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THE NEURAL CIRCUITRY UNDERLYING REINSTATEMENT OF HEROIN-SEEKING BEHAVIOR IN AN ANIMAL MODEL OF RELAPSE

J.L. ROGERS, S. GHEE, and R.E. SEE*

Department of Neurosciences, Medical University of South Carolina, Charleston, SC 29425, USA

Abstract

Reinstatement of extinguished drug-seeking has been utilized in the study of the neural substrates of relapse to drugs of abuse, particularly cocaine. However, limited studies have examined the circuitry that drives the reinstatement of heroin-seeking behavior in the presence of conditioned cues, or by heroin itself. In order to test the hypothesis that the circuitry underlying reinstatement in heroin-experienced animals would show overlapping, yet distinct differences from cocaine-experienced animals, we used transient inhibition of several cortical, striatal, and limbic brain regions during reinstatement of heroin-seeking produced by heroin-paired cues, or by a single priming dose of heroin. Rats lever pressed for i.v. heroin discretely paired with a conditioned stimulus (CS) during daily 3-hr sessions for a period of 2 weeks, followed by daily extinction of lever responding. Subsequent reinstatement of heroin-seeking was measured as lever responding in the absence of heroin reinforcement. The first set of reinstatement tests involved response-contingent CS presentations following bilateral intracranial infusion of either a combination of GABA receptor agonists (baclofen-muscimol, B/M) or vehicle (saline) into one of thirteen different brain regions. The second set of reinstatement tests involved a single heroin injection (0.25 mg/kg, s.c.) following either B/M or vehicle infusions. Our results showed that vehicle infused animals reinstated to both CS presentations and a priming injection of heroin, while B/M inactivation of several areas known to be important for the reinstatement of cocaine-seeking also attenuated heroin-seeking in response to CS presentations and/or a priming dose of heroin. However, as predicted, inactivation of areas previously shown to not affect cocaine-seeking significantly attenuated heroin-seeking, supporting the hypothesis that the circuitry underlying the reinstatement of heroin-seeking is more diffusely distributed than that for cocaine.

Keywords

Extinction; Heroin; Neurocircuitry; Reinstatement; Relapse; Self-administration

Introduction

Relapse to drug-taking following detoxification and/or abstinence is a major impediment in the treatment of addiction. The subjective desires that drive the behavioral output towards relapse can be elicited by interactions with stimuli previously associated with the drug

*Corresponding author Ronald E. See, Department of Neurosciences, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC 29425, Telephone: 843-792-2487, Fax: 843-792-4423, E-mail: seere@musc.edu.

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(Childress et al., 1999; Sell et al., 2000; Coffey et al., 2002), exposure to the drug itself (Jaffe et al., 1989), or external and internal stressors (Sinha et al., 2000). Experimentally, relapse can be studied using the reinstatement model in animals, whereby exposure to previously drug-paired cues, stress, or a drug priming injection ‘reinstates’ extinguished drug-seeking behavior, operationally defined as responding on a previously drug-paired operandum in the absence of primary reinforcement (de Wit and Stewart, 1983; Shaham et al., 1996; Meil and See, 1997). The neural circuitry for relapse to cocaine-seeking has been fairly well elucidated for conditioned-cued (See, 2002, 2005), stress-induced (Lu et al., 2003; McFarland et al., 2004), and cocaine-primed reinstatement (Kalivas and McFarland, 2003; Schmidt et al., 2005). In contrast, while some limited studies have looked at brain region specific regulation of the reinstatement of heroin-seeking produced by discrete cues (Fuchs and See, 2002; Zhou et al., 2007), contextual cues (Bossert et al., 2004; Bossert et al., 2006), or heroin priming injections (Fuchs and See, 2002), a systematic examination of the neurocircuitry underlying relapse to heroin-seeking has never been accomplished.

Opiates, including heroin, act as direct agonists at μ , δ , and/or κ opioid receptors (De Vries and Shippenberg, 2002). Stimulation of the μ receptor is largely responsible for the reinforcing effects of opiates, although some data suggests that δ receptors also play a role in opiate reinforcement (Self and Stein, 1992; Mello and Negus, 1996; De Vries and Shippenberg, 2002). In addition to the immediate reinforcing effects, μ receptor activity appears to be critical in some forms of drug-induced relapse, since blockade of the μ receptor has been shown to attenuate reinstatement of cocaine-seeking (Gerrits et al., 2005; Tang et al., 2005) and alcohol-seeking (Ciccocioppo et al., 2002; Oslin et al., 2006) in animal models. Mu opioid receptors are ubiquitous in the central nervous system, with extensive expression most notably in the ventral tegmental area (VTA), striatum, bed nucleus of the stria terminalis (BNST), and amygdala (German et al., 1993; Mansour and Watson, 1995; Svingos et al., 1996). Mu receptor activation modulates neuronal activity in three primary ways: postsynaptic inhibition, axoaxonic inhibition, and via presynaptic autoreceptors (Yaksh, 1997). Unlike psychostimulants, which directly increase dopamine (DA) activity by reuptake blockade or reverse transport, activation of μ opioid receptors by heroin and other opiates disinhibits GABA neurons in the VTA, consequently increasing DA release in the nucleus accumbens and prefrontal cortex (Kelley et al., 1980).

