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Steady-State Methadone Blocks Cocaine Seeking and Cocaine-Induced Gene Expression Alterations in the Rat Brain

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Abstract

To elucidate the effects of steady-state methadone exposure on responding to cocaine conditioned stimuli and on cocaine-induced alterations in central opioid, hypocretin/orexin, and D2 receptor systems, male Sprague-Dawley rats received intravenous infusions of 1 mg/kg/inf cocaine paired with an audiovisual stimulus over three days of conditioning. Then, mini pumps releasing vehicle or 30 mg/kg/day methadone were implanted (SC), and lever pressing for the stimulus was assessed in the absence of cocaine and after a cocaine prime (20 mg/kg, IP). It was found that rats treated with vehicle, but not methadone, responded for the cocaine conditioned stimulus and displayed elevated mu-opioid receptor mRNA expression in the nucleus accumbens core and basolateral amygdala, reduced hypocretin/orexin mRNA in the lateral hypothalamus, and reduced D2 receptor mRNA in the caudate-putamen. This is the first demonstration that steady-state methadone administered after cocaine exposure blocks cocaine-induced behavioral and neural adaptations.

Keywords

Methadone; cocaine; mu-opioid receptor; hypocretin/orexin; dopamine receptor

Introduction

Our laboratories have been exploring whether steady-state methadone (SSM) exposure can alter behaviors motivated by cocaine, and whether these alterations co-occur with alterations in gene expressions in regions of the brain known to play a role in addictive behaviors. In one study, we found that SSM exposure blocked cocaine seeking as assessed by the conditioned place preference procedure, as well as cocaine-induced up-regulation of mu-opioid receptors (MOP-r) mRNA in the nucleus accumbens core (Leri et al. 2006). The effect of cocaine on MOP-r mRNA was consistent with several other reports of cocaine-induced alterations of the endogenous opioid system (Azaryan et al. 1998; Cohen et al. 1991; Hurd et al. 1992; Izenwasser et al. 1996; Spangler et al. 1993; Turchan et al. 2002; Unterwald 2001). Furthermore, because the changes in MOP-r mRNA expression were selectively found in neural areas involved in the regulation of incentive motivation and addictive behaviors (Di Chiara 1995; Koob 1992;

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Wise 1987), our findings provided further evidence for a role of cocaine-induced modification of MOP-r function in the development and maintenance of cocaine seeking (Gorelick et al. 2005; Kreek 1996; Schroeder et al. 2007; Zubieta et al. 1996).

However, in our aforementioned study, cocaine was administered during SSM exposure, and therefore methadone could have interfered with some direct pharmacological action of cocaine. In addition, although the test of place preference was administered during SSM exposure, MOP-r mRNA was measured only 10 days after withdrawal from methadone. Finally, because we did not measure mRNA expression of the endogenous ligands of the MOP-r, we did not clarify whether changes in MOP-r could have reflected alterations in pre-synaptic opioid-mediated neural transmission.

The first objective of the present study was to address these limitations. Hence, SSM was administered after the period of cocaine exposure, and mRNA expressions of MOP-r, pro-opiomelanocortin (POMC), prepro-enkephalin (ppEnk), and prepro-dynorphin (ppDyn) in mesolimbic and nigrostriatal regions was investigated right after the behavioral test given during SSM exposure. Furthermore, to specifically investigate the effect of SSM on the expression of responses to cocaine conditioned stimuli, we employed a behavioral method based on a conditioned reinforcement procedure (Davis and Smith 1987) recently adapted to study cocaine seeking in rats (Goddard and Leri 2006). This method is conceptually similar to place conditioning in that rats associate environmental stimuli with the effects of cocaine administered by the experimenter and then, in a drug-free state, emit an unlearned response motivated by these stimuli (Calcagnetti and Schechter 1993). Its advantage over other conditioned reinforcement procedures (Di Ciano and Everitt 2004) or typical models of cue-induced drug seeking based on intravenous self-administration (Capriles et al. 2003; Kruzich et al. 2001; Shalev et al. 2002) is that rats never learn a response for the primary reinforcer and hence the neural changes measured during acquisition/performance of this task cannot reflect the formation of stimulus-response habits. Another advantage over traditional self-administration procedures is that rats receive drug infusions passively and, therefore, all animals in a given group receive identical amounts of drug. This is particularly important because the scope of our present research is to compare gene expression in groups of rats exposed to cocaine alone or in combination with methadone. Finally, this model has advantages over place conditioning in that it is more sensitive to variations in cocaine dosages (Bardo et al. 1995; Goddard and Leri 2006) and rats can be exposed to larger quantities of drug over short periods of time.

The second objective of this study was to quantify, in the same animals, mRNA expression of two other genes implicated in addictive behaviors. The first is the hypocretin/orexin (Hcrt) gene in the lateral hypothalamus (de Lecea et al. 1998; Sakurai et al. 1998) which is co-localized with dynorphin (Chou et al. 2001) and MOP-r (Georgescu et al. 2003), and which is known to play a role in the regulation of goal-directed behaviors (DiLeone et al. 2003; Harris et al. 2005; Narita et al. 2006; Scammell and Saper 2005; Sutcliffe and de Lecea 2002) including cocaine seeking (Boutrel et al. 2005). The second is the dopamine D2 receptor (DR2) gene in the dorsal striatum. DR2 agonists are known to precipitate cocaine seeking (De Vries et al. 2002; Self et al. 1996) and low DR2 availability in the ventral and dorsal striatum predict vulnerability to cocaine reinforcement (Dalley et al. 2007; Nader and Czoty 2005). Furthermore, in the dorsal striatum, DR2 is co-localized with the MOP-r (Ambrose et al. 2004), and reduced availability of DR2 binding in this region is associated with cocaine addiction and cocaine cravings (Volkow et al. 2006; 2007). The effect of SSM on mRNA expression of these two genes in rats exposed to cocaine has never been investigated.

Additional quantifications targeted the hypothalamic-pituitary-adrenal (HPA) hormonal levels, which are altered in drug abusers and normalized by methadone maintenance (Kreek 1992; Kreek et al. 2002).

Experimental procedures

Subjects

Subjects were adult male Sprague-Dawley rats (Charles River, QC) weighing 225-250g at the beginning of all experiments. They were singly housed and maintained on a reverse light/dark cycle (8:00 lights off; 20:00 lights on) with free access to food and water except during behavioral testing. 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 & Apparatuses

Osmotic mini pumps—Chronic exposure to SSM 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 haemostat 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.

