

A Single Amphetamine Infusion Reverses Deficits in Dopamine Nerve-Terminal Function Caused by a History of Cocaine Self-Administration

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There are ~1.6 million people who meet the criteria for cocaine addiction in the United States, and there are currently no FDA-approved pharmacotherapies. Amphetamine-based dopamine-releasing drugs have shown efficacy in reducing the motivation to self-administer cocaine and reducing intake in animals and humans. It is hypothesized that amphetamine acts as a replacement therapy for cocaine through elevation of extracellular dopamine levels. Using voltammetry in brain slices, we tested the ability of a single amphetamine infusion *in vivo* to modulate dopamine release, uptake kinetics, and cocaine potency in cocaine-naïve animals and after a history of cocaine self-administration (1.5 mg/kg/infusion, fixed-ratio 1, 40 injections/day × 5 days). Dopamine kinetics were measured 1 and 24 h after amphetamine infusion (0.56 mg/kg, *i.v.*). Following cocaine self-administration, dopamine release, maximal rate of uptake (V_{max}), and membrane-associated dopamine transporter (DAT) levels were reduced, and the DAT was less sensitive to cocaine. A single amphetamine infusion reduced V_{max} and membrane DAT levels in cocaine-naïve animals, but fully restored all aspects of dopamine terminal function in cocaine self-administering animals. Here, for the first time, we demonstrate pharmacologically induced, immediate rescue of deficits in dopamine nerve-terminal function in animals with a history of high-dose cocaine self-administration. This observation supports the notion that the DAT expression and function can be modulated on a rapid timescale and also suggests that the pharmacotherapeutic actions of amphetamine for cocaine addiction go beyond that of replacement therapy.

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INTRODUCTION

The number of people addicted to cocaine in the United States has remained relatively stable over the past several decades and ranges between 1.0 and 1.7 million (Substance Abuse and Mental Health Services Administration (SAMHSA), 2008). In 2012, over half of this population sought treatment for their addiction to cocaine (Substance Abuse and Mental Health Services Administration (SAMHSA), 2008). The relatively stable prevalence of cocaine addiction despite large numbers seeking treatment is due, in part, to the fact that there is currently no FDA-approved pharmacotherapy for the treatment of cocaine addiction.

Some of the more promising pharmacotherapeutic agents from the basic and clinical research literatures are amphetamine-based dopamine releasers (Rush and Stoops, 2012). Dopamine releasers serve as replacement therapies for the dopamine elevating and primary reinforcing effects of cocaine (Rothman *et al*, 2006, 2008) and may acutely offset dopamine hypofunction observed in abstinent cocaine users.

Indeed, cocaine-dependent humans exhibit reduced stimulated dopamine release (Volkow *et al*, 1997; Malison *et al*, 1999; Martinez *et al*, 2007) and reduced extracellular dopamine levels. Reduced extracellular levels and reduced stimulated release may be mechanistically related; however, changes in the function of the dopamine transporter (DAT) may also account for shifts in dopamine tone. Clinical and preclinical work demonstrate mixed results with respect to cocaine-induced changes in levels of the DAT, with the majority of investigations indicating increased DAT levels in cocaine-dependent or newly abstinent individuals (Narendran and Martinez, 2008; Little *et al*, 1993; Malison *et al*, 1998). Moreover, [¹¹C]cocaine binding to DAT is reduced in human addicts measured by PET (Volkow *et al*, 1996a), and psychostimulants are less effective at elevating extracellular dopamine levels which corresponds with reduced euphoric effects (Volkow *et al*, 1996b).

We have demonstrated that a prominent preclinical rodent model of cocaine dependence, produced by extended access to cocaine (Ahmed and Koob, 1998), exhibits nearly identical characteristics of striatal dopamine system hypofunction during abstinence as compared with the clinical literature. The extended access model (Ahmed and Koob, 1998), in which animals are allowed daily access to cocaine in 6 h sessions (or a maximum of 40 injections per session that yields the same effects), results in escalated cocaine intake

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over days (Ferris *et al*, 2012; Calipari *et al*, 2014a, b), blunted electrically stimulated dopamine release, blunted cocaine-induced dopamine release measured with voltammetry and microdialysis (Mateo *et al*, 2005; Ferris *et al*, 2011, 2012, 2013a; Calipari *et al*, 2014a), reduced basal extracellular dopamine levels (Mateo *et al*, 2005; Ferris *et al*, 2011), mixed results with respect to shifts in dopamine uptake by the DAT depending on the schedule and pattern of intake (Mateo *et al*, 2005; Ferris *et al*, 2011, 2012, 2013a; Calipari *et al*, 2013a), reduced total DAT levels (Calipari *et al*, 2014b), reduced D2 autoreceptor function (Mateo *et al*, 2005), and a reduced ability of cocaine and other blockers to inhibit dopamine uptake (Mateo *et al*, 2005; Ferris *et al*, 2011, 2012, 2013a; Calipari *et al*, 2013a, 2014a).

The impetus for the current investigation came from observations that the pharmacological effects of amphetamine on the dopamine system, unlike cocaine and DAT blockers, remained intact and were not disrupted by cocaine self-administration (Ferris *et al*, 2011, 2012). Given (1) the interest in amphetamine as a pharmacotherapeutic agent to acutely offset a hypodopaminergic state during cocaine abstinence, (2) the fact that cocaine and amphetamine are differentially affected by cocaine self-administration, and (3) the well-documented mechanistic differences between cocaine and amphetamine, a thorough investigation of the lack of cross-tolerance is important. We discovered that the pharmacological effects of amphetamine are not only unaffected by cocaine self-administration (Ferris *et al*, 2011), but a single administration of amphetamine is capable of fully reversing dopamine system hypofunction caused by an extended history of cocaine self-administration. Most notably, this full reversal remains present even after amphetamine is cleared from the system. Thus, we outline a newly discovered characteristic of amphetamine's effects on dopamine neurochemistry that has profound implications for the regulation of dopamine terminal and DAT function in general, and for amphetamine as a pharmacotherapeutic agent for cocaine addiction.

MATERIALS AND METHODS

Subjects and General Procedure

Male, Sprague-Dawley rats (350–375 g; Harlan Laboratories) were used as subjects. All animals were maintained according to the National Institutes of Health guidelines in accredited facilities. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Wake Forest School of Medicine.

