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The limbic circuitry underlying cocaine seeking encompasses the PPTg/LDT

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Abstract

The direct glutamatergic projection from the medial prefrontal cortex (mPFC) to the nucleus accumbens plays a critical role in mediating the reinstatement of cocaine-seeking behavior. The mPFC also sends glutamatergic projections to the pedunculopontine tegmental nucleus (PPTg)/laterodorsal tegmental nucleus (LDT), which in turn sends glutamatergic and cholinergic efferents to the ventral tegmental area (VTA) where they synapse on dopaminergic cells that innervate limbic structures including the nucleus accumbens. The goal of these experiments was to examine a potential role for the PPTg/LDT in the reinstatement of cocaine seeking. All rats were trained to self-administer cocaine (0.25 mg, i.v.) on a fixed-ratio 5 (FR5) schedule of reinforcement. Cocaine self-administration behavior was extinguished and a series of subsequent pharmacological experiments were performed to assess the potential role of the mPFC, PPTg/LDT and VTA in the reinstatement of cocaine seeking. Administration of the D1-like dopamine receptor agonist SKF-81297 (1.0 μg) directly into the mPFC produced a small, but statistically significant, increase in cocaine-seeking behavior. Furthermore, microinjection of the ionotropic glutamate receptor antagonist CNQX (0.3 μg) into the PPTg/LDT attenuated the reinstatement of drug seeking induced by a priming injection of cocaine (10 mg/kg, i.p.). Intra-VTA administration of CNQX, the nicotinic receptor antagonist mecamylamine (10.0 μg) or the muscarinic receptor antagonist scopolamine (24.0 μg) also blocked cocaine seeking. Taken together, these results suggest that cocaine priming-induced reinstatement of drug seeking is mediated in part by a serial polysynaptic limbic subcircuit encompassing the mPFC, PPTg/LDT and VTA.

Keywords

medial prefrontal cortex; ventral tegmental area; nucleus accumbens; dopamine; glutamate; acetylcholine

Introduction

A growing literature indicates that afferents from the medial prefrontal cortex (mPFC) play critical roles in priming-induced reinstatement of cocaine seeking (Kalivas et al., 2005; Schmidt et al., 2005). For example, activating the cortico-accumbal glutamatergic pathway reinstates cocaine seeking (Park et al., 2002; McFarland et al., 2003). The mPFC may also

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influence the activity of the dopaminergic projections from the VTA to the nucleus accumbens, which are critically involved in the reinstatement of cocaine seeking (see Anderson & Pierce, 2005; Schmidt *et al.*, 2005). However, the regulation of the mesoaccumbens dopaminergic projections by the mPFC must be indirect since glutamatergic afferents from the mPFC to the VTA do not synapse on dopaminergic cells that project to the nucleus accumbens (Carr & Sesack, 2000).

The mesopontine tegmentum, which is composed of the rostral pedunculpontine tegmental nucleus (PPTg) and the more caudal laterodorsal tegmental nucleus (LDT), receives excitatory input from limbic sites in the forebrain including the mPFC (Sesack *et al.*, 1989; Semba & Fibiger, 1992) and sends glutamatergic and cholinergic afferent projections to the VTA, where they synapse on dopaminergic neurons some of which project to the nucleus accumbens (Hallanger & Wainer, 1988; Cornwall *et al.*, 1990; Semba & Fibiger, 1992; Steininger *et al.*, 1992; Futami *et al.*, 1995; Charara *et al.*, 1996; Oakman *et al.*, 1999; Omelchenko & Sesack, 2005). Thus, the PPTg/LDT is positioned anatomically to receive information from the mPFC and relay it to dopaminergic cells in the VTA. Consistent with this notion, stimulation of the PPTg/LDT activates dopaminergic cells in the VTA (Kelland *et al.*, 1993; Floresco *et al.*, 2003; Lodge & Grace, 2006b; a) and leads to increased dopamine release in the striatum/accumbens (Floresco *et al.*, 2003; Forster & Blaha, 2003). Moreover, activation of the mPFC increased extracellular dopamine in the accumbens, an effect that was blocked by administration of glutamate receptor antagonists into the VTA but not the nucleus accumbens (Murase et al., 1993; Taber et al., 1995; Karreman et al., 1996). Collectively, these results suggest that the mPFC modulates the activity of the mesoaccumbal dopaminergic pathway indirectly via a polysynaptic circuit encompassing the PPTg/LDT.

Several lines of evidence indicate that the PPTg/LDT processes information related to both drug and natural reinforcers (Winn, 2006). Thus, lesioning the PPTg attenuated the conditioned rewarding properties of drugs of abuse (Bechara & van der Kooy, 1989; Olmstead *et al.*, 1998) as well as natural reinforcers including food (Bechara & van der Kooy, 1992) and sex (Kippen & van der Kooy, 2001). Lesions of the PPTg (Alderson et al., 2003) or the LDT (Nelson et al., 2007) also attenuated the development of behavioral sensitization to d-amphetamine. Moreover, pharmacological inactivation of the PPTg reduced cocaine self-administration maintained on fixed ratio and progressive ratio schedules of reinforcement (Corrigall et al., 1999; Corrigall et al., 2002). It seems plausible, therefore, that the PPTg/LDT complex might also play an important role in the reinstatement of cocaine seeking, an animal model of relapse, but this hypothesis has not yet been examined.

The current experiments used pharmacological methodologies to assess a potential role for the PPTg/LDT, as well as its afferent and efferent connections, in cocaine priming-induced reinstatement of drug seeking. Our results are consistent with the activation of a serial circuit encompassing the mPFC, PPTg/LDT, VTA and nucleus accumbens playing a critical role in the reinstatement of cocaine seeking.

Materials and Methods

Animals and Housing

Male Sprague Dawley rats (*Rattus norvegicus*) weighing 250–300 g were obtained from Taconic Laboratories (Germantown, N.Y., USA). Animals were single-housed with food and water available *ad libitum* (rats undergoing food reinstatement experiments were placed on restricted diets, as outlined below). All animals were housed in a colony maintained on a 12-hr/12-hr light/dark cycle with the lights on at 7:00 a.m. All experimental procedures were

performed during the light phase. All experimental protocols were in accordance with the guidelines set forth by the National Institutes of Health and were approved by the Boston University School of Medicine Institutional Animal Care and Use Committee.

Materials

All behavioral experiments were conducted in ventilated, sound attenuating operant chambers purchased from Med-Associates Inc. (East Fairfield, VT). Each operant chamber was equipped with both inactive and active response levers, a food pellet dispenser as well as an automated injection pump for administering drug or vehicle solutions intravenously.

Surgery

Rats were allowed one week to acclimate to their home cages upon arrival. Prior to surgery, the rats were anesthetized with 80 mg/kg ketamine and 12 mg/kg xylazine (Sigma/RBI, St. Louis, MO). An indwelling catheter (CamCaths; Cambridge, UK) was inserted into the right, external jugular vein and sutured securely in place. The catheter was connected to a mesh backmount, which was implanted subcutaneously above the shoulder blades. In order to prevent infection and to maintain patency, catheters were flushed daily with 0.3 ml of a solution of the antibiotic Timentin (0.93 mg/ml) dissolved in heparinized saline. When not in use, the catheters were sealed with plastic obturators.

