

Effects of High-Dose Methadone Maintenance on Cocaine Place Conditioning, Cocaine Self-Administration, and Mu-Opioid Receptor mRNA Expression in the Rat Brain

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Methadone maintenance at appropriate doses can effectively reduce cocaine abuse in heroin-dependent individuals. In the present studies, we investigated the effect of high-dose methadone maintenance cocaine conditioned place preference (CPP) and cocaine intravenous self-administration. Rats implanted with methadone-filled osmotic mini-pumps (20 and 55 mg/kg/day, SC) and conditioned with cocaine (1, 5, and 20 mg/kg, i.p.) did not express cocaine CPP. Similarly, rats implanted with methadone pumps (55 mg/kg/day) after cocaine conditioning (20 mg/kg) displayed neither spontaneous nor cocaine-precipitated (20 mg/kg, i.p.) CPP. In contrast, methadone maintenance (30 and 55 mg/kg/day, SC) did not alter the intravenous self-administration (continuous schedule of reinforcement) of various doses of cocaine (0.1, 0.5, and 2.0 mg/kg/inf). To explore neuropharmacological interactions between methadone maintenance and cocaine conditioning, we quantitatively measured mRNA levels of mu-opioid receptor (MOR) and proopiomelanocortin genes 10 days after methadone maintenance. MOR mRNA levels in both the nucleus accumbens core and frontal cortex were significantly elevated in rats exposed to cocaine during CPP conditioning. However, upregulation of MOR mRNA levels in the nucleus accumbens core were reduced by methadone maintenance in a dose-dependent manner. In conclusion, our results suggest that high-dose methadone maintenance does not alter the direct reinforcing effect of cocaine, but blocks spontaneous and cocaine-precipitated cocaine-seeking, possibly by preventing MOR alterations in the nucleus accumbens core induced by cocaine conditioning.

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INTRODUCTION

Cocaine abuse is commonly observed in individuals enrolled in methadone maintenance programs (Grella *et al*, 1997; Hartel *et al*, 1995; Kidorf and Stitzer, 1993; Kosten *et al*, 1987; Levin *et al*, 1996; Magura *et al*, 1998), and it is associated with significant health risks (Foltin and Fischman, 1996) and with negative treatment outcomes, including relapse (Dolan *et al*, 2001; Kosten *et al*, 1988; Perez *et al*, 1997). Contingency management interventions have been found to be efficacious in reducing cocaine abuse (Sigmon *et al*, 2004), but this approach appears to be effective in about 50–60% of methadone patients (Silverman *et al*, 1996). Those who do not profit from cognitive–

behavioral interventions may require higher methadone dosages (Stine *et al*, 1992; Strain *et al*, 1994).

There is some evidence, in fact, that at appropriate doses, methadone maintenance can decrease cocaine use in heroin–cocaine co-users. In a clinical trial by Strain *et al* (1993), it was found that rates of cocaine use were lower among patients maintained on 50 mg/day (52.6%) compared to those maintained on 20 mg/day (62.4%). Similarly, Borg *et al* (1999) found that 69% of patients stopped regular cocaine abuse when maintained on average methadone dose of 67.1 mg/day (± 2.1). Stine *et al* (1991) developed a contingency treatment program in which the patient's methadone dose increased by 5 mg in response to each cocaine-positive urine screen, up to a maximum dose of 120 mg/day. Under these conditions, when the methadone dose achieved an average of 115 mg/day, cocaine-positive urine samples decreased by 89.2%. Finally, Schottenfeld *et al* (2005) recently reported that methadone maintenance (65–85 mg/day), even without formal contingency management, promoted retention in treatment, significantly reduced opioid and cocaine use, and enhanced the length of abstinence from both drugs.

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Higher doses of methadone may be required in patients who display severe cocaine abuse because cocaine exposure can alter mu-opioid receptors in the central nervous system (Azaryan *et al*, 1996; Yuferov *et al*, 1999; Unterwald, 2001). In rats, 14 days of binge-pattern cocaine administration increased mu-opioid receptor density in striatal-cortico-limbic areas, including the nucleus accumbens and subregions of the frontal cortex (FC) (Unterwald *et al*, 1992). Using PET technology, upregulation of mu-opioid receptor binding has been observed in cocaine-dependent individuals, and it has been associated with cocaine cravings (Gorelick *et al*, 2005; Zubietta *et al*, 1996). Thus, heavy cocaine users may necessitate more aggressive methadone titration to compensate for cocaine-induced upregulation of mu-opioid receptor density in regions of the brain involved in drug-taking and drug-seeking behaviors (Self, 2004).

Paradoxically, pre-clinical studies performed in human subjects have found that methadone maintenance at doses ranging between 50 and 100 mg/day do not modify, and can even enhance, subjective reports of liking and stimulation induced by acute cocaine challenges (Foltin *et al*, 1995; Foltin and Fischman, 1996; Preston *et al*, 1996). These results suggest the hypothesis that methadone maintenance, even at high doses, may not alter the direct reinforcing and stimulatory effects of cocaine, but may still reduce cocaine abuse by attenuating cocaine-seeking behavior. In support of this idea, it has been found that methadone maintenance in rats does not reduce the acute stimulatory effect of cocaine on locomotion, but significantly reduces both heroin- and cocaine-seeking behavior, as indexed by rates of lever pressing in extinction conditions (Leri *et al*, 2004). The interpretation of this latter result, however, is complicated by findings in primates suggesting that chronic methadone exposure has a significant effect on rates of operant responding *per se* (Negus and Mello, 2004).

Therefore, the present experiments in rats were designed to investigate the effect of high-dose methadone maintenance on cocaine-seeking behavior measured using a procedure that does not rely on operant responding, namely the conditioned place preference (CPP). In the first experiment, rats were maintained on methadone during cocaine conditioning and CPP testing. In the second experiment, rats were maintained on methadone during CPP testing, but not during conditioning. The absence of a CPP in both experiments would indicate that methadone can inhibit the expression of cocaine CPP without interfering with the direct reinforcing properties of cocaine. To directly test this hypothesis, we also studied the effect of methadone maintenance on intravenous self-administration of cocaine on a continuous schedule of reinforcement.

Furthermore, because the neuropharmacological consequences of long-term withdrawal from cocaine and methadone are virtually unknown, we removed the osmotic pumps in animals tested in the first CPP experiment, and extracted brains and plasma after a 14-day withdrawal period from cocaine/10-day withdrawal period from methadone. We quantified mu-opioid receptor (MOR) mRNA expression in the frontal cortex, nucleus accumbens core, caudate-putamen, amygdala, and hypothalamus. These regions were selected because of their known involvement in various behavioral and motivational aspects of drugs of

abuse (Everitt and Wolf, 2002; Koob, 1992), and because they constitutively express MOR mRNA (Mansour *et al*, 1994). In addition, the arcuate nucleus of the hypothalamus contains neurons that express proopiomelanocortin (POMC) and which are presumably the sources of beta-endorphins input to the nucleus accumbens (Finley *et al*, 1981). As the release of beta-endorphins in the nucleus accumbens is critical to drug- and non-drug-motivated behaviors (Roth-Deri *et al*, 2004; Zangen and Shalev, 2003), we also measured expression of POMC mRNA in the hypothalamus.

