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Reinforcing Doses of Intravenous Cocaine Produce Only Modest Dopamine Uptake Inhibition

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Abstract

The reinforcing efficacy of cocaine is thought to stem from inhibition of the dopamine transporter (DAT) and subsequent increases in extracellular dopamine concentrations in the brain. In humans, this hypothesis has generally been supported by positron emission tomography imaging studies where the percent of DAT occupied by cocaine is used as a measure of cocaine activity in the brain. Interpretation of these studies, however, often relies on the assumption that measures of DAT occupancy directly correspond with functional DAT blockade. In the current studies, we used in vivo and in vitro fast scan cyclic voltammetry in mice to measure dopamine uptake inhibition following varying doses of cocaine as well as two high affinity DAT inhibitors. We then compared dopamine clearance rates following these drug treatments to dopamine clearance obtained from DAT knockout mice as a proxy for complete DAT blockade. We found that administration of abused doses of cocaine resulted in approximately 2% of maximal DAT blockade. Overall our data indicate that abused doses of cocaine produce a relatively modest degree of DA uptake inhibition, and suggest that the relationship between DAT occupancy and functional blockade of DAT is more complex than originally posited.

Keywords

Dopamine transporter; Fast scan cyclic voltammetry; Occupancy; WF23; PTT

Introduction

An extensive literature demonstrates that cocaine interacts with monoamine transporters to inhibit the uptake of dopamine (DA), norepinephrine, and serotonin¹. While each of these neurotransmitter systems plays a role in the psychotropic effects of cocaine², several lines of evidence indicate that the reinforcing actions of cocaine primarily stem from inhibition of the DA transporter (DAT). For instance, multiple studies indicate that the rewarding and reinforcing effects of cocaine and phenyltropane cocaine analogs are highly correlated with their affinities for the DAT^{1, 3–7} and associated elevations in extracellular DA^{8, 9}. Consistent

with this, mice engineered to express cocaine-insensitive DATs display reduced conditioned place preference for cocaine¹⁰.

These observations generated the long-standing hypothesis that the reinforcing effects of cocaine are derived from enhancement of DA signaling that occurs due to inhibition of the DAT^{11–13}. This perspective has generally been supported by positron emission tomography (PET) studies. For example, PET has been used to demonstrate that psychostimulants increase striatal DA levels in humans^{14, 15}, and that the percentage of DATs occupied by cocaine correlates with the subjective experience of ‘high’¹⁶. PET studies in humans and non-human primates have also shown that low doses of cocaine that do not produce a significant ‘high’, yield approximately 50% DAT occupancy^{16, 17}, and that the administration of a single, commonly abused dose of cocaine (0.6 – 1.0 mg/kg) results in approximately 70 – 80% DAT occupancy^{16, 17}. This result has been replicated across several species, using both PET imaging and binding assays^{16–19}, and this has led to the conclusion that most DAT are occupied following a single 0.6 – 1.0 mg/kg dose of cocaine in humans, baboons, and mice^{16–18}.

Based on conclusions drawn from PET imaging and DAT binding studies, it might be expected that doses of cocaine greater than 1.0 mg/kg or repeated dosing of cocaine would produce a negligible increase in DA uptake inhibition relative to a single 1.0 mg/kg dose²⁰. Importantly, however, cocaine doses greater than 1.0 mg/kg are abused in some human populations²¹, and rats are known to self-administer doses considerably higher than 1.0 mg/kg^{22, 23}. Moreover, it has repeatedly been shown that rats will self-administer cocaine at very rapid rates at the onset of cocaine self-administration sessions, a phenomenon that has been referred to as ‘loading’^{24–27}. During this loading phase, rats will rapidly self-administer multiple injections of cocaine at doses that would be predicted to achieve 70 – 80% DAT occupancy following a single dose. For example, rats given access to 2.0 mg/kg cocaine will administer 2–3 injections within the first 10 min of cocaine access^{22, 23}, likely elevating brain concentrations to levels that exceed a single 2.0 mg/kg injection²⁷.

Given that individuals will exceed cocaine intake to levels beyond those that produce 70 – 80 % occupancy, it has been suggested that the reinforcing nature of high or repeated doses of cocaine might stem from mechanisms that extend beyond cocaine inhibition of the DAT¹⁷. This interpretation, however, relies on the assumption that measures of DAT occupancy correspond to functional DAT blockade. For instance, it has been suggested that doses of cocaine that produce 70 – 80% DAT occupancy should produce an approximate 70 – 80% reduction in overall DAT function²⁰. Despite this assumption, the extent to which DAT occupancy relates to functional changes in DA uptake remains unclear. In the present studies, we first used in vivo voltammetry to determine the degree of DA uptake inhibition produced by multiple doses of cocaine (0.3 – 3.0 mg/kg). We then used in vitro and in vivo voltammetry to determine the magnitude of uptake inhibition produced by cocaine, and two separate high affinity DAT inhibitors, relative to estimates of complete DAT blockade obtained from DAT knockout (KO) mice.

Results and Discussion

Increasing doses of cocaine produce increasing DA uptake inhibition

We first sought to determine the degree of DA uptake inhibition produced by three doses of cocaine administered intravenously (i.v.). For these experiments, we measured DA uptake in the dorsal caudate of anesthetized mice following stimulation of DA cell bodies in the ventral tegmental area. Baseline DA uptake rate was determined before delivery of a single i.v. injection of either 0.3, 1.0, or 3.0 mg/kg cocaine. The 0.3 and 1.0 mg/kg cocaine doses were chosen to compare with previous studies examining similar dose ranges on DAT occupancy¹⁸ and because these doses exert robust reinforcing effects^{18, 22}. The selection of 3.0 mg/kg cocaine and 5.0 mg/kg for PTT and WF-23 was based on preliminary observations indicating that these were the highest, non-lethal doses possible in mice.

