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Behavioral sensitization to cocaine in rats: Evidence for temporal differences in dopamine D₃ and D₂ receptor sensitivity

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Abstract

Rationale—Cocaine-induced changes in D₂ receptors have been implicated in the expression of sensitized behavioral responses and addiction-like behaviors, however, the influence of D₃ receptors is less clear.

Objectives—To characterize the effects of repeated cocaine administration on the sensitivity of rats to D₂- and D₃-mediated behaviors, as well as the binding properties of ventral striatal D₂-like and D₃ receptors.

Methods—Pramipexole was used to assess the sensitivity of rats to D₃/D₂ agonist-induced yawning, hypothermia, and locomotor activity, 24h, 72h, 10d, 21d and 42d after repeated cocaine or saline administration. The locomotor effects of cocaine (42d), and the binding properties of ventral striatal D₂-like and D₃ receptors (24h and 42d) were also evaluated.

Results—Cocaine-treated rats displayed an enhanced locomotor response to cocaine, as well as a progressive and persistent leftward/upward shift of the ascending limb (72h–42d), and leftward shift of the descending limb (42d) of the pramipexole-induced yawning dose-response curve. Cocaine treatment also decreased B_{max} and K_d for D₂-like receptors, and increased D₃ receptor binding at 42d. Cocaine treatment did not change pramipexole-induced hypothermia or locomotor activity, or yawning induced by cholinergic or serotonergic agonists.

Conclusions—These studies suggest that temporal differences exist in the development of cocaine-induced sensitization of D₃ and D₂ receptors, with enhancements of D₃-mediated behavioral effects observed within 72h, and enhancements of D₂-mediated behavioral effects apparent 42d after cocaine. These findings highlight the need to consider changes in D₃ receptor function when thinking about the behavioral plasticity that occurs during abstinence from cocaine use.

Introduction

Early studies on the effects of repeated cocaine administration described a common behavioral sequelae in monkeys, dogs, and rats in which the behavioral and physiologic effects of cocaine were observed sooner, and with greater intensity following repeated, intermittent cocaine administration (Downs and Eddy 1932a; b; Post and Rose 1976; Tatum and Seevers 1929), and the progressive and persistent nature of these effects, frequently referred to as *behavioral sensitization*, are now well established in rodents. Although some important differences exist between effects of experimenter- and self-administered cocaine (Jacobs et al. 2003), a variety of progressive and time-dependent neuropharmacologic and behavioral changes have been shown to correspond to the expression of the sensitized locomotor response to cocaine, including decreases in dopamine transporter (DAT) density, and enhancements of cocaine-induced increases in extracellular dopamine, regardless of whether cocaine is experimenter- (Heidbreder et al. 1996; Henry and White 1995; Kalivas and Duffy 1993; Pilotte et al. 1994; Wilson et al. 1994), or self-administered (Ahmed and Cador 2006; Ferrario et al. 2005; Hooks et al. 1994; Phillips and Di Ciano 1996; Zapata et al. 2003). The repeated administration of psychostimulants is also known to affect a variety of neurotransmitter systems (e.g., cholinergic, dopaminergic, GABAergic, glutamatergic, opioidergic, and serotonergic), and it is thought that the neuroadaptations that underlie the development and expression of the sensitized behavioral responses are also important for the development of addiction-like behaviors in animals (for reviews see; Kalivas and Stewart 1991; Pierce and Kalivas 1997; Robinson and Berridge 1993; Vanderschuren and Kalivas 2000; Vezina 2004). An important theory of the basis of drug addiction posits that drug-induced behavioral sensitization reflects sensitized brain circuits that, in turn, enhance the incentive value of the drugs and the cues associated with them (Robinson and Berridge, 1993). Among the data that support this theory are findings that cocaine-induced sensitization of D₂-like (D₂, D₃, and D₄) receptors within the nucleus accumbens plays a role in the increased effectiveness of D₂-like agonists in inducing cocaine-directed responding (i.e., drug-seeking) following the cessation of cocaine self-administration (De Vries et al. 2002; Dias et al. 2004; Edwards et al. 2007). What is not specified in the theory or demonstrated in these data are the respective roles of the D₂, D₃, and D₄ receptors in mediating either the behavioral sensitization or the corresponding neurological changes.

There is, nevertheless, growing evidence to suggest that cocaine, either experimenter- or self-administered, has differential effects on the density/availability/expression of D₂-like (D₂, D₃ and D₄), with decreases in D₂ receptor levels reported in rodents (Bailey et al. 2008; Conrad et al. 2010; Goeders and Kuhar 1987; Maggos et al. 1998), and rhesus monkeys (Nader et al. 2006), and increases in D₃ receptor levels observed in human cocaine overdose fatalities (Segal et al. 1997; Staley and Mash 1996), and rats with a history of experimenter- (Le Foll et al. 2005), or self-administered cocaine (Conrad et al. 2010; Neisewander et al. 2004).

