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Stimulation of Medial Prefrontal Cortex Serotonin 2C (5-HT_{2C}) Receptors Attenuates Cocaine-Seeking Behavior

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Serotonin 2C receptor (5-HT_{2C}R) agonists administered systemically attenuate both cocaine-primed and cue-elicited reinstatement of extinguished cocaine-seeking behavior. To further elucidate the function of these receptors in addiction-like processes, this study examined the effects of microinfusing the 5-HT_{2C}R agonist MK212 (0, 10, 30, 100 ng/side/0.2 μl) into the medial prefrontal cortex (mPFC) on cocaine self-administration and reinstatement of extinguished cocaine-seeking behavior. Male Sprague–Dawley rats were trained to self-administer cocaine (0.75 mg/kg, i.v.) paired with light and tone cues. Once responding stabilized, rats received MK212 microinfusions before tests for maintenance of cocaine self-administration. Next, extinction training to reduce cocaine-seeking behavior, defined as responses performed without cocaine reinforcement available, occurred until low extinction baselines were achieved. Rats then received MK212 microinfusions before tests for reinstatement of extinguished cocaine-seeking behavior elicited by cocaine-priming injections (10 mg/kg, i.p.) or response-contingent presentations of the cocaine-associated cues; operant responses during cocaine-primed reinstatement tests produced no consequences. MK212 microinfusions into the prelimbic and infralimbic, but not anterior cingulate, regions of the mPFC dose-dependently attenuated both cocaine-primed and cue-elicited reinstatement of extinguished cocaine-seeking behavior, but did not reliably affect cocaine self-administration. A subsequent experiment showed that the effects of MK212 (100 ng/side/0.2 μl) on reinstatement of extinguished cocaine-seeking behavior were blocked by co-administration of the 5-HT_{2C}R antagonist SB242084 (200 ng/side/0.2 μl). MK212 administered alone into the mPFC as a drug prime produced no discernable effects on cocaine-seeking behavior. These findings suggest that stimulation of 5-HT_{2C}Rs in the mPFC attenuates the incentive motivational effects produced by sampling cocaine or exposure to drug-paired cues.
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INTRODUCTION

A major difficulty in treating cocaine dependence is the high incidence of relapse (O'Brien, 2005; Wallace, 1992), which may occur even after prolonged periods of abstinence. Factors contributing to relapse include incentive motivational effects produced by either sampling cocaine or exposure to drug-related cues (Davis and Smith, 1976; de Wit and Stewart, 1981). Incentive motivation for cocaine is measured in animals using the extinction/reinstatement model (de Wit and Stewart, 1981), whereby animals are initially trained to perform an operant response reinforced with cocaine, followed by extinction training during which responses produce no consequences. Responding in the absence of drug reinforcement is referred to as cocaine-seeking behavior and provides a measure of incentive motivation for cocaine. Measuring reinstatement of extinguished cocaine-seeking behavior elicited by cocaine or cocaine-associated cues provides an animal model to study the mechanisms involved in the incentive motivational effects of these stimuli (Epstein *et al*, 2006).

Serotonin (5-HT) systems are critical in mediating the incentive motivational effects of cocaine and cocaine-paired cues in both animals and humans (Bubar and Cunningham, 2008; Hyman *et al*, 2006; Rothman *et al*, 2008). In humans, acute 5-HT depletion reduces self-reports of craving elicited by cocaine-associated cues (Satel *et al*, 1995) and reduces euphoric effects of intranasal cocaine (Aronson *et al*, 1995). However, increasing 5-HT with the reuptake inhibitor fluoxetine also reduces cocaine-induced positive subjective effects and craving (Batki *et al*, 1993; Walsh *et al*, 1994), although others report an increase in 'likely to use cocaine' (Harris *et al*, 2004). In rats, 5-HT depletion or elevation with

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indirect agonists attenuates reinstatement of cocaine-seeking behavior after exposure to cocaine-paired cues (Tran-Nguyen *et al*, 1999, 2001). In contrast, indirect agonists fail to reliably alter effects of cocaine priming (Baker *et al*, 2001; Burmeister *et al*, 2003).

The inconsistency of effects elictied by cocaine priming is likely related to the complexity of 5-HT systems. There are 14 different 5-HT receptor (5-HTR) subtypes that have diverse effects (Barnes and Sharp, 1999) and are differentially regulated (Cunningham *et al*, 1992; Neumaier *et al*, 2002; Rocha *et al*, 1993) and/or affected functionally (Baumann and Rothman, 1995, 1996, 1998; Darmani *et al*, 1992; King *et al*, 1993; Lee and Meltzer, 1994; Levy *et al*, 1992, 1994) by chronic cocaine, other indirect agonists, or 5-HT depletion. Moreover, 5-HT may produce similar or opposite effects through stimulation of different 5-HTRs. For example, actions at 5-HT$_{2A}$ and 5-HT$_{2C}$Rs have opposite effects on cocaine-seeking behavior. Agonist stimulation of 5-HT$_{2C}$Rs decreases cue- and cocaine-primed reinstatement of extinguished cocaine-seeking behavior (Burbassi and Cervo, 2008; Fletcher *et al*, 2008; Grottick *et al*, 2000; Neisewander and Acosta, 2007), whereas blocking 5-HT$_{2A}$Rs with selective antagonists decreases cue- (Burmeister *et al*, 2004; Filip, 2005; Nic Dhonnchadha *et al*, 2009) and cocaine-primed reinstatement (Filip, 2005; Fletcher *et al*, 2002a).

Little is known about the neural circuitry underlying the inhibitory effects of 5-HT$_{2C}$R agonists on reinstatement of extinguished cocaine-seeking behavior. The medial prefrontal cortex (mPFC) is likely involved given that it is densely populated with 5-HT$_{2C}$R mRNA and protein (Liu *et al*, 2007; López-Giménez *et al*, 1997; Pompeiano *et al*, 1994) and the neural circuitries mediating the incentive motivational effects of cocaine priming *vs* cues converge in the mPFC (Neisewander *et al*, 2000; Peters *et al*, 2009; Robbins and Everitt, 2002; Weiss, 2005). Furthermore, 5-HT$_{2C}$Rs in the mPFC mediate other stimulant-induced behavior. For instance, the 5-HT$_{2C}$R agonist MK212 microinfused into the mPFC inhibits both cocaine-induced hyperactivity and discriminative-stimulus effects without altering spontaneous locomotion (Filip and Cunningham, 2003) and decreases MDMA-induced behavioral sensitization (Ramos *et al*, 2005).