An additional quantification targeted components of the hypothalamic-pituitary-adrenal (HPA) axis. We assessed plasma adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) because they are known to be altered by chronic exposure to opiates and cocaine (Schluger *et al*, 2001; Zhou *et al*, 1999, 2003). Finally, because glucocorticoids exert a negative feedback control on the release of ACTH and beta-endorphins by the anterior pituitary, we quantified mRNA expression of their precursor (POMC) in this region.

MATERIALS AND METHODS

Subjects

Subjects were adult male Sprague-Dawley rats (Charles River, QC) weighing 225–250 g at the beginning of all experiments. They were singly housed and maintained on a reverse light/dark cycle (0800 h lights off; 2000 h lights on) with free access to food and water except during behavioral testing, which always occurred during the dark cycle. All experiments were approved by the Animal Care Committee of the University of Guelph and were carried out in accordance with the recommendations of the Canadian Council on Animal Care.

Surgery

Osmotic mini-pumps. Methadone maintenance was achieved by implanting osmotic mini pumps SC (Alzet model 2ML2, 0.5 µl per hour for 14 days, Durect Corporation, Cupertino, CA). Isoflurane was used to anaesthetize the rats and a small incision between the scapulae was made in the skin. Subcutaneous connective tissues were spread apart using a hemostat to make a small pocket for the pump. Osmotic pumps were placed into the pocket with flow moderator directed away from the incision. Wound clips kept the incision closed. An identical procedure was carried out for the animals receiving sham surgery.

Intravenous self-administration. Rats were surgically implanted with intravenous silastic catheters (Dow Corning, Midland, MI) in the right jugular vein, under general anesthesia induced by a combination of sodium pentobarbital (18.5 mg/kg i.p., MTC Pharmaceutical, Cambridge, ON), morphine (5 mg/kg s.c., Ontario Veterinary College, Guelph, ON) and diazepam (1 mg/kg s.c., Sabex Inc., Boucherville, QC). Rats were given atropine sulfate (4.5 mg/kg s.c., Ontario Veterinary College, Guelph, ON) just before surgery and depocillin (300 000 IU, 0.1 ml/rat IM, Intervet Canada, Whitby, ON) immediately following

surgery. The catheter was secured to the vein with silk sutures and was passed s.c. to the top of the skull, where it exited into a connector (a 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 when not in use. Catheters were flushed daily with saline and every second day with 0.1 ml of a saline-heparin solution (0.2 mg/ml Hepalean 1.000 IU, Organon, Toronto, ON).

Testing Apparatus

Conditioned place preference. Six, custom-made (University of Guelph, ON, Canada), place conditioning boxes were used in these experiments. The boxes were located in the center of the laboratory room. Each conditioning box was made of dark gray PVC, and comprised of three compartments: two large ($30 \times 40 \times 26 \text{ cm}^3$) and one smaller, middle ($23 \times 30 \times 26 \text{ cm}^3$) compartment. Removable inserts, with or without small arch-way openings ($10 \times 10 \text{ cm}^2$), formed the center compartment. The two large compartments differed primarily in visual cues; one large compartment was dark gray, while the other had a white wall and a 10-cm white stripe painted along the top of the other walls. In addition, there were cues that provided spatial information external to the compartments, such as posters on walls, benches, and lights. In this apparatus, rats do not display a significant spontaneous preference for any of the compartments (ie, the apparatus is balanced). The entire apparatus was covered by black wire mesh to allow video tracking of the rats during testing. The tracking software employed was EthoVision (version 3, Noldus Information Technology, The Netherlands). This system was used to record two dependent variables: time (s) spent in each compartment during test for place preference, and locomotor activity (total cm) during conditioning and during place preference test.

Intravenous self-administration. In all, 20 Plexiglas operant chambers (model ENV-008CT, Med Associates, Lafayette, IN) were each enclosed in larger sound-attenuating plywood chambers (model ENV-018M, Med Associates). Each operant box had a house light (28 V) and two levers, one retractable and one stationary, located 10 cm apart and 8 cm above the floor of the box. The retractable lever (active lever) was connected to an infusion pump for the delivery of drugs (Razel Scientific Instruments, Stamford, CT), positioned outside the sound-insulating chamber. The stationary lever served to control for baseline, nonreinforced operant behavior; pressing this lever had no consequence (inactive lever), but all presses were recorded. A white light (28 V) stimulus located 3 cm above the active lever was illuminated for 30 s at the beginning of the session, and for the duration of each drug infusion (10 s), serving as a discrete stimulus for drug delivery.

Biochemical Assays

Solution hybridization ribonuclease (RNase) protection-trichloroacetic acid (TCA) precipitation assay. Immediately following decapitation, brains were extracted and the hypothalamus (H) and anterior pituitary (AP) dissected out.

All tissue was rapidly frozen in dry ice, and stored at -40°C for subsequent measurements of POMC mRNA in the H and AP. Mu-opioid receptor (MOR) mRNA was quantified in the hypothalamus, the frontal cortex (FC), the caudate putamen (CP), the amygdala core (NACc), and the nucleus accumbens. Except for the hypothalamus, each structure was dissected out bilaterally by razor blade or hole-punch. For the nucleus accumbens, the hole-punches were centered on its core component (NACc), and therefore our results are limited to this subregion.

The solution hybridization RNase protection-TCA precipitation protocol has been described in detail in earlier reports (Branch et al, 1992). A 2100 bp fragment from the rat MOR cDNA (a kind gift from Dr G Uhl of NIDA, MD) and a 538 bp fragment from the rat POMC cDNA (a kind gift from Dr JL Roberts of Mount Sinai Medical Center in New York, NY) were cloned into the polylinker region of the pSP64 plasmid (Promega, Madison, WI) in both the sense and antisense orientations. The plasmid pS/E (a pSP65 derivative; a kind gift from Drs T Nilsen and P Maroney of Case Western University) was used to synthesize riboprobe for the 18S rRNA to determine total RNA. ^{33}P -labeled cRNA antisense probes and unlabeled RNA sense standards were synthesized using an SP6 transcription system. A denaturing agarose gel containing 1.0 M formaldehyde showed that a single full-length transcript had been synthesized from each plasmid.

RNA extracts were dried in 1.5 ml Eppendorf tubes and re-suspended in 30 μl that contained 150 000–300 000 c.p.m. of a probe in $2 \times \text{TESS}$ (10 mM *N*-Tris[hydroxy-methyl]-methyl-2-aminoethane sulfonic acid, pH 7.4; 10 mM EDTA; 0.3 M NaCl; 0.5% SDS). Samples were covered with mineral oil and hybridized overnight at 75°C . For RNase treatment, 250 μl of a buffer that contained 0.3 M NaCl; 5 mM EDTA; 10 mM Tris-HCl (pH 7.5), 40 $\mu\text{g/ml}$ RNase A (Worthington, Biochemicals, Freehold, NJ), and 2 $\mu\text{g/ml}$ RNase T1 (Calbiochem, San Diego, CA) were added and each sample was incubated at 30°C for 1 h. TCA precipitation was effected by the addition of 1 ml of a solution that contained 5% TCA and 0.75% sodium pyrophosphate. Precipitates were collected onto a filter in sets of 24 by using a cell harvester (Brandel, Gaithersburg, MD) and were measured in a scintillation counter with liquid scintillant (Beckman Instruments, Palo Alto, CA).