Immediately following implantation of the indwelling catheter, some rats were mounted in a stereotaxic apparatus (Kopf Instruments, CA) and bilateral guide cannulae (14 mm 24 gauge tubing, Small Parts Inc., Roanoke, VA) were implanted 2 mm dorsal to the mPFC, 1 mm dorsal to the PPTg/LDT or 1 mm dorsal to the VTA according to the following stereotaxic coordinates from the atlas of Paxinos and Watson (1997): mPFC: +2.5 mm anteroposterior (A/P, relative to bregma), ±0.5 mm mediolateral (M/L, relative to bregma) and −2.0 mm dorsoventral (D/V, relative to dura): PPTg/LDT: −7.8 mm A/P, ±2.0 mm M/L and −6.2 mm D/V: VTA: −5.8 mm A/P,±0.5 mm M/L and −7.0 mm D/V. Guide cannulae were cemented in place by affixing dental acrylic to three stainless steel screws fastened to the skull. Obturators (14 mm, 33 gauge stainless steel wire, Small Parts Inc., Roanoke, VA) were inserted into each guide cannula in order to prevent occlusion.

Cocaine Self-Administration

After surgery, rats were allowed seven days to recover before behavioral testing commenced. Initially, rats were placed in operant chambers daily and allowed to lever press for intravenous cocaine (0.25 mg cocaine/59 μl saline, infused over a 5 sec period) on a fixed-ratio 1 (FR1) schedule of reinforcement. Each session began with the i.v. administration of 59 μl cocaine (0.25 mg) to fill the catheter. Rats were allowed to self-administer a maximum of 30 injections per 120-minute operant session. Stable responding on the FR1 schedule was defined as less than 15% variation in response rates over three consecutive self-administration days. After stable responding was achieved, animals were switched to a fixed-ratio 5 (FR5) schedule of reinforcement. The maximum number of injections was again limited to 30 per daily self-administration session under the FR5 schedule. For both the FR1 and FR5 schedules, a 20 second time-out period followed each cocaine infusion, during which time active lever responses were tabulated but had no scheduled consequences. Responses made on the inactive lever, which had no scheduled consequences, were also recorded during both the FR1 and FR5 training sessions.

Extinction and reinstatement of cocaine seeking

Following approximately 21 days of daily cocaine self-administration sessions, drug-seeking behavior was extinguished by replacing the cocaine with 0.9% saline. Daily two-hour

extinction sessions continued until responding on the active lever was <15% of the response rate maintained by cocaine self-administration under the FR5 schedule of reinforcement. Typically, it took approximately 7 days for rats to meet this criterion.

The FR5 schedule of reinforcement was used throughout the reinstatement phase of the experiment. When an animal met the response requirement (five presses on the active lever) an intravenous infusion of saline was administered. Using a between-session reinstatement paradigm, each daily reinstatement session was followed by extinction days until responding was less than 15% of the maximum number of responses maintained by cocaine self-administration. In general, it took 1–2 days of extinction for each animal to reach criterion between reinstatement sessions. Using this experimental design, subjects underwent a series of extinction and reinstatement sessions that lasted approximately 16 days. During this period, animals may lose the ability to reinstate active lever responding following a priming injection of cocaine. However, we have previously shown that reinstatement of cocaine seeking persists for at least 20 days after the initial extinction of cocaine self-administration (Park et al., 2002; Anderson et al., 2003). Moreover, we were able to assess the magnitude of reinstatement by randomly administering priming injections of cocaine throughout the reinstatement phase of the experiment. All animals displayed stable drug seeking, which was operationally defined as greater than 30 active lever responses per 2-hr operant session, during the reinstatement phase of the experiment.

Once self-administration behavior was extinguished, the ability of a priming injection of cocaine (10 mg/kg, i.p.) or its vehicle (0.9% saline) to reinstate cocaine seeking was assessed. On subsequent test days, the selective D1-like dopamine receptor agonist R(+)-SKF-81297 hydrobromide (1.0 μg/0.5 μl, Sigma/RBI, St. Louis, MO) or its vehicle (0.9% saline) was microinjected into the mPFC in order to test its ability to reinstate cocaine-seeking behavior. Animals were placed into the operant chambers immediately following the intra-mPFC microinfusion of SKF-81297 and the 2-hr reinstatement session began. Additional experiments assessed the ability of an intra-PPTg/LDT microinjection of an ionotropic glutamate receptor antagonist or an intra-VTA microinjection of a nicotinic acetylcholine receptor antagonist, muscarinic acetylcholine receptor antagonist or ionotropic glutamate receptor antagonist to attenuate reinstatement of drug seeking elicited by a priming injection of cocaine (10 mg/kg, i.p.). The ionotropic glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX; 0.03 or 3.0 μg/0.5 μl, Tocris, Ellisville, MS) was administered directly into the PPTg/LDT ten minutes prior to a priming injection of cocaine (10 mg/kg, i.p.). Furthermore, CNQX (0.03 or 3.0 μg/0.5 μl), (−)-scopolamine hydrochloride (2.4 or 24.0 μg/0.5 μl, Sigma/RBI, St. Louis, MO) or mecamylamine hydrochloride (1.0 or 10.0 μg/0.5 μl, Sigma/RBI, St. Louis, MO) was microinjected into the VTA ten minutes prior to a systemic priming injection of cocaine (10 mg/kg, i.p.) in order to assess their ability to attenuate cocaine priming-induced reinstatement.

The dose ranges for each of the aforementioned pharmacological compounds were based on the following rat microinjection experiments: SKF-81297 (Beyer & Steketee, 2002; Cools *et al.*, 2002; Schmidt *et al.*, 2006), CNQX (Cornish et al., 1999; See et al., 2001; Park et al., 2002), (−)scopolamine hydrochloride (Ikemoto & Goeders, 2000; Corrigall *et al.*, 2002; See *et al.*, 2003; Pratt & Kelley, 2005) and mecamylamine hydrochloride (Sziraki *et al.*, 2002; Forster & Blaha, 2003; Sharf & Ranaldi, 2006).

Microinjection Procedures

Obturators were removed from the guide cannulae and 33 gauge stainless steel microinjectors (Small Parts Inc.) were inserted. Microinjectors were cut to a length that extended 2 mm (for microinjections targeting the mPFC) or 1 mm (for microinjections

targeting the PPTg/LDT or VTA) below the ventral end of the guide. Bilateral infusions were performed simultaneously over a 120 second time period in a total volume of 0.5 μl per side. Microinjectors were left in place for 60 seconds following the microinfusions, in order to allow the drug solution or vehicle to diffuse away from the tips of the cannulae, before they were removed. Animals were placed immediately into the operant chambers following microinjection of SKF-81297 or its vehicle (0.9 % saline) and the reinstatement session began without delay. Animals pretreated intra-cranially with CNQX, scopolamine, mecamylamine or their vehicle (0.9% saline) 10 minutes prior to a priming injection of cocaine (10 mg/kg, i.p.) were placed in the operant chambers immediately following the cocaine priming injection.

The goal of the experimental design was to have each animal serve as its own control and receive up to six microinjections per brain region (i.e. two doses plus vehicle for two drugs for a maximum of 6 microinjections per brain region). However, we were frequently forced to deviate from this experimental design when technical difficulties (i.e. blocked microinjection cannulae or loss of catheter patency) made it impossible to test all doses of a compound plus vehicle in an entire cohort of subjects. In every case, however, an animal received a minimum treatment of one drug dose and its vehicle. In order to control for potential rank order effects of drug or vehicle treatments, the agonist, antagonists and vehicle were counterbalanced across reinstatement sessions and within cohorts of animals. Using this experimental design, no order effects of drug treatments were observed.