DAT-mediated uptake rate was determined by fitting DA overflow curves to the exponential decay model tau (66.6 % return to baseline; units in secs). We calculated DA uptake inhibition by measuring tau at baseline and comparing this to tau following cocaine delivery. Similar to our previous reports²⁸, examination of the time course of cocaine effects indicated that peak uptake inhibition was reached between 30 and 60 sec post-injection (Supplementary Table I). We used the peak uptake inhibition value for all subsequent analyses. As expected, we found significant DA uptake inhibition following injection of 0.3 (paired t-test: $t_{(4)} = 3.053$, $p < 0.05$), 1.0 (paired t-test: $t_{(5)} = 3.166$, $p < 0.05$) and 3.0 mg/kg cocaine (paired t-test: $t_{(5)} = 5.128$, $p < 0.01$) (Fig 1A, B). To compare the degree of DA uptake inhibition across cocaine doses, we expressed peak uptake inhibition as a percent of baseline tau (Figure 1C)²⁹. We found a significant effect of dose (One-way ANOVA: $F_{(2,14)} = 5.750$, $p < 0.05$), and as expected, post-hoc analysis indicated that DA uptake inhibition produced by the 3.0 mg/kg dose was greater than that produced by 0.3 mg/kg. We also found that despite producing a similar degree of DAT occupancy in mice¹⁸, 3.0 mg/kg cocaine also produced greater inhibition of DA uptake than 1.0 mg/kg cocaine. These experiments suggest that the relationship between cocaine dose and DA uptake inhibition is different than the reported relationship between cocaine dose and DAT occupancy^{16–18}.

Repeated administration of cocaine results in increasing DA uptake inhibition

Our single dose experiments (Figure 1) demonstrate a dissociation between the effects of cocaine on DA uptake inhibition and measures of DAT occupancy. We next sought to determine if repeated injections of cocaine that mimic patterns of cocaine self-administration produce DA uptake inhibition that exceeds that of a single dose of cocaine. To achieve this, we administered 10 i.v. injections of 1.0 mg/kg cocaine at inter-injections intervals based on rates of cocaine intake under a fixed ratio 1 (FR1) self-administration schedule (1 injection every 7 min)^{30, 31}. We found that repeated injections of cocaine resulted in cumulative increases in DA uptake inhibition across the 10 injections (One-way ANOVA: $F_{(4, 36)} = 37.01$, $p < 0.0001$), and that the tenth injection yielded the greatest degree of DA uptake inhibition (Figure 2). This indicates that repeated administration of reinforcing doses of cocaine that mimic behaviorally-relevant conditions can produce DA uptake inhibition that exceeds that of a single dose of cocaine.

Complete pharmacological DAT blockade can be achieved in vitro

The percentage of DAT occupied by cocaine has generally been thought to correspond with the percentage of functional DAT blockade^{17, 20}. However, the results presented above bring this assumption into question. We next used in vitro FSCV to determine the maximal degree of functional DAT blockade that can be produced by pharmacological inhibition. To do this, we compared DA uptake inhibition produced by cocaine and the high affinity DAT inhibitors 2beta-propanoyl-3beta-(4tolyl) tropane (PTT) and 2β-propanoyl-3β-(2-naphthyl)-tropane (WF-23) to DA clearance observed in DAT knockout mice (Table I).

Bath application of cocaine resulted in a characteristic increase in stimulated DA release to approximately 200% of baseline at 1 – 10 μM cocaine, with a contrasting inhibition of DA release at 30 – 60 μM cocaine (Figure 3A)^{32, 33}. As in previous reports, cocaine at concentrations greater than 30 μM resulted in almost complete inhibition of DA release in response to a single electrical pulse³². To characterize DA uptake inhibition produced by these high concentrations of cocaine we applied a stronger stimulation to elicit release (5 pulses at 10 Hz), but this was only effective at concentrations up to 60 μM cocaine. Inhibition of DA release at high concentrations prevented measurement of plateau levels of cocaine-induced DA uptake inhibition, and thus the maximum observed level of DA uptake inhibition was 1624 ± 247.3 percent of baseline tau at 60 μM cocaine (Figure 3B). In contrast, inhibition of DA release below baseline levels was not observed with PTT or WF-23 (Figure 3A), and thus, we were able to measure plateau levels of DA uptake inhibition, which reached 2828 ± 293.9 and 4226 ± 864.61 percent of baseline tau, respectively (Figure 3B).

We next examined how the level of uptake inhibition produced by cocaine, PTT and WF-23 compared to DA clearance in DAT KO mice as a proxy for complete DAT blockade. To properly make this comparison, we compared tau across maximal DA uptake inhibition-producing concentrations of cocaine, PTT, and WF-23 as well as for DAT KO mice as previously described. Using this approach we found a significant effect of condition (One-way ANOVA: $F_{(3, 16)} = 8.80$, $p < 0.01$) and post-hoc tests revealed that tau did not statistically differ between PTT, WF-23, and DAT KO mice, despite a modest trend for faster uptake in DAT KO relative to maximally effective concentrations of PTT and WF-23. In contrast, DA clearance rates produced by 60 μM cocaine were faster than that produced by PTT, WF-23, and in DAT KO mice (Figure 3C). These data indicate that complete DAT blockade can be achieved by pharmacological inhibition of the DAT using high affinity inhibitors in vitro, however, this level of uptake inhibition cannot be readily obtained with cocaine.

Cocaine produces only modest uptake inhibition in vivo

We next examined the relative magnitude of DA uptake inhibition produced by i.v. cocaine, PTT, and WF-23 in vivo. In these studies, we compared peak uptake inhibition following a single bolus dose of 0.3, 1.0 or, 3.0 mg/kg cocaine, or following the 10th administration of 1.0 mg/kg cocaine. Further, we examined the effects of a single bolus dose of 5.0 mg/kg PTT or WF-23.

