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Treatment-like steady-state methadone in rats interferes with incubation of cocaine sensitization and associated alterations in gene expression

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Abstract

In a previous study, steady-state methadone treatment was found to prevent associative cocaine learning, as well as related decreases in mRNA expression of preprohypocretin/preproorexin (ppHcr) in the lateral hypothalamus (LH) and dopamine D2 receptor (DR2) in the caudate-putamen (CP), and increases in mu-opioid receptor in the ventral striatum of rats. To investigate whether the same regimen of methadone exposure could prevent the incubation of cocaine sensitization and related alterations in gene expression, male Sprague–Dawley rats received 45 mg/kg/day steadydose “binge” cocaine administration (IP) for 14 days followed by mini-pumps releasing 30 mg/kg/day methadone (SC). After 14 days of methadone, and a subsequent 10-day drug-free period, all rats were tested for sensitization (cocaine test dose: 15 mg/kg) and brain tissue was collected to quantify mRNA expression. Rats exposed to cocaine displayed cocaine-induced stereotypy at test, as well as enhanced ppHcr mRNA in the LH and reduced DR2 mRNA in the CP. Importantly, these alterations were significantly reduced in rats treated with methadone following cocaine. These results suggest that steady-state methadone can interfere with the incubation of neuroadaptations underlying changes in behavioral responses to cocaine and cocaine-associated stimuli, and that these effects can be observed even after withdrawal from methadone.

Keywords

Cocaine; Methadone; Sensitization; Stereotypy; Gene expression; Preprohypocretin/preproorexin; Dopamine D2 receptor; Lateral hypothalamus; Striatum

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Contributors

Francesco Leri and Yan Zhou designed the study, wrote the protocol, managed the literature searches and analyses, undertook the statistical analysis, and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

All authors declare that they have no conflicts of interest.

1. Introduction

Methadone, a synthetic opioid with high affinity for the muopioid receptor (MOP-r) and low affinity for delta and kappa receptors, is a highly effective maintenance intervention for the treatment of heroin dependence (Kreek, 1973) and, at appropriate dosages, it has also been reported to significantly reduce cocaine abuse in heroin–cocaine poly-drug users (Borg et al., 1999; Peles et al., 2006).

Our laboratories have been conducting studies in animals to gain insight into the mechanisms underlying the purported beneficial effect of methadone on cocaine abuse. In rats, steady-state chronic methadone treatment (via subcutaneous osmotic mini-pumps) was found to reduce a variety of cocaine-motivated behaviors. For example, methadone attenuated cocaine-induced reinstatement of cocaine seeking, reduced cocaine self-administration on a progressiveratio schedule of reinforcement, and blocked cocaine place preference (Leri et al., 2006). These effects are probably attributable to methadone's direct agonistic action at MOP-r receptors, and consequent interactions with cocaine at neural sites controlling expression of responses to cocaine and cocaine-associated stimuli (Wise, 1989).

More recent evidence, however, suggested the possibility that reduction of cocaine seeking by methadone in rats may also be due to an interference with the development of cocaine-induced alterations (Hyman et al., 2006; Zweifel et al., 2008) in neural systems that control cocaine-directed responses. In fact, a study was performed in which there was no direct pharmacological overlap between the two drugs: chronic methadone was administered *after* a series of conditioning sessions during which intravenous infusions of cocaine were paired with an audiovisual cue. That is, methadone was administered during a period in which neurochemical alterations underlying “incubation” of drug seeking are known to develop (Lu et al., 2004; 2006; Li et al., 2008; Koya et al., 2009). At test, it was found that the cocaine-associated stimulus maintained lever-pressing behavior in vehicle but not methadone-treated rats. In addition, gene expression in particular regions of the brain was compared between treatment groups, and it was found that unlike control animals, methadone-treated rats did not show decreased levels of preprohypocretin/preproorexin (ppHcrt) mRNA in the lateral hypothalamus (LH), decreased dopamine D2 receptors (DR2) mRNA levels in the caudateputamen (CP), and increased levels of MOP-r mRNA in the ventral striatum and basolateral amygdala (Leri et al., 2009).