The mPFC is anatomically and functionally heterogeneous. The ventromedial (vm) PFC, consisting of the ventral prelimbic (PrL) and infralimbic (IL) cortices, is preferentially connected with the nucleus accumbens shell (NAcsh) and core (NAcc), amygdala, and ventral tegmental area (VTA), whereas the dorsomedial PFC, consisting of the dorsal PrL and anterior cingulate cortices, is predominantly linked with the NAcc (Ding *et al*, 2001; Gabbott *et al*, 1997, 2005; McFarland and Kalivas, 2001; Zahm and Brog, 1992). Inactivation of the PrL, but not IL, cortex reduces context-, stress-, cue-, and cocaine-primed reinstatement of extinguished cocaine-seeking behavior (Capriles *et al*, 2003; Fuchs *et al*, 2005; McFarland and Kalivas, 2001; McLaughlin and See, 2003; Peters *et al*, 2009). However, lesions of either the PrL (Zavala *et al*, 2003) or IL (Tzschentke and Schmidt, 1999) cortices reduce cocaine-conditioned place preference and behavioral sensitization to cocaine. The mPFC also has a function in cocaine reinforcement as animals will self-administer cocaine directly into this region (Goeders and Smith, 1984).

This study investigated whether 5-HT$_{2C}$Rs located within the mPFC inhibit cue- and cocaine-primed reinstatement of extinguished cocaine-seeking behavior by microinfusing MK212 into the PrL and IL cortices before testing. Additional tests examined the behavioral (effects of intra-mPFC MK212 microinfusions on maintenance of cocaine self-administration), pharmacological (effects of co-infusion with the 5-HT$_{2C}$R antagonist SB242084), and anatomical (effects of anterior cingulate cortex MK212 microinfusions) specificity of the MK212 effects.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats ($n = 77$) weighing 275–325 g at the time of surgery were individually housed under a 12-h reversed light/dark cycle. All husbandry and experimentation adhered to the Guide for the Care and Use of Laboratory Animals (1996).

Surgery

Catheters were implanted into jugular veins under isoflurane (2–3%) anesthesia as detailed previously (Pentkowski *et al*, 2009). Next, each rat was placed into a stereotaxic instrument, the scalp was incised and retracted, and the head was positioned with Bregma and Lambda at the same DV coordinate. Holes were drilled into the skull to bilaterally insert steel guide cannulae (26G, Plastics One, Roanoke, VA) into the mPFC using the following coordinates derived from previous research (Filip and Cunningham, 2003): +2.10 (Cg2) or +2.70 (PrL, IL) mm anterior to bregma, ±0.75 mm from the midline, and −3.00 mm ventral from the surface of the skull (Paxinos and Watson, 2007). The guide cannulae and metal end of the catheter were secured to the skull and anchor screws using dental acrylic. Stylets (30G, Plastics One) were placed into the guide cannulae to maintain patency. Rats were returned to their home cages for 5–6 days recovery. Catheters were flushed daily with 0.1 ml saline containing heparin sodium (70 U/ml; APP Pharmaceuticals, Schaumburg, IL), Abbokinase (20 mg/ml; ImaRx Therapeutics, Tucson, AZ), and Timentin (66.7 mg/ml; GlaxoSmithKline, Research Triangle Park, NC) to maintain patency. Proper catheter function was tested periodically by administering 0.05 ml methohexital sodium (16.7 mg/ml; JHP Pharmaceuticals, Rochester, MI), a dose that produces brief anesthetic effects only when administered i.v.

Drugs

Cocaine hydrochloride (RTI International, Research Triangle Park, NC) dissolved in saline was filtered through 0.2 μm membranes. MK212 and SB242084 (Tocris Cookson, Ellisville, MO) were dissolved in artificial cerebrospinal fluid (aCSF) and adjusted to a pH of 7.4. Injections were administered i.p. at a volume of 1 ml/kg or intracranially at a volume of 0.2 μl/side. Microinfusions were delivered over a 1-min period using bilateral injection cannulae (30G, Plastics One) connected through polyethylene 50 tubing (Becton Dickinson, Sparks, MD) to 25-μl syringes (Hamilton,

Reno, NV) mounted in an infusion pump (CMA Microdialysis, North Chelmsford, MA). Injection cannulae extended exactly 1 (Cg2, PrL) or 2 (IL) mm below guide cannulae tips. Movement of an air bubble the correct distance in the injection tubing confirmed accurate infusion volume. Injection cannulae remained in place for 1 min before removal, and then the stylets were replaced.

Experimental Phases

An outline of each experimental phase and a timeline for the first experiment is shown in Figure 1. Each phase is detailed below.

Self-Administration Training

Self-administration training occurred in operant conditioning chambers (30 × 25 × 25 cm; Med Associates, St Albans, VT) equipped with active and inactive levers, a cue light 4 cm above the active lever, a tone generator (500 Hz, 10 dB above background noise), and a house light on the top center of the wall opposite the levers. Each chamber was housed within a larger ventilated sound-attenuating chamber. Infusion pumps (Med Associates) were connected to liquid swivels (Instech, Plymouth Meeting, PA) located above the chambers. Swivels were fastened to catheters through polyethylene 50 tubing encased inside metal spring leashes (Plastics One).

Rats were trained to self-administer cocaine (0.75 mg/kg/0.1 ml, i.v.) 6 days/week during 2-h sessions. Schedule completions on the active lever resulted in the simultaneous activation of the cue light and tone generator followed 1 s later by a 6-s cocaine infusion. The light and tone cues were inactivated with the termination of the infusion and the house light switched on for 20 s to signal a timeout period, during which lever presses were recorded, but produced no consequences. Inactive lever presses were recorded, but produced no consequences. To facilitate acquisition of cocaine self-administration, rats were restricted to 16 g of food/day beginning 2 days before training and then maintained on food restriction (16–22 g/day) until they progressed from a fixed ratio (FR) 1 to a variable ratio (VR) 5 schedule of reinforcement. We chose a VR5 schedule of reinforcement because this schedule produces relatively high reinstatement response rates, thereby increasing sensitivity to detect MK212-induced decreases in reinstatement of extinguished cocaine-seeking behavior (Acosta *et al*, 2008). After reaching criterion for 5 consecutive days on a VR5 schedule, rats were given *ad libitum* access to food throughout the rest of training (ie at least 5 more sessions) and testing.

