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Role of Mu- and Delta-Opioid Receptors in the Nucleus Accumbens in Cocaine-Seeking Behavior

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Earlier studies suggest that opioid receptors in the ventral tegmental area, but not the nucleus accumbens (NAc), play a role in relapse to drug-seeking behavior. However, environmental stimuli that elicit relapse also release the endogenous opioid β-endorphin in the NAc. Using a within-session extinction/reinstatement paradigm in rats that self-administer cocaine, we found that NAc infusions of the mu-opioid receptor (MOR) agonist DAMGO moderately reinstated responding on the cocaine-paired lever at low doses (1.0–3.0 ng/side), whereas the delta-opioid receptor (DOR) agonist DPDPE induced greater responding at higher doses (300–3000 ng/side) that also enhanced inactive lever responding. Using doses of either agonist that induced responding on only the cocaine-paired lever, we found that DAMGO-induced responding was blocked selectively by pretreatment with the MOR antagonist, CTAP, whereas DPDPE-induced responding was selectively blocked by the DOR antagonist, naltrindole. Cocaine-primed reinstatement was blocked by intra-NAc CTAP but not naltrindole, indicating a role for endogenous MOR-acting peptides in cocaine-induced reinstatement of cocaine-seeking behavior. In this regard, intra-NAc infusions of β-endorphin (100–1000 ng/side) induced marked cocaine-seeking behavior, an effect blocked by intra-NAc pretreatment with the MOR but not DOR antagonist. Conversely, cocaine seeking elicited by the enkephalinase inhibitor thiorphan (1–10 μg/side) was blocked by naltrindole but not CTAP. MOR stimulation in more dorsal caudate-putamen sites was ineffective, whereas DPDPE infusions induced cocaine seeking. Together, these findings establish distinct roles for MOR and DOR in cocaine relapse and suggest that NAc MOR could be an important therapeutic target to neutralize the effects of endogenous β-endorphin release on cocaine relapse.
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INTRODUCTION

Drug addiction involves dysregulation in brain reward circuitry leading to compulsive drug use (Dackis and O'Brien, 2001; Kalivas and Volkow, 2005; Koob and Le Moal, 2001). In addition to drug reward, the mesolimbic dopamine system plays an integral role in relapse to drug-seeking behavior, as stimuli that elicit drug seeking also activate dopamine neurons in the ventral tegmental area (VTA) leading to dopamine release in forebrain regions, such as the nucleus accumbens (NAc) (Phillips *et al*, 2003; Pruessner *et al*, 2004; Self and Nestler, 1998; Shalev *et al*, 2002; Spealman *et al*, 1999; Stewart, 2000). Opioid receptors also play a role in relapse to cocaine seeking in animal models, as systemic treatment with naltrexone inhibits cocaine seeking elicited by exposure to cocaine-associated cues (Burattini *et al*, 2008). Similarly, cocaine-primed cocaine seeking is blocked by systemic administration of the partial mu-opioid receptor (MOR) agonist buprenorphine, the delta-opioid receptor (DOR) antagonist naltrindole, or the nonspecific opioid antagonist naltrexone (Comer *et al*, 1993; Gerrits *et al*, 2005).

Stewart (1984) and colleagues found that opioid receptors in the VTA play a role in reinstatement of cocaine and heroin seeking, as intra-VTA morphine treatments trigger drug seeking in an extinction/reinstatement paradigm, an animal model of relapse. This effect is thought to involve local disinhibition of dopamine neurons in the VTA, leading to dopamine release in the NAc (Ford *et al*, 2006; Johnson and North, 1992; Leone *et al*, 1991). In contrast, earlier studies suggest that opioid receptors localized in the NAc do not play a role in drug seeking, as intra-NAc morphine treatments fail to reinstate cocaine or heroin seeking (Stewart and Vezina, 1988; Tang *et al*, 2005), and blockade of NAc MOR with the selective MOR antagonist CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2) failed to significantly alter cocaine-primed reinstatement of

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cocaine seeking (Tang *et al*, 2005). However, endogenous opioid peptides, such as β-endorphin, are released in the NAc by cocaine, and stressful situations (Roth-Deri *et al*, 2004, 2003; Zangen and Shalev, 2003), events that trigger the reinstatement of cocaine seeking, and intra-NAc morphine infusions induce a conditioned place preference (van der Kooy *et al*, 1982).

Opioid receptors are highly expressed by NAc neurons (Mansour *et al*, 1995, 1987), and local opioid infusions in the NAc modulate behavior in a biphasic manner. Thus, microgram doses of (D-Ala^2,N-Me-Phe^4,glycinol^5)-enkephalin (DAMGO) (MOR agonist) or morphine infused in the NAc initially suppress locomotion but subsequently induce hyper locomotion (Cunningham and Kelley, 1992; Meyer *et al*, 1994). Lower doses of DAMGO decrease the latency for hyper locomotion to occur (Meyer *et al*, 1994). As doses of agonists used in these locomotor studies are similar to those used in prior reinstatement studies, it is possible that the behavioral suppressive effects masked the potential of NAc opioid receptor stimulation to trigger the reinstatement of drug-seeking behavior. Moreover, NAc infusions of opioid agonists induce feeding behavior but also with a prolonged latency to initiate feeding (Bakshi and Kelley, 1993; Kelley *et al*, 2005). Similarly, intra-NAc infusions of DAMGO increase the motivation for food on a progressive ratio reinforcement schedule, and where response breakpoints are obtained after some delay (Zhang *et al*, 2003). Therefore, it is possible that these delayed motivational effects reflect the initial suppressive effects of high-dose MOR agonist infusions.

In this study, we investigated the role of NAc opioid receptors in the reinstatement of cocaine-seeking behavior using MOR- and DOR-selective ligands and endogenous opioid peptides. We found that NAc infusions of MOR and DOR agonists effectively reinstate cocaine seeking through selective actions at their respective receptors. Stimulation of NAc opioid receptors by the endogenous peptides, β-endorphin and enkephalins, also induced cocaine-seeking behavior. The results clearly establish that either MOR or DOR stimulation in the NAc is sufficient to elicit cocaine-seeking behavior, and that MOR receptors play an important role in cocaine-primed relapse. These findings also suggest that persistent neuroadaptations in NAc opioid receptors following chronic cocaine use could contribute to drug-seeking behavior in prolonged abstinence.

MATERIALS AND METHODS

Animals and Housing Conditions

Male Sprague–Daley rats weighing 225–275 g (Charles River Laboratories, Kingston, NY) were individually housed in wire cages with food and water available *ad libitum*, except during lever press training. Experiments were conducted during the light cycle of a 12 : 12h light/dark cycle (lights on at 0700 hours) in accordance with guidelines established by the National Institute of Health and the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

Sucrose Lever Press Training and Surgery

Lever-press training, self-administration, and reinstatement testing were performed in operant test chambers (Med-Associates, East Fairfield, VT). Chambers were equipped with two response levers and an infusion pump as described earlier (Edwards *et al*, 2007). Animals were food-restricted to prevent weight gain and trained to lever-press for sucrose pellets on a fixed ratio 1 (FR1) reinforcement schedule until an acquisition criteria of 100 sucrose pellets consumed for 3 consecutive test days was reached. Following lever-press training, animals were fed *ad libitum* for at least 1 day before surgery. Animals were anesthetized and implanted with a chronic indwelling catheter into the jugular vein that exited subcutaneously on the back. An intracranial, 26-guage bilateral guide cannula was aimed at the NAc ($\pm$ 1.5 mm lateral; 1.7 mm anterior to bregma; $-$5.7 ventral to dura with the level skull) or caudate putamen (CPu) ($\pm$ 1.5 mm lateral; 1.7 mm anterior to bregma; $-$3.2 mm ventral to dura) (Paxinos and Watson, 1998). Dummy and infusion cannulae (33 gauge) were cut to extend 1 mm beyond the guide cannulae tip, and dummy cannulae remained in place until the day of intracranial drug infusion. Animals were allowed 5–7 days to recover before starting the experiment.