Intravenous catheterization—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 IP, MTC Pharmaceutical, Cambridge, ON), morphine (5 mg/kg SC, Ontario Veterinary College, Guelph, ON) and diazepam (1 mg/kg SC, Sabex Inc., Boucherville, QC). Rats were given atropine sulfate (4.5 mg/kg SC, 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 subcutaneously 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).

Activity chambers—Twelve, custom made (University of Guelph, ON), activity chambers were used to measure locomotion activity. The boxes were located in the center of the laboratory room. Each chamber (30 × 40 × 26 cm) was made of dark gray PVC and 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).

Operant chambers—Twenty-six Plexiglas operant chambers (model ENV-008CT, Med Associates, Georgia, VT) were each enclosed in larger sound-attenuating plywood chambers (model ENV-018M, Med Associates). Each chamber had a house light (28 V), and two levers, one retractable and one stationary, located 10 cm apart and 8 cm above the floor. Presses on the retractable lever (active lever) activated a white light (28 V) and a 65 dB buzzer located 3 and 8 cm above the lever, respectively. The stationary lever served to control for non-specific lever responding; pressing this lever had no consequence (inactive lever), but all presses were recorded. Infusion pumps (Razel Scientific Instruments, Stamford, CT) for the delivery of drug solutions during the period of conditioning were positioned outside the sound-attenuating chamber.

Solution hybridization RNase protection-TCA precipitation, immunoreactivity and gas liquid chromatography

The frontal cortex (FC), caudate-putamen (CP), core subdivision of the nucleus accumbens (NAcC), shell subdivision of the nucleus accumbens (NAcS), central amygdala (CeA), basolateral amygdala (BLA), ventral tegmental area (VTA), medial hypothalamus (MH), lateral hypothalamus (LH), and anterior pituitary (AP) were dissected on ice, homogenized in guanidinium thiocyanate buffer and extracted with acidic phenol and chloroform. The mRNA levels for POMC, ppEnk, ppDyn, MOP-r, Hcrt and DR2 were quantified in specific regions on the basis of known levels of constitutive expression as well as previous results showing expression changes as a result of exposure to cocaine (Zhou et al. 2002; 2008). The solution hybridization RNase protection-TCA precipitation protocol has been described in detail in earlier reports (Branch et al. 1992; Zhou et al. 2006). A 2100 bp fragment from the rat MOP-r cDNA, a 860 bp fragment from the rat DR2 cDNA, a 538 bp fragment from the rat POMC cDNA, a 935 bp fragment from the rat ppEnk cDNA, or a 1700 bp fragment from the rat ppDyn cDNA, was cloned into the polylinker region of the pSP64 plasmid (Promega, Madison, WI) in both the sense and antisense orientations. A 531 bp fragment from rat Hcrt cDNA was cloned into the polylinker region of pBC SK+ (Stratagene, La Jolla, CA). To determine the total attomoles of each mRNA in each extract, the amounts calculated from the standard curves were multiplied by 5.71 for MOP-r, 2.91 for DR2, 2.04 for POMC, 1.58 for ppEnk, 1.4 for ppDyn, or 1.3 for Hcrt to correct for the difference in length between the sense transcript (2100, 860, 538, 935, 1700 or 531 bases for the MOP-r, DR2, POMC, ppEnk, ppDyn or Hcrt, respectively) and the full-length mRNA (12, 2.5, 1.1, 1.5, 2.4, or 0.7 k base for the MOP-r, DR2, POMC, ppEnk, ppDyn or Hcrt).

Trunk blood was collected immediately after decapitation, and plasma was separated. Levels of corticosterone (CORT) and adrenocorticotrophic hormone (ACTH) immunoreactivity were assayed using kits from MP Biomedicals (Costa Mesa, CA) and DiaSorin Inc. (Stillwater, MN). All CORT and ACTH values were determined in duplicate in a single assay. Plasma methadone levels were determined by gas liquid chromatography at Bendiner & Schlesinger Inc. Cutoff point for plasma detective limit was 300 ng/ml (Borg et al. 1999).

Behavioral testing

Behavioral testing began after intravenous surgery and consisted of six phases. Table 1 represents these phases as well as the experimental groups, sample size, and drug treatments.

Phase 1: Test of baseline level of responding for a novel audiovisual stimulus —Rats were placed in the operant chambers, and following a delay of 5 min, a 3h session started with the activation of a house light, and 10 sec later, the entry of an active lever and the activation of a light-buzzer compound stimulus for 45 sec. Subsequent presses on this lever led to the activation of the light-buzzer compound stimulus for 10 sec. This baseline session was necessary because our pilot studies highlighted the existence of large individual differences in spontaneous tendency to respond for a novel light-buzzer compound stimulus. Therefore, individual scores on this variable were used to assign rats to different drug treatments (see Table 1).

Phase 2: Pavlovian conditioning—On three Pavlovian conditioning sessions (1 × 2h and 2 × 4h) given over 3 consecutive days, rats received passive intravenous infusions of 1 mg/kg/inf cocaine (or vehicle) accompanied by the presentation of the light-buzzer compound stimulus. The duration of the first session was shorter in order to reduce possible aversive effects of intense cocaine exposure in cocaine-naïve rats. During all conditioning sessions, the stimulus was activated 5 sec before, and during the 10 sec intravenous infusion (300 μ l). Rats

received one stimulus-infusion pairing every 4 min. During this phase, the active lever was not introduced in the chamber.

Phase 3: Implantation of osmotic mini-pumps and test of locomotion—Two days following the last conditioning session, rats were implanted with 30 mg/kg/day methadone (or vehicle)-filled osmotic mini pumps and, after four days of recovery from surgery and adaptation to methadone, animals received a 2-h long test of locomotor activity in order to evaluate possible methadone-induced motor impairments.

Phase 4: Tests of responding reinforced by the cocaine conditioned stimulus—The following day, lever pressing for the cocaine conditioned buzzer-light stimulus was reassessed by introducing the active lever in the chamber and allowing the rats to freely press it in the absence of cocaine. In other words, contingencies present in Phases 1 and 4 were identical. Each test session lasted 3 hours, and rats received a total of 5 test sessions over 5 consecutive days. Because during this phase cocaine was not infused, each of these test sessions also served to extinguish the conditioned reinforcing property of the cocaine conditioned stimulus.

Phase 5: Reinstatement—Just prior to the second-last test of responding for the cocaine conditioned stimulus, all rats received an injection of vehicle (Prime I) and, the following day just prior to the last test session (3h), they received a priming injection of 20 mg/kg (IP) cocaine (or vehicle; Prime II - see Table 1). All rats were sacrificed immediately after the conclusion of Prime II reinstatement session. At the time of euthanasia, animals were still exposed on methadone (or vehicle).