These actions of methadone on gene expression may have been central to its behavioral effect. In fact, ppHcrt in the LH (de Lecea et al., 1998; Sakurai et al., 1998), which is colocalized with preprodynorphin (ppDYN) (Chou et al., 2001) and MOP-r (Georgescu et al., 2003), is known to regulate motivated behaviors (Aston-Jones et al., 2010) including cocaine seeking (Boutrel et al., 2005). For example, systemic administration of the hypocretin 1 receptor antagonist SB-334867, reduced the motivation to self-administer cocaine and attenuated cocaine-induced enhancement of dopamine signaling (España et al., 2010). Furthermore, the DR2 gene in the dorsal striatum, which is co-localized with preproenkephalin (ppENK) and MOP-r (Ambrose et al., 2004), is involved in vulnerability to cocaine addiction and cocaine cravings (Volkow et al., 2007; Volkow et al., 2006). Finally, cocaine-induced alterations in MOP-r expression/function in striatal and limbic regions have been implicated in the development and maintenance of cocaine seeking (Gorelick et al., 2005; Kreek, 1996; Schroeder et al., 2003; Unterwald, 2001).

The present study was designed to evaluate behavioral and neurochemical interactions between steady-state methadone and a form of cocaine-induced non-associative learning: sensitization. Behavioral sensitization refers to the progressive augmentation of behavioral responses to psychomotor stimulants that develops as a result of their repeated

administration, and which persists even after long periods of withdrawal (Stewart and Badiani, 1993). Cocaine sensitization is partially dependent on functional glutamate NMDA receptors (Vanderschuren and Kalivas, 2000; Wolf, 1998), and its expression (both enhanced locomotion and rise in accumbal dopamine levels) was blocked by the partial MOP-r agonist buprenorphine (Placenza et al., 2008).

There are four novel aspects to the current study. First, it was designed to selectively investigate the effect of steady-state methadone on the incubation of cocaine sensitization. That is, as in the case of drug seeking, there is a cascade of neural adaptations that occur after cessation of drug administration and which ultimately lead to persistent drug hyperresponsiveness (Vanderschuren and Kalivas, 2000). Therefore, in the current experiment, methadone was administered postcocaine treatment, when the neural adaptations required for the expression of behavioral sensitization are believed to occur. Second, the test of behavioral responsiveness to a cocaine challenge, as well as collection of brain tissue, was performed after 10 days of withdrawal from methadone. Thus, animals were methadone-free when expression of sensitization was assessed. This design was selected to impart translational value to the study. In fact, at a clinical level, pharmacological treatments for drug addiction are likely administered after, and not prior or during, periods of chronic drug intake. Third, mRNA expression analysis focused on the *Hcrt* and the *DR2* genes, and co-localized genes including *ppDYN*, *ppENK*, and *MOP-r*. Alterations in expression of these genes have never been explored within the context of interaction between methadone and cocaine-induced sensitization. Finally, this study employed the steady-dose “binge” model of cocaine exposure to induce sensitization. This regimen was selected because it mimics the dosing pattern of human abusers who binge on cocaine over several hours (Koob and Kreek, 2007). In addition, the steady-dose “binge” administration of 45 mg/kg/day $\times$ 14 days has been found to sensitize rodents to the stimulatory effects of cocaine (Spangler et al., 1997), and to alter central expression of stress-related genes (Zhou et al., 2010) and *DR2* (Maggos et al., 1998).

2. Methods

2.1. Subjects

Adult male Sprague–Dawley rats (Charles River, St-Constant, QC) weighing 225–250 g at the beginning of the study were used as subjects. All rats were individually housed and maintained on a reverse light/dark cycle (8 AM lights off; 8 PM lights on) with free access to food and water except during testing. All procedures 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.

2.2. Apparatus

Twelve custom-made (University of Guelph, ON) activity chambers were used to measure horizontal and vertical locomotion activities. Each chamber (30 $\times$ 40 $\times$ 26 cm) was made of semitransparent Plexiglas and covered by black wire mesh to allow video tracking of the rats during testing (Ethovision, Noldus Information Technology, The Netherlands). A second video camera was used to record body movements, and hence allow assessment of stereotypical behavior (see below).

2.3. Surgery

Subcutaneous osmotic mini-pumps (Alzet model 2ML2, 0.5 μ l/h for 14 days, Durect Corporation, Cupertino, CA) were used for chronic delivery of methadone and vehicle. See Leri et al. (2009) for further surgical details.