Cocaine Self-Administration and Within-Session Reinstatement Testing

Animals were tested in a within-session extinction/reinstatement paradigm as described earlier (Bachtell *et al*, 2005). Briefly, animals self-administered cocaine (0.5 mg/kg in 0.1 ml over 5 s, time-out period 15 s) in daily 4 h sessions for 5–6 days/week until a criteria of 3 consecutive days of $<$10% variance in mean cocaine intake was reached ($\sim$3 weeks). During the 5s injection, a cue light above the lever was illuminated, whereas the house light was turned off for the entire injection and time-out period. Subsequently, animals were trained in the within-session extinction paradigm that consisted of 1 h cocaine availability followed by 3 h extinction conditions in which only response-contingent injection cues were available. Animals extinguished responding to criteria of $\leqslant$5 responses at either the drug-paired or the inactive lever for the final hour of the session for at least three consecutive sessions, while maintaining a minimum of 15 self-administered cocaine injections with $\pm$ 10% variability in the first hour of the session. Mean cocaine self-administration on the test day was 25 $\pm$ 0.74 (NAc) and 26 $\pm$ 0.95 (CPu) injections/h. Test days were conducted with an intra-NAc infusion of MOR and DOR agonists alone (0.5 μl/side over 2 min) or in combination as sequential antagonist/agonist infusions (1.0 μl/side total volume) immediately before the final hour of the test session. For cocaine priming experiments, animals received NAc or CPu antagonist infusions followed immediately by iv priming with saline (0.4 ml) or cocaine (2.0 mg/kg in 0.4 ml). Following each test day, animals returned to within-session extinction training until stable self-administration and extinction criteria were reached for at least two consecutive sessions before the next test. Animals received a maximum of eight intracranial test infusions.

Locomotor Testing

Some animals trained in the within-session reinstatement paradigm were given 1 week off from cocaine self-administration and tested for locomotor responses to agonist infusions in the NAc or CPu using peak doses for reinstatement that were selective for the drug-paired lever. The locomotor testing apparatus consisted of a circular-shaped plexiglass arena with 12 cm wide metal floors (Med-Associates) with four pairs of photocells located at 90° intervals around the 1.95 m perimeter to record locomotor activity. Animals were habituated for 2 h in the dark followed by an intra-NAc or intra-CPu drug infusion and returned to the locomotor chambers for 2 h of subsequent testing. Testing of each drug was randomized and performed on consecutive days. Animals received five injections in locomotor tests.

Histological Confirmation of Injection Sites

Animals were anesthetized with chloral hydrate, and cresyl violet (0.3 μl) was infused into the NAc or CPu through the guide cannula. Animals were immediately decapitated and brains removed. Slices (0.8 mm thick) were collected throughout the forebrain and analyzed under a dissecting microscope for the location of the infusion sites according to the coordinates of Paxinos and Watson (1998).

Drugs

Drugs used were DAMGO, DPDPE ((D-Pen^2,D-Pen^5)-enkephalin), CTAP, β-endorphin, met-enkephalin, and thiorphan (Bachem Bioscience Inc., King of Prussia, PA), and naloxone and naltrindole (Sigma-Aldrich, Atlanta, GA). Ligands were dissolved in 0.9% sterile saline except thiorphan, which was dissolved in 1 : 4 DMSO/saline. Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (Research Triangle Park) and dissolved in 0.9% sterile saline.

Statistical Analysis

As not all animals completed an experiment, data were analyzed using a two-factor mixed regression analysis (SAS 9.1.3) of treatment × lever, followed by main effects analysis of each lever separately. *Post hoc* tests utilized one- or two-tailed Dunnett's tests where appropriate for comparison with controls and Tukey's honestly significant difference test for pair-wise comparison where appropriate. Locomotor data were analyzed by one-way repeated measures ANOVA on treatment for each hour tested. *Post hoc* tests utilized Dunnett's one-tailed test for comparison with controls.

RESULTS

MOR and DOR Involvement in Reinstatement

We first determined whether NAc infusions of the MOR-selective agonist, DAMGO, and the DOR-selective agonist, DPDPE, could reinstate non-reinforced drug-paired lever responding following extinction of cocaine seeking. Intra-NAc infusions of DAMGO produced an inverted U-shaped dose–response curve (Figure 1a) for non-reinforced responding on the drug-paired but not inactive lever (dose × lever: $F_{6,161} = 2.37$, $p = 0.032$), with a main effect of both dose ($F_{6,161} = 3.52$, $p = 0.003$) and lever ($F_{1,161} = 46.01$, $p < 0.001$). DAMGO induced moderate peak rates of responding at very low doses (1–3 ng/side) when compared with vehicle infusions without increasing inactive lever responding, whereas higher doses (10 ng/side) led to reduced responding (drug-paired lever: $F_{6,66} = 3.33$, $p = 0.006$; inactive lever: $F_{6,66} = 1.13$, $p =$ NS). Similarly, intra-NAc infusions of DPDPE produced an inverted U-shaped dose–response curve (Figure 1b), but induced greater responding at higher doses of 300–3000 ng/side (dose: $F_{6,113} = 12.09$, $p < 0.001$; lever: $F_{1,113} = 28.16$, $p < 0.001$). Unlike DAMGO, DPDPE induced substantial and significant lever pressing of both drug-paired and inactive levers compared with vehicle (drug-paired lever: $F_{6,40} = 11.37$, $p < 0.001$; inactive lever: $F_{6,40} = 3.34$, $p = 0.009$). Inactive lever responding increased significantly only at the peak dose for drug-paired lever responding (1000 ng/side).

Antagonist Inhibition of Agonist-Mediated Reinstatement

To determine whether DAMGO-stimulated reinstatement of cocaine seeking was mediated by MOR stimulation in the

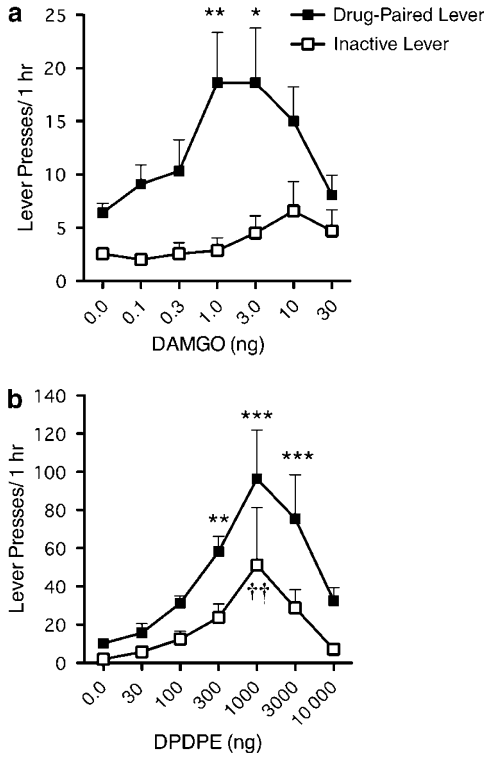

Figure 1 Intra-NAc infusions of (a) the mu-opioid receptor-selective agonist DAMGO or (b) the delta-opioid receptor-selective agonist DPDPE increase non-reinforced drug-paired lever responding in a within-session reinstatement procedure. Data represent the mean ± SEM for doses of DAMGO ($n = 9$–27 animals/treatment) and DPDPE ($n = 5$–22 animals/treatment). Symbols indicate that drug-paired lever (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) or inactive lever ($^{\dagger\dagger}p < 0.01$) differs from vehicle-infused controls by Dunnett's *post hoc* tests.

