Behavioral/Cognitive

# Amphetamine Reverses Escalated Cocaine Intake via Restoration of Dopamine Transporter Conformation

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**Cocaine abuse disrupts dopamine system function, and reduces cocaine inhibition of the dopamine transporter (DAT), which results in tolerance. Although tolerance is a hallmark of cocaine addiction and a DSM-V criterion for substance abuse disorders, the molecular adaptations producing tolerance are unknown, and testing the impact of DAT changes on drug taking behaviors has proven difficult. In regard to treatment, amphetamine has shown efficacy in reducing cocaine intake; however, the mechanisms underlying these effects have not been explored. The goals of this study were twofold; we sought to (1) identify the molecular mechanisms by which cocaine exposure produces tolerance and (2) determine whether amphetamine-induced reductions in cocaine intake are connected to these mechanisms. Using cocaine self-administration and fast-scan cyclic voltammetry in male rats, we show that low-dose, continuous amphetamine treatment, during self-administration or abstinence, completely reversed cocaine tolerance. Amphetamine treatment also reversed escalated cocaine intake and decreased motivation to obtain cocaine as measured in a behavioral economics task, thereby linking tolerance to multiple facets of cocaine use. Finally, using fluorescence resonance energy transfer imaging, we found that cocaine tolerance is associated with the formation of DAT-DAT complexes, and that amphetamine disperses these complexes. In addition to extending our basic understanding of DATs and their role in cocaine reinforcement, we serendipitously identified a novel therapeutic target: DAT oligomer complexes. We show that dispersion of oligomers is concomitant with reduced cocaine intake, and propose that pharmacotherapeutics aimed at these complexes may have potential for cocaine addiction treatment.**

*Key words:* agonist therapy; behavioral economics; nucleus accumbens; self-administration; tolerance; voltammetry

**Significance Statement**

**Tolerance to cocaine's subjective effects is a cardinal symptom of cocaine addiction and a DSM-V criterion for substance abuse disorders. However, elucidating the molecular adaptions that produce tolerance and determining its behavioral impact have proven difficult. Using cocaine self-administration in rats, we link tolerance to cocaine effects at the dopamine transporter (DAT) with aberrant cocaine-taking behaviors. Further, tolerance was associated with multi-DAT complexes, which formed after cocaine exposure. Treatment with amphetamine deconstructed DAT complexes, reversed tolerance, and decreased cocaine seeking. These data describe the behavioral consequence of cocaine tolerance, provide a putative mechanism for its development, and suggest that compounds that disperse DAT complexes may be efficacious treatments for cocaine addiction.**

## Introduction

Drug addiction is one of the most damaging public health problems, resulting in close to 600,000 deaths per year and a societal cost of over $500 billion in the United States alone (McGinnis et al., 1999; O'Connor et al., 2014). Cocaine use results in disrupted neuronal function, especially of dopaminergic systems (Volkow et al., 1996, 1997; Dackis and O'Brien, 2001), which can lead to escalated and uncontrolled drug intake. In particular, studies in

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cocaine-dependent humans have revealed that cocaine addiction is associated with a reduction in the ability of cocaine to inhibit the dopamine transporter (DAT), increase dopamine levels, and produce euphoric effects (Volkow et al., 1996, 1997, 2014). Preclinical literature has identified the DAT as the main site of action for cocaine, necessary for both the euphoric effects and self-administration behavior (Roberts et al., 1977; Ritz et al., 1987; Chen et al., 2006; Thomsen et al., 2009); thus, changes in cocaine effects at the DAT have been hypothesized to be a major component in the development of addiction. Despite the implications of cocaine tolerance and DAT alterations in addiction, the molecular changes that underlie tolerance have remained elusive, and directly testing the effects of tolerance on drug taking and seeking behavior has been a long-standing challenge.

Amphetamine (AMPH) has shown efficacy in reducing cocaine intake in human addicts in multiple clinical trials (Grabowski et al., 2001, 2004; Rush and Stoops, 2012; Nuijten et al., 2016). Currently, AMPH is categorized as an agonist replacement therapy (i.e., a medication that acts on the same primary target as the abused compound), which typically must be administered indefinitely on a daily maintenance schedule to be effective (Rush and Stoops, 2012); however, there have been no investigations of the cellular mechanisms underlying AMPH's efficacy in reducing cocaine intake. Here we hypothesized that AMPH's therapeutic effects may be due to interactions with the DAT that result in changes in cocaine potency and dopamine neurotransmission (Ferris et al., 2015). Thus, we sought to (1) identify the molecular alterations to DATs that underlie cocaine tolerance, (2) determine the behavioral impact of cocaine tolerance, and (3) determine whether AMPH interferes with these mechanisms. To address these questions, we used the long- and short-access cocaine self-administration procedure in which animals are given daily access to cocaine for 1 h (short-access; ShA) or 6 h (long-access; LgA) per day. LgA conditions result in cardinal sequelae of addiction, including escalation of cocaine intake, compulsive cocaine seeking, and increased motivation to administer cocaine (Ahmed and Koob, 1998; Grimm et al., 2001; Paterson and Markou, 2003). This is in contrast to animals given access to cocaine under ShA conditions, which do not develop aberrant cocaine-taking behaviors over time. Comparisons of these two conditions provides a powerful model for determining the molecular underpinnings of cocaine addiction pathology. By combining drug self-administration with fast-scan cyclic voltammetry (FSCV), and fluorescence resonance energy transfer (FRET) imaging, we systematically identify the molecular alterations in the DAT that may underlie drug seeking.

We found that LgA cocaine self-administration produces neurochemical tolerance to cocaine effects concomitant with dysregulated cocaine intake. AMPH, when administered via mini-pump at a low dose during or after cocaine self-administration, ameliorated cocaine tolerance, prevented escalation of cocaine intake, and reduced maladaptive drug seeking even after cessation of treatment. Using behavioral economic analyses, we found that the increased motivation to administer cocaine after LgA was also prevented by AMPH treatment, thus linking DAT alterations to multiple aberrant cocaine-taking behaviors. Importantly, we show that cocaine exposure produced tolerance to cocaine effects concomitantly with the formation of DAT oligomer complexes, and that AMPH reversed cocaine tolerance as well as returning DATs to a monomer configuration. Thus, AMPH's ability to reduce cocaine intake likely results from more complex mechanisms than previously hypothesized. These findings provide a putative molecular mechanism underlying cocaine tolerance and provide a link between dopamine system dysfunction and aberrant cocaine-taking behaviors.

## Materials and Methods

*Animals.* Male Sprague Dawley rats (350–400 g at the start of experiments; Harlan Laboratories) were maintained on a 12:12 h reverse light/dark cycle (3:00 A.M. lights off; 3:00 P.M. lights on) with food and water *ad libitum*. All animals were maintained according to the National Institutes of Health guidelines in Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Wake Forest School of Medicine.

*Self-administration surgery and training.* Rats were anesthetized and implanted with chronic indwelling jugular catheters as previously described (Siciliano et al., 2015). Animals were singly housed, and all sessions took place in the home cage during the active/dark cycle (9:00 A.M. to 3:00 P.M.). After a 2 day recovery period, animals underwent a training paradigm within which animals were given access on a fixed ratio 1 (FR1) schedule to a cocaine-paired lever, which, upon responding, initiated an intravenous injection of cocaine (0.75 mg/kg, infused over 4 s). After each response/infusion, the lever was retracted and a stimulus light was illuminated for a 20 s timeout period. Training sessions were terminated after a maximum of 20 infusions or 6 h, whichever occurred first. Acquisition occurred when an animal responded for 20 injections for 2 consecutive days and a stable pattern of infusion intervals was present.

*Osmotic mini-pumps.* Following completion of the final acquisition session, animals were anesthetized briefly with isoflurane and osmotic mini-pumps (Alzet) containing AMPH (5 mg/kg/d) or saline were implanted subcutaneously just posterior to the shoulder blades. This dose is well below doses shown to have neurotoxic effects on dopamine neurons (Jonsson and Nwanze, 1982).

*Extended access cocaine self-administration.* Following acquisition, animals were allowed to administer cocaine on an FR1 schedule of reinforcement (0.75 mg/kg, infused over 4 s) with unlimited injections for a total of 6 h (LgA) or 1 h (ShA) per day for a total of 14 d. Animals were treated with mini-pump delivered AMPH or saline throughout the 14 d of self-administration.

A separate group animals were allowed to self-administer under LgA or ShA conditions for 14 d before undergoing a 7 d abstinence period. In this group, animals were treated with mini-pump delivered AMPH or saline throughout the 7 d abstinence period. In all groups, pumps were removed following the final self-administration session, ~18 h before being euthanized for voltammetric recordings.