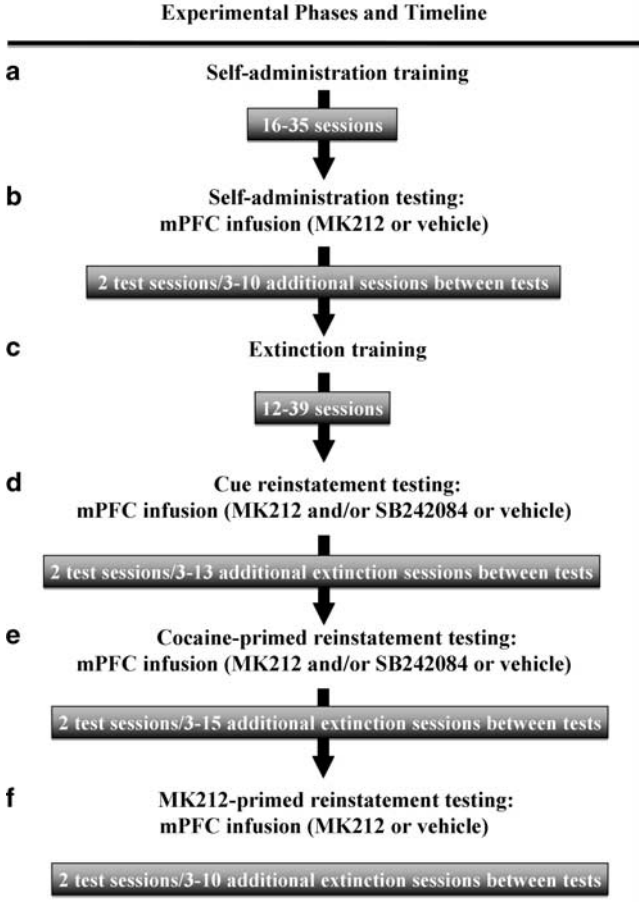

Figure 1 Timeline depicting the order of experimental stages for rats included in the MK212 and MK212 + SB242084 experiments: (a) self-administration training; (b) effects of MK212 on maintenance of cocaine self-administration (0.75 mg/kg/0.1 ml, i.v.); (c) extinction training; (d) effects of MK212 or MK212 + SB242084 on cue-elicited reinstatement; (e) effects of MK212 or MK212 + SB242084 on cocaine-primed reinstatement; and (f) effects of MK212 priming on reinstatement. The sequence of stages (d), (e), and (f) were counterbalanced for order of presentation to control for order effects. The specific number of sessions at each phase varied depending on individual performance. Across all testing phases, rats received a maximum of eight intracranial microinfusions.

Effects of MK212 on Self-Administration

After reaching a stability criterion of $<10\%$ variability in the number of cocaine infusions/session across three consecutive sessions on the VR5 schedule, without upward or downward trends, rats were assigned to MK212 dosage groups (10, 30, 100 ng/0.2 μl/side) counterbalanced for previous cocaine intake ($n = 6$–10/group). They were tested for the effects of MK212 on self-administration of cocaine (0.75 mg/kg, i.v.), receiving an intracranial vehicle (aCSF) microinfusion before one test and their assigned MK212 dose before the other test, with order of these treatments counterbalanced. The test sessions began 1–2 min after the microinfusions, and at least three training sessions intervened test days to re-establish stable self-administration rates.

Extinction Training

Extinction training began the day after the last self-administration session and consisted of daily 1-h exposures to the self-administration environment. During extinction, rats were connected to the tethers, and active and inactive lever responses were recorded, but produced no consequences (ie the cocaine infusion pump was not activated, and the discrete light and tone cues were not presented). Responding on the active lever in the absence of cocaine reinforcement is the operational definition of cocaine-seeking behavior. Extinction training continued until response rates

on the active lever declined to 20% of the highest rate observed during extinction or to <20 responses per hour. Once responses declined to criterion levels, cocaine-seeking behavior was considered to be extinguished.

Effects of MK212 on Reinstatement

After reaching the extinction criterion, rats were re-assigned to MK212 dosage groups (10, 30, 100 ng/0.2 μl/side) counterbalanced for earlier cocaine intake ($n = 6$–8/group). Rats received the same assigned dose of MK212 throughout the reinstatement test phase. There were a total of six, 1-h reinstatement tests. The first two tests assessed MK212 effects on extinction, and the other four tests included two cue-elicited and two cocaine-primed reinstatement tests, with the order of test type counterbalanced. For each test type, rats received an intracranial microinfusion of vehicle (aCSF) before one test and their assigned dose of MK212 before the other test, with order of these treatments counterbalanced. Rats were connected to the tethers 1–2 min after the microinfusions. At least three extinction sessions intervened between test days to re-establish baseline response rates. For MK212-primed and cocaine-primed reinstatement tests, responses on the levers produced no consequences (ie no cues were presented). For cocaine-primed reinstatement tests, rats received cocaine (10 mg/kg, i.p.) primes immediately before placement into the self-administration chambers. To control for injection stress, rats were given i.p. saline injections immediately before the two extinction sessions preceding cocaine-primed reinstatement testing, and response rates during these sessions served as the extinction baseline. During cue-induced reinstatement tests, active lever responses on an FR1 schedule resulted in presentations of the stimulus complex previously paired with cocaine infusions (ie, light and tone cues, infusion pump). If a rat did not respond within the first 5 min, a non-contingent cue was presented.

Effects of SB242084 on MK212-Induced Decreases in Reinstatement

A separate cohort of rats was used to examine whether the 5-HT$_{2C}$R antagonist SB242084 blocked the effects of MK212 on reinstatement. Rats were assigned to one of two groups counterbalanced for cocaine intake during self-administration: MK212/SB242084 or vehicle/SB242084 ($n = 8$–10/group). Rats were tested twice for cue-induced reinstatement and twice for cocaine-primed reinstatement, with order of test type counterbalanced. For each test type, rats in the MK212/SB242084 group were pretreated with MK212 (100 ng/0.2 μl/side) before one test and MK212 (100 ng/0.1 μl/side) + SB242084 (200 ng/0.1 μl/side) before the other test; rats in the vehicle/SB242084 group were pretreated with vehicle before one test and SB242084 (200 ng/0.2 μl/side) before the other test. Order of respective pretreatments was counterbalanced. All other aspects of training and testing were identical to the previous experiment.

Histology

Rats were killed with sodium pentobarbital (100 mg/kg, i.p.) and then 0.2 μl/side of 1% methylene blue was infused intracranially to verify cannulae tip placements. Placements were determined from coronal sections (40 μm) under a microscope by an observer blind to experimental conditions. Sections were then thionin stained to assess possible neurotoxicity.

Statistical Analyses

Infusion and response rates were analyzed using separate ANOVAs with dosage group as a between-subjects variable, when appropriate, and test session (baseline, vehicle pretreatment, and MK212 and/or SB242084 pretreatment) as the repeated-measures factor. *Post hoc* Newman–Keuls tests provided subsequent pairwise comparisons. Baseline response rates for these measures were defined as the average response rate during the self-administration or extinction sessions that preceded vehicle and drug pretreatment tests. Reinstatement for both the cue- and cocaine-primed tests was operationally defined as a minimum of 10 active lever responses and at least a doubling of baseline response rate during either the vehicle (aCSF) or drug (MK212 and/or SB242084) pretreatment test session; animals that failed to meet these criteria on both test days were excluded from the analyses as 'non-reinstaters.'

RESULTS

Histology

Figure 2 presents serial histological reconstructions and representative photomicrographs of injector tip placements within the subregions of the mPFC for rats included in the analyses. Three rats were excluded due to placements outside the mPFC and five were eliminated because of catheter failure. Microscopic examination of thionin-stained coronal sections indicated a lack of tissue damage aside from typical amounts sustained from cannulae implantation. Neurotoxicity, indicated by neuronal cell loss filled with gliosis, was not observed. Furthermore, the stable and consistent levels of reinstatement elicited by cocaine-priming injections and exposure to cocaine-paired cues (see below) after intracranial vehicle microinfusions are consistent with a lack of neurotoxicity.

Self-Administration Training

Depending on individual performance, rats tested for the effects of MK212 on cocaine reinforcement and reinstatement of extinguished cocaine-seeking behavior received a minimum of 16–35 self-administration sessions before testing and a maximum of 22–42 total sessions, including those that occurred during the training and testing phases; rats in the antagonist experiment received 26 training sessions. Total cocaine intake and the average number of infusions across the last 5 days of VR5 self-administration training did not differ across groups in either experiment, nor was there a difference in response rates (Table 1).