Given that heroin administration activates μ opioid receptors and indirectly stimulates DA release, we hypothesized that the neurocircuitry underlying relapse to heroin-seeking would have overlapping, yet distinct differences from the previously established circuitry for relapse to cocaine-seeking induced by cocaine priming injections (McFarland and Kalivas, 2001) or conditioned cues (McLaughlin and See, 2003; Fuchs et al., 2004b). This notion builds upon our previous limited evidence (Fuchs and See, 2002) and that of other studies suggesting qualitative differences in the motivational and reinforcing aspects of heroin vs. cocaine (Ettenberg et al., 1982; Ettenberg and Geist, 1993). Determining the similarities of brain structures underlying reinstatement of drug-seeking for opiates vs. psychostimulants is a key issue for understanding the fundamental neurobiology that drives relapse across drugs of different pharmacological classes. Elucidation of distinct differences in relapse circuitries could lead to different approaches for creating effective treatments to preventing relapse. In order to assess this issue, the present study examined the neural structures previously shown to mediate relapse to cocaine-seeking in order to determine if the same neural circuitry also underlies heroin-seeking triggered by previously heroin-paired cues or a single priming injection of heroin itself.

EXPERIMENTAL PROCEDURES

Subjects

Male, Sprague-Dawley rats ($n = 140$, initial weight 250–275 g; Charles River, Wilmington, MA, USA) were individually housed in a temperature- and humidity-controlled vivarium on a 12-h reversed light-dark cycle (lights off 6 AM to 6 PM). Animals were given water *ad libitum* and were maintained on 25 g of standard rat chow (Harlan, Indianapolis, IN, USA) per day for the duration of each experiment. Rats were acclimated to handling and allowed to adapt for a minimum of four days prior to the start of the experiment. Housing and care of the rats were carried out in accordance with the “Guide for the Care and Use of Laboratory Rats” (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, 1996) and experimental procedures were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina.

Lever response training

Rats were trained to lever press in standard self-administration chambers (30 × 20 × 20 cm) linked to a computerized data collection program (MED-PC, Med Associates, Inc., St. Albans, VT, USA). The chambers were equipped with two retractable levers, a white stimulus light above each lever, a food pellet dispenser between the levers, a tone generator (ENV-223HAM, Med Associates), and a house light on the wall opposite the levers. Each chamber was contained within a sound-attenuating cubicle equipped with a ventilation fan. Rats were food deprived overnight and trained to lever press on a fixed ratio (FR) 1 schedule of food reinforcement (45 mg pellets; Noyes, Lancaster, NH, USA) during a 15-h overnight training session in the absence of explicit conditioned stimulus (CS) presentation (i.e., active lever presses resulted in the delivery of a food pellet only). Lever presses on an inactive lever were recorded, but had no programmed consequences. Following lever response training, food dispensers were permanently removed from the test chambers.

Surgery

Two days after lever training, rats were anesthetized using a mixture of ketamine hydrochloride and xylazine (66 and 1.33 mg/kg, respectively), followed by equithesin (0.5 ml/kg of a solution of 9.72 mg/ml pentobarbital sodium, 42.5 mg/ml chloral hydrate, and 21.3 mg/ml magnesium sulfate heptahydrate dissolved in 44% propylene glycol, 10% ethanol solution, i.p.). Surgical procedures were conducted using aseptic techniques. Catheters were constructed using previously described methods (Fuchs et al., 2004b) and consisted of external guide cannulae with screw-type connectors (Plastics One, Inc., Roanoke, VA, USA), Silastic tubing (10 cm; ID = 0.64 mm; OD = 1.19 mm; Dow Corning, Midland, MI, USA), prolite polypropylene monofilament mesh (2 cm diameter, Atrium Medical Corporation, Hudson, NH, USA), and cranioplastic cement. A small incision was made on the back and chest of the rat 5 mm above the area where the jugular vein enters the rib cage. The external guide cannula exited from the incision on the rat's back and the open end of the Silastic tubing was passed subcutaneously to the area of the jugular vein. The free end of the tubing was inserted 33 mm into the right jugular vein and secured with 4.0 silk sutures. Both incisions were sutured with 4.0 sterile surgical thread.