NAc, we tested the ability of the MOR-selective antagonist, CTAP, to block DAMGO-primed reinstatement using the lowest effective dose from the earlier experiment (1 ng/side). Intra-NAc pretreatment of CTAP dose dependently blocked DAMGO-primed reinstatement (Figure 2a; dose × lever: $F_{6,172} = 7.82$, $p < 0.001$), with a main effect of dose ($F_{6,172} = 7.78$, $p < 0.001$) and lever ($F_{1,172} = 123.36$, $p < 0.001$). Non-reinforced responding at the drug-paired lever was blocked with maximally effective doses as low as 0.1 ng/side of CTAP (drug-paired lever: $F_{6,67} = 8.59$, $p < 0.001$; inactive lever: $F_{6,67} = 1.19$, $p =$ NS). Similarly, we tested the DOR-selective antagonist, naltrindole, against the lowest effective dose for DPDPE-induced reinstatement that did not increase inactive lever responding (300 ng). Figure 2b shows that intra-NAc treatment of naltrindole reduced DPDPE-primed reinstatement in a dose-dependent manner, achieving control levels at 1000 ng/side (dose × lever: $F_{4,139} = 2.85$, $p = 0.026$; dose: $F_{4,139} = 11.35$, $p < 0.001$; lever: $F_{1,139} = 55.54$, $p < 0.001$). Drug-paired lever responding was significantly attenuated starting at 300 ng/side, with the maximal suppression at 1000 ng/side ($F_{4,58} = 11.63$, $p < 0.001$). Naltrindole produced some mild suppression of responding on the inactive lever (inactive lever: $F_{4,58} = 2.48$, $p = 0.05$).

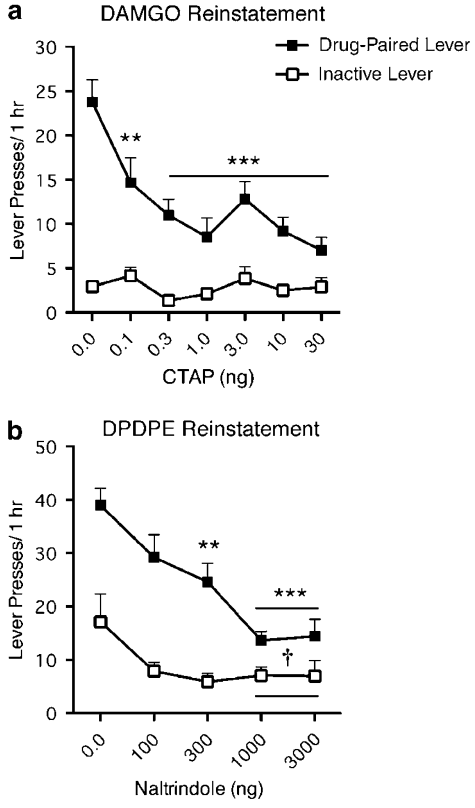

Figure 2 Intra-NAc pretreatment with (a) the mu-opioid receptor-selective antagonist CTAP followed by 1 ng DAMGO and (b) the delta-opioid receptor-selective antagonist naltrindol followed by 300 ng DPDPE dose dependently attenuates reinstatement of cocaine seeking. Data represent the mean ± SEM for DAMGO/CTAP ($n = 13$–22 animals/treatment) and DPDPE/naltrindole ($n = 18$–20 animals/treatment) combinations. Symbols indicate that drug-paired lever (**$p < 0.01$, ***$p \leqslant 0.001$) or inactive lever ($^{\dagger}p < 0.05$) differs from agonist/vehicle-infused controls by Dunnett's *post hoc* tests.

Drug Specificity and Antagonist Inhibition of Cocaine-Mediated Reinstatement

To determine whether DAMGO- and DPDPE-induced cocaine seeking was specific to MOR or DOR blockade, MOR and DOR agonists and antagonists were tested in a cross-blockade experimental design. Animals were given intra-NAc infusions of DAMGO (1 ng/side) or DPDPE (300 ng/side) following pretreatment with maximally effective doses of CTAP (30 ng/side), naltrindole (1000 ng/side), or vehicle. Figure 3a shows that DAMGO-induced reinstatement of drug-paired lever responding was selectively blocked by CTAP, but not naltrindole, when compared with vehicle (treatment × lever: $F_{2,82} = 5.09$, $p = 0.008$; treatment: $F_{2,82} = 4.84$, $p = 0.01$; lever: $F_{1,82} = 68.65$, $p < 0.001$). CTAP significantly attenuated drug-paired lever responding ($F_{2,29} = 5.61$, $p = 0.009$), with no effect on inactive lever responding ($F_{2,29} = 0.40$, $p =$ NS). Conversely, Figure 3b shows that DPDPE-induced reinstatement was selectively blocked by naltrindole but not CTAP (treatment: $F_{2,40} = 8.83$, $p < 0.001$; lever: $F_{1,40} = 39.01$, $p < 0.001$), with attenuation mainly on the drug-paired lever ($F_{2,13} = 5.65$, $p = 0.017$) and a trend for reduction in lower responding on the inactive lever ($F_{2,13} = 2.87$, $p = 0.092$). Together, these results indicate that a selective stimulation of either MOR or DOR in the NAc is sufficient to independently trigger cocaine-seeking behavior.

Given that cocaine injections are known to increase endogenous opioid release in the NAc, we tested whether MOR or DOR in the NAc plays a role in cocaine-primed reinstatement of cocaine-seeking behavior. Animals were given NAc pretreatments with vehicle, CTAP, or naltrindole immediately before an iv cocaine injection (2 mg/kg) in the reinstatement paradigm. As the peak dose of CTAP (30 ng against 1 ng DAMGO) had no effect on cocaine-primed reinstatement (data not shown), we tested a higher dose of CTAP (3 μg/side) more commonly used in intracranial studies (Soderman and Unterwald, 2008; Tang *et al*, 2005), along with the 1μg/side dose of naltrindole. The affinity of CTAP for MOR (2.36 ± 0.46 nM) is 15.7 times lower than that of naltrindole for DOR (0.15 ± 0.01 nM) (Bonner *et al*, 2000; Clayson *et al*, 2001; Pelton *et al*, 1986; Portoghese *et al*, 1988), indicating that relatively higher amounts of CTAP than naltrindole may be required to inhibit endogenous opioid activity at MOR than DOR. Furthermore, the doses of CTAP and naltrindole used were roughly molar equivalents (5.4 and 4.8 μM, respectively). Intra-NAc pretreatment with CTAP significantly reduced cocaine-primed reinstatement compared with vehicle pretreatment (Figure 3c), whereas pretreatment with naltrindole did not (treatment × lever: $F_{2,82} = 4.17$, $p = 0.019$; treatment: $F_{2,82} = 5.12$, $p = 0.008$; lever: $F_{1,82} = 62.51$, $p = 0.001$). CTAP significantly attenuated drug-paired lever responding in response to an iv cocaine prime without affecting inactive lever responding (drug-paired lever: $F_{2,17} = 7.08$, $p = 0.006$; inactive lever: $F_{2,17} = 0.38$, $p =$ NS). These findings indicate that endogenous opioid release in the NAc contributes to cocaine-primed reinstatement of cocaine seeking through the activation of MOR but not DOR.