Despite these apparent differences in the effects of cocaine on D₂ and D₃ receptor levels, the lack of agonists or antagonists with high degrees of selectivity for the D₂ or D₃ receptor has made it difficult to parse the relative contributions of these receptor subtypes to the expression of sensitized behavioral responses or addiction-like behaviors. One approach that

has been useful in determining the involvement of different dopamine receptor subtypes has been to evaluate the capacity of cocaine to sensitize animals to the selective behavioral effects of D1-like (D₁, and D₅) or D2-like (D₂, D₃, and D₄) receptor agonists. Accordingly, increases in SKF-38393-induced tongue protrusions (Neisewander et al. 1996) have been interpreted as a cocaine-induced sensitization of D1-like receptors, whereas enhancements of quinpirole-, or quinlorane-induced sniffing, head movements, or locomotor stimulation (De Vries et al. 2002; Dias et al. 2004; Edwards et al. 2007; Ujike et al. 1990) have been attributed to a sensitization of D₂ receptors. It should be noted, however, that these latter studies do not permit a discrimination between effects produced through the D₂ as opposed to the D₃ receptors as the majority of these effects are observed at doses of quinpirole or quinlorane that are 50- to 250-fold higher than those required to induce behaviors that have been attributed to the selective activation of D₃ receptors, such as yawning (Collins et al. 2007; Collins et al. 2005), and 5- to 25-fold higher than those required to produce D₂-mediated effects, such as hypothermia (Chaperon et al. 2003; Collins et al. 2007).

Support for these sub-type-specific roles of the D₃, and D₂ receptors in the regulation of yawning and hypothermia has been provided by antagonist interaction studies in which D₃-selective antagonists have been shown to produce dose-dependent and selective rightward shifts of the ascending limb of the dose-response curve for D2-like agonist-induced yawning, whereas D₂-preferring antagonists have been shown to produce selective rightward shifts of the descending limb of the yawning dose-response curve for D2-like agonist-induced yawning at doses that also inhibit the induction of hypothermia (Baladi et al. 2009; Collins et al. 2008; Collins et al. 2007; Collins et al. 2009; Collins et al. 2005). Based on these differential roles of the D₃ (induction) and D₂ (inhibition) receptors in the mediation of yawning, we have hypothesized that cocaine-induced increases in D₃ receptor density and/or sensitivity would result in leftward shifts of the ascending limb of the yawning dose-response curve, whereas decreases in D₂ receptor density would result in rightward shifts of the descending limb of the yawning dose-response curve. Conversely, an increase in the sensitivity of D₂ receptors should result in a leftward shift of the descending limb of the yawning dose-response curve.

In the current experiments, rats were first given a regimen of once daily cocaine, or saline injections prior to assessing the sensitivity of rats to the D₃- and D₂-mediated behavioral effects of the D₃-preferring agonist, pramipexole (and ~30-fold selective for D₃ over D₂ in vivo; Collins et al. 2007; ~90-fold selective for D₃ over D₂ in vitro; Millan et al. 2002). Dose-response curves for pramipexole-induced yawning, locomotor activity, and hypothermia were obtained 24h, 72h, 10d, 21d, and 42d after the cessation of cocaine or saline administration to allow for evaluation of the development of cocaine-induced changes in D₃- and/or D₂-mediated behavioral effects. To assess the selectivity of cocaine's effects for dopaminergic systems, dose-response curves for yawning induced by the indirect cholinergic agonist, physostigmine, and the 5-HT₂ receptor agonist, 3-Trifluoromethylphenylpiperazin (TFMPP), were also obtained after 24–72h, and 41–43d of cessation from cocaine, or saline administration. In parallel, radioligand binding studies were performed to assess the effects of repeated cocaine administration on the binding properties of ventral striatal D₂ and D₃ receptors 24h and 42d after the cessation of cocaine or saline administration.

Methods

Subjects

Male Sprague-Dawley rats (250–300 g) were obtained from Harlan (Indianapolis, IN), and individually housed for the duration of the study in a temperature- (21–23 °C), and humidity-controlled environment, on a 12-hr dark/light cycle with lights on at 7:00 AM.

Except during behavioral observations, all animals had free access to standard Purina rodent chow and tap water throughout the course of the experiment. All experimental procedures were approved by the University of Michigan Committee on the Use and Care of Animals and performed in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health.

Sensitization Paradigm

In order to establish cocaine-induced behavioral sensitization, cocaine was administered once daily in the rat's home cage for a period of seven days according to the following schedule: days 1–2: 15 mg/kg; i.p., and days 3–7: 30 mg/kg; i.p. Saline-treated rats received once-daily i.p. injections of saline in their home cage. All injections were administered between 1:00 and 1:30 PM. Behavioral observations, locomotor/core body temperature studies, and tissue collection began all 24h after the last cocaine or saline injection.

Behavioral Observations of Yawning

Yawning behavior was defined as a prolonged (~1s), broad opening of the mouth followed by a rapid closure of the mouth. Prior to experimental sessions, rats were weighed and transferred from their home cage to a clear test chamber (48 × 23 × 20 cm, with standard cob bedding and ventilated lid). A mirror was placed behind the test chamber to allow for the simultaneous observation of two individual rats by a trained observer. Rats were allowed a 30-min habituation period prior to the administration of successive doses of saline and test compounds. Dose-response curves for pramipexole-, TFMPP-, and physostigmine-induced yawning were generated using a multiple-dose procedure as previously described (Collins et al. 2008). Briefly, the total numbers of yawns were counted for the 30-min period immediately following injections of saline followed by five doses of pramipexole (0.01, 0.032, 0.1, 0.32, and 1.0 mg/kg; s.c.), three doses of TFMPP (1.0, 3.2, and 10.0 mg/kg; s.c.), or three doses of physostigmine (0.032, 0.1, and 0.32 mg/kg; i.p.), with successive injections administered at 30-min intervals. Dose-response curves for pramipexole-induced yawning were generated in one group of cocaine- and one group of saline-treated rats (n=8/treatment) tested 24h, 72h, 10d, 21d, and 42d after the cessation of cocaine/saline injections. Dose-response curves for TFMPP- and physostigmine-induced yawning were generated in a separate group of cocaine- and saline-treated rats (n=8/treatment). Half the rats were exposed to physostigmine, and half were exposed to TFMPP at the 24h time-point, with the treatments counterbalanced at the 72h time-point, and again at the 41d and 43d time-points so that all rats were tested with both physostigmine and TFMPP within 3-day periods corresponding to the short and long periods of cessation. All test sessions were conducted between 12:00 PM and 5:00 PM.