2.4. Drugs, treatment regimens and doses

Cocaine HCL (Dumex, Toronto, ON) and methadone HCL (Pharmascience, Montreal, QC) were dissolved in physiological saline (i.e., vehicle). The dose of cocaine used for locomotion testing (15 mg/kg) was selected because it reliably increases locomotion activity in drug naive rats (Kalivas and Stewart, 1991). Thirty mg/kg/day methadone was used because it has been found to prevent the development of cocaine-induced conditioned reinforcement and related alterations of ppHcrt mRNA in the LH and DR2 mRNA in the CP (Leri et al., 2009). In rats, steady-state administration of 30 mg/kg/day methadone by osmotic pumps achieves a mean plasma level of about 450 ng/ml (Leri et al., 2009), which is comparable to levels achieved and sustained at 24 h after last administration during chronic methadone maintenance at high doses in humans (Kreek, 1996).

2.5. Procedure

Rats were allowed 7 days to habituate to the animal facility and were handled twice for approximately 10 min. Table 1 represents the experimental phases, groups, sample sizes, and drug treatments. The study consisted of six phases.

2.5.1. Phase 1—Habituation—On the first day of the experiment (i.e., Day 1), all rats received a vehicle injection and their activity was monitored for 1 h. This was performed to habituate the subjects to the injection procedure and to the testing apparatus.

2.5.2. Phase 2—Locomotion Test 1—In the AM of the day after habituation, all rats received an injection of vehicle followed by a “baseline” locomotion test (1 h). In the PM of the same day, rats received another test of locomotion (1 h) after an acute injection of cocaine (15 mg/kg), or vehicle.

2.5.3. Phase 3—Sensitization treatment—The day after Locomotion Test 1, rats were given 3 injections of cocaine (15 mg/kg each, total of 45 mg/kg/day), or vehicle, 1 h apart beginning at 09:30 AM, and this was repeated for 14 days. During the entire treatment period, there were no tests of locomotion, and animals were placed in their home cage immediately after each injection.

2.5.4. Phase 4—Mini-pump treatment—The day following the last cocaine injection, rats received 3 methadone injections (0.2, 0.4, and 0.8 mg/kg, SC) or vehicle, in order to build-up tolerance to the depressant effects of chronic methadone on respiratory functions. The following day, osmotic mini pumps releasing 30 mg/kg/day methadone, or vehicle, were surgically implanted. During the entire treatment period, there were no tests of locomotion.

2.5.5. Phase 5—Withdrawal—After 14 days of chronic methadone exposure, the pumps were removed and rats underwent a period of withdrawal from all drugs that lasted 10 days. Again, during the entire withdrawal period, there were no tests of locomotion.

2.5.6. Phase 6—Locomotion Test 2 and stereotypy—In the AM of the 11th day after removal of the mini-pumps, and 26th day after the last sensitizing injection of cocaine, all rats were given an injection of vehicle followed by a “baseline” locomotion test (1 h). In the PM of the same day, rats received another test of locomotion (1 h) after an acute injection of cocaine (15 mg/kg), or vehicle. During this second test, animals were also videotaped to score stereotypy. Scoring was performed by an observer blind to drug treatments, using a modified version of the Creese and Iversen (1974) and Daunais and McGinty (1995) scales. More specifically, because this steady-dose “binge” model of cocaine exposure has been previously found (Spangler et al., 1997; Schlussman et al., 2005)

to primarily increase “sniffing or rearing maintained in one location” (# 4 on Creese and Iversen's scale) and “intense sniffing in one location” (# 6 on Daunais and McGinty's scale), in the present study we only quantified the amount of time spent engaging in repetitive sniffing and/or high-frequency, low-amplitude, stationary head movements in one location of the locomotion chamber.