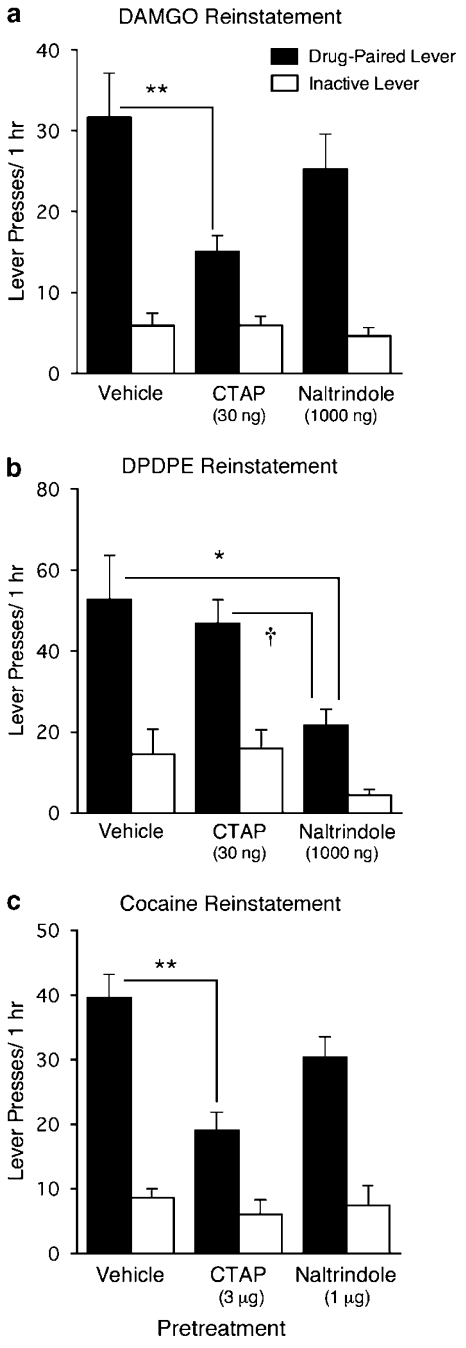

Figure 3 (a) Reinstatement induced by 1 ng DAMGO is blocked by intra-NAc pretreatment with CTAP and not naltrindole. (b) Reinstatement induced by 300 ng DPDPE is blocked by intra-NAc pretreatment with naltrindole and not CTAP. (c) Reinstatement induced by intravenous cocaine priming (2 mg/kg) is blocked by intra-NAc pretreatment with CTAP and not naltrindole. Data represent the mean ± SEM agonist/antagonist combinations ($n = 12$–40 animals/treatment). Symbols indicate that drug-paired lever responses differ from agonist/vehicle-infused controls (*$p < 0.05$, **$p < 0.01$) or CTAP differs from naltrindole ($^{\dagger}p < 0.05$) by Tukey's HSD *post hoc* tests.

Endogenous Opioid Peptides Reinstate Cocaine Seeking in the NAc

The next set of experiments determined the ability of endogenous opioids to reinstate cocaine seeking using intra-NAc infusions of β-endorphin and met-enkephalin. Intra-NAc infusions of β-endorphin dose dependently reinstated responding on the cocaine-paired lever, with effective doses ranging from 100 to 1000 ng/side (Figure 4a; dose × lever: $F_{4,103} = 4.07$, $p = 0.004$; dose: $F_{4,103} = 12.11$, $p < 0.001$; lever: $F_{1,103} = 51.27$, $p < 0.001$). β-Endorphin infusions significantly increased drug-paired lever responding ($F_{4,42} = 8.82$, $p < 0.001$), with a minor increase in inactive lever responding at the highest dose (inactive lever: $F_{4,42} = 7.32$, $p < 0.001$). In contrast, intra-NAc infusions of met-enkephalin failed to reinstate cocaine seeking up to doses as high as 10 μg/side (Figure 4b; $F_{5,102} = 0.44$, $p =$ NS).

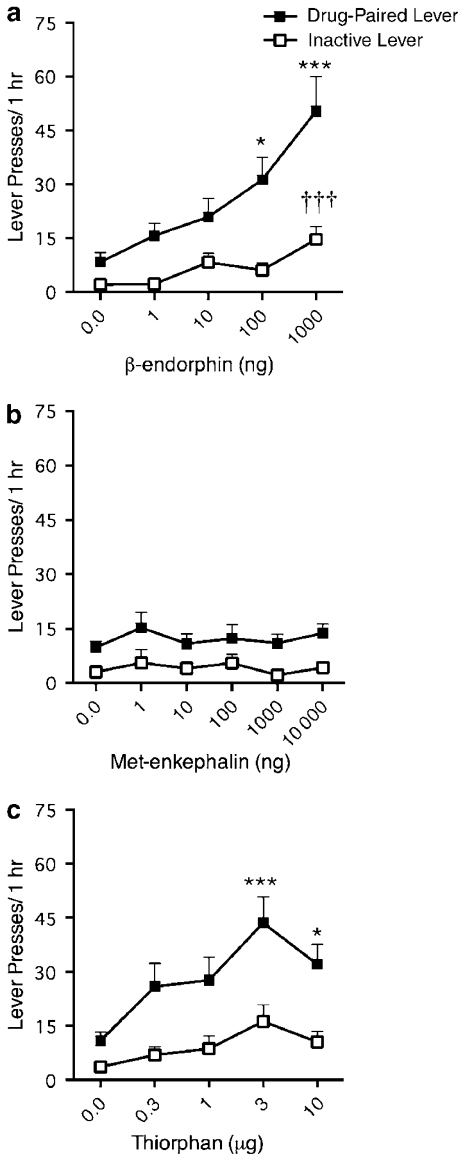

Figure 4 Intra-NAc infusion of (a) the endogenous opioid peptide β-endorphin dose dependently reinstates drug-paired lever responding, (b) whereas met-enkephalin has no effect. (c) Intra-NAc infusion of the enkephalinase inhibitor thiorphan significantly increases drug-paired lever responding. Data represent the mean ± SEM for doses of β-endorphin ($n = 10$–15 animals/treatment), met-enkephalin ($n = 7$–11 animals/treatment), and thiorphan ($n = 10$–15 animals/treatment). Symbols indicate that drug-paired lever (*$p < 0.05$, ***$p < 0.001$) or inactive lever ($^{\dagger\dagger\dagger}p < 0.001$) differs from vehicle-infused controls by Dunnett's *post hoc* tests.