*Threshold procedure.* In a separate group of animals, the threshold procedure was used to determine LgA-induced changes in cocaine self-administration. The threshold procedure was performed following acquisition, again following 14 d of LgA with saline or AMPH mini-pump treatment, and a final assessment was made 7 d following cessation of LgA and treatment. The threshold procedure is a behavioral economics approach to assessing drug taking and reinforcing efficacy. Using behavioral economic analyses first requires generating a demand function, or in other words, measuring the consumption of a reinforcer across varying prices. Here, price was defined as responses required to obtain 1 mg of cocaine; thus, price can be manipulated in two ways: (1) by changing the response requirement per infusion or (2) by changing the amount of cocaine received with each infusion. Both approaches have been used to generate demand functions for drugs (Wade-Galuska et al., 2007; Oleson and Roberts, 2012). The threshold procedure, used here, consists of generating a demand curve by giving rats access to a descending series of 11 unit doses of cocaine (421, 237, 133, 75, 41, 24, 13, 7.5, 4.1, 2.4, and 1.3 $\mu$g/injection) available on an FR1 schedule of reinforcement. Each dose is available for 10 min, with each bin presented consecutively across the 110 min session. Animals performed this procedure for 3 consecutive days, and responding was averaged to derive the values used. Completion of the procedure produces a within-session dose–response curve, depicted in Figure 2*B*. Shifts in responding across the dose–response curve can be analyzed using behavioral economics principles, as described below.

Behavioral economic analysis was used to determine the maximal price paid ($P_{max}$) for cocaine (Oleson et al., 2011; Oleson and Roberts,

2012). Briefly, $P_{max}$ was derived mathematically using a demand curve. Demand curves were generated by curve-fitting individual animals' intake using an equation: $\log(Q) = \log(Q_0) + k \times (e - \alpha \times Q_0 \times C - 1)$[43]. In this equation, $P_{max}$ was determined to be the unit price at which the first derivative point slope of the function $= -1$. The value $k$ was set to 2 for all animals, whereas $Q_0$ and $\alpha$, which represent the acceleration of the function in response to changing price, were estimated to achieve best fit (Hursh and Silberberg, 2008; Bentzley et al., 2013). This measure is explained in detail below.

*$Q_0$*: $Q_0$ is a measure of the animals' preferred level of cocaine consumption. This can be measured when the dose is high and cocaine is available at low effort, or a minimally constraining price. This preferred level of consumption is established in the early bins of the threshold procedure.

*$P_{max}$*: Price is expressed as the responses emitted to obtain 1 mg of cocaine; thus, as the dose is decreased in each consecutive bin of the threshold procedure, price increases. During the initial bins of the procedure, when the price is low, the animal is able to obtain a preferred level of cocaine intake with minimal responding. As the price is increased across bins, the animal must increase responding to maintain consistent intake. $P_{max}$ is the price at which the animal no longer emits enough responses to maintain intake and consumption decreases. Thus, animals with higher $P_{max}$ will increase responding to maintain cocaine levels farther into the dose–response curve; in other words, they will pay a higher price for cocaine. Here, we reported standardized $P_{max}$ which was derived by normalizing each animals' $P_{max}$ value to their $Q_0$.

Ex vivo *FCSV.* Animals were euthanized for FSCV experiments, as previously described (Siciliano et al., 2014a), the morning following the final self-administration session (~18 h), when no drug was present. FSCV was used to characterize presynaptic dopamine system kinetics, dopamine autoreceptor sensitivity, and the ability of cocaine to inhibit dopamine uptake in the NAc core. A vibrating tissue slicer was used to prepare 400-$\mu$m-thick coronal brain sections containing the NAc core. The tissue was immersed in oxygenated aCSF containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 $NaH_2PO_4$, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 11 glucose, 0.4 L-ascorbic acid, pH adjusted to 7.4. Once sliced, the tissue was transferred to the testing chambers containing bath aCSF (32°C), which flowed at 1 ml/min. A carbon fiber microelectrode (100–200 $\mu$m length, 7 $\mu$m diameter) and bipolar stimulating electrode were placed into the core of the NAc. Dopamine release was evoked by a single electrical pulse (350 $\mu$A, 4 ms, monophasic) applied to the tissue every 3 min. Extracellular dopamine was recorded by applying a triangular waveform (−0.4 to 1.2 to −0.4 V vs Ag/AgCl, 400 V/s). Once the extracellular dopamine response was stable, quinpirole (3–300 nM) or cocaine (0.3–30 $\mu$M) was applied cumulatively to different brain slices.

*Data analysis.* For all analysis of FSCV data, Demon Voltammetry and Analysis software was used (Yorgason et al., 2011). Recording electrodes were calibrated by recording responses (in electrical current; nA) to a known concentration of dopamine (3 $\mu$M) using a flow-injection system. To evaluate drug potency, evoked levels of dopamine were modeled using Michaelis-Menten kinetics as described previously (Siciliano et al., 2014b).

*[$^3$H]Dopamine uptake.* DAT was stably expressed in neuroblastoma N2A cells (N2A-DAT) as described previously (Chen et al., 2005). N2A-DAT cells were treated with vehicle or freshly made cocaine (10 $\mu$M) every day for 3 consecutive days. On day 4, culture medium was replaced with KRH buffer composed of the following (in mM): 25 HEPES, 125 NaCl, 4.8 KCl, 1.2 $KH_2PO_4$, 1.3 $CaCl_2$, 1.2 $MgSO_4$, and 5.6 glucose. AMPH (10 $\mu$M) or vehicle was added to the cells for 1 h followed by extensively washing with cold KRH three times. Dopamine uptake was initiated by adding 10 nM [$^3$H]dopamine (specific activity: 23.5 Ci/mmol; PerkinElmer Life and Analytical Sciences) with or without cocaine (0.01–10 $\mu$M) in KRH for 10 min at 22°C as previously described (Chen et al., 2005). The reaction was terminated by washing cells twice with cold KRH, and cells were dissolved in 0.1% Triton. The amount of [$^3$H]dopamine accumulated in the cells was determined by scintillation fluid in a liquid scintillation counter (Topcount, Packard Instrument). All experiments were performed in triplicate. A sigmoidal curve of cocaine competition for [$^3$H]dopamine binding was generated using three-parameter nonlinear regression analysis. The $IC_{50}$ values were extrapolated from the curve.

*FRET.* Parental N2A cells were plated on poly-D-lysine-coated glass-bottom dishes (Mattek no. 1.5) at ~20%–30% confluency. When the cells reached 40%–50% confluency, they were transfected with YFP-DAT (yellow fluorescent protein tagged DAT), CFP-DAT (cerulean fluorescent protein tagged DAT), using the Calcium Phosphate transfection kit (Invitrogen, K2780–01). After 12–15 h, the cells were gently washed 3 times with warm external solution (146 mM NaCl, 5 mM KCl, 30 mM dextrose, 5 mM HEPES, 1.2 mM $MgCl_2 \cdot 6H_2O$, and 2.5 mM $CaCl_2 \cdot 2H_2O$, pH 7.4, osmolarity 290–300 mOsm) and then again 3 times with warm media. This was followed by cocaine (10 $\mu$M) or vehicle treatment for 3 d in the incubator with media change containing fresh cocaine daily. On the day of imaging, the cells were treated with external solution or external solution containing amphetamine (10 $\mu$M) for 30 min at 37°C. The cells were then washed once in external solution and fixed with 3.7% PFA for 5 min at room temperature followed by three 5 min washes with DPBS and imaged using the Nikon Eclipse Ti confocal microscope. A similar experimental approach was used for positive and negative control experiments where FRET8 plasmid, or empty plasmids expressing either YFP or CFP, were expressed in the cells. FRET8 is a plasmid encoding tandem YFP-CFP and a generous gift from Dr. David Piston (Washington University, St. Louis).

FRET measurements were done using the method called acceptor photobleaching as previously described (Butler et al., 2015). The "dequenching" of the donor fluorophore was evaluated in the presence of an acceptor fluorophore by measuring the intensity of the fluorescence of the donor fluorophore in the same cell, preacceptor and postacceptor photobleaching. FRET was considered as positive when there was an increase in fluorescence intensity of donor fluorophore after photobleaching of the acceptor fluorophore. The microscope was configured to detect emission from the donor CFP (at 464–499 nm after excitation at 457 nm) and emission from the acceptor YFP (at 552–617 nm after excitation at 514 nm). After an initial baseline image acquisition, the acceptor (YFP) photobleaching was obtained using the 514 nm laser at 100% power for 2 s followed by another acquisition phase. Changes in the fluorescence of the donor (CFP) were quantified by subtracting post-bleach images from the prebleach images after subtraction of background intensities. Quantified FRET efficiencies were calculated using the mean fluorescent intensity in the region of interest covering the plasma membrane of the cells.