Similar to previous reports, both PTT (One-way ANOVA: $F_{(8,48)} = 4.97$, $p = 0.001$) and WF-23 (One-way ANOVA: $F_{(8,40)} = 8.39$, $P < 0.001$) produced significant DA uptake inhibition³⁴. Examination of the time-course of effects indicated that the average peak uptake inhibition was reached at 50 – 60 min post injection for both PTT and WF-23 (Supplementary Table I). We found a significant difference in peak uptake inhibition produced by the treatments administered (One-way ANOVA: $F_{(5, 35)} = 11.38$, $p < 0.001$), and that WF-23 produced significantly more robust DA uptake inhibition at peak effect than all other treatments (Figure 4). These data indicate that the highest capacity for pharmacological DA uptake inhibition in vivo is far greater than that produced by a dose of cocaine that is proposed to produce near maximal DAT occupancy. Further, these data show that the highest tolerable dose of cocaine elicits only a fraction of the possible DA uptake inhibition produced by high affinity DAT inhibitors. These observations are consistent with previous studies indicating that a single i.v. injection of the high affinity DAT inhibitor GBR-12909 (3.0 mg/kg) produces DA uptake inhibition that is tenfold higher than that produced by 0.75 mg/kg injection of cocaine²⁸.

We next sought to estimate the magnitude of DA uptake inhibition produced by cocaine, PTT and WF-23 relative to complete DAT blockade as estimated by measures of DA clearance in DAT KO mice. We measured in vivo DA clearance in anesthetized DAT KO mice, and then calculated the relative percent of uptake inhibition following cocaine, PTT and WF-23. This analysis revealed that DA clearance in DAT KO mice was significantly slower than DA clearance obtained from all pharmacological manipulations (One-way ANOVA: $F_{(6, 33)} = 20.25$, $p < 0.001$). As shown in figure 5, PTT and WF23 produced only 13.7 % and 29.3 % of DAT independent DA clearance observed in DAT KO mice. For cocaine, clearance rates varied across dose of cocaine (0.92% at 0.3mg/kg; 1.3 % at 1 mg/kg; 1.8 % at 3mg/kg) and was further increased when rats received repeated injections of 1mg/kg cocaine (2.6%). Together, these data indicate that maximally tolerable doses of several DAT inhibitors produce only modest functional DAT blockade in vivo.

Results Summary

While DAT occupancy studies show that a single 1.0 mg/kg dose of cocaine occupies 70 – 80% of available DAT^{16–18}, our current results demonstrate that these doses of cocaine produce only modest DA uptake inhibition. Our initial studies revealed that the relationship between cocaine dose and DA uptake inhibition is different than the relationship between cocaine dose and DAT occupancy^{16–18}. Specifically, since 1.0 mg/kg cocaine occupies 70 – 80% of DAT, it has been predicted that larger doses would not produce significantly greater ‘DAT blockade’^{17, 18}, however, we found that 3.0 mg/kg cocaine produced greater uptake inhibition than 0.3 and 1.0 mg/kg cocaine. Further, we found that repeated administration 1.0 mg/kg cocaine, following a regimen that mimics cocaine self-administration, resulted in increased DA uptake inhibition beyond that produced by a single 1.0 mg/kg dose.

These initial studies led us to investigate the effects of cocaine relative to a 100% functional DAT blockade. For these studies, we used DA clearance in DAT KO mice as a proxy for complete DAT blockade. These results revealed that, although complete blockade of the

DAT is achievable in vitro, the highest tolerable doses of cocaine and high affinity DAT inhibitors produced only a fraction of complete DAT blockade in vivo.

Methodological Considerations

In these studies we used DA clearance in DAT KO mice as a proxy for maximal DAT inhibition. The DAT KO mouse has a genetic deletion of the DAT, and thus DA clearance measured in these mice is independent of DAT uptake mechanisms. In the absence of DAT, it is likely that DA clearance is largely a product of diffusion away from release sites³⁵, although other mechanisms of DA clearance such as uptake through the organic cation transporter-3 (OTC-3)³⁶ or extracellular metabolism³⁷ may contribute to the rate of DA clearance in DAT KO mice. The existence of these alternative DA clearance mechanisms raises the question of whether or not DA clearance in these mice accurately mirrors maximal DAT inhibition. Indeed, these mice express dramatic DA system adaptations^{38, 39}, and thus it is possible that compensatory increases in OTC-3 mediated uptake or the rate of extracellular metabolism might influence the reliability of DA clearance rates measured in DAT KO mice used as a proxy for maximum DAT inhibition. Secondly, the comparability of DA clearance in the presence of DAT inhibitors versus DA clearance in DAT KO mice may be influenced differences in DA release amplitude between groups. Stimulated DA release is lower in DAT KO mice^{38, 40}, and thus measures of tau may be artificially influenced by potential differences in DA release. If indeed our measures were influenced by compensatory increases in DA clearance rate that occur in DAT KO mice or by differences in DA release amplitude, this would result in underestimation of maximal inhibition of uptake in the current studies. Such an underestimation would diminish the observed differences in our analysis, but would not produce a false positive result.

We measured DA uptake rates before and after the administration of several DAT inhibitors, and this unveiled a discrepancy between reports of DAT blockade as inferred from DAT occupancy studies and our real time measurements of DA uptake rate. DA uptake rate is influenced by both the number of available DATs, and the apparent affinity of DA for the DAT⁴¹. Given that the measure of DA uptake used in the current studies cannot differentiate alterations in DA uptake rate due to changes in DAT availability versus changes in the affinity of DAT for DA, it is possible that changes in DAT availability have influenced our results. While multiple studies have indeed shown that acute application of the class of DAT inhibitors referred to as 'releasers' or 'substrates' (i.e. amphetamine) can produce rapid changes in plasmalemmal DAT availability^{42, 43}, these same effects have not been observed following acute application of non-substrate DAT inhibitors including cocaine and cocaine analogues such as those used in these studies^{44, 45}. Moreover, acute pre-incubation with the non-substrate DAT inhibitor cocaine has been shown to block substrate-induced internalization of DAT⁴². Based on these observations, it seems unlikely that changes in DAT availability following inhibitor administration could have influenced our measurements of DA uptake inhibition, or the comparability of uptake inhibition in our studies to measures of DAT occupancy.