In sum, as illustrated in Table 1, this study included 6 groups of rats. The three groups most critical to the study of the effect of methadone on responding reinforced by the cocaine conditioned cues (darkened rows in Table 1) received: vehicle during conditioning, vehicle pumps and vehicle on Prime II (VVV group; n=8); cocaine during conditioning, vehicle pumps, and cocaine on Prime II (CVC group; n=10); and cocaine during conditioning, methadone pumps, and cocaine on Prime II (CMC group; n=8). Three additional groups were created to interpret the gene expression data. Animals in these groups underwent the same phases of behavioral testing, but received different combinations of cocaine conditioning/methadone/cocaine priming treatments. Thus, the methadone only group (VMV; n=8) was exposed to vehicle during conditioning, methadone pumps and vehicle on Prime II; the cocaine conditioning only group (CVV; n=7) received cocaine during conditioning, vehicle-pumps, and vehicle on Prime II; and, finally, the cocaine prime only group (VVC; n=8) received vehicle during conditioning, vehicle pumps, and cocaine on Prime II.

Drugs and doses

Cocaine HCL (Dumex, Toronto, On) and methadone HCL (Pharmascience, Montreal, Qc) were dissolved in physiological 0.9% saline. The doses of cocaine employed during Pavlovian conditioning (1 mg/kg/inf) and priming (20 mg/kg) were selected on the basis of a previous study that employed an identical methodology which demonstrated substantial conditioned responding and reinstatement (Goddard and Leri 2006). Total quantity of cocaine exposure achieved through conditioning with 1 mg/kg/inf is similar to levels achieved in rats that actively self-administered the same dose (Leri et al. 2004). Thirty mg/kg/day methadone was used because this methadone dose has been previously shown to block cocaine induced reinstatement of cocaine seeking (Leri et al. 2004), and to block formation and expression of cocaine place conditioning (Leri et al. 2006) without producing general locomotor and other behavioral deficits (Leri et al. 2007).

Statistical analyses

A two-way mixed design ANOVA (factors: Test Session and Group) was used to compare operant responding emitted at the different phases of behavioral testing. Separate one-way ANOVAs were used to compare mRNA expression levels in the different groups for each gene. The Bonferroni correction was applied to the p value when multiple ANOVAs were performed on the same gene measured in various brain regions (MOP-r, POMC, ppEnk, ppDyn). Alternative statistical approaches are reported in the Results sections. Individual measurements deviating more than 2 standard deviations from the group mean were removed from the analyses (plasma hormonal levels: ACTH = 3, CORT = 1 values removed; mRNA levels: LH Hcrt = 3, MH POMC = 3, MH MOP-r = 2, NAcC POMC = 2, NAcC ppEnk = 3, NAcC MOP-r = 1, CP ppDyn = 1, CP DR2 = 4, BLA MOP-r = 1, CE ppDyn = 3, CE MOP-r = 1, FC ppEnk = 1, FC MOP-r = 1, values removed). In case of significant interactions or significant main effects, multiple comparisons were performed using the Newman Keul's method to identify individual mean differences ($\alpha = 0.05$). In general, the specific values of negative findings are not reported. All statistical analyses were performed using SigmaStat (version 3.0 for Windows, SPSS Inc).

Results

Behavior

Panel A of Figure 1 represents responses on the active lever across all test sessions in all groups, but only the comparison between the VVV, CVC and the CMC groups is of empirical interest (darkened rows in Table 1). The ANOVA revealed a significant interaction between Test session and Group [$F(35,301)=1.95$, $p<0.005$], as well as significant main effects of Test session [$F(7,301)=28.17$, $p<0.001$] and Group [$F(5,43)=3.05$, $p<0.05$]. Multiple comparisons indicated that, in comparison to drug-naïve animals (VVV group), rats conditioned with cocaine and implanted with vehicle-filled pumps (CVC group) displayed significantly higher levels of responding maintained by the cocaine conditioned stimulus. Furthermore, in these rats, the priming injection of cocaine was effective in reinstating responding after extinction induced by repeated testing. In contrast, rats conditioned with cocaine and implanted with methadone-filled pumps (CMC group) showed no maintenance of responding for the cocaine conditioned stimulus, and showed no reinstatement following the cocaine prime. Importantly, this lack of operant responding was not the result of motor sedation as rats implanted with methadone pumps displayed normal locomotor activity during the test of locomotion administered before the beginning of operant testing (see panel B of Figure 1; one-factor independent group ANOVA: [$F(2,23)=0.71$, $p=0.50$]). No notable Test session or Group effects were observed on responding on the inactive lever responding, which was generally low.

Plasma hormonal levels

No significant group differences were found in CORT or ACTH levels (all groups compared; Table 2).

Plasma methadone levels

No differences in plasma methadone levels were found between the VMV and the CMC groups (see Table 2).

mRNA expression levels

Statistical analyses revealed that mRNA expression levels of most genes measured in the various regions of the brain did not differ significantly among experimental groups (see Table 2). These genes/regions included: POMC in the MH, AP, and NAcC; ppEnk in NAcC and FC; MOP-r in MH, NAcS, CP, CA, and VTA; and ppDyn in the CA and FC.

However, as illustrated in Figure 2, expression of Hcrt mRNA in the LH was significantly reduced in animals conditioned and primed with cocaine (CVC group), but not in rats similarly exposed to cocaine and implanted with methadone pumps (CMC group; [$F(5,40) = 2.68$, $p<0.05$]). Further, multiple comparisons revealed that hypocretin/orexin mRNA in this region was not altered in rats that received cocaine conditioning only (CVV group) or cocaine priming only (VVC group).

Further, as illustrated in Panel A of Figure 3, expression of MOP-r mRNA in the NAcC was significantly elevated in animals conditioned and primed with cocaine (CVC group), but not in rats conditioned and primed with cocaine and implanted with methadone pumps (CMC group; [$F(2,42) = 8.25$, $p<0.006$; significant after Bonferroni correction]). Multiple comparisons further revealed that MOP-r mRNA in this region was not altered in rats that received cocaine conditioning only (CVV group) or cocaine priming only (VVC group).

A somewhat different pattern of group differences was found for expression of MOP-r mRNA in the BLA. That is, as illustrated in Panel B of Figure 3, expression level was significantly elevated in animals conditioned and primed with cocaine (CVC group), but not in rats similarly exposed to cocaine and implanted with methadone pumps (CMC group; [$F(5, 42) = 6.28$, $p<0.006$; significant after Bonferroni correction]). However, MOP-r mRNA in this region was also significantly elevated in rats that received cocaine priming only (VVC group).