Core Body Temperature and Locomotor Activity

Core body temperature and locomotor activity were measured using radio-telemetric probes (E-4000 E-Mitter, Mini-Mitter, Bend, OR, USA) implanted in the abdominal cavity of rats. Prior to surgery, rats were anesthetized with ketamine (90mg/kg i.p.) and xylazine (15 mg/kg i.p.) and their abdominal area was shaved and cleaned with alternating applications of Betadine and alcohol. A small rostral-caudal incision was made in the abdominal wall to allow for implantation of a radio telemetric probe. After implantation, the abdominal wall was sutured with absorbable 4-0 chromic gut sutures and the skin was sutured using 5-0 Ethilon® sutures. Rats were allowed a minimum of seven days to recover prior to the start of the sensitization paradigm. On test day, rats were weighed and returned to their home cages, which were placed on a receiving pad to allow for the real-time collection of locomotor activity and core body temperature with measures taken every minute. At least 45-min of baseline body temperature and locomotor activity were recorded before successive injections of saline and the test compound of interest. Dose-response curves for

pramipexole-induced changes in locomotor activity and core body temperature were generated using a multiple-dose procedure as previously described (Collins et al. 2008). Briefly, injections of saline, 0.01, 0.032, 0.1, 0.32, and 1.0 mg/kg; s.c. pramipexole were separated by 30-min. Dose-response curves for pramipexole-induced changes in locomotor activity and core body temperature were generated in one group of cocaine- and one group of saline-treated (n=6/treatment) rats 24h, 72h, 10d, and 21d after the cessation of cocaine/saline injections. Food and water were freely available and all experiments were conducted between the hrs of 11:00 AM and 6:00 PM.

In addition, these two groups of cocaine- and saline-treated rats were assessed for the development of a sensitized locomotor response to cocaine at the 42d time-point. Following a 45-min baseline period, all rats received an i.p. injection of saline followed 60-min later by an i.p. injection of 15 mg/kg cocaine. Saline- and cocaine-induced locomotor activity was collected for 60 minutes following each injection.

D2-like and D₃ Receptor Binding

Independent groups of cocaine- and saline-treated (n=10/treatment) rats were exposed to the regimen of cocaine (or saline) injections and sacrificed by decapitation either 24h (n=5/treatment) or 42d (n=5/treatment) after the cessation of cocaine or saline administration. In order to control for the possible influence of experimental or drug history on the D₃ and D₂ receptor-mediated effects, both the saline- and cocaine-treated rats were exposed to the same experimental procedures that the rats used in the yawning studies were exposed to. Thus, the 24h groups did not receive any pramipexole injections and were not placed in an observation chamber, whereas the 42d groups were placed in test chambers at 12:30 PM at the 24h, 72h, 10d, and 21d time-points with injections of saline, 0.01, 0.032, 0.1, 0.32, and 1.0 mg/kg; s.c. pramipexole administered at 30-min intervals. The only difference was that behaviors were not scored. At the specified times, brains were removed and frozen upside down on dry ice prior to being stored at -80°C. Ventral striatum (nucleus accumbens and olfactory tubercle) was isolated by freehand dissection on ice according to a modification of the methods of Glowinski and Iversen (1966). Dissected brain tissue was homogenized in 20 vol. (w/v) of buffer (50 mM Tris; pH 7.4 at 23° C) using a PRO homogenizer (setting 4 for 10 s). The crude homogenate was centrifuged twice (15 min at 48,000 × g), and pellets were resuspended each time in 20 vol. of buffer. The final pellet was resuspended in the appropriate concentration for each assay in the respective assay buffer.

The affinity and density of D2-like receptors were assessed using [³H]spiperone (GE Healthcare; 96 Ci/mmol; 0.03– 1.0 nM) in an equilibrium, filtration assay as previously described (Levant 2003). The assay buffer was 50 mM Tris, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, pH 7.4 @ 23°C. Nonspecific binding was defined in the presence of 1 mM (+)-butaclamol. Assay tubes were incubated at 23° C for 90 min. The reaction was terminated by rapid vacuum filtration. Bound radioactivity was quantified using a Beckman LS-6500 liquid scintillation counter. Membrane protein concentrations were determined by the BCA method (Pierce, Rockford, IL) and were ~50 mg/tube. Specific binding is expressed as fmol/mg protein and analyzed for K_D and B_{max} using SigmaPlot (v. 8.0.2).