2.6. Experimental groups

The two groups central to this study (i.e., cCB/M_C and cCB/V_C) received cocaine on Locomotion Test 1, steady-dose cocaine “binge” (CB) to induce sensitization, chronic methadone (M; $n=15$) or vehicle (V; $n=13$), and cocaine prior to Locomotion Test 2 (see Table 1). These two groups were necessary to reveal a possible effect of chronic methadone on behavioral sensitization to acute cocaine challenges induced by steady-dose “binge” cocaine administration. In order to ascertain the development of behavioral sensitization, an additional group of animals received cocaine challenge injections on locomotion tests, but no sensitizing regimen of cocaine exposure and no methadone treatment (cVB/V_C ; $n=9$). Also, in order to ascertain whether chronic methadone produced sensitization to cocaine, a fourth group received the cocaine challenges on locomotion tests, as well as the methadone treatment, but no sensitizing regimen of cocaine exposure (cVB/M_C ; $n=9$). Finally, in order to interpret the results of the neurobiological assays, two additional control groups were included. The first group (Control) did not receive any drug: just vehicle injections and vehicle-filled pumps ($n=9$); the second group received the sensitizing regimen of cocaine exposure (vCB/V_V ; $n=12$), and vehicle on all other phase of the study. A group treated only with methadone was not included because 30 mg/kg/day for 14 days did not alter expression of genes quantified in our study (Leri et al., 2009). A sample size of 9 animals is typically appropriate in these experiments to generate enough power to detect significant group differences. But, because only 12 activity chambers were available for use, and because protocols that involve high cocaine and methadone doses can lead to loss of subjects (2 rats lost during the cocaine-binge treatment), this particular study was carried out in separate runs of 6 or 9 rats per run. If the subjects were not lost, their data were included in the appropriate group even if total n per group was larger than 9. This explains unequal sample size in this study.

2.7. Brain dissections and preparation of RNA extracts

Rats were sacrificed by decapitation after a brief exposure to CO_2 (within 15 s) within 1 h after Locomotion Test 2. Brain was removed from the skull and rapidly frozen with dry ice for storage at $-80^\circ C$. Subsequently, brains were placed in a chilled rat brain matrix (ASI Instruments, Houston, Texas), and coronal sections containing regions of interest were removed from the matrix and placed on a chilled Petri dish. Dissection was carried out using razor blades and forceps under a dissecting microscope. Brain regions of interest were identified according to the atlas of Paxinos and Watson (2005), as described in detail by Zhou et al. (2006). The lateral hypothalamus, caudateputamen and nucleus accumbens were dissected, homogenized in guanidinium thiocyanate buffer and extracted with acidic phenol and chloroform. After the final ethanol precipitation step, each extract was re-suspended in DEPC-treated H_2O and stored at $-80^\circ C$.

2.8. Solution hybridization ribonuclease (RNase) protection-trichloroacetic (TCA) precipitation assay

The mRNA levels of MOP-r, ppENK, ppDYN, ppHcrt 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., 2010). The solution hybridization RNase protection-TCA precipitation protocol has been described in detail in earlier reports (Zhou et al., 2006). Briefly, a 2100 bp fragment from the rat MOP-r

cDNA, a 935 bp fragment from the rat ppENK cDNA, a 1700 bp fragment from the rat ppDYN cDNA, and a 860-bp fragment from the rat DR2 cDNA were 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). The plasmid pS/E (a pSP65 derivative) was used to synthesize riboprobe for the 18S rRNA to determine total RNA. ³³P-labeled cRNA antisense probes and unlabeled cRNA sense standards were synthesized using a SP6, T3 or T7 transcription system. RNA extracts were re-suspended in 30 µl of 2× 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) that contained 150 K to 300 K cpm of a probe. Samples were hybridized overnight at 75 °C. For RNase treatment, 250 µl of a buffer containing 0.3 M NaCl; 5 mM EDTA; 10 mM Tris-HCl (pH 7.5), 40 µg/ml RNase A (Worthington, Biochemicals, Freehold, NJ) and 2 µg/ml RNase T1 (Calbiochem, San Diego, CA) was 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 using a cell harvester (Brandel, Gaithersburg, MD) and were measured in a scintillation counter with liquid scintillant (Beckman, Palo Alto, CA). The procedure to measure mRNA levels involved a comparison of values obtained from experimental samples (brain extracts) to those obtained for a set of calibration standards.