Immediately following catheter surgery, animals were placed into a stereotaxic frame (Stoelting, Wood Dale, IL). Bilateral stainless steel guide cannulae (26 gauge; Plastics One, Inc.) were inserted in one of 12 target brain regions (Table 1). Three small screws and cranioplastic cement secured the guide cannulae to the skull. Stylets (Plastics One, Inc.) were placed into the guide cannulae and catheter to prevent occlusions. To maintain catheter patency, catheters were flushed once daily for 4 days after surgery with 0.1 ml each of an antibiotic solution of cefazolin (100 mg/ml; Schein Pharmaceuticals, Florham Park, NJ, USA) dissolved

in heparinized saline (70 U/ml; Elkins-Sinn, Cherry Hill, NJ, USA) and heparinized saline. For the duration of the experiment, each subject then received 0.1 ml of heparinized saline (10 U/ml) immediately prior to self-administration sessions and the cefazolin and 70 U/ml heparinized saline regimen following each session. To verify catheter patency, rats occasionally received a 0.12 ml infusion of methohexital sodium (10.0 mg/ml i.v.; Eli Lilly and Co., Indianapolis, IN, USA), a short-acting barbiturate that produces a rapid loss of muscle tone when administered intravenously.

Heroin self-administration

Five to seven days after surgery, rats began self-administration of heroin (diacetylmorphine HCl, provided by the National Institute on Drug Abuse, Research Triangle Park, NC). Using previously established methods (Fuchs and See, 2002; Bossert et al., 2004), rats self-administered along a FR1 schedule at an initial dose of 50 µg/50 µl/infusion for 2 days, followed by 10–12 days of self-administration at a dose of 25 µg/50 µl/infusion. At the start of each 3-h session, the catheter was connected to a liquid swivel (Instech, Plymouth Meeting, PA) via polyethylene 20 tubing that was encased in steel spring leashes (Plastics One, Inc.). The swivels were suspended above the operant conditioning chamber and were connected to infusion pumps (model PHM-100, Med-Associates). The house light signaled the initiation of the session and remained illuminated throughout the entire session. Active lever responses resulted in a 2 s activation of the infusion pump and a 5 s presentation of a conditioned stimulus complex (CS), which consisted of a cue light above the active lever and a tone (78 dB, 4.5 kHz). Following each infusion, responding on the active lever had no consequences during a 20 s time-out period. Inactive lever presses also had no consequences, but were recorded.

Extinction

After the last day of self-administration, rats experienced daily 3-h extinction sessions. On the first session, the catheter was connected to the swivel; however, no drug was administered. On subsequent sessions, rats were placed into the chamber without being attached to the swivel. Throughout extinction training, the house light signaled the initiation of the session and remained illuminated during the session. Responses on either the active or inactive lever were recorded, but resulted in no programmed consequences (i.e., no infusion and no CS presentation). Animals continued under extinction conditions until they reached a criterion of a minimum of 10 days and ≤ 25 lever presses per session for two consecutive days.

Intracranial infusions

For intracranial infusions, stainless steel injection cannulae (33 gauge, Plastics One) were inserted to a depth of 2 mm below the tip of the guide cannulae immediately prior to placement into the chamber. The injection cannulae were connected to 10-µl Hamilton syringes (Hamilton Co., Reno, Nev., USA) mounted on an infusion pump (Harvard Apparatus, South Natick, Mass., USA). Baclofen/muscimol (B/M; 1.0/0.1 mM) or phosphate buffered saline vehicle (pH=7.0 for both) were infused bilaterally over a 2 min time period. Dose-response analyses have shown that this concentration of B/M site-selectively attenuates cocaine-primed (McFarland and Kalivas, 2001) or conditioned-cued reinstatement (Fuchs et al., 2004b) of cocaine-seeking after extinction training. The injection cannulae were left in place for 1 min prior to and after the infusion.

Reinstatement testing

Following extinction, rats underwent four reinstatement tests, using a counterbalanced, within subjects design, with a minimum of 2 days of extinction between each test. Immediately prior to each reinstatement test, the rat received either intracranial vehicle or B/M. During the first two reinstatement tests, rats were placed into the chambers for 3 h, during which the house

light was illuminated and each active lever press resulted in a 5 s CS presentation in the absence of any drug reinforcement, followed by a 20 s time out period. For heroin-primed reinstatement tests, a single, non-contingent dose of heroin (0.25 mg/kg, s.c.) was administered immediately prior to the rat entering the chamber for a 3 h session, during which the house light was illuminated and lever responses had no programmed consequences (i.e., no CS presentation).