The procedure to measure mRNA levels involved comparison of values obtained from experimental samples (brain extracts) to those obtained for a set of calibration standards. The calibration standards had known amounts of an *in vitro* sense transcript whose concentration was determined by optical absorbance at 260 nm. To determine the total picograms of each mRNA in each extract, the amounts calculated from the standard curves were multiplied by 5.71 for MOR and by 2.04 for POMC to correct for the difference in length between the sense transcript (2100 and 538 bases for MOR and POMC respectively) and the full-length mRNA (12 and 1.1 k base for MOR and POMC, respectively). Total cellular RNA concentrations were measured by hybridization of diluted extracts to a ^{33}P -labeled probe complementary to 18S rRNA at 75°C . The calibration standards for this curve contained 10 μg of *Escherichia coli* tRNA plus either 0.0, or from 2.5 to 40 ng of total RNA from rat brain, whose concentration was determined by optical absorbance at 260 nm.

Radioimmunoassay. Trunk blood was collected immediately after decapitation, stored in tubes, placed on ice, and spun in a centrifuge. Plasma was separated and stored at -40°C for CORT measurement by radioimmunoassay. CORT levels were assayed using a rat CORT ^{125}I kit from ICN Biomedicals (Costa Mesa, CA). ACTH immunoreactivity levels were assayed using a kit from Nichols Institute (San Juan Capistrano, CA). All CORT and ACTH values were determined in duplicate in a single assay, and intra-assay coefficients of variation for CORT and ACTH were 1.5 and 4.1%, respectively.

Drugs

Cocaine HCL (Dumex, Toronto, ON) and Methadone HCL (Pharmascience, Montreal, QC) were dissolved in physiological saline.

Experiment 1: Effect of Methadone Maintenance on Formation and Expression of Cocaine CPP

Procedure. Rats were allowed 6 days to habituate to the animal facility and were handled twice for approximately 10 min before the beginning of the experiment. The place conditioning procedure included three phases: habituation, conditioning, and testing.

Habituation: On this day, the inserts with openings were used, and rats had free access to the three compartments for 20 min. The main purposes of this phase were to allow the rats to become accustomed to the apparatus, and to measure levels of spontaneous preference for each large compartment. The following day, animals were randomly assigned to three different methadone groups ($n = 24$ each) and implanted with osmotic mini-pumps. Methadone doses were 0, 20, and 55 mg/kg/day. In rats, it has been demonstrated that 10 mg/kg/day results in a mean plasma level of 123 ng/ml (range: 100–150 ng/ml) (Zhou *et al*, 1996). In humans, a dose of 100 mg/day results in an average daily concentration of about 240 ng/ml (Kreek, 2000). Thus, in this experiment, we used a methadone dose (ie, 20 mg/kg/day) corresponding to the high end of typical therapeutic ranges (Dole, 1988; Maxwell and Shinderman, 1999), and a dose more than twice as high (ie, 55 mg/kg/day).

Conditioning: This phase began 3 days following implant of methadone mini-pumps. During conditioning, the inserts with openings were replaced with solid inserts to fully separate the compartments. Rats underwent 3 days of conditioning, and each day they received two 30-min conditioning sessions, one in the morning and the other in the afternoon (morning session: between 0900 h and 1200 h; afternoon session: between 1400 h and 1700 h). Each day, rats received one conditioning session with cocaine (see doses below) and the other with vehicle. The specific compartment chosen to be associated with cocaine was counterbalanced across rats. In addition, the time of cocaine sessions was counterbalanced across rats and, for each rat, across days of conditioning. Injections were administered immediately before confinement in one of the two large compartments. Rats in each methadone dose group ($n = 24$ each) were randomly assigned to three different cocaine doses ($n = 8$ each): 1, 5, or 20 mg/kg. Previous pilot studies using this apparatus and this conditioning procedure

demonstrated that, in contrast to 1 and 5 mg/kg, 20 mg/kg produces a robust place preference. Cocaine doses of 1 and 5 mg/kg were included to evaluate the possibility that methadone maintenance may potentiate the rewarding efficacy of lower cocaine doses. It should be noted that, in order to interpret the biochemical analyses, an additional group of rats ($n = 8$) was included. These rats received identical behavioral manipulations, but were exposed neither to methadone (ie, received vehicle-filled pumps) nor to cocaine (received only vehicle injections during conditioning). The behavioral data from this control group are not included in the Results section.

Testing: Place preference was assessed on a test given 3 days following the last day of conditioning. For this test, the solid inserts were replaced by those with openings, and rats were given 20 min of free access to all compartments. At this time, rats were still maintained on methadone.

The day following this test, methadone pumps were removed using the anesthetic protocol described, and, after 10 days of withdrawal, all rats were euthanized using CO_2 , rapidly decapitated, and serum and brains collected for subsequent neurochemical analyses. This particular period of withdrawal was selected for two reasons. First, we have observed that 10 days are required for rats to recover body-weight loss caused by withdrawal from heroin exposure via minipumps (Leri *et al*, 2003). Second, euthanasia occurred 14 days after the last cocaine conditioning injection, a time point relatively unexplored as most studies of cocaine-induced alterations in MOR mRNA levels have been limited to periods of acute (3 h) and early withdrawal (1–7 days).

Statistical analyses. In a three-compartment apparatus, place preference typically results from a decrease in time spent in the vehicle- and an increase in time spent in the drug-paired compartments (Leri and Rizos, 2005). Thus, our statistical analysis involves the comparison between these times within each group on the test day, a method used by a number of other laboratories employing the unbiased CPP procedure (Bardo *et al*, 1995; Everitt *et al*, 1991; Hoffman, 1989; Leri and Franklin, 2000; Mueller *et al*, 2002; Parker and McDonald, 2000). Typically, animals also spend a substantial amount of time in the middle compartment, both during habituation and during the test for CPP. However, this variable is not significantly altered by place conditioning and, therefore, it is not included in the behavioral analyses.

Place preference during test and locomotor activity during conditioning (average of conditioning sessions with vehicle and with cocaine) were analyzed using three-factor (methadone dose, cocaine dose, compartment/injection) mixed-design ANOVA (compartment/injection was within subjects). Locomotor activity during the test of preference was analyzed using a two-factor (methadone dose and cocaine dose) ANOVA with independent groups. Changes in body weight induced by spontaneous withdrawal from methadone were analyzed using a three-factor (methadone dose, cocaine dose, withdrawal day) mixed design ANOVA (withdrawal day was within subjects).

Plasma ACTH, plasma CORT, POMC in H, POMC in AP, and MOR mRNA in the FC, NACc, CP, A, and H were all

analyzed separately. For each measure, a two-factor (methadone dose and cocaine dose) ANOVA with independent groups was performed first. Subsequently, a one-factor ANOVA with independent groups was performed to compare the control group (ie, no methadone and no cocaine) to the groups that received 1, 5, or 20 mg/kg cocaine. Separate analyses were performed for each group of rats that received different methadone doses (ie, vehicle, 20 and 55 mg/kg/day).

In case of significant interactions or significant main effects, multiple comparisons were performed using the Holm-Sidak method in order to identify individual mean differences. The alpha level was set to 0.05.

Experiment 2: Effect of Methadone Maintenance on Expression and Reinstatement of Cocaine CPP

Procedures. Twelve rats were habituated and conditioned (20 mg/kg cocaine) as described above, but in this experiment the methadone pumps (0 mg/kg/day, $n = 6$; 55 mg/kg/day, $n = 6$) were implanted *after* the last session of conditioning. Thus, there was no overlap between cocaine conditioning and methadone exposure. At 3 days after the implantation of the mini-pumps, animals received a place preference test as described above.