2.9. Statistical analyses

Because the focus of the study was on how locomotion changed at the various tests within each treatment group, separate two-way repeated measures ANOVAs (repeated factors: Test and Session time) were used to analyze locomotion activity in cVB/V_C , cCB/V_C , cVB/M_C and cCB/M_C groups. Activity measured on baseline vehicle tests given in the AM prior to PM cocaine Locomotion Tests 1 and 2 did not change significantly in any group, and therefore it was averaged to obtain a single “vehicle baseline” (see Fig. 1). One-way ANOVAs were used to compare stereotypy in cVB/V_C , cCB/V_C , cVB/M_C and cCB/M_C groups, as well as mRNA expression levels in all groups included in the study. Individual mRNA measurements deviating more than 2 standard deviations from the group mean were removed from the analyses. In case of significant interactions or significant main effects, multiple comparisons were performed using the Newman-Keul's method to identify individual mean differences ($\alpha = 0.05$). The specific values of negative findings are not reported. All statistical analyses were performed using Sigma-Stat (version 3.0 for Windows, SPSS Inc).

3. Results

3.1. Behavior

Locomotion activity in the four relevant groups is represented in Fig. 1 (main panels). The challenge dose of cocaine (15 mg/kg) elevated locomotion activity for nearly 1 h in rats that did not receive the sensitizing regimen of cocaine exposure (cVB/V_C panel; Test by Session time interaction [$F(22, 176) = 2.11$, $p < 0.01$]; main effect of Test [$F(2, 16) = 46.3$, $p < 0.001$] and Session time [$F(11, 88) = 21.18$, $p < 0.001$]) and, in this group, multiple comparisons revealed no significant differences between the two cocaine tests (p ranging between 0.07 and 0.178). More importantly, however, the second cocaine challenge induced a significant reduction in locomotion activity during the initial 40 min of the session in animals treated with steady-dose cocaine “binge” (cCB/V_C panel; Test by Session time interaction [$F(22, 264) = 4.18$, $p < 0.001$]; main effect of Test [$F(2, 24) = 23.19$, $p < 0.001$] and Session time [$F(11, 132) = 19.69$, $p < 0.001$]), but not in animals that received methadone following the cocaine treatment (cCB/M_C panel; main effect of Test [$F(2, 28) = 12.67$, $p < 0.001$] and

Session time [$F(11, 154)=22.64, p<0.001$]). This decrease in locomotion activity was probably due to the development of cocaine-induced stereotypy, which occurred during large part of Test 2 in the cCB/V_C group, but was significantly less persistent in the cCB/M_C group (inserts in Fig. 1; [$F(3, 42)=132.5, p<0.001$]). In other words, the cocaine binge treatment induced sensitization to the effect of cocaine on stereotypy, but this form of behavioral sensitization was much less pronounced in the group exposed to cocaine followed by methadone (inserts in Fig. 1: multiple comparisons revealed a significant difference between mean time in stereotypy displayed by the cCB/V_C and all other groups, as well as a significant difference between the cCB/M_C and the cVB/V_C groups; $p<0.05$). Finally, as it can be seen from the main panels of Fig. 1, chronic methadone exposure alone (cVB/M_C group) did not produce crosssensitization to cocaine (Test by Session time interaction [$F(22, 176)=3.24, p<0.001$]; main effect of Test [$F(2, 16)=22.48, p<0.001$] and Session time [$F(11, 88)=24.14, p<0.001$]).

3.2. Gene expression

The pattern of mRNA expression was affected by drug treatments, and differed across the genes studied. Fig. 2 represents expression of ppHcrt, MOP-r and ppDYN mRNAs in the LH. Only the ppHcrt gene differed across groups ($[F(5, 55)=3.56, p<0.01]$), and multiple comparisons indicated that ppHcrt mRNA was significantly elevated over control only in the cCB/V_C group. That is, rats challenged with cocaine that received binge cocaine followed by methadone (cCB/M_C) displayed unaltered expression and, similarly, expression in rats that received binge cocaine but vehicle at all other phases of the study (vCB/V_V) did not differ from control.

Fig. 3 represents expression of DR2 mRNA in the CP, as well as ppENK mRNA in the CP and NAc. Only the DR2 gene differed significantly across groups ($[F(5, 54)=6.13, p<0.001]$). In the case of this gene, however, multiple comparisons indicated a significant decrease in the groups that received binge cocaine and cocaine (cCB/V_C) or vehicle (vCB/V_V) on tests of locomotion. Again, rats that received binge cocaine followed by methadone displayed gene expression (cCB/M_C) that was within levels observed in control rats.