In the case of MOP-r mRNA in the FC (Panel C of Figure 3), expression levels were higher in rats conditioned and primed with cocaine whether maintained on vehicle (CVC group) or methadone (CMC group; [$F(5,41) = 4.24$, $p<0.005$; not significant after Bonferroni correction]). Also, multiple comparisons indicated that MOP-r mRNA was elevated in rats that received cocaine conditioning only (CVV group), but not cocaine priming only (VVC group).

In the CP, expression of ppDyn mRNA (Figure 4) was elevated in rats conditioned and primed with cocaine whether maintained on vehicle (CVC group) or methadone (CMC group; [$F(5,41) = 4.24$, $p<0.016$; significant after Bonferroni correction]). Also ppDyn mRNA in this region was significantly elevated in rats that received cocaine priming only (VVC group), but not cocaine conditioning only (CVV group).

Finally, expression of DR2 mRNA in the CP (Figure 5) was significantly reduced in animals conditioned and primed with cocaine (CVC group), but not in rats similarly exposed to cocaine and implanted with methadone pumps (CMC group; [$F(5,39) = 6.11$, $p<0.001$]). Further, multiple comparisons indicated that DR2 mRNA was also significantly reduced in rats that received cocaine conditioning only (CVV group), but not cocaine priming only (VVC group).

Discussion

In this study, rats exposed to cocaine during conditioning and, subsequently, to steady-state methadone, did not display significant operant responding reinforced by a cocaine conditioned stimulus, and cocaine priming had no effect on their behavior. In addition, rats exposed to cocaine and SSM did not display alterations in MOP-r, Hcrt and DR2 mRNA expression in regions of the brain known to play a role in addictive behaviors. To our knowledge, this is the first demonstration that SSM administered after cocaine exposure blocks cocaine-induced alterations in behavior and gene expression in the rat brain.

Our present results are consistent with findings from other studies on the application of a conditioned reinforcement procedure to study cocaine seeking in rats (Goddard and Leri 2006) and effects of cocaine conditioning on mRNA expressions in the rat brain (Leri et al. 2006). In fact, animals trained to associate an audiovisual stimulus with intravenous infusions of cocaine displayed significant levels of responding reinforced solely by this conditioned

stimulus during extinction tests, as well as during a test of reinstatement induced by a priming injection of cocaine (CVC group). Also, in comparison to drug-naïve animals (VVV group), these rats displayed site-specific mRNA expression differences in the endogenous opioid system, hypocretin/orexin system, and D2 receptors. More specifically, in CVC rats, we observed a down-regulation of Hcrt mRNA expression in the LH, up-regulations of MOP-r mRNA expression in the NAcC, BLA and FC (but not significant after Bonferroni correction), up-regulation of ppDyn mRNA in the CP, and down-regulation of DR2 mRNA in the CP.

SSM blocked the expression of responding reinforced by the cocaine conditioned stimulus, both during the tests in extinction conditions and after the priming injection of cocaine (CMC group). This effect of SSM could not have been completely caused by direct pharmacologic interactions between methadone and cocaine because the two drugs were administered at different times; that is, methadone pumps were implanted after the period of cocaine conditioning. Only during priming was cocaine administered to animals on SSM, but even in this case a pharmacokinetic interference between SSM and cocaine is doubtful. In fact, acute cocaine administration is known to up-regulate ppDyn mRNA expression in the CP (Zhou et al. 2002; Ziolkowska et al. 2006) and this alteration was found in animals sacrificed 3 hours after the cocaine prime, whether exposed to SSM or not (CMC and CVC groups). Similarly, an interference of SSM on cognitive abilities required to display cocaine seeking is unlikely as rats exposed to 30 mg/kg/day have been found to express foot-shock induced reinstatement (Leri et al. 2004).

The effect of SSM on operant responding maintained by the cocaine conditioned cue is also not explainable by generalized motor deficits because SSM exposed rats displayed normal levels of locomotion when tested in activity chambers prior to the beginning of operant testing. This may appear at odds with the findings that methadone decreases operant responding in monkeys (Negus and Mello 2004) and produces sedation in humans (Walsh et al. 1995). However, in our laboratory rats, motor sedation induced by doses equal or above 30 mg/kg/day SSM administered by SC mini pumps dissipate within a few hours from implantation of pumps. Furthermore, 2-4 days following mini pump surgery, animals on SSM display dose-dependent elevations, not decreases, in locomotion activity (Leri et al. 2004) and normal lever pressing behavior (Leri et al. 2007). Finally, we have reported that 30 mg/kg/day SSM administered via mini pump does not reduce behaviors motivated by food, and does not reduce basal dopamine concentrations in the NAc (Leri et al. 2007).

Accordingly, the results of the present study indicate that 30 mg/kg/day SSM reduced the conditioned motivational value of the cocaine conditioned stimulus, and therefore reduced its ability to serve as a reinforcer and maintain operant behavior. This is consistent with the observation that 30 mg/kg/day SSM also reduced approach to a cocaine conditioned environment, as well as responding on a cocaine conditioned lever during extinction and after cocaine priming (Leri et al. 2004; 2006).

The results of our gene expression data provide novel evidence that can be helpful in understanding this behavioral effect of SSM. In fact, rats implanted with methadone-filled pumps after cocaine conditioning (CMC group) displayed levels of Hcrt mRNA in the LH, MOP-r mRNA in the NAcC and BLA, and DR2 mRNA in the CP that were within control levels (VVV group). Importantly, because the delivery of cocaine infusions during conditioning was controlled by a computer, the lack of alterations in gene expression levels observed in CMC rats cannot be attributed to lower quantity, or different regimen, of cocaine exposure. Interestingly, SSM did not reduce cocaine-induced elevation of MOP-r mRNA expression in the FC (but not significant after Bonferroni correction) and, by itself (VMV group), did not alter any of the plasma hormonal levels and gene expressions measured in this study.