Animals were implanted with an intravenous jugular (i.v.) catheter and were single-housed in cages that served as both a home cage and their self-administration chamber. In the initial set of studies, a noncontingent infusion of cocaine (1.5 mg/kg in 0.1 ml saline), amphetamine (0.56 mg/kg in 0.1 ml saline), or saline (0.1 ml) was infused through the i.v. catheter 24 h following the start of their final self-administration session. This design yielded five groups: naive rats with saline infusion through i.v. catheters (Naive+Saline), cocaine self-administration rats with saline infusion (Coc SA+Saline), naive rats with amphetamine infusion (Naive+AMPH), cocaine self-administration rats with amphetamine infusion (Coc SA+AMPH), and cocaine self-administration rats with

cocaine infusion (Coc SA+Coc). Animals were then killed for voltammetry studies 1 h after the noncontingent infusion of drug. In a follow-up study, a noncontingent infusion of amphetamine (0.56 mg/kg in 0.1 ml saline) was infused through the i.v. catheter exactly as described above, but animals were killed 24 h after the drug infusion. We selected this dose of amphetamine to be consistent with our earlier studies on amphetamine and cocaine (Ferris *et al*, 2011) and to match the amount of extracellular dopamine elicited in response to amphetamine to the amount of dopamine elicited by the dose of cocaine used in self-administration. In other words, our goal was to match each drug's ability to elicit similar amounts of dopamine overflow.

Self-Administration Procedures

The procedures for cocaine self-administration have been described previously (Ferris *et al*, 2011, 2012) and are repeated herein for clarity. Each animal was maintained on a reverse light cycle (0300 h lights off; 1500 h lights on), and all self-administration procedures occurred during the active/dark cycle (0900 to 1500 h). Each lever press resulted in the delivery of 1.5 mg/kg cocaine over 4 s. This dose was chosen because it is the most reinforcing dose, at the top of the dose–response curve in rats responding under a progressive-ratio schedule of reinforcement, and preferred over lower doses in choice studies (Richardson and Roberts, 1996). Concurrent with the start of each injection, the lever retracted and a stimulus light located on the lever was activated for 20 s to signal a time-out period. The session was terminated after 40 injections or after 6 h, whichever occurred first. Once the animals reached the maximum number of injections allowed in a single session (ie, 40), they were required to self-administer 40 injections per day for five consecutive days before administration of the single, noncontingent drug infusion and subsequent voltammetry studies.

Fast-Scan Cyclic Voltammetry

All voltammetry experiments were conducted in the morning (active/dark cycle) following the final self-administration session (24 h from the start of the final session). Multiple coronal slices (400 μ M) containing the nucleus accumbens (NAc) were prepared from each animal with a vibrating tissue slicer while immersed in oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (126), KCl (2.5), NaH₂PO₄ (1.2), CaCl₂ (2.4), MgCl₂ (1.2), NaHCO₃ (25), glucose (11), L-ascorbic acid (0.4), and pH adjusted to 7.4. Once sliced, tissue was transferred to the testing chambers containing bath aCSF (32 °C) that flowed at 1 ml/min. After a 30-min equilibration period, a cylindrical carbon fiber microelectrode (100–200 μ M length, 7 μ M radius) and a bipolar stimulating electrode were placed into the core of the NAc. We chose the NAc because it is rich in dopamine nerve terminals and heavily implicated in goal-directed behavior, reinforcement, reward, drug addiction, and important for cocaine self-administration. Note that the effects of cocaine self-administration in our previous work are similar between the NAc core and NAc shell (Mateo *et al*, 2005). Dopamine was evoked by a single, rectangular, electrical pulse (300 μ A, 4 ms), applied every 5 min. Extracellular dopamine was monitored at the carbon-fiber electrode every 100 ms by

applying a triangular voltage waveform (-0.4 to $+1.2$ to -0.4 V vs Ag/AgCl, 400 V/s). Once the extracellular dopamine response was stable, cocaine (0.3 – 30 μM) was applied cumulatively to the brain slice. Immediately following the completion of each concentration–response curve, recording electrodes were calibrated by recording their response (in electrical current; nA) to a known concentration of dopamine in aCSF (3 μM). All voltammetry data were collected and modeled using Demon Voltammetry and Analysis Software (Yorgason *et al*, 2011) using Michaelis–Menten kinetics. The three voltammetry parameters used in the current investigation were the peak height of the signal (in μM concentration), the maximal rate of dopamine uptake (V_{max}), and apparent K_m (*app* K_m) to study the effects of release, uptake, and cocaine-induced uptake inhibition, respectively. These parameters are described in detail in previous work (Ferris *et al*, 2013b), and described briefly here for clarity. V_{max} is a rate term (measured as [dopamine] per second) that is inversely related to the time it takes dopamine to be cleared when no drug is present, and is measured on the descending slope of the drug-free dopamine curve. *App* K_m measures the rightward shift/slowness of the descending limb of the dopamine trace following equilibration of each concentration of cocaine. Data were collected for 15 s during baseline collections and for 30 s following administration of cocaine in order to capture the entire descending limb of the curve. Two-way ANOVA with experimental group and concentration of the drug as factors was performed. When significant main effects were obtained ($p < 0.05$), differences between groups at each dose were tested using Bonferroni or Tukey's *post hoc* tests.

Western Blot Analyses of Fractionated Striatal Samples

Striatal tissues were processed by centrifugation to separate the cytosolic and the membrane fractions as described previously (Calipari *et al*, 2014c). Tissue was homogenized in 50 mM Tris Cl buffer (pH 7.4) containing 150 mM NaCl, 10% sucrose, and a cocktail of protease inhibitors (Sigma-Aldrich). A portion of the homogenates was saved, lysed in RIPA buffer, and used as the total homogenate. The remaining homogenates were centrifuged at 20 000 g for 1 h. The supernatant was collected and stored at -80 °C for further analyses of the cytosol-associated protein levels. The pellets were collected and resuspended by sonication in 20 mM Tris buffer (pH 8, containing 1 mM EDTA, 100 mM NaCl, 1% sodium deoxycholate, 1 mM dithiothreitol, and a cocktail of protease and phosphatase inhibitors) and lysed for 1 h at 4 °C. The lysate was centrifuged at 100 000 g for 60 min and the supernatants were collected for analyses of membrane-associated protein levels. Protein concentrations were measured using a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). Samples containing 10 μg protein were loaded onto 8% SDS-polyacrylamide gel. Gels were transferred electrophoretically onto nitrocellulose membranes. After incubation with the blocking buffer containing 5% nonfat milk in phosphate-buffered saline (PBS), the membranes were probed overnight at 4 °C with rabbit anti-DAT antibody (Millipore). The membranes were then incubated with goat anti-rabbit secondary antibodies conjugated with peroxidase. Actin was probed with the

primary antibody goat anti-actin (Santa Cruz) followed by incubation with donkey anti-goat (Sigma-Aldrich). The enhanced chemiluminescence substrate solution (Pierce Chemical) was applied to the membrane and then exposed to X-ray film. Films were analyzed densitometrically using ImageJ (NIH). The band density was normalized to its respective actin level and then each group normalized to the control group (Naive+Saline). Data were represented relative to the control group.