4. Discussion

This study generated three main findings. First, chronic methadone (30 mg/kg for 14 days) administered after cocaine “binge” treatment (45 mg/kg/day for 14 days) significantly attenuated hyper-sensitivity to the effects of an acute cocaine challenge (15 mg/kg) on locomotion and stereotypy. This result is notable because the cocaine and the methadone treatments did not pharmacologically overlap, and animals were tested after a 10-day period of withdrawal from methadone. Second, cocaine sensitized animals displayed elevated preprohypocretin/preproorexin mRNA expression in the lateral hypothalamus, as well as reduced mRNA expression of dopamine D2 receptors in the caudate-putamen. However, while the elevation of ppHcrt mRNA required a cocaine challenge (15 mg/kg) to be expressed, the reduction of DR2 mRNA did not. Expression of MOP-r, ppDYN and ppENK, genes measured because they are co-expressed with ppHcrt and DR2, did not differ between groups. Finally, alterations in both ppHcrt and DR2 genes were not observed in animals that received chronic methadone after the sensitizing regimen of cocaine exposure.

The present effect of methadone on cocaine sensitization is parallel to the effect of buprenorphine reported by Placenza et al. (2008). In fact, although our animals became more sensitive to cocaine-induced stereotypy rather than to cocaine-induced locomotor stimulation, chronic treatment with both drugs after cocaine administration reduced sensitization. But, in the current study, rats were tested methadone-free, and therefore reduced sensitivity to cocaine-induced stereotypy could not have been the result of direct

MOP-r modulation of neurochemical systems (Leri et al., 2007; Placenza et al., 2008; Sorge and Stewart, 2006) involved in the expression of sensitization (Vanderschuren and Kalivas, 2000). Rather, our current finding suggests that chronic methadone interfered with the development of neuro-adaptations (Kauer and Malenka, 2007; Nestler, 2001, 2002) underlying incubation of cocaine sensitization (Stewart and Badiani, 1993; Thomas et al., 2008). In fact, the mesolimbic dopamine system is critical to the initiation and incubation of behavioral sensitization (Kalivas and Stewart, 1991), and endogenous opioids are known to modulate dopaminergic activity and synaptic plasticity at several levels within this system (Nugent et al., 2007; Spanagel, 1995). Furthermore, methadone is a non-competitive NMDA antagonist (Ebert et al., 1998; Gorman et al., 1997), and NMDA receptors within the mesocorticolimbic dopamine system are known to change during the incubation of cocaine sensitization (Li et al., 1997).

Hypocretin-1/orexin A and hypocretin-2/orexin B are two neuropeptides that have been implicated in arousal, feeding, and more recently, addiction (Aston-Jones et al., 2010; Boutrel and de Lecea, 2008). They are synthesized in neurons in LH, and project widely through the brain (Peyron et al., 1998; Sakurai et al., 1998), including to the ventral tegmental area (VTA) (Balcita-Pedicino and Sesack, 2007). It is through the VTA that these peptides are believed to modulate the activity of the mesolimbic dopamine system (Narita et al., 2006; Vittoz and Berridge, 2006), and consequently alter cellular and behavioral responses to drugs of abuse, including cocaine (España et al., 2010; 2011). For example, the orexin 1 receptor antagonist SB 334867 blocked cocaine-induced synaptic plasticity in the VTA and prevented locomotor sensitization (Borgland et al., 2006). Pilot studies conducted in our laboratories found that LH ppHcrt mRNA expression was unaltered immediately after acute (2.5 h) “binge” cocaine (45 mg/kg), and immediately or after 1 day of withdrawal from 14 days of chronic steadydose (45 mg/kg/day) cocaine (data not shown). Consistent with these observations, the present study indicated that basal ppHcrt mRNA expression was unaltered 24 days after withdrawal from cocaine (vCB/V_V group). However, a significant up-regulation of ppHcrt mRNA expression in the LH was noted in the sensitized rats that were challenged with cocaine about 2 h earlier (cCB/V_C group) before tissue collection (see Fig. 2).

