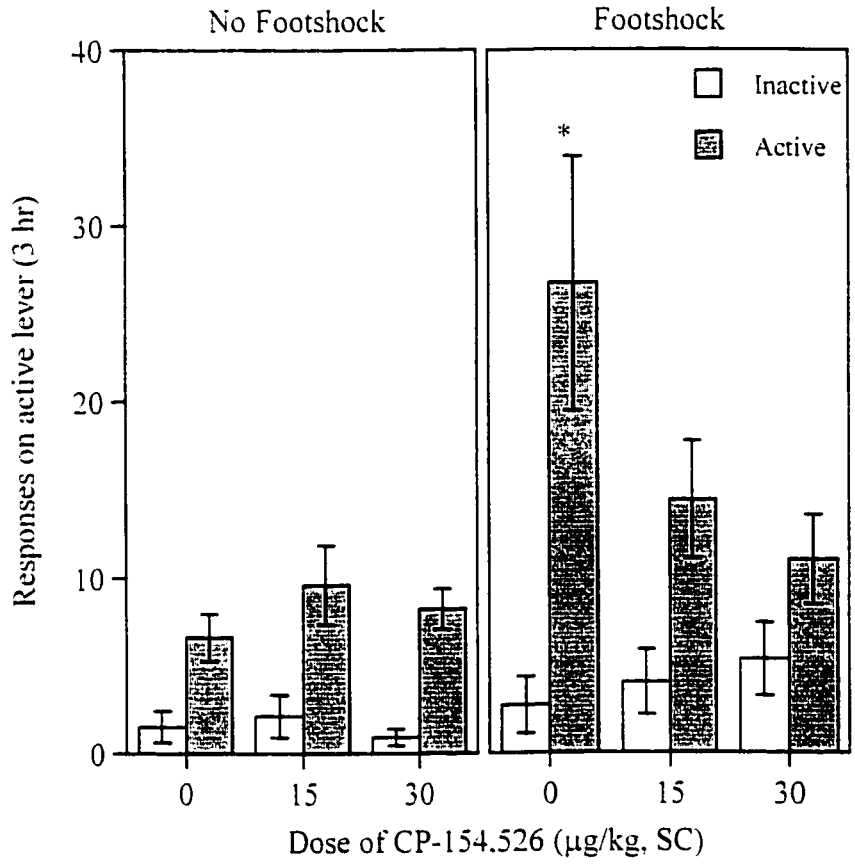
Training and extinction

The mean number of infusions of 1.0 mg/kg cocaine made in the 3-hour sessions on the last day of training was and 19.7 ± 1.9. The mean (±SEM) number of responses (infusions + time out responses) made on the active lever on the first day of extinction was 41.7 ± 8.88. By the last extinction session, all animals had reached the criterion of 10 or fewer responses.

Tests for reinstatement

Figure 9 shows the mean number of responses made on the active lever (saline infusions + time-out responses) during the 3-hour tests for reinstatement under vehicle and CP-154,526 pretreatment in the *Footshock* and *No Footshock* test conditions. It can be seen that both doses of CP-154,526 blocked the footshock-induced reinstatement of responding observed following vehicle pretreatment. A Friedman analysis conducted for all combinations of pretreatment and test conditions only approached significance ($\chi^2[5]=10.6$, $p=.06$). However, Wilcoxon comparisons conducted between *No Footshock* and *Footshock* test conditions at each dose were significant only for the vehicle condition (Figure 9, $p<.05$). Thus, systemic pretreatment with a non-peptide CRF-receptor antagonist, selective for the CRF_1 receptor, effectively blocked the footshock-induced reinstatement of cocaine seeking. The magnitude of the effect was similar to that observed following central pretreatment with a peptide CRF-receptor antagonist.

Figure 9 *Systemic CP-154,526:* Mean (±SEM) number of responses (infusions + timeout responses) on the inactive and previously active levers in 3-hour tests for reinstatement after exposure to *No Footshock* or *Footshock* (intermittent footshock, 15 min; 0.6 mA; 0.5 sec on; mean off period of 40 sec; immediately before the start of the session). Animals were tested in both test conditions after each of three doses of CP-154,526 (n= 10; 0 , 20, 40 mg/kg).
* Different from other conditions (active lever): ps<.05.


No Footshock
Footshock
Inactive
Active
*
Responses on active lever (3 hr)
40
30
20
10
0
0
15
30
0
15
30
Dose of CP-154.526 (µg/kg, SC)

GENERAL DISCUSSION (Chapter 2)

Stress-induced relapse

ICV and systemic injections of CRF-receptor antagonists

One of the primary findings from the experiments presented in this chapter is that the ICV administration of the potent CRF-receptor antagonist, D-Phe CRF_{12-41}, abolishes the footshock-induced reinstatement of cocaine seeking in intact animals (Experiment 1) and in adrenalectomized animals given corticosterone replacement (Experiment 3). These data suggest that CRF can act directly in the brain, independent of its effects on the HPA axis, to mediate the effects of footshock on relapse. In addition, it was found that systemic injections of a non-peptide CRF_1 receptor antagonist, CP-154,526, were also effective in suppressing the footshock-induced reinstatement of cocaine seeking in intact animals (Experiment 5).

These effects of CRF-receptor antagonists on the footshock-induced reinstatement of cocaine seeking extend those of studies conducted with heroin-trained rats in which it was found that ICV administration of the peptide CRF-receptor antagonist, alpha-helical CRF_{9-41}, and systemic injections of CP-154,526 attenuated the footshock-induced reinstatement of heroin seeking (Shaham, Erb, Leung, Buczek, & Stewart, 1998; Shaham et al., 1997b). It is important to note, however, that pharmacological blockade of the CRF system seems to exert a stronger effect on footshock-induced reinstatement in cocaine-trained rats than heroin-trained rats. In heroin-trained rats, antagonism of the CRF system by both the peptide and non-peptide CRF antagonists decreased footshock-induced reinstatement by approximately 50 percent; responding, however was

significantly above baseline levels (Shaham et al., 1998; Shaham et al., 1997b). In contrast, in cocaine-trained rats, both peptide (Experiment 1) and non-peptide (Experiment 5) CRF-receptor antagonists blocked footshock-induced reinstatement; that is, following pretreatment with either antagonist, animals showed a comparable level of responding in footshock and no footshock tests for reinstatement. Thus, it seems that, for reasons that are yet to be elucidated, the CRF system is more critically involved in stress-induced reinstatement in subjects previously exposed to cocaine than in those exposed to heroin.

A second finding from the present series of experiments is that footshock did not induce reinstatement of cocaine seeking in adrenalectomized animals (Experiment 2). This lack of response was reversed, however, when adrenalectomized animals were given basal levels of corticosterone. These findings indicate that although the footshock-induced reinstatement can occur in the absence of a stress-induced rise in corticosterone, some minimal level of corticosterone is necessary for the footshock stressor to induce reinstatement in cocaine-trained animals. The data are different from those obtained in the studies in heroin-trained rats in which adrenalectomy had no effect on footshock-induced reinstatement (Shaham et al., 1997b).

The differential effect of adrenalectomy on stress-induced reinstatement in cocaine- and heroin-trained animals may arise from the different effects of repeated exposure to these drugs on the response of the HPA axis. There is evidence that the cocaine-induced rise in corticosterone and ACTH does not diminish in animals given repeated intermittent injections of cocaine (Torres & Rivier, 1992). In the case of opioid agonists, however, acute injections increase plasma corticosterone and ACTH levels

whereas repeated exposure leads to a complete tolerance of these effects (Pechnick, 1993). For example, Buckingham and Cooper (1984) showed that acute injection of a moderate dose of morphine (20 mg/kg, IP) increased plasma levels of ACTH and corticosterone. However, after nine daily injections, plasma levels of the hormones were significantly below control levels. This difference in the effects of opioid agonists and cocaine on the HPA axis could have consequences of relevance for understanding the differential effect of adrenalectomy in cocaine- and heroin-trained rats.

One possible consequence is that the self-administration of heroin and cocaine becomes differentially dependent on the presence of corticosterone during training and maintenance of the behavior; that is, responding becomes differentially state-dependent. Since in the case of cocaine, responding for the drug is consistently accompanied by increases in corticosterone, some minimal level of corticosterone may be required to maintain responding, especially in the absence of cocaine and after extinction of the drug-related cues. In the case of heroin, on the other hand, repeated self-administration of the drug is likely to result in suppressed levels of corticosterone (this is an especially likely outcome in the studies conducted with heroin-trained animals in which a high unit dose of heroin, 0.1 mg/kg/infusion, was self-administered for nine hours per day (Shaham et al., 1997b)); thus, in the case of heroin-trained animals, a minimal level of corticosterone may not be required to maintain responding and, as a result, adrenalectomy might have minimal consequences on responding during tests for reinstatement.

Another possible explanation for the effect of adrenalectomy on footshock-induced reinstatement in cocaine-trained rats might arise from the effect of adrenalectomy on the functioning of the extrahypothalamic CRF system. Recent studies

suggest that glucocorticoids regulate CRF expression in, for example, the AMG. Makino et al. (1994a; 1994b) reported that adrenalectomy modestly decreased, whereas high doses of corticosterone increased, CRF mRNA in the central nucleus of the AMG (CeA) (Swanson & Simmon, 1989). These studies suggest that CRF functioning in the AMG may be attenuated in adrenalectomized rats. It is possible, therefore, that in adrenalectomized animals, the CRF response to footshock in regions such as the AMG is reduced, resulting in decreased responsiveness to the stressor in tests for reinstatement.

Two potential weakness of the above argument should, however, be noted. First, adrenalectomy failed to attenuate stress-induced reinstatement in heroin-trained rats (Shaham et al., 1997b). However, as mentioned above, the effect of blockade of CRF receptors on stress-induced reinstatement is considerably greater in cocaine-trained rats than in heroin-trained rats, suggesting that CRF systems may be more critical in animals with a history of cocaine taking. Second, adrenalectomy does not affect the expression of CRF mRNA in the BNST (Makino et al., 1994b), a region that in Experiment 4 was found, in contrast to the AMG, to be critically involved in the effects of CRF on stress-induced reinstatement of cocaine seeking. It has been found, however, that administration of corticosterone in intact animals does modestly increase CRF mRNA in the BNST, suggesting that glucocorticoids are capable of modifying CRF expression in this region. Furthermore, it is worthwhile noting that the CeA, which provides a major CRF input to the BNST (Sakanaka, Shibasaki, & Lederis, 1986), may be a principle source of CRF to that region during stress. Therefore, changes in the sensitivity of CRF systems in the CeA could conceivably play a more important role in CRF receptor activation in the BNST than CRF systems intrinsic to the BNST, itself (see Chapter 4).

Intra-BNST and intra-AMG injections of a CRF-receptor antagonist

In Experiment 4, it was found that intra-BNST administration of a CRF-receptor antagonist abolished footshock-induced reinstatement of cocaine seeking in the rat. In contrast, intra-AMG injections of D-Phe CRF_{12-41} were completely without effect. Furthermore, it was found that local administration of CRF into the BNST, but not the AMG, induced reinstatement of cocaine seeking, mimicking, at least in part, the effects of footshock on relapse.

These findings are consistent with those of Davis and colleagues suggesting that the BNST, and not the AMG, is responsible for the unconditioned emotional changes induced by CRF. Lee and Davis (1997) showed, for example, that lesions of the BNST and not the AMG interfered with the effects of CRF administered ICV, and that intra-BNST injections of CRF enhanced startle responses. Furthermore, the present findings support the view that the BNST is preferentially involved in mediating behavioral responses to unconditioned stress (Gewirtz et al., 1998; Walker & Davis, 1997); neither the footshock stressor nor the CRF injections had been paired previously with the self-administration context, and were, as such, unconditioned stressors.

Injections of CRF and its receptor antagonist into the BNST could mimic or block the effects of CRF released locally from cells intrinsic to this structure (Veinante, Stoekel, & Freund-Mercier, 1997). It must be kept in mind, however, that although injections of CRF and D-Phe CRF_{12-41} into the AMG neither induced reinstatement nor interfered with footshock-induced reinstatement, the AMG cannot be ruled out as a component of the circuitry mediating the effect of stress. As mentioned, a CRF-

containing projection from the CeA to the BNST has been identified (Sakanaka et al., 1986) and stress-induced activation of this pathway could be responsible, in part, for the increases in CRF found in the BNST following exposure to stress (Lee & Davis, 1997).

Finally, it is worthwhile noting that although D-Phe CRF_{12-41} is a non-selective CRF-receptor antagonist (Menzaghi et al., 1994a), CRF_1 and CRF_2 receptors are differentially distributed in the brain (Chalmers et al., 1995) and are functionally dissociable (e.g., Heinrichs, Lapsansky, Lovenberg, De Souza, & Chalmers, 1997; Radulovic, Rühmann, Liepold, & Spiess, 1999). Although both receptors are present in the BNST, in the lateral division of the BNST there are more CRF_1 than CRF_2 receptors and it is there that they are most abundant (Chalmers et al., 1995). It would appear, therefore, that CRF_1 receptors are especially important for mediating the effects of footshock on relapse. Consistent with this possibility, is the finding from Experiment 5 that systemic injections of CP-154, 526, a CRF-receptor antagonist selective for the CRF_1 receptor, blocked the footshock-induced reinstatement of cocaine seeking as effectively as ICV injections of a peptide CRF-receptor antagonist (Experiment 1).

Cocaine-induced relapse

In Experiment 1, it was found that pretreatment with the CRF-receptor antagonist, D-Phe CRF_{12-41}, had some effect on cocaine-induced reinstatement; responding was mildly attenuated at the lowest (0.1 μg) and highest (1.0 μg) doses tested but, unaccountably, not at the intermediate dose. These findings suggest that although the activation of CRF receptors is not critical for cocaine-induced relapse, CRF plays a modulatory role in reinstatement by cocaine, possibly via interactions with the midbrain

DA system known to be involved in cocaine-induced reinstatement. Activation of midbrain DA systems has, as discussed in Chapter 1, been shown to mediate reinstatement by both stimulant and opioid drugs (De Vries et al., 1999; Self, Barnhart, Lehman, & Nestler, 1996; Stewart, 1984; Stewart & Vezina, 1988), and there is evidence that CRF has effects on this system. For example, CRF has been shown to increase sensitivity to the effects of D-amphetamine on locomotor activity (Cador, Cole, Koob, Stinus, & Le Moal, 1993), to increase DA use in terminal regions of the mesolimbic DA system (Lavicky & Dunn, 1993), and to directly activate DA cells in the VTA (Cameron & Nalivaiko, 1999). However, it has been found that maintenance of cocaine self-administration is not affected by blockade of CRF receptors (Ahmed et al., 1996).