Histology and data analysis

After completion of reinstatement testing, the rats were deeply anesthetized with equithesin and transcardially perfused with PBS and 10% formaldehyde solution. The brains were dissected and stored in 10% formaldehyde solution prior to sectioning. Using a vibratome (Technical Products International, St. Louis, MO, USA), brains were sectioned in the coronal plane (75 μ m thickness), mounted on gelatin-coated slides, and stained for Nissl substance with cresyl violet (Kodak, Rochester, NY, USA). The sections were examined with light microscopy using 10 $\times$ magnification. The most ventral point of the microinjector tips were mapped onto schematics from a rat brain atlas (Paxinos and Watson, 1997). Analyses of lever responding and heroin intake during self-administration, as well as lever responses during extinction, CS-induced, and heroin-primed reinstatement testing were analyzed using analysis of variance (ANOVA). Post-hoc analyses were conducted using Tukey tests. Analyses were considered statistically significant at $P < 0.05$. All data are presented as mean $\pm$ SEM. Finally, to test for correlations of responding between the different reinstatement tests, correlations were generated using Pearson's product correlations.

RESULTS

Histology

Schematic representations of all the cannulae placements are shown in Fig. 1. Of the animals that started the study, ten rats had cannulae located outside of the target brain areas. These areas included the supramammillary nucleus, horizontal/diagonal band of Broca, deep mesencephalic nucleus, deep white layer of the superior colliculus, ventral olfactory nucleus, central gray, and red nucleus. We utilized these animals collectively as anatomical controls. In addition, based on clear differences in cannulae placements, we divided the BNST group into two distinct subgroups: the medial posterior group (BNSTmp) and the ventral group (BNSTv). Final group sizes were: dmPFC (n=12), vmPFC (n=9), SS (n=7), BLA (n=9), CEA (n=11), BNSTv (n=7), BNSTmp (n=8), NAcore (n=8), NAcshell (n=8), VTA (n=9), dlCPu (n=9), SN (n=15), VP (n=8), and Controls (n=10).

Self-administration and extinction of heroin-seeking

All animals, regardless of cannulae placement, responded equally during heroin self-administration, with no group differences in lever responding during chronic heroin self-administration on the active ($F_{13,123}=0.51$, $P > 0.05$) or inactive lever ($F_{13,123}=0.89$, $P > 0.05$). As shown in Fig. 2A, responding for the last 3 days of self-administration on the active lever was 58.8 ± 11.6 and responding on the inactive lever was 11.6 ± 4.9 . The daily number of heroin infusions for the last 3 days of self-administration was 19.1 ± 1.2 (approximately 1.3 mg/kg/session) across the 3 h session. When heroin was no longer available, lever responding readily declined across daily extinction sessions, as seen in Figure 2B. Finally, days-to-criterion prior to the first and second set of tests (i.e., the number of extinction sessions between the cue-induced and heroin-primed reinstatement tests) was 2.1 ± 0.9 .

Conditioned cue-induced reinstatement

Fig. 3 shows responding on the previously heroin-paired lever during the last 3 days of extinction (Fig. 3A) and during conditioned cue-induced reinstatement after intracranial

infusions of vehicle (Fig. 3B) or B/M (Fig. 3C). Compared to extinction level responding, responding on the previously heroin-paired lever in the presence of cues significantly reinstated drug-seeking following vehicle infusion. A two way ANOVA with brain region as the between subjects factor and intracranial drug infusion (vehicle or B/M) as the within subjects factor revealed significant main effects for region ($F_{13,252} = 3.92$, $P < 0.001$) and drug infusion ($F_{1,252} = 55.45$, $P < 0.001$). Furthermore, there was a significant interaction between region and drug infusion ($F_{13,252} = 2.08$, $P < 0.05$). Post-hoc analyses (Tukey) revealed that inactivation of the medial prefrontal cortex (PFC - both dorsal and ventral), amygdala (BLA and CEA), medial posterior BNST, nucleus accumbens core (NAcore), dorsolateral caudate-putamen (dlCPu), substantia nigra, or ventral pallidum attenuated heroin-seeking behavior in the presence of conditioned cues. Although there was a significant main effect for brain region, post-hoc analyses revealed no group differences during cue-induced reinstatement following vehicle infusion.