Potential mechanisms for the discrepancy between DA uptake inhibition and DAT occupancy

One possible explanation for the discrepancy between our studies on DA uptake inhibition and studies of DAT occupancy may be differences in timing of the measurements taken. In our studies, we primarily used peak uptake inhibition as a measure of inhibitor effectiveness, and we have found that peak uptake inhibition produced by cocaine occurs within the first 60 sec of cocaine administration in rats²⁸ as well as in the current mouse studies. Measures of DAT occupancy using PET in humans and non-human primates has most often been determined over the first 5–20 min post cocaine injection^{16, 17, 46}, and DAT occupancy in these studies is generally in accordance with levels of DAT occupancy measured in mice using autoradiography following sacrifice at 5 min post-injection¹⁸. Thus, one possibility is that differences in DA uptake inhibition and DAT occupancy may be a result of changes in DAT inhibition that occur within the first 5 min after cocaine injections. This is an unlikely explanation, however, because although DA uptake inhibition peaks within 30–60 seconds post injection, the decrease in uptake inhibition that occurs over the first 5 min post-injection is not significant^{28, 33, 47}. The modest change in DA uptake inhibition across the first 5 min post-injection, thus, cannot account for the large discrepancy observed between reports of DAT occupancy and the levels of functional uptake inhibition observed in the current studies.

An alternative explanation for differences between our findings and those obtained from PET studies is that the relationship between DAT occupancy and DA uptake inhibition is not direct. For example, it is possible that DAT occupancy levels of approximately 50% (i.e., 0.3 mg/kg cocaine) result in a level of DAT ligand interaction that produces only modest uptake inhibition. By comparison, when DAT occupancy levels exceed 50% (i.e., >1 mg/kg cocaine) DA uptake inhibition accelerates in a sigmoidal fashion. Our current in vitro voltammetry observations largely support this hypothesis. At low concentrations, PTT and WF-23 produced negligible DA uptake inhibition. When concentrations were increased, however, a sigmoidal increase in uptake inhibition was observed. The observations, along with others showing a disconnect between occupancy and microdialysis levels of DA in the striatum⁴⁸, offer the likelihood that PET measures of occupancy may represent aspects of DAT ligand interactions that do not directly relate to functional DA uptake inhibition.

Insights into the role of DAT inhibition in the reinforcing efficacy of psychostimulants

In the current studies we found that the degree of DA uptake inhibition produced by abused doses of cocaine is only a fraction of DAT independent DA clearance. Nonetheless, we found that all doses tested did indeed produce a significant degree of uptake inhibition, and this is in line with multiple studies that have shown such doses increase extracellular DA levels by using either PET imaging or microdialysis^{15, 23}. Further, we found that a single maximally tolerable dose of the high affinity cocaine analogues PTT and WF-23 produce far greater DA uptake inhibition than the maximally tolerable dose of cocaine. While PTT and WF-23 show a reinforcing efficacy that is comparable to that of cocaine^{49, 50}, differences in speed of onset⁵¹, duration of effects^{49, 52, 53}, and off-target affinities of the drugs tested⁴⁹ make it difficult to directly determine a relationship between DA uptake inhibition and reinforcing efficacy. Nevertheless, our studies show that larger or repeated doses of cocaine

increase DA uptake inhibition beyond that produced by a single dose that yields 70 – 80% DAT occupancy^{16–18}, and thus we provide evidence against the hypothesis that the reinforcing nature of high or repeated doses of cocaine must stem from mechanisms that extend beyond cocaine inhibition of the DAT¹⁷.

Conclusion

In these studies we found a large discrepancy between reports of DAT occupancy by cocaine as measured in PET imaging studies and the effects of cocaine on DA uptake inhibition. PET imaging studies have provided key insights into the pharmacological effects of psychostimulants, and the interpretation of these studies has been critical in shaping our understanding of the addictive properties of these drugs. The data presented herein provides important insight into the relationship between PET imaging outcome measures and the pharmacological effects of cocaine. Specifically, although abused doses of cocaine produce up to 80% DAT occupancy^{16–18}, we found that these same doses results in less than 2% of complete DAT blockade. Therefore, the common assumption that DAT occupancy observed in PET studies is equivalent to functional DAT blockade was not supported in our studies, with the two measures painting very different pictures of DAT-cocaine interactions. We therefore conclude that caution should be used when drawing parallels between DAT occupancy and the inhibition DA uptake.

Methods

Animals

Adult wild-type C57BL6J or DAT KO mice (25–29g) on a mixed background C57BL6J/129SvJ (bred in house for >10 generations) were housed on a 12:12 h light:dark cycle with food and water available ad libitum. All animals were maintained according to the National Institutes of Health (NIH) 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 Committees at Drexel University College of Medicine and Wake Forest University Health Sciences.

Chemicals

Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (Rockville, MD, USA). PTT and WF-23 were donated by Huw M. L. Davies (Emory University, Atlanta, GA, USA). PTT and WF-23 have previously been shown to produce robust and long-lasting stimulant activity in vivo^{52, 54}. All drugs were dissolved in 0.9% saline. Table I shows published binding affinities for the DAT inhibitors examined in this manuscript.

In vivo voltammetry

We used voltammetry in anesthetized mice for these studies, as opposed to voltammetry in freely moving mice, for two reasons. First, an anesthetized preparation allows for the application of a larger electrical current when compared to freely moving subjects. This larger electrical current is often necessary to elicit DA release to levels that fully saturate DAT at baseline, which is required to properly model the kinetics of DA release and

uptake⁵⁵. Second, the high doses of cocaine, PTT and WF-23 used herein would likely exert adverse behavioral effects that could impact the fidelity of voltammetry recordings and thereby might confound our results, especially considering that our interest was in the purely pharmacological effects of cocaine and other DAT inhibitors. Urethane-anesthetized mice were used to avoid potential alterations in DA uptake kinetics that can occur with other anesthetics^{33, 56}. On the day of testing, mice were anesthetized with urethane (1.5 g/kg, i.p.; Sigma-Aldrich, St. Louis, MO, USA) and for experiments involving i.v. injections of DAT inhibitors, mice were also implanted with an i.v. catheter as previously described²⁸. Mice were subsequently placed in a stereotaxic apparatus, a stimulating electrode was lowered into the VTA (−3.0 A/P, +1.0 M/L, −4.5 – 5.0 D/V), a carbon fiber electrode was lowered into the dorsal caudate (+1.3 A/P, +1.3 M/L, −3.0 D/V) and a reference electrode was implanted in the contralateral cortex (+1.5 A/P, −1.5 M/L, −2.0 D/V)⁵⁷. We elected to measure DA dynamics in the dorsal caudate to better relate our findings to previous PET and binding studies that have focused on the ‘striatum’ as an entire structure^{16–18, 58}. DA release was elicited via electrical stimulation of the VTA using 0.4 – 1 sec, 60 Hz monophasic (4 ms; ~400 μ A) stimulation trains. The length of stimulation was varied to ensure sufficient DA release across experiments.