Another important finding from the present series of experiments is that corticosterone appeared to be only minimally involved in reinstatement induced by priming injections of cocaine. Adrenalectomy did not block reinstatement by the priming injections of cocaine (Experiment 2). Although there was a modest attenuation of responding in adrenalectomized animals, the number of responses was well above that seen after the priming injections of saline (Figure 4, *top*). Furthermore, the fact that corticosterone replacement had little effect in adrenalectomized animals suggests that, in contrast to footshock-induced reinstatement, minimal levels of corticosterone are not required for cocaine-induced reinstatement.

It is somewhat surprising that adrenalectomy had little effect on cocaine-induced reinstatement, given that adrenalectomy and administration of synthesis inhibitors of corticosterone have been shown to reduce the reinforcing effects of cocaine and cocaine-induced locomotion. Both adrenalectomy and synthesis inhibitors of corticosterone

interfere with the initiation and maintenance of cocaine self-administration (Deroche et al., 1997; Goeders & Guerin, 1996a; Goeders & Guerin, 1996b; Piazza et al., 1994) and reduce the acute psychomotor effects of cocaine (Deroche et al., 1997; Goeders & Guerin, 1996a; Goeders & Guerin, 1996b; Marinelli et al., 1997b; Piazza et al., 1994). Piazza and colleagues have argued on the basis of these data that corticosterone tonically enhances DA transmission in the midbrain DA system (Piazza et al., 1996a; Piazza et al., 1996c). It may well be, therefore, that the effects of adrenalectomy on cocaine-induced behaviors will be less obvious at doses of the drug that are sufficiently high to overcome any effect of reduced DA tone. Thus, it is possible that adrenalectomy would attenuate reinstatement by a lower priming dose of cocaine than that used in Experiment 2. On the other hand, experiments conducted with rhesus monkeys have failed to demonstrate a relationship between cortisol and cocaine-reinforced responding (Broadbear, Winger, & Woods, 1999; Broadbear, Winger, Cicero, & Woods, 1999), findings that are consistent with the apparent lack of effect of glucocorticoid manipulations on cocaine-induced relapse in the present experiments.

A possible change in the sensitivity of CRF systems with chronic exposure to cocaine: Implications for relapse

Animals with a history of drug taking appear to be highly sensitive to footshock-induced relapse. It has been shown in several studies, using similar procedures to those described here, that animals trained to self-administer either food, sucrose pellets, or sucrose solutions show little evidence of stress-induced reinstatement of these behaviors (Ahmed & Koob, 1997; Buczek et al., 1999). On the basis of the data presented in this

chapter, it would seem reasonable to suggest that the reactivity of brain CRF systems may change as a consequence of chronic exposure to drugs.

Recent reports indicate that CRF is involved in anxiogenic and aversive symptoms of withdrawal from several drugs of abuse, including cocaine (Sarnyai et al., 1995), opioids (Heinrichs, Menzaghi, Schulteis, Koob, & Stinus, 1995), and alcohol (Menzaghi et al., 1994b; Rassnick, Heinrichs, Britton, & Koob, 1993). These effects of CRF are probably related to its extra-hypothalamic effects in limbic structures such as the AMG (for a review see Koob, 1996). For example, injection of a CRF-receptor antagonist into the CeA has been found to attenuate a withdrawal-induced conditioned place aversion in morphine-dependent rats (Heinrichs et al., 1995) and to reverse anxiogenic effects of ethanol withdrawal (Menzaghi et al., 1994b; Rassnick et al., 1993). These data on the role of CRF in withdrawal from drugs, together with data from this laboratory on the involvement of CRF in reinstatement of drug seeking induced by footshock stress, suggest that brain CRF may be involved in processses underlying excessive drug use.

It should be noted, however, that although changes in CRF activity occur during withdrawal (see Menzaghi et al., 1994b Heinrichs, 1995 #1782; Rassnick et al., 1993; Sarnyai et al., 1995), the relevance of these changes to relapse to drug use induced by stress after prolonged drug-free periods is not obvious. For example, there is no evidence that increased activity of the CRF system seen after withdrawal from drugs persists for more than 24 to 48 hours. In a recent study, it was reported that behavioral anxiety exhibited 48 hours after withdrawal from cocaine was accompanied by reduction in tissue levels of CRF immunoreactivity in the hypothalamus, AMG, and basal forebrain (Sarnyai

et al., 1995). In other studies, however, in which aspects of the CRF system were examined after longer periods of withdrawal, no changes were found. Ambrosio et al (1997) reported changes in CRF_1 receptor binding in the basolateral AMG immediately after the last injection of cocaine, but not after 10 days of withdrawal. In a study of CRF mRNA in the brain after a "binge" pattern of cocaine administration, increases were seen in the hypothalamus and the AMG on the first two days of cocaine administration, but these levels returned to baseline after 10 days of withdrawal (Zhou et al., 1996). It should be noted, however, that there are no studies on the reactivity of the CRF system to stressors after prolonged drug-free periods. It is possible that the reactivity of the CRF system changes as a consequence of chronic exposure to drugs, even if there are no changes in basal measures.

Summary

The experiments presented in this chapter lend support to the view that the neural and hormonal mechanisms underlying stress-induced reinstatement are not identical to those underlying drug-induced reinstatement (Shaham et al., 1997b). Furthermore, the data strongly suggest that CRF acting directly in the brain, independent of its actions on the HPA axis, mediates stress-induced relapse to cocaine seeking. One question that arises from these data is what other neurotransmitter systems are involved in or interact with CRF systems in mediating the effects of footshock on relapse? One system that is very likely to play a role in stress-induced relapse and that is known to interact with CRF systems in the brain is NE (e.g., Li, Takeda, Tsuji, Liu, & Matsumiya, 1998; Shimizu, Nakane, Hori, & Hayashi, 1994; Smagin, Zhou, Harris, & Ryan, 1997; Valentino et al.,

1991; Valentino & Wehby, 1988). Consideration of a possible role for NE in the footshock- and cocaine-induced reinstatement of cocaine seeking is the focus of the next chapter.

CHAPTER 3

A ROLE FOR NORADRENALINE IN STRESS-, BUT NOT COCAINE-, INDUCED RELAPSE TO COCAINE SEEKING

Release of the catecholamines NE and DA within the brain, in addition to activation of CRF systems and the release of glucocorticoids, is a hallmark feature of the stress response. In response to an acute stressor, release of NE in the brain contributes to sympathetic activation, including increases in heart rate and blood pressure, and participates in the regulation of the HPA axis by stimulating the release of CRF in the PVN (Pacak, Miklos, Kopin, & Goldstein, 1995; Sapolsky, 1992).

The NE system is comprised of a dorsal stream of neurons originating in the LC and a ventral stream of neurons originating in the lateral tegmental nuclei (Aston-Jones, Shipley, & Grzanna, 1995; Moore & Bloom, 1979). Although the stress-related functions of the ventral projections have been only partially characterized (Cole & Robbins, 1987; Hansen, Stanfield, & Everitt, 1980), NE projections from the LC have been directly implicated in a number of stress-related responses. For example, electrical stimulation of the LC induces anxiety and results in activation of the autonomic nervous system, whereas local injections of clonidine, which act to inhibit NE cell firing (Aghajanian & VanderMaelen, 1982) and release (Carter, 1997), decrease physiological and behavioral responses to stressors (Bremner, Krystal, Southwick, & Charney, 1996; Redmond & Huang, 1979).

Interactions between NE and CRF systems in the brain have been implicated in a number of physiological responses to acute stress. For example, injections of CRF into the lateral ventricles or LC increase, as does exposure to stress, the discharge rate of LC neurons; these effects are prevented by ICV or local injections of a CRF-receptor antagonist (Valentino et al., 1991; Valentino & Wehby, 1988). Similarly, ICV injections

of a CRF-receptor antagonist attenuate stress-induced NE release in the prefrontal cortex (Shimizu et al., 1994; Smagin et al., 1997), a structure innervated by LC neurons, and stress-induced NE turnover in AMG and lateral septum (Li et al., 1998), structures innervated by both the ventral and dorsal bundles (Aston-Jones et al., 1995; Moore & Bloom, 1979).

Interactions between brain NE and CRF systems may underlie stress-induced relapse to drug seeking. As demonstrated by findings reported in Chapter 2, administration of CRF-receptor antagonists blocks stress-induced relapse to cocaine seeking. Recently, it has been found that systemic or ICV injections of the alpha-2 adrenergic receptor agonist, clonidine, acting through the alpha-2 autoreceptor, blocks footshock-induced reinstatement of heroin seeking (Shaham, Leung, Buczek, & Stewart, in press). The similarities between the effects of attenuation of NE functioning and blockade of CRF receptors points to a possible interaction between the systems in the mediation of stress-induced relapse.

There is also reason to think that NE may play a role in relapse to cocaine seeking induced by cocaine itself, in spite of the fact that the CRF-receptor antagonist had only a modest effect on cocaine-induced relapse. As mentioned previously, the effects of priming injections of psychostimulant and opioid drugs on relapse to drug seeking have been shown to be mediated by the midbrain DA system (De Vries et al., 1999; Self et al., 1996; Stewart, 1984; Stewart & Vezina, 1988). It is interesting, therefore, to note that inhibition of NE systems by injections of the alpha-2 adrenergic-receptor agonist, clonidine, is known to have effects on the functioning of midbrain DA neurons (Lategan, Marien, & Colpaert, 1990). For example, it has been shown that systemic injections of

alpha-2 adrenergic-receptor agonists decrease DA release in the nucleus accumbens (Murai et al., 1998) and the frontal cortex (Gobert et al., 1998). These effects may be due to the effect of these compounds on DA cell firing. Grenhoff and Svensson (1989) have shown that clonidine regularizes midbrain DA cell firing in anesthetized rats, an effect that should reduce DA release from terminals (Roth, Wolf, & Deutch, 1987). Thus, NE may play a role in the cocaine-induced reinstatement of cocaine seeking through an interaction with midbrain DA systems.

The experiments presented in this chapter were conducted to assess the role of NE in footshock- and cocaine-induced relapse to cocaine seeking. The effects of a series of alpha-2 adrenergic-receptor agonists, including clonidine, lofexidine, and guanabenz, on relapse were determined. The alpha-2 adrenergic-receptor agonists, at the doses used in these experiments, are known to act primarily at the alpha-2 autoreceptor to inhibit NE cell firing (Aghajanian & VanderMaelen, 1982) and release (Carter, 1997). Thus, if activation of NE cells and/or release of NE are necessary conditions for footshock- or cocaine-induced relapse to cocaine seeking, alpha-2 adrenergic-receptor agonists should interfere in relapse induced by these events.

EXPERIMENT 1: EFFECTS OF CLONIDINE ON FOOTSHOCK- AND COCAINE-INDUCED REINSTATEMENT

Of the available alpha-2 adrenergic-receptor agonists, clonidine is probably the most well-characterized, both pharmacologically and behaviorally. Clonidine has been shown to decrease physiological and behavioral responses to stress (Bremner et al., 1996;

Redmond & Huang, 1979) and also to decrease the aversive effects of opioid withdrawal in rats (Aston-Jones, Delfs, Druhan, & Zhu, 1999; Roth, Elsworth, & Redmond, 1982; Van der Laan, 1985) and in humans (Lin, Strang, Su, Tsai, & Hu, 1997; Warner, Kosten, & O'Connor, 1997; Washton & Resnick, 1982). In Experiment 1, the effects of systemic injections of clonidine on the footshock- and cocaine-induced reinstatement of cocaine seeking were assessed.

MATERIALS AND METHODS

Subjects

The subjects were 25 male Long-Evans rats (350-425g; Charles River laboratories, Canada). The animals were housed in a temperature- and humidity-controlled colony room on a reverse light/dark cycle (lights on from 1730 to 0530 hr).

Surgery

Animals were prepared with IV catheters, as described in the General Methods (Chapter 1).

Apparatus

Described in the General Methods (Chapter 1).

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 IP (0, 20, and 40 μg/kg).

Procedure

A *within-days extinction and testing* procedure, described in detail in the General Methods (Procedures: Chapter 1), was used in this experiment. Briefly, the experiment was run in three phases: self-administration training (0.5 mg/kg per infusion cocaine HCl), drug-free period, and extinction and testing for reinstatement. During training and testing sessions, the active lever was inserted into the chambers for one 3-hour session per day, during which time lever presses resulted in drug infusions (training phase) or were without consequence (test phase). During extinction sessions, the active lever was inserted into the chambers for several 1-hour sessions each day, during which time lever presses were without consequence.

On Day 1 of the extinction and testing phase, all animals were given three 1-hour self-administration sessions with 15-minute intervening periods, followed by a 40-minute intervening period and then two additional 1-hour self-administration sessions with a 40-minute intervening period. On Days 2 to 4 of Phase 2, animals were given 1-hour self-administration sessions with 15-minute intervening periods until all animals responded 20 or fewer times in one hour on the active lever; this criterion was reached within two to three sessions. The criterion session was followed by a 40-minute intervening period

after which further 1-hour self-administration sessions were given preceded by a 40-minute intervening period, until the level of responding reached 15 or fewer responses on the active lever; this criterion was reached within one to two sessions. When all animals reached the baseline criterion for that day, a test for reinstatement was given.

Separate groups of animals were pretreated with either 0, 20, or 40 μg/kg, IP, clonidine before each of three tests for reinstatement (saline, cocaine, and footshock), given on consecutive days and in a counterbalanced order. Clonidine was injected 40 minutes before the lever was inserted. The doses of clonidine were chosen on the basis of reinstatement experiments conducted with heroin-trained rats (Shaham et al., in press). See the General Methods (Chapter 1) for a detailed description of the test conditions.

Statistical analyses

Test condition (saline, footshock, cocaine) was a within-subjects factor; dose of clonidine (0, 20, 40 μg/kg) was a between-subjects factor. 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 non-related (Kruskal-Wallis and Mann-Whiney) samples were used.

RESULTS AND DISCUSSION

Training and Extinction

The mean (±SEM) number of infusions of 0.5 mg/kg cocaine made in the 3-hour session on the last two days of training was 32.24 (±2.77) and 25.76 (±2.96), respectively. The mean (±SEM) number of responses (infusions + time out responses) made during the first three 1-hour extinction sessions on Day 1 of Phase 2, was 28.92 (±2.81), 13.16 (±2.03), and 9.68 (±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.