Food Reinstatement

Potential nonspecific rate-suppressing effects of the pharmacological compounds tested were evaluated by assessing the influence of intra-PPTg/LDT CNQX or intra-VTA CNQX, scopolamine or mecamylamine on reinstatement of food-reinforced responding. Rats were trained initially to lever press for sucrose pellets (Research Diets, Inc., New Brunswick, NJ) on a FR1 schedule of food reinforcement during 1-hour operant sessions. Once animals achieved stable responding for food (defined as <15% variation in responding over two consecutive days) on the FR1 schedule of reinforcement, the response requirement was increased to an FR5 schedule of reinforcement. Animals were limited to 30 sucrose pellets within a 1-hour operant session and were food restricted to four pellets of lab chow (Harlan Teklad, Wilmington, DE) in their home cages for the duration of the experiment.

After two weeks of food-maintained responding on the FR5 schedule of reinforcement, responding was extinguished by inactivating the food dispenser so that every 5 lever presses had no scheduled consequences. Once lever responding decreased to <15% of the maximum number of responses completed during food self-administration, animals proceeded to reinstatement testing. Bilateral microinjections of CNQX (0.3 μg/0.5 μl) or vehicle into the PPTg/LDT or scopolamine (24.0 μg/0.5 μl), mecamylamine (10.0 μg/0.5 μl), CNQX (0.3 μg/0.5 μl) or vehicle into the VTA were administered ten minutes prior to the beginning of the reinstatement session. The experimenter remotely administered one sucrose pellet every 2 minutes for the first 10 minutes of the reinstatement session. A between-session paradigm was used so that each daily 1-hour reinstatement session was followed by an extinction session the following day until responding was again <15% of the response rate maintained by food.

Histology and Verification of Cannulae Placements

After the microinjection experiments were concluded, animals were overdosed with a systemic injection of pentobarbital (100 mg/kg) and perfused intracardially with 60 ml of 0.9% saline followed by 60 ml of 10% formalin. The brains were removed and stored in 10% formalin. Subsequently, 100–150 μm thick coronal sections were taken at the level of

the mPFC, PPTg/LDT or VTA with a Vibratome (Technical Products Int., St. Louis, MO.). These sections were mounted on gel-coated slides and stained with Cresyl violet. An individual blind to the animals' behavioral responses determined cannulae placements as well as potential drug- or cannula-induced neuronal damage. Light microscopy was used to determine cannulae placements as well as the presence and extent of cell death and associated gliosis.

Behavioral Data Analyses

Total active and inactive lever responses during the reinstatement phase of experiments with SKF-81297 in the mPFC were analyzed with unpaired t-tests. For the experiments utilizing CNQX in the PPTg/LDT, and CNQX, scopolamine and mecamylamine in the VTA, the total mean active and inactive lever responses during the reinstatement phase were analyzed with one-way analyses of variance (ANOVAs) or unpaired t-tests. Pairwise comparisons were made with Tukey's HSD ($P<0.05$).

Drugs

Cocaine was obtained from the National Institutes of Drug Abuse (NIDA) and dissolved in sterile 0.9% saline. R(+)-SKF-81297 hydrobromide (1.0 μg/0.5 μl), (−)-scopolamine hydrochloride (2.4 or 24.0 μg/0.5 μl), and mecamylamine hydrochloride (1.0 or 10.0 μg/0.5 μl) were purchased from Sigma/RBI (St. Louis, MO) and dissolved in sterile 0.9% saline. CNQX disodium salt (0.03 or 3.0 μg/0.5 μl) was purchased from Tocris (Ellisville, MS) and dissolved in sterile 0.9% saline.

Results

The mean (±S.E.M.) total active and inactive lever responses during the last day of cocaine self-administration for all subjects used in the following experiments are included in Table 1.

Microinjection of the D1-like dopamine receptor agonist SKF-81297 into the medial prefrontal cortex reinstated cocaine seeking in rats

The cannulae placements in this experiment are presented in Figure 1A. Microinjections were targeted to the dorsal mPFC including the dorsal prelimbic cortex and the anterior cingulate cortex. The data depicted in Figure 1B show the cumulative number of responses (mean±S.E.M.) on both the active and inactive levers during the reinstatement phase of the experiment. Animals were administered saline or 1.0 μg SKF-81297 into the medial prefrontal cortex prior to the start of the reinstatement session. Active lever data were analyzed with an unpaired t-tests and a significant difference was found between the saline and 1.0 μg SKF-81297 treatments [$t(14)=4.642$, $p<0.0004$]. No significant difference was found between saline and 1.0 μg SKF-81297 treatments in terms of inactive lever responding [$t(14)=1.995$, $p<0.0658$]. Figure 1B displays responses made on the active lever during each of the 10-minute components of the 2-hr operant session for the intra-mPFC saline and 1.0 μg SKF-81297 treatments.

Microinjection of the AMPA/kainate receptor antagonist CNQX into the PPTg/LDT dose-dependently attenuated cocaine priming-induced reinstatement of drug seeking but had no influence on the reinstatement of food-seeking behavior in rats

Animals were administered saline ($n = 9$), 0.03 ($n = 6$) or 0.3 μg ($n = 9$) CNQX directly into the PPTg/LDT prior to a priming injection of cocaine (10 mg/kg i.p.). The total active lever responses (mean±S.E.M.) during the reinstatement session are shown in Figure 2A. These data were analyzed with a one-way ANOVA, which revealed a significant main effect of

treatment [$F(2,21)=17.208$, $p<0.0001$]. Subsequent pairwise analyses (Tukey's HSD, $P<0.05$) showed total active lever responses were significantly different between the saline and both the 0.03 μg CNQX as well as 0.3 μg CNQX treatments. Inactive lever responses (mean±S.E.M.) following intra-PPTg/LDT administration of saline, 0.03 or 0.3 μg CNQX prior to a priming injection of cocaine (10 mg/kg, i.p.) were analyzed with a one-way ANOVA and plotted in Figure 2A. No significant differences in responses made on the inactive lever induced by a 10 mg/kg priming injection of cocaine were revealed following microinjection of saline, 0.03 or 0.3 μg CNQX into the PPTg/LDT [$F(2,21)=1.493$, $p<0.2477$]. The active lever responses (mean±S.E.M.) made during each of the 10-minute components in the intra-PPTg/LDT saline + cocaine (10 mg/kg, i.p.), 0.03 μg CNQX + cocaine (10 mg/kg, i.p.) and 0.3 μg CNQX + cocaine (10 mg/kg, i.p.) treatments are shown in Figure 2B.