D₃ receptor affinity was determined in membranes prepared from ventral striatum (nucleus accumbens and olfactory tubercle) using [³H]7-OH-DPAT (GE Healthcare; 163 Ci/mmol; 0.18 nM) according to a modification of previously described methods (Bancroft et al. 1998). Due to the small size of the ventral striatum and the low density of D₃ sites, as well as to allow for independent determinations to be made within individual animals, binding analyses were performed using a single concentration of radioligand. Assays were performed as described above except the assay buffer was 50 mM Tris, 1 mM EDTA; pH

7.4 at 23° C, nonspecific binding was defined by 1 μ M spiperone, the membrane protein concentration was ~200 mg/tube, and the incubation time was 2h.

Drugs

Cocaine was obtained from the National Institute on Drug Abuse (Bethesda, MD). Pramipexole was purchased from APAC Pharmaceutical, LLC (Columbia, MD). Physostigmine was obtained from Tocris Chemical Company (Ellisville, Mo), and TFMPP was obtained from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in physiologic saline and administered in a volume of 1.0 ml/kg. Cocaine, physostigmine, and their vehicle controls were administered i.p., whereas pramipexole, TFMPP, and their vehicle controls were administered s.c.

Data Analysis

Dose-response curves for agonist-induced yawning were generated using eight saline-treated, and eight cocaine-treated rats, whereas dose-response curves for pramipexole-induced locomotor activity and changes in core body temperature, as well as cocaine-induced locomotor activity, were generated using six saline-treated, and six cocaine-treated rats. Binding studies were conducted on tissue from ten saline-treated, and ten cocaine-treated (five of each condition at the 24h and 42d time-points). All yawning data are expressed as the mean $\pm$ standard error of the mean (SEM) number of yawns observed during the 30 min immediately after injection. Pramipexole-induced locomotor activity represents the mean $\pm$ SEM of the total locomotor activity for the 30 min period following each injection, whereas pramipexole-induced changes in core body temperature represent the mean $\pm$ SEM difference in the core body temperature as measured 30 min after each injection compared to the core body temperature measured 1 min before the saline injection. $B_{\max}$ (fmol/mg protein) and K_D (nM) represent the mean $\pm$ SEM fmol/mg as determined by scatchard plot analysis of ^3H -spiperone for D2-like receptor determinations. ^3H -7-OH-DPAT for D₃ receptor determinations represents the mean $\pm$ SEM of single-point binding density presented as fmol/mg protein.

Significant main effects of treatment on agonist-induced yawning, locomotor activity, and changes in core body temperature between the cocaine- and saline-treated groups were determined by two-way, repeated measures analysis of variance (ANOVA) with post-hoc Bonferroni tests (GraphPad Prism; GraphPad Software Inc., San Diego, CA). Significant main effects of time on agonist-induced yawning, locomotor activity, and changes in core body temperature within the cocaine- and saline-treated groups were determined by two-way, repeated measures analysis of variance (ANOVA) with post-hoc Bonferroni tests. Significant main effects of treatment on saline- and cocaine-induced locomotor activity between the cocaine- and saline-treated groups were determined by two-way, repeated measures analysis of variance (ANOVA) with post-hoc Bonferroni tests. Two-tailed, paired t-tests were used to determine significant differences between the cocaine- and saline-treated groups with respect to the total locomotor activity observed over the 60-min following saline and cocaine injections. Significant differences in $B_{\max}$ and K_d between the saline- and cocaine-treated groups were determined by two-tailed, paired t-tests.

Results

Effects of Cocaine Treatment on Cocaine-Induced Locomotor Activity

As shown in Figure 1, the locomotor response to 15.0 mg/kg; i.p. cocaine was significantly enhanced [$F(1,110)=5.38$; $p<0.05$] in rats that were treated with cocaine as compared to saline 42d prior to testing. In addition, although this enhancement of cocaine-induced locomotor activity was observed even when the locomotor effects of cocaine were summed

over 5-min or 60-min ($p < 0.05$) bins, there was no effect of prior cocaine treatment on locomotor response to saline.

Effects of Cocaine Treatment on Pramipexole-Induced Behavioral Effects

As shown in Figure 2, a progressive increase in the amount of yawning induced by pramipexole was observed 24h to 42d after seven days of cocaine administration (15 mg/kg; i.p. $\times$ 2 days and 30 mg/kg; i.p. $\times$ 5 days). Pramipexole produced significant dose-dependent increases in yawning in both the cocaine- and saline-treated groups at all experimental time-points (main-effect of dose: all $F > 13.18$, and all $p < 0.001$). Although there were no differences between the saline- and cocaine-treated rats with respect to pramipexole-induced yawning 24h after the cessation of cocaine or saline treatment, significant differences in the number of yawns induced by a low dose of pramipexole (0.032 mg/kg) were observed at the 10d ($p < 0.05$), 21d ($p < 0.001$), and 42d ($p < 0.001$) time-points. Between group comparisons revealed a significant main-effect of treatment at 21d [$F(1,70) = 8.87$; $p < 0.01$], and 42d [$F(1,70) = 10.38$; $p < 0.05$] after the cessation of cocaine. Within-subjects analyses revealed a significant main-effect of time from treatment for the cocaine-treated group [$F(4,168) = 3.31$; $p < 0.05$], but not the saline-treated rats. Cocaine-treatment resulted in a progressive increase in the number of yawns induced by a low dose of pramipexole (0.032 mg/kg), with significantly greater amounts of yawning observed 72h ($p < 0.01$), 10d ($p < 0.01$), 21d ($p < 0.001$), and 42d ($p < 0.001$) after cessation of cocaine treatment as compared to the 24h time-point; a significant decrease in number of yawns induced by 0.1 mg/kg pramipexole ($p < 0.05$) was also observed at the 42d compared to the 24h time-point. With the exception of the 72h time-point (0.1 mg/kg pramipexole; $p < 0.05$) pramipexole-induced yawning was no different than the 24h time-point for the saline-treated group.