As earlier studies used enkephalin derivatives, suggesting that enkephalins are degraded too rapidly to produce effects in behavioral tests (Kalivas and Bronson, 1985; Phillips *et al*, 1983), we used the enkephalinase inhibitor thiorphan to determine if the accumulation of endogenously released enkephalins would reinstate cocaine seeking. Intra-NAc thiorphan infusions effectively reinstated responding to levels similar to β-endorphin (Figure 4c; dose: $F_{4,92} = 6.77$, $p < 0.001$; lever: $F_{1,92} = 63.27$, $p < 0.001$). Thiorphan induced prominent responding on the drug-paired lever ($F_{4,36} = 4.55$, $p = 0.004$), with minor increases in responding on the inactive lever at the peak dose of 3.0 μg that approached significance ($F_{4,36} = 2.48$, $p = 0.061$). These findings indicate that either MOR-preferring (β-endorphin) or DOR-preferring (enkephalins) endogenous opioid peptides in the NAc are capable of eliciting cocaine-seeking behavior.

Receptor Specificity of Endogenous Opioid-Induced Reinstatement of Cocaine Seeking

Although β-endorphin and enkephalins preferentially interact with MOR and DOR respectively, they also interact with other opioid receptors. We tested the ability of 3 μg CTAP, 1 μg naltrindole, and the less-specific opioid antagonist naloxone to block β-endorphin- and thiorphan-induced reinstatement. Animals were given NAc infusions of maximal effective doses of β-endorphin (1 μg/side) or thiorphan (3 μg/side) immediately following vehicle, CTAP (3 μg/side), naltrindole (1 μg/side), or naloxone (10 μg/side) pretreatments. Figure 5a shows that β-endorphin-induced reinstatement of cocaine seeking was selectively attenuated by CTAP or naloxone, but not naltrindole (treatment × lever: $F_{3,74} = 4.83$, $p = 0.004$; treatment: $F_{3,74} = 10.45$, $p < 0.001$; lever: $F_{1,74} = 66.94$, $p < 0.001$), specifically reducing responding on the drug-paired lever ($F_{3,27} = 12.63$, $p < 0.001$) and not on the inactive lever ($F_{3,27} = 0.94$, $p =$ NS). Conversely, Figure 5b shows that reinstatement elicited by the enkephalinase inhibitor thiorphan was blocked selectively by naltrindole or naloxone, but not significantly by CTAP (treatment: $F_{3,69} = 5.55$, $p = 0.002$; lever: $F_{1,69} = 15.19$, $p < 0.001$). Naltrindole and naloxone reduced thiorphan-induced responding on the drug-paired and not inactive lever (drug-paired lever: $F_{3,26} = 4.45$, $p = 0.012$; inactive lever: $F_{3,26} = 2.23$, $p =$ NS). Thus, the endogenous opioid peptide, β-endorphin, reinstates cocaine seeking through the selective activation of NAc MOR, whereas elevations in endogenous enkephalin levels trigger cocaine seeking primarily through DOR activation, consistent with their preference for these receptors.

Regional Specificity For MOR- But Not DOR-Induced Reinstatement of Cocaine-Seeking Behavior

To determine whether MOR and DOR stimulation of cocaine seeking was specific to the NAc, or due to potential spread up the cannulae shaft, we infused effective doses of all agonists 2.5 mm dorsal to the NAc site in the CPu, a region shown to have similar expression patterns of opioid receptors as the NAc. Although none of the MOR-acting agonists or the enkephalinase inhibitor induced reinstatement in the CPu (Figure 6a), CPu infusions of DPDPE were

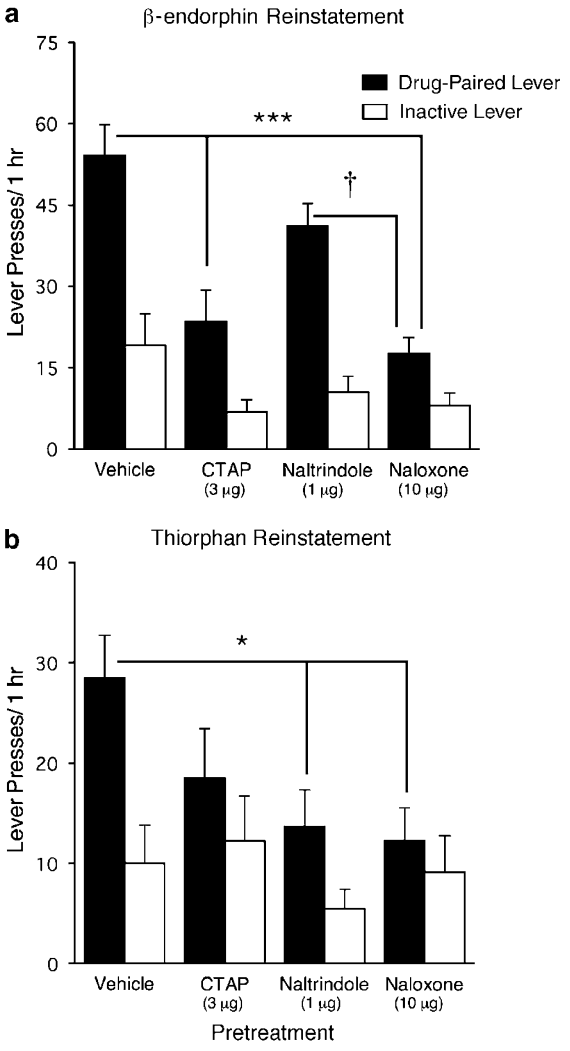

Figure 5 (a) Reinstatement induced by 1 μg β-endorphin is blocked by intra-NAc pretreatment with CTAP or naloxone, but not naltrindole. (b) Reinstatement induced by 3 μg thiorphan is blocked by intra-NAc pretreatment with naltrindole or naloxone, but not CTAP. Data represent the mean ± SEM for β-endorphin/antagonist ($n = 9$–17 animals/treatment) and thiorphan/antagonist ($n = 8$–14 animals/treatment) combinations. Symbols indicate that drug-paired lever responses differ from agonist/vehicle-infused controls (*$p < 0.05$, ***$p < 0.001$) or differs from naltrindole ($^{\dagger}p < 0.05$) by Tukey's HSD *post hoc* tests.

sufficient to stimulate responding (treatment × lever: $F_{4,97} = 2.41$, $p = 0.05$; treatment: $F_{4,97} = 6.51$, $p < 0.001$; lever: $F_{1,97} = 40.91$, $p < 0.001$), with increases in responding on both the drug-paired lever ($F_{4,38} = 4.61$, $p = 0.004$) and inactive lever ($F_{4,38} = 3.43$, $p = 0.017$). It should be noted, however, that the 300 ng/side dose of DPDPE elicited twice as much responding in the NAc than in the CPu. In addition, intra-CPu pretreatment with CTAP at a dose that blocked cocaine-primed reinstatement in the NAc failed to alter cocaine seeking when infused into the CPu (Figure 6b) compared with vehicle-pretreated animals (treatment × lever: $F_{1,14} = 0.08$, $p =$ NS; treatment: $F_{1,14} = 0.09$, $p =$ NS; lever: $F_{1,14} = 10.21$, $p < 0.01$). Together, these data indicate that MOR involvement in reinstatement of cocaine seeking is specific to the NAc, whereas DORs in both sites are capable of triggering this behavior.