Extinction Training

Depending on individual performance, rats tested for the effects of MK212 and/or SB242084 on reinstatement of

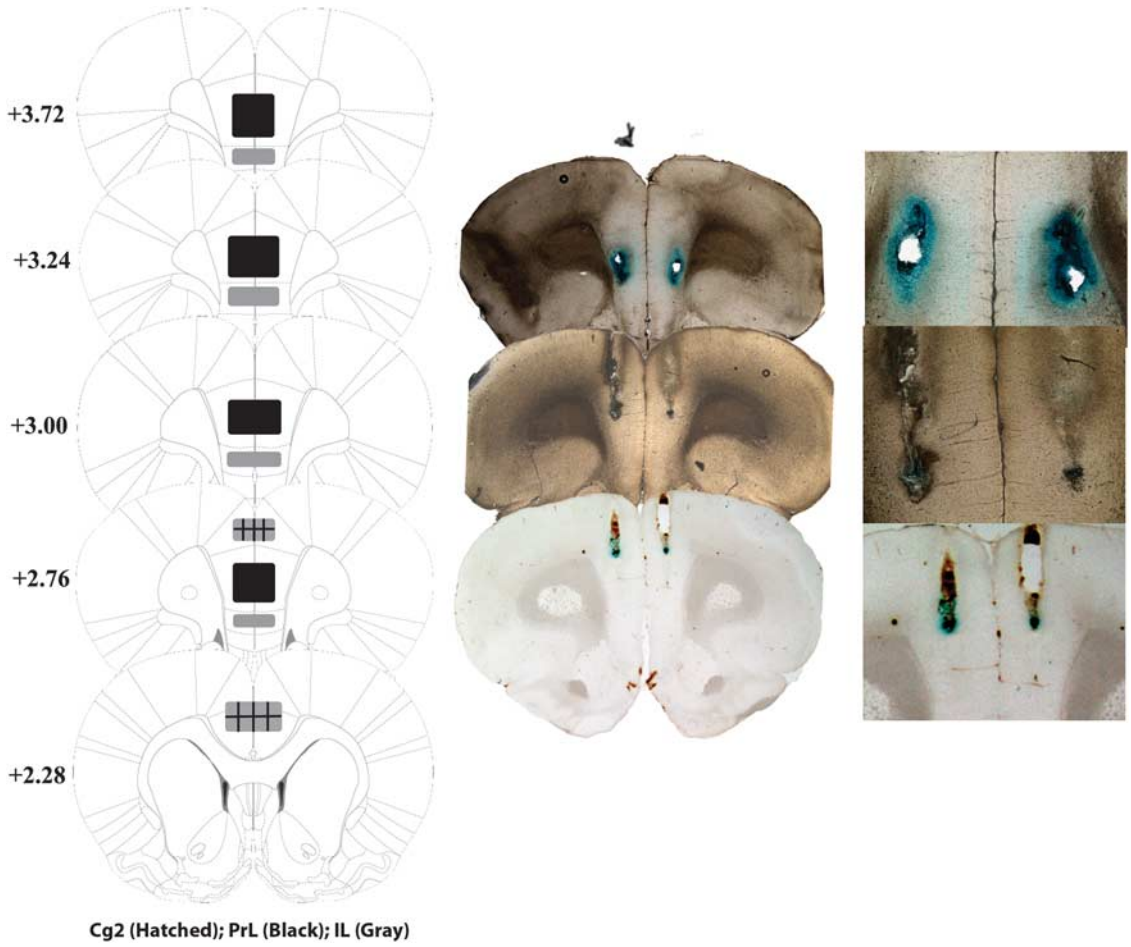

Figure 2 Histological reconstructions (left) presenting injector tip placements within the PrL (black), IL (gray), and Cg2 (hatched) subregions of the mPFC of rats included in the analysis; schematic representations (Paxinos and Watson, 2007) were used with permission from Elsevier. Representative photomicrographs showing methylene blue microinfusions for each mPFC subregion taken at magnifications of × 1 (middle) and × 10 (right).

Table 1 Cocaine Reinforcers and Response Rates (Mean ± SEM) During Self-Administration (SA) and Extinction

Brain region/drug	Infusions/session (last 5 days)	Total infusions	Active lever presses/hour	
			Last SA day	First extinction day
PrL				
10 ng/side MK212 ($n=7$)	26.11 ± 0.82	642.86 ± 11.29	91.57 ± 14.74	135.14 ± 34.17
30 ng/side MK212 ($n=8$)	26.43 ± 0.94	602.00 ± 38.09	81.56 ± 12.92	82.88 ± 10.12
100 ng/side MK212 ($n=7$)	27.17 ± 1.59	718.57 ± 62.86	78.29 ± 13.76	109.71 ± 15.68
IL				
10 ng/side MK212 ($n=6$)	23.70 ± 1.24	663.33 ± 76.27	64.17 ± 6.88	119.18 ± 16.13
30 ng/side MK212 ($n=7$)	29.31 ± 2.25	698.29 ± 69.76	119.43 ± 43.43	85.86 ± 19.96
100 ng/side MK212 ($n=7$)	25.17 ± 1.26	687.57 ± 47.49	67.64 ± 4.91	137.14 ± 14.85
Cg2				
100 ng/side MK212 ($n=7$)	27.34 ± 2.18	658.00 ± 58.50	67.00 ± 4.10	112.00 ± 31.57
PrL/IL				
SB242084/vehicle ($n=10$)	30.76 ± 1.84	521.50 ± 52.48	90.65 ± 10.24	98.50 ± 13.45
MK212/MK212+SB242084 ($n=10$)	29.66 ± 2.01	496.80 ± 40.03	78.80 ± 7.88	97.60 ± 11.53

Each i.v. cocaine infusion contained 0.75 mg/kg/0.1 ml.

extinguished cocaine-seeking behavior received a minimum of 12–39 extinction sessions before testing and a maximum of 36–68 total sessions, including those that occurred during the training and testing phases. Active lever presses decreased during extinction training, and there were no group differences on the first day of extinction (Table 1) or across the 12 days of extinction prior to reinstatement testing (data not shown).

Effects of MK212 on Cocaine Self-Administration

Figure 3 illustrates the effects of intra-mPFC MK212 microinfusions on the number of cocaine infusions obtained during self-administration testing. MK212 failed to produce a dosage group by test-session interaction, but the ANOVA indicated a main effect of test session [$F(2, 34) = 5.4$, $p < 0.001$]. When collapsed across MK212 dosage groups, *post hoc* Newman–Keuls revealed a decrease in responding on both the vehicle and drug pretreatment tests relative to baseline. There was no difference between vehicle and drug groups when collapsed across dose, suggesting that these effects resulted from injection stress rather than stimulation of 5-HT$_{2C}$Rs.