Heroin-primed reinstatement

Fig. 4 shows the responding on the previously heroin-paired lever during the last 3 days of extinction (Fig. 4A) and during heroin-primed reinstatement after site selective infusions of vehicle (Fig. 4B) or B/M (Fig. 4C). Similar to heroin-paired cues, a priming injection of heroin (0.25 mg/kg, s.c.) produced robust reinstatement as measured by responding on the previously heroin-paired lever in the absence of any contingent reinforcement following bilateral vehicle infusions. In general, responding following heroin-priming was somewhat greater than responding for cues, as previously reported (Fuchs and See, 2002). A two way ANOVA with brain region as the between subjects factor and intracranial drug infusion as the within subjects factor revealed significant main effects for region ($F_{13,234} = 1.81$, $P < 0.05$) and drug infusion ($F_{1,252} = 100.47$, $P < 0.001$). Furthermore, there was a significant interaction between region and drug infusion ($F_{13,234} = 1.78$, $P < 0.05$). In contrast to conditioned-cued reinstatement, post-hoc analyses revealed that inactivation of all regions, except the SS and the anatomical controls, attenuated heroin-primed reinstatement. Although there was a significant main effect for brain region, post-hoc analyses revealed no group differences during heroin-primed reinstatement following vehicle infusion.

Order effects and correlations

Although the order of intracranial drug infusions (i.e., Vehicle-B/M; B/M-Vehicle) was counterbalanced among groups of animals for each set of reinstatement tests, we also verified that there were no order effects during cue-induced reinstatement ($F_{13,123} = 0.04$, $P > 0.05$) or heroin-primed reinstatement ($F_{13,117} = 0.48$, $P > 0.05$). In order to determine the relationship of responding during heroin self-administration and at the time of reinstatement, we conducted a Pearson product correlation analysis on the heroin-paired lever presses during self-administration, cue-induced reinstatement, and heroin-primed reinstatement. In accordance with our prediction, high-responding during heroin self-administration was correlated with greater heroin-primed reinstatement responding, as seen by significant correlations between self-administration and heroin-primed reinstatement after either vehicle ($r = 0.47$, $P < 0.01$) or B/M ($r = 0.23$, $P < 0.01$) infusions. In contrast, no such relationship was found between responding during heroin self-administration and cue-induced reinstatement after either vehicle ($r = -0.07$, $P > 0.05$) or B/M ($r = 0.05$, $P > 0.05$) infusions. In addition to these correlations, we also found that levels of responding after vehicle or B/M infusions were significantly correlated across groups during both cue-induced reinstatement ($r = 0.19$, $P < 0.05$) and heroin-primed reinstatement ($r = 0.30$, $P < 0.01$). Thus, animals with high response rates following vehicle infusion were more likely to show higher responses following B/M infusion, even though the level of responding decreased across most groups as a whole.

DISCUSSION

The present results demonstrate that both heroin-paired cue and heroin-induced reinstatement of drug-seeking depends upon a complex limbic-cortical-striatal circuit involving several neural structures, including midbrain DA cell regions, amygdala, prefrontal cortex, and nucleus accumbens. Many of these same nuclei have also been implicated in cue-induced (Shalev et al., 2002; See, 2005) and cocaine-induced (McFarland and Kalivas, 2001; Schmidt et al., 2005) reinstatement of cocaine-seeking. Thus, B/M inactivation of several brain areas critical for the reinstatement of cocaine-seeking also reduced heroin-seeking behavior to CS presentations and/or a priming dose of heroin. However, as predicted, additional brain areas were involved in mediating reinstatement of heroin-seeking, namely the vmPFC, NAc shell, CEA, and BNSTmp. The current findings support previous work suggesting that neuroadaptations produced by chronic psychostimulant vs. opiate administration show overlapping, yet divergent properties (Ettenberg et al., 1982; De Vries and Shippenberg, 2002).

As mentioned above, μ opioid receptors stimulate DA release by disinhibiting GABA neurons in the VTA (Kelley et al., 1980). Thus, although heroin and cocaine both stimulate DA release, the divergent mechanisms of action (i.e., DA uptake blockade vs. μ receptor activation) likely play a key role in the differences in adaptations produced by chronic drug exposure. As μ receptors are quite ubiquitous in the CNS, we hypothesized that heroin would engage a more distributed neural circuit than that for cocaine at the time of reinstatement. In support of this hypothesis, a reduction in heroin-induced brain activity was found using functional magnetic resonance imaging in rats 8–9 days after heroin withdrawal in several brain regions, including the PFC and nucleus accumbens (Luo et al., 2004). The authors suggest that this persistent reduction in the opiate pathways relates to relapse propensity. In opiate-dependent human subjects, positron emission tomography scanning showed that the pattern of functional connectivity in brain regions such as the PFC reflected the activation of attentional and memory circuits following the presentation of drug-paired stimuli (Daglish et al., 2003). These authors found that there was no specific circuit for heroin relapse, per se, but rather a “normal” diffuse circuit that was activated to a greater degree. Further evidence of differences between cocaine and heroin comes from single unit recordings in the rat (Chang et al., 1998). This study revealed that in both medial PFC or nucleus accumbens neurons, overlapping populations were active during cocaine and heroin self-administration; however, a majority of neurons responded differently for cocaine and heroin. Studies using conditioned place preference (CPP) paradigms have reported that 6-OHDA lesions of the VTA or nucleus accumbens attenuated morphine-induced reinstatement of a CPP (Wang et al., 2003), and that depletion of norepinephrine in the PFC decreased morphine-induced DA release in the nucleus accumbens and blocked CPP reinstatement (Ventura et al., 2005). Taken together, previous research supports the current results indicating that relapse to heroin-seeking relies upon a complex, distributed network, including most previously identified structures of the motivation circuitry (Koob, 2000; Robbins and Everitt, 2002).