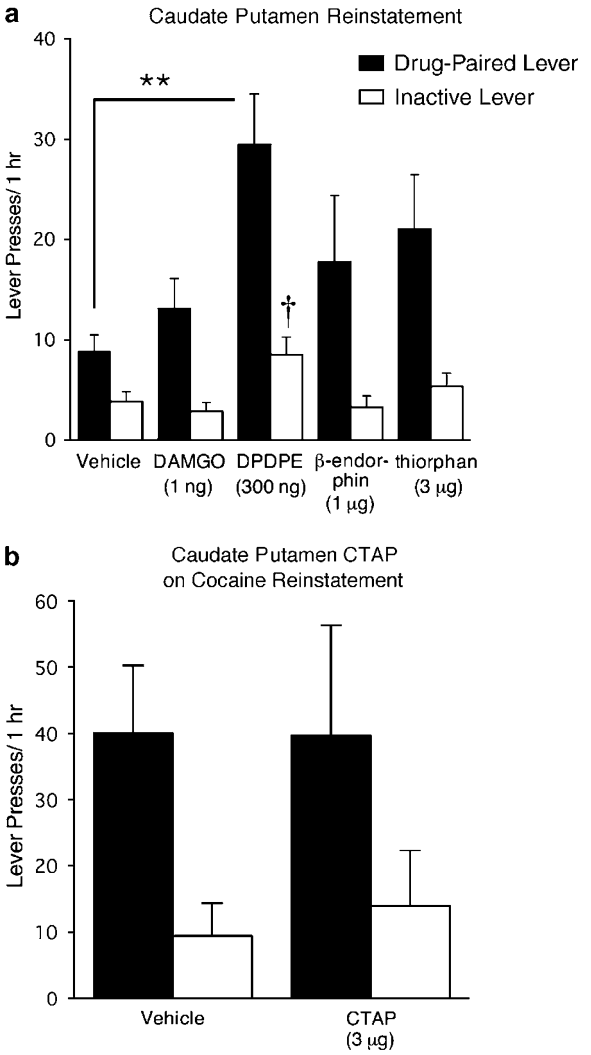

Figure 6 (a) Effective NAc doses of DAMGO, β-endorphin, and thiorphan are ineffective at reinstatement when infused in the CPu, whereas intra-CPu DPDPE induces significant drug-paired and inactive lever responding. (b) Pretreatment with intra-CPu infusions of CTAP has no effect on reinstatement induced by intravenous cocaine priming (2 mg/kg). Data represent the mean ± SEM for doses of agonists ($n = 7$–18 animals/treatment) and cocaine/antagonist ($n = 6$–8 animals/treatment) combinations. Symbols indicate that drug-paired lever responses (**$p < 0.01$) or inactive lever responses ($^{\dagger}p < 0.05$) differ from vehicle-infused controls by Dunnett's *post hoc* tests.

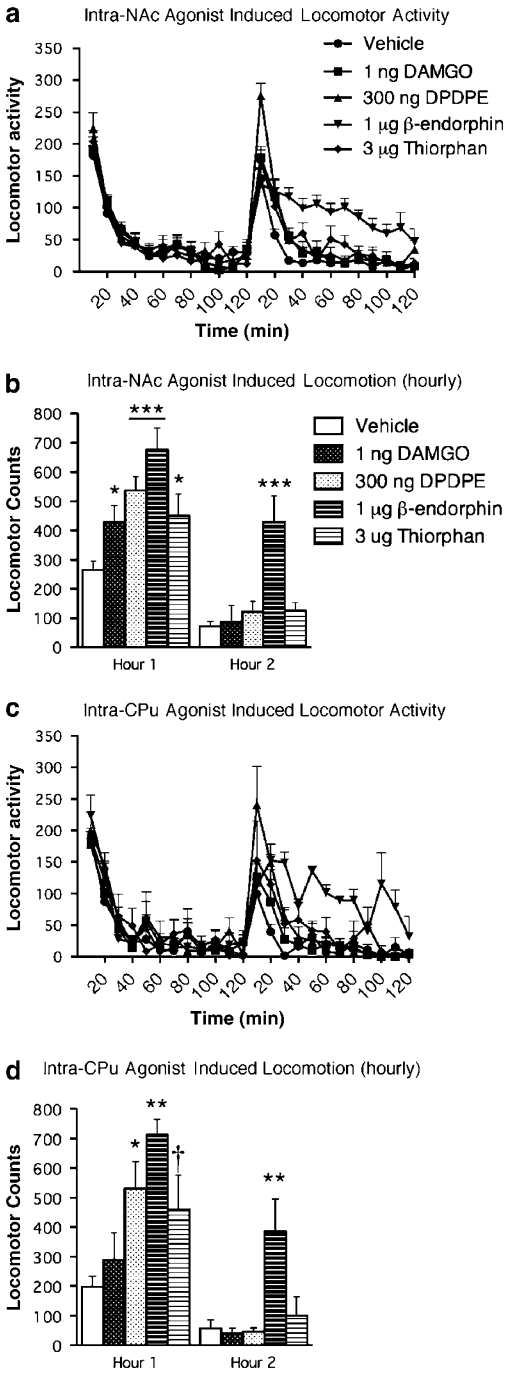

Figure 7 Effects of reinstating doses of agonist treatments on horizontal locomotion in cocaine-trained animals. (a) Timeline of locomotor behavior during habituation for 2 h and following intra-NAc infusion of opioid agonist. (b) All agonists increase locomotor behavior for 1 h when infused in the NAc whereas β-endorphin activity remains elevated during the second hour of testing. (c) Timeline of locomotor behavior in response to the intra-CPu infusion of opioid agonists. (d) Only DPDPE and β-endorphin increased locomotor responding in the CPu, with a trend for thiorphan to increase locomotion. Data represent the mean ± SEM for NAc ($n = 7$–13 animals/treatment) and CPu ($n = 4$–5 animals/treatment). Symbols indicate (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, $^{\dagger}p = 0.059$) lever differs from vehicle-infused controls by Dunnett's *post hoc* tests.

Opioid Agonist Induction of Locomotor Behavior in Cocaine-Trained Animals

Following 1-week withdrawal from cocaine self-administration and reinstatement testing, the locomotor response to intracranial infusions of DAMGO, DPDPE, β-endorphin and thiorphan was tested using doses that produced peak and primarily drug-paired lever responding when infused in the NAc. Figure 7a and b shows that all treatments increased locomotion for 1 h after infusion into the NAc when compared with vehicle infusions ($F_{4,46} = 8.429$, $p < 0.001$), whereas only β-endorphin increased locomotion for at least 2 h after infusion ($F_{4,41} = 8.258$, $p < 0.001$). Thus, the lower doses of DAMGO and DPDPE that triggered cocaine seeking produced psychomotor effects without the delay typically observed with higher doses in earlier studies. Figure 7c and d shows that very similar locomotor responses were

produced by infusions of DPDPE and β-endorphin in the CPu ($F_{4,16} = 5.427$, $p = 0.006$), with a trend for thiorphan to increase locomotor activity during the first hour ($p = 0.059$). In contrast, intra-CPu infusions of DAMGO failed to significantly increase locomotion. Together, these findings suggest that although psychomotor activation may accompany reinstatement of cocaine seeking with NAc infusions, similar locomotor responses with CPu infusions are dissociated from cocaine seeking in many cases.

Injection Sites

Figure 8 illustrates the localization of all infusion sites in the NAc and CPu used in this study. Fourteen animals were eliminated from NAc studies, and three animals were eliminated from CPu studies, due to misplacement of one or both cannulae.