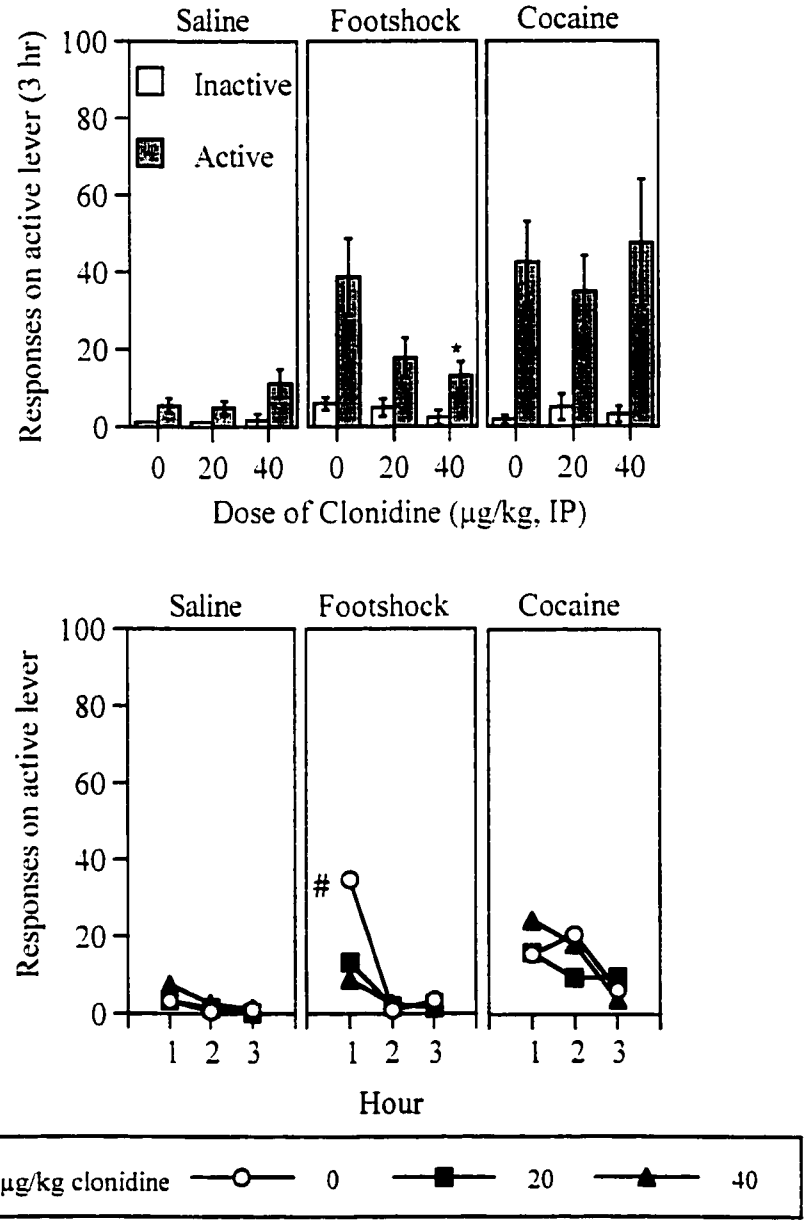
Tests for reinstatement

The number of responses made on the active and inactive levers during each 3-hour test for reinstatement following pretreatment with clonidine is shown in Figure 10 *(top)*. Figure 10 (*bottom*) shows the number of responses on the active lever during each hour of testing. It can be seen that pretreatment with 20 or 40 μg/kg clonidine blocked footshock-induced relapse 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 (ps<.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 10 (*top*) shows that responding on the inactive lever

Figure 10 *Systemic clonidine: Top* Mean (±SEM) number of responses (infusions + timeout responses) on the inactive and previously active levers in the 3-hour tests for reinstatement after *Saline* (physiological saline, 1.0 ml/kg, IP; 5 min before the start of the session), *Footshock* (intermittent footshock, 15 min; 0.6 mA; 0.5 sec on; mean off period of 40 sec; immediately before the start of the session), and *Cocaine* (20 mg/kg, IP; 5 min before the start of the session). *Bottom,* Mean number of responses (saline infusions + time-out responses) on the active lever in each hour of each of the 3-hour tests for reinstatement. Rats were pretreated with vehicle (0) (n=9) or 20 (n=8) or 40 (n=8) µg/kg clonidine, IP

* Different from vehicle (0) dose in the *Footshock* condition; $p<.05$.

\# Different from other doses, Hour 1, *Footshock* condition; $ps<.05$.


Saline
Footshock
Cocaine
Responses on active lever (3 hr)
100
80
60
40
20
0
Inactive
Active
*
0 20 40 0 20 40 0 20 40
Dose of Clonidine (µg/kg, IP)
Responses on active lever
#
1 2 3 1 2 3 1 2 3
Hour
µg/kg clonidine
0
20
40

was low under all pretreatment and test conditions. This was also the case in Experiments 2 and 3 below, where there were no significant effects on the number of responses on the inactive lever.

Thus, clonidine is effective in preventing footshock-induced reinstatement of cocaine seeking, implicating NE systems in the mediation of stress-induced relapse to cocaine seeking.

EXPERIMENT 2: EFFECTS OF LOFEXIDINE ON FOOTSHOCK- AND COCAINE-INDUCED REINSTATEMENT

Experiment 2 was conducted to determine whether the effects of clonidine observed in Experiment 1 would generalize to another alpha-2 adrenergic-receptor agonist, lofexidine. Although the pharmacological profile for clonidine has been better characterized than for lofexidine, both compounds seem to be associated with similar behavioral effects. For example, lofexidine, like clonidine, has been shown in rats to attenuate aversive effects of opioid withdrawal (Shearman, Lal, & Ursillo, 1980; Van der Laan, 1985; Van der Laan, Van Veenendaal, Voorthuis, Weick, & Hillen, 1985) and both compounds have been used with some success in humans in the treatment of opioid withdrawal (e.g., Carnwath & Hardman, 1998). Lofexidine, however, has been reported in humans to be associated with fewer adverse side effects than clonidine, in particular fewer hypotensive effects (Carnwath & Hardman, 1998; Kahn, Mumford, Rogers, & Beckford, 1997; Lin et al., 1997), a finding that is of potential clinical significance.

MATERIALS AND METHODS

Subjects

The subjects were 49 male Long-Evans rats (350-400 g; 34 from Charles River laboratories, Canada; 15 from Harlan Sprague Dawley, USA). The animals were maintained as described in Experiment 1.

Drugs

Lofexidine HCl was generously supplied by Keith Davies, Britannia Pharmaceuticals Ltd., UK. The drug was dissolved in physiological saline and injected IP (0, 50, 100, 150, or 200 μg/kg).

Procedure

As in Experiment 1, a *within-days extinction and testing* reinstatement procedure, described in detail in the General Methods (Procedures; Chapter 1), was used. On Day 1 of extinction and testing, animals were given four 1-hour extinction sessions separated by 60-minute intervening periods. On Days 2 to 4, when testing occurred animals received 1-hour self-administration sessions with 60-minute intervening periods until all animals responded 15 or fewer times on the active lever in a 1-hour session; this criterion was reached within two to three sessions.

Separate groups of animals were pretreated with either 0, 50, 100, 150, or 200 μg/kg, IP, lofexidine before each of three tests for reinstatement (saline, footshock,

cocaine), given on consecutive days and in a counterbalanced order. See the General Methods (Procedure: Chapter 1) for a detailed description of the test conditions. Lofexidine was injected 60 minutes before the insertion of the lever. The doses of lofexidine were chosen on the basis of results obtained in an experiment that was conducted to assess the effects of the drug on high rates of responding for sucrose. In that experiment, it was found that 80 to 200 μg/kg lofexidine was without effect, when compared with the vehicle condition, on the number of responses made for sucrose. At the highest dose, animals made approximately 100 responses in 30 minutes for a 30 percent sucrose solution.

Statistical analyses

Test condition (saline, footshock, and cocaine) was a within-subjects factor; dose of lofexidine (0, 50, 100, 150, or 200 μg/kg) was a between-subjects factor. Data were analyzed using the non-parametric tests for related (Wilcoxon) and non-related (Kruskal-Wallis and Mann-Whiney) samples.

RESULTS AND DISCUSSION

Training and Extinction

The mean (±SEM) number of cocaine infusions made in the 3-hour session on the last two days of training was 30.07 (±2.37) and 30.91 (±2.06), respectively. On Day 1 of extinction, the mean (±SEM) number of responses made in the first three 1-hour extinction sessions (infusions + time out responses) was 31.32 (±3.22), 16.84 (±3.29),

and 16.23 (±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

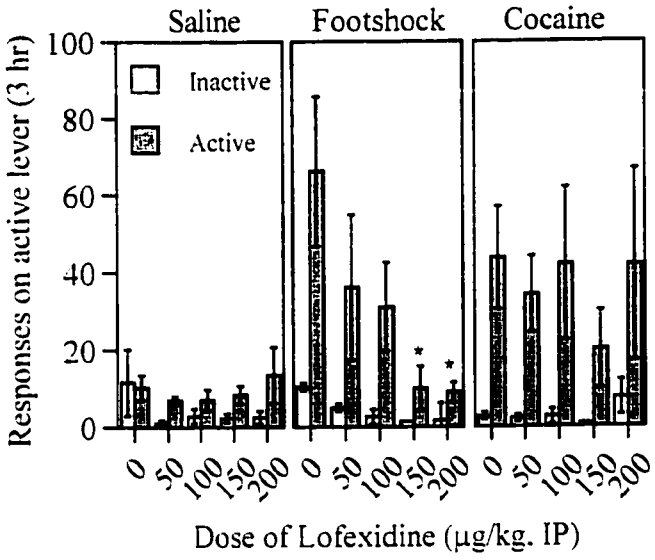
Figure 11 (*top*) shows the number of responses made on the active and inactive levers during each 3-hour test for reinstatement following pretreatment with vehicle (0 µg/kg) or lofexidine. Figure 11 (*bottom*) shows the number of responses on the active lever during each hour of testing. It can be seen that both footshock and priming injections of cocaine induced reinstatement of cocaine seeking. Only the effect of footshock was attenuated by lofexidine. This is confirmed by the Kruskal-Wallis tests conducted for the 3-hour tests for reinstatement where the only significant effect was found in the *Footshock* condition (χ^2 [4]=10.50, p<.05). Subsequent Mann-Whitney comparisons revealed a significant difference between the 0 and 150 µg/kg and 0 and 200 µg/kg lofexidine doses (ps<.05); furthermore, the response to footshock after 150 and 200 µg/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 (χ^2 [4]=17.31, p<.01, *Footshock*), when all doses of lofexidine blocked the footshock effect (ps<.05).

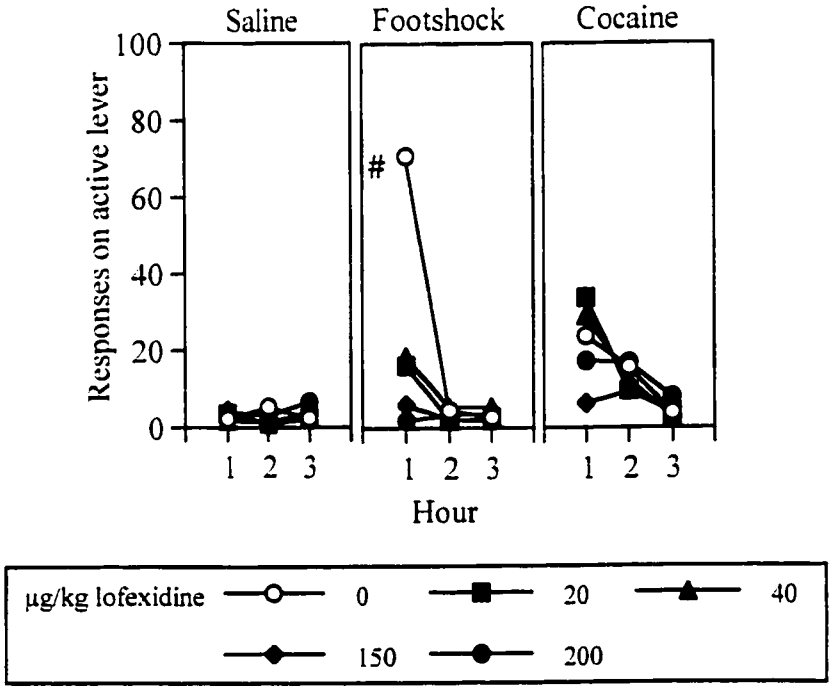
Thus, the effect of clonidine on stress-induced reinstatement observed in Experiment 1 generalized to another alpha-2 adrenergic-receptor agonist, lofexidine. Furthermore, like clonidine, lofexidine was without effect on the reinstatement of cocaine seeking induced by a priming injection of cocaine. The fact that lofexidine was as

Figure 11 *Systemic lofexidine: Top,* Mean (±SEM) number of responses (infusions + timeout responses) on the inactive and previously active levers in the 3-hour tests for reinstatement after *Saline* (physiological saline, 1.0 ml/kg, IP; 5 min before the start of the session), *Footshock* (intermittent footshock, 15 min; 0.6 mA; 0.5 sec on; mean off period of 40 sec; immediately before the start of the session), and *Cocaine* (20 mg/kg, IP; 5 min before the start of the session). *Bottom,* Mean number of responses (saline infusions + time-out responses) on the active lever in each hour of each of the 3-hour tests for reinstatement. 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

* Different from vehicle (0) condition: ps<.05.

\# Different from other doses, Hour 1, *Footshock* condition: ps<.05.


Saline
Footshock
Cocaine
Inactive
Active
Responses on active lever (3 hr)
100
80
60
40
20
0
0 50 100 150 200 0 50 100 150 200 0 50 100 150 200
Dose of Lofexidine (µg/kg, IP)


Saline
Footshock
Cocaine
Responses on active lever
100
80
60
40
20
0
#
1 2 3 1 2 3 1 2 3
Hour
µg/kg lofexidine
0
20
40
150
200

effective as clonidine in preventing the footshock-induced reinstatement of cocaine seeking, is of potential clinical significance in that, as mentioned, lofexidine is associated with fewer adverse side effects in humans than is clonidine (Carnwath & Hardman, 1998; Kahn et al., 1997; Lin et al., 1997).