When using receptor antagonists that decrease reinstatement of cocaine-seeking behavior, such as CNQX, general behavioral suppression is a concern. In the present experiments, two measures were used to evaluate potential nonspecific rate suppressing effects of intra-cranial microinjections of CNQX. First, each operant chamber was equipped with an inactive lever, responses on which are often used as a measure of nonspecific alterations in lever responding. While intra-PPTg/LDT administration of CNQX had no significant effect on inactive lever responding, one could argue that responses were uniformly too low to meaningfully assess potential rate suppressant effects of drug treatment. Therefore, we also assessed the ability of intra-PPTg/LDT microinjections of CNQX to alter food reinstatement, where noncontingent administration of food reinstates responding previously maintained by food reinforcement. Intra-PPTg/LDT infusion of the same dose of CNQX that attenuated cocaine priming-induced reinstatement did not affect food reinstatement (see Figure 2C). Animals were administered 0.3 μg CNQX ($n = 6$) or saline ($n = 6$) directly into the PPTg/LDT 10 minutes prior to a 1 hour food reinstatement test session. The active lever responses (mean±S.E.M.) obtained following microinjection of saline or 0.3 μg CNQX into the PPTg/LDT are depicted in Figure 2C. These data were analyzed with an unpaired t-test, which did not reveal a significant difference between saline or 0.3 μg CNQX treatments [$t(10)=0.425$, $p<0.6801$]. These data suggest that attenuation of drug seeking induced by a priming injection of cocaine was not due to general motor impairment.

The location of all the cannula placements in the PPTg/LDT are shown in Figure 2D. The PPTg/LDT is located in the mesopontine tegmentum adjacent to the following nuclei: deep mesencephalic nucleus, microcellular tegmentum nucleus, retrorubral nucleus, pontine reticular nucleus, paralemniscal nucleus, subpendencular tegmental nucleus, epirubrospinal nucleus, reticulotegmental nucleus and the lateral tegmentum nucleus (Paxinos & Watson, 1997). Given the small size of the PPTg/LDT, it is conceivable that drug solutions microininjected into the PPTg/LDT diffused into these areas. Repeated microinjections into the PPTg/LDT resulted in no cases of excessive mechanical damage or cell death.

Microinjection of the AMPA/kainate receptor antagonist CNQX into the VTA dose-dependently attenuated cocaine priming-induced reinstatement of drug seeking

Total active lever responses (mean±S.E.M.) following intra-VTA administration of CNQX prior to a 10 mg/kg priming injection of cocaine during the reinstatement phase of the experiment are plotted in Figure 3A. The active lever data from Figure 3A were analyzed using a one-way ANOVA, which revealed a significant main effect of treatment [$F(2,27)=34.711$, $p<0.0001$]. Pairwise analyses revealed a significant difference in responding on the active lever between the saline ($n=10$) or 0.03 μg ($n=10$) and 0.3 μg ($n=10$) CNQX treatments (Tukey's HSD, $p<0.05$). Total inactive lever responses (mean ±S.E.M.) following a systemic priming injection of cocaine (10 mg/kg i.p.) in animals pretreated with intra-VTA administration of saline ($n = 10$), 0.03 ($n = 10$) or 0.3 ($n = 10$) μg

CNQX are plotted in Figure 3A. These data were analyzed using a one-way ANOVA. No significant difference on inactive lever responding was found between treatments [$F(2,27)=1.362$, $p<0.2723$]. The active lever response rate for each 10-minute component of the operant session during the reinstatement phase of the experiment for intra-VTA saline, 0.03 and 0.3 μg CNQX treatments is shown in Figure 3B.

Ten minutes prior to a 1 hour food reinstatement test session animals received intra-VTA microinfusions of 0.3 μg CNQX (n=6) or saline (n=6). The active lever responses (mean ±S.E.M.) obtained following microinjection of saline or 0.3 μg CNQX into the VTA are depicted in Table 2. These data were analyzed with an unpaired t-test, which did not reveal a significant difference between saline or 0.3 μg CNQX treatments [$t(10)=1.192$, $p<0.2607$]. This finding is consistent with a previous study, which demonstrated that intra-VTA microinfusions of the non-selective ionotropic glutamate receptor antagonist kynurenate did not affect food-seeking behavior (Sun et al., 2005). Collectively, these data indicate that attenuation of cocaine seeking by intra-VTA CNQX was not due to general motor impairment. The cannula placements for all of the microinjections into the VTA (from Figures 3 and 4) are shown in Figure 3C.

Microinjection of mecamylamine or scopolamine into the VTA dose-dependently attenuated cocaine priming-induced reinstatement of drug seeking

Total active lever responses (mean±S.E.M.) following intra-VTA administration of the muscarinic acetylcholine receptor antagonist scopolamine prior to a 10 mg/kg priming injection of cocaine during the reinstatement phase of the experiment are plotted in Figure 4A. A one-way ANOVA was used to analyze the active lever data, which revealed a significant main effect of treatment [$F(2,24)=15.223$, $p<0.0001$]. Further pairwise analyses revealed a significant difference in responding on the active lever between the saline (n=10) or 2.4 μg (n=8) and 24.0 μg (n=9) scopolamine treatments (Tukey's HSD, P<0.05). Total inactive lever responses (mean±S.E.M.) following a systemic priming injection of cocaine (10 mg/kg i.p.) in animals pretreated with microinfusions of saline (n = 10), 2.4 (n = 8) or 24.0 (n = 9) μg scopolamine in the VTA also are plotted in Figure 4A. These data were analyzed using a one-way ANOVA. No significant difference on inactive lever responding was found between saline, 2.4 or 24.0 μg scopolamine treatments [$F(2,24)=0.499$, $p<0.613$].

Animals were administered saline (n=10), 1.0 (n=10) or 10.0 μg (n=8) of the nicotinic receptor antagonist mecamylamine directly into the VTA prior to a priming injection of cocaine (10 mg/kg i.p.). The total active lever responses (mean±S.E.M.) during the reinstatement session are shown in Figure 4B. These data were analyzed with a one-way ANOVA, which revealed a significant main effect of treatment [$F(2,25)=9.003$, $p<0.0011$]. Subsequent pairwise analyses (Tukey's HSD, P<0.05) showed total active lever responses were significantly different between the saline or 1.0 μg mecamylamine and 10.0 μg mecamylamine treatments. Inactive lever responses (mean±S.E.M.) following intra-VTA administration of saline, 1.0 or 10.0 μg mecamylamine prior to a priming injection of cocaine (10 mg/kg, i.p.) were analyzed using a one-way ANOVA and plotted in Figure 4B. No significant difference was found on inactive lever responding following a 10 mg/kg priming injection of cocaine in animals pretreated with microinjections of saline, 1.0 or 10.0 μg mecamylamine into the VTA [$F(2,25)=0.551$, $p<0.5834$).

The active lever response rate for each 10-minute component of the operant session during the cocaine (10 mg/kg, i.p.) priming-induced reinstatement phase of the experiment for animals pretreated with intra-VTA saline, 10.0 μg mecamylamine or 24.0 μg scopolamine is shown in Figure 4C. The cannula placements for all of the microinjections into the VTA (from Figures 3 and 4) are shown in Figure 3C. Repeated microinjections into the VTA did not cause excessive mechanical damage or cell death in any cases.

Ten minutes prior to a 1 hour reinstatement test session, where reinstatement of lever pressing maintained by food reinforcement was initiated by noncontingently administered sucrose pellets, animals received intra-VTA microinfusions of 24.0 μg scopolamine (n=6) or saline (n=6). The active lever responses (mean±S.E.M.) obtained following microinjections of saline or 24.0 μg scopolamine into the VTA are depicted Table 2. These data were analyzed with separate unpaired t-tests, which did not reveal a significant difference between saline or 24.0 μg scopolamine treatments [$t(10)= 0.503$, $p<0.6257$] or between saline or 10.0 μg mecamylamine treatments [$t(10)= 0.067$, $p<0.9476$]. This data suggests that attenuation of cocaine seeking by intra-VTA administration of acetylcholine antagonists was not due to general motor impairment.