DISCUSSION

This study found that the selective stimulation of either MOR or DOR in the NAc is sufficient to reinstate cocaine-seeking behavior in rats following extinction of cocaine self-administration. Thus, NAc infusions of either the MOR-selective agonist, DAMGO, or the DOR-selective agonist, DPDPE, effectively elicited cocaine-seeking responses on the drug-paired lever that delivered cocaine injections during prior self-administration. The threshold dose for reinstating cocaine seeking was 300 times lower with DAMGO (1 ng/side) than that with DPDPE (300 ng/side), whereas DPDPE induced greater peak rates of responding and was associated with generalized but lower rates of responding on the inactive lever. This latter effect with DPDPE could be related to psychomotor activation rather than motivation for cocaine, or an inability to appropriately discriminate the drug-paired from inactive levers with increased DOR stimulation, although a lower dose of DPDPE selectively induced responding on the drug-paired lever. Both of these metabolically stable opioid peptide agonists produced an inverted U-shaped dose–response curve, indicating that higher doses were ineffective, potentially explaining the failure to detect morphine-induced reinstatement of drug seeking at microgram doses used in earlier studies (Stewart and Vezina, 1988; Tang *et al*, 2005).

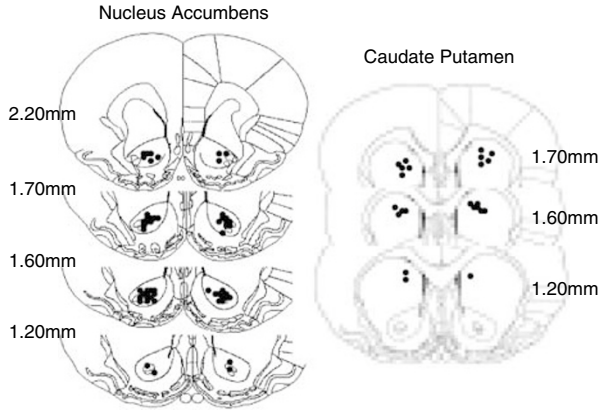

Figure 8 Localization of infusion sites in the medial NAc core and CPu (+1.2 through +2.2 mm from Bregma, Paxinos and Watson, 1998).

In contrast, intra-NAc infusions of the endogenous opioid peptide β-endorphin induced cocaine seeking with a monophasic dose–response curve up to 1 μg/side, possibly reflecting the sensitivity of this peptide to metabolic degradation. Importantly, the reinstating effects of both DAMGO and β-endorphin were blocked by the MOR-selective antagonist CTAP, and not by the DOR-selective antagonist naltrindole. The ability of DAMGO and β-endorphin to reinstate cocaine seeking was localized to the NAc, as the failure of more dorsal CPu infusions to reinstate responding negates the possibility of diffusion along the cannulae shaft or into the cerebral ventricles. These data firmly establish that MORs in the NAc mediate relapse to cocaine-seeking behavior. Although the stimulation of MOR in dorsomedial CPu is ineffective, dorsolateral CPu sites could be involved in cocaine seeking, given that the inactivation of this site reduces cocaine-seeking behavior (See *et al*, 2007).

In addition, infusions of CTAP into the NAc, but not the CPu, attenuated cocaine-primed reinstatement, possibly relating to the ability of cocaine to increase endogenous β-endorphin release in the NAc (Olive *et al*, 2001; Roth-Deri *et al*, 2003). Higher doses of CTAP (3 μg) were required to attenuate cocaine-primed reinstatement than DAMGO-primed reinstatement (30 ng), possibly reflecting higher concentrations of cocaine-induced β-endorphin release relative to the very low doses of DAMGO that were effective (1–3 ng/side). The high dose of CTAP that attenuated cocaine-primed reinstatement also blocked β-endorphin-primed reinstatement that required a higher dose range (0.1–1 μg) than found with DAMGO.

Cocaine-induced β-endorphin release in the NAc is blocked by dopamine receptor antagonist infusions in the arcuate nucleus of the hypothalamus (Doron *et al*, 2006), the primary source for β-endorphin innervation of the NAc. β-Endorphin release in the NAc also is induced by exposure to footshock stress, or the unmet expectation of cocaine reward under extinction conditions (Roth-Deri *et al*, 2003; Zangen and Shalev, 2003), situations that elicit cocaine-seeking behavior. Thus, taken together with our findings, β-endorphin stimulation of MOR in the NAc could contribute to cocaine seeking elicited by cocaine priming, exposure to cocaine-associated environments, and stressful events. In contrast, an earlier study found that NAc pretreatment with CTAP does not block cocaine-primed reinstatement using longer acting intraperitoneal cocaine-priming injections (Tang *et al*, 2005), whereas an effective blockade was found using shorter acting intravenous cocaine priming in our study. Another difference could involve the use of the within- *vs* between-session extinction/reinstatement paradigms.

Contrary to DAMGO and β-endorphin, cocaine seeking induced by DPDPE was blocked by pretreatment with DOR- but not the MOR-selective antagonist in the NAc. NAc infusions of the enkephalinase inhibitor thiorphan (to elevate endogenous enkephalins) also reinstated cocaine seeking, and the effect was blocked by DOR antagonist pretreatment, although marginal (nonsignificant) attenuation was found with the MOR antagonist potentially relating to enkephalin activity at MOR. DOR stimulation in more dorsal CPu sites with DPDPE also induced a moderate degree of cocaine seeking, but with greater

efficacy in the NAc. Moreover, the reinstating effect of DPDPE in the CPu was accompanied by a significant inactive lever responding, an effect not found with this DPDPE dose in the NAc, and potentially relating to psychomotor activation, as discussed above. In this regard, infusions of the enkephalinase inhibitor thiorphan in the CPu failed to reinstate cocaine seeking and had no effect on inactive lever responding in either striatal site. Together, the double dissociation with MOR- and DOR-selective ligands clearly indicates that MORs and DORs in the NAc mediate cocaine seeking through distinct and independent mechanisms.

Interestingly, blockade of DOR in the NAc failed to attenuate cocaine-primed reinstatement of cocaine seeking. Whether cocaine increases extracellular enkephalins in the NAc is unknown, but cocaine acutely increases preproenkephalin expression throughout the striatum (Hurd and Herkenham, 1992), although this acute effect is diminished with chronic cocaine administration (Arroyo *et al*, 2000; Mantsch *et al*, 2004). One study found that systemic administration of naltrindole decreases cocaine self-administration but only at doses that also suppressed locomotor behavior (de Vries *et al*, 1995). Another study showed reduced lever pressing for cocaine irrespective of reinforcement schedule (Reid *et al*, 1995), and intra-NAc infusions of an irreversible DOR-alkylating analog of naltrindole (Portoghese *et al*, 1990) decreased responding for cocaine on a more demanding progressive ratio schedule of reinforcement (Ward and Roberts, 2007), suggesting generalized effects on motor performance. In contrast, icv administration of the naltrindole analog strongly reduced heroin self-administration while only modestly decreasing cocaine self-administration on a less-demanding fixed-ratio reinforcement schedule (Martin *et al*, 2000), suggesting that endogenous DOR activity plays little role in the effects of cocaine. Similarly, our results are consistent with the notion that endogenous release of enkephalins in the NAc does not contribute to cocaine-primed reinstatement of cocaine seeking, but further tests are needed to determine whether cocaine seeking induced by stress or cocaine-associated cues involves endogenous enkephalinergic activity at NAc DOR.