Nevertheless, caution should be used when interpreting the relationships between SSM, behavior and mRNA expression because of at least two reasons. First, we did not measure protein levels, and thus our findings are limited to protein biosynthesis and/or release. Second, the effects of cocaine on mRNA expression were quite diverse. That is, when levels of gene expression in drug-naïve rats (VVV group) was compared to levels measured in rats conditioned and primed with cocaine (CVC group), conditioned only (CVV group) or primed only (VVC group), three patterns of results emerged. First, changes in Hcrt mRNA expression in the LH and MOP-r mRNA expression in the NAcC were observed only in cocaine conditioned animals primed with cocaine (CVC group); animals that received either treatment alone (CVV or VVC groups) showed unaltered expression levels. This observation has two implications. First, it suggests that cocaine priming produced rapid (i.e., within 3 hours) down-regulation of Hcrt and up-regulation of MOP-r mRNA expressions in animals that responded for the cocaine conditioned stimulus. Second, it indicates that the changes in mRNA expression are likely linked to cocaine exposure during conditioning rather than to learning occurring during conditioning. Further, changes in MOP-r mRNA expression in the BLA and ppDyn in the CP were observed in animals primed with cocaine that did not display behavioral evidence of conditioning because they were never conditioned (VVC group). This implies that the rapid expression of these genes was the result of an acute action of cocaine which was not influenced by past cocaine exposure or cocaine conditioning, and which was probably not related to the activation of behavior by cocaine conditioned stimuli. Finally, the decrease in DR2 mRNA expression in the CP was observed in rats that received cocaine conditioning, whether primed with cocaine (CVC group) or not (CVV group). Because these rats displayed significant operant responding during the extinction tests, and because decrease in D2 receptor binding/function in the striatum of animals and humans is not limited to cocaine exposure or cocaine addiction (Fehr et al. 2008;Heinz et al. 2005;Sun et al. 2003;Zijlstra et al. 2008), it is likely that reduced DR2 mRNA in the CP resulted from conditioning and that it played a role in the expression of behavior motivated by the cocaine conditioned stimulus.

Although our gene expression analysis was limited to mRNA levels, and tissue was collected after the behavioral tests, there is experimental evidence suggesting that the behavior observed in rats exposed to SSM could indeed reflect unaltered expression of MOP-r, and/or hypocretin/orexin, and/or DR2 genes in the regions investigated. In fact, the medium-size spiny neurons in the NAcC that project to the dorsolateral ventral pallidum (Heimer et al. 1991; Zahm and Brog 1992) express MOP-r (Herkenham and Pert 1981; Mansour et al. 1995; Napier and Mitrovic 1999). These neurons are important components of a “motive” circuit (Mogenson et al. 1980) regulating the expression of motor responses to motivationally significant stimuli (Kalivas et al. 1999), including cocaine conditioned stimuli (Hollander and Carelli 2007). Hypocretin/orexin neurons in the LH, possibly because of reciprocal anatomical connections with the NAc and the VTA (Alberto et al. 2006; Scammell and Saper 2005; Yoshida et al. 2006), are known to play a role in the regulation of goal-directed behaviors (DiLeone et al. 2003; Harris et al. 2005; Narita et al. 2006; Sutcliffe and de 2002) including cocaine seeking and sensitization (Borgland et al. 2006; Boutrel et al. 2005). The decrease in LH Hcrt mRNA levels observed in rats conditioned and primed with cocaine (CVC group) is consistent with a recent observation of similar decreases in rats that expressed a cocaine place preference (Zhou et al. 2008). Decreased brain reward function after cocaine administration, as reflected by increases in intracranial self-stimulation thresholds (ICSS), is considered central to cocaine addiction (Koob 2008). Central infusion of hypocrein-1 (orexin A) has been found to elevate ICSS thresholds in the LH, indicating a decrease in excitability of brain reward systems (Boutrel et al. 2005). Therefore, decreased hypocretin/orexin gene expression (and therefore possibly decreased biosynthesis and release) observed in our cocaine conditioning studies may be associated with hypocretin/orexin inhibition resulting from hyper-activated brain reward systems. Finally, neurons in the CP that project to the ventral pallidum express predominantly D2 receptors (Robertson and Jian 1995), and reduced availability of DR2 binding in this region

has been consistently associated to cocaine addiction and cocaine cravings (Volkow et al. 2006; 2007). In this study, we measured plasma levels of prolactin in groups of rats not exposed to methadone measured by radioimmunoreactivity and found significantly lower levels in rats conditioned and primed with cocaine in comparison to VVV rats (data not shown). Because levels of prolactin in plasma are normally inhibited by the activation of DR2 by dopamine released in the hypothalamic-hypophyseal portal system (Leebaw et al. 1978), it is possible that the down-regulation in DR2 mRNA observed in the CP of cocaine conditioned rats represented a homeostatic adaptation to a hyper-active/hyper-responsive dopaminergic system.

It is unclear is how these findings in rats speak to effectiveness of SSM maintenance in reducing cocaine addiction in humans, as clinical studies on this subject have yielded inconsistent findings (Bux et al. 1995; Dunteman et al. 1992; Foltin et al. 1995; Foltin and Fischman 1996; Kosten et al. 1986; Maremmani et al. 2007; Peles et al. 2006). Also unclear is how SSM prevented the expression of neural adaptations associated with cocaine exposure and cocaine conditioning. Because we found no significant alterations in measures of mRNA expression of endogenous ligands of the MOP-r, we suspect that the changes observed did not reflect alterations in pre-synaptic opioid-mediated neural transmission. Therefore, steady-state methadone exposure may have blocked other pre/post-synaptic mechanisms of neuronal plasticity (Kauer and Malenka 2007; Nestler 2001; Nestler 2002) either because of its agonist action at MOP-r (Nugent et al. 2007), or because of its non-competitive antagonist action at glutamate NMDA receptors (Davis and Inturrisi 1999; Ebert et al. 1995; Ebert et al. 1998; Gorman et al. 1997). A role for altered glutaminergic transmission in the striatum is likely as steady state administration of buprenorphine, a partial MOP-r agonist, has been found to increase levels of glutamate in the nucleus accumbens and to block expression of sensitization to the locomotor-activating effects of cocaine (Placenza et al. 2008). Finally, our study does not clarify whether the effect of steady-state methadone on responses to cocaine associated cues is attributable to its pharmacodynamic or to its pharmacokinetic. However, the observations that rats display larger cocaine place preference after bolus injections of methadone (Bilsky et al. 1992) and that monkeys prefer methadone–cocaine combinations over cocaine alone (Wang et al. 2001) suggest that methadone pharmacokinetic is a critical determinant of its behavioral effect.

Nevertheless, our study clearly demonstrates that exposure to steady-state methadone in rats blocks the development/expression of responding to cocaine conditioned cues, as well as cocaine-induced alterations of Hcrt mRNA in the LH, MOP-r mRNA in the NAcC and BLA, and DR2 mRNA in the CP. We propose that additional work should be carried out to explore the molecular mechanisms mediating these effects of SSM because, to our knowledge, this is the first evidence of blockade of cocaine-induced neural adaptations by a drug treatment administered after cocaine exposure, and by a substance that is already clinically available in many countries of the world (World Health Organization 2008).