EXPERIMENT 3: EFFECTS OF GUANABENZ ON FOOTSHOCK- AND COCAINE-INDUCED REINSTATEMENT

It is possible that the effects of clonidine and lofexidine observed in Experiments 1 and 2 could have been mediated through their actions at imodazoline type-1 (I_1) receptors rather than alpha-2 receptors. The nM affinity of clonidine for the alpha-2 receptor is 28±2.6 and for the I_1 receptor is 0.99±0.43 (Ernsberger, Giuliano, Willette, & Reis, 1990); the affinity of lofexidine for these receptors is almost identical (Buccafusco, Lapp, Westbrooks, & Ernsberger, 1995). Alpha-2 and I_1 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, Halaris, & Ernsberger, 1994). For example, in drug discrimination studies, alpha-2 receptors appear to be responsible for a clonidine-induced cue, for which other alpha-2 adrenergic-receptor agonists, including lofexidine, substitute dose-dependently (Bennett & Lal, 1982; Jordan, Jackson, Nutt, & Handley, 1993; Lal & Yaden, 1985). On the other hand, I_1 receptors of the medulla are thought to mediate the antihypertensive actions of these drugs (Buccafusco et al., 1995; but see Guyenet, 1997).

The present experiment was conducted to attempt to clarify the contribution of alpha-2 versus I_1 receptors in the effects observed with clonidine and lofexidine in Experiments 1 and 2. In this experiment, the effects of guanabenz, an alpha-2 adrenergic-receptor agonist with high affinity for the alpha-2 receptor (K_i=7.2±0.6 nM) but only very low affinity for the I_1 receptor (K_i= >1,000,000nM) (Buccafusco et al., 1995), on the footshock-induced reintatement of cocaine seeking were assessed. It was hypothesized that if I_1 receptors mediate the effects of clonidine and lofexidine, then guanabenz, which has very low affinity for the I_1 receptor, should not interfere in the footshock-induced reinstatement of cocaine seeking. If, on the other hand, the effects of clondine and lofexidine are in fact due to the actions of these drugs at the alpha-2 receptor, then guanabenz should also be effective in blocking the footshock-induced reinstatment of cocaine seeking.

MATERIALS AND METHODS

Subjects

The subjects were 18 male Long-Evans rats (350-400g; Charles River laboratories, Canada). 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 IP (0, and 640 μg/kg).

Procedure

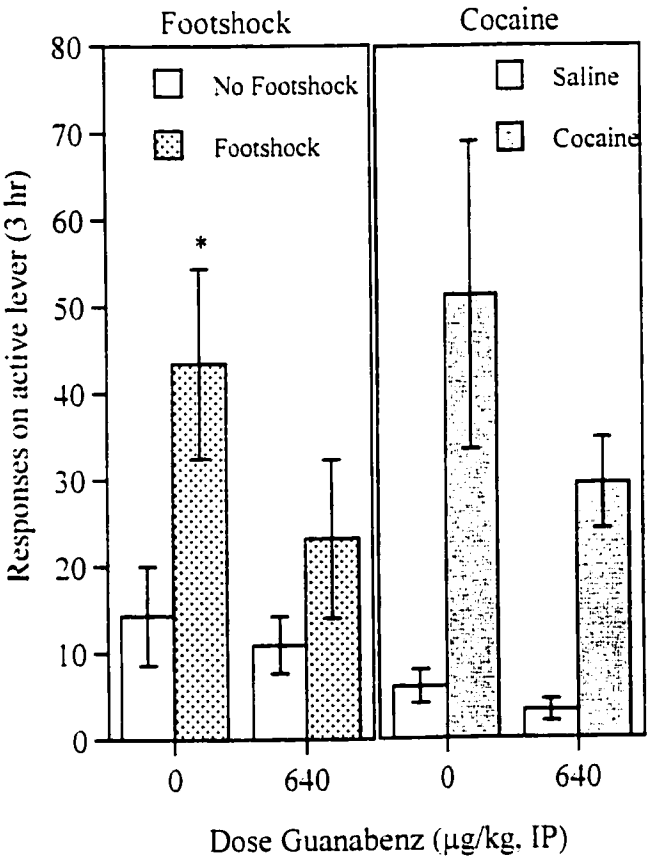
Animals were trained to self-administer cocaine under the conditions described in Experiment 1. On Day 1 of Phase 2, animals were given four 1-hour self-administration sessions separated by 60-minute periods. On Days 2 to 4, animals received 1-hour self-administration sessions with 60 minute intervening periods until all animals responded 15 or fewer times on the active lever in a 1-hour session; this criterion was reached within two to three sessions.

All animals were pretreated with 0 or 640 μg/kg guanabenz, IP, before each of two tests for reinstatement (no footshock, footshock or saline, cocaine), given on consecutive days and in a counterbalanced order. See the General Methods (Procedure: Chapter 1) for a detailed description of the test conditions. Guanabenz was injected 60 minutes before the insertion of the lever. The dose of guanabenz was chosen on the basis of its substitutability for 40 μg/kg clonidine in a drug discrimination (Bennett & Lal, 1982).

Statistical analyses

Test condition (no footshock and footshock or saline and cocaine) was a within-subjects factor; dose of guanabenz (0 or 640 μg/kg) was a within-subjects factor for animals tested in the no footshock and footshock conditions and a between-subjects factor for animals tested in the saline and cocaine conditions. Data were analyzed using the non-parametric tests for related (Friedman and Wilcoxon) and non-related (Kruskal-Wallis and Mann-Whiney) samples.

Figure 12 *Systemic guanabenz:* Mean (±SEM) number of responses (infusions + time out responses) on the previously active lever during the 3-hour tests for reinstatement after *No Footshock* and *Footshock* (intermittent footshock, 15 min; 0.6 mA; 0.5 sec on; mean off period of 40 sec; immediately before the start of the session) or *Saline* (physiological saline, 1.0 ml/kg, IP; 5 min before the start of the session) and *Cocaine* (20 mg/kg, IP; 5 min before the start of the session). Rats were pretreated with 0 or 640 µg/kg guanabenz.
* Different from other *Footshock* conditions: ps<.05.


Footshock
Cocaine
No Footshock
Footshock
Saline
Cocaine
*
Responses on active lever (3 hr)
80
70
60
50
40
30
20
10
0
0
640
0
640
Dose Guanabenz (µg/kg, IP)

RESULTS AND DISCUSSION

Figure 12 shows the number of responses made on the active lever in the *No Footshock, Footshock, Saline,* and *Cocaine* tests for reinstatement after pretreatment with either 0 or 640 μg/kg guanabenz. It can be seen that guanabenz blocked the effect of footshock, but had no effect on cocaine-induced reinstatement. A Friedman analysis on the *Footshock* and *No Footshock* tests after pretreatment with vehicle (0) or guanabenz 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 μg/kg) condition differed from the others ($p< .05$). Thus, guanabenz had a similar effect on the footshock-induced reinstatement of cocaine seeking to that of clonidine and lofexidine. The fact that guanabenz, an alpha-2 adrenergic-receptor agonist with very low affinity for the I_1 receptor, was effective makes it appear unlikely that the attenuation of the footshock-induced reinstatement of cocaine seeking by this compound, or by clonidine or lofexidine, was I_1-receptor mediated.

EXPERIMENT 4: EFFECTS OF SYSTEMIC INJECTIONS OF ST-91 ON FOOTSHOCK-INDUCED RELAPSE TO COCAINE SEEKING

It is possible that clonidine, lofexidine, and guanabenz blocked the footshock-induced reinstatement of cocaine seeking via their actions at peripheral rather than central NE receptors. Experiment 4 was conducted to addresss this possibility. In this experiment, the effects of systemic injections of ST-91 on the footshock-induced reinstatement of cocaine seeking were assessed. ST-91 is a charged analog of clonidine

that has a very low capacity to cross the blood brain barrier. Blockade of footshock-induced reinstatement by this compound would, therefore, suggest a peripheral site of action of alpha-2 adrenergic-receptor agonists in preventing reinstatement by footshock; a lack of effect of ST-91, on the other hand, would support the view that the effects of the alpha-2 adrenergic-receptor agonists observed in Experiments 1 to 3 were mediated centrally.

MATERIALS AND METHODS

Subjects

The subjects were 16 male Long-Evans rats (350-400g; Charles River laboratories, Canada). The animals were maintained as described in Experiment 1.

Drug

ST-91 (2-[2,6-diethylphenylamino]-2-imidazole) was generously supplied by Boehringer Ingelheim (Ridgefield, CT) and was dissolved in physiological saline and injected IP (0 and 40 μg/kg).

Procedure

Animals were trained to self-administer cocaine under the conditions described in Experiment 1. On Day 1 of Phase 2, animals were given four 1-hour self-administration sessions separated by 45-minute intervening periods. On Days 2 to 4, animals received

1-hour self-administration sessions with 45 minute intervening periods until all animals responded 15 or fewer times on the active lever in a 1-hour session; this criterion was reached within two to three sessions.

Different groups of animals were pretreated with 0 or 40 μg/kg ST-91, IP, before each of two tests for reinstatement (no footshock, footshock), given on consecutive days and in a counterbalanced order. See the General Methods (Procedure: Chapter 1) for a detailed description of the test conditions. ST-91 was injected 45 minutes before the insertion of the lever.

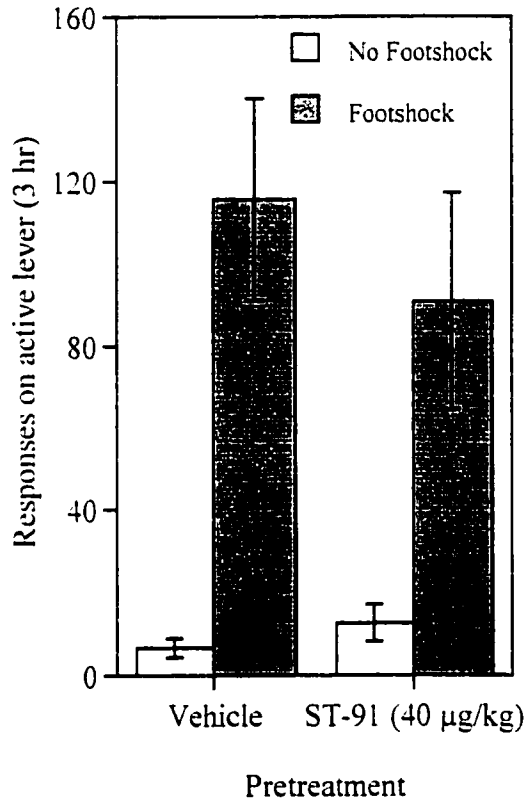
Statistical analyses

Test condition (no footshock and footshock) was a within-subjects factor; dose of ST-91 (0 or 40 μg/kg) was a between-subjects factor. Data were analyzed using the non-parametric tests for related (Wilcoxon) and unrelated (Mann-Whiney) samples.

RESULTS AND DISCUSSION

Figure 13 shows the number of responses made on the active lever in the *No Footshock* and *Footshock* tests for reinstatement after pretreatment with either 0 or 40 μg/kg ST-91. It can be seen that animals in both pretreatment groups showed a comparable magnitude of footshock-induced reinstatement. A Mann-Whitney comparison conducted between pretreatment groups in the *Footshock* condition was not significant ($U=24.0$, $p=0.44$). A Wilcoxon test conducted between the *No Footshock* and

Figure 13 *Systemic ST-91:* Mean (=SEM) number of responses (infusions + time out responses) on the previously active lever during the 3-hour tests for reinstatement after *No Footshock* and *Footshock* (intermittent footshock, 15 min: 0.6 mA; 0.5 sec on; mean off period of 40 sec: immediately before the start of the session) tests for reinstatement. Rats were pretreated with 0 or 40 µg/kg ST-91.


160
120
80
40
0
No Footshock
Footshock
Responses on active lever (3 hr)
Vehicle
ST-91 (40 µg/kg)
Pretreatment

Footshock conditions was significant ($Z=2.98$, $p< .05$). Since systemic injections of ST-91 did not interfere in the footshock-induced reinstatement of cocaine seeking, it would appear unlikely that activation of peripheral alpha-2 receptors mediates the effects of clonidine, lofexidine, and guanabenz on footshock-induced relapse.

GENERAL DISCUSSION (Chapter 3)

Three major findings emerge from the present series of experiments. First, systemic injections of clonidine and lofexidine attenuated the footshock-induced reinstatement of cocaine seeking, but had 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 attenuated footshock-induced reinstatement, but had no effect on reinstatement induced by priming injections of cocaine. Third, systemic injections of ST-91, the charged analog of clonidine with a low capacity to cross the blood brain barrier, did not interfere in the footshock-induced reinstatement of cocaine seeking.

The finding that clonidine, lofexidine, and guanabenz attenuated footshock- but not cocaine-induced reinstatement was not unexpected in view of previous findings, including those presented in Chapter 1, showing that stress-induced and drug-induced relapse can be dissociated pharmacologically. In Chapter 1 data were presented showing that CRF-receptor antagonists block footshock-induced, but not cocaine-induced relapse. Likewise, it has been reported that CRF-receptor antagonists strongly attenuate footshock-induced reinstatement of heroin seeking but have only modest effects on

heroin-induced reinstatement (Shaham et al., 1998; Shaham et al., 1997b). From these data it seems logical to consider that footshock may have its effects on reinstatement through an interaction between the CRF and NE systems. Although the issue of where in the brain this interaction might take place has not yet been addressed directly, studies reported in a paper by Shaham and colleagues (in press) bear on this question. After showing that either systemic or ICV injections of clonidine blocked stress-induced reinstatement of heroin seeking, they administered clonidine or ST-91 directly into the LC and found that it was ineffective. In contrast, 6-OHDA lesions of the ventral NE pathway attenuated footshock-induced reinstatement of heroin seeking, suggesting that clonidine acts through NE neurons of the ventrolateral tegmental nuclei of the pons and medulla, and not the LC, to interfere in footshock-induced reinstatement.