RESULTS

Cocaine Self-Administration Reduced Dopamine Release and Uptake and Blunted the Ability of Cocaine to Inhibit Dopamine Uptake

Consistent with a large amount of previous work using this model (Ferris *et al*, 2012; Calipari *et al*, 2014a, b), rats increased their rate of responding across all 5 days of cocaine self-administration (day 1 = 7.25 ± 0.45 infusions per h vs day 5 = 8.76 ± 0.47 infusions per h, $p < 0.05$), indicative of escalation of cocaine intake (mean \pm SEM). Figure 1a (left and middle panels) shows that a 5-day history of cocaine self-administration reduced electrically stimulated dopamine release ($t_{(18)} = 1.91$, $p < 0.05$). Figure 1a (left and right panels) shows decreased V_{max} in cocaine self-administration animals ($t_{(18)} = 2.54$, $p < 0.01$). To ensure that different magnitudes of dopamine release did not influence our measure of V_{max} , we tested whether there was a difference in V_{max} between the highest and lowest quartiles of dopamine release. There was no difference in V_{max} based on a quartile split of release ($p > 0.05$), indicating no influence of release on our measure of V_{max} . Furthermore, our V_{max} data are consistent with membrane DAT expression (see Figure 3), indicating our measure of V_{max} is a pure measure of uptake and not influenced by release magnitude. Representative pseudocolor plots of cocaine-induced uptake inhibition at 30 μM are shown in Figure 1b. Cocaine extends dopamine signaling (z axis) further out in time by as much as 10 to 15 s (x axis) in the Naive+Saline group (top panel) relative to Coc SA+Saline group (bottom panel). Grouped data plotted across all concentrations in Figure 1c show that cocaine inhibited dopamine uptake for both groups ($F_{(4, 10)} = 236.0$, $p < 0.0001$), but this effect was blunted following a 5-day history of cocaine self-administration (main effect of group: $F_{(1, 10)} = 19.94$, $p < 0.001$, group \times concentration: $F_{(4, 40)} = 19.69$, $p < 0.0001$). Bonferroni comparisons indicated that the Coc SA+Saline group showed reduced uptake inhibition at the 10 μM ($p < 0.01$) and 30 μM ($p < 0.001$) concentrations relative to Naive+Saline group.

A Single Amphetamine Infusion Reversed Deficits in Dopamine Terminals Caused by a History of Cocaine Self-Administration

We performed voltammetry in brain slices 1 h after a single amphetamine infusion (0.56 mg/kg, i.v.) in naive and cocaine self-administering animals. Electrically stimulated dopamine release under drug-free bath conditions trended toward decreases following an infusion of amphetamine in both naive and cocaine self-administering animals in a manner that did not appear to deviate from Coc SA+Saline animals

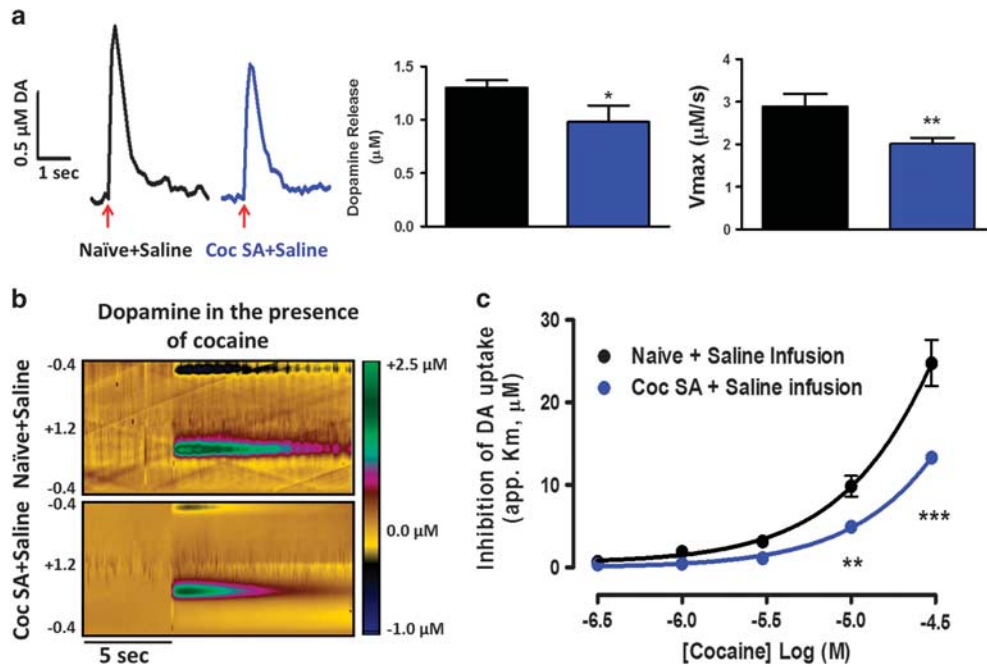


Figure 1 Cocaine self-administration (Coc SA) produces dopamine system hypofunction in the nucleus accumbens core as measured by voltammetry in brain slices. (a) Coc SA+Saline infusion animals (blue) showed reduced electrically stimulated dopamine release (left and middle panels) and maximal rate of dopamine uptake (V_{max} , left and right panels) relative to Naive+Saline infusion animals (black), as indicated by its oxidation +0.6 V and reduction at -0.2 V (y axis), across time (x axis) is represented as pseudocolor plots. Representative plots demonstrate that the ability of cocaine to inhibit uptake, when applied directly to brain slices, is blunted in Coc SA+Saline animals relative to Naive+Saline animals. (c) Grouped data show a significant reduction in the ability of cocaine to inhibit dopamine uptake following a history of cocaine self-administration. *** $P < 0.001$, ** $p < 0.01$, * $p < 0.05$.