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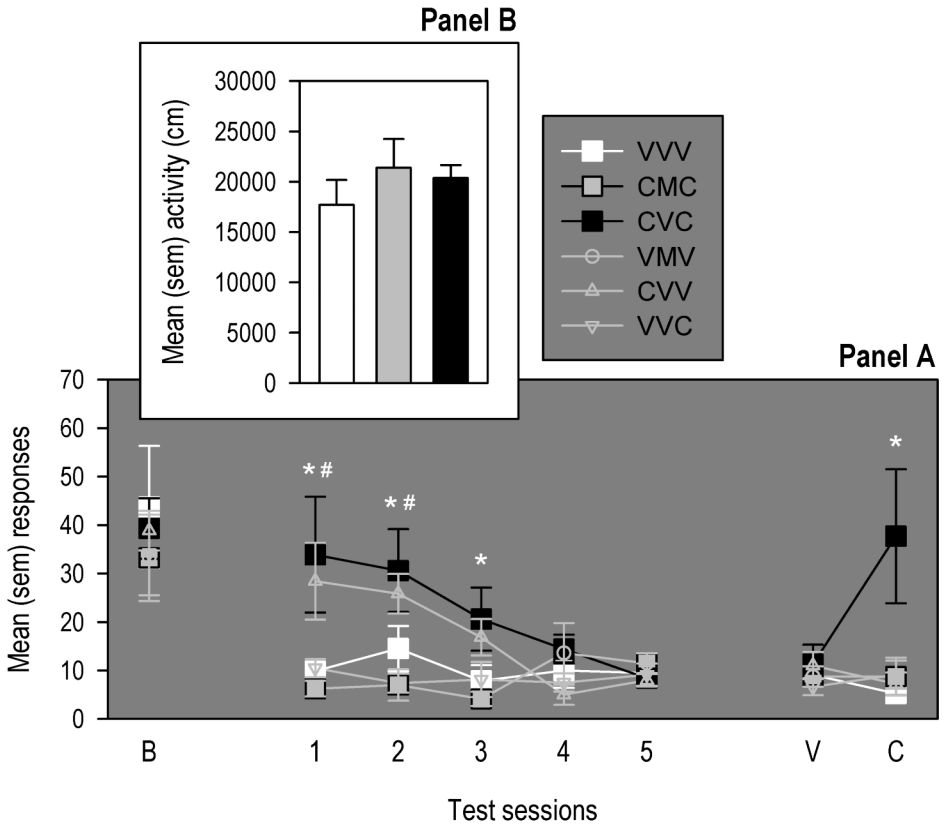

Figure 1.

Panel A: mean (SEM) responses made on the active lever at each test of responding for the activation of an audiovisual compound stimulus before (i.e., Baseline; B) and after (Tests 1-5 Primes I & II) Pavlovian conditioning of this stimulus with IV infusions of cocaine (1 mg/kg/infusion). On Prime I session, all rats were injected with vehicle (V). On Prime II session, some rats (CVC, CMC and VVC) were injected with a 20 mg/kg cocaine prime (C). The * indicates a significant difference between VVV and CVC groups. The # indicates a significant difference between VVV and CVV groups. Panel B: mean (SEM) horizontal distance (cm) travelled by drug-naïve rats (VVV group) and cocaine conditioned/primed groups implanted with 30 mg/kg/day methadone-(CMC group) and vehicle-(CVC) filled mini-pumps. This test of locomotion activity was administered before the period of conditioning.

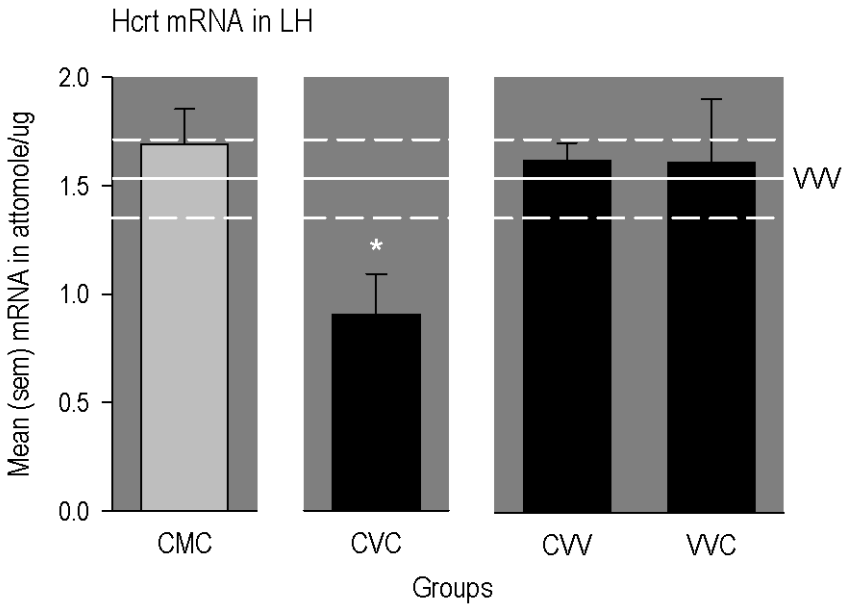

Figure 2.

Mean (SEM) concentrations of different mRNAs in different regions of the rat brain. The horizontal line represents mean levels measured in drug-naïve rats (VVV group). The upper and lower dashed lines represent positive and negative SEMs. The * indicates a significant difference from VVV group. CMC group = conditioned with cocaine, implanted with methadone-filled mini-pumps; primed with cocaine. CVC group = conditioned with cocaine, implanted with vehicle-filled mini-pumps; primed with cocaine. CVV group = conditioned with cocaine, implanted with vehicle-filled mini-pumps; primed with vehicle. VVC group = conditioned with vehicle, implanted with vehicle-filled mini-pumps; primed with cocaine.