As stated above, differences and similarities have been reported in the neurobiological substrates for psychostimulants and opiates. Based on previous data from reinstatement studies after cocaine self-administration (Fuchs and See, 2002; Kalivas and McFarland, 2003; Schmidt et al., 2005) and the current results for reinstatement of heroin-seeking, Fig. 5 shows a schematic representation of the circuitries underlying relapse to either heroin- (Fig. 5A) or cocaine-seeking behavior (Fig. 5B). In general, the results suggest that the reinstatement circuitry underlying heroin-seeking engages a wider neural network than that for cocaine-seeking. For example, inactivation of the dmPFC, but not the vmPFC, attenuated cocaine-primed (McFarland and Kalivas, 2001) or conditioned-cued (McLaughlin and See, 2003) reinstatement. In contrast, the present results indicate that both the dorsal (i.e., anterior

cingulate and prelimbic cortex) and ventral (i.e., infralimbic cortex) components of the medial PFC mediate both cue- and heroin-induced reinstatement. In accordance with this, increased *zif/268*, a marker of neuronal activity, has been reported in the vmPFC following cue-induced reinstatement of heroin-seeking (Koya et al., 2006). Anatomically, these subregions of the mPFC express differential neuronal projections, with the dmPFC primarily projecting to the BNST, amygdala, and substantia nigra, whereas the vmPFC has a more predominant innervation with the nucleus accumbens, medial striatum, amygdala, VTA, and substantia nigra (Vertes, 2004). Furthermore, the dmPFC and vmPFC differentially project to the nucleus accumbens, with the dmPFC projecting selectively to the NAc core and the vmPFC to the NAc shell (Beckstead, 1979; Newman and Winans, 1980; Groenewegen et al., 1982; Berendse et al., 1992; Voorn et al., 2004). Studies of these differential projections, including *in vivo* neuroimaging, have led some researchers to suggest functional dissociations in the medial PFC in regards to stress responsivity (Sullivan, 2004), decision making (Volz et al., 2006), and regulation of affect (Urry et al., 2006), factors that influence the propensity of relapse to drug-seeking behavior (Bossert et al., 2005).

In regards to limbic circuitry, the current study replicated our earlier finding that BLA inactivation via sodium channel blockade attenuated both cue- and heroin-primed reinstatement (Fuchs and See, 2002). In contrast, BLA inactivation had no effect upon cocaine-primed reinstatement (McFarland and Kalivas, 2001). In addition to the BLA, the present results suggest that intact CEA function is necessary for both cue-induced and heroin-primed reinstatement. Similar to the effects on heroin-seeking, inactivation of the CEA also attenuated cue-induced reinstatement of cocaine-seeking (Kruzich and See, 2001). Anatomically, sensory information enters the amygdala through the BLA which, in turn, projects to the CEA (Pitkanen, 2000). Additionally, the BLA receives projections from the mPFC, orbital and temporal cortices, VTA, substantia nigra, mediodorsal thalamus, and midbrain monoamine neurons. The CEA, in turn, projects to the nucleus accumbens, BNST, substantia nigra, mediodorsal thalamus, reticular formation, lateral hypothalamus, VTA, medial PFC, as well as orbital and temporal cortices (Pitkanen, 2000). Perhaps related to these differential projections, it has been suggested that the CEA modulates the drive-state of the animal, whereas the BLA modulates the coordination of specific behaviors related to incentive stimuli (Phillips et al., 2003).

For the nucleus accumbens, inactivation of the NAc core, but not NAc shell, attenuated cocaine-primed (McFarland and Kalivas, 2001) or conditioned-cue (Fuchs et al., 2004b) reinstatement. The present findings diverged somewhat from cocaine-seeking as inactivation of the NAc shell attenuated heroin-primed reinstatement, but had no effect on conditioned-cue reinstatement. DA neurons innervating the NAc shell originate from the medial/posterior VTA (Ford et al., 2006), which may account for the important role of this structure in heroin-seeking after a heroin prime. In addition, the NAc shell has been implicated in opioid-mediated feeding behavior (Ward et al., 2006). Thus, although the NAc shell may not be necessary for cocaine-seeking, the current results suggest a selective role of the NAc shell in heroin-primed reinstatement. Finally, in regards to the caudate-putamen (an area with dense opiate receptor expression), it has been demonstrated that inactivation for the dlCPu disrupted cocaine-seeking following either explicit extinction training or abstinence from the drug-paired environment (Fuchs et al., 2006). The present results implicate a similar role of the dlCPu in both heroin cue-induced and heroin-primed reinstatement.