(Figure 2a, left panel). There was, however, a main effect of treatment on V_{max} ($F_{(3,29)} = 6.75$, $p < 0.01$; Figure 2a, right panel). Tukey's test indicated that Coc SA+AMPH animals had significantly elevated V_{max} relative to all three other groups, suggesting an interactive effect between a history of cocaine self-administration and amphetamine infusion. Representative pseudocolor plots of cocaine-induced uptake inhibition at 30 μM (Figure 2b) show that the ability of cocaine to extend dopamine signaling (z axis) in time (x axis), which is blunted in the Coc SA+Saline group (middle panel) relative to Naive+Saline group (top panel), is restored following single amphetamine infusion (bottom panel). Grouped data plotted across all concentrations in Figure 2c revealed a main effect of treatment ($F_{(3,18)} = 8.59$, $p < 0.0001$), a main effect of cocaine concentration ($F_{(4,18)} = 139.2$, $p < 0.0001$) and a treatment \times concentration interaction ($F_{(12,72)} = 4.53$, $p < 0.0001$). Bonferroni comparisons indicated a significant decrease in *app* K_m for Coc SA+Saline relative to Naive+Saline at 10 μM ($p < 0.01$) and 30 μM ($p < 0.001$) concentrations and an increase in *app* K_m for Coc SA+AMPH group relative to all three groups at both 10 and 30 μM concentrations. The Naive+AMPH group was not different from the Naive+Saline group.

We also performed voltammetry in brain slices from animals that received a single infusion of cocaine (1.5 mg/kg) through their i.v. catheters. This group controlled for the possibility of generalized dopamine elevation or any stimulant causing a reversal of cocaine's effects following self-administration. Supplementary Figure 1 shows that a single cocaine infusion did not modulate reductions in dopamine release, uptake, or the sensitivity of cocaine to DAT following a history of cocaine self-administration.

A Single Amphetamine Infusion Reversed Deficits in Membrane-Associated Expression of the Dopamine Transporter Caused by a History of Cocaine Self-Administration

To test whether cocaine self-administration with and without an amphetamine infusion altered V_{max} via redistribution of the DAT to and from the membrane, we performed western blot analyses on fractionated striatal samples from naive and cocaine self-administering animals with and without amphetamine infusion 1 h before killing. Data in Figure 3 are consistent with V_{max} from voltammetry experiments in Figure 2a, right panel. Namely, there was a main effect in both total ($F_{(3,21)} = 4.85$, $p < 0.01$; Figure 3a and b) and membrane ($F_{(3,21)} = 5.35$, $p < 0.001$; Figure 3a and d) DAT levels, but the trend toward increased cytosolic DAT levels in Coc SA+Saline and Naive+AMPH groups yielded no main effects (Figure 3a and c). Cocaine self-administration reduced total DAT levels, and a single amphetamine infusion restored DAT levels equivalent to those observed in Naive+Saline-treated animals (Figure 3b). The effect on the total DAT level (Figure 3b) appeared to be attributable to the significant reductions in membrane DAT levels following cocaine self-administration and the amphetamine-induced restoration of membrane DAT levels in cocaine self-administration animals (Figure 3d).

Rescue of Dopamine Terminal Function Following Amphetamine Infusion Was Stable

We next tested whether restoration of dopamine terminal function by amphetamine remained 24 h following