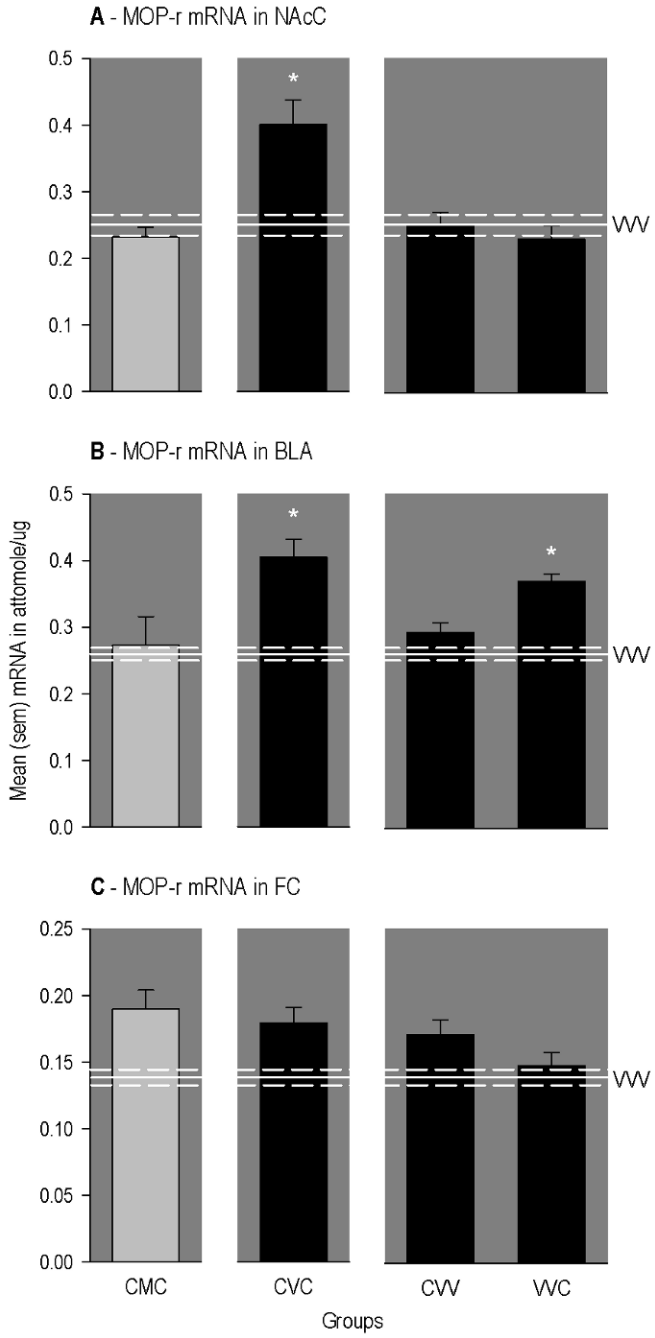

Figure 3.

Mean (SEM) concentrations of different mRNAs in different regions of the rat brain. The horizontal line represents mean levels measured in drug-naïve rats (VVV group). The upper and lower dashed lines represent positive and negative SEMs. The * indicates a significant difference from VVV group. CMC group = conditioned with cocaine, implanted with methadone-filled mini-pumps; primed with cocaine. CVC group = conditioned with cocaine, implanted with vehicle-filled mini-pumps; primed with cocaine. CVV group = conditioned with cocaine, implanted with vehicle-filled mini-pumps; primed with vehicle. VVC group = conditioned with vehicle, implanted with vehicle-filled mini-pumps; primed with cocaine.

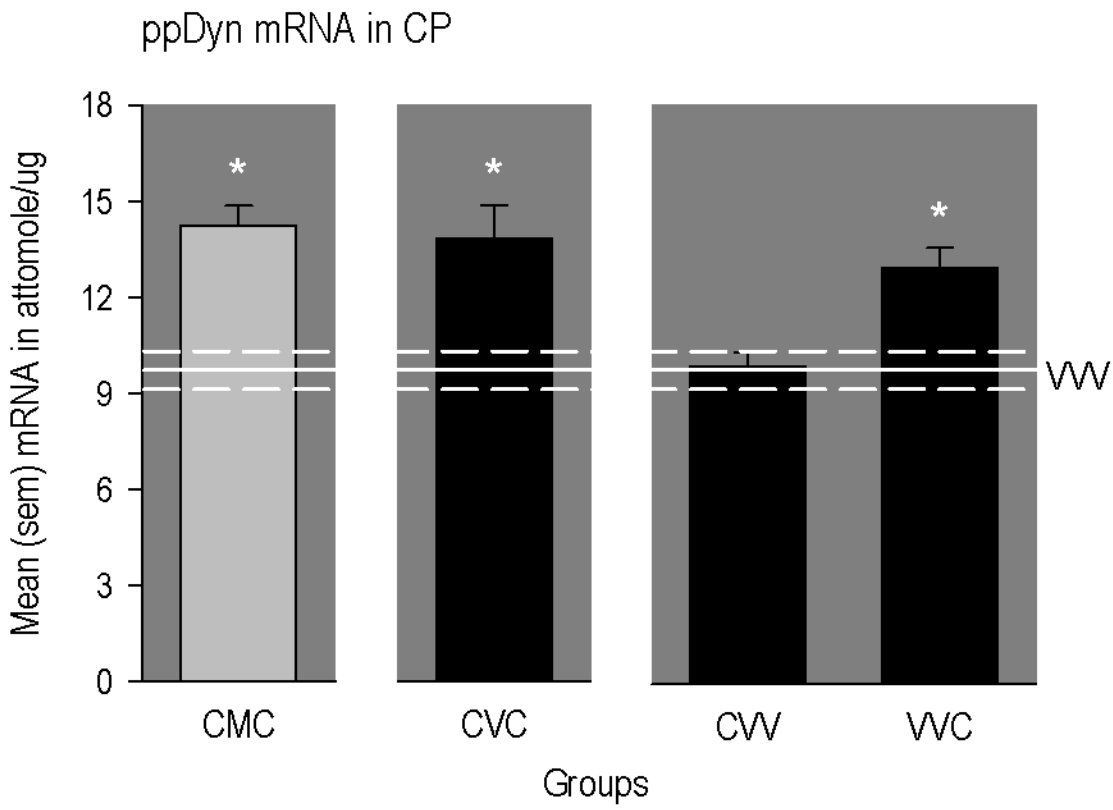

Figure 4.

Mean (SEM) concentrations of different mRNAs in different regions of the rat brain. The horizontal line represents mean levels measured in drug-naïve rats (VVV group). The upper and lower dashed lines represent positive and negative SEMs. The * indicates a significant difference from VVV group. CMC group = conditioned with cocaine, implanted with methadone-filled mini-pumps; primed with cocaine. CVC group = conditioned with cocaine, implanted with vehicle-filled mini-pumps; primed with cocaine. CVV group = conditioned with cocaine, implanted with vehicle-filled mini-pumps; primed with vehicle. VVC group = conditioned with vehicle, implanted with vehicle-filled mini-pumps; primed with cocaine.