The VTA is a heterogeneous structure composed of at least two anatomically (Swanson, 1982; German & Manaye, 1993; Olson *et al.*, 2005) and functionally (Ikemoto *et al.*, 1997a; Ikemoto *et al.*, 1997b; 1998; Carlezon *et al.*, 2000; Olson *et al.*, 2005) distinct subregions differentiated across the rostral-caudal axis. As shown in Figure 3C, the microinjections in these experiments tended to be in more rostral region of the VTA, although there were many microinjections in the caudal VTA as well.

Discussion

While a growing body of evidence indicates that the mPFC, VTA and nucleus accumbens are critically involved in cocaine seeking (Kalivas et al., 2005; Schmidt et al., 2005), a potential role for the PPTg/LDT region in the reinstatement of cocaine seeking has received relatively little attention to date. The present results include three novel findings: i) administration of a D1-like dopamine receptor agonist into the mPFC reinstated cocaine seeking behavior; ii) intra-PPTg/LDT administration of the AMPA/kainate receptor antagonist CNQX dose-dependently attenuated the ability of a priming injection of cocaine to reinstate drug seeking; and iii) administration of AMPA, nicotinic or muscarinic receptor antagonists into the VTA dose-dependently attenuated cocaine priming-induced reinstatement of drug seeking. Collectively, these results indicate that increases in glutamatergic transmission in the PPTg/LDT and enhancement of glutamatergic and cholinergic release in the VTA promote cocaine-priming induced reinstatement of drug seeking. Taken together, the present findings suggest that the PPTg/LDT region may function as an intermediate nucleus relaying information from the mPFC to the mesoaccumbal dopamine system during the reinstatement of cocaine seeking.

The role of medial prefrontal cortex dopamine in cocaine priming-induced reinstatement of drug seeking

Previous microinjection studies demonstrated that transient pharmacological inactivation of the dorsal mPFC (anterior cingulate and dorsal prelimic cortex), but not the ventral mPFC (i.e. ventral prelibic and infralimbic cortices), with GABAergic agonists, tetrodotoxin or dopamine receptor antagonists attenuated cocaine priming-induced reinstatement of drug seeking in rats (McFarland & Kalivas, 2001; Park *et al.*, 2002; Capriles *et al.*, 2003; Sun & Rebec, 2005). Moreover, administration of cocaine, amphetamine or dopamine into the dorsal mPFC reinstated cocaine seeking (McFarland & Kalivas, 2001; Park *et al.*, 2002). The present results showed that administration of the D1-like dopamine receptor agonist, SKF-81297, into the dorsal mPFC reinstated cocaine seeking (albeit at relatively low levels compared to responding maintained on the last day of cocaine self-administration). Previous studies have demonstrated that the mPFC regulates cocaine-seeking behavior through extralimbic circuits encompassing the basolateral amygdala and dorsal hippocampus (Fuchs *et al.*, 2007) and that different subregions of the mPFC play critical roles in drug seeking depending upon the type of contextual cue present during cocaine self-administration (Di Pietro *et al.*, 2006). The low levels of cocaine seeking following administration of

SKF-81297 directly into the mPFC may highlight the importance of this brain region in regulating cocaine-associated contextual cues (the present study did not incorporate contextual cues in its design) and/or the weak ability of mPFC D1-like dopamine receptors to modulate information processing through amygdala- and hippocampus-dependent circuits during cocaine-priming induced reinstatement. Therefore, the complete contribution of stimulating D1-like dopamine receptors in the mPFC on cocaine reinstatement cannot be determined by the present study because a full dose-response curve for intra-mPFC SKF-81297 was not determined. Moreover, a trend toward increased responding on the inactive lever was observed following intra-mPFC administration of SKF-81297, which makes it difficult to establish a firm conclusion based on these findings. However, these results are consistent with previous studies demonstrating a role for prefrontal cortex D1-like dopamine receptors in cocaine reinstatement (Sun & Rebec, 2005) (however see, Capriles *et al.*, 2003). Taken together, these results indicate that increases in dopamine transmission in the dorsal mPFC play a critical role in cocaine-priming induced reinstatement of drug seeking.

A role for the PPTg/LDT in cocaine priming-induced reinstatement of drug seeking

As reviewed above, the mPFC efferents include a glutamatergic projection to the PPTg/LDT region (Sesack *et al.*, 1989; Semba & Fibiger, 1992). The current results demonstrated that microinjection of the AMPA/kainate receptor antagonist CNQX into the PPTg/LDT attenuated the ability of a priming injection of cocaine to reinstate drug seeking. The mesopontine tegmentum consists of the rostral PPTg and the more caudal LDT, which are considered morphologically and physiologically homogenous nuclei (Steriade *et al.*, 1990; Clements *et al.*, 1991; Semba & Fibiger, 1992). Both the PPTg and LDT receive glutamatergic projections from the mPFC and send cholinergic and glutamatergic projections that synapse on dopaminergic neurons in the VTA (Clements *et al.*, 1991; Semba & Fibiger, 1992; Charara *et al.*, 1996; Oakman *et al.*, 1999; Omelchenko & Sesack, 2005). Projections from the mesopontine tegmentum to the midbrain are topographically organized in the rodent brain, such that the LDT and caudal PPTg primarily innervate the VTA, whereas more rostral regions of the PPTg innervate the more medial substantia nigra (Oakman *et al.*, 1995; Blaha *et al.*, 1996; Forster & Blaha, 2000; Maskos, 2008). Although the microinjections of CNQX in the present study did not discriminate among subregions of the mesopontine tegmentum, it seems likely that the attenuation of the reinstatement of cocaine seeking was due to the modulation of activity in the VTA by afferents from the LDT, which receives inputs from the PPTg (Semba & Fibiger, 1992).

The role of AMPA/kainate glutamate receptors in the VTA in cocaine priming-induced reinstatement of drug seeking

A growing body of evidence indicates that the stimulation of dopaminergic neurons in the VTA reinstates cocaine seeking. For example, intra-VTA administration of compounds known to increase the firing rates of dopaminergic neurons promotes cocaine-seeking behavior (Stewart, 1984; Vorel et al., 2001; Placenza et al., 2004). Moreover, microinjection of the ionotropic gluamate receptor antagonist, kynurenate, into the VTA attenuated the reinstatement of drug seeking induced by a priming injection of cocaine as well as cocaine-associated cues (Sun et al., 2005). Consonant with these findings, the present results showed that intra-VTA administration of an AMPA/kainate receptor antagonist dose-dependently attenuated cocaine priming-induced reinstatement of drug seeking. Taken together, these findings demonstrate that increased glutamate transmission in the VTA promotes the reinstatement of cocaine seeking, presumably by increasing dopamine transmission in projection regions such as the nucleus accumbens (Schmidt *et al.*, 2005; Schmidt & Pierce, 2006b; a).