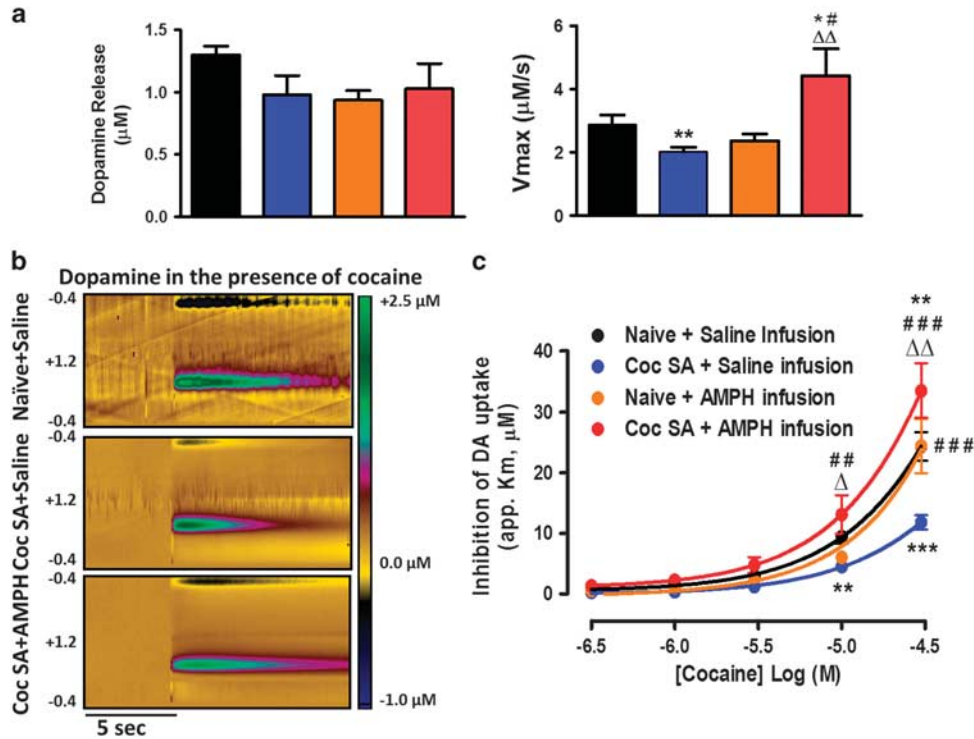


Figure 2 A single infusion of amphetamine (AMPH) decreases the maximal rate of dopamine uptake (V_{max}) with no effect on cocaine potency in naive animals, but increases V_{max} and restores the ability of cocaine to inhibit dopamine uptake in cocaine self-administration (Coc SA) animals within 1 h. (a) AMPH infusion in naive animals (orange) produced a trend toward decreased dopamine release (left panel) and decreased V_{max} (right panel), similar to levels observed following Coc SA (blue). AMPH after 1 h does not influence release in Coc SA animals (red), but does significantly increase V_{max} above Naive + Saline-treated animals (black). (b) Dopamine (z axis), as indicated by its oxidation +0.6 V and reduction at -0.2 V (y axis), across time (x axis) is represented as pseudocolor plots. Representative plots demonstrate that the ability of cocaine to inhibit uptake, when applied directly to brain slices, is blunted in Coc + Saline animals (middle panel) relative to Naive + Saline animals (top panel), and fully restored 1 h after AMPH infusion (bottom panel). (c) AMPH (red) reverses the reduced ability of cocaine to inhibit dopamine uptake after cocaine self-administration, with no effect in Naive animals (orange). *** $P < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs Naive + Saline; ### $p < 0.001$, ## $p < 0.01$ vs Coc SA + Saline; $\Delta p < 0.01$, $\Delta p < 0.05$ vs Naive + AMPH.

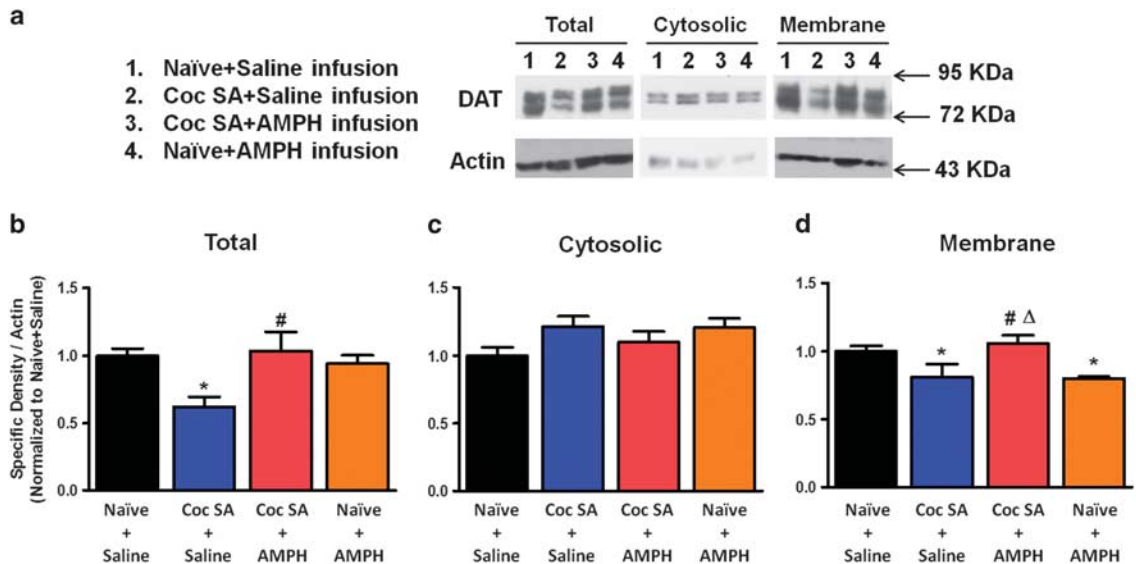


Figure 3 Amphetamine (AMPH) reverses the decrease in membrane-associated expression of the dopamine transporter that follows cocaine self-administration (Coc SA) as measured by western blot analysis. (a) Representative blots of all four treatment groups. (b) Total DAT expression shows significant decreases following Coc SA that is reversed by an AMPH infusion. (c) No significant shift in cytosolic DAT levels across all four groups. (d) Membrane-associated DAT localization shows significant decreases following Coc SA that is reversed by an AMPH infusion, in addition to decreases in naive animals following AMPH infusion. * $P < 0.05$ vs Naive + Saline; # $p < 0.05$ vs Coc SA + Saline; $\Delta p < 0.05$ vs Naive + AMPH.