Following this test, the two groups underwent extinction of place preference. Extinction was similar to conditioning in that it was carried out over 3 days, with two 30-min conditioning sessions each day. However, during extinction, rats received vehicle injections before confinement in *both* compartments. Finally, two place preference tests were given over two consecutive days. Just prior to the first test, animals received a vehicle injection (ie, Vehicle Prime), while prior to the second test they received cocaine (ie, Cocaine Prime, 20 mg/kg, i.p.). No neurochemical analyses were performed on plasma or brains at the conclusion of this experiment.

Statistical analyses. The main focus of this experiment was to determine how preference changed within each methadone group (0 and 55 mg/kg/day) across tests (CPP test, and tests given after extinction following vehicle and cocaine injections). Therefore, two separate two-factors repeated-measures ANOVAs (both test and compartment within subjects) were performed. Finally, a two-factors (methadone dose and priming injection) mixed-design ANOVA (priming injection was within subjects) was used to compare locomotion during the two post-extinction tests in the two groups.

Experiments 3A and 3B: Effect of Methadone Maintenance on Intravenous Cocaine Self-Administration

Procedure. Rats were placed in the chambers and their connectors attached to the infusion lines. Each session started with the activation of the house light, the entry of the retractable lever, and the illumination of the light stimulus for 30 s. Subsequently, lever presses on the active lever led to drug infusions according to a continuous schedule of reinforcement. Drug was infused in a volume of 300 μ l over a 10-s period, and during this period the light stimulus was

illuminated. Responses on the active lever made during the infusion were recorded, but did not lead to further infusions. Drug concentration was adjusted for differences in body weight.

In Experiment 3A, six rats were initially trained to self-administer 2.0 mg/kg/infusion cocaine for five consecutive daily sessions, each lasting 3 h. After this period, methadone-filled osmotic mini-pumps (0 and 55 mg/kg/day, $n = 3$ each) were implanted and, following a 6-day recovery period, self-administration for the same dose of cocaine was evaluated during methadone maintenance on two test sessions given over two consecutive days.

In Experiment 3B, we increased the sample size and we performed a within-subjects dose-response analysis of cocaine self-administration in methadone-maintained rats. For this experiment, we reduced the methadone dose to 30 mg/kg/day because we had noticed toxicity (ie, rapid loss of body weight) in the rats tested in the previous self-administration study. This methadone dose has previously been shown to decrease cocaine-seeking in animals trained to self-administer both heroin and cocaine (Leri et al, 2004).

Therefore, 12 rats were initially trained to self-administer 0.5 mg/kg/infusion cocaine for five consecutive daily sessions, each lasting 3 h. After this period, methadone-filled osmotic mini-pumps (0 and 30 mg/kg/day, $n = 6$ each) were implanted and, following a 6-day recovery period, self-administration for the same dose of cocaine was evaluated during methadone maintenance on two self-administration test sessions given over two consecutive days. Subsequently, half of the animals in each group were transferred to a lower cocaine dose (0.1 mg/kg/infusion) and the other half to a higher cocaine dose (2 mg/kg/infusion), which they self-administered for 2 days during methadone maintenance. The doses were reversed for the two final days of cocaine self-administration, always under methadone maintenance.

Statistical analyses. In both experiments, two-factor mixed-design ANOVAs were used to compare cocaine infusions (average of two days with the same dose) obtained before and after the implantation of methadone pumps.

RESULTS

Experiment 1

Conditioned place preference. Animals ($n = 72$) displayed no significant spontaneous preference for either compartment during habituation (mean (SEM): vehicle-to-be-conditioned compartment = 350.3 (8.7) s; cocaine-to-be-conditioned compartment = 332.4 (9.2) s). However, after conditioning, significant methadone dose by cocaine dose by compartment interaction ($F(63, 143) = 2.59$, $p < 0.05$) and significant cocaine dose by compartment interaction ($F(2, 143) = 3.16$, $p < 0.05$) were found. As it can be seen in Figure 1, animals conditioned with 20 mg/kg cocaine and not maintained on methadone (0 mg/kg/day methadone) displayed a significant preference for the cocaine-paired compartment. Animals conditioned with 20 mg/kg cocaine and maintained on 20 mg/kg methadone showed a reduced CPP ($p = 0.05$). Finally, animals conditioned with 20 mg/kg cocaine and maintained on 55 mg/kg methadone showed no CPP. Thus, methadone blocked cocaine CPP in a dose-

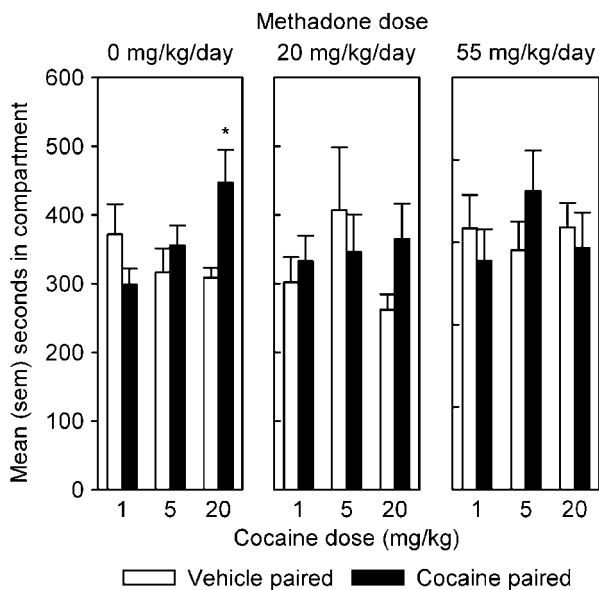


Figure 1 Mean (SEM) seconds spent in the vehicle- and the cocaine-paired compartments on a 20-min place preference test. During conditioning and during test, different groups of animals ($n=24$ each) were maintained on 0, 20, or 55 mg/kg/day methadone via osmotic minipumps. During conditioning, different subgroups of rats ($n=8$) in each methadone dose group received 1, 5, or 20 mg/kg cocaine. * Denotes a significant difference between time in the vehicle- and the cocaine-paired compartments.

dependent manner. Methadone did not seem to potentiate the reinforcing efficacy of lower cocaine doses (1 and 5 mg/kg), which, in fact, were ineffective in inducing a significant place preference in all groups.

Methadone maintenance did not alter locomotor responses to acute cocaine injections given during conditioning. In fact, as illustrated in Figure 2, 5 and 20 mg/kg cocaine elevated locomotion activity equally in all methadone groups (cocaine dose by compartment interaction ($F(2,143)=86.6$, $p<0.001$); main effect of cocaine dose ($F(2,63)=61.4$, $p<0.001$); and main effect of compartment ($F(1,72)=244.4$, $p<0.001$)). Also, although methadone maintenance blocked the expression of cocaine CPP induced by 20 mg/kg, it did not significantly alter the level of locomotor activity during the test of preference (for example: mean (SEM) activity in group 20 mg/kg cocaine—0 mg/kg/day methadone = 8211.2 (289.8); mean (SEM) activity in group 20 mg/kg cocaine—55 mg/kg/day methadone = 8078.8 (465.3)).