As shown in Figure 3, there were no significant differences between the cocaine- and saline-treated groups with respect to pramipexole-induced changes in core body temperature, or locomotor activity at any time after cessation of treatment. Although pramipexole induced dose-dependent decreases in core body temperature at all experimental time-points (main-effect of Dose: all $F > 11.67$, and all $p < 0.001$), a significant effect of time from treatment was observed for both the cocaine- [$F(3,90) = 14.65$; $p < 0.001$] and saline-treated groups [$F(3,90) = 11.15$; $p < 0.001$]. Additionally, slight differences in the hypothermic effects of pramipexole were observed throughout the duration of the experiment, as compared to the respective 24h time-points [(Saline-treated: 72h – 0.32 mg/kg, $p < 0.01$; 21d – 1.0 mg/kg, $p < 0.05$), and (Cocaine-treated: 10d – 1.0 mg/kg, $p < 0.05$; 21d – 0.1 mg/kg, $p < 0.001$ and 1.0 mg/kg, $p < 0.01$)]. Unlike pramipexole-induced yawning and hypothermia, dose-dependent decreases in locomotor activity were only observed 24h after cessation of treatment [$F(5,50) = 4.19$; $p < 0.01$], and there was no significant effect of time from treatment in either the cocaine- or saline-treated groups. A dose of 1.0 mg/kg pramipexole did induce significantly more locomotor activity in the saline-treated group at the 10d time-point as compared to the locomotor response in the same group at the 24h time-point.

Effects of Cocaine Treatment on Physostigmine- and TFMPP-Induced Behavioral Effects

Although physostigmine and TFMPP produced dose-dependent increases in yawning in both the cocaine- and saline-treated groups (main effect of dose: all $F > 3.39$ and all $p < 0.05$), treatment with cocaine did not alter the capacity of physostigmine or TFMPP to induce yawning (main effect of treatment: all $F < 2.8$, and all $p > 0.12$) during the 24/72h, or 41/43d time-points (Table 1). Moreover, a significant effect of time from treatment was only observed for TFMPP-induced yawning in the saline-treated group [$F(1,28) = 24.08$; $p < 0.001$]; an effect that was primarily due to a significant reduction in the number of yawns induced by 3.2 mg/kg TFMPP at the 41/43d time-point compared to the 24/72h time-point ($p < 0.001$).

Effects of Cocaine Treatment on Dopamine D2-like and D₃ Receptor Binding

The effects of cocaine treatment on D2-like density and affinity, as determined from membranes prepared from the ventral striatum (nucleus accumbens and olfactory tubercle), are shown in Table 2. Although there were no significant effects of treatment on D2-like density or affinity at the 24h time-point, significant differences were observed 42d after cessation of cocaine/saline treatment. Not only did cocaine treatment result in a significant decrease in the density of ventral striatal D2-like receptors compared to the 24h cocaine-treated group ($p < 0.05$), but cocaine treatment also resulted in a decrease in ventral striatal D2-like receptor density as compared to the saline-treated group at the 42d time-point ($p < 0.05$). In addition to the effects of cocaine treatment on D2-like receptor density, ventral striatal samples taken from the cocaine-treated rats at the 42d time-point also showed a significant increase in D2-like receptor affinity as compared to the 42d saline-treated group ($p < 0.001$). Although it was possible to assess changes to both D2-like receptor density and affinity, the relatively low levels of expression, and limited amount of ventral striatal samples precluded similar analyses with regard to D₃ receptors; however, as shown in Figure 4, there was a significant effect of cocaine treatment on D₃ receptor binding as determined by a single-point binding assay. Although D₃ receptor binding within the ventral striatum did not differ between the saline- and cocaine-treated groups at the 24h time-point, cocaine treatment did result in a significant increase in D₃ receptor binding at the 42d time-point.

Discussion

In agreement with the majority of previous studies, cocaine-treated rats displayed an enhanced locomotor response to cocaine 42d after the cessation of cocaine, suggesting the current regimen of cocaine administration was sufficient to produce a long-lasting behavioral sensitization to cocaine. In addition, although cocaine treatment failed to alter pramipexole-induced locomotor activity or hypothermia, cocaine-treated rats displayed a progressive and persistent leftward and/or upward shift in the ascending limb of the dose-response curve for pramipexole-induced yawning, as well as a leftward shift in the inhibition of yawning that comprises the descending limb of the dose-response curve for yawning. The temporal differences in the development of these two effects suggest that increases in the sensitivity of rats to D₃-mediated behavioral effects become apparent shortly after the cessation of cocaine with progressive and persistent enhancements observed for up to 42d, whereas increases in the sensitivity of rats to D₂-mediated behavioral effects do not emerge until ~42d after the cessation of cocaine.