The FRET efficiency was calculated at the plasma membrane using the following formula:

$$\text{FRET efficiency} = \frac{\{(\text{Donor}_{post} - \text{Background}_{post}) - (\text{Donor}_{pre} - \text{Background}_{pre})\} * 100}{(\text{Donor}_{post} - \text{Background}_{post})}$$

The $\text{Donor}_{post}$ is the intensity of the fluorescence of the donor (CFP) after photobleaching of the acceptor (YFP), and $\text{Donor}_{pre}$ is the intensity of the fluorescence of the donor before photobleaching of the acceptor.

*Statistics.* Graph Pad Prism (version 6) was used to statistically analyze datasets and create graphs. Escalation data, behavioral economics data, burst frequency response curves, and concentration response curves for cocaine and quinpirole were subjected to a two-way repeated-measures ANOVA with session, burst frequency, or concentration as the within-subjects factor and experimental group as the between-subjects factor. Dopamine uptake data and FRET efficiencies between groups were compared using one-way ANOVAs. Differences between groups were tested using a Bonferroni *post hoc* test. All $p$ values of $<0.05$ were considered to be statistically significant.

## Results

### AMPH treatment during cocaine self-administration prevents escalation of intake and augmented reinforcing efficacy of cocaine

We first assessed the effects of AMPH treatment during LgA (6 h access/d) and ShA (1 h access/d) cocaine self-administration (Fig. 1*A*). Increased intake over days occurred only in LgA animals treated with saline (LgA+saline), but not ShA treated with saline

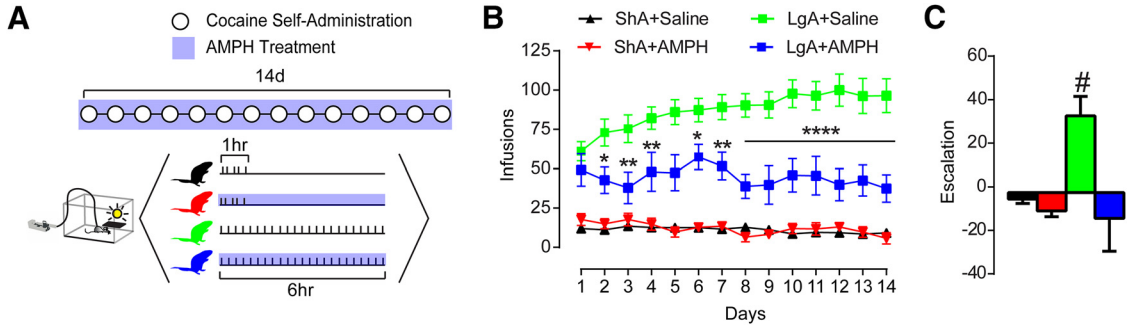


**Figure 1.** AMPH treatment prevented escalation of cocaine self-administration. ***A***, Timeline showing conditions across days (top) and within days (bottom). ***B***, Total infusions per session were lowered by AMPH treatment during long-access, but not short-access, self-administration. ***C***, Session 14 minus session 1 infusions reveal that LgA + Saline animals escalate intake over time, whereas all other groups do not. Error bars indicate ± SEM. $^{*}p < 0.05$ versus LgA + Saline. $^{**}p < 0.01$ versus LgA + Saline. $^{****}p < 0.0001$ versus LgA + Saline. $^{\#}p < 0.05$ versus 0. ShA + Saline, $n = 6$; ShA + AMPH, $n = 4$; LgA + Saline, $n = 7$; LgA + AMPH, $n = 7$.

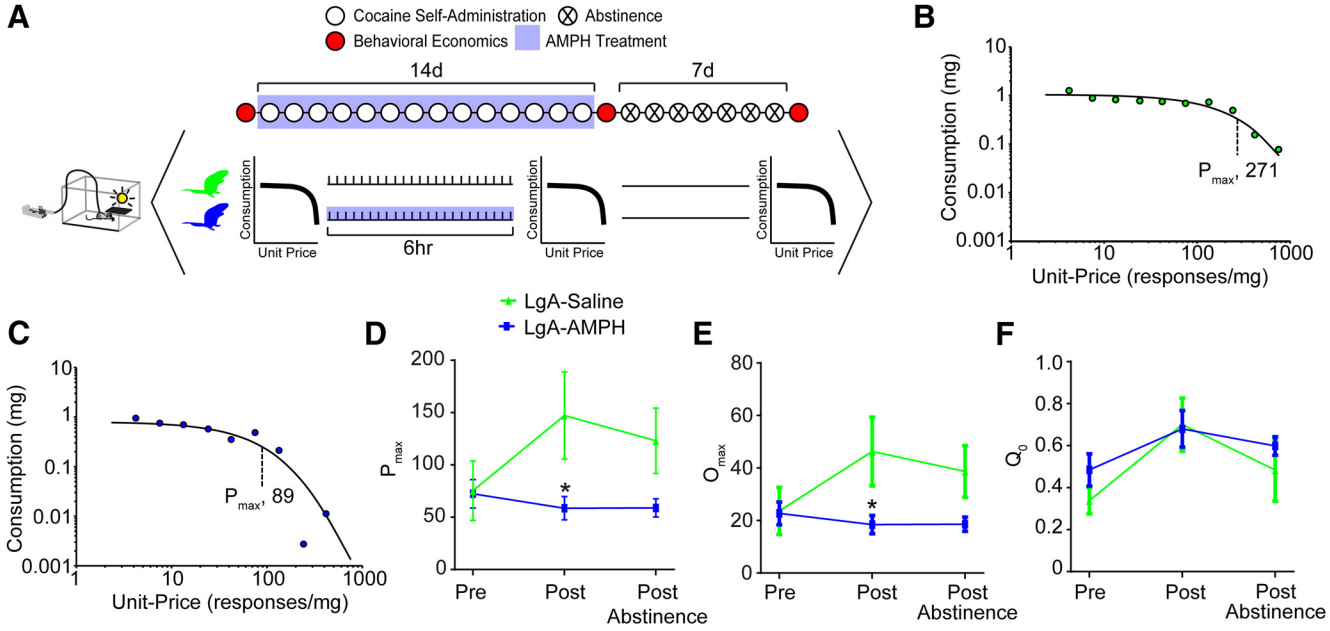


**Figure 2.** AMPH treatment prevented increase in motivation to administer cocaine. ***A***, Experimental timeline showing when assessment of motivation to obtain cocaine (threshold procedure) was performed. Representative data from LgA + Saline (***B***) and LgA + AMPH (***C***) animals showing that the reinforcing efficacy of cocaine ($P_{max}$) is decreased by AMPH treatment. ***D***, LgA-induced increases in reinforcing efficacy are prevented by AMPH treatment during LgA. Importantly, reinforcing efficacy stays depressed for up to 7 d following cessation of treatment. Similar results were seen with $O_{max}$ (***E***), but not $Q_0$ (***F***). Error bars indicate ± SEM. $^{*}p < 0.05$ versus LgA + Saline. LgA + Saline, $n = 5$; LgA + AMPH, $n = 7$.

(ShA + saline). Intake was decreased by AMPH treatment (5 mg/kg/d, delivered continuously via subcutaneous osmotic mini-pump) in LgA animals (LgA + AMPH), but not ShA animals (ShA + AMPH) (Fig. 1*B*): repeated-measures two-way ANOVA, treatment group × session, group ($F_{(3,20)} = 45.31$, $p < 0.0001$), interaction ($F_{(39,260)} = 1.572$, $p = 0.0216$). Escalation, as defined by last session minus first session infusions, was greater than zero only in the LgA + Saline animals, demonstrating that AMPH selectively blocked escalation in LgA animals (Fig. 1*C*; one sample t-test, LgA + Saline vs 0, $p = 0.01$).

We then used a behavioral economics approach to assess drug taking longitudinally across multiple points in the animal's self-administration history: at baseline, after LgA, and after an abstinence period (Fig. 2*A*). This approach, termed the "threshold procedure," allows for the assessment of an animal's motivation to obtain cocaine (maximal price paid [$P_{max}$]) (Fig. 2*B*,*C*). In LgA + saline animals, the motivation to administer cocaine was increased, which is a critical component of addiction in humans (Fig. 2*D*): repeated-measures two-way ANOVA (treatment group × time), group ($F_{(1,26)} = 7.762$, $p = 0.0098$). Amphetamine treatment, delivered by osmotic mini-pump during LgA self-administration, blocked the increased motivation to administer cocaine (Fig. 2*D*,*E*). Importantly, AMPH mini-pumps were removed 18 h before the assessment of reinforcing efficacy; thus, these results are a consequence of prior AMPH treatment, rather than an acute AMPH effect. Also, in animals treated with AMPH, the reinforcing efficacy of cocaine did not rebound even 1 week following cessation of treatment (Fig. 2*D*), prompting us to hypothesize that AMPH effects go beyond simple agonist replacement therapy. We then aimed to determine the neurochemical mechanism underlying escalation of cocaine intake, and if amphetamine is able to reverse these deficiencies.