Baseline DA response parameters were collected in 5 min intervals for a minimum of 30 min prior to drug injection. Once a stable baseline of three consecutive collections was obtained (defined as DA peak height within 15%), mice received a single 2 sec, ~200 μ L i.v. injection of cocaine (0.3 mg/kg n=5, 1.0 mg/kg n=7, and 3.0 mg/kg n=6), WF-23 (5.0 mg/kg n=6), or PTT (5.0 mg/kg n=6) or 10 repeated injections of cocaine (1.0 mg/kg n=5). Inter-injection intervals for repeated cocaine injections were based on rates of cocaine intake observed in self-administration experiments in rats^{30, 31}. DA response parameters were acquired at 30 and 60 sec post injection and every 5 min thereafter until a peak effect was established.

In vitro voltammetry

Mice were anesthetized with isoflurane before being sacrificed by decapitation since this procedure does not alter terminal DA release and uptake kinetics³³. Following decapitation, brains were removed and then transferred into oxygenated ice-cold artificial cerebral spinal fluid (aCSF; In mM equivalents: 126 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 2.4 CaCl₂, 1.2 MgCl₂, 25 NaHCO₃, 11 glucose, 0.4 l-ascorbic acid, pH adjusted to 7.4). A vibrating microtome was used to produce 300 μ m thick coronal sections containing the NAc, and these slices were transferred into a testing chamber and flushed with aCSF (32 °C). After an equilibration of approximately 30 min, a carbon fiber microelectrode (150–200 μ m length $\times$ 7 μ m diameter) and a bipolar stimulating electrode (Plastics One, Roanoke, VA) were placed in the dorsal caudate. DA release was elicited every 5 min using a single electrical pulse (400 μ A, 4 ms, monophasic), and was recorded as described below. For DAT KO animals, DA release and uptake values were determined from the first three stable baseline stimulations (less than 10% variation). For pharmacological inhibition experiments, three stable baseline responses were recorded before cocaine (1 – 60 μ M), PTT (0.1 – 30 μ M), or WF-23 (0.01 – 3 μ M) was cumulatively applied. For these experiments, we recorded three stable responses (less than 10% variation) for each drug concentration before applying the next concentration³³.

Data acquisition

The electrode potential was scanned from -0.4 to 1.2 and back to -0.4 V vs Ag/AgCl. Cyclic voltammograms were recorded every 100 ms using a scan rate of 400 V/s by means of a voltammeter/ampereometer (Chem-Clamp, Dagan Corporation, MN). The magnitude of electrically-evoked DA release and transporter-mediated uptake were monitored and DA overflow curves were fitted to an exponential decay model and expressed as tau (66.6 % return to baseline; units in secs) using Demon Voltammetry and Analysis software²⁹ written in Labview (National Instruments, Austin, TX).

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References

1. Ritz MC, Lamb RJ, Goldberg SR, Kuhar MJ. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science*. 1987; 237:1219–1223. [PubMed: 2820058]
2. Uhl GR, Hall FS, Sora I. Cocaine, reward, movement and monoamine transporters. *Molecular psychiatry*. 2002; 7:21–26. [PubMed: 11803442]
3. Calligaro DO, Eldefrawi ME. High affinity stereospecific binding of [3H] cocaine in striatum and its relationship to the dopamine transporter. *Membr Biochem*. 1987; 7:87–106.
4. Bergman J, Madras BK, Johnson SE, Spealman RD. Effects of cocaine and related drugs in nonhuman primates. III. Self-administration by squirrel monkeys. *J Pharmacol Exp Ther*. 1989; 251:150–155. [PubMed: 2529365]
5. Kuhar MJ, Ritz MC, Boja JW. The dopamine hypothesis of the reinforcing properties of cocaine. *Trends Neurosci*. 1991; 14:299–302. [PubMed: 1719677]
6. Cline EJ, Terry P, Carroll FI, Kuhar MJ, Katz JL. Stimulus generalization from cocaine to analogs with high in vitro affinity for dopamine uptake sites. *Behavioural pharmacology*. 1992; 3:113–116. [PubMed: 11224107]
7. Wilcox KM, Paul IA, Woolverton WL. Comparison between dopamine transporter affinity and self-administration potency of local anesthetics in rhesus monkeys. *European journal of pharmacology*. 1999; 367:175–181. [PubMed: 10078990]
8. Hurd YL, Ungerstedt U. Cocaine: an in vivo microdialysis evaluation of its acute action on dopamine transmission in rat striatum. *Synapse*. 1989; 3:48–54. [PubMed: 2537539]
9. Butcher SP, Liptrot J, Aburthnott GW. Characterisation of methylphenidate and nomifensine induced dopamine release in rat striatum using in vivo brain microdialysis. *Neurosci Lett*. 1991; 122:245–248. [PubMed: 1902922]
10. Chen R, Tilley MR, Wei H, Zhou F, Zhou FM, Ching S, Quan N, Stephens RL, Hill ER, Nottoli T, Han DD, Gu HH. Abolished cocaine reward in mice with a cocaine-insensitive dopamine transporter. *Proc Natl Acad Sci U S A*. 2006; 103:9333–9338. [PubMed: 16754872]
11. Volkow ND, Wang GJ, Fischman MW, Foltin R, Fowler JS, Franceschi D, Franceschi M, Logan J, Gatley SJ, Wong C, Ding YS, Hitzemann R, Pappas N. Effects of route of administration on cocaine induced dopamine transporter blockade in the human brain. *Life Sci*. 2000; 67:1507–1515. [PubMed: 10983846]
12. Desai RI, Kopajtic TA, French D, Newman AH, Katz JL. Relationship between in vivo occupancy at the dopamine transporter and behavioral effects of cocaine, GBR 12909 [1-{2-[bis-(4-fluorophenyl)methoxy]ethyl}-4-(3-phenylpropyl)piperazine], and benztropine analogs. *The Journal of pharmacology and experimental therapeutics*. 2005; 315:397–404. [PubMed: 16014753]
13. Horn AS. Dopamine uptake: a review of progress in the last decade. *Prog Neurobiol*. 1990; 34:387–400. [PubMed: 2192393]