As mentioned previously, whereas footshock-induced reinstatement may be mediated by the NE and CRF systems, the effects of priming injections of drugs on relapse could potentially involve an interaction between NE and midbrain DA systems. In the experiments presented here, however, manipulations of NE systems were without any effect on the cocaine-induced reinstatement of cocaine seeking. It is interesting to note that systemic injections of clonidine have been shown to interfere in heroin-induced reinstatement of heroin seeking (Y. Shaham, unpublished data). These differential effects of clonidine in cocaine- and heroin-trained rats may reflect differences in the pharmacological actions of the two drugs. In the face of cocaine-induced blockade of DA reuptake, any changes in DA transmission induced by clonidine (see, for example, Grenhoff & Svensson, 1989; Lategan et al., 1990; Murai et al., 1998) may not have been sufficient to prevent increases in extracellular DA in the nucleus accumbens. It is also

possible that previous exposure to cocaine during the self-administration phase of the study served to increase DA availability in the nucleus accumbens, thereby increasing sensitivity to a priming injection of the drug (Kalivas & Duffy, 1993a). In the case of heroin, on the other hand, which acts in the VTA to increase DA cell firing, changes in DA transmission induced by clonidine may have been sufficient to interfere in heroin-induced reinstatement of heroin seeking.

Two 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, Press, & Raffa, 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. Sucrose-trained rats have been shown to respond nearly 100 times in 20 minutes following injection of 40 μg/kg clonidine and 100 times in 30 minutes following 200 μg/kg lofexidine (Erb et al., Submitted; Shaham et al., in press).

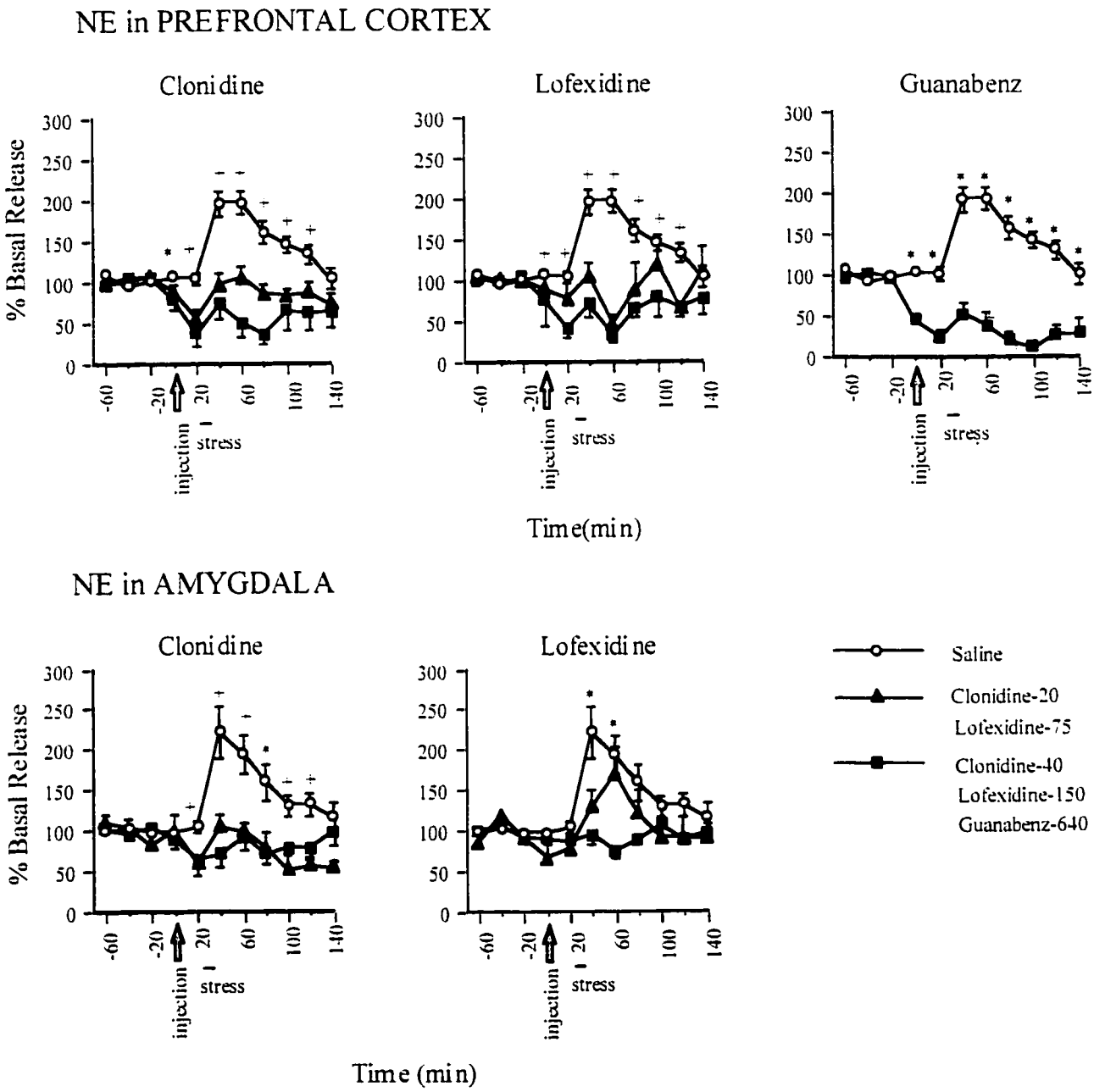
The possibility that the effects observed in the present study were due to the actions of the alpha-2 adrenergic-receptor agonists at peripheral rather than central

receptors also seems unlikely since systemic injections of ST-91 did not interfere in the footshock-induced reinstatement of cocaine seeking (Experiment 4). In addition, in a study conducted with heroin-trained rats it was found that ICV injections of clonidine were as effective as systemic injections in blocking footshock-induced reinstatement (Shaham et al., in press). 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 & Engel, 1988).

Using in vivo microdialysis and similar dose and footshock parameters to those used in the present experiments, it has been shown that clonidine, lofexidine, and guanabenz are all effective in suppressing footshock-induced rises in NE in the prefrontal cortex and the AMG (see Figure 14, in Erb et al., Submitted). Although these results show that at the doses used in the tests for reinstatement the alpha-2 adrenergic-receptor agonists are effective in blocking stress-induced rises in NE, it should be pointed out that the animals used in this study were drug naïve. It is possible that a history of drug-taking could sensitize the NE response to footshock and, conceivably, alter the effectiveness with which alpha-2 adrenergic-receptor agonists block footshock-induced rises in NE;

Figure 14 *Microdialysis - Prefrontal cortex and AMG:* Mean (±SEM) NE concentrations in 20 min dialysate samples in prefrontal cortex and amygdala. Animals were injected with either clonidine (0, 20, or 40 μg/kg, IP), lofexidine (0, 75, or 150 μg/kg, IP), or guanabenz (0 or 640 μg/kg, IP) at Time 0, and 40 min later were exposed to 10 minutes of intermittent footshock stress (0.6 mA). Significant Time by Dose interactions were obtained for the prefrontal cortex and AMG after clonidine ($F(16,192)=4.40$, $p<.0.01$ and $F(16,168)=2.03$, $p<0.03$, respectively), lofexidine ($F(16,168)=5.09$ $p<.0.01$ and $F(16,192)=2.09$, $p<.03$, respectively), and guanabenz ($F(8,120)=6.22$, $p<.001$).
* Different from high dose only; $ps<.05$.
+ Different from both other doses; $ps<.05$.


NE in PREFRONTAL CORTEX
Clonidine
Lofexidine
Guanabenz
% Basal Release
300
250
200
150
100
50
0
-60
-20
20
60
100
140
injection
stress
Time(min)
NE in AMYGDALA
Clonidine
Lofexidine
% Basal Release
Time (min)
Saline
Clonidine-20
Lofexidine-75
Clonidine-40
Lofexidine-150
Guanabenz-640

this is a possibility that is explored in Chapter 4. Nevertheless, the fact that the drugs were effective behaviorally, suggests that suppression of NE release and/or inhibition of NE cell firing may underlie the effects of the alpha-2-receptor agonists in the present experiments.

Clearly, the microdialysis and reinstatement test data are correlative. Therefore, any interpretations of one set of data derived from the other must be considered cautiously. Indeed, it is possible that the effects of the alpha-2 adrenergic-receptor agonists observed in the present series of experiments were in fact due to their actions at postsynaptic alpha-2 receptors, rather than at autoreceptors. There is evidence that alpha-2 adrenergic-receptor agonists have biochemical and behavioral effects via actions at postsynaptic receptors (Fendt, Koch, & Schnitzle, 1994; Mongeau, Blier, & de Montigny, 1997; Taylor, Punch, & Elsworth, 1998). It seems unlikely, however, that the effects observed here were in fact due to postsynaptic actions since higher doses of the compounds than those used in the present experiments are generally needed to activate the less-sensitive postsynaptic NE receptors (see Cooper, Bloom, & Roth, 1996).

It cannot be said at this point what brain sites or circuitry contribute to the effects of alpha-2 adrenergic receptor agonists on the footshock-induced reinstatement of cocaine seeking. Based on the finding, however, that injections of clonidine or ST-91 into the cell body region of the dorsal NE bundle (i.e., the LC) do not interfere with footshock-induced reinstatement of heroin seeking (see Shaham et al., in press), it would appear that the prefrontal cortex, whose sole source of NE input is the LC (Korf, Aghajanian, & Roth, 1973), is unlikely to play a critical role. The AMG and BNST, on

the other hand, also receive projections from the lateral tegmental NE neurons via the ventral NE bundle (Aston-Jones et al., 1995; Moore & Bloom, 1979). This observation, in combination with findings implicating these areas in a number of physiological and behavioral responses to stress (Davis, Walker, & Lee, 1997; Lee & Davis, 1997), suggests that they may be part of the circuitry underlying stress-induced relapse to drug use. Indeed, the BNST was found to be an important site for the action of the CRF-receptor antagonist, D-Phe CRF12-41, in the blockade of stress-induced reinstatement of cocaine seeking (Chapter 1, Experiment 4). A possible interaction between NE and CRF systems in the BNST and AMG will be considered more closely in the next chapter.

CHAPTER 4

A GENERAL DISCUSSION

On the basis of the data presented in preceding chapters, two general conclusions can be made concerning the neurobiological substrates of footshock- and cocaine-induced relapse to cocaine seeking. First, both CRF and NE are important systems underlying the footshock-induced reinstatement of cocaine seeking. Second, the pathways underlying footshock- and cocaine-induced reinstatement are not identical, inasmuch as manipulations of CRF and NE systems that completely blocked the effects of footshock stress were with little or no effect on reinstatement induced by priming injections of cocaine.

The focus of the following discussion will be on characterizing, behaviorally and neurobiologically, the effect of footshock on the reinstatement of drug seeking. In addition, future research directions and possible implications of the findings for treatment will be discussed.

THE PHENOMENOLOGY OF FOOTSHOCK-INDUCED RELAPSE TO DRUG SEEKING

Before considering what neurobiological pathways might underlie footshock-induced reinstatement, it would seem important to address more generally the question of why footshock reinstates drug seeking. This is a complex question and, as yet, one can only speculate about possible answers. It is known that the phenomenon is one that is quite specific to drug seeking. As mentioned before, footshock does not reinstate food seeking (Ahmed & Koob, 1997) and although there is some evidence that it reinstates

sucrose seeking, this effect is much weaker than for the reinstatement of drug seeking (Buczek et al., 1999; Lê et al., 1998). Likewise, it does not appear that the effect of footshock reflects a generalized state of arousal in the animal; for example, sexual arousal does not induce the reinstatement of heroin seeking (Shaham, Puddicombe, & Stewart, 1997c). Another unlikely explanation is that footshock reinstates drug seeking by inducing a fear state in the animal. Using fear conditioning procedures, it has been found that reinstatement is not induced by a discrete conditioned stimulus (CS) previously paired with footshock, nor is it induced by exposure to predator (fox) odor (S. Erb, Y. Shaham, & J. Stewart, unpublished).

An answer to the question of why footshock reinstates drug seeking may be found in a consideration of the nature of contextual conditioning. During the training and extinction phases of the experiments reported here, the animal learns at least two different things in the self-administration context. First, during the training phase, it learns that in this context the presentation of a lever into the self-administration chamber and simultaneous 30-second illumination of a white light above that lever signals the availability of drug reinforcement (unconditioned stimulus; US), contingent upon pressing the lever. Thus, during training, excitatory conditioning occurs to the combined lever and white light CS (recall that each lever press also results in illumination of the white light for the 10 or 20 second drug infusion period). During the extinction phase, on the other hand, the animal learns that in this context the presentation of a lever into the self-administration chamber and simultaneous 30-second illumination of a white light above that lever signals the non-availability of response-contingent drug reinforcement

(i.e., absence of US). Thus, following extinction, the lever-light CS has two meanings for the animal.

It has been argued that non-contingent re-exposure to a US, following extinction of conditioned responding to a discrete CS with which the US had been paired previously, can condition "excitation" to the context in which re-exposures are presented and, thereby, "reinstate" conditioned responding to the CS (Bouton & Peck, 1989). Such an analysis can explain reinstatement of drug seeking following priming injections of the self-administered drug and may also provide an explanation for reinstatement following exposure to footshock. Albeit a very different stimulus from the drug US, footshock might also serve to condition contextual excitation and, thereby, interefere in inhibitory processes associated with extinction (see also Bouton & Swartzentruber, 1991).

Another way to look at this might be to say that footshock reinstates drug seeking by "disinhibiting" neuronal processes underlying extinction (Bouton & Swartzentruber, 1991). Indeed, extinction is considered an active inhibitory process whereby animals learn to suppress a behavior when reinforcers are no longer available (Pavlov, 1927). In support of a disinhibition hypothesis, there are recent data showing that temporary blockade of neuroconduction by infusion of the neurotoxin tetrodotoxin into the medial septum, a region known to be involved in inhibitory responses (see, for example, Gray & McNaughton, 1983; Grossman, 1977), mimics the effect of footshock on reinstatement and that presentation of footshock before extinction sessions increases resistance to extinction (Highfield et al., submitted).

Another possible explanation for footshock-induced reinstatement, that also focuses on the nature of contextual conditioning, comes from so-called *renewal* studies. Bouton and his collaborators (Bouton, 1993; Bouton, 1988; Bouton & Peck, 1989) have demonstrated that the effectiveness of an extinguished, but not non-extinguished, excitatory CS is highly sensitive to context. Using *renewal* procedures, they found that if excitatory appetitive or aversive conditioning occurs in one context (Context A) and extinction occurs in a different context (Context B), then returning the animal to context A *renews* responding to the CS. On the other hand, presentations of a non-extinguished CS in a context different from the original conditioning context has little or no effect on responding to the CS (Bouton & Peck, 1989). These findings have led to the argument that contextual cues do not simply summate with the CS during conditioning to become part of a complex of conditioned stimuli (a possibility that would predict a change in responding to a non-extinguished CS with a change in context), but rather, contextual cues determine the meaning of an ambiguous CS. Thus, when the animal is returned to Context A following extinction in Context B, Context A signals retrieval of the original CS-US association.