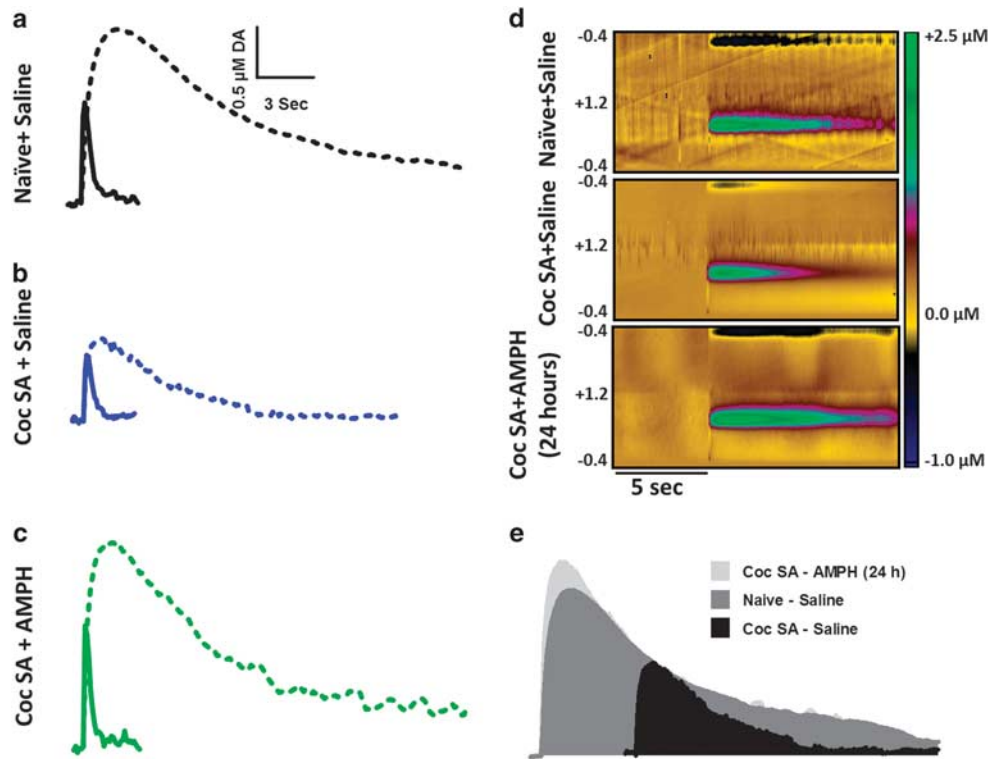


Figure 4 Representative dopamine traces demonstrate reduced dopamine function across multiple indices (release, uptake, and cocaine inhibition of uptake) following cocaine self-administration (Coc SA) that is fully restored 24 h following a single amphetamine (AMPH) infusion. Dopamine release and uptake under drug-free conditions (solid lines in a–c) and following bath application of 30 μM cocaine (dashed lines in a–c) in Naïve+Saline (a, black), Coc SA + Saline (b, blue), and Coc SA+AMPH after 24 h (c, green). (d) Dopamine (z axis), as indicated by its oxidation +0.6 V and reduction at –0.2 V (y axis), across time (x axis) is represented as pseudocolor plots. Representative plots demonstrate that the ability of cocaine to inhibit uptake, when applied directly to brain slices, is blunted in Coc SA+Saline animals (middle panel) relative to Naïve+Saline animals (top panel), and fully restored 24 h after AMPH infusion (bottom panel). (e) To better represent blunted cocaine effects at the dopamine transporter, the representative dopamine trace for the Coc SA+Saline group is shifted later in time to match the peak height dopamine concentration of the Naïve+Saline and Coc SA+AMPH groups (black vs gray and light gray).

amphetamine administration. Figures 4a–c show representative dopamine traces under drug-free conditions (solid traces) and following bath application of 30 μM cocaine (dashed traces) for Naïve+Saline (black traces, top), Coc SA+Saline (blue traces, middle), and Coc SA+AMPH after 24 h (green traces, bottom). The ability of cocaine to facilitate dopamine overflow was substantially reduced in animals with a history of cocaine self-administration (blue vs black traces), whereas a single amphetamine infusion was able to fully restore dopamine signaling elicited under conditions in which aCSF was drug free and when it contained cocaine (green vs black traces). Figure 4d and e (area under the curve following cocaine application) shows that both decreased peak height and decreased rightward shift on the descending limb of the curve accounted for the reduced ability of cocaine to increase dopamine following cocaine self-administration. These parameters were restored 24 h after a single amphetamine infusion. In particular, the reduced ability of cocaine to inhibit dopamine uptake after cocaine self-administration and the full reversal after amphetamine infusion is exemplified in Figure 4e. Even when the curve for Coc SA+Saline is shifted so that the peak height matches the dopamine concentration of the Naïve+Saline group (Figure 4e), the descending curve reaches baseline many seconds before naive animals.

There was no main effect of treatment group on dopamine release, with a trend toward restored dopamine release 24 h

after amphetamine infusion in cocaine self-administering animals (Figure 5a). There was a main effect of treatment group on V_{\max} ($F_{(3,35)} = 9.58$, $p < 0.0001$; Figure 5b), with Tukey's analyses indicating significantly faster V_{\max} in the group tested 24 h after amphetamine infusion relative to Coc SA+Saline animals but not Naïve+Saline animals. Cocaine-induced uptake inhibition in Figure 5c shows a main effect of treatment ($F_{(3,20)} = 11.60$, $p < 0.0001$), main effect of cocaine concentration ($F_{(4,20)} = 262.5$, $p < 0.0001$), and a treatment \times concentration interaction ($F_{(12,80)} = 7.85$, $p < 0.0001$). Bonferroni analysis indicates that amphetamine infusion 24 h before voltammetry testing fully restored cocaine-induced uptake inhibition to naive levels. Cocaine-induced increases in peak height of the signal in Figure 5d shows a main effect of treatment ($F_{(3,20)} = 5.30$, $p < 0.01$), main effect of concentration, ($F_{(4,20)} = 58.43$, $p < 0.0001$), and a treatment \times concentration interaction ($F_{(12,80)} = 2.08$, $p < 0.05$). Thus, amphetamine infusion 24 h before voltammetry testing fully restored cocaine-induced facilitation of release to naive levels.

DISCUSSION

In the current investigation and in work over the course of 10 years, we have established that multiple models that demonstrate *increases* in specific behavioral characteristics

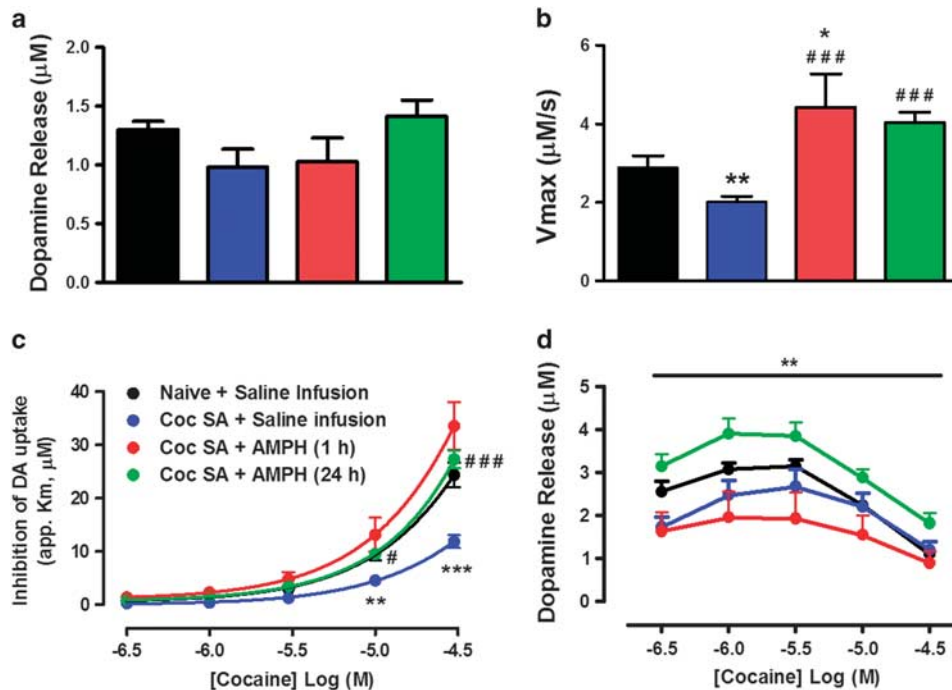


Figure 5 A single infusion of amphetamine (AMPH) restores dopamine uptake and the ability of cocaine to inhibit dopamine uptake in cocaine self-administration (Coc SA) animals within 24 h. (a) Although not significant, AMPH infusion reverses the trend of decreased dopamine release following cocaine self-administration to naive levels (black) after 24 h (green). (b) The maximal rate of dopamine uptake (V_{max}) is restored and not significantly different than Naive+Saline-treated animals. (c) AMPH after 24 h (green) reverses the reduced ability of cocaine to inhibit dopamine uptake after cocaine self-administration, and the slight overshoot after 1 h (red) is fully resolved. (d) AMPH after 24 h (green) reverses the reduced ability of bath-applied cocaine to facilitate electrically stimulation dopamine release across the entire cocaine concentration response curve. *** $P < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs Naive+Saline; ### $p < 0.001$, # $p < 0.05$ Coc SA+AMPH(24 h) vs Coc SA+Saline.