Effects of MK212 Alone on Reinstatement

Figure 4 illustrates the effects of intra-mPFC MK212 microinfusions on extinguished active lever responding. The ANOVAs of these data for each PFC subregion failed to uncover significant effects.

Effects of MK212 on Cue-Elicited Reinstatement

Of the 49 rats tested for cue-elicited reinstatement, 2 rats from the IL and 1 from the Cg2 group failed to meet the reinstatement criteria and were omitted. Figure 5 illustrates the effects of intra-mPFC MK212 microinfusions on active lever responding during FR1 cue-elicited reinstatement of extinguished cocaine-seeking behavior. ANOVAs of active lever responses revealed dosage group by test-session interactions for the PrL [$F(4, 38) = 4.1$, $p < 0.01$] and IL [$F(4, 30) = 3.3$, $p < 0.05$] cortices, and a main effect of day for the Cg2 [$F(2, 12) = 8.7$, $p < 0.005$] region. Lever presses on the inactive lever were negligible and there was no effect of MK212 in any of the mPFC subregions on inactive lever responding (data not shown). *Post hoc* comparisons indicated an increase in responding during the vehicle pretreatment test when response-contingent cues were available, relative to baseline when active lever responses produced no consequences (Newman–Keuls, $p < 0.05$), indicating cue-elicited reinstatement of extinguished cocaine-seeking behavior in all groups pretreated with vehicle regardless of drug-dosage group or region. MK212 dose and region dependently decreased cue-elicited reinstatement of extinguished cocaine-seeking behavior. In both the PrL and IL cortices, the 10 ng/side dose failed to alter reinstatement

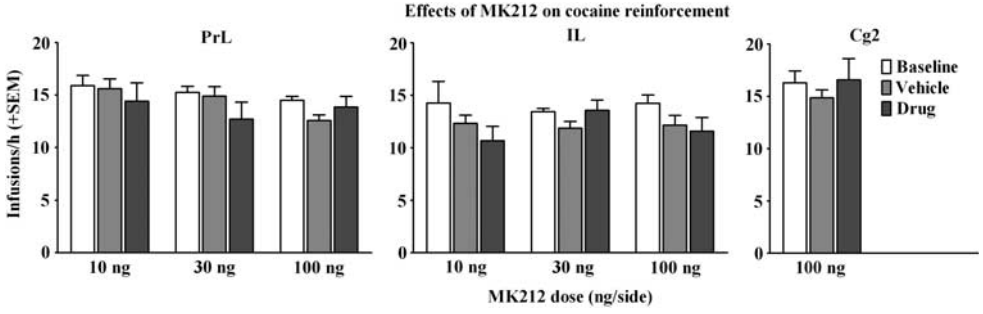

Figure 3 Effects of MK212 microinfused into the PrL, IL, and Cg2 subregions of the mPFC (10, 30, 100 ng/side) on the mean number of reinforcers (+SEM) obtained on a VR5 schedule of cocaine (0.75 mg/kg, i.v.) reinforcement during a 1-h test session. Baselines (white bars) represent mean infusions during the self-administration sessions preceding each test. Rats ($n = 6$–10/group) were pretreated with vehicle (gray bars) before one test and their assigned dose of MK212 (black bars) before the other test, with order of these treatments counterbalanced. IL microinfusions produced a main effect of day regardless of MK212 dosage group. *Post hoc* comparisons collapsed across dosage groups indicated that the mean (+SEM) number of infusions after vehicle (12.10 ± 0.44) or drug (12.00 ± 0.72) was lower than that obtained during baseline (13.95 ± 0.65; Newman–Keuls, $P < 0.05$).

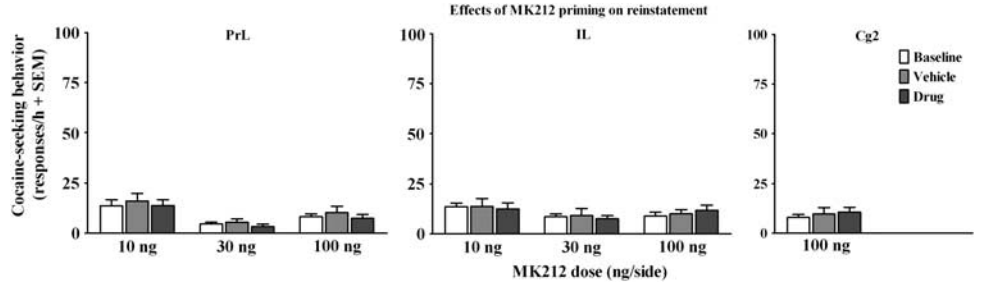

Figure 4 Effects of MK212 (10, 30, 100 ng/side) microinfused alone into the PrL, IL, and Cg2 subregions of the mPFC on extinguished cocaine-seeking behavior expressed as the mean number of active lever responses (+SEM) during a 1-h test session. Baselines (white bars) represent mean responses during the extinction sessions preceding each test. Rats ($n = 6$–8/group) were pretreated with vehicle (gray bars) before one test and their assigned dose of MK212 (black bars) before the other test, with order of these treatments counterbalanced; responses produced no scheduled consequences during testing nor did rats receive cocaine on the test day.

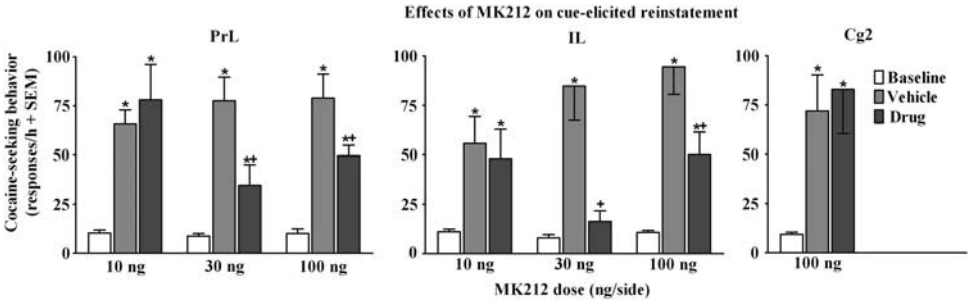

Figure 5 Effects of MK212 (10, 30, 100 ng/side) microinfused into the PrL, IL, and Cg2 subregions of the mPFC on cue-elicited reinstatement of extinguished cocaine-seeking behavior expressed as the mean number of active lever responses (+SEM) during a 1-h test session. Baselines (white bars) represent mean responses during the extinction sessions preceding each test. Rats ($n = 6$–8/group) were pretreated with vehicle (gray bars) before one test and their assigned dose of MK212 (black bars) before the other test, with order of these treatments counterbalanced. Cues were available response contingently during the test session on an FR1 schedule. Asterisk (*) represents a difference from baseline (Newman–Keuls, $p < 0.05$). Plus sign (+) represents a difference from vehicle pretreatment test day (Newman–Keuls, $p < 0.05$).