As previously found with cocaine-primed reinstatement (McFarland and Kalivas, 2001), inactivation of the VP or SN attenuated both cue-induced and heroin-primed reinstatement. Previously, Tang et al. (2005) reported that intra-VP infusions of the μ -receptor antagonist, CTAP, attenuated cocaine-seeking; however, intra-VP infusions of morphine reinstated cocaine-seeking (Tang et al., 2005). Furthermore, heroin administration increases extracellular

enkephalin in the VP (Olive and Maidment, 1998; Caille and Parsons, 2004). The role of the SN is not unexpected, since μ -receptors are highly expressed in the SN (Mansour et al., 1987; Yaksh, 1997) and that opiate receptors are concentrated on nigral afferents from the forebrain, which affect both locomotor activity and/or reinforcement (Ostrowski and Pert, 1995).

One unique finding that arose from the current study was the differential pattern that emerged in subregions of the BNST. Anatomically, the BNST connects with the NAc shell (Brog et al., 1993), VTA (Georges and Aston-Jones, 2001), as well as the CEA and BLA (Dong and Swanson, 2006), leading some anatomists to describe the BNST as part of the “extended amygdala” (Heimer and Van Hoesen, 2006). Within the BNST, research has focused on the ventrolateral (subcommissural) aspect (i.e., BNSTv), given its implications in opiate abuse (Delfs et al., 2000; Walker et al., 2000). In support of this notion, stimulation of the BNSTv increased VTA DA release (Georges and Aston-Jones, 2001). Whereas our results suggest that the BNSTv plays a role only in heroin-primed reinstatement, the medial posterior component is necessary for both forms of relapse to heroin-seeking. The BNSTmp not only projects to the BNSTv, but also is involved in regulating orofacial responses, body-water homeostasis, neuroendocrine function, and thalamocortical feedback (Dong and Swanson, 2006). Furthermore, the BNSTmp is a part of the vomeronasal (VN) organ, a system that relays pheromone signals to the CNS and influences mating and social behavior (Guillamon et al., 1988; Hosokawa and Chiba, 2005; Witt and Wozniak, 2006). Although beyond the scope of this mapping study to delineate a mechanism by which the BNSTmp is involved in heroin-seeking, it is possible that the BNSTmp plays a unique role in the interoceptive experience of heroin. Clearly more work is needed to elucidate these findings in the BNST.

It is important to note that not every brain region examined plays a role in mediating relapse to heroin-seeking. We selected brain regions that have been previously implicated in motivational behavior with drugs of abuse. Importantly, the anatomical controls (animals for which the cannulae fell into regions outside the targeted motivational circuitry), and animals with cannulae in the somatosensory cortex (SS) showed no differences in responding after B/M vs. vehicle infusions for either form of reinstatement to heroin-seeking behavior. These controls demonstrate a selectivity of our effects by showing that simply inactivating any brain region does not attenuate heroin-seeking behavior. The use of B/M microinfusion allows for highly discrete separation of function, even in adjacent brain areas (McFarland and Kalivas, 2001; Fuchs et al., 2004b; Fuchs et al., 2004a), so it cannot simply be the case that B/M diffused in a widely dispersed pattern following intracranial infusion. Furthermore, it is unlikely that the attenuation of lever responding was due to motor impairment. Inactivation via intracranial infusions of B/M or TTX has been shown to have no effect on locomotor activity when infused into the SN or dICPu (See et al., 2007); VTA, VP, dmPFC, or NAc core (McFarland and Kalivas, 2001); CEA, BNSTv, or NAc shell (McFarland et al., 2004); or the BLA, vmPFC, or SS (Fuchs et al., 2005). The fact that inactivation of these regions attenuates reinstatement of drug-seeking behavior without adversely affecting general locomotor activity further supports the critical role for this circuit in relapse behavior.