Although it has been well established that repeated, experimenter- or self-administered cocaine results in a progressive and persistent enhancement of the locomotor stimulatory effects of subsequent cocaine challenges (Heidbreder et al. 1996; Henry and White 1995; Kalivas and Duffy 1993), it is important to note that cross-sensitization between the locomotor stimulatory effects of cocaine and direct D2-like agonists has been reported with self-administered cocaine (De Vries et al. 2002; Dias et al. 2004; Edwards et al. 2007), but not experimenter-administered cocaine (e.g., Djano and Martin-Iverson 2000). The inability of cocaine to cross-sensitize rats to the locomotor stimulatory effects of pramipexole in the present studies provides further support for this dichotomy between experimenter- and self-administered cocaine. However, it is possible that cross-sensitization was not observed due to the relatively low doses of pramipexole that were used, the relatively short period of time that pramipexole-induced locomotor activity was measured, or some combination thereof (De Vries et al. 2002; Dias et al. 2004; Edwards et al. 2007). Alternatively, it has also been suggested that the development (Henry et al. 1998; Hoffman and Wise 1993), and expression (Djano and Martin-Iverson 2000; Kiyatkin 1994) of cross-sensitization between the locomotor stimulatory effects of experimenter-administered cocaine and D2-like

agonists depends upon an activation of both D1- and D2-like receptors, raising the possibility that basal extracellular dopamine levels were insufficient to activate D1-like receptors during the locomotor tests. Nevertheless, when taken together with previous reports of food restriction-induced enhancements of the locomotor stimulatory effects of pramipexole under identical experimental conditions (Collins et al. 2008), the current findings suggest that locomotor assays are insufficient to detect changes in D₂ and/or D₃ receptor sensitivity following experimenter-administered cocaine in rats.

Despite this inability of prior cocaine administrations to alter the locomotor effects of pramipexole, cocaine-treated rats displayed a progressive and persistent leftward and upward shift of the D₃-mediated (Collins et al. 2007; Collins et al. 2005), ascending limb of the dose-response curve for pramipexole-induced yawning, as well as an increased sensitivity to the D₂-mediated (Collins et al. 2007; Collins et al. 2005), inhibitory effects of pramipexole on yawning when tested after the cessation of cocaine administration. These findings not only suggest that yawning assays are sensitive enough to differentiate between cocaine-induced changes in the D₃- and D₂- mediated behavioral effects of pramipexole, but also that there are temporal differences in these effects. Progressive enhancements of D₃-mediated behaviors emerged shortly after the cessation of cocaine administration (~72h) and persisted for at least 42d, whereas increases in the sensitivity of rats to some D₂-mediated behaviors do not become apparent until after a prolonged period of time since the cessation of cocaine administration (~42d). Importantly, cocaine treatment failed to alter the induction of yawning by the indirect cholinergic agonist, physostigmine, or the 5-HT₂ receptor agonist, TFMPP, suggesting that the cocaine-induced changes in pramipexole-induced yawning represent changes in the sensitivity and/or expression of the D₃ and D₂ receptors that mediate yawning, and not a more general enhancement of the signaling pathways within the paraventricular nucleus that regulate yawning.

Although the relatively low levels of D₂ and D₃ receptor expression, and small volume of the paraventricular nucleus precluded the ability to accurately assess changes in the binding properties of D2-like receptors within this brain region, the results of radioligand binding studies performed on ventral striatal tissue provide molecular evidence to support these behavioral changes. Importantly, although ³H-spiperone binds to both the D₂ and D₃ receptor subtypes, its preferential affinity for D₂ compared to D₃ receptor (~ 80-fold selective for D₂ over D₃ in rat brain using the current in vitro assay conditions; Levant and DeSouza 1993), combined with the differential levels of expression for the D₂ and D₃ receptors (D₂ expressed at ~100-fold higher levels than D₃; Levesque et al. 1992), means that the cocaine-induced changes in the B_{max} and K_d obtained with ³H-spiperone in the current studies can be primarily attributed to changes in the binding properties of D₂ receptors. Similar to what was observed with pramipexole-induced yawning at the 24h time-point, cocaine treatment failed to alter the binding properties of D₃ or D2-like receptors at this early time point. Although previous studies in mice, rats, and rhesus monkeys have suggested that cocaine is capable of altering D2-like receptor levels within a few hours of the last cocaine administration (Bailey et al. 2008; Le Foll et al. 2005; Maggos et al. 1998; Nader et al. 2002; Nader et al. 2006; Tsukada et al. 1996), it is important to note that differences in the dosing regimen, route of administration, and length of time since cocaine treatment are all known to affect changes in D2-like receptor density (e.g., Anderson and Pierce 2005). Consistent with the cocaine-induced changes in pramipexole-induced yawning that developed over the course of the study, significant increases in D₃ receptor binding, as well as significant decreases in D2-like receptor density, and increases in D2-like receptor affinity were observed in the ventral striatum of cocaine-treated rats 42d after the cessation of cocaine. Not only are these cocaine-induced changes in D₃ receptor binding similar to the time-dependent increases in D₃ receptor binding and cell surface expression that have been reported following periods of cocaine self-administration in rats (Conrad et al. 2010;

Neisewander et al. 2004), but the observed decreases in D₂-like receptor density are also similar to recent reports of decreases in D₂ receptor expression in rats with a history of cocaine self-administration (Conrad et al. 2010). It should be noted, however, that similar decreases in D₂ and increases in D₃ receptor densities have also been reported following the repeated administration of high doses of D₂-like agonists (Stanwood et al. 2000). Although an interaction between cocaine pretreatment and pramipexole cannot be ruled out, the fact that repeated pramipexole administration failed to alter the binding properties of either D₂-like or D₃ receptors in the saline-treated group suggests that the observed changes in D₂-like and D₃ receptor binding resulted from the repeated administration of cocaine, and not pramipexole.