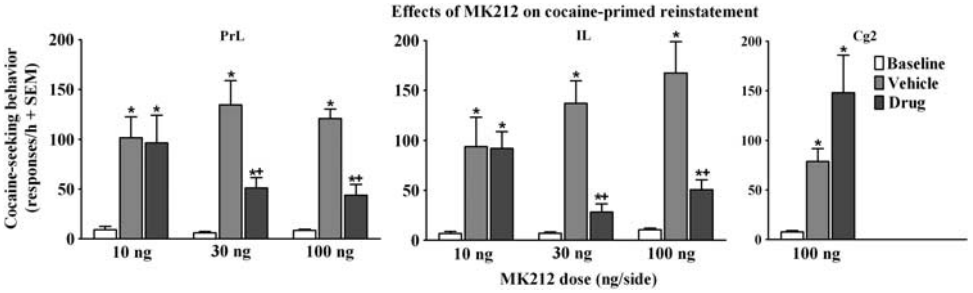

Figure 6 Effects of MK212 (10, 30, 100 ng/side) microinfused into the PrL, IL, and Cg2 subregions of the mPFC on cocaine-primed (10 mg/kg, i.p.) reinstatement of extinguished cocaine-seeking behavior expressed as the mean number of active lever responses (+SEM) during a 1-h test session. Baselines (white bars) represent mean responses during the extinction sessions preceding each test. Rats ($n = 6$–8/group) were pretreated with vehicle (gray bars) before one test and their assigned dose of MK212 (black bars) before the other test, with order of these treatments counterbalanced. The cocaine prime was administered after the intra-mPFC microinfusions and immediately before testing. No cues were presented during the test sessions. Asterisk (*) represents a difference from baseline (Newman–Keuls, $p < 0.05$). Plus sign (+) represents a difference from vehicle pretreatment test day (Newman–Keuls, $p < 0.05$).

as responding increased relative to baseline (Newman–Keuls, $p < 0.05$) and did not differ from vehicle pretreatment. In the PrL cortex, rats pretreated with the 30 and 100 ng/side doses exhibited increased responding relative to baseline, but also attenuated reinstatement of responding relative to vehicle (Newman–Keuls, $p < 0.05$). In the IL cortex, the 30 ng/side dose blocked reinstatement as responding did not differ from baseline and was decreased relative to vehicle pretreatment, whereas the 100 ng/side dose attenuated reinstatement as responding was increased relative to baseline, but was also decreased relative to vehicle pretreatment (Newman–Keuls, $p < 0.05$). In the Cg2 region, the 100 ng/side dose failed to alter reinstatement as responding did not differ relative to vehicle pretreatment and was increased relative to baseline (Newman–Keuls, $p < 0.05$).

Effects of MK212 on Cocaine-Primed Reinstatement

Of the 49 rats tested for cocaine-primed reinstatement, 1 rat from the IL group failed to meet the reinstatement criteria and was omitted. Figure 6 illustrates the effects of intra-mPFC MK212 microinfusions on active lever responding during the cocaine-primed (10 mg/kg, i.p.) reinstatement tests. The ANOVAs of active lever responses revealed dosage group by test-session interactions for the PrL [$F(4, 38) = 4.2$, $p < 0.01$] and IL [$F(4, 30) = 6.7$, $p < 0.001$] cortices, and a main effect of day for the Cg2 [$F(2, 10) = 9.3$, $p < 0.01$] region. Lever presses on the inactive lever were negligible and there was no effect of MK212 in any of the mPFC subregions on inactive lever responding (data not shown). *Post hoc* comparisons indicated an increase in responding on the vehicle pretreatment test when cocaine-priming injections were given, relative to baseline when saline-priming injections were administered (Newman–Keuls, $p < 0.05$), indicating cocaine-primed reinstatement of extinguished cocaine-seeking behavior in all groups pretreated with vehicle regardless of drug dose or region. MK212 dose and region dependently decreased cocaine-primed reinstatement. In the PrL and IL cortices, the 10 ng/side dose failed to alter reinstatement as responding increased relative to baseline and did not differ from vehicle pretreatment, whereas the 30 and 100 ng/side doses attenuated reinstatement as responding increased relative to baseline, but was decreased relative to vehicle pretreatment (Newman–Keuls, $p < 0.05$). In the Cg2 region, the 100 ng/side dose failed to alter reinstatement as responding did not differ relative to vehicle pretreatment and was increased relative to baseline (Newman–Keuls, $p < 0.05$).

Effects of SB242084 on MK212-Induced Decreases in Cue-Elicited Reinstatement

Of the 20 rats tested for cue-elicited reinstatement, 2 rats from the vehicle/SB242084 group failed to meet reinstatement criteria and were omitted. Figure 7 illustrates the

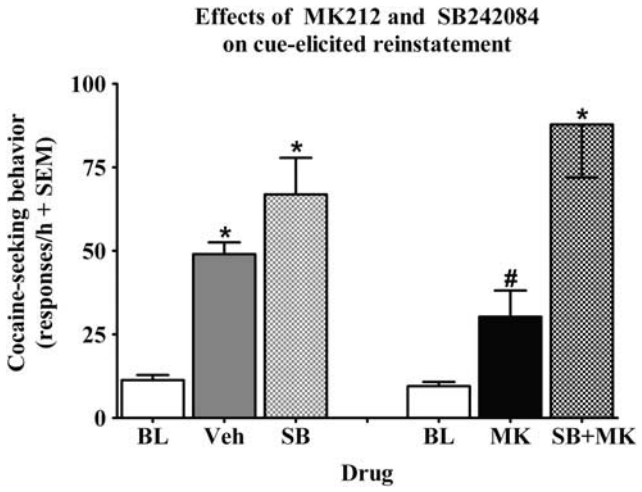

Figure 7 Effects of SB242084 (200 ng/side) on the MK212 (100 ng/side)-induced decrease in cue-elicited reinstatement of extinguished cocaine-seeking behavior expressed as the mean number of active lever responses (+SEM) during a 1-h test session. Baselines (white bars) represent mean responses during the extinction sessions preceding each test. Rats in the vehicle/SB242084 group ($n=8$–10/group) were pretreated with vehicle (gray bars) before one test and SB242084 (checkered gray bars) before the other test, whereas rats in the MK212/SB242084 group were pretreated with MK212 (black bars) before one test and MK212 + SB242084 (checkered black bars) before the other test, with order of these treatments counterbalanced. Cues were available response contingently on an FR1 schedule. Asterisk (*) represents a difference from baseline (Newman–Keuls, $p<0.05$). Pound sign (#) represents a difference from MK212 + SB242084 pretreatment test day (Newman–Keuls, $p<0.05$).