### AMPH treatment during cocaine self-administration prevents cocaine-induced DAT alterations

To determine how the development of addictive behaviors and reversal by AMPH relate to cocaine effects at the DAT, animals were killed for *ex vivo* FSCV recordings in the NAc core 18 h

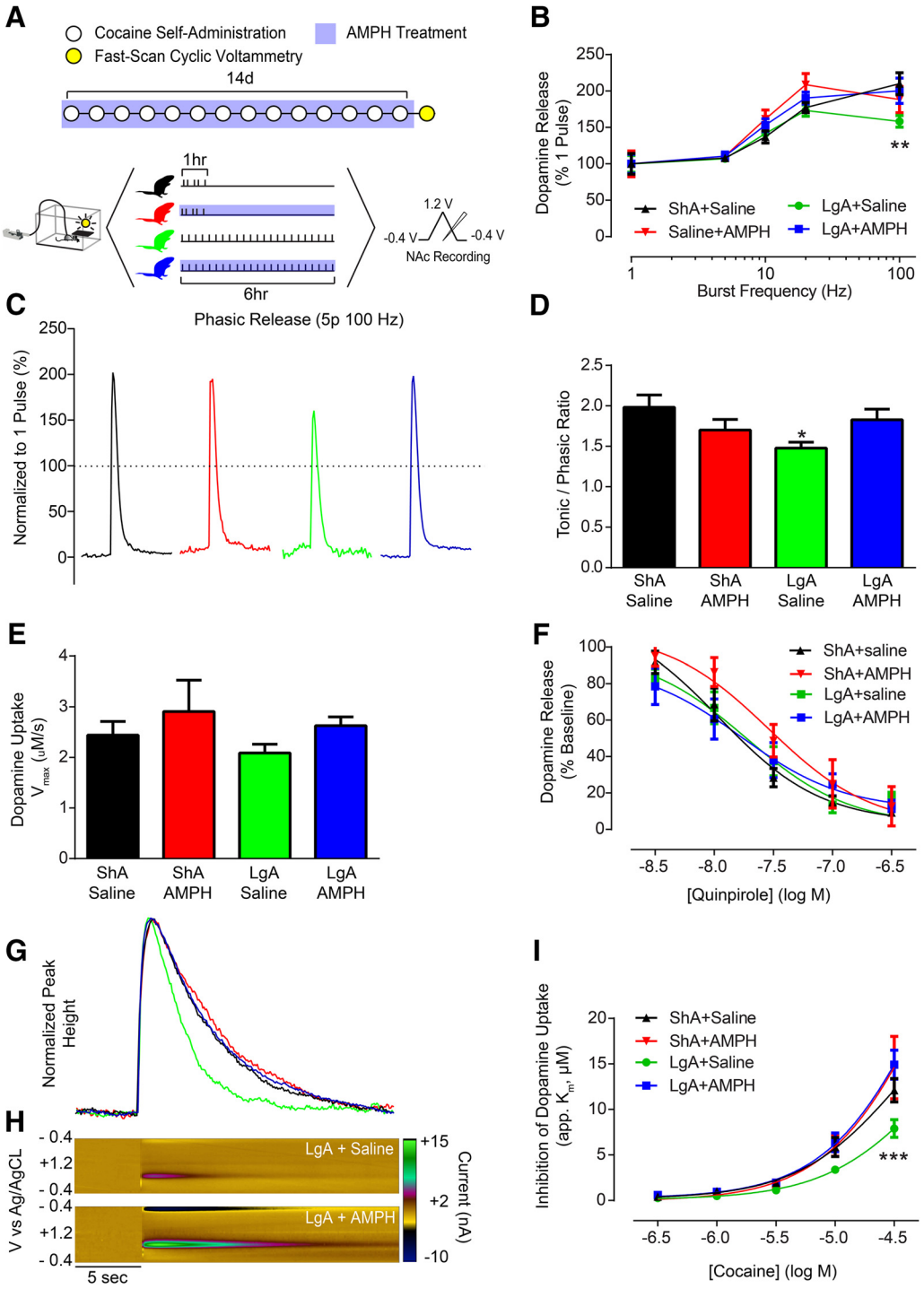


**Figure 3.** AMPH treatment prevented cocaine-evoked alterations to rapid dopamine signaling and cocaine potency. ***A***, Timeline showing conditions across days (top) and within days (bottom). ***B***, Dopamine release elicited by phasic-like stimulations (5 pulse, 100 Hz) is reduced in LgA animals, and this effect is prevented by treatment with AMPH during cocaine self-administration. ***C***, Representative traces elicited by 100 Hz stimulation trains indicating that phasic dopamine release is attenuated in LgA + Saline animals, and that this effect is prevented by AMPH treatment. ***D***, Phasic/tonic ratio (100 Hz/5 Hz) is reduced in LgA animals, and this effect is prevented by AMPH treatment. ***E***, Maximal rate of dopamine uptake ($V_{max}$) is unaffected in all treatment groups when AMPH treatment is given during cocaine self-administration. ***F***, Quinpirole concentration response curves show no change in autoreceptor sensitivity across all treatment groups. ***G***, Representative traces following bath application of 30 $\mu$M cocaine. ***H***, Pseudo-color plots for LgA + saline (top) and LgA + AMPH (bottom) animals following bath application of 30 $\mu$M cocaine. ***I***, Group data demonstrating that LgA cocaine self-administration results in a reduction in the ability of cocaine to inhibit the DAT, and that this effect is selectively prevented by AMPH treatment. Error bars indicate ± SEM. *$p < 0.05$ versus ShA + Saline. **$p < 0.01$ versus ShA + Saline. ***$p < 0.001$ versus ShA + Saline. ShA + Saline, $n = 6$; ShA + AMPH, $n = 4$; LgA + Saline, $n = 6$; LgA + AMPH, $n = 7$.

following cessation of the final cocaine self-administration session and AMPH (or saline) treatment (Fig. 3*A*). Before examining cocaine effects, we first determined whether basal dopamine terminal function was altered. To probe differences in dopamine release magnitude, we used 5 pulse stimulations and varied stimulation frequency to mimic the within-burst firing rates of dopamine neurons (Schultz, 1986; Hyland et al., 2002). LgA reduced phasic dopamine release (i.e., 20 or 100 Hz stimulations) (Fig.

3*B*,*C*), and this decrease was selectively prevented by treatment with AMPH (Fig. 3*B*): repeated-measures two-way ANOVA (treatment group × burst frequency), burst frequency ($F_{(4,136)} = 49.46$, $p < 0.0001$). Phasic/tonic ratios (100 Hz/5 Hz) revealed that LgA reduced the contrast between tonic and phasic signaling and that all other groups were unaffected (Fig. 3*D*): one-way ANOVA ($F_{(3,34)} = 2.416$, $p = 0.0834$); Bonferroni post-test: ShA+Saline vs LgA+Saline ($p < 0.05$). Importantly, we saw no effect on maximal rate of dopamine uptake ($V_{max}$) in any group (Fig. 3*E*), and alterations in dopamine release were not driven by changes in D2-type autoreceptor activity (Fig. 3*F*).

Despite basal dopamine uptake being unaltered, bath application of cocaine to brain slices (Fig. 3*G*,*H*) revealed that LgA resulted in marked tolerance to the ability of cocaine to inhibit the DAT compared with ShA animals; however, the development of tolerance was completely prevented by AMPH treatment (Fig. 3*I*): repeated-measures two-way ANOVA (treatment group × concentration), group ($F_{(3,19)} = 4.168$, $p = 0.0199$), concentration ($F_{(4,76)} = 144.6$, $p < 0.0001$), interaction ($F_{(12,76)} = 2.732$, $p = 0.0040$). The fact that there was no effect of cocaine self-administration or AMPH treatment on dopamine uptake rate under drug-free bath conditions suggests that alterations in cocaine potency and rescue by AMPH treatment occur independently from canonical DAT function. These results prompted us to hypothesize that AMPH-induced decreases in cocaine intake are due to reversal of cocaine tolerance.

### AMPH treatment during abstinence reverses the effects of chronic cocaine on the DAT

If AMPH's actions as a pharmacotherapeutic agent are indeed due to a selective amelioration of cocaine-induced dopamine deficits, it should reverse cocaine effects when given during abstinence, in addition to blocking cocaine effects when given during use. To test this hypothesis, animals were allowed 14 d of cocaine self-administration without treatment and were treated during a 7 d abstinence period (Fig. 4*A*). Following treatment, identical voltammetry experiments were performed to determine the ability of AMPH treatment during abstinence to reverse cocaine-induced dopaminergic deficits. Surprisingly, in contrast to decreased phasic dopamine signaling induced by LgA, following abstinence, phasic dopamine signaling was increased (Fig. 4*B*,*C*). Importantly, treatment with AMPH during abstinence selectively attenuated LgA-induced increases in phasic signaling while having no effect on ShA animals treated with AMPH (Fig. 4*B*): repeated-measures two-way ANOVA (treatment group × burst frequency), burst frequency ($F_{(4,104)} = 38.18$, $p < 0.0001$). Calculation of phasic/tonic ratios revealed that LgA and withdrawal increased the dynamic range that can be encoded between tonic and phasic signaling and that all other groups were unaffected (Fig. 4*D*): one-way ANOVA ($F_{(3,26)} = 2.879$; $p = 0.0552$); Bonferroni post-test: ShA+Saline vs LgA+Saline ($p < 0.05$). Similar to animals treated during cocaine self-administration, we found no effects on dopamine uptake rate (Fig. 4*E*), and no changes were observed in dopamine autoreceptor sensitivity (Fig. 4*F*).