Curiously, unlike the rapid sensitization of rats to the D₂-mediated effects of pramipexole that has been observed with food restriction (Collins et al. 2008), cocaine treatment appeared to have a delayed effect on the D₂-mediated effects of pramipexole as no changes in the sensitivity of rats to the inhibition of yawning, induction of hypothermia, and locomotor stimulation were observed throughout the first 21d after the cessation of cocaine administration. Importantly, however, increases in the sensitivity of rats to the inhibitory effects of pramipexole on yawning corresponded to a significant decrease in k_d for [³H]-spiperone at D₂-like receptors at 42d, suggesting that the observed behavioral changes were mediated by an increased affinity of pramipexole at the D₂ receptor. Although the current studies were not designed to differentiate between the high- and low-affinity states of D₂ receptors, it is possible that the increased sensitivity of the cocaine-treated rats to the D₂-mediated inhibition of pramipexole-induced yawning was also influenced by an increased proportion of D₂ receptors in the high-affinity state as has been reported in rats following cocaine self-administration (Briand et al. 2008), or sensitization to caffeine (Simola et al. 2008), or amphetamine (Seeman 2009). In addition to providing convergent evidence to support a cocaine-induced sensitization of the D₂ receptor, the current studies also suggest that increases in D₃ receptor binding may underlie the progressive increase in the sensitivity of cocaine-treated rats to the D₃-mediated induction of yawning by low doses of pramipexole. However, the limitations of single-point assays make it impossible to determine whether these behavioral changes resulted from an increase in D₃ receptor density, D₃ receptor affinity, or some combination of the two. Interestingly, Hamilton and colleagues (2010) have recently reported a similar enhancement of D₂-like agonist-induced yawning in male monkeys that were exposed to cocaine in utero. Not only was this effect positively correlated with the maximal daily dose of cocaine received during gestation, but this enhanced yawning response was observed 13 years later, suggesting that cocaine exposure may result in long lasting, if not permanent enhancements of the function and/or sensitivity of D₃ receptors.

In addition to altering the potency and/or effectiveness of drugs (e.g., psychostimulants or D₂-like agonists) to stimulate locomotor activity or elicit other behavioral effects, the neuroadaptations that underlie behavioral sensitization are also thought to play a crucial role in the development of addiction-like behaviors in animals (e.g., Kalivas and Stewart 1991; Robinson and Berridge 1993; Vezina 2004), such as the increased capacity of cocaine and direct D₂-like agonists to increase responding that was previously reinforced by cocaine (De Vries et al. 1998; De Vries et al. 2002; Dias et al. 2004; Edwards et al. 2007). Although these studies suggest that increases in responding are mediated by a sensitization of D₂-like receptors in general, there is growing evidence to support a role for the D₃ receptor, specifically, in the capacity of cocaine-paired cues to reinstate, or maintain responding following periods of cocaine self-administration (Cervo et al. 2007; Collins and Woods 2009; Gal and Gyertyan 2006; Gilbert et al. 2005). Given this putative role for the D₃ receptor, it is interesting to note that the capacity of these cues to reinstate responding appears to be positively correlated with the duration of abstinence insofar as progressive and

persistent increases in responding have been observed over the first three months of abstinence (e.g., Grimm et al. 2001; Lu et al. 2004). This effect appears to be similar in magnitude and time course to the increases in D₃-mediated yawning observed in the current studies.

In summary, these studies provide convergent behavioral and molecular evidence to support a cocaine-induced enhancement of both D₃- and D₂-mediated elicited behavioral effects in rats. The temporal differences in the development of these effects suggest that increases in the sensitivity of rats to the D₃-mediated effects of pramipexole emerge as early as 72h after the cessation of cocaine administration with progressive and persistent enhancements observed for at least 42d, whereas increases in the sensitivity of rats to the D₂-mediated behavioral effects of pramipexole do not become apparent until after longer periods of time have passed. In addition to suggesting that D₂-like agonist-induced yawning may provide a valuable tool for the evaluation of the functional changes in D₃ and D₂ receptors that occur following cocaine administration, when taken together with previous reports (Conrad et al. 2010; Hamilton et al. 2010; Neisewander et al. 2004) these studies provide further support for the notion that the progressive and persistent increases in the density, function, and/or sensitivity of the D₃ receptor that occur during periods of abstinence or withdrawal may contribute to the enduring vulnerability to relapse commonly observed in human drug abusers.

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Figure 1.

Locomotor activity induced by i.p. saline (Left) and 15.0 mg/kg; i.p. (Right) in cocaine- and saline-treated rats 42d after the cessation of repeated cocaine or saline administration. Data represent the mean $\pm$ S.E.M. ($n=6$ per group) of the total locomotor activity for each 5-min bin, and for each 60-min bin (inset panels) following administration of saline and cocaine. *, $p<0.05$; **, $p<0.01$. Significant differences in the amount of locomotor activity for cocaine- and saline-treated groups were determined by two-way, repeated-measures ANOVA with post-hoc Bonferroni tests (5-min bins) and by two-tailed, paired t-tests.