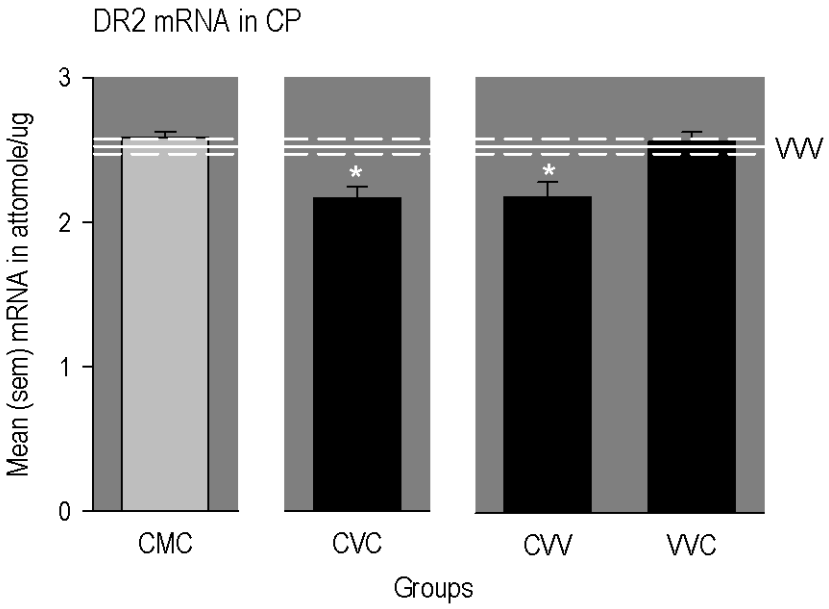

Figure 5.

Mean (SEM) concentrations of different mRNAs in different regions of the rat brain. The horizontal line represents mean levels measured in drug-naïve rats (VVV group). The upper and lower dashed lines represent positive and negative SEMs. The * indicates a significant difference from VVV group. CMC group = conditioned with cocaine, implanted with methadone-filled mini-pumps; primed with cocaine. CVC group = conditioned with cocaine, implanted with vehicle-filled mini-pumps; primed with cocaine. CVV group = conditioned with cocaine, implanted with vehicle-filled mini-pumps; primed with vehicle. VVC group = conditioned with vehicle, implanted with vehicle-filled mini-pumps; primed with cocaine.

Table 1

Groups, sample size, experimental phase and corresponding drug treatment. Darkened rows highlight groups important for the behavioral assessment of steady-state methadone (SSM) exposure.

Groups	Sample Size	Test of baseline responding for a novel audiovisual stimulus 1 session (3h)	Pavlovian conditioning 1 (2h) and 2 (4h) sessions	Implantation of osmotic mini-pumps and subsequent tests of responding for the cocaine conditioned stimulus 5 sessions (3h)	Reinstatement tests (2 3h sessions) of responding for the cocaine conditioned stimulus following:	
					Prime I	Prime II
VVV	8	No injections/drugs	Vehicle	Vehicle	Vehicle	Vehicle
VMV	8		Vehicle	Methadone	Vehicle	Vehicle
CMC	8		Cocaine	Methadone	Vehicle	Cocaine
CVC	10		Cocaine	Vehicle	Vehicle	Cocaine
CVV	7		Cocaine	Vehicle	Vehicle	Vehicle
VVC	8		Vehicle	Vehicle	Vehicle	Cocaine

Table 2

Groups, treatments, and results of the radioimmunoassay, gas liquid chromatography (for plasma methadone) and solution hybridization RNase TCA precipitation assay. The values represent the means and SEM. mRNA measured in attomole/μ g total RNA. The values omitted from darkened rows are represented graphically in appropriate figure. N/A = not assayed.

Group		VVV	VMV	CMC	CVC	CVV	VVC
Pavlovian conditioning		Vehicle		Cocaine		Cocaine	Vehicle
Osmotic mini pumps		Vehicle	Methadone	Methadone	Vehicle	Vehicle	
Prime II		Vehicle		Cocaine		Vehicle	Cocaine
Plasma Methadone (ng/ml)		N/A	451 (14)	491 (10)	N/A	N/A	N/A
Plasma	ACTH (pg/ml)	461 (56)	419 (52)	434 (56)	382 (49)	467 (43)	450 (52)
	CORT (ng/ml)	343 (56)	394 (65)	416 (122)	238 (34)	300 (51)	462 (96)
Lateral Hypothalamus (LH) Medial Hypothalamus (MH)	Hcrt mRNA	Figure 2					
	POMC mRNA	15.2 (1.7)	16.0 (1.8)	16.3 (2.2)	15.7 (2.5)	17.0 (0.9)	14.2 (2.1)
	MOP-r mRNA	0.20 (0.02)	0.20 (0.02)	0.20 (0.03)	0.18 (0.03)	0.20 (0.02)	0.19 (0.03)
Anterior Pituitary (AP)	POMC mRNA	410 (18)	430 (25)	383 (14)	425 (18)	394 (28)	391 (29)
Nucleus Accumbens Core (NAcC)	POMC mRNA	1.23 (0.15)	1.23 (0.18)	1.35 (0.14)	0.94 (0.18)	1.25 (0.07)	1.87 (0.53)
	ppEnk mRNA	59.0 (9.2)	62.6 (7.5)	64.5 (7.3)	73.6 (11.4)	62.8 (4.7)	67.0 (11.0)
	MOP-r mRNA	Figure 3, Panel A					
Nucleus Accumbens Shell (NAcS)	MOP-r mRNA	0.40 (0.03)	0.41 (0.06)	0.41 (0.06)	0.42 (0.03)	0.42 (0.02)	0.44 (0.04)
Caudate-Putamen (CP)	ppDyn mRNA	Figure 4					
	MOP-r mRNA	0.12 (0.01)	0.13 (0.01)	0.13 (0.01)	0.18 (0.03)	0.13 (0.01)	0.14 (0.02)
	DR2 mRNA	Figure 5					
Basolateral Amygdala (BLA)	MOP-r mRNA	Figure 3, Panel B					
Central Amygdala (CE)	ppDyn mRNA	6.31 (0.63)	5.21 (1.17)	7.14 (0.42)	6.57 (0.52)	7.02 (1.03)	5.38 (0.57)
	MOP-r mRNA	1.54 (0.10)	1.50 (0.09)	1.47 (0.14)	1.50 (0.13)	1.50 (0.06)	1.49 (0.08)
Ventral Tegmental Area (VTA)	MOP-r mRNA	0.37 (0.02)	0.40 (0.07)	0.42 (0.04)	0.39 (0.02)	0.38 (0.01)	0.40 (0.03)
Frontal Cortex (FC)	ppDyn mRNA	0.89 (0.07)	0.96 (0.07)	0.98 (0.06)	1.01 (0.07)	0.91 (0.06)	0.89 (0.15)
	ppEnk mRNA	32.5 (3.6)	31.7 (6.0)	35.9 (5.1)	24.9 (1.2)	30.7 (2.7)	31.8 (2.4)
	MOP-r mRNA	Figure 3, Panel C					