But, although these data suggest that the Hcrt gene may have been “sensitized” by cocaine exposure, a direct link to behavioral sensitization does not appear likely. In fact, treatments that induce locomotor sensitization can also increase the motivational properties of psychostimulants (Vezina, 2004), but elevations of central hypocretin-1 levels lead to decreased sensitivity of brain reward systems (Boutrel et al., 2005). Furthermore, Hcrt has been found to mediate morphine place preference, but not morphine-induced hyperactivity and sensitization (Sharf et al., 2010). Finally, the Hcrt gene in the LH appears hypo-active during responses to cocaine-associated stimuli because mRNA expression was decreased after expression of cocaine place preference (Zhou et al., 2008), and after cocaine-primed reinstatement of responding for a cocaine-conditioned stimulus (Leri et al., 2009). Therefore, from the results of the current study, it is concluded that a sensitizing regimen of cocaine administration increased the responsiveness of hypothalamic Hcrt gene expression to acute administration of cocaine, but this may not be the critical mechanism underlying behavioral hypersensitivity to the stimulatory, and possibly motivational, effects of the drug.

On the other hand, mRNA alterations of the DR2 gene in the CP appear more directly related to cocaine sensitization and cocaine seeking. In fact, decreased DR2 mRNA expression was observed 24 days after the last injection of the cocaine treatment, and this effect was not modulated by a cocaine challenge (compare cCB/V_C and vCB/V_V groups in Fig. 3). These data replicate the findings of our previous study of cocaine conditioned reinforcement (Leri et al., 2009), and the findings of a pilot study suggesting that basal CP

DR2 mRNA expression was decreased 1 day after 14 days of steady-dose chronic cocaine administration (data not shown). The functional significance of these changes is supported by other studies. In fact, cocaine induces longlasting cellular adaptation in striatal DA receptors (Gifford and Johnson, 1992; King et al., 1994) including reduction of DR2 availability/function (Nader and Czoty, 2005; Nader et al., 2006; Tsukada et al., 1996), and Thompson et al. (2010) demonstrated that degradation of striatal DR2 in mice is a significant contributing factor to cocaine-induced behavioral sensitization (increased locomotion). Furthermore, in DR2 knockout mice, acute cocaine caused an exaggerated increase of striatal extracellular DA levels, probably due to loss of DR2-mediated presynaptic autoreceptor function (Rougé-Pont et al., 2002). These mice display an increase of stereotypy with a reduction of locomotion (Welter et al., 2007), similar to those observed in cocaine-withdrawn rats following acute cocaine. Finally, there is substantial evidence suggesting that down-regulation of striatal DR2 maybe one neural marker common to addiction-like behaviors in rodents (Dalley et al., 2007; Johnson and Kenny, 2010; Nader and Czoty, 2005; Nader et al., 2006; Parada et al., 1995; Tsukada et al., 1996; Volkow et al., 2002; Volkow et al., 1993).

Although interesting and novel, the results of the current study should be interpreted with caution because of at least four reasons. First, no methadone dose–response was performed to ascertain the pharmacological profile of the effect obtained in this study. However, we previously tested a range of methadone doses (20–55 mg/kg) on a number of other cocaine-motivated behaviors in rats (Leri et al., 2006, 2007, 2009, 2004) and found significant inhibition of cocaine CPP expression, cocaine self-administration on a progressive ratio schedule, cocaine operant conditioned reinforcement, and cocaine reinstatement, only at doses equal or higher than 30 mg/kg/day. Second, we did not measure peptide or protein levels, and thus our findings are limited to gene expression for biosynthesis. Third, it may not be appropriate to infer causal relationships between changes in gene expression and changes in behavior from this study because animals were sacrificed 60 min after behavioral testing. Finally, and related to the previous point, there may be other genes that were not measured which may also be involved in the effect of methadone on cocaine sensitization.

It is not completely clear how these findings in rats speak to effectiveness of methadone maintenance in curbing heroin–cocaine co-addiction, as clinical studies on this subject have yielded variable findings (Borg et al., 1999; Bux et al., 1995; Duntzman et al., 1992; Foltin et al., 1995; Foltin and Fischman, 1996; Kosten et al., 1986; Maremmani et al., 2007; Peles et al., 2006). Nevertheless, this study in rats demonstrates that exposure to methadone reduced cocaine sensitization, as well as associated cocaine-induced alterations of ppHcrt mRNA in the LH and DR2 mRNA in the CP. Importantly, these effects are attributable to an interference with the incubation, but not expression, of cocaine-induced sensitization because methadone was administered after the period of cocaine administration, and rats were tested methadone-free. We propose that additional work should be carried out to explore the molecular mechanisms mediating these effects of methadone because, to our knowledge, this is the first evidence of reduction of cocaine-induced sensitization and associated 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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Fig. 1.