CONCLUSIONS

Taken together, the present results suggest that the circuitry underlying relapse to heroin-seeking behavior shows overlapping, yet distinct differences from the circuitry underlying cocaine-seeking. As predicted, inactivation of additional brain regions attenuated reinstatement to heroin-seeking as compared to reinstatement of cocaine-seeking, implying a more diffuse central nervous system network underlying relapse to opiates. At a cellular level, the diffuse network is likely regulated by neuroadaptations driven by long-lasting alterations in the μ opioid receptor (Bart et al., 2004; Drakenberg et al., 2006). These findings add to our current

understanding on opiate addiction and relapse. Given the high degree of interconnectivity among the brain regions examined in the current study, follow-up studies will focus on the interactions necessary to elicit relapse to heroin-seeking and the selective pharmacological dynamics underlying reinstatement in several key cortical and limbic areas.

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Abbreviations

B/M	Baclofen-Muscimol
BLA	Basolateral Amygdala
BNSTmp	Bed Nucleus Stria Terminalis medial posterior
BNSTv	Bed Nucleus Stria Terminalis ventral
CEA	Centromedial Amygdala
CS	Conditioned stimulus
DA	Dopamine
dmPFC	Dorsomedial Prefrontal Cortex
dlCPu	Dorsolateral Striatum
EXT	Extinction
FR	Fixed Ratio
Nacore	Nucleus Accumbens core
Nashell	Nucleus Accumbens shell
SN	Substantia Nigra
VP	Ventral Pallidum
VTA	

Ventral Tegmental Area

vmPFC

Ventromedial Prefrontal Cortex

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Fig. 1.

Microinfusion cannula placement as verified on cresyl violet-stained sections. The symbols represent the most ventral point of the infusion cannula tract for each rat on coronal sections based on the atlas of Paxinos and Watson (1997). Numbers indicate the distance from bregma (mm).

Fig. 2. Responses on the active and inactive levers (mean $\pm$ SEM) during the last three days of self-administration (panel A) and the ten days of extinction sessions (panel B).

Fig. 3.

Conditioned cue-induced reinstatement. Lever responses (mean±SEM) on the previously heroin-paired lever during the last two days of extinction prior to testing (panel A), and following vehicle (panel B) or B/M (panel C) infusions at the time of reinstatement, which consisted of CS presentations in the absence of heroin infusions. Significant differences between vehicle and B/M are indicated (Tukey test, $*P < 0.05$)

Fig. 4.

Heroin-primed reinstatement. Lever responses (mean±SEM) on the previously heroin-paired lever during the last two days of extinction prior to testing (panel A), and following vehicle (panel B) or B/M (panel C) infusions at the time of reinstatement immediately following an injection of heroin (0.25 mg/kg, s.c.) in the absence of cues or heroin infusions. Significant differences between vehicle and B/M are indicated (Tukey test, $*P < 0.05$)

Fig. 5.

(A) The proposed circuitry mediating relapse to heroin-seeking based on the findings above (figure adapted from Kelley, 2004). Compared with cocaine, reinstatement of heroin-seeking, both cue and heroin induced, engages a more diffuse network of neural regions, most notably the vmPFC, CEA, NAcshell, and BNSTmp. (B) The proposed circuitry mediating relapse to cocaine-seeking (based on Kalivas and McFarland, 2003). These authors propose that the projections from the dmPFC to the NAc core represents “a final common pathway” for all priming stimuli. The remaining neural regions may selectively be engaged during specific forms of reinstatement (for example, the BLA is necessary for cue reinstatement, but not for cocaine or stress induced reinstatement). No shading represents neural regions necessary for cue reinstatement only, whereas, striped gray shading represents neural regions necessary for drug-primed reinstatement only. Finally, gray shading refers to neural regions necessary for both cue and drug-primed reinstatement.

Table 1

Stereotaxic coordinates for intracranial cannulae.

BRAIN REGION	Guide Cannulae Coordinates*		
	A/P	M/L	D/V
Dorsomedial Prefrontal Cortex (dmPFC)	+3.0	±0.7	-1.0
Ventromedial Prefrontal Cortex (vmPFC)	+3.0	±0.7	-2.0
Somatosensory Cortex (SS)	+1.2	±5.0	-2.2
Basolateral Amygdala (BLA)	-2.5	±4.7	-6.5
Centromedial Amygdala (CEA)	-2.1	±4.0	-6.0
Bed Nucleus Stria Terminalis ventral (BNST)	-0.4	±3.5	-6.2
Nucleus Accumbens core (NAcore)	+1.2	±1.6	-4.5
Nucleus Accumbens shell (NAshell)	+1.5	±0.6	-4.5
Ventral Tegmental Area (VTA)**	-5.2	±0.85	-6.15
Dorsolateral Striatum (DLS)	+1.2	±3.8	-3.4
Substantia Nigra (SN)	-5.2	±2.1	-6.0
Ventral Pallidum (VP)	-0.25	±2.4	-5.9

* A/P: relative to Bregma; M/L: relative to midline suture; D/V: relative to skull

** VTA cannulae angled 6° from the midline