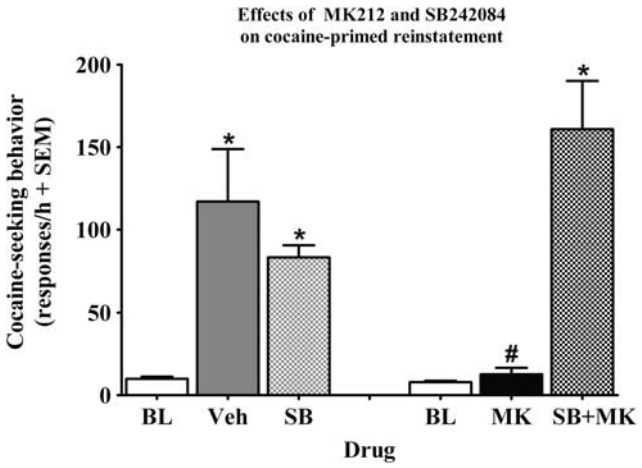

Figure 8 Effects of SB242084 (200 ng/side) on the MK212 (100 ng/side)-induced decrease in cocaine-primed reinstatement of extinguished cocaine-seeking behavior expressed as the mean number of active lever responses (+SEM) during a 1-h test session. Baselines (white bars) represent mean responses during the extinction sessions preceding each test. Rats in the vehicle/SB242084 group ($n=8$–10/group) were pretreated with vehicle (gray bars) before one test and SB242084 (checkered gray bars) before the other test, whereas rats in the MK212/SB242084 group were pretreated with MK212 (black bars) before one test and MK212 + SB242084 (checkered black bars) before the other test, with order of these treatments counterbalanced. For cocaine-primed reinstatement, the cocaine prime (10 mg/kg, i.p.) was administered immediately before testing and no cues were presented during the test sessions. Asterisk (*) represents a difference from baseline (Newman–Keuls, $p<0.05$). Pound sign (#) represents a difference from MK212 + SB242084 pretreatment test day (Newman–Keuls, $p<0.05$).

effects of SB242084 on the MK212-induced decrease in active lever responding during cue-elicited reinstatement testing. The ANOVAs of active lever responses revealed drug group by test-session interactions for both the MK212/SB242084 [$F(2,18)=17.7$, $p<0.001$] and vehicle/SB242084 [$F(2,14)=15.8$, $p<0.001$] groups. Lever presses on the inactive lever were negligible and there was no effect of intra-mPFC microinfusions on inactive lever presses (data not shown). *Post hoc* comparisons indicated an increase in responding during the vehicle, SB242084, and MK212 + SB242084 pretreatment test sessions when response-contingent cues were available, relative to baseline when active lever responses produced no consequences (Newman–Keuls, $p<0.05$), indicating cue-elicited reinstatement of extinguished cocaine-seeking behavior regardless of drug-dosage group. MK212 blocked cue-elicited reinstatement as rats receiving 100 ng/side of MK212 did not differ from their respective baselines and exhibited a reduction in active lever responses after exposure to cocaine-paired cues compared with MK212 + SB242084 pretreatment (Newman–Keuls, $p<0.05$).

Effects of SB242084 on MK212-Induced Decreases in Cocaine-Primed Reinstatement

Of the 20 rats tested for cocaine-primed reinstatement, 1 rat from the vehicle/SB242084 drug group failed to meet criteria and was omitted. Figure 8 illustrates the effects of SB242084 on the MK212-induced decrease in active lever responding during cocaine-primed reinstatement testing. The ANOVAs of active lever responses revealed drug-dosage group by test-session interactions for both the MK212/SB242084 [$F(2,18)=24.5$, $p<0.001$] and vehicle/SB242084 [$F(2,16)=9.2$, $p<0.005$] groups. Lever presses on the inactive lever were negligible and there was no effect of intra-mPFC microinfusions on inactive lever presses (data not shown). *Post hoc* comparisons indicated an increase in responding during the vehicle, SB242084, and MK212 + SB242084 pretreatment test sessions when cocaine-priming injections were given, relative to baseline when saline-priming injections were administered (Newman–Keuls, $p<0.05$), indicating cocaine-primed reinstatement of extinguished cocaine-seeking behavior regardless of drug-dosage group. MK212 blocked cocaine-primed reinstatement as rats receiving 100 ng/side of MK212 did not differ from their respective baselines, and exhibited a reduction in active lever responses after cocaine-priming injections compared with MK212 + SB242084 pretreatment (Newman–Keuls, $p<0.05$).

DISCUSSION

The results from this study show that microinfusions of the selective 5-HT$_{2C}$R agonist MK212 into the PrL and IL subregions of the mPFC dose-dependently attenuated cue-elicited and cocaine-primed reinstatement of extinguished cocaine-seeking behavior. Although other studies have evaluated the effects of intra-vmPFC MK212 microinfusions on the stimulant and discriminative stimulus effects of

cocaine (Filip and Cunningham, 2003), this is the first study to show a function for mPFC 5-HT$_{2C}$Rs in mediating the incentive motivation for cocaine. These effects seem to be region specific as microinfusions into the neighboring Cg2 subregion of the anterior cingulate cortex did not alter cue- or cocaine-primed reinstatement. Furthermore, the reduction in cocaine-seeking behavior seems to be behaviorally specific as the number of cocaine reinforcers obtained during self-administration testing at the training dose of cocaine (0.75 mg/kg, i.v.) was not altered by MK212 microinfusions into any of the three subregions of the mPFC, although the microinfusion procedure itself produced a small decrease in self-administration rate regardless of whether drug or vehicle was infused.

MK212 binds to 5-HT$_{2C}$Rs with the highest affinity compared with other receptors; however, it also has affinity for 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_3$Rs (Cussac *et al*, 2002; Glennon *et al*, 1989; Kennett, 1993; Porter *et al*, 1999). To verify that the effects of MK212 on cue-elicited and cocaine-primed reinstatement were 5-HT$_{2C}$R mediated, we showed that co-administration of the 5-HT$_{2C}$R antagonist SB242084 blocked the agonist effects. SB242084 has a high affinity for 5-HT$_{2C}$Rs (pKi = 9) relative to 5-HT$_{2B}$Rs and 5-HT$_{2A}$Rs, with a 150-fold selectivity over the 2A and 2B receptors (Kennett *et al*, 1997). The ability of SB242084 to block the effects of MK212 suggests that these effects are 5-HT$_{2C}$R mediated. Moreover, because SB242084 administered alone as a drug prime did not alter cocaine-seeking behavior, it seems unlikely that stimulation of mPFC 5-HT$_{2C}$Rs through tonic levels of 5-HT modulates incentive motivation for cocaine. Furthermore, increasing stimulation of 5-HT$_{2C}$Rs above tonic levels with MK212 in the absence of a reinstating stimulus did not alter cocaine-seeking behavior, but attenuated responding elicited by cues and cocaine primes, providing evidence that increased stimulation of 5-HT$_{2C}$Rs within the mPFC inhibits the incentive motivational effects of these stimuli. Collectively, these results strongly suggest that the effects of intra-mPFC MK212 microinfusions on cue-elicited and cocaine-primed reinstatement of extinguished cocaine-seeking behavior are 5-HT$_{2C}$R mediated.