We then sought to determine whether AMPH treatment during abstinence reversed cocaine tolerance at the DAT. Following cocaine self-administration and abstinence (Fig. 4*A*), concentration-response curves for cocaine (Fig. 4*G*,*H*) revealed that LgA animals remained tolerant to cocaine's effects during abstinence. In support of our hypothesis, this effect was reversed by AMPH treatment (Fig. 4*I*): repeated-measures two-way ANOVA (treatment group × concentration), concentration ($F_{(4,64)} = 152.5$; $p < 0.0001$), interaction ($F_{(12,64)} = 2.472$, $p = 0.0101$). Together, these results demonstrate that AMPH selectively prevents or reverses cocaine-induced plasticity of DATs and ameliorates aberrant cocaine-taking behaviors.

### Cocaine tolerance is concomitant with formation of DAT oligomers

Our data demonstrate a surprising effect of AMPH whereby both the neurochemical and behavioral deficits induced by escalated cocaine self-administration are prevented or reversed by AMPH treatment. We next sought to determine the molecular mechanisms underlying this amelioration. Previous studies have shown that DAT can be present at the membrane in complexes containing multiple DAT molecules, or DAT oligomers (Hastrup et al., 2001; Torres et al., 2003). Interestingly, these complexes may display altered inhibition by uptake inhibitors compared with DAT monomers (Chen and Reith, 2007), and thus may underlie the cocaine tolerance observed in the current study. Given that AMPH interacts with multiple signaling cascades that affect DAT and could alter its conformation (Fog et al., 2006; Moritz et al., 2015), we hypothesized that the development of cocaine tolerance and restoration by AMPH may be due to the formation/dispersion of DAT oligomer complexes.

To test this hypothesis, we first confirmed that the phenomenon of cocaine tolerance and reversal by AMPH could be observed in a reduced preparation where it is possible to detect DAT-DAT interactions. We exposed the N2A-DAT cells to cocaine (10 $\mu$M) for 3 d before measuring cocaine-induced inhibition of [$^3$H]dopamine uptake. We found that, indeed, pretreatment with cocaine markedly reduced the ability of cocaine to inhibit DAT uptake activity without changing baseline uptake rates (Fig. 5*A*), mirroring our findings following self-administration of cocaine. Further, the addition of AMPH to the buffer fully reversed this effect back to control levels: repeated-measures two-way ANOVA (treatment group × concentration), concentration ($F_{(5,30)} = 664.1$; $p < 0.0001$), group ($F_{(2,6)} = 11.42$; $p = 0.009$), interaction ($F_{(10,30)} = 9.453$; $p < 0.0001$). Calculation of $IC_{50}$ concentrations for effects of cocaine on uptake revealed large cocaine pretreatment-induced increases (i.e., decreased potency) and restoration by AMPH (Fig. 5*B*): one-way ANOVA ($F_{(2,6)} = 104.6$, $p < 0.0001$).

To test the hypothesis that cocaine tolerance and its reversal by AMPH are associated with an oligomeric form of DAT complexes, we used a similar tolerance-inducing protocol in DAT-expressing cells. Here, however, we used FRET microscopy, a quantitative microscopy approach that allows examination of interactions between proteins that exist in close proximity to each other (Fig. 5*C*) (Schmid et al., 2001; Sorkina et al., 2003; Biener et al., 2005). We investigated the FRET efficiency between CFP-DAT, the donor molecule, and YFP-DAT, the acceptor molecule at the stacked membrane. This FRET pair has been extensively used and characterized in the literature (Oliveria et al., 2003; Rizzo et al., 2006; Grabowska et al., 2014; Butler et al., 2015). As shown in Figure 5*D*, we measured a positive FRET signal between YFP-DAT and CFP-DAT at or near the membrane. No FRET signal was detected in control experiments (Fig. 4*D*). The control experiments were performed in cells that: (1) coexpressed YFP empty vector and CFP-DAT or (2) coexpressed CFP empty vector and YFP-DAT. Furthermore, consistent with the literature (Rizzo et al., 2006; Grabowska et al., 2014), we measured a large FRET signal in cells expressing a tandem YFP-CFP (FRET8) (Fig. 5*D*), which served as a positive control group.