Main panels: Mean (sem) horizontal and vertical distance (cm) traveled in 1 h on tests of locomotion induced by injections of: single dose of 15 mg/kg cocaine immediately before Locomotion Test 1 on Day 1 for each panel; single dose of 15 mg/kg cocaine immediately before Locomotion Test 2 on Day 42 for each panel; and single dose of vehicle (saline) on the baseline Locomotion Tests 1 and 2 (average) for each panel. A—The c_{VB}/V_c group ($n = 9$) did not receive the sensitizing regimen of cocaine exposure. B—The c_{CB}/V_c group ($n = 13$) received steady-dose cocaine “binge” (45 mg/kg/day×14days) treatment to induce sensitization. C—The c_{VB}/M_c group ($n = 9$) received steady-state 30 mg/kg/day×14 days methadone via SC mini-pump. D—Finally, the c_{CB}/M_c group ($n = 15$) received the

sensitizing regimen of cocaine exposure followed by the methadone treatment. The * represents a significant difference (<0.05) between tests 1 and 2. Inserts: Mean (sem) total time (min) spent engaging in repetitive sniffing and/or high-frequency, low-amplitude, head movements in one location of the locomotion chamber on Test 2. The * represents a significant difference (<0.05) between the cCB/Vc and all other groups. The # represents a significant difference (<0.05) between the cCB/M_C and cVB/V_C groups.

Fig. 2.

Mean (sem) levels of preprohypocretin (ppHcrtr), mu opioid receptor (MOP-r) and preprodynorphin (ppDYN) mRNAs in the lateral hypothalamus. The horizontal line and grayed area represent mean (sem) levels measured in drug-naïve rats (vVB/Vv group; n=9). See Table 1 for description of drug treatments received by the different groups. The * indicates a significant difference from vVB/Vv group.

Fig. 3.

Mean (sem) levels of dopamine D2 receptor (DR2), preproenkephalin (ppEnk) mRNAs in the caudate-putamen (CP) and nucleus accumbens (NAc). The horizontal line and grayed area represent mean (sem) levels measured in drug-naïve rats (vVB/Vv group; n=9). See Table 1 for description of drug treatments received by the different groups. The * indicates a significant difference from vVB/Vv group.

Table 1

Groups, sample sizes, experimental phases and corresponding drug treatments. The “Fig. 1” column specifies the panel (A–D) in which the behavior (locomotion and stereotypy) of the group is represented. The sequence of letters used to identify groups (i.e., XX/XX) should be read as follows: v=vehicle injection prior to PMTest 1 of locomotion or c = cocaine injection prior to PM Test 1 of locomotion; VB=vehicle binge treatment or CB = cocaine binge treatment; V = vehicle pumps or M=methadone pumps; v=vehicle injection prior to PM Test 2 of locomotion or c = cocaine injection prior to PM Test 2 of locomotion.

Group	Fig. 1	n	Phase 1		Phase 2		Phase 3		Phase 4		Phase 5		Phase 6	
			Habituation		Locomotion Test 1		Sensitization treatment		Mini-pump treatment		Withdrawal		Locomotion Test 2 and stereotypy	
			Day 1	Day 2 AM	Day 2 AM	PM	Days 3 to 16	Days 17 to 31	Days 32 to 41	Day 42 AM	PM			
cVB/V _c	Panel A	9	Vehicle only	Vehicle	Vehicle	Cocaine	Vehicle	Vehicle	No injections	Vehicle	Cocaine	Cocaine		
cCB/V _c	Panel B	13				Cocaine	Cocaine	Vehicle			Cocaine	Cocaine		
cVB/M _c	Panel C	9				Cocaine	Vehicle	Methadone			Cocaine	Cocaine		
cCB/M _c	Panel D	15				Cocaine	Cocaine	Methadone			Cocaine	Cocaine		
Control		9				Vehicle	Vehicle	Vehicle			Vehicle	Vehicle		
vCB/V _v		12				Vehicle	Cocaine	Vehicle			Vehicle	Vehicle		