Although in the experiments reported here, training and extinction took place in the same physical context, it is important to note that training occurred in the presence of drug (in that the animal was engaging in drug self-administration). It would seem reasonable to suppose, therefore, that the drug could have altered the perception and meaning of the environment to the animal to create an internal state or context that was distinct from the extinction context. Indeed, it has been shown that altering an animal's internal state between conditioning and extinction can serve to create contexts that are

psychologically different from one another. For example, animals that undergo fear conditioning in a sober state and extinction in an intoxicated state show renewed responding to the CS if tested in a sober state (Cunningham, 1979). It is not immediately clear how such differences in internal state between training and testing could explain the ability of footshock stress to reinstate drug seeking behavior. One possibility is that footshock, an excitatory stimulus, induces an internal state that is more compatible with that present during training than extinction. A difficulty with this explanation is that the findings of the present experiments, and those of other experiments (e.g., Shaham et al., 1997b; Shaham et al., 1996; Shaham & Stewart, 1996), show that footshock does not mimic the effect of drug on the reinstatement of drug seeking; that is, different neurobiological mechanisms underlie footshock- and drug-induced reinstatement. An alternative explanation is that footshock, itself, induces a sufficient change in context to make it distinct from both the training and extinction contexts. Indeed, in renewal experiments, it has been shown that if animals undergo conditioning in Context A and extinction in Context B, renewed responding to the CS is exhibited if animals are tested in a third context, i.e., Context C (see Bouton, 1988).

An explanation of footshock-induced reinstatement of drug seeking based on contextual conditioning hypotheses would need to account for findings that, relative to animals trained to self-administer drug, footshock stress induces no or comparatively weak reinstatement in animals trained to self-administer non-drug reinforcers (Ahmed & Koob, 1997; Buczek et al., 1999; Lê et al., 1998). One explanation for this discrepancy may be that the long-term neuronal changes induced by chronic drug exposure in self-administering animals act to effectively lower the threshold required for footshock to

induce disinhibition of responding. Alternatively, if the hypothesis is true that exposure to footshock at test creates a context that is distinct from the training and extinction contexts, a different explanation can be posited. Although it has been found that fear conditioning in Context A and extinction in Context B leads to renewed responding to the fear-eliciting CS in Context C (i.e., A, B, C), little or no renewed responding in Context C occurs if both excitatory conditioning and extinction occur in the *same* context (i.e., A, A, C) (see Bouton, 1988). From this perspective one could argue that, as in the case of a fear conditioning preparation, self-administration of food or sucrose, unlike drug, does not induce a sufficient change in an animal's internal state to render the training and extinction contexts distinct. Thus, although footshock may create a distinct context at test, it is possible that the stressor does not renew responding for non-drug reinforcers because training and extinction were carried out in the *same* context.

The phenomenology of the footshock-induced reinstatement of drug seeking is at present not well understood. The possibility, however, that contextual conditioning plays an important role in the phenomenon is one that warrants further investigation.

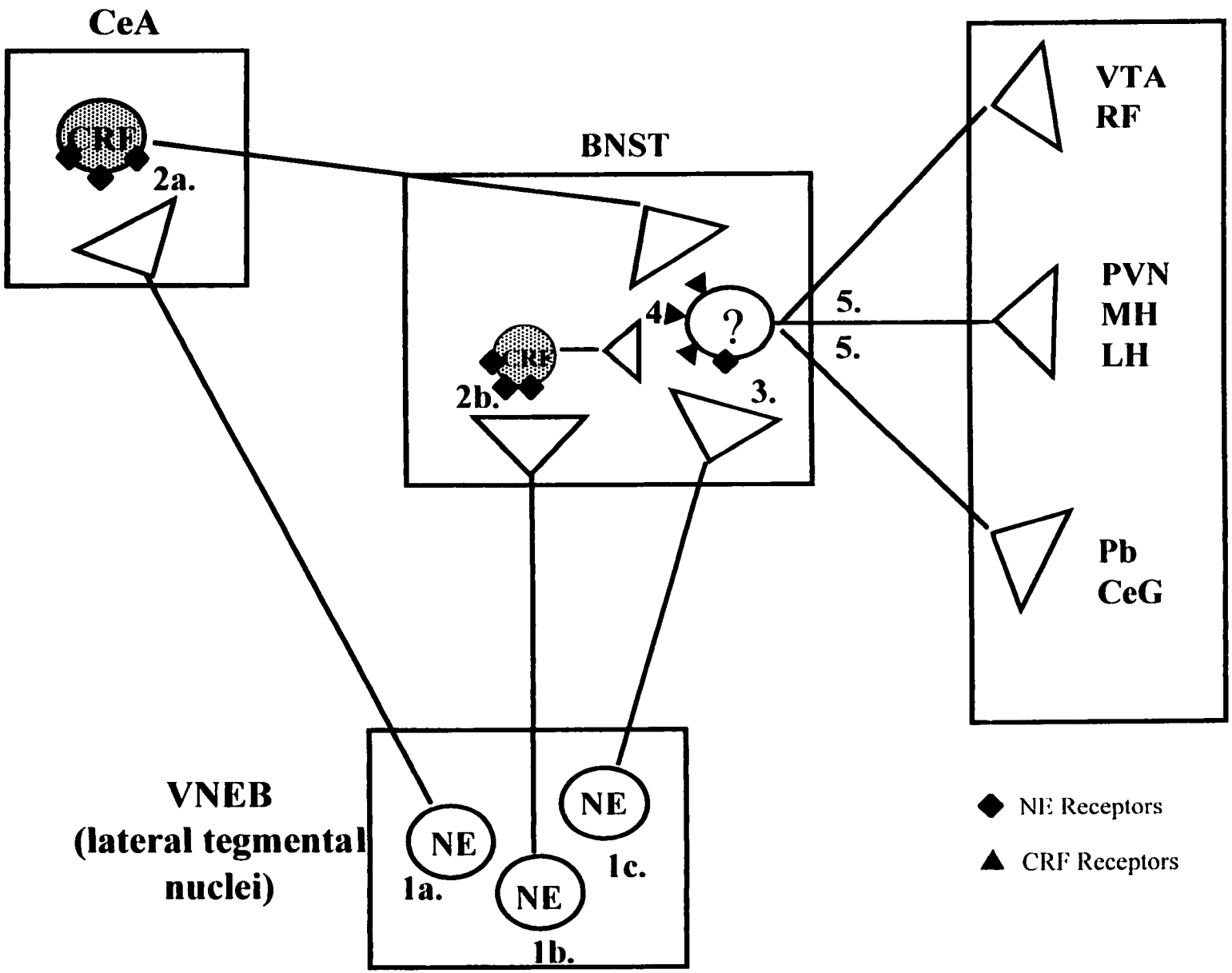
PATHWAYS TO STRESS-INDUCED RELAPSE

A summary of the major findings presented in preceding chapters is a useful starting point for speculating about pathways that might be involved in the effects of footshock on relapse. First, it was found that ICV administration of a CRF-receptor antagonist completely blocked the footshock-induced reinstatement of cocaine seeking and that the BNST, but not the AMG, was an important site of action for this effect. Second, it was found that several alpha-2 adrenergic receptor agonists, that act to inhibit

NE cell firing and release (Aghajanian & VanderMaelen, 1982; Carter, 1997), also blocked the footshock-induced reinstatement of cocaine seeking, suggesting an important role for NE in the effects of footshock on relapse. Thus, the ventral NE bundle, which provides a major input to the BNST and the CeA (Aston-Jones et al., 1995; Moore & Bloom, 1979), appears to play an important role in mediating the effects of footshock stress on relapse (Shaham et al., in press).

On the basis of these and other findings (see below), it can be hypothesized that the footshock-induced reinstatement of drug seeking involves an interaction between CRF and NE systems within a circuitry referred to as the extended AMG, of which the CeA and BNST are considered primary structures (de Olmos & Heimer, 1999). A preliminary model of the neurobiology of footshock-induced relapse to drug seeking that is based on this hypothesis is presented in Figure 15. According to this model, there are two sites of interaction between NE and CRF systems, the BNST and CeA, that may be of functional importance for stress-induced relapse. It is proposed that following exposure to footshock stress, NE neurons of the ventral NE bundle are activated (no. 1a-c), inducing release of NE in the CeA and BNST (no. 2a, 2b, and 3). Subsequently, NE, acting via receptors on CRF-containing cell bodies in the CeA and/or BNST (no. 2a and b), induces the release of CRF in the BNST (no. 4). CRF then acts at receptors located on efferent neurons of the BNST that are, as yet, unidentified (see no. 5 for possible targets of BNST efferents) to initiate the behaviors involved in relapse. It is also possible that NE released in the BNST acts directly on the same efferent neurons acted upon by CRF to facilitate relapse (no. 3).

Figure 15. A model of the neurobiology of stress-induced relapse. It is proposed that in response to footshock stress, NE neurons of the ventral NE bundle (VNEB) are activated (no. 1 a-c). Subsequently, NE is released in the CeA (no. 2a) and/or the BNST (no. 2b) where it interacts at receptors located on CRF cell bodies. CRF released in the BNST (no. 4) then acts on neurons in the BNST, that are as yet unidentified (no. 5), to initiate the behaviors involved in relapse. It is also possible that NE released in the BNST acts directly on the same neurons acted upon by CRF (no. 3). As depicted in the diagram, possible targets of CRF in the BNST are the retrorubral field (RF), ventral tegmental area (VTA), lateral hypothalamus (LH), medial (MH) hypothalamus, and various brainstem regions, including the central gray (CeG) and parabrachial nucleus (Pb).


CeA
CRF
2a.
BNST
VTA
RF
PVN
MH
LH
Pb
CeG
4
?
5.
5.
CRF
2b.
3.
VNEB
(lateral tegmental
nuclei)
NE
1a.
NE
1b.
NE
1c.
NE Receptors
CRF Receptors

In order to evaluate the plausibility of the general model just summarized, and of the individual mechanisms of interaction between NE and CRF neurons depicted in Figure 15, several questions must be addressed in detail. First, what is the evidence that NE and CRF cells can and do interact within the extended AMG? Second, if NE and CRF cells do interact within the extended AMG, what is the nature of this interaction? Third, on what efferent pathways of the extended AMG, in particular of the BNST, might CRF act to initiate the behaviors involved in relapse? Before discussing these questions, however, two additional questions will be addressed in order to provide a general context for the model. These questions are, 1) what is the extended AMG? and 2) how is the extended AMG organized and what is the source of CRF release in the BNST following stress?

What is the extended AMG?

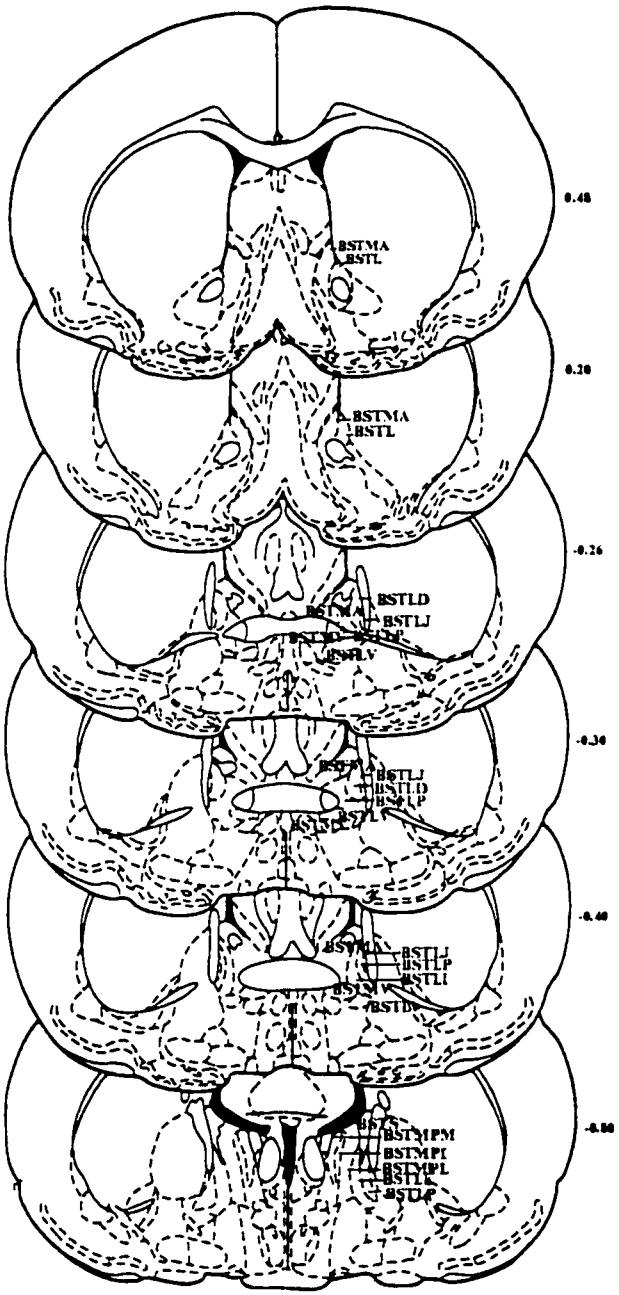
The concept of the extended AMG is a controversial one because it is difficult to define anatomically and because of its questionable usefulness conceptually (see, for example, de Olmos & Heimer, 1999). In general, the extended AMG is represented as a neuronal continuum, based on similarities in regional cytoarchitecture and histochemistry, that extends from the AMG to the BNST, the ventral region of the substantia innominata, and possibly the shell of the nucleus accumbens (Alheid & Heimer, 1988; de Olmos & Heimer, 1999; Kelley, 1999). Particular attention has been paid to similarities that exist between the CeA and BNST, considered the major components of the extended AMG. The CeA and BNST share similarities in extrinsic

connectivity; both structures have a number of efferent and afferent projections in common, including strong reciprocal connections with the parabrachial nucleus (Arluison et al., 1994; Moga, Saper, & Gray, 1989; Veening, Swanson, & Sawchenko, 1984) and the nucleus of the solitary tract (Gray & Magnusson, 1987; Ricardo & Koh, 1978). The CeA and BNST also express similar neuropeptides, including CRF, enkephalin, neurotensin, somatostatin, and Substance P (e.g., Arluison et al., 1994; Day, Curran, Watson, & Akil, 1999; Gray & Magnuson, 1992). Finally, striking similarities exist between the two structures in terms of their suborganization. Five major subdivisions have been identified in the CeA based on differences in cell morphology, organization of efferent projections, and neuropeptide localization. The organization of the BNST appears to be more complex, and delineation of the various subregions remains a controversial issue; however, similar differences in cell morphology, efferent projections, and histochemistry found in the different subregions of the CeA have also been identified in specific regions of the BNST (see Sun, Roberts, & Cassell, 1991). It should be noted that in the following discussion, the general mapping of the BNST that appears in the atlas of Paxinos and Watson (1997) will be followed (see Figure 16 for a diagram).