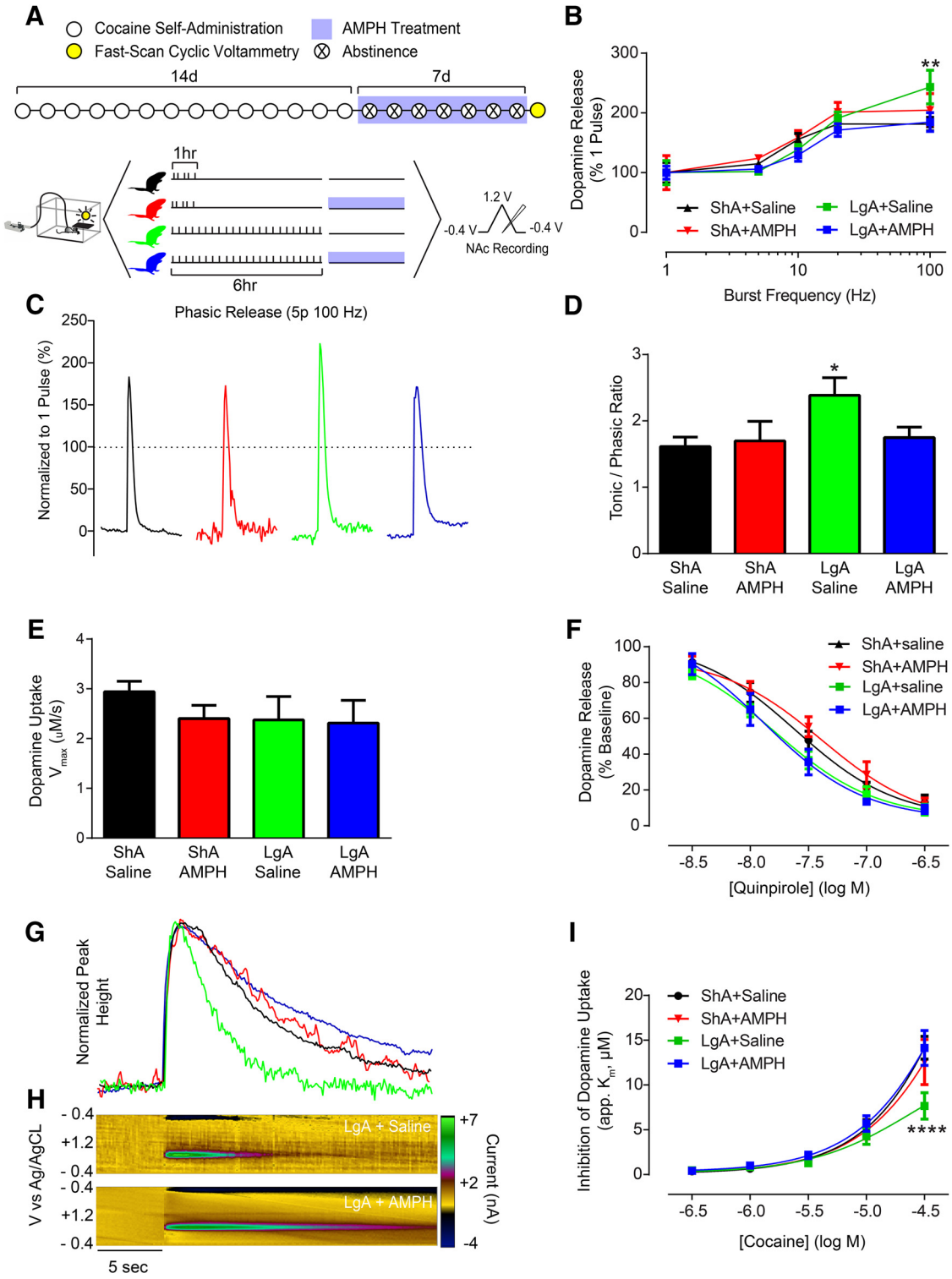


**Figure 4.** AMPH treatment reversed cocaine-induced changes to dopamine signaling. ***A***, Experimental timeline depicting 14 d of cocaine self-administration (LgA or ShA) followed by a 7 d abstinence period. During the abstinence period, animals were treated with either saline or AMPH delivered via osmotic mini-pump. ***B***, Dopamine release elicited by tonic-like frequency stimulations (5 pulse, 5 or 10 Hz) is unchanged in any treatment group. Dopamine release elicited by phasic-like frequency stimulations (5 pulse, 100 Hz) is increased in LgA animals after a 7 d abstinence period, and this effect is reversed by treatment with AMPH for 7 d following LgA. ***C***, Representative traces indicating that phasic dopamine release is augmented in LgA animals after an abstinence period, and that this effect is reversed by AMPH treatment. ***D***, Phasic/tonic ratio (100 Hz/5 Hz) is increased in LgA animals after a 7 d abstinence period, and this effect is reversed by AMPH treatment. ***E***, Maximal rate of dopamine uptake ($V_{max}$) is unaffected in all treatment groups when AMPH treatment is given during abstinence. ***F***, Quinpirole concentration response curves show no change in autoreceptor sensitivity across all treatment groups. ***G***, Representative traces following bath application of 30 $\mu$M cocaine indicating that cocaine is less effective at inhibiting dopamine uptake following LgA cocaine self-administration and abstinence. ***H***, Pseudo-color plots for LgA + Saline (top) and LgA + AMPH (bottom) animals following bath application of 30 $\mu$M cocaine. ***I***, Group data demonstrating that LgA cocaine self-administration animals remain tolerant to cocaine's effects following a 7 d abstinence period, and that this effect is reversed by AMPH treatment during abstinence. Error bars indicate ± SEM. *$p < 0.05$ versus ShA + Saline. **$p < 0.01$ versus ShA + Saline. ****$p < 0.0001$ versus ShA + Saline. ShA + Saline, $n = 6$; ShA + AMPH, $n = 5$; LgA + Saline, $n = 4$; LgA + AMPH, $n = 5$.

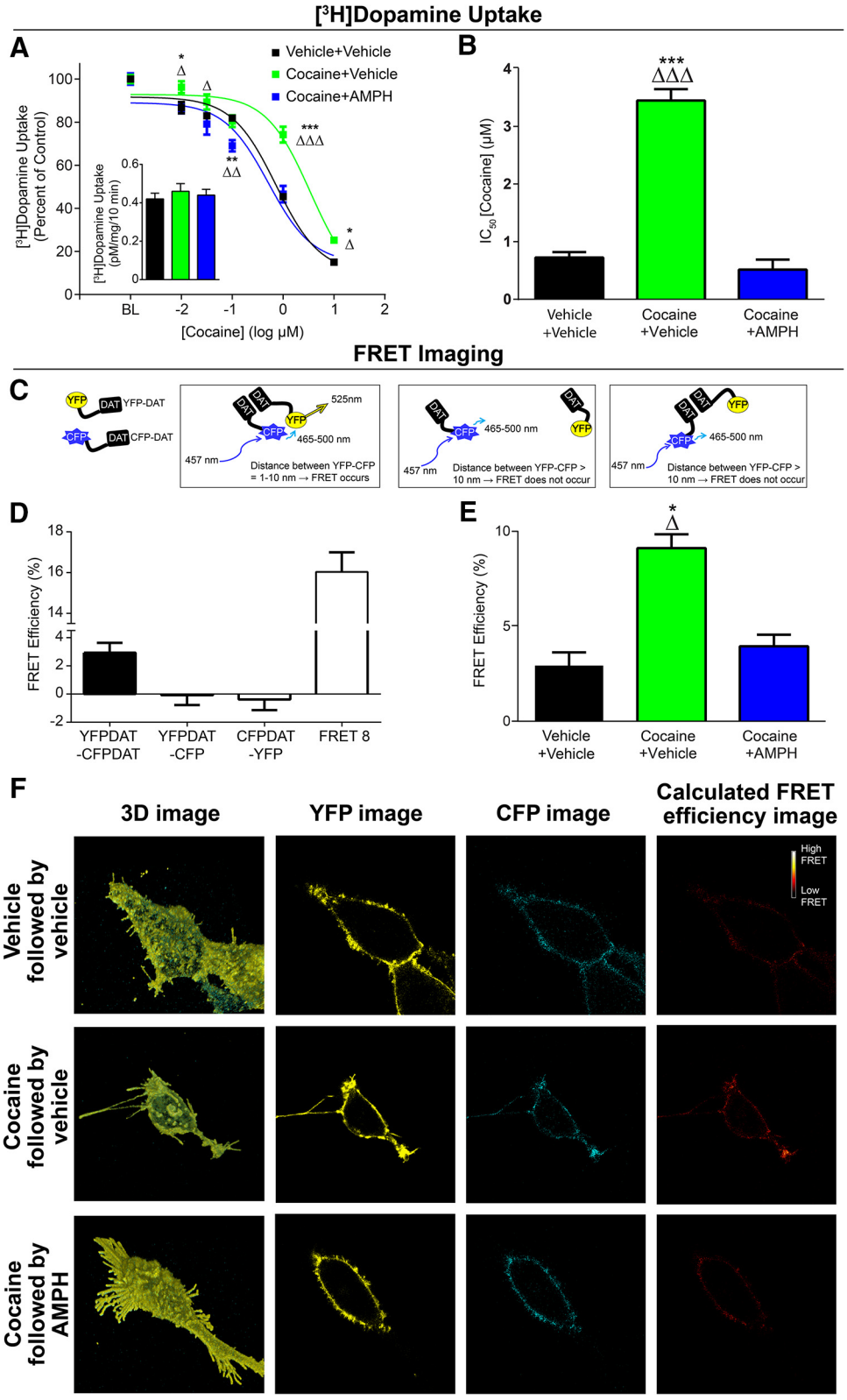


**Figure 5.** DAT oligomers are concomitant with cocaine tolerance. ***A***, N2A cells were treated for 3 d with cocaine (10 $\mu$M) followed by AMPH (10 $\mu$M) on the fourth day before assessing cocaine-induced inhibition of [$^3$H]dopamine uptake. We found that cocaine exposure produced marked tolerance to cocaine effects, that this effect was ameliorated by AMPH. Inset, Baseline uptake rate for 10 nM [$^3$H]dopamine was unchanged in any group. ***B***, $IC_{50}$ values for cocaine effects on dopamine uptake across groups. ***C***, Schematic describing the use of FRET imaging to measure DAT-DAT interactions. Cells were transfected with YFP-DAT and CFP-DAT or appropriate control constructs as described in Materials and Methods. ***D***, Control conditions for FRET measurements. ***E***, Group data showing that cocaine exposure increases FRET efficiency, and this was decreased by AMPH treatment. ***F***, Representative computed FRET images generated by Nikon NIS Elements software using the FRET efficiency equation described above, showing effects of cocaine exposure and AMPH treatment on FRET efficiency between YFPDAT/CFPDAT. BL, baseline. Error bars indicate ± SEM. *$p < 0.05$ versus Vehicle+Vehicle. **$p < 0.01$ versus Vehicle+Vehicle. ***$p < 0.001$ versus Vehicle+Vehicle. $^{\triangle}p < 0.05$ versus Cocaine+AMPH. $^{\triangle\triangle}p < 0.01$ versus Cocaine+AMPH. $^{\triangle\triangle\triangle}p < 0.001$ versus Cocaine+AMPH. ***A***, ***B***, $n = 3$ per group. ***C–F***, YFPDAT-CFP, $n = 15$; CFPDAT-YFP, $n = 15$; FRET8, $n = 29$; Vehicle+Vehicle, $n = 26$; Cocaine+Vehicle, $n = 28$; Cocaine+AMPH, $n = 25$.