14. Volkow ND, Fowler JS, Gatley SJ, Dewey SL, Wang GJ, Logan J, Ding YS, Franceschi D, Gifford A, Morgan A, Pappas N, King P. Comparable changes in synaptic dopamine induced by methylphenidate and by cocaine in the baboon brain. *Synapse*. 1999; 31:59–66. [PubMed: 10025684]
15. Volkow ND, Wang GJ, Fowler JS, Logan J, Gatley SJ, Wong C, Hitzemann R, Pappas NR. Reinforcing effects of psychostimulants in humans are associated with increases in brain dopamine and occupancy of D(2) receptors. *The Journal of pharmacology and experimental therapeutics*. 1999; 291:409–415. [PubMed: 10490931]
16. Volkow ND, Wang GJ, Fischman MW, Foltin RW, Fowler JS, Abumrad NN, Vitkun S, Logan J, Gatley SJ, Pappas N, Hitzemann R, Shea CE. Relationship between subjective effects of cocaine and dopamine transporter occupancy. *Nature*. 1997; 386:827–830. [PubMed: 9126740]
17. Volkow ND, Gatley SJ, Fowler JS, Logan J, Fischman M, Gifford AN, Pappas N, King P, Vitkun S, Ding YS, Wang GJ. Cocaine doses equivalent to those abused by humans occupy most of the dopamine transporters. *Synapse*. 1996; 24:399–402. [PubMed: 10638828]
18. Gatley SJ, Volkow ND, Gifford AN, Fowler JS, Dewey SL, Ding YS, Logan J. Dopamine-transporter occupancy after intravenous doses of cocaine and methylphenidate in mice and humans. *Psychopharmacology (Berl)*. 1999; 146:93–100. [PubMed: 10485970]
19. Rothman RB, Grieg N, Kim A, De Costa BR, Rice KC, Carroll FI, Pert A. Cocaine and GBR12909 produce equivalent motoric responses at different occupancy of the dopamine transporter. *Pharmacology, biochemistry, and behavior*. 1992; 43:1135–1142.
20. Gatley SJ, Volkow ND, Gifford AN, Ding YS, Logan J, Wang GJ. Model for estimating dopamine transporter occupancy and subsequent increases in synaptic dopamine using positron emission tomography and carbon-11-labeled cocaine. *Biochemical pharmacology*. 1997; 53:43–52. [PubMed: 8960062]
21. Verebey K, Gold MS. From coca leaves to crack: the effects of dose and routes of administration in abuse liability. *Psychiatric Annals*. 1988; 18:513–520.
22. Richardson NR, Roberts DCS. Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy. *J Neurosci Methods*. 1996; 66:1–11. [PubMed: 8794935]
23. Wise RA, Newton P, Leeb K, Burnette B, Pocock D, Justice JB Jr. Fluctuations in nucleus accumbens dopamine concentration during intravenous cocaine self-administration in rats. *Psychopharmacology (Berl)*. 1995; 120:10–20. [PubMed: 7480530]
24. Oleson EB, Talluri S, Childers SR, Smith JE, Roberts DC, Bonin KD, Budygin EA. Dopamine uptake changes associated with cocaine self-administration. *Neuropsychopharmacology*. 2009; 34:1174–1184. [PubMed: 18923398]
25. Ahmed SH, Koob GF. Long-lasting increase in the set point for cocaine self-administration after escalation in rats. *Psychopharmacology (Berl)*. 1999; 146:303–312. [PubMed: 10541731]
26. Wee S, Specio SE, Koob GF. Effects of dose and session duration on cocaine self-administration in rats. *J Pharmacol Exp Ther*. 2007; 320:1134–1143. [PubMed: 17202405]
27. Zimmer BA, Oleson EB, Roberts DC. The motivation to self-administer is increased after a history of spiking brain levels of cocaine. *Neuropsychopharmacology*. 2012; 37:1901–1910. [PubMed: 22453139]
28. España RA, Roberts DCS, Jones SR. Short-acting cocaine and long-acting GBR-12909 both elicit rapid dopamine uptake inhibition following intravenous delivery. *Neuroscience*. 2008; 155:250–257. [PubMed: 18597947]
29. Yorgason JT, España RA, Jones SR. Demon Voltammetry and Analysis software: Analysis of cocaine-induced alterations in dopamine signaling using multiple kinetic measures. *J Neurosci Methods*. 2011
30. España RA, Oleson EB, Locke JL, Brookshire BR, Roberts DCS, Jones SR. The hypocretin-orexin system regulates cocaine self-administration via actions on the mesolimbic dopamine system. *Eur J Neurosci*. 2010; 31:336–348. [PubMed: 20039943]
31. España RA, Melchior JR, Roberts DCS, Jones SR. Hypocretin 1/orexin A in the ventral tegmental area enhances dopamine responses to cocaine and promotes cocaine self-administration. *Psychopharmacology*. 2011:1–12.