Spontaneous methadone withdrawal. As it can be seen in Figure 3, 24 h following the removal of the methadone-filled mini-pumps, rats in the 20 and the 55 mg/kg/day groups lost a significant amount of body weight regardless of the cocaine dose received during conditioning (methadone dose by withdrawal day interaction ($F(4,126)=10.2$, $p<0.001$); main effect of methadone dose ($F(2,63)=23.8$, $p<0.0001$); main effect of withdrawal day ($F(2,126)=40.55$, $p<0.0001$)). By the time of euthanasia, 10 days following removal of the pumps, the body weight of rats in the 55 mg/kg/day group was still significantly lower than the body weight of rats in the 0 mg/kg/day group.

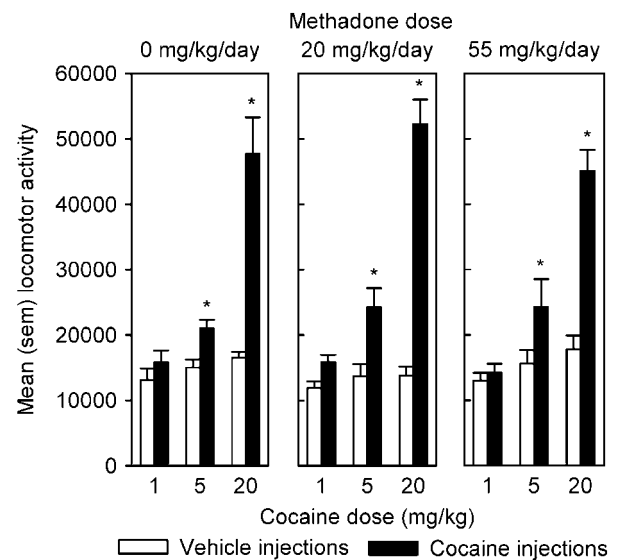


Figure 2 Mean (SEM) locomotor activity (average of the three sessions with vehicle and three sessions with cocaine) measured in each experimental group during place conditioning. * Denotes a significant difference between activity induced by vehicle and by cocaine injections.

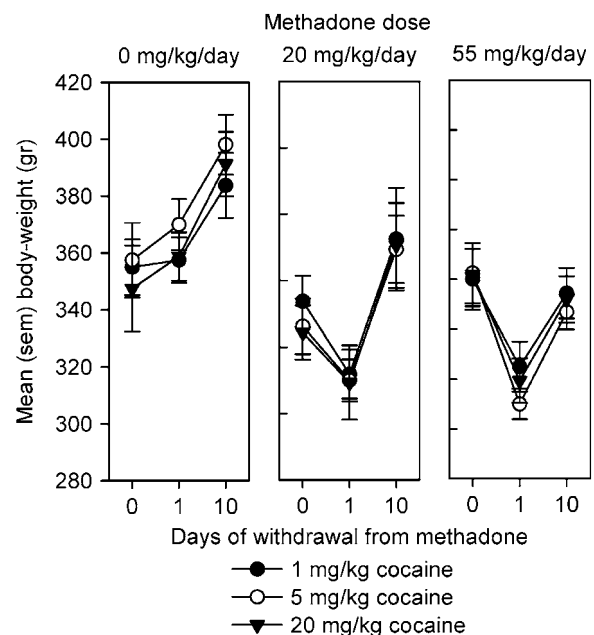


Figure 3 Mean (SEM) body weight measured in each experimental group upon removal of the methadone-filled minipumps (withdrawal day 0), 24 h (withdrawal day 1), and 9 days later (withdrawal day 10).

HPA axis activity. ACTH in plasma: There was a significant interaction between methadone dose and cocaine dose ($F(4,62)=2.55$, $p=0.05$). Multiple comparisons indicated that rats exposed to 5 mg/kg cocaine during CPP and maintained on 20 and 55 mg/kg/day methadone showed significantly higher ACTH levels than rats exposed to 5 mg/kg cocaine only (see Table 1). When ACTH levels were compared within each methadone group to the control group (ie, no methadone and no cocaine), it was found that: in the 0 mg/kg/day methadone, animals previously exposed to 20 mg/kg cocaine showed significantly higher ACTH levels ($F(3,26)=3.2$, $p<0.05$); in the 20 mg/kg/day

Table 1 HPA Axis Activity in Experimental Subjects Killed 10 Days Following the Removal of the Methadone-Filled Osmotic Minipumps

Methadone maintenance dose (mg/kg/day)									
0			20				55		
Cocaine conditioning dose (mg/kg)									
0	1	5	20	1	5	20	1	5	20
Mean (SEM) ACTH plasma (pg/ml)									
425.0 (60)	626.31 (140)	618.4 (89)	927.5 (131) [#]	810.1 (96) [#]	1116.5 (142) ^{##}	731.9 (127)	812.3 (86) [#]	962.6 (85) ^{##}	839.9 (70) [#]
Mean (SEM) CORT plasma (pg/ml)									
204.2 (22.3)	177.5 (35)	147.3 (25)	250.0 (54)	199.6 (30)	205.1 (32)	141.5 (26)	149.9 (36)	216.1 (46)	150.4 (29)
Mean (SEM) POMC mRNA in AP (pg/μg total RNA)									
124.31 (20.50)	85.06 (10.73) [#]	67.66 (7.25) [#]	63.30 (6.06) [#]	79.48 (11.87) [#]	72.78 (11.47) [#]	69.66 (9.49) [#]	64.92 (7.81) [#]	68.42 (11.90) [#]	72.54 (9.23) [#]
Mean (SEM) POMC mRNA in H (pg/μg total RNA)									
6.61 (0.69)	5.74 (0.87)	5.42 (0.95)	5.47 (0.52)	5.69 (1.07)	6.43 (1.24)	6.06 (0.88)	6.73 (0.68)	6.51 (0.97)	5.81 (0.91)

ACTH = adrenocorticotrophic hormone; CORT = corticosterone; POMC = pro-opiomelanocortin; AP = anterior pituitary; H = hypothalamus.

[#]Significant difference from the control group.

^{##}Significant difference from the same cocaine dose group in the 0 mg/kg/day methadone group.

methadone group, although ACTH levels were elevated in each cocaine dose group, only levels in the 1 and 5 mg/kg cocaine groups were significantly different from controls ($F(3,27) = 6.0$, $p < 0.005$); finally, in the 55 mg/kg group, animals in all cocaine dose groups showed higher ACTH levels than controls ($F(3,27) = 8.5$, $p < 0.001$).

CORT in plasma: No significant differences were found (see Table 1).

POMC in the AP: There was no interaction between methadone dose and cocaine dose on POMC mRNA expressed in the anterior pituitary. Animals in all cocaine dose groups showed significant lower expression levels than controls, and this was observed in all methadone groups (Table 1: 0 mg/kg/day group ($F(3,25) = 5.6$, $p < 0.005$); 20 mg/kg/day group ($F(3,24) = 3.2$, $p < 0.05$); 55 mg/kg/day group ($F(3, 23) = 4.5$, $p < 0.05$)).

POMC in the H: No significant differences were found (see Table 1).

MOR mRNA. FC: There was no interaction between methadone dose and cocaine dose on MOR mRNA expressed in the FC. Animals in all cocaine dose groups showed significantly higher expression levels than controls, and this was observed in all methadone groups (Table 2: 0 mg/kg/day group ($F(3,27) = 3.9$, $p < 0.05$); 20 mg/kg/day group ($F(3,27) = 9.7$, $p < 0.001$); 55 mg/kg/day group ($F(3, 28) = 4.7$, $p < 0.01$)).