A recent *in vitro* study demonstrated that stimulation of the PPTg increases acetylcholine release in the VTA and modulates activity of VTA neurons, in part, through an AMPA receptor-mediated mechanism of action (Good & Lupica, 2009). Moreover, PPTg-evoked synaptic currents in the VTA are decreased by an alpha7-nicotinic acetylcholine receptor (α7-nAchR) antagonist, suggesting that stimulation of the PPTg increases acetylcholine release in the VTA, which activates presynaptic α7-containing nicotinic acetylcholine autoreceptors and thereby decreases extracellular levels of glutamate in the VTA (Good & Lupica, 2009). Furthermore, tonic input from the LDT is required for glutamate-mediated burst firing in VTA dopamine neurons (Lodge & Grace, 2006b). These results are consistent with the present findings demonstrating that administration of AMPA or nicotinic acetylcholine receptor antagonists directly into the VTA attenuates cocaine priming-induced reinstatement, effects that are likely mediated by postsynaptic and presynaptic receptors, respectively.

Although the VTA receives glutamatergic afferents from several nuclei, few of these projections synapse directly onto dopaminergic neurons. Indeed, the primary glutamatergic afferents to dopaminegic neurons in the VTA arise from the PPTg/LDT (Charara *et al.*, 1996; Maskos, 2008) and the bed nucleus of the stria terminalis (BNST) (Georges & Aston-Jones, 2001; 2002). While the role of the BNST in cocaine-primed reinstatement has not been determined, the BNST does play a role in stress-induced reinstatement of cocaine seeking (Erb & Stewart, 1999; McFarland *et al.*, 2004). Together with the present results, these findings suggest that cocaine priming-induced reinstatement is mediated in part by glutamatergic projections from the PPTg/LDT, and perhaps the BNST, that synapse directly on dopaminergic neurons in the VTA.

The role of VTA acetylcholine in cocaine priming-induced reinstatement of drug seeking

Cholinergic projections that synapse on dopaminergic neurons in the VTA arise mainly from the caudal PPTg and LDT (Hallanger & Wainer, 1988; Oakman *et al.*, 1995; Omelchenko & Sesack, 2005). Consistent with these anatomical findings, increased cholinergic transmission in the VTA was shown to activate dopaminergic neurons and promote dopamine release in the nucleus accumbens (Westerink *et al.*, 1996; Forster & Blaha, 2000; Gronier *et al.*, 2000). Therefore, we next wanted to assess the influence of nicotinic and muscarinic acetylcholine receptors, which are abundantly expressed on dopaminergic neurons in the VTA (Weiner et al., 1990; Klink et al., 2001), on the reinstatement of cocaine seeking. Our results indicate that administration of nicotinic (mecamylamine) or muscarinic (scopolamine) acetylcholine antagonists into the VTA attenuated the drug seeking induced by a cocaine priming injection. These results suggest that cocaine priming-induced reinstatement of drug seeking is mediated in part by increased cholinergic transmission in the VTA, which may lead to increased dopamine transmission in the nucleus accumbens. To our knowledge, our results are the first to implicate VTA acetylcholine in the reinstatement of cocaine seeking, which is surprising given that mecamylamine has been shown to reduce drug craving in human cocaine addicts (Reid et al., 1999).

Recent evidence indicates that the PPTg/LDT is comprised of distinct subpopulations of cholinergic, glutamatergic and GABAergic neurons (Wang & Morales, 2009). While a growing literature suggests that PPTg/LDT cholinergic and glutamatergic afferents regulate activity of dopamine neurons in the VTA (Overton & Clark, 1997; Forster & Blaha, 2000; Floresco & Grace, 2003; Lodge & Grace, 2006b), the precise role of PPTg/LDT GABAergic afferents on VTA dopamine neurons remains unknown. The present results indicate that both cholinergic and glutamatergic subpopulations of projection neurons in the PPTg/LDT play critical roles in the reinstatement of cocaine seeking.

Summary and Conclusions

The present data indicate a novel role for the PPTg/LDT in the circuitry mediating cocaine priming-induced reinstatement of drug seeking. As reviewed above, the PPTg/LDT receives excitatory glutamatergic projections from the mPFC and in turn sends excitatory glutamatergic and cholinergic projections to the VTA where they synapse on dopaminergic neurons. Our results indicate that increased dopamine transmission in the mPFC, enhanced glutamate transmission in the PPTg/LDT and augmented glutamate and acetylcholine release in the VTA collectively promote cocaine-seeking behavior in rats. However, future studies using more sophisticated disconnection experiments are required to determine the precise role of glutamatergic and cholinergic projections to the VTA from the PPTg and other nuclei mediating cocaine-seeking behavior. Collectively, these findings suggest that during the reinstatement of cocaine seeking the PPTg/LDT relays information from the mPFC to the mesoaccumbens dopaminergic system.

A simplification of the limbic circuitry underlying the reinstatement of cocaine seeking is presented in Figure 5. There is strong evidence supporting a role for the direct glutamatergic projection from the mPFC to the nucleus accumbens in the reinstatement of cocaine seeking (McFarland & Kalivas, 2001;Park *et al.*, 2002;McFarland *et al.*, 2003). The reciprocal connections between the mPFC and VTA also have been implicated in this process (Sun et al., 2005). The present findings further suggest that a serial polysynaptic subcircuit encompassing the mPFC, PPTg/LDT, VTA and nucleus accumbens plays a critical role in cocaine priming-induced reinstatement of drug seeking.

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Abbreviations

mPFC	medial prefrontal cortex
PPTg	pedunculopontine tegmental nucleus
LDT	laterodorsal tegmental nucleus
FR	fixed ratio
VTA	ventral tegmental area
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
i.v.	intravenous
ANOVA	analysis of variance
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
BNST	bed nucleus of stria terminalis

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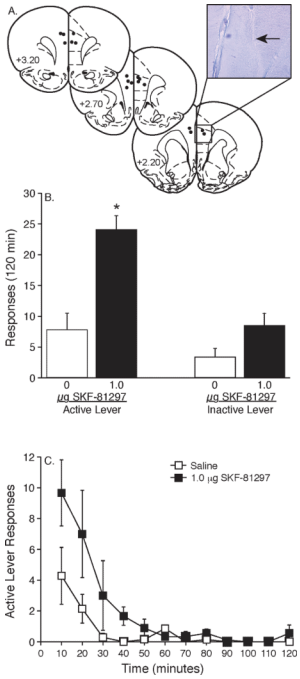

Figure 1. Microinjection of the D1-like dopamine receptor agonist SKF-81297 into the medial prefrontal cortex reinstated cocaine seeking in rats

(A) Ventral tips of the microinjection cannulae, as indicated by the closed circles, targeted the dorsal mPFC. Numbers on the left side of each coronal section denote distance from bregma in the anteroposterior direction. The inset displays a representative coronal section stained with 0.1% cresyl violet and viewed under 4x magnification using a light microscope. The arrow indicates a microinfusion site within the dorsal mPFC. **(B)** Total number of responses (mean±S.E.M.) on the active and inactive levers during the reinstatement session following intra-mPFC administration of saline (*n*=7) or 1.0 μg SKF-81297 (*n*=9). The asterisk denotes a significant difference on active lever responding between 1.0 μg SKF-81297 and saline treatments (unpaired t-test, P<0.0004). **(C)** The time courses of active lever responses (mean±S.E.M.) for the data summarized in panel B.