The decrease in cocaine-seeking behavior after intra-mPFC MK212 microinfusions may have involved several mechanisms, including sensory or motor systems, learning and memory, or motivation. In our view, however, it is unlikely that learning and memory systems were responsible for the present behavioral effects, because the rats' self-administration behavior, which involves memory, was not affected. Moreover, lesions of the PrL cortex have been shown to attenuate cocaine-primed reinstatement of extinguished cocaine-conditioned place preference without altering acquisition or extinction of this behavior (Zavala *et al*, 2003). Earlier research has shown that microinfusions of MK212 into the vmPFC block cocaine-induced hyperactivity without altering spontaneous locomotion (Filip and Cunningham, 2003). In light of the latter findings and our results showing that MK212 did not impair operant behavior during cocaine self-administration testing, it seems unlikely that the observed effects of MK212 on cocaine-seeking behavior resulted from non-specific motor impairments. Finally, the present findings are complementary to previous research showing that MK212 microinfusions into the vmPFC attenuate the discriminative stimulus effects of cocaine (Filip and Cunningham, 2003). Although drug discrimination and reinstatement of extinguished cocaine-seeking behavior are not different behavioral expressions of a unitary neurobiological process, there is a considerable degree of overlap between reinstatement of drug-seeking behavior and drug discrimination (for a review see Spealman *et al*, 1999). From the above discussion, we favor the explanation that enhanced signaling through 5-HT$_{2C}$Rs located within the PrL and IL, but not Cg2, subregions of the mPFC attenuates cocaine-induced and cocaine-conditioned incentive motivation for cocaine.

It is important to note that the purpose of examining the effects of intra-mPFC MK212 microinfusions on cocaine self-administration was to provide a control manipulation to examine the specificity of potential effects on cocaine-seeking behavior, rather than to investigate the function of cortical 5-HT$_{2C}$Rs in cocaine reinforcement, *per se*. This study is, therefore, limited because only one dose of cocaine (0.75 mg/kg, i.v.) was examined. However, the 0.75 mg/kg, i.v. dose of cocaine that was tested falls midway on the descending limb of the cocaine dose-effect function, thus we likely had the sensitivity to detect either an increase or decrease in reinforcement rates during self-administration testing. Nevertheless, we cannot rule out the possibility that mPFC 5-HT$_{2C}$Rs may influence cocaine reinforcement at other doses as peripheral administration of the 5-HT$_{2C}$R agonist Ro600175 reduces maintenance of cocaine self-administration, as well as break points for cocaine on a progressive ratio schedule of reinforcement (Grottick *et al*, 2000). Furthermore, systemic administration of the selective 5-HT$_{2C}$R antagonist SB242084 increases responding for low (0.0625 and 0.125, but not 0.25 mg) doses of cocaine (Fletcher *et al*, 2002b), and 5-HT$_{2C}$R-deficient mice self-administer more cocaine reinforcers on a progressive ratio schedule compared with wild-type controls (Rocha *et al*, 2002). In light of these findings, we speculate that 5-HT$_{2C}$Rs in regions other than the mPFC likely modulate the reinforcing effects of cocaine. For instance, microinfusions of the 5-HT$_{2C}$R agonist Ro600175 into the VTA reduce the reinforcing effects of self-administered cocaine on both fixed and progressive ratios (Fletcher *et al*, 2004), suggesting that the VTA may be responsible for the reduction of cocaine self-administration by systemically administered 5-HT$_{2C}$R agonists.

Neuroanatomically, the reduction in cocaine-seeking behavior following intra-mPFC MK212 microinfusions may have resulted from inhibition of the mesolimbic dopamine (DA) system through 5-HT$_{2C}$R stimulation of a mPFC afferent pathway. A fundamental feature of addiction is the inability to self-regulate chronic drug-seeking and -taking behaviors (Jentsch and Taylor, 1999; Kalivas, 2008), and these deficits in self-regulation are associated with functional 'hypofrontality' in the PFC (Childress *et al*, 1999; Goldstein and Volkow, 2002). The mPFC is densely innervated by 5-HT neurons (Van Bockstaele *et al*, 1993) and 5-HT$_{2C}$Rs provide modulatory influence over PFC function (Clemett *et al*, 2000; Lopez-Gimenez *et al*, 2001; Pompeiano *et al*, 1994). 5-HT$_{2C}$Rs within the mPFC are localized postsynaptically (Clemett *et al*, 2000) on both GABA interneurons (Liu *et al*, 2007) that form numerous synapses on glutamate pyramidal projection neurons, and on glutamatergic output neurons directly (Carr *et al*, 2002;

Vysokanov *et al*, 1998), thus regulating mPFC output (Eyles *et al*, 2002). Pyramidal projections terminate in subcortical regions, including the VTA and NAc, which are the origin and terminal regions, respectively, of the DA mesoaccumbens pathway (Gabbott *et al*, 2005). Thus, stimulation of 5-HT$_{2C}$Rs located on mPFC GABA interneurons or glutamate projection neurons may reduce or increase, respectively, excitatory output from the mPFC to the mesoaccumbens pathway, providing a mechanism for mPFC 5-HT$_{2C}$Rs to regulate glutamate and DA levels in the NAc.

In support of the above hypothesis, GABA agonist microinfusions into the mPFC inhibit glutamate release in the VTA and NAc (Harte and O'Connor, 2005), whereas GABA antagonist microinfusions into mPFC increase DA release in the NAc (Karreman and Moghaddam, 1996). These effects could involve 5-HT$_{2C}$R mediation as intra-mPFC iontophoretic application of mCPP, a drug possessing 5-HT$_{2C}$R agonist properties, suppresses spontaneous and glutamate-activated firing of PFC neurons (Bergqvist *et al*, 1999). In addition, 5-HT$_{2C}$R mutant mice exhibited higher baseline extracellular DA levels in the NAc (Abdallah *et al*, 2009) and increased cocaine-induced DA release in the NAc compared with wild-type controls (Rocha *et al*, 2002). In contrast, mPFC electrical stimulation increases glutamate levels in the VTA (Rossetti *et al*, 1998) and NAc, as well as DA release in the NAc (You *et al*, 1998). Thus, the results obtained from MK212 microinfusions into the mPFC may have resulted from activation of 5-HT$_{2C}$Rs located on GABA neurons, leading to a reduction in DA release in the NAcsh and an accompanying decrease in cocaine-seeking behavior. Importantly, GABA agonists infused into the PrL cortex block cocaine-primed reinstatement (McFarland and Kalivas, 2001), whereas GABA agonists infused into the vmPFC encompassing both the PrL and IL cortices block incubation of cue-elicited craving (Koya *et al*, 2009), suggesting that the effects of MK212 in the present study involve increases in GABA neurotransmission. However, the reduction in cocaine-seeking behavior after MK212 microinfusions into the IL cortex may have resulted from direct stimulation of glutamatergic projection neurons as microinfusions of the glutamate agonist AMPA into this region reduce cocaine-seeking behavior as well (Peters *et al*, 2008).

In conclusion, this study provides strong evidence for an inhibitory function of mPFC 5-HT$_{2C}$Rs in reinstatement of extinguished cocaine-seeking behavior produced by either drug-paired cues or cocaine-priming injections. Further research is needed to determine the neural circuitry through which mPFC 5-HT$_{2C}$Rs produce inhibitory effects on cocaine-seeking behavior. Elucidating the neural mechanisms underlying the incentive motivational effects produced by sampling cocaine and exposure to cocaine-associated cues is important for developing pharmacological treatments to help prevent cocaine craving and relapse.

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DISCLOSURE

The authors declare no conflict of interest.

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