NACc: As illustrated in Figure 4, MOR mRNA expression was elevated in rats conditioned with 5 and 20 mg/kg cocaine, and methadone maintenance reduced this effect in a dose-dependent manner ($F(2,58) = 8.81$, $p < 0.001$). Thus, when compared to controls, MOR mRNA was significantly elevated in animals exposed to 5 and 20 mg/kg cocaine and maintained on 0 mg/kg/day methadone ($F(3,25) = 5.8$, $p < 0.005$). This elevation was reduced in animals maintained on 20 mg/kg/day methadone, but it was still

significant ($F(3,26) = 3.7$, $p < 0.05$). However, in rats maintained on 55 mg/kg/day methadone, MOR mRNA levels were undistinguishable from controls regardless of the dose of cocaine given during CPP.

CP: No significant differences were found (see Table 2).

A: No significant differences were found (see Table 2).

H: No significant differences were found (see Table 2).

Experiment 2

Two rats in the 0 mg/kg/day methadone group experienced post-surgical complications, and were thus removed from the study. The ANOVA performed within this group identified a significant main effect of compartment ($F(1,9) = 22.2$, $p < 0.001$). As it can be seen in panel a of Figure 5, these rats ($n = 4$) displayed a significant place preference for the cocaine-paired compartment (20 mg/kg) on the initial test. Extinction training eliminated such a preference and, in fact, following the vehicle prime, there was no significant difference in times spent in the two conditioning compartments. Finally, a priming injection of 20 mg/kg cocaine just prior to the final test effectively reinstated the original cocaine place preference. Interestingly, when the same analysis was performed in the 55 mg/kg/day methadone group (panel c, Figure 5), no significant differences were found. In other words, these animals showed no preference on all CPP tests. It is notable, however, that the cocaine prime increased locomotor activity equally in both groups of animals (panels b and d, Figure 5; significant main effect of priming injection ($F(1,8) = 28.8$, $p < 0.001$) only).

Experiment 3A

By the fourth and fifth days of self-administration, rats were responding almost exclusively on the active lever producing

Table 2 Expression of MOR mRNA in Experimental Subjects Killed 10 Days Following the Removal of the Methadone-Filled Osmotic Minipumps

Methadone maintenance dose (mg/kg/day)									
0				20			55		
Cocaine conditioning dose (mg/kg)									
0	1	5	20	1	5	20	1	5	20
Mean (SEM) MOR mRNA in FC (pg/μg Total RNA)									
2.11 (0.12)	3.01 (0.27) [#]	3.26 (0.26) [#]	3.08 (0.29) [#]	3.14 (0.23) [#]	3.04 (0.26) [#]	3.65 (0.22) [#]	3.52 (0.47) [#]	3.14 (0.36) [#]	3.64 (0.28) [#]
Mean (SEM) MOR mRNA in CP (pg/μg total RNA)									
3.75 (0.58)	5.06 (0.52)	5.20 (0.94)	5.16 (0.31)	5.45 (0.45)	5.61 (0.90)	6.38 (0.90)	5.26 (0.43)	4.87 (0.56)	6.38 (0.80)
Mean (SEM) MOR mRNA in A (pg/μg total RNA)									
10.47 (0.56)	8.42 (0.83)	8.90 (0.98)	8.70 (0.77)	8.83 (1.02)	7.77 (0.49)	7.53 (0.44)	9.86 (1.38)	8.15 (0.74)	8.85 (0.95)
Mean (SEM) MOR mRNA in H (pg/μg total RNA)									
3.43 (0.56)	3.77 (0.23)	4.51 (0.38)	3.68 (0.26)	3.51 (0.40)	3.64 (0.17)	3.80 (0.26)	3.50 (0.19)	3.83 (0.26)	3.36 (0.31)

FC = frontal cortex; CP = caudate-putamen; A = amygdala; H = hypothalamus.
[#]Significant difference from the control group.

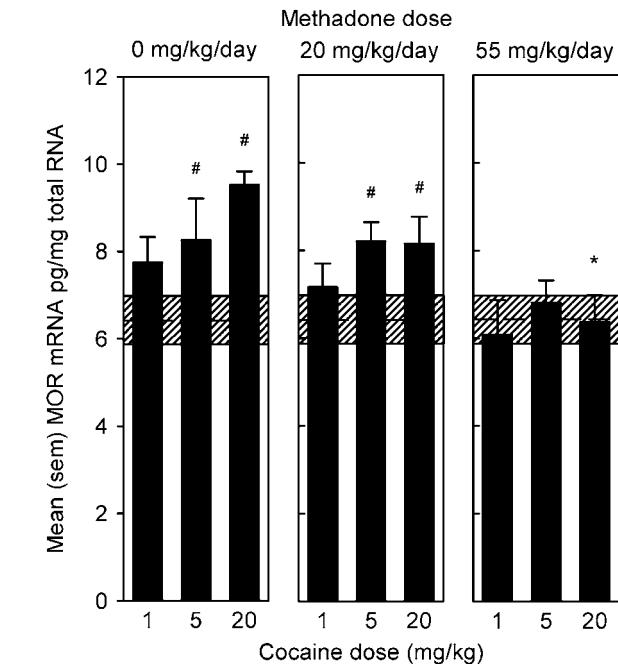


Figure 4 Mean (SEM) MOR mRNA expressed in the nucleus accumbens core of experimental subjects killed 10 days following the removal of the methadone-filled osmotic minipumps. The shaded area in each panel represents the data from the control group ($n=8$) that received no methadone and no cocaine. The upper and lower borders represent the SEM, while the central horizontal line represents the group mean. *Denotes a significant difference from the same cocaine dose group in the 0 mg/kg/day methadone group. # Denotes a significant difference from the control group.

intravenous infusions of cocaine (96 and 95% of total responding, respectively). As indicated in Figure 6a, there was no significant change in mean number of cocaine

infusions obtained during the two sessions preceding, and the two sessions following, the implantation of the methadone-filled mini-pumps.

Experiment 3B

By the fourth and fifth days of self-administration, rats were responding almost exclusively on the active lever producing intravenous infusions of cocaine (93 and 99% of total responding, respectively). One animal from the methadone group had to be excluded from the experiment because of poor health. The ANOVA revealed a main effect of dose ($F(3, 27)=15.4$, $p<0.001$), but no group differences. In other words, animals took more infusions when the dose of cocaine was lowered from 0.5 to 0.1 mg/kg/infusion, and fewer infusions when the cocaine dose was increased to 2.0 mg/kg/infusion, but these shifts in self-administration were similar in animals maintained on 0 and 30 mg/kg/day methadone (see Figure 6b).

DISCUSSION

These studies were designed to: (1) explore the effects of high-dose methadone maintenance (20 and 55 mg/kg/day) on cocaine-seeking behavior assessed by the CPP procedure; (2) explore the effects of high-dose methadone maintenance (30 and 55 mg/kg/day) on cocaine-taking behavior assessed by the intravenous self-administration procedure using a continuous schedule of reinforcement; (3) explore the effect of methadone maintenance on the expression of mu-opioid receptor mRNA in mesocortico-limbic structures in animals that underwent cocaine CPP; and (4) explore the effect of methadone maintenance on the activity of the HPA axis in animals that underwent cocaine CPP.