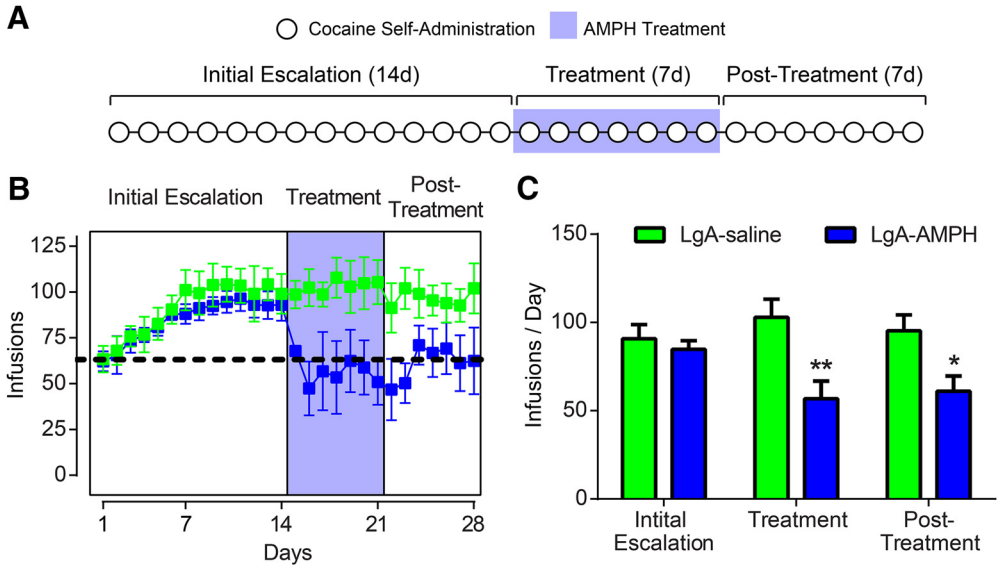


**Figure 6.** Cocaine reverses escalated cocaine intake. ***A***, Experimental timeline of 28 d of cocaine self-administration split into three epochs: (1) 14 d of self-administration with no treatment (initial escalation period), (2) 7 d of AMPH treatment during cocaine self-administration (treatment period), and (3) 7 d of self-administration after cessation of treatment (post-treatment period). ***B***, Average infusions per day over the 28 d of self-administration. Dotted line indicates average intake across groups on day 1. Shaded area represents the AMPH treatment period. ***C***, Cocaine intake was decreased by AMPH treatment during the treatment and post-treatment periods. Error bars indicate ± SEM. *$p < 0.05$ versus LgA + Saline. **$p < 0.01$ versus LgA + Saline. LgA + Saline, $n = 5$; LgA + AMPH, $n = 5$.

YFP-DAT/CFP-DAT-expressing cells were subjected to cocaine exposure followed by AMPH treatment as described in the [$^3$H]dopamine uptake assay. We found that 3 d of cocaine exposure significantly increased the FRET efficiency between YFP-DAT and CFP-DAT (Fig. 5*E*,*F*), which suggests increased YFP-DAT/CFP-DAT oligomers. AMPH treatment following the 3 d cocaine exposure decreased cocaine-induced increase in FRET efficiency back to control levels (Fig. 5*E*,*F*): one-way ANOVA ($F_{(2,76)} = 24.16$, $p < 0.0001$). These data are consistent with the interpretation that AMPH disperses cocaine-induced DAT oligomers and reverses cocaine-induced tolerance.

### AMPH treatment reverses escalated cocaine use

Thus far, our data suggest that AMPH effects on self-administration of cocaine are due to AMPH-induced reversal of complex formations of the DAT, which produce functional alterations in signaling and resulting behavior. Further, these results are in opposition to proposed hypotheses stating that AMPH's efficacy as a pharmacotherapeutic agent are due to simple elevations in extracellular dopamine (Rush and Stoops, 2012). If AMPH effects are indeed due to reversal of plasticity, AMPH treatment should (1) reverse escalated cocaine consumption back to initial levels and (2) have lasting benefit beyond the treatment period. We tested for these qualities by allowing animals to escalate their cocaine intake over a 14 d self-administration period (initial escalation) before receiving AMPH treatment during an additional 7 d of self-administration (treatment period). Finally, animals were allowed to self-administer cocaine for 7 more days following the cessation of treatment (post-treatment period) (Fig. 6*A*). We found that AMPH treatment was able to reverse escalated cocaine intake back to pre-escalation levels. Remarkably, intake remained low during the 7 d post-treatment period (Fig. 6*B*,*C*): two-way ANOVA (treatment group × epoch), treatment group ($F_{(1,23)} = 16.41$, $p = 0.0005$). Together, these data show AMPH-induced reversal of cocaine-evoked plasticity on a molecular, physiological, and behavioral level (Fig. 7).

## Discussion

Here we outline molecular adaptations to the DAT produced by chronic cocaine exposure, and define their physiological impact on dopamine signaling and behavioral actions of cocaine. We show that escalated cocaine self-administration produces neurochemical tolerance to cocaine effects that lasts at least 7 d. Further, using AMPH to reverse tolerance, we show that preventing or reversing cocaine tolerance prevents or reverses escalation of cocaine intake, respectively. We provide a putative molecular mechanism for tolerance to cocaine effects, which has broad implications for the cocaine addiction field. Finally, we outline an unexpected mechanism for AMPH's pharmacotherapeutic actions, which may lead to the development of more targeted therapeutics, potentially avoiding the abuse liability associated with AMPH.

### Specificity of AMPH effects and methodological considerations

One important finding of the current study is that the LgA model of cocaine self-administration accurately models the dopaminergic profile of human cocaine addicts. Indeed, PET imaging studies of cocaine-dependent individuals have revealed that they exhibit decreased dopaminergic responsiveness and tolerance to the dopamine-elevating effects of cocaine (Volkow et al., 1996, 1997, 2014), effects that were both observed following LgA in the current study (but see Ahmed et al., 2003). It is hypothesized that decreased cocaine potency is an integral component of the neurochemical consequences of cocaine use, which underlies maladaptive behaviors of cocaine addicts, such as increased intake to compensate for decreased subjective effects (Dackis and O'Brien, 2001). Our data support these hypotheses by providing a mechanistic link between cocaine potency at the DAT and the motivation to administer cocaine. We show that AMPH regimens that ameliorate cocaine-induced changes to the DAT also prevented escalation of intake, and suppressed LgA-induced increases in the

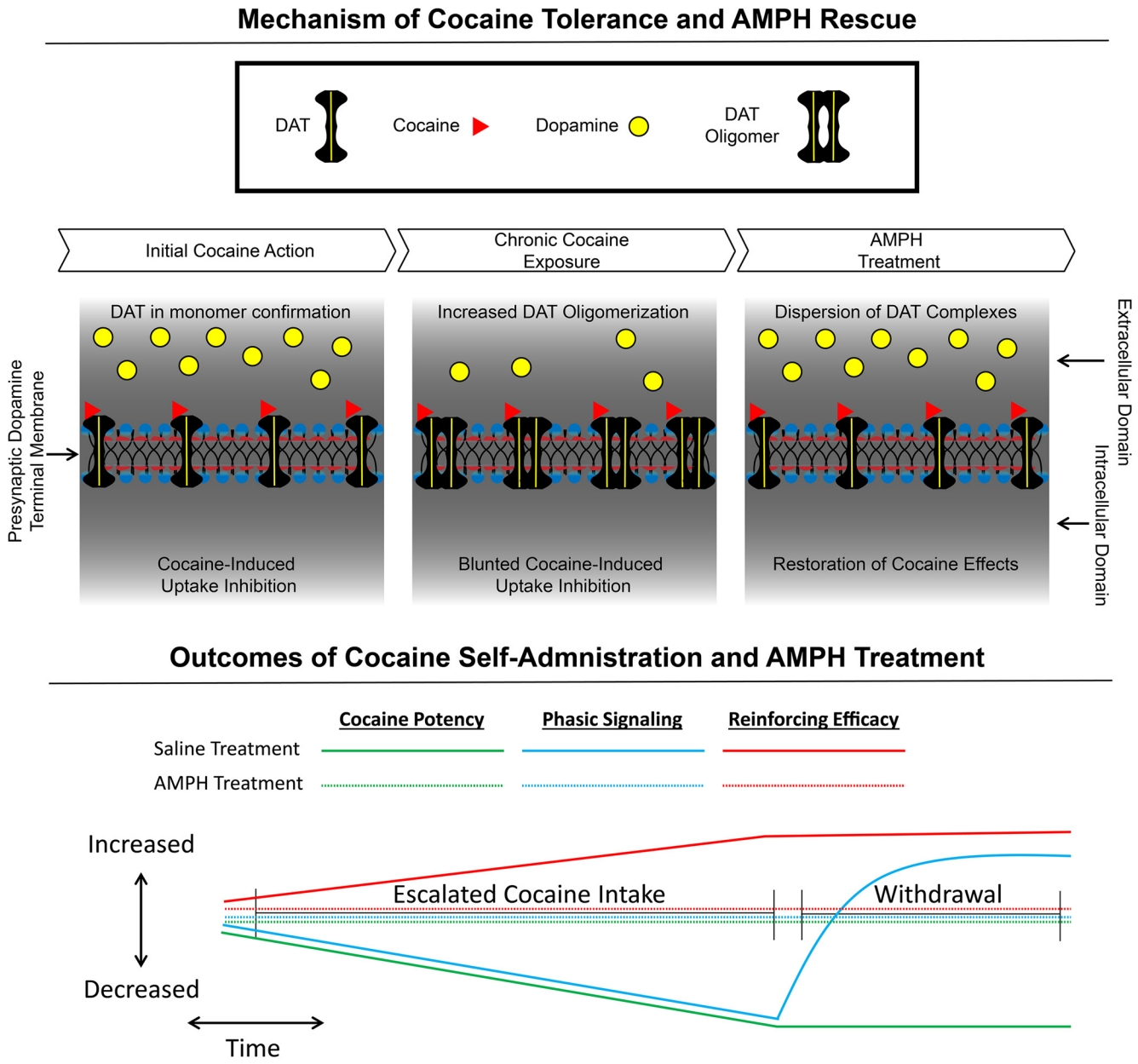


**Figure 7.** Summary of findings. Top, Model of the molecular underpinnings of the development of cocaine tolerance and restoration by AMPH. Bottom, Model of cocaine-induced changes to phasic dopamine signaling, cocaine potency, and reinforcing efficacy of cocaine over the course of LgA and abstinence. All three measures are normalized to control levels by AMPH.