and responses for cocaine, such as escalation of cocaine intake/motivation on fixed and progressive ratios, result in a hypodopaminergic resting state of dopamine terminal function in addition to a reduced ability of cocaine to inhibit dopamine uptake and correspondingly increase dopamine in the extracellular space (Ferris *et al*, 2012; Calipari *et al*, 2014b; however, see Ahmed *et al*, 2003). Phillips and colleagues (Willuhn *et al*, 2014) have mechanistically linked dopamine system deficits with escalation of cocaine intake and showed that restoration of phasic dopamine release eliminated escalated intake of cocaine. This body of work strongly suggests that ‘sensitization’ of mesolimbic dopamine in response to cocaine is not a prerequisite for escalation of intake or escalation in the amount of work an animal will put forth to receive drug, and instead reduced dopamine function may be related to tolerance to the rate-decreasing effects of cocaine thought to be associated with escalation of cocaine intake (Zernig *et al*, 2007). Furthermore, this work is consistent with work indicating that D1 or D2 receptor blockade in the NAC core facilitates escalation of cocaine intake (Suto *et al*, 2009) whereas combined infusion of D1/D2 agonists into the NAC core promotes satiety in animals that are self-administering cocaine, effectively blocking escalation (Suto and Wise, 2011). Indeed, animals’ titration of responses across shifting cocaine doses or during self-stimulation in order to meet satiety thresholds is a well-documented characteristic of cocaine self-administration (see, eg, Zimmer *et al*, 2011, 2013; Norman *et al*, 2014).

Here, we describe the rescue of deficits in dopamine function by a single infusion of amphetamine. Specifically, a single i.v. infusion of amphetamine rapidly reversed cocaine

self-administration-induced decreases in membrane and total DAT expression, V_{max} and the ability of cocaine to inhibit dopamine uptake within 1 h. This restoration of DAT function persisted for at least 24 h after amphetamine infusion, and was followed at the 24-h time point by a rescue of dopamine release elicited by electrical stimulation. We have previously published that restoration of dopamine nerve-terminal function and the ability of cocaine to inhibit dopamine uptake does not occur until 14 days following cessation of cocaine self-administration in animals that do not receive an amphetamine infusion (Ferris *et al*, 2011). This demonstrates that the amphetamine-induced reversal of dopamine deficits at the 1 and 24 h time points is not simply due to withdrawal from cocaine self-administration.

One surprising observation was a differential effect of amphetamine on the presynaptic dopamine terminal in animals with a history of cocaine self-administration as compared with cocaine-naive controls. Cocaine self-administration decreased DAT membrane expression and V_{max} , and these decreases were completely restored (ie, increased) to control levels 1 h following a single amphetamine infusion. However, in naive animals, a single amphetamine infusion decreased DAT membrane expression and V_{max} , demonstrating that amphetamine effects on these endpoints are substantially modulated by previous experience with cocaine. Although membrane expression of the DAT as measured by western blotting in the current investigation is not necessarily indicative of DAT cell-surface expression *per se*, a reduction in membrane expression following amphetamine infusion in naive animals is consistent with a large body of

literature on amphetamine-induced DAT internalization (Fleckenstein *et al*, 1997, 1999; Saunders *et al*, 2000; Boudanova *et al*, 2008; Richards and Zahniser, 2009; Hong and Amara, 2013). Indeed, previous studies have reported substrate-induced downregulation of surface DAT expression anywhere from 1 min to hours after administration in cultured cell models or in synaptosomes (Fleckenstein *et al*, 1999; Saunders *et al*, 2000; Richards and Zahniser, 2009).

A mechanism for this differential effect of amphetamine on expression and function based on a history of cocaine is unclear. Although cocaine and amphetamine binding sites largely overlap at the DAT (Beuming *et al*, 2008), they may activate different second messenger signals. A history of cocaine experience may modulate amphetamine's signaling cascade before amphetamine ever being administered, leading to the marked increase in membrane-associated DAT levels and DAT function observed in the present studies. As a precautionary note, it should be pointed out that the effect of cocaine self-administration history on DAT expression remains equivocal with examples of increased, decreased, and no change in DAT expression in all species investigated (Narendran and Martinez, 2008). For example, our observation of decreased DAT expression and V_{\max} following cocaine self-administration, despite being replicated numerous times in our model, differs to some degree from the majority of human studies (Narendran and Martinez, 2008; Little *et al*, 1993; Malison *et al*, 1998), and even some rat (Oleson *et al*, 2009; Calipari *et al*, 2013a) and nonhuman primate work (Letchworth *et al*, 2001). These examples show either increases or no change in DAT expression following a history of cocaine self-administration. There is typically at least one discrepancy (if not numerous discrepancies) between research that shows decreased DAT expression and V_{\max} compared with others that show increased DAT expression and V_{\max} . Perhaps any one discrepancy alone is sufficient to mediate the differential effects on DAT expression and/or V_{\max} . One difference includes the degree of abstinence from cocaine. Indeed, we previously observed an increase in V_{\max} following extended withdrawal in rats that perhaps better resembles the human data (Ferris *et al*, 2011). Another difference includes pattern of cocaine intake. We previously observed increases in V_{\max} following intermittent access self-administration despite having almost no period of abstinence (Calipari *et al*, 2013a). Intermittent patterns of intake may more closely resemble human patterns of intake as it is unlikely humans self-administer high levels of cocaine continuously for 6 h per day over the course of 1 week. Even if humans binge in a pattern similar to our model, it is possible that there are periods of abstinence followed by additional binging patterns over the period of several years. Therefore, understanding the effects of amphetamine following cocaine self-administration on DAT expression is not fully possible without more work to test the effects of amphetamine infusion under different self-administration conditions, including protracted withdrawal from cocaine, alternative access paradigms, and following different doses of cocaine.