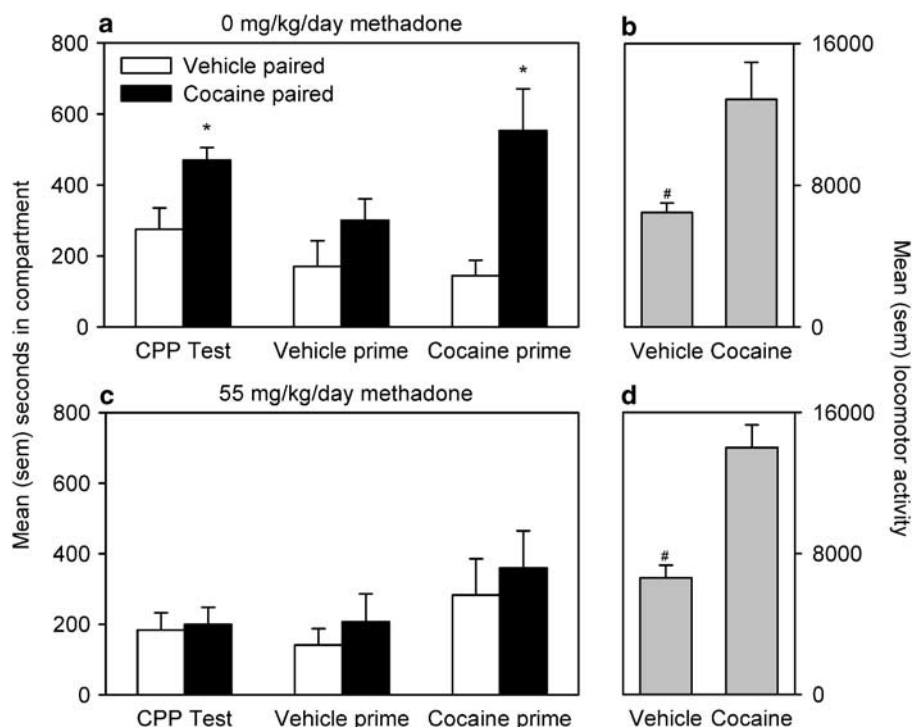


Figure 5 Mean (SEM) seconds spent in the vehicle- and the cocaine-paired compartments on three 20-min place preference tests. Prior to the first test (CPP test), different groups of animals were implanted with 0 ($n = 4$; panel a, b) and 55 ($n = 6$; panel c, d) mg/kg/day methadone-filled osmotic minipumps. During conditioning, all rats received 20 mg/kg cocaine. Following extinction training, rats were tested after a vehicle injection (Vehicle prime), and on the subsequent day, after an injection of cocaine (20 mg/kg; cocaine prime). * Denotes a significant difference between time in the vehicle- and the cocaine-paired compartments. Panels b and d illustrate the mean (SEM) locomotor activity displayed by the 0 and the 55 mg/kg/day methadone groups during the reinstatement tests, respectively. # Denotes a significant increase in locomotion between the vehicle and the cocaine prime tests.

The CPP procedure was used to model both spontaneous (Bardo and Bevins, 2000) and cocaine-precipitated (Mueller and Stewart, 2000) cocaine seeking. In the first CPP experiment, animals were maintained on methadone (0, 20, and 55 mg/kg/day) during both conditioning and testing. Although methadone maintenance did not alter locomotor responses to cocaine injections given during the course of conditioning, it blocked the expression of cocaine (20 mg/kg) CPP in a dose-dependent manner. The lack of preference was particularly marked in rats maintained on the highest methadone dose (ie, 55 mg/kg/day), and was not due to methadone-induced inhibition/excitation of basal locomotor activity as no group differences in locomotion were observed during CPP test. In the same experiment, no evidence of potentiation of cocaine CPP by methadone was observed, as low cocaine doses (1 and 5 mg/kg) failed to induce a significant place preference in all groups (ie, 0, 20 and 55 mg/kg/day methadone).

In the second CPP experiment, the methadone pumps (0 and 55 mg/kg/day) were implanted after cocaine (20 mg/kg) conditioning. Consistent with the results of the previous study, animals maintained on methadone did not express a significant place preference for the cocaine-paired compartment. Furthermore, it was found that priming injections of cocaine (20 mg/kg) had no effect on the expression of place preference in methadone-maintained rats, although locomotor activation produced by the cocaine prime was unaltered by methadone maintenance.

There are several possible explanations as to why high-dose methadone maintenance blocked cocaine CPP. For

example, it could be argued that the expression of cocaine CPP was influenced by state-dependent learning. However, this interpretation cannot account for the results of Experiment 1 where rats were both conditioned and tested while maintained on methadone. It could also be argued that methadone blocked the expression of CPP because it produced a memory impairment, resulting in complete forgetting of associations learned during conditioning. It has been found that methadone produces spatial learning and working memory impairments in rats (Hepner *et al*, 2002), but this was observed after acute injections, not during chronic maintenance.

In contrast to the results of the CPP studies, it was found that methadone maintenance (30 and 55 mg/kg/day) did not alter the intravenous intake of various doses of cocaine available on a continuous schedule of reinforcement. Furthermore, Leri *et al* (2004) found that stress, unlike cocaine and heroin primes, was effective in reinstating drug-seeking behavior in methadone-maintained rats. Therefore, taken together, these results indicate that the direct reinforcing effects of cocaine are not altered by methadone maintenance, and they further suggest that methadone maintenance does not block the expression of all goal-motivated behaviors because of general cognitive or motivational impairments. It is important to note that these results may be in part attributable to the chronic pattern of methadone exposure. In fact, unlike maintenance, acute injections of methadone do enhance cocaine CPP in rats (Bilsky *et al*, 1992) and monkeys prefer methadone–cocaine combinations over cocaine alone (Wang *et al*, 2001).

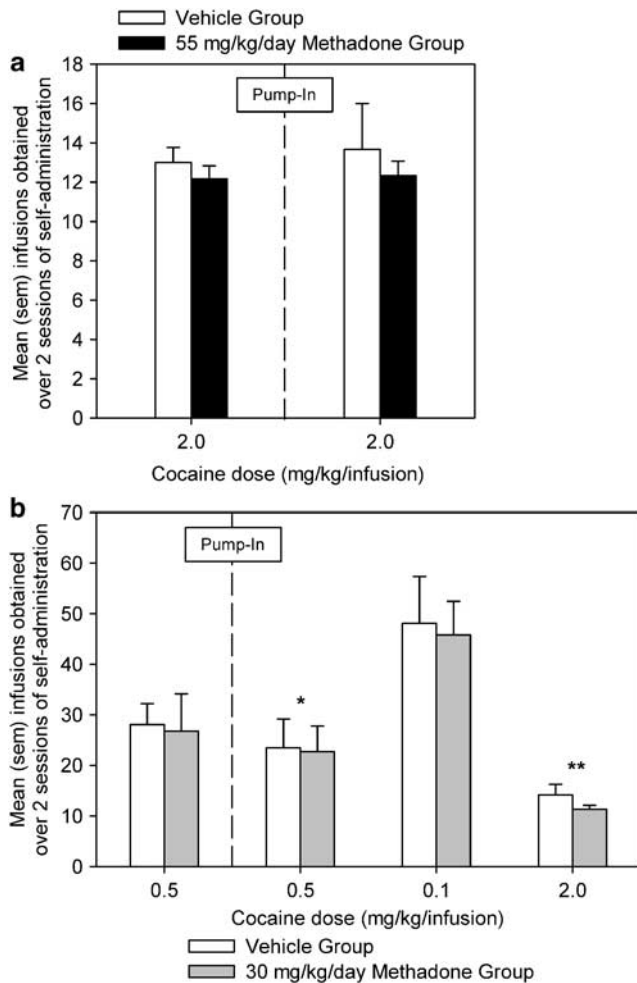


Figure 6 Panel a: Mean (SEM) infusions obtained over two sessions of intravenous self-administration of cocaine (2 mg/kg/infusion) before and after the implantation of methadone-filled (0 and 55 mg/kg/day) osmotic mini-pumps. Panel b: Mean (SEM) infusions obtained over two sessions of intravenous self-administration of cocaine before and after the implantation of methadone-filled (0 and 30 mg/kg/day) osmotic minipumps. Animals were trained with 0.5 mg/kg/infusion cocaine and, after pump-implantation, were tested with 0.5, 0.1, and 2 mg/kg/infusion cocaine. * Denotes a significant increase in infusions from 0.5 to 0.1 mg/kg/infusion, and ** denotes a significant decrease in infusions from 0.1 to 2 mg/kg/infusions.