Figure 2.

Dose-response curves for pramipexole-induced yawning in cocaine- and saline-treated rats as assessed 24hr, 72hr, 10d, 21d, and 42d after the cessation of repeated cocaine or saline administration. Data represent the mean $\pm$ S.E.M. (n=8 per group) number of yawns observed during the 30-minute period following each injection. *, $p < 0.05$; ***, $p < 0.001$. Significant effect of cocaine treatment on yawning compared to the saline-treated group at that particular time point as determined by two-way, repeated-measures ANOVA with post-hoc Bonferroni tests. +, $p < 0.05$. Significant difference in the amount of yawning observed for the saline-treated group compared to the 24hr saline-treated time point as determined by two-way, repeated-measures ANOVA with post-hoc Bonferroni tests. ##, $p < 0.01$; ###, $p < 0.001$. Significant difference in the amount of yawning observed for the cocaine-treated group compared to the 24hr cocaine-treated time point as determined by two-way, repeated-measures ANOVA with post-hoc Bonferroni tests.

Figure 3. Dose-response curves for pramipexole-induced changes in core body temperature (Left Panels) and locomotor activity (Right Panels) in cocaine- and saline-treated rats as assessed 24hr, 72hr, 10d, and 21d after the cessation of repeated cocaine or saline administration. Data represent the mean $\pm$ S.E.M. (n=6 per group) change in core body temperature as measured 30 minutes after each injection compared to the core body temperature 1 minute prior to the first injection, and mean $\pm$ S.E.M. (n=6 per group) locomotor activity counts summed over the 30 minutes following each injection. +, p<0.05; ++, p<0.01. Significant difference in the change in core body temperature or locomotor activity for the saline-treated group compared to the 24hr saline-treated time point as determined by two-way, repeated-measures ANOVA with post-hoc Bonferroni tests. #, p<0.05; ##, p<0.01; ###, p<0.001. Significant difference in the change in core body temperature or locomotor activity for the cocaine-treated group compared to the 24hr cocaine-treated time point as determined by two-way, repeated-measures ANOVA with post-hoc Bonferroni tests.

Figure 4. Quantification of [³H]7-OH-DPAT bound to ventral striatal tissue collected from cocaine- and saline-treated rats 24hr and 42d after the cessation of repeated cocaine or saline administration. ***, $p < 0.001$. Significant effect of treatment as determined by two-tailed paired t-tests.

Physostigmine- and TFMPP-induced yawning in cocaine- and saline-treated rats 24–72h, and 41–43d after the cessation of repeated cocaine or saline treatment.

Table 1

Mean Yawns/30 min (± SEM)					
		Saline-Treated		Cocaine-Treated	
Compound	Dose	24/72 hr	41/43 d	24/72 hr	41/43 d
Physostigmine	Vehicle	1.4 (1.0)	2.3 (1.6)	1.8 (1.0)	2.3 (0.9)
	0.032 mg/kg	4.0 (1.4)	2.9 (0.8)	2.5 (1.1)	2.8 (1.0)
	0.1 mg/kg	6.0 (3.2)	7.9 (2.3)	3.6 (1.3)	4.6 (1.2)
	0.32 mg/kg	5.6 (2.2)	1.5 (0.5)	7.1 (2.1)	2.0 (0.8)
TFMPP	Dose				
	Vehicle	1.6 (0.7)	1.0 (0.5)	2.8 (1.1)	2.6 (1.0)
	1.0 mg/kg	4.9 (1.2)	2.6 (1.2)	3.6 (1.2)	4.8 (2.1)
	3.2 mg/kg	11.8 (2.5)	3.3 (0.8)+++	8.4 (1.5)	3.9 (0.7)
	10.0 mg/kg	3.6 (1.0)	0.0 (0.0)	3.0 (1.4)	0.3 (0.2)

+++ p<0.001. Significant difference in the amount of yawning observed for the saline-treated group compared to the 24hr saline-treated time point as determined by two-way, repeated-measures ANOVA with post-hoc Bonferroni tests.

Table 2

Binding properties for D2-like receptors as determined by scatchard analysis of [3H]-spiperone binding to ventral striatal tissue collected from cocaine- and saline-treated rats 24hr and 42d after the cessation of repeated cocaine or saline administration.

Treatment	Bmax (fmol/mg): mean ($\pm$ SEM)		Kd (nM): mean ($\pm$ SEM)	
	24 hr	42 d	24 hr	42 d
Saline	334.0 (44.7)	290.4 (22.2)	0.17 (0.05)	0.14 (0.1)
Cocaine	321.9 (34.4)	227.9 (12.0) *,#	0.12 (0.03)	0.05 (0.004) ###

* $p < 0.05$. Significant effect of cocaine treatment on Bmax compared to the saline-treated group at the 42d time point as determined by two-tailed, paired t-tests.

$p < 0.05$;

$p < 0.001$. Significant difference in Bmax or Kd for the cocaine-treated group at the 42d time point compared to the cocaine-treated group at the 24hr time point as determined by two-tailed, paired t-tests.