Organization of the extended AMG and potential sources of CRF release in the BNST following stress

There is an extensive system of intrinsic interconnections between the CeA and BNST and also between various subregions of the CeA (Cassell, Freedman, & Shi, 1999; Sun et al., 1991). Neuroanatomical and immunocytochemical studies have pointed to the inhibitory neurotransmitter, gamma aminobutyric acid (GABA), as the primary

Figure 16. Coronal sections showing the subregions of the bed nucleus of the stria terminalis; adapted from the atlas of Paxinos & Watson (1997). Values at right of figures represent mm from bregma. Abbreviations: **BSTIA** bed nucleus of the stria terminalis, intraamygdaloid division; **BSTL** bed nucleus of the stria terminalis, lateral division; **BSTLD** bed nucleus of the stria terminalis, lateral division, dorsal part; **BSTLI** bed nucleus of the stria terminalis, lateral division, intermediate part; **BSTLJ** bed nucleus of the stria terminalis, lateral division, juxtacapsular part; **BSTLP** bed nucleus of the stria terminalis, lateral division, posterior part; **BSTLV** bed nucleus of the stria terminalis, lateral division, ventral part; **BSTMA** bed nucleus of the stria terminalis, medial division, anterior part; **BSTMPI** bed nucleus of the stria terminalis, medial division, posterointermediate part; **BSTMPL** bed nucleus of the stria terminalis, medial division, posterolateral part; **BSTMPM** bed nucleus of the stria terminalis, medial division, posteromedial part; **BSTMV** bed nucleus of the stria terminalis, medial division, ventral part; **BSTS** bed nucleus of stria terminalis, supracapsular part.


0.48
BSTMA
BSTL
0.20
BSTMA
BSTL
-0.26
BSTLD
BSTLJ
-0.30
BSTLJ
BSTLD
-0.40
BSTLJ
BSTLP
BSTLI
BSTMPM
BSTMPI
BSTMPL
-0.80

neurotransmitter of the extended AMG, acting to maintain neurons within this circuitry under strong, tonic inhibitory control (Sun & Cassel, 1993; Sun, Yi, & Cassell, 1994). Additionally, experiments using electron microscopy have shown that these intrinsic connections serve as an interface between the inputs and outputs of the extended AMG (Sun et al., 1994).

The present discussion will focus on connections between the CeA and lateral BNST, regions that according to de Olmos and Heimer (1999) comprise the central extended AMG. Since the lateral BNST is the region into which injections of CRF and its receptor antagonist were made and were effective (Chapter 2 ,Experiment 4), and since the lateral BNST receives a direct CRF projection from the CeA (Sakanaka et al., 1986), this circuitry would seem particularly relevant to the present discussion.

Using combined immunocytochemical and anterograde/retrograde tracing techniques, it has been shown that GABA-containing cell bodies in the lateral aspect of the CeA project to the dorsolateral and ventrolateral aspects of the BNST and that GABA-containing cell bodies in the dorsolateral BNST project to the ventrolateral and posterior BNST, and to a lesser degree to the CeA (Sun et al., 1994). Whereas most GABA terminals in the CeA are thought to arise from local connections within the nucleus, the majority of GABA terminals in the BNST appear to arise from 'long' intrinsic connections from the CeA , with only a few connections originating in the BNST itself (Sun et al., 1991; Sun et al., 1994).

As mentioned earlier, GABA neurons of the extended AMG are known to synthesize a variety of neuropeptides, including CRF, enkephalin, neurotensin,

somatostatin, and Substance P (e.g., Arluison et al., 1994; Day et al., 1999; Gray & Magnuson, 1992). Over 90 percent of the GABA neurons in the CeA and BNST co-localize neurotensin. One subpopulation of these neurons also synthesizes enkephalin and another subpopulation also synthesizes CRF (Day et al., 1999; Veinante et al., 1997). Of particular relevance to the present discussion is the prevalence of CRF-containing neurons in the CeA and BNST. Veinante et al. (1997) found that, in the dorsolateral BNST, 40 percent of all glutamic acid decarboxylase (GAD; a GABA-synthesizing enzyme)-IR neurons are also CRF-IR and that 45 percent of all CRF-IR neurons are also GAD-IR; GAD and CRF immunoreactivities are colocalized in a similar proportion of cells in the CeA. Although some GAD-IR cells are seen in the ventrolateral BNST, it has not been established whether GABA and CRF colocalize in this region. However, based on striking histochemical similarities between the CeA and ventrolateral BNST and the known pervasiveness of GABA throughout the extended AMG, it would seem reasonable to suppose that GABA and CRF are also colocalized in this region of the circuitry.

Within the extended AMG, CRF-IR neurons are found primarily in the lateral CeA and lateral BNST (Ju, Swanson, & Simerly, 1989; Phelix & Paull, 1990; Sakanaka et al., 1986; Swanson, Sawchenko, Rivier, & Vale, 1983). Although it is known that at least some of these neurons project to various hypothalamic and brainstem regions (Cummings, Elde, Ells, & Lindall, 1983; Moga & Gray, 1985; Moga et al., 1989; Sakanaka et al., 1986; Swanson et al., 1983), others are intrinsic to the extended AMG . For example, evidence of a projection from the CeA to the lateral BNST comes from a study in which, following electrolytic lesions of the CeA, a decrease in CRF-immunoreactivity was found in the lateral BNST (Sakanaka et al., 1986). It is also

thought that short intrinsic CRF projections exist within the lateral BNST, itself, most likely projecting from the dorsal to ventral region (see Phelix & Paull, 1990; Sun et al., 1991; Veinante et al., 1997).

It is known that CRF (Chappell et al., 1986), as well as GAD (Bowers, Cullinan, & Herman, 1998), levels increase in the BNST following stress. Questions of importance for an understanding of the role of CRF release in the BNST following stress are which cells of the BNST are sensitive to CRF and to which regions of the brain do these cells project? Before considering these questions, however, the possibility that there is an interaction between CRF and NE systems in the BNST will be discussed.

An interaction between CRF and NE systems in the extended AMG may underlie the footshock-induced reinstatement of cocaine seeking

Neuroanatomical evidence for a NE-CRF interaction in the extended AMG

The CeA and BNST receive major NE inputs from the ventral NE bundle, originating in the lateral tegmental nuclei, and minor NE inputs from the dorsal NE bundle, originating in the LC (Aston-Jones et al., 1995; Moore & Bloom, 1979). Because neurotransmission within the ventral NE pathway (Shaham et al., in press) and CRF release in the BNST (Chapter 2) are important events underlying the footshock-induced reinstatement of drug seeking, the possibility of a direct interaction between NE and CRF systems within the extended AMG is intriguing.

There is clear neuroanatomical evidence for direct synaptic connections between NE terminals and CRF cell bodies in the ventrolateral BNST. Using dual

immunocytochemistry for CRF and DA beta-hydroxylase (DBH), a major NE synthesizing enzyme, Hornby and Piekut (1989) have identified two populations of CRF-IR neurons, one in the dorsolateral and one in the ventrolateral BNST. Surrounding CRF cell bodies in the ventral region, there is a massive network of DBH-IR stained fibres, whereas in the region of CRF cell bodies in the dorsal region relatively few DBH-IR fibres are observed. These findings, which are supported by independent reports of similar distinct anatomical groupings of CRF cell bodies in the BNST (Ju et al., 1989; Phelix & Paull, 1990)) and dense NE terminals in the ventral BNST (Aston-Jones et al., 1995; Terenzi & Ingram, 1995), provided some of the first evidence for an interaction between CRF and NE systems in this region. This interaction has since been more fully characterized by Phelix et al (1994) who, using combined light and electron microscopy, demonstrated the existence of direct synaptic interactions between NE axons and the dendrites of CRF-containing cell bodies in the ventrolateral BNST (see also Phelix, Liposits, & Paull, 1992).

Evidence for a direct interaction between NE and CRF systems in the ventrolateral BNST might appear, at least at first glance, to provide an explanation for the similarity of the effects of CRF-receptor antagonists (Chapter 2, see also Shaham et al., 1997b) and alpha-2 adrenergic receptor agonists (Chapter 3, see also Shaham et al., in press) on the footshock-induced reinstatement of drug seeking. On second thought, however, it becomes clear that in order to attribute the effects of both CRF and NE to their actions in the BNST, at least some of the CRF neurons on which NE terminals act would have to be interneurons, providing local release within this region (i.e., Figure 15; no. 2b). This requirement is based on the assumption that, since CRF-receptor

antagonists were effective in blocking footshock-induced reinstatement and CRF induced reinstatement when injected into the ventrolateral region of the BNST, and since this is the primary region of interaction between NE and CRF neurons in the BNST, CRF must be released here following stress. Although the possibility cannot be ruled out, there is at present no evidence that interneurons within the ventral BNST are CRF-containing. On the other hand, there is considerable evidence that CRF neurons in the lateral BNST project to various hypothalamic and brainstem regions, regions that may in fact be involved in the effects of stress on relapse (see below). If, however, all ventral BNST CRF neurons are projection neurons, how can the actions of CRF and its receptor antagonist in the BNST be explained by a direct effect of NE and CRF neurons in this region? A possible explanation is that NE acts on the same BNST neurons upon which CRF acts (Figure 15; no. 1c and 3), and this effect is reduced by the presynaptic effects of the alpha-2 agonist. Alternatively, the alpha-2 agonist might act directly at the post-synaptic cell (Figure 15; no. 5) to interfere with the effects of CRF.

Another possibility that must be considered is that NE and CRF neurons interact in the CeA to exert their effects on BNST neurons (Figure 15; no. 1a and 2a). Such an interaction could account both for the effects of CRF and its receptor antagonists and for the effects of alpha-2 adrenergic-receptor agonists on footshock-induced reinstatement. Since the ventral NE bundle is known to project to the CeA (Aston-Jones et al., 1995; Moore & Bloom, 1979) and since the CeA is known to provide a CRF projection to the BNST (Sakanaka et al., 1986), it follows that interfering with transmission of either pathway could interfere in stress-induced reinstatement (see Figure 15; no. 1a, 2a, and 4). It has not been established whether NE terminals in the CeA synapse on CRF cell bodies

in the region. Based on numerous similarities in the organization of inputs and outputs to the CeA and lateral BNST, however, it is not unreasonable to speculate that such interactions do exist in the CeA, as they do in the lateral BNST.

Mechanism of interaction between NE and CRF systems in the extended AMG

Even if an interaction between NE and CRF systems in the extended AMG does underlie the footshock-induced reinstatement of drug seeking, it remains unclear how it works. In general, NE has been found to have inhibitory effects on cell firing in the BNST (Bassant, Joly, Nisson, Bjorklund, & Lamour, 1988; Casada & Dafny, 1993; Sawada & Yamamoto, 1981). Casada and Dafny (1993) showed that iontophoretic application of NE in the BNST inhibited the spontaneous firing rate of 70 percent, and induced excitation in only two percent, of cells recorded in the region. Furthermore, they reported that over 90 percent of the cells recorded were in the ventral region of the BNST. Although evidence concerning the postsynaptic effects of NE in the CeA is less clear, it appears overall that, as in the BNST, NE inhibits spontaneous cell firing in this nucleus (Freedman & Aghajanian, 1985; Perkins, Demaine, & Whitehead, 1977).

Although there is no direct evidence that NE has an inhibitory effect on CRF neurons per se in the BNST or CeA, the fact that NE appears primarily to inhibit spontaneous cell firing in these regions would seem problematical for the hypothesis that the footshock-induced reinstatement of cocaine seeking is mediated by NE-induced release of CRF within these regions. It should be noted, however, that the effects of iontophoretic application of NE in a given brain region on spontaneous cell firing may be quite different from the effects of NE on stress-induced cell firing in that region. It has

been shown in the somatosensory cortex, for example, that although iontophoretic application of NE inhibits spontaneous cell firing, it enhances the excitatory response of these cells to tactile stimulation or to subthreshold doses of glutamate or acetylcholine (Foehring, Schwindt, & Crill, 1989; Law-Tho, Crepel, & Hirsch, 1993; Mouradian, Sessler, & Waterhouse, 1991; Radisavljevic, Cepeda, Peacock, Buchwald, & Levine, 1994; see also Sawada & Yamamoto, 1981). Similar findings have been reported for the prefrontal cortex and have led to the interpretation that activation of NE receptors may actually facilitate neurotransmission postsynaptically in the prefrontal cortex by enhancing the signal-to-noise ratio (Mantz, Milla, Glowinski, & Thierry, 1988; Thierry, Godbout, Mantz, & Glowinski, 1990). It is possible that similar principles of NE function apply in the extended AMG and could provide a mechanism whereby stress-induced activation of NE receptors in the BNST or CeA in fact facilitates CRF transmission.

How and via what efferent pathways does CRF in the BNST initiate the behaviors involved in relapse?

The model of stress-induced relapse to drug seeking developed so far implies that CRF released in the ventrolateral BNST in response to stress acts postsynaptically (Figure 15; no. 4), on efferent neurons, to facilitate the behaviors involved in relapse. Two further questions that need to be addressed in order to assess the plausibility of this hypothesis are 1) what effects does CRF have on cell firing in the BNST and 2) on which efferent neurons of the BNST does CRF act?