Taken together, our studies in rats suggest that high-dose methadone maintenance does not alter the direct stimulatory and reinforcing properties of cocaine, but effectively reduces spontaneous and cocaine-precipitated cocaine seeking. Such results appear to parallel findings in opiate-dependent individuals maintained on 50–100 mg/day methadone who report unaltered (Foltin and Fischman, 1996), or enhanced (Foltin *et al*, 1995; Preston *et al*, 1996), cocaine-induced stimulation and cocaine liking, and yet consume less cocaine (Borg *et al*, 1999; Schottenfeld *et al*, 2005; Stine *et al*, 1992). A critical question that we are currently exploring is whether high-dose methadone maintenance can block seeking behaviors motivated by stimuli associated with other rewards such as palatable food or sexual behavior.

Methadone-maintained animals that underwent cocaine CPP in the first experiment were killed 10 days after removal of the methadone-filled minipumps. This time

point was also selected to reduce the possibility that MOR were still being directly activated by residual levels of methadone in the brain. In humans, active methadone maintenance downregulates opioid-binding sites, and lowers both affinity and receptor availability (Kling *et al*, 2000). In the present study, we found a significant upregulation of MOR mRNA expression in the NAC core and FC of cocaine-conditioned rats maintained on vehicle. However, animals maintained on 20 and 55 mg/kg/day methadone displayed a dose-dependent blockade of upregulation of MOR mRNA in the NAC core. The mechanisms by which methadone maintenance modulated MOR mRNA levels in the nucleus accumbens core are not clear. In fact, such an effect may result either from methadone's agonistic action at MOR (Kreek *et al*, 2002) or from its antagonistic action at NMDA glutamate receptors (Gorman *et al*, 1997). Two lines of evidence, however, support the former possibility. First, we have found that chronic exposure to morphine (0, 10, and 20 mg/kg/day), an agent that has no direct antagonist action at NMDA receptors, dose dependently blocked cocaine (10 mg/kg) CPP (unpublished observation). Second, Zhou *et al* (2004) have found that chronic exposure to morphine leads to alterations in mRNA for MOR in the nucleus accumbens measured 12 h after the last morphine injection.

Our analysis of MOR mRNA expression suggests the possibility that methadone maintenance may have blocked the expression of cocaine CPP, at least in part, by preventing upregulation of MOR in the nucleus accumbens core induced by cocaine conditioning. The nucleus accumbens core mediates the motivational influence of Pavlovian stimuli on goal-directed behavior (Hall *et al*, 2001) and can therefore control drug-seeking activity. In fact, various manipulations of this region have been found to block cocaine CPP, operant responding for cocaine-conditioned cues, cocaine-induced reinstatement of cocaine seeking, cocaine self-administration on a second-order schedule, and acquisition/conditioning of drug seeking maintained by cocaine-conditioned stimuli (Baker *et al*, 1998; Cornish and Kalivas, 2000; Duvauchelle *et al*, 2000; Fuchs *et al*, 2004; Grimm *et al*, 2003; Ito *et al*, 2000, 2004; Kaddis *et al*, 1995; McFarland and Kalivas, 2001; Wise, 2004). Also, our observation of lack of effect of methadone on MOR mRNA upregulation in the FC, and lack of effect of methadone on cocaine self-administration supports the idea that the FC mediates the subjective motivational properties of cocaine, while the nucleus accumbens core is involved in stimulated drug-seeking activity (Everitt and Wolf, 2002). However, because of the design of our study, it is not possible to attribute the effects of methadone on cocaine-seeking behavior to the alterations of MOR mRNA we detected 14 days following behavioral testing. Nevertheless, this is a likely hypothesis which we are currently testing by measuring MOR mRNA expression on the same day of behavioral testing.

It is fairly well established that exposure to drugs of abuse produce dynamic changes in the state of activation of the HPA axis (Kreek *et al*, 2002). In rats, acute injections of cocaine increase plasma levels of ACTH and CORT (Moldow and Fischman, 1987), possibly via a stimulation of corticotropin-releasing factor in the hypothalamus. The effects of chronic cocaine exposure on HPA axis activity are more variable and influenced by the specific regimen of

cocaine exposure (Torres and Rivier, 1992; Zhou *et al*, 2003). More importantly, several of these cocaine-induced alterations of the HPA axis appear to persist for some period of time following cocaine exposure, an observation that may explain the long-lasting vulnerability to stress-induced relapse in abstinent cocaine addicts (Goeders, 2002; Shaham *et al*, 2000; Sinha *et al*, 2000). The results obtained in the current study are certainly consistent with the suggestion that cocaine induces alterations of the HPA which outlast the period of active drug exposure. In fact, it was found that cocaine CPP dose-dependently increased both plasma ACTH and CORT, although CORT values did not reach statistical significance. This is a notable effect because animals only received three cocaine injections (5 or 20 mg/kg each) over 3 days of conditioning, and because ACTH and CORT were measured 14 days after the last cocaine injection.

Arguably, however, a surprising result was the limited effect of high-dose methadone maintenance on the HPA axis. In fact, when we analyzed the plasma and brain of rats 10 days following the removal of methadone-filled mini pumps, the only significant effect was on ACTH; methadone maintenance potentiated cocaine-induced increases in ACTH. This synergy, however, probably resulted from the effects of methadone withdrawal which, similarly to withdrawal from other opioids, induces hyperactivity of the HPA axis (Kreek, 2000). We suspect that this may be the case because removal of the pumps precipitated a state of opiate withdrawal as indicated by a rapid loss of body weight (Leri *et al*, 2003), which, in the 55 mg/kg/day methadone group, was not fully recovered by the time of euthanasia. Besides this effect on ACTH, high-dose methadone maintenance did not alter plasma CORT or POMC mRNA expression in the anterior pituitary or the hypothalamus. These results extend previous findings by Zhou *et al* (1996), who found that POMC mRNA expression in the anterior pituitary and hypothalamus as well as circulating levels of CORT were not altered during methadone maintenance (10 mg/kg/day).

In summary, the present experiments in rats show that high-dose methadone maintenance can effectively reduce spontaneous and cocaine-precipitated cocaine-seeking without altering the direct stimulatory and reinforcing effects of cocaine. In addition, our results suggest the hypothesis that methadone maintenance blocks cocaine seeking by inhibiting the upregulation of MOR mRNA expression in the nucleus accumbens core induced by cocaine conditioning.

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