In addition to shifts in DAT membrane expression and V_{\max} following cocaine self-administration, we also observed decreases in the ability of cocaine to inhibit dopamine uptake. We have speculated that a cocaine-induced conformational change of surface DATs, rather than a shift in

DAT surface level, is responsible for reduced cocaine effects (Ferris *et al*, 2011). This is similar to how specific DAT mutations can reduce cocaine potency in mice while sparing substrate potency (Chen *et al*, 2006; Tilley *et al*, 2009). Reduced cocaine binding with no change in DAT density has been demonstrated in humans (Volkow *et al*, 1996a, b). Our reasoning for ruling out trafficking for reduced cocaine potency stems from the fact that V_{\max} is tightly linked to DAT levels in this study and in others (Calipari *et al*, 2013b), and we have shown that V_{\max} is not mechanistically linked to cocaine-induced decreases in cocaine potency (Mateo *et al*, 2005; Ferris *et al*, 2012; Calipari *et al*, 2013b). Nevertheless, there is a distinct possibility that amphetamine causes an insertion of DATs at the membrane that have had no prior direct exposure to cocaine, and therefore would not be modified. These newly inserted DATs might be equally sensitive to cocaine as DATs from naive animals, thus reversing the reduced cocaine effects at the DAT. This hypothesis is consistent with recent reports showing that inducible expression of wild-type DATs can reestablish cocaine potency in mice that initially only express cocaine-insensitive DATs (O'Neill *et al*, 2014). Further investigation is needed to test this hypothesis particularly since there was no significant fluctuation in cytosolic DAT expression across our groups. This suggests that redistribution between the membrane and cytosol may not be responsible for shifts in DAT levels and V_{\max} and that amphetamine-induced introduction of 'new' DATs may not be at play. On the contrary, a shift in membrane DAT expression with no shift in cytosolic DAT expression supports a different hypothesis than what we have suggested in previous work. Specifically, we have hypothesized that a history of cocaine self-administration could be promoting the formation of DAT oligomers or higher-order DAT complexes with other proteins (Ferris *et al*, 2012). The functional effects of DAT oligomerization have yet to be fully elucidated, but early work hypothesizes that formation of DAT oligomers could lead to many of the same functional outcomes found in the current work. For example, oligomer formation may reduce monomers, reduce DAT function, and reduce the affinity of certain ligands (Chen and Reith, 2007; Sitte *et al*, 2004). This is consistent with our observation of reduced membrane monomer DAT expression, V_{\max} , and reduced ability of cocaine to inhibit dopamine uptake. Moreover, amphetamine has been shown to dissociate DAT oligomers in cell culture studies (Chen and Reith, 2008). Therefore, a single amphetamine infusion may be reestablishing DAT monomer expression, increasing V_{\max} , and restoring the ability of cocaine to inhibit dopamine uptake via dissociating DAT oligomers or higher-order complexes that were formed by cocaine self-administration.

In addition to the effects of amphetamine after 1 h, we also show that cocaine-induced reductions in V_{\max} and cocaine potency remained reversed 24 h after amphetamine infusion, and that an additional restoration of dopamine release manifested only at this later time point. Thus, this time point not only confirmed that these effects are likely not transient, but showed that amphetamine can reverse deficits in dopamine release caused by cocaine self-administration, although in a delayed manner relative to reversal of other nerve-terminal functions. The delayed restoration of release suggests that the amphetamine-induced reexpression of DAT

monomers on the membrane may be necessary for, and lead to, increases in the amount of releasable dopamine. For example, it was recently discovered that DAT expression can determine, at least in part, the amount of readily releasable dopamine (Egana *et al*, 2009; Ferris *et al*, 2014). However, if DAT expression leads to refilling of vesicles, it is clear that some amount of time longer than 1 h, but less than 24 h, is required for this process.

The inverse relationship between mesolimbic dopamine function and cocaine intake demonstrated here is important, because the putative mechanism for amphetamine as an agonist therapy has its foundation in decreasing cocaine intake by increasing extracellular dopamine tone during the cocaine self-administration session (Rothman *et al*, 2006, 2008). Continuous amphetamine administration delivered during cocaine self-administration sessions is effective at reducing breakpoints on a progressive-ratio schedule (Negus and Mello, 2003a; Chiodo *et al*, 2008; Czoty *et al*, 2010; Zimmer *et al*, 2014), reducing second-order responding for cocaine (Negus and Mello, 2003b), and reducing preference for cocaine in a choice paradigm (Negus, 2003) in both rats and/or nonhuman primates. Similarly, oral d-amphetamine treatment has been shown to reduce cocaine use in human addicts (Grabowski *et al*, 2001, 2004a, b). We show here, for the first time, that a single amphetamine infusion also reverses cocaine-induced deficits in dopaminergic terminals. A reversal of cocaine's effects after direct pharmacological effects of amphetamine have dissipated is consistent with behavioral studies showing reduced progressive ratio responding following discontinuation of amphetamine treatment that can last for days (Chiodo *et al*, 2008; Czoty *et al*, 2010), and even weeks in some animals (Czoty *et al*, 2011), well after the acute pharmacological effects of amphetamine have passed. It is unclear, however, whether the effects of cocaine are reestablished following administration of cocaine that occurs after amphetamine treatment, and this will be an important determinant of the therapeutic potential of amphetamine to reverse cocaine-induced adaptations to dopamine systems. Moreover, additional studies are underway to investigate whether the reversal of cocaine's effects is a pharmacotherapeutic characteristic that can be generalized to other releasers as well—perhaps one with less inherent risk of abuse than amphetamine. In addition, it is presently unclear whether the dose of amphetamine infusion dictates this reversal, and whether the effects we see here underlie the efficacy of amphetamine at reducing cocaine self-administration behavior. If such is the case, then not only does this research document a novel characteristic of amphetamine function, but it also expands amphetamine's pharmacotherapeutic mechanisms from a simple replacement therapy to a multifaceted therapy that includes reversal of cocaine-induced plasticity of dopamine function.

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