Intra-NAc infusions of MOR and DOR agonists at doses that effectively reinstated cocaine seeking also increased horizontal locomotion, with β-endorphin infusions producing prolonged effects over 2 h of testing. Infusions of all treatments into the dorsomedial CPu also increased locomotion to similar levels, with the exception of the MOR agonist DAMGO, whereas only the DOR agonist DPDPE triggered cocaine seeking in this region. Although these data support the notion that DPDPE-induced reinstatement may be related to psychomotor activation, the dissociation of locomotor activity and cocaine seeking with infusions of β-endorphin and thiorphan in the CPu suggests that the reinstating effects of these treatments in the NAc are not related to generalized psychomotor activation. Moreover, although it could be argued that DAMGO-induced reinstatement is related to psychomotor activation, the lack of increases in inactive lever responding with DAMGO infusions suggests that reinstatement reflects motivational rather than motor effects. In contrast to reinstatement of cocaine seeking, NAc infusions of higher doses of DAMGO (0.25–2.5 μg) induce a delayed increase in locomotion and preference for sucrose and high-fat foods often after a period of behavioral suppression (Cunningham and Kelley, 1992; Meyer *et al*, 1994; Zhang and Kelley, 1997; Kelley *et al*, 2005), whereas we found that very low doses induce locomotion and cocaine seeking without delay. These findings suggest that lower doses of DAMGO could be employed to elicit appetitive behavior without delay in future studies.

Although MOR and DOR are coupled to similar intracellular signaling pathways, their distinct involvement in modulating drug-seeking behavior can be attributed to differences in their subanatomical distribution. MORs are largely expressed extrasynaptically on dendrites and dendritic shafts of GABAergic and cholinergic cells within striatal patches (Svingos *et al*, 1997; Wang and Pickel, 1998) in which they modulate excitatory and GABAergic input to NAc neurons (Gracy *et al*, 1997). Presynaptic MOR can also modulate the release of GABA onto NAc neurons (Svingos *et al*, 1997). DOR can either directly or indirectly modulate dopamine release through expression on dopamine terminals or on GABAergic terminals apposed to dopamine terminals. DOR can also modulate postsynaptic responses in spiny neurons that receive dopamine input (Svingos *et al*, 1999). MOR colocalizes predominantly with preprotachykinin-positive neurons in patch compartments that constitute the direct striatal output, and more rarely with preproenkephalin-positive neurons of the striatal matrix that constitute the indirect output (Furuta *et al*, 2002). The differential expression patterns of MOR and DOR lend them different mechanisms of action, with DOR more frequently modulating inhibitory and dopaminergic input to the NAc and MOR primarily modulating NAc GABAergic neurons themselves (Svingos *et al*, 1999, 1997; Wang and Pickel, 1998).

Cocaine-primed reinstatement of cocaine seeking requires glutamatergic neurotransmission in the NAc core (Cornish and Kalivas, 2000; McFarland *et al*, 2003) and dopaminergic neurotransmission in the NAc shell (Anderson *et al*, 2003), although direct dopamine receptor stimulation in the medial NAc core elicits greater cocaine seeking than that in the shell or lateral core region (Bachtell *et al*, 2005, Schmidt *et al*, 2006). Although we did not compare core with shell subregions in this study, the ability of the MOR antagonist CTAP to block cocaine-primed cocaine seeking suggests that β-endorphin is released in the vicinity of the medial NAc. Given that the locomotor-activating effects of intra-NAc MOR- and DOR-selective agonists are not attenuated by dopamine depletion or chronic dopamine receptor blockade (Stinus *et al*, 1986; Churchill and Kalivas, 1992), it is likely that cocaine seeking elicited by MOR and DOR stimulation is mediated independent of dopamine release in the NAc. Furthermore, dopamine depletion leads to supersensitivity to MOR but not DOR agonist infusions in locomotor tests (Churchill and Kalivas, 1992).

Chronic cocaine administration modulates opioid receptor expression in the NAc (for review see Boutrel, 2008; Kreek, 2001), suggesting that changes in these receptors could alter the propensity for relapse during cocaine withdrawal. Free β-endorphin levels are decreased in the NAc and other brain regions within 1 day withdrawal from cocaine self-administration, potentially reflecting the

depletion of endogenous stores (Sweep *et al*, 1989). Similarly, opioid receptor binding decreases immediately after and before the next scheduled cocaine self-administration session (Gerrits *et al*, 1999), possibly reflecting the release of endogenous opioids during cocaine self-administration. Chronic cocaine administered in a daily binge pattern transiently increases MOR but not DOR density and MOR-stimulated [35S]GTPγS binding in the NAc (Schroeder *et al*, 2003; Unterwald *et al*, 1992). However, the ability of DOR, but not MOR, stimulation to inhibit adenylyl cyclase activity is impaired in the NAc following chronic cocaine (Unterwald *et al*, 1993), and this impairment persists for at least 1 day of cocaine withdrawal (Perrine *et al*, 2008), coinciding with increased internalization of DOR in NAc neurons (Ambrose-Lanci *et al*, 2008). Although these changes could modify the ability of MOR and DOR to trigger cocaine relapse in early cocaine withdrawal, we reported that MOR, and not DOR, levels in the NAc core progressively increase from 1 to 6 weeks of withdrawal from chronic cocaine self-administration (Self *et al*, 2004), and the effect is accompanied by increases in the precursor for β-endorphin, pro-opiomelanocortin, in the arcuate nucleus of the hypothalamus (Smagula *et al*, 2005). These findings suggest that progressive increases in MOR signaling in the NAc contribute to time-dependent increases in cocaine-seeking behavior in cocaine withdrawal when animals are exposed to cocaine-paired environments or stressful conditions (Grimm *et al*, 2001; Sorge and Stewart, 2005; Tran-Nguyen *et al*, 1998).

Human studies also support a relationship between increased MOR and cocaine craving in abstinence. Thus, MOR binding measured by positron emission tomography is increased in striatal and cortical regions in abstinent cocaine addicts and positively correlates with measures of cocaine craving (Gorelick *et al*, 2005; Zubieta *et al*, 1996). In subsequent studies, the upregulation in MOR binding was found to persist for up to 12 weeks of abstinence and positively correlate with the amount of prior cocaine use (Gorelick *et al*, 2005). Moreover, the upregulation of MOR in abstinence served as an independent predictor of time to relapse in cocaine addicts and positively correlated with the amount of cocaine use during the first month of relapse (Gorelick *et al*, 2008). Although limitations in detection precluded examination of MOR exclusively in the NAc, our animal data suggest that such long-lasting increases in MOR could functionally increase the propensity for cocaine relapse (Self *et al*, 2004).

In this regard, treatment with the opioid receptor antagonist naltrexone in combination with behavioral therapy decreased cocaine use over time (Schmitz *et al*, 2001). When threefold higher doses of naltrexone were utilized in combination with psychosocial treatment, the severity of cocaine use decreased (Pettinati *et al*, 2008). In response to an acute cocaine dose, addicts reported decreased 'good effects' and 'crash' when treated with naltrexone (Kosten *et al*, 1992; Sofuoglu *et al*, 2003), although naltrexone reportedly does not decrease subjective reports of craving elicited by cocaine-associated cues (Modesto-Lowe *et al*, 1997). Our findings suggest that blockade of MOR and DOR in the NAc contributes to the therapeutic potential of naltrexone in the treatment of cocaine addiction.

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CONFLICT OF INTEREST

The authors declare that over the past 3 years, D Self has received compensation from Teva Pharmaceutical Industries, and D Simmons has received no financial support from any individual or corporate entity. There are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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