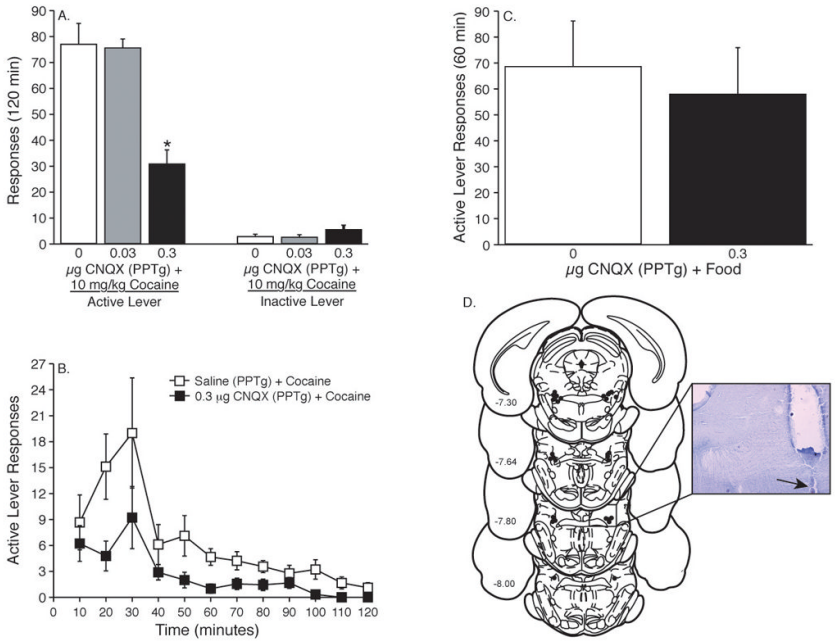

Figure 2. Microinjection of the AMPA/kainate receptor antagonist CNQX into the PPTg/LDT dose-dependently attenuated reinstatement of drug seeking induced by a priming injection of cocaine

(A) Data in panel A depict the total responses (mean±S.E.M.) on the active and inactive levers following a systemic priming injection of cocaine (10 mg/kg, i.p.) in animals pretreated with microinfusions of saline (*n* = 9), 0.03 (*n* = 6) or 0.3 μg (*n* = 9) CNQX into the PPTg/LDT. The asterisk represents a significant difference between responses on the active lever following 0.3 μg CNQX and saline or 0.03 μg CNQX pretreatments (Tukey's HSD, $P<0.05$). No significant differences in responding on the inactive lever (mean±S.E.M.) were found during the reinstatement phase of the experiment when saline, 0.03 or 0.3 μg CNQX were administered into the PPTg/LDT 10 minutes prior to a systemic injection of cocaine (10 mg/kg, i.p.). **(B)** The time courses of active lever responding (mean±S.E.M.) following intra-PPTg/LDT administration of saline (*n* = 9) or 0.3 μg CNQX (*n* = 9) prior to a systemic priming injection of cocaine (10 mg/kg i.p.). **(C)** Intra-PPTg/LDT administration of CNQX did not affect food-seeking behavior in rats. Animals microinjected with saline (n = 6) or 0.3 μg CNQX (n = 6) directly into the PPTg/LDT prior to the reinstatement of food seeking displayed no difference in total active lever responses between treatments. (**D**) Coronal sections depicting microinjection sites, as indicated by the closed circles, targeting the PPTg/LDT. Numbers on the left side of the coronal sections denote distance from bregma in the anteroposterior direction. The insert displays a magnified (4x) microinfusion site within the PPTg (the ventral tip of the microinjector is indicated by the arrow).

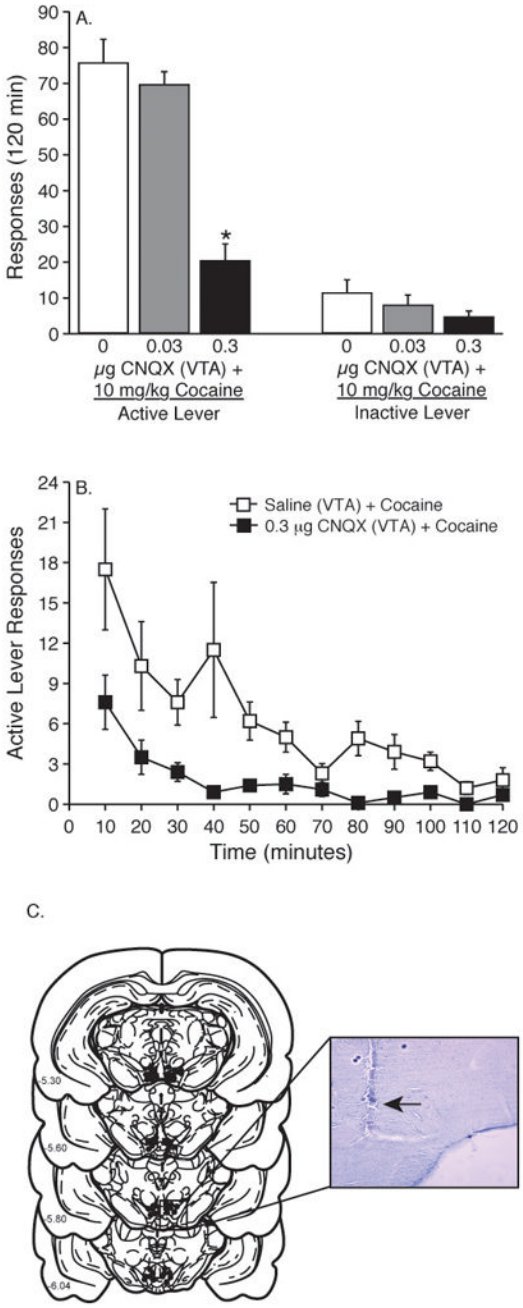

Figure 3. Intra-VTA administration of the AMPA/kainate receptor antagonist CNQX dose-dependently attenuated cocaine priming-induced reinstatement of drug seeking

(A) Total number of responses (mean±S.E.M.) on the active lever during the reinstatement session following a 10 mg/kg priming injection of cocaine in animals pretreated with saline (*n* =10), 0.03 (*n* = 10) or 0.3 (*n* = 10) μg CNQX into the VTA. The asterisk indicates a significant difference between 0.3 μg CNQX and 0.03 μg CNQX or saline treatments with regards to total active lever responses (Tukey's HSD, P<0.05). Total number of responses (mean±S.E.M.) on the inactive lever during the reinstatement phase of the experiment following a systemic priming injection of cocaine (10 mg/kg i.p.) in animals pretreated with animals receiving intra-VTA administration of saline (*n* =10), 0.03 (*n* = 10) or 0.3 (*n* = 10) μg CNQX. No significant difference in responding on the inactive lever was found between

treatments. **(B)** The time courses of active lever responses (mean±S.E.M.) for animals given a priming injection of cocaine (10 mg/kg i.p.) following intra-VTA administration of saline (n = 10) or 0.3 μg CNQX (n = 10). **(C)** Closed circles denote cannula placements for all the microinjections into the VTA (from Figure 3 & 4). Numbers on the left side of each coronal section indicate distance from bregma in the anteroposterior direction. The arrow displayed within the insert designates a representative microinfusion site within the VTA.