reinforcing efficacy of cocaine. It is important to note that these changes are likely specific to cocaine reinforcement, as responding for food has been shown to be unchanged by AMPH using the same dose as in the current study (Chiodo et al., 2008). Additionally, studies in nonhuman primates have shown that AMPH treatment either does not affect, or transiently decreases, food consumption, whereas decreases in responding for cocaine are consistent and long-lasting (Negus et al., 2003a, b; Czoty et al., 2010, 2011). In the current study, neither cocaine self-administration nor AMPH treatment produced changes in basal dopamine uptake rate, demonstrating that changes in cocaine potency are independent of alterations to orthosteric function of the transporter (i.e., interactions with native substrates are unchanged). This is supported by previous work demonstrating that genetically increasing expression of the DAT has no effect on cocaine potency (Salahpour et al., 2008; Calipari et al., 2013), and is particularly important as it demonstrates that AMPH can modulate cocaine potency without disrupting basal DAT function. Further, this led us to hypothesize that cocaine tolerance induced by cocaine self-administration, and reversal by AMPH, were acting through an allosteric mechanism.

The observation that changes in cocaine potency are independent of orthosteric DAT function led us to explore possible conformational changes that could affect cocaine binding without altering basal uptake. Previous literature in cultured cells has suggested that the formation of DAT complexes can alter the effects of DAT blockers on uptake (Chen and Reith, 2007). Based on these studies, we explored the possibility that cocaine may affect the preferred conformation of the DAT, and found that cocaine exposure increased formation of oligomeric DAT complexes as indicated by increased FRET efficiency between YFP and CFP-tagged DATs. It is important to note that, although FRET microscopy reliably identifies close association of FRET pairs (~100 Å), it does not

distinguish the number of molecules within the oligomers and cannot tell us whether dimers, trimers, tetramers, or other groupings are formed, or whether there are additional proteins complexed with the DATs. In addition, our reported cocaine-induced increase in YFP-DAT/CFP-DAT FRET efficiency likely underestimates cocaine-induced DAT oligomerization because FRET does not occur between other combinations of oligomers, such as YFP-DAT/YFP-DAT or CFP-DAT/CFP-DAT oligomers. Unfavorable orientations of the two fluorophores will not result in FRET occurrence, thus further underestimating the actual magnitude of DAT-DAT interactions.

**Mechanisms of DAT oligomerization**

This is the first demonstration that chronic cocaine exposure can stimulate the formation of DAT oligomers. Importantly, these oligomers were dispersed by brief exposure to AMPH, similar to the restoration of cocaine effects observed *in vivo*. It is possible that AMPH is altering cocaine-induced oligomerization of DAT via mobilization of intracellular signaling cascades or proteins that could ultimately affect DAT conformation. For example, AMPH is known to interact with a number of different proteins that can also affect DAT, including $\alpha$-synuclein (Mortensen et al., 2003; Wersinger et al., 2006; Butler et al., 2015) and dopamine D2 autoreceptors (Sevak et al., 2007; Chen et al., 2009, 2015; Bowton et al., 2010; Owens et al., 2012), as well as alter signaling pathways, including calmodulin kinases, phosphatidylinositol 3-kinase, Rho, and protein kinase A and C$\beta$ (Carvelli et al., 2002; Fog et al., 2006; Wei et al., 2007; Lute et al., 2008; Saunders and Galli, 2015; Wheeler et al., 2015). Given that these pathways/proteins can result in differential phosphorylation, palmitoylation, and conformations of the DAT (e.g., inward- vs outward-facing DAT) and differential AMPH-induced DAT efflux (Khoshbouei et al., 2004; Cervinski et al., 2005; Foster et al., 2006, 2008, 2012; Robertson et al., 2009; Gaffaney et al., 2014; Moritz et al., 2015), it is possible that these alterations may result in restructuring of DAT-DAT interactions and return them to monomer conformation.

**Alterations to presynaptic dopamine release**

It is important to note that cocaine self-administration also resulted in changes in presynaptic dopamine release, which may play an important role in cocaine-seeking behaviors. Phasic dopamine release is involved in encoding the salience of rewards as well as encoding the probability that they will occur, given particular environmental cues (Waelti et al., 2001; Phillips et al., 2003; Tobler et al., 2005; Nicola, 2010; Steinberg et al., 2013). As such, phasic dopamine signaling is integral to selecting goal-oriented behaviors that produce advantageous outcomes, and its dysregulation by abused drugs has been hypothesized to be a critical component of maladaptive decision making in drug abusers. Here we show a time-dependent and biphasic effect of cocaine-induced alterations to phasic signaling at the level of the dopamine terminal. Initially, cocaine self-administration results in a reduction of dopamine terminal responsivity to phasic-like stimulations. This is consistent with previous results showing that phasic dopamine release decreases over the course of LgA proportionally with escalated cocaine intake (Willuhn et al., 2014). Combined with reduced postsynaptic dopamine receptors (Volkow et al., 1990; Nader et al., 2006), reductions in dopamine release are likely to result in a greatly decreased range of dopaminergic modulation of striatal outputs during decision-making in the acute withdrawal period. Conversely, following abstinence, phasic signaling was increased. Increased activation of NAc core output neurons by cocaine-associated cues is thought to underlie cocaine craving and seeking (Hollander and Carelli, 2007), which may be mediated, in part, by increased cue-elicited phasic dopamine signaling (Phillips et al., 2003). Thus, augmented phasic signaling during abstinence may contribute to the time-dependent increase in cocaine-seeking behaviors, termed "incubation," which occurs following extended access cocaine self-administration (Grimm et al., 2001). It will be interesting to see whether the observed increase in phasic-like release potential *ex vivo* will translate to increased dopamine release to cocaine-associated cues *in vivo*. We found that low-dose continuous AMPH treatment ameliorated both decreases in phasic signaling observed immediately following LgA and increases in phasic signaling during abstinence.

The mechanisms governing cocaine-induced changes in release are currently unknown. One possible explanation is that release changes are also downstream of cocaine-induced alterations to the DAT. Because the DAT is responsible for reuptake and repacking of dopamine into vesicles, DAT function is tightly linked to vesicular dopamine content and storage, and changes in dopamine uptake often result in changes in dopamine release magnitude (Ferris et al., 2014). Although we did not see a change in uptake rate following LgA or AMPH treatment, vesicular repackaging also relies on membrane-bound formations of multiple proteins, including DAT, vesicular monoamine transporter-2, and scaffolding proteins, such as synaptogyrin-3 (Egaña et al., 2009; Cartier et al., 2010; Eriksen et al., 2010). Thus, it is possible that the formation of DAT oligomers may disrupt normal docking to vesicular monoamine transporter-2, thereby altering dopamine repacking and release. If this is the case, it would also explain why AMPH treatment rescued cocaine self-administration-induced changes in dopamine release.

In conclusion, we delineate a mechanistic link between cocaine-induced dopaminergic deficits and addictive behaviors. We show that escalation and increased motivation are associated with alterations in cocaine potency; and by reversing this tolerance, normal behavioral function is restored. A key aspect of the AMPH-induced restoration of dopamine system function is that cocaine self-administration remained attenuated even 7 d after cessation of treatment, suggesting that AMPH may have long-lasting benefits in human cocaine addicts after short treatment periods, rather than daily maintenance, which has been used with replacement therapies for heroin (Trussell and Gollance, 1970). Further, we demonstrate that the formation of DAT oligomers and subsequent restoration to monomers underlie the development of cocaine tolerance and AMPH reversal. Finally, one major reason that AMPH has not been approved for use in cocaine addicts despite extensive evidence of its efficacy (Grabowski et al., 2001, 2004; Negus et al., 2003a, b, 2007; Chiodo et al., 2008; Czoty et al., 2010, 2011; Rush and Stoops, 2012; Banks et al., 2015; Nuijten et al., 2016) is its inherent abuse liability (Negus and Henningfield, 2015). However, by elucidating the therapeutic action of AMPH, these findings may lead to the development of novel therapeutic compounds that have similar properties to AMPH in regard to stabilization of the dopamine system, but without abuse liability.

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