First, it is not clear whether CRF in the BNST has excitatory or inhibitory effects on cell firing in the region. In general, CRF has been found to have different effects in different brain regions. For example, iontophoretic application of CRF in the thalamus and lateral septum inhibits spontaneous cell firing, whereas excitation occurs in the cortex and hypothalamus (Eberly, Dudley, & Moss, 1983). In the LC, ICV CRF increases the spontaneous discharge rate of cells, but both decreases the excitatory and increases the inhibitory component of sensory-elicited responses of these cells. These findings suggest that CRF, like NE, can act to change the signal-to-noise ratio (Valentino & Foote, 1988). In the AMG, CRF has different effects on cell firing in different nuclei; in the basolateral nucleus of the AMG, CRF increases cell firing, whereas in the CeA it decreases the rate of firing (Rainnie et al., 1992). It is not known what the direction of the effects of CRF are in the BNST. Again, however, because of the similarities between these regions, it would seem reasonable that, as in the CeA, it might induce inhibition. Nevertheless, it should be kept in mind that, as discussed above, CRF might have a different net effect on spontaneous cell firing than on sensory-elicited cell firing.

Not only is the direction of the postsynaptic effects of CRF unclear, but so too is the question of which efferent cells of the ventrolateral BNST are targets of CRF. As mentioned, BNST neurons send projections to various midbrain and brain stem regions (see Figure 15; no. 5), including the medial hypothalamus, lateral hypothalamus, PVN, lateral septum, VTA, retrorubral field, mesopontine nucleus, medulla oblongata, periaqueductal gray, midbrain central gray, nucleus of the solitary tract, and ventrolateral medulla (de Olmos & Heimer, 1999; Gray & Magnuson, 1992; Holstege, Meiners, & Tan, 1985; Moga & Saper, 1994; Numan & Sheehan, 1997; Tazi et al., 1987). Some of

these projection neurons are CRF-containing, such as those targeting the midbrain central gray (Gray & Magnuson, 1992), parabrachial nucleus (Moga & Gray, 1985), PVN (Moga & Saper, 1994) and, possibly, the lateral and medial hypothalamus (see Phelix et al., 1994), the nucleus of the solitary tract, and the nuclei of the ventrolateral medulla (Holstege et al., 1985). There is considerable evidence that the major outputs of the extended AMG are inhibitory, including GABA projections to medulla nuclei (Jia, Rao, & Shi, 1997) and the nucleus of the solitary tract (Pickel et al, 1995). Thus, the major effect of CRF in the BNST following stress might be to release projection neurons from inhibition, allowing for the disinhibition of prepotent behaviors.

As mentioned before, one can only speculate about which projections of the BNST are involved in the role that CRF plays in relapse. It is interesting to consider, however, some of the goal-directed behaviors in which efferent projections of the BNST have been implicated (see Figure 15: no. 5). For example, projections from the dorsolateral and ventrolateral BNST to the lateral and medial hypothalamus and to brain stem nuclei are involved in agonistic behaviors, including quiet biting attack, defensive posturing, and defensive activation of the sympathetic nervous system (Shaikh, Brutus, Siegel, & Siegel, 1985; Shaikh, Brutus, Siegel, & Siegel, 1986). Likewise, the lateral division of the BNST is associated with stress-induced fighting, presumably through its projections to hypothalmic regions and the midbrain central gray (see Albert & Walsh, 1982; Gray & Magnuson, 1992; Watson, Edinger, & Siegel, 1983). Thus, one way in which CRF in the BNST could act to allow the initiation of behaviors involved in relapse is through projections to sites that have been implicated in "aggressive", but essentially active approach, behaviors.

Alternatively, or in addition, stress-induced relapse to drug seeking could involve activation of systems that subserve, more obviously, appetitive behaviors such as feeding (e.g., Devenport & Balagura, 1971; Kelley, 1999; Stanley, Willett, Donias, Dee, & Duva, 1996) and maternal behavior (e.g., Numan & Numan, 1996; Numan & Numan, 1997; Numan & Sheehan, 1997). The lateral hypothalamus, for example, which receives a major projection from the lateral BNST, is strongly implicated in the control of feeding (Devenport & Balagura, 1971; Stanley et al., 1996). Likewise, the nucleus accumbens, which receives a direct projection from the ventral BNST (Brog, Salyapongse, Deutch, & Zahm, 1993; Numan & Numan, 1996), is implicated in a variety of reward-related behaviors, including feeding, drinking, sex, exploration, and response-reinforcement learning (Everitt et al., 1999; Hitchcott & Phillips, 1998; Holahan, Kalin, & Kelley, 1997; Kelley, Smith-Roe, & Holahan, 1997; Robledo, Robbins, & Everitt, 1996). The BNST also projects directly to the VTA which, through its projection to the nucleus accumbens, has been shown to be critically involved in the rewarding effects of a variety of motivationally-significant stimuli, including drugs of abuse (see, for example, Wise, 1982a). In addition, neurons of the "preoptic locomotor region", which extends to and overlaps with the ventrolateral region of the BNST, have been found to induce forward locomotion and approach behaviors, possibly through a projection to the VTA (Sinnamon, 1992). In the case of maternal behavior, Numan and colleagues have shown an important role for the ventral BNST and its projections to the medial hypothalamus and the A8 (retrorubral field; RF) and A10 (VTA) DA cell body regions in the neural control of maternal behaviors, such as pup retrieval, nursing, and pup grooming (Numan & Numan, 1996; Numan & Numan, 1997; Numan & Sheehan, 1997). These maternal

behaviors involve approach toward motivationally-significant conditioned and unconditioned stimuli, as does drug seeking. Thus, it is conceivable that projections from the BNST to any number of sites subserving appetitive behaviors could be involved in the initiation of drug seeking during relapse.

Whatever the projections of the BNST involved in stress-induced relapse, it is proposed here that CRF acting postsynaptically on these neurons is a critical event mediating the effects of footshock on relapse. It is clear, however, that considerable research is still required to determine how and via which output systems CRF in the BNST acts to initiate the behaviors involved in relapse.

Summary: Implications of a proposed model of stress-induced reinstatement for future research

In the preceding discussion, a model of footshock-induced reinstatement of drug seeking, summarized in Figure 15, has been developed that focuses on an interaction between NE and CRF systems in the extended AMG and on possible postsynaptic effects of CRF in the ventrolateral BNST. The model is based on three lines of evidence: 1) evidence of an interaction between NE and CRF systems in the BNST and the possibility of such an interaction in the CeA; 2) evidence that the ventral NE bundle, which provides the majority of NE input to the BNST and CeA (Aston-Jones et al., 1995; Moore & Bloom, 1979), is the pathway that plays an important role in mediating the effects of NE on the footshock-induced reinstatement of drug seeking (Shaham et al., in press); and 3) the demonstration of a critical role for the BNST in mediating the effects of CRF on stress-induced relapse (Chapter 2).

It is important to emphasize that the model proposed here is a *preliminary* one. Other neurotransmitter and neuropeptide systems are likely to interact or act in parallel with CRF and NE systems to mediate the effects of footshock on relapse. For example, it has been found that sustained treatment with a non-selective DA receptor antagonist, flupenthixol, blocked the footshock-induced reinstatement of heroin seeking, suggesting that some level of DA activity is necessary for the effects of footshock on relapse (Shaham & Stewart, 1996). It has also been found that the medial septum, which is reciprocally connected with the AMG and BNST, may play an important role in footshock-induced reinstatement (Jakab & Laranth, 1996; Meibach & Siegel, 1977; Weller & Smith, 1982); inactivation of the medial septum with tetrodotoxin robustly reinstates heroin seeking, possibly by disinhibiting systems within the AMG and BNST, and stimulation of the structure blocks footshock-induced reinstatement (Highfield et al., submitted).

In addition to being preliminary with respect to identifying systems directly involved in footshock-induced reinstatement, the proposed model is also preliminary with respect to determining its generalizability to all drugs of abuse. All of the findings presented in Chapters 2 and 3 were obtained in animals with a history of cocaine taking, and although some of these experiments have been conducted in animals with a history of heroin or alcohol taking, others have not. Likewise, some experiments conducted in animals with a history of heroin or alcohol taking have been conducted with cocaine-trained animals, whereas others have not. It can be said, however, that when similar studies targeting CRF and NE systems have been conducted in cocaine- and heroin-trained animals, similar outcomes have been observed. Clearly, further experimentation

is required to more fully characterize the pathways involved in stress-induced relapse and to test the generalizability of the proposed model to different drugs of abuse.

FUTURE DIRECTIONS

Behavioral studies using the reinstatement procedure

In order to more fully characterize the phenomenology and neurobiology of the footshock-induced reinstatement, three areas of investigation should be pursued. One area is the characterization of the role of context in the effect of footshock on reinstatement. A variety of manipulations of training, extinction, and testing contexts could be made to determine whether, or to what extent, the footshock-induced reinstatement of drug seeking involves contextual conditioning processes. Another area is the identification of other stressors that reinstate drug seeking. Stressors that may be interesting to test include social defeat stress, something found to increase the rate of responding during the maintenance phase of cocaine self-administration (Miczek & Mutschler, 1996), tail pinch or tail shock stress, swim stress, and hemodynamic stress. The development of a battery of stressors that do and do not induce reinstatement could help to shed more light on both the behavioral and neurobiological processes underlying stress-induced relapse. A third area of investigation is the further characterization of the neurobiological substrates of footshock-induced reinstatement. As mentioned previously, it is likely that neurochemical systems in addition to CRF and NE underlie the footshock-induced reinstatement of drug seeking. Pharmacological and lesion studies, similar to those already done manipulating CRF and NE systems, could be conducted to investigate

the involvement of other systems, including serotonin, DA, acetylcholine, GABA, and neurotensin.

Neurochemical and neuroanatomical studies

Earlier it was hypothesized that neuronal changes induced by chronic exposure to drug might lower the threshold required for footshock to induce reinstatement and may explain why footshock is more effective in inducing reinstatement in drug-trained animals than in animals trained with non-drug reinforcers. An extension of this hypothesis is that the neurochemical systems mediating footshock-induced reinstatement become sensitized in animals with a history of drug self-administration. To test these hypotheses, microdialysis procedures could be used to determine whether footshock stress results in greater release of NE and/or CRF in animals trained to self-administer drug versus saline. Analysis of CeA and BNST microdialysates may help to shed light on how NE and CRF in these regions are involved in the effects of footshock on relapse. Similarly, changes in the responsivity of CRF systems in animals with a drug history could be assessed using various molecular techniques, including immunocytochemistry and in situ hybridization, to measure CRF protein and message within the extended AMG. Finally, it is conceivable that the effectiveness of NE and/or CRF on their receptors or second messengers is enhanced following chronic exposure to drug. This hypothesis could be tested using techniques designed to assess receptor binding characteristics and extracellular levels of cyclic AMP, a second messenger activated by CRF (Chalmers, Lovenberg, Grigoriadis, Behan, & De Souza, 1996; De Souza, 1995) and NE (Cooper et al., 1996).

IMPLICATIONS OF FINDINGS FOR THE TREATMENT OF ADDICTION AND PREVENTION OF RELAPSE

Findings from studies carried out in laboratory animals and humans suggest that chronic drug exposure renders individuals highly vulnerable to relapse, even after extended drug-free periods, and this problem remains the most difficult challenge for treatment. The findings reported in this thesis may, from this perspective, have a number of important implications.

The differential effects of the CRF-receptor antagonist and alpha-2 adrenergic-receptor agonists on reinstatement induced by stress and by priming injections of cocaine lends support to the view that the neuronal and hormonal mechanisms underlying stress-induced reinstatement are not identical to those underlying drug-induced reinstatement (Shaham et al., 1997b). In the experiments reported in Chapters 2 and 3, footshock-induced reinstatement was blocked by CRF-receptor antagonists and by alpha-2 adrenergic receptor agonists. In these experiments, however, antagonism of CRF receptors had minimal effects and the alpha-2 adrenergic receptor agonists were without effect on reinstatement induced by priming injections of cocaine.

The observed dissociations between the neurobiological events mediating stress- and cocaine-induced relapse to cocaine seeking may have important clinical implications. First and foremost, the dissociations suggest that no single treatment approach, pharmacological or otherwise, will be effective in preventing all instances of relapse. What may be an effective method for decreasing the probability or magnitude of a relapse

episode induced by a stressful life event may not be an effective treatment strategy once drug has been ingested. The findings do suggest, however, that pharmacological manipulations targeting CRF and NE systems may be effective in preventing stress-related relapse episodes in drug-free abstaining individuals.

The data presented in Chapter 2 provide conclusive evidence that CRF exerts its effects on stress-induced relapse by its actions on extrahypothalamic brain sites, in particular the BNST. These data, together with those from recent studies indicating an important role for extrahypothalamic CRF in the aversive effects of drug withdrawal (Heinrichs et al., 1995; Koob, 1996; Menzaghi et al., 1994a; Richter & Weiss, 1999; Sarnyai et al., 1995), indicate that alterations in the CRF system in the brain may play a major role in compulsive drug use. Thus, pharmacological interventions aimed at targeting CRF systems may hold promise in the treatment of addiction and prevention of relapse, particularly in those individuals experiencing significant stress. In this regard, the continued development and clinical assessment of systemically-injectable CRF-receptor antagonists, such as that reported on in Chapter 2 (Experiment 5), may be of particular clinical relevance.

As the results presented in Chapter 2 provide a rationale for the use of CRF-receptor antagonists in the prevention of relapse, the results presented in Chapter 3 provide a rationale for the use of alpha-2 adrenergic receptor agonists. Currently, alpha-2 adrenergic receptor agonists are being used with some success in the short-term treatment of opioid withdrawal (Lin et al., 1997; Warner et al., 1997). The findings presented in Chapter 3 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 & O'Brien, 1997). It is

also interesting to note that the experiments reported in Chapter 3 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 & Hardman, 1998; Kahn et al., 1997; Lin et al., 1997).

CONCLUSION

The present findings, and those of other studies (e.g., Lê et al., 1998; Shaham et al., 1996; Shaham & Stewart, 1995; Shaham & Stewart, 1996), show that at least two events, re-exposure to self-administered drugs and exposure to footshock stress, are highly effective in provoking relapse to drug seeking in rats after extinction and prolonged drug-free periods. Furthermore, the results of the present experiments show a clear dissociation between the neurobiological mechanisms mediating stress- and drug-induced relapse to drug seeking. The findings point to a critical role for CRF and NE systems within the extended AMG, and a possible interaction between these systems, in the mediation of stress-induced relapse to drug seeking. In contrast, the data indicate that CRF and NE systems are only minimally, if at all, involved in drug-induced relapse. These findings underscore the complexity of relapse processes, both neurobiologically and phenomonologically. Although the experiments reported on in this thesis represent an important step toward understanding the processes involved in relapse, there are many questions remaining and a more complete understanding of the phenomenon awaits further experimentation.

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