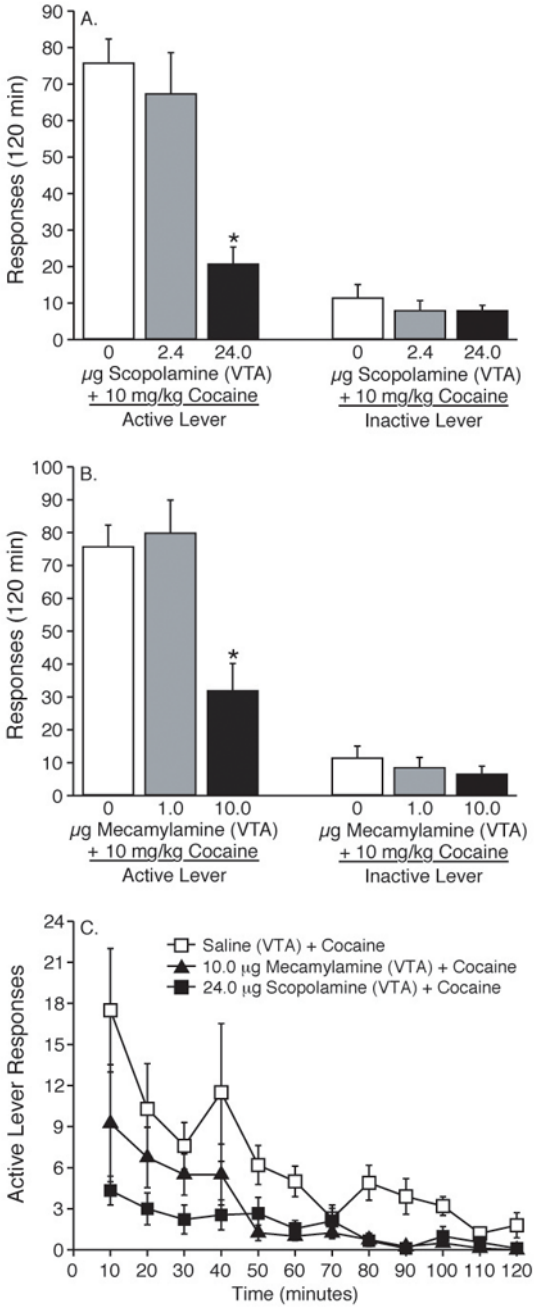

Figure 4. Microinjection of the muscarinic acetylcholine receptor antagonist scopolamine or the nicotinic acetylcholine receptor antagonist mecamylamine into the VTA dose-dependently attenuated reinstatement of drug seeking elicited by a priming injection of cocaine

(A) Total number of responses (mean±S.E.M.) on the active and inactive levers during the reinstatement session following a 10 mg/kg priming injection of cocaine in animals pretreated with saline (n =10), 2.4 (n = 8) or 24.0 (n = 9) μg scopolamine into the VTA. The asterisk indicates a significant difference between 24.0 μg scopolamine and 2.4 μg scopolamine or saline treatments with regards to total active lever responses (Tukey's HSD, $P<0.05$). **(B)** Data in panel B depict the total responses (mean±S.E.M.) on the active lever following a systemic priming injection of cocaine (10 mg/kg, i.p.) in animals pretreated with

microinfusions of saline (*n* = 10), 1.0 (*n* = 10) or 10.0 μg (*n* = 8) mecamylamine into the VTA. The asterisk represents a significant difference between the 10.0 μg mecamylamine and saline or 1.0 μg mecamylamine treatments (Tukey's HSD, $P<0.05$). Total responses (mean±S.E.M.) on the inactive lever following a priming injection of cocaine (10 mg/kg i.p.) in animals pretreated with saline, 1.0 or 10.0 μg mecamylamine into the VTA. No significant difference was found between treatments with regard to inactive lever responses during the 2-hour reinstatement session. **(C)** The time courses of active lever responses (mean±S.E.M.) for data collected from animals pretreated with intra-VTA microinfusions of saline, 24.0 μg scopolamine or 10.0 μg mecamylamine prior to a priming injection of cocaine.

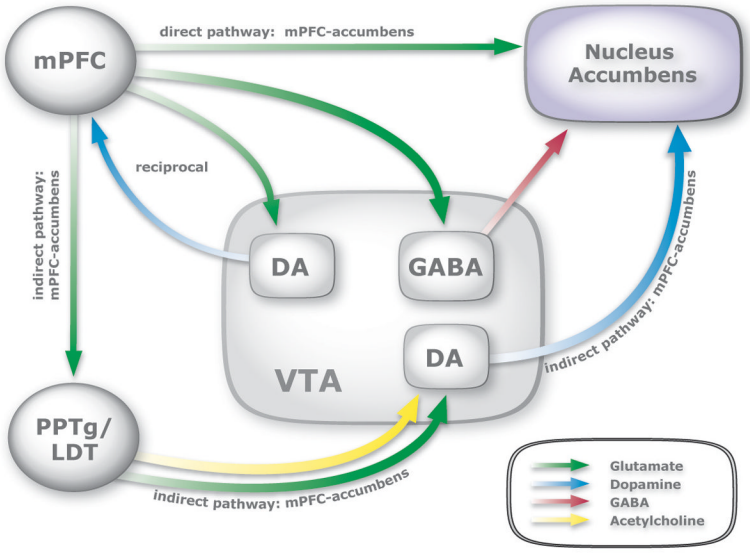

Figure 5. In addition to a direct glutamatergic projection, the mPFC modulates dopamine transmission in the nucleus accumbens through a serial polysynaptic circuit involving the PPTg/ LDT and VTA

The mPFC plays a critical role in cocaine reinstatement through direct glutamatergic projections to the nucleus accumbens and reciprocal connections with the VTA. Here we show that the mPFC also modulates dopamine transmission in the nucleus accumbens through a limbic circuit comprised of the PPTg/LDT and VTA. Thus, the mPFC plays a pivotal role in the limbic circuitry underlying cocaine reinstatement through both direct projections to the nucleus accumbens and VTA as well as an indirect, polysynaptic limbic circuit involving the PPTg/LDT and VTA.

Table 1

Total active and inactive lever responses during the last day of cocaine self-administration for all subjects receiving microinfusions of vehicle or drug directly into discrete nuclei of the brain during reinstatement trials in the present study.

Brain Region	Treatment	n/treatment	Total Active Lever Responses (mean ± S.E.M.)	Total Inactive Lever Responses (mean ± S.E.M.)
mPFC	Vehicle & 1.0 μg SKF-81297	7–9	125.00 ±7.04	3.89 ±2.47
PPTg/LDT	Vehicle, 0.03 & 0.3 μg CNQX	6–9	114.92 ±9.23	9.17 ±3.16
VTA	Vehicle, 0.03 & 0.3 μg CNQX	10	135.87 ±9.17	8.25 ±2.94
VTA	Vehicle, 2.4 & 24.0 μg Scopolamine	8–10	121.79 ±8.27	9.36 ±3.91
VTA	Vehicle, 1.0 & 10.0 μg Mecamylamine	10	142.00 ±9.48	5.08 ±2.44

Table 2

Intra-VTA administration of CNQX, mecamylamine, or scopolamine does not affect food-seeking behavior in rats

Animals microinjected with 3.0 μg CNQX, 10.0 μg mecamylamine or 24.0 μg scopolamine directly into the VTA prior to the reinstatement of food seeking displayed no significant difference in total active lever responses relative to the saline control treatment.

Intra-VTA Treatment	n	Total Active Lever Responses (mean ± S.E.M.)
Saline	6	95.50 ± 10.19
0.3 μg CNQX	6	113.00 ± 11.70
10.0 μg Mecamylamine	6	93.33 ± 14.00
24.0 μg Scopolamine	6	86.50 ± 12.21