32. John CE, Jones SR. Voltammetric characterization of the effect of monoamine uptake inhibitors and releasers on dopamine and serotonin uptake in mouse caudate-putamen and substantia nigra slices. *Neuropharmacology*. 2007; 52:1596–1605. [PubMed: 17459426]
33. Brodnik ZD, España RA. Dopamine uptake dynamics are preserved under isoflurane anesthesia. *Neurosci Lett*. 2015; 606:129–134. [PubMed: 26321152]
34. Yorgason JT, Jones SR, España RA. Low and high affinity dopamine transporter inhibitors block dopamine uptake within 5 sec of intravenous injection. *Neuroscience*. 2011; 182:125–132. [PubMed: 21402130]
35. Mateo Y, Budygin EA, John CE, Banks ML, Jones SR. Voltammetric assessment of dopamine clearance in the absence of the dopamine transporter: no contribution of other transporters in core or shell of nucleus accumbens. *J Neurosci Methods*. 2004; 140:183–187. [PubMed: 15589348]
36. Graf EN, Wheeler RA, Baker DA, Ebben AL, Hill JE, McReynolds JR, Robble MA, Vranjkovic O, Wheeler DS, Mantsch JR, Gasser PJ. Corticosterone acts in the nucleus accumbens to enhance dopamine signaling and potentiate reinstatement of cocaine seeking. *J Neurosci*. 2013; 33:11800–11810. [PubMed: 23864669]
37. Waymunt HK, Schenk JO, Sorg BA. Characterization of extracellular dopamine clearance in the medial prefrontal cortex: role of monoamine uptake and monoamine oxidase inhibition. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2001; 21:35–44. [PubMed: 11150317]
38. Jones SR, Gainetdinov RR, Jaber M, Giros B, Wightman RM, Caron MG. Profound neuronal plasticity in response to inactivation of the dopamine transporter. *Proc Natl Acad Sci U S A*. 1998; 95:4029–4034. [PubMed: 9520487]
39. Jones SR, Gainetdinov RR, Hu XT, Cooper DC, Wightman RM, White FJ, Caron MG. Loss of autoreceptor functions in mice lacking the dopamine transporter. *Nat Neurosci*. 1999; 2:649–655. [PubMed: 10404198]
40. Zhuang X, Oosting RS, Jones SR, Gainetdinov RR, Miller GW, Caron MG, Hen R. Hyperactivity and impaired response habituation in hyperdopaminergic mice. *Proc Natl Acad Sci U S A*. 2001; 98:1982–1987. [PubMed: 11172062]
41. Ferris MJ, Calipari ES, Yorgason JT, Jones SR. Examining the complex regulation and drug-induced plasticity of dopamine release and uptake using voltammetry in brain slices. *ACS Chem Neurosci*. 2013; 4:693–703. [PubMed: 23581570]
42. Johnson LA, Furman CA, Zhang M, Guptaroy B, Gnegy ME. Rapid delivery of the dopamine transporter to the plasmalemmal membrane upon amphetamine stimulation. *Neuropharmacology*. 2005; 49:750–758. [PubMed: 16212991]
43. Furman CA, Chen R, Guptaroy B, Zhang M, Holz RW, Gnegy M. Dopamine and amphetamine rapidly increase dopamine transporter trafficking to the surface: live-cell imaging using total internal reflection fluorescence microscopy. *J Neurosci*. 2009; 29:3328–3336. [PubMed: 19279270]
44. Chi L, Reith ME. Substrate-induced trafficking of the dopamine transporter in heterologously expressing cells and in rat striatal synaptosomal preparations. *The Journal of pharmacology and experimental therapeutics*. 2003; 307:729–736. [PubMed: 12975490]
45. Gorentla BK, Vaughan RA. Differential effects of dopamine and psychoactive drugs on dopamine transporter phosphorylation and regulation. *Neuropharmacology*. 2005; 49:759–768. [PubMed: 16181646]
46. Volkow ND, Fowler JS, Wang GJ. Imaging studies on the role of dopamine in cocaine reinforcement and addiction in humans. *Journal of psychopharmacology (Oxford, England)*. 1999; 13:337–345.
47. Prince CD, Rau AR, Yorgason JT, España RA. Hypocretin/Orexin regulation of dopamine signaling and cocaine self-administration is mediated predominantly by hypocretin receptor 1. *ACS chemical neuroscience*. 2015; 6:138–146. [PubMed: 25496218]
48. Kimmel HL, Nye JA, Voll R, Mun J, Stehouwer J, Goodman MM, Votaw JR, Carroll FI, Howell LL. Simultaneous measurement of extracellular dopamine and dopamine transporter occupancy by cocaine analogs in squirrel monkeys. *Synapse*. 2012; 66:501–508. [PubMed: 22237864]

49. Roberts DCS, Phelan R, Hodges LM, Hodges MM, Bennett B, Childers S, Davies H. Self-administration of cocaine analogs by rats. *Psychopharmacology (Berl)*. 1999; 144:389–397. [PubMed: 10435412]
50. Lile JA, Morgan D, Birmingham AM, Wang Z, Woolverton WL, Davies HM, Nader MA. The reinforcing efficacy of the dopamine reuptake inhibitor 2beta-propanoyl-3beta-(4-tolyl)-tropane (PTT) as measured by a progressive-ratio schedule and a choice procedure in rhesus monkeys. *J Pharmacol Exp Ther*. 2002; 303:640–648. [PubMed: 12388646]
51. Samaha AN, Robinson TE. Why does the rapid delivery of drugs to the brain promote addiction? *Trends Pharmacol Sci*. 2005; 26:82–87. [PubMed: 15681025]
52. Daunais JB, Hart SL, Smith HR, Letchworth SR, Davies HM, Sexton T, Bennett BA, Childers SR, Porrino LJ. Long-acting blockade of biogenic amine transporters in rat brain by administration of the potent novel tropane 2beta-propanoyl-3beta-(2-Naphthyl)-tropane. *The Journal of pharmacology and experimental therapeutics*. 1998; 285:1246–1254. [PubMed: 9618429]
53. Bennett BA, Wichems CH, Hollingsworth CK, Davies HM, Thornley C, Sexton T, Childers SR. Novel 2-substituted cocaine analogs: uptake and ligand binding studies at dopamine, serotonin and norepinephrine transport sites in the rat brain. *J Pharmacol Exp Ther*. 1995; 272:1176–1186. [PubMed: 7891330]
54. Porrino LJ, Migliarese K, Davies HM, Saikali E, Childers SR. Behavioral effects of the novel tropane analog, 2 beta-propanoyl-3 beta-(4-toluy)l)-tropane (PTT). *Life Sci*. 1994; 54:PL511–517. [PubMed: 8208059]
55. Wightman RM, Zimmerman JB. Control of dopamine extracellular concentration in rat striatum by impulse flow and uptake. *Brain Res Brain Res Rev*. 1990; 15:135–144. [PubMed: 2282449]
56. Greco PG, Garris PA. In vivo interaction of cocaine with the dopamine transporter as measured by voltammetry. *Eur J Pharmacol*. 2003; 479:117–125. [PubMed: 14612143]
57. Shaw JK, Ferris MJ, Locke JL, Brodnik ZD, Jones SR, España RA. Hypocretin/orexin knock-out mice display disrupted behavioral and dopamine responses to cocaine. *Addict Biol*. 2016
58. Volkow ND, Wang GJ, Fowler JS, Gatley SJ, Ding YS, Logan J, Dewey SL, Hitzemann R, Lieberman J. Relationship between psychostimulant-induced “high” and dopamine transporter occupancy. *Proc Natl Acad Sci U S A*. 1996; 93:10388–10392. [PubMed: 8816810]
59. Davies HM, Saikali E, Sexton T, Childers SR. Novel 2-substituted cocaine analogs: binding properties at dopamine transport sites in rat striatum. *European journal of pharmacology*. 1993; 244:93–97. [PubMed: 8420793]
60. Davies HM, Saikali E, Huby NJ, Gilliatt VJ, Matasi JJ, Sexton T, Childers SR. Synthesis of 2 beta-acyl-3 beta-aryl-8-azabicyclo[3.2.1]octanes and their binding affinities at dopamine and serotonin transport sites in rat striatum and frontal cortex. *J Med Chem*. 1994; 37:1262–1268. [PubMed: 8176704]

Figure 1.

Increasing doses of cocaine produce increasing DA uptake inhibition. **(A)** Example traces of peak uptake inhibition following 0.3, 1.0, and 3.0 mg/kg cocaine. **(B)** DA uptake inhibition relative to baseline uptake rate for 0.3, 1.0, and 3.0 mg/kg cocaine. **(C)** Uptake rate as a percent of baseline tau for 0.3, 1.0, and 3.0 mg/kg cocaine. Data are expressed as a mean $\pm$ SEM; $n = 5-6$ for all experimental groups. Paired t-test: * $p < 0.05$, ** $p < 0.01$. Bonferroni post-hoc test: # $p < 0.05$. BL, baseline.

Figure 2.

Repeated administration of 1.0 mg/kg cocaine results in increasing DA uptake inhibition.

(A–C) Example traces of repeated administration of 1.0 mg/kg cocaine. (D) Average uptake rate following the first through tenth injection of 1.0 mg/kg cocaine. Data are expressed as a mean $\pm$ SEM; $n = 5$ – 6 for all experimental groups. Dunnet's post-hoc tests versus the first 1.0 mg/kg injection: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. BL, baseline.

Figure 3.

In vitro characterization of DA uptake inhibition produced by cocaine, PTT, and WF-23. **(A)** DA release as a percent of baseline following application of cocaine (1 – 60 μ M), PTT (0.1 – 30 μ M), and WF-23 (0.01 – 3 μ M). **(B)** DA uptake rate as a percent of baseline tau following the application of cocaine (1 – 60 μ M), PTT (0.1 – 30 μ M), and WF-23 (0.01 – 3 μ M). **(C)** Colorplots showing DA uptake inhibition following cocaine, PTT, and WF-23 as well as DAT-independent clearance in DAT KO mice. Current is depicted in pseudocolor plotted against the applied potential and time. **(D)** DA clearance rates (tau) at maximally effective concentrations of cocaine, PTT, and WF-23 compared to DA clearance rate in DAT KO mice. Data are expressed as a mean $\pm$ SEM; $n = 5$ for all experimental groups. Bonferroni post-hoc: ** $p < 0.01$, * $p < 0.05$ vs. cocaine.

Figure 4.

Peak DA uptake inhibition produce by cocaine, PTT and WF-23 in vivo. DA uptake rate as a percent of baseline tau for 0.3, 1.0, 3.0 mg/kg and the tenth injection of 1.0 mg/kg cocaine, as well as following 5.0 mg/kg PTT and WF-23. Data are expressed as a mean $\pm$ SEM; n = 5–6 for all experimental groups. Bonferroni post-hoc: *** p < 0.001, ** p < 0.01 vs. WF-23.

Figure 5.

Peak DA uptake inhibition produced by cocaine, PTT and WF-23 relative to DA clearance in DAT KO mice in vivo. **(A)** Example traces of DA clearance following the first and tenth injection of 1.0 mg/kg cocaine, following 5.0 mg/kg PTT and WF-23, and from DAT KO mice. **(B)** DA clearance rate as a percent of baseline tau for 0.3, 1.0, 3.0 mg/kg and the tenth injection of 1.0 mg/kg cocaine, as well as 5.0 mg/kg PTT and WF-23, and from DAT KO mice. Data are expressed as a mean $\pm$ SEM; $n = 5-7$ for all experimental groups. Bonferroni post-hoc: *** $p < 0.001$, ** $p < 0.01$ vs. DAT KO.

Table I

Affinity for Cocaine and DAT inhibitors

	Cocaine	PTT	WF-23
DA uptake inhibition	150 nM IC ₅₀ ⁵³	3.2 nM IC ₅₀ ⁵³	0.65 nM IC ₅₀ ⁵³
[¹²⁵I]RTI-55 Binding Displacement	173 nM IC ₅₀ ⁵³	8.2 nM IC ₅₀ ^{53, 59, 60}	0.12 – nM IC ₅₀ ^{53, 60}

DA uptake inhibition [³H]DA and binding [¹²⁵I]RTI-55 displacement data shows the relative affinities of cocaine, PTT, and WF-23 for the DAT. PTT is highly selective for DAT and the norepinephrine transporter (NET) with little activity at the serotonin transporter (SERT). WF-23 is a non-selective, and virtually irreversible monoamine transporter inhibitor with equi-affinity for the DAT, NET and SERT^{52, 53}.