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Abstract

Amphetamine, a highly addictive drug with therapeutic efficacy, exerts paradoxical effects on the fundamental communication modes employed by dopamine neurons in modulating behavior. While amphetamine elevates tonic dopamine signaling by depleting vesicular stores and driving non-exocytotic release through reverse transport, this psychostimulant also activates phasic dopamine signaling by up-regulating vesicular dopamine release. We hypothesized that these seemingly incongruent effects arise from amphetamine depleting the reserve pool and enhancing the readily releasable pool. This novel hypothesis was tested using in vivo voltammetry and stimulus trains of varying duration to access different vesicular stores. We show that amphetamine actions are stimulus dependent in the dorsal striatum. Specifically, amphetamine up-regulated vesicular dopamine release elicited by a short-duration train, which interrogates the readily releasable pool, but depleted release elicited by a long-duration train, which interrogates the reserve pool. These opposing actions of vesicular dopamine release were associated with concurrent increases in tonic and phasic dopamine responses. A link between vesicular depletion and tonic signaling was supported by results obtained for amphetamine in the ventral striatum and cocaine in both striatal sub-regions, which demonstrated augmented vesicular release and phasic signals only. We submit that amphetamine differentially targeting dopamine stores reconciles the paradoxical activation of tonic and phasic dopamine signaling. Overall, these results further highlight the unique and region-distinct cellular mechanisms of amphetamine and may have important implications for its addictive and therapeutic properties.

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Introduction

Amphetamine (AMPH) is both addictive, with several notable episodes of widespread abuse worldwide, and therapeutic, for treating narcolepsy, attention deficit hyperactivity disorder, obesity, and traumatic brain injury [1,2]. While there is little debate that behavioral effects of this important psychostimulant are associated with a hyperdopamine state [3–6], the underlying mechanisms by which this condition manifests have been the subject of intense study. Two, what ostensibly appear to be mutually exclusive, views have emerged. On the one hand, AMPH enhances tonic dopamine signaling by reversing dopamine transporter (DAT) direction, leading to a non-exocytotic, action potential-independent type of release or "efflux" that is driven by vesicular depletion and the redistribution of dopamine to the cytosol [7,8]. On the other hand, AMPH enhances phasic dopamine signaling by promoting burst firing of dopamine neurons [9,10], inhibiting dopamine uptake [11,12], and up-regulating vesicular dopamine release [13,14]. How AMPH concurrently activates tonic and phasic dopamine signaling, the two fundamental modes of communication used by dopamine neurons [15], yet elicits opposing actions on vesicular dopamine stores is perplexing and unresolved.

Presynaptic neurotransmitter vesicles are functionally and anatomically segregated into at least three distinct pools, readily releasable, recycling, and reserve, that are interrogated by electrical stimulation of short, intermediate, and long duration, respectively [16]. Distinct vesicular stores have also been proposed to contribute to exocytotic dopamine release in a stimulus-dependent manner [17–20]. At the cellular level, AMPH exerts differential actions on dopamine vesicle populations [21–23]. Moreover, although not systematically evaluated to assess distinct vesicular stores, AMPH effects on electrically evoked levels of extracellular dopamine in the striatum *in vivo* are stimulus-dependent, with increases revealed by short trains and decreases by long trains [24,25]. It is thus interesting to speculate that AMPH depleting the reserve pool drives tonic dopamine signaling by providing a source of cytosolic dopamine for efflux, but enhancing the readily releasable pool drives phasic dopamine signaling by augmenting vesicular dopamine release.

Here we use *in vivo* voltammetry and vary stimulus duration to test the novel hypothesis that AMPH elicits opposing actions on dopamine stores. In support of this hypothesis, we show in the dorsal striatum that AMPH increased exocytotic dopamine release evoked by a short train, which interrogates the readily releasable pool, but decreased release evoked by a long train, which interrogates the reserve pool. A concurrent augmentation of tonic and phasic dopamine signaling was also observed. Vesicular depletion and enhanced tonic signaling appear to be linked because these effects were specific to AMPH and not cocaine, and

to the dorsal but not ventral striatum, whereas activation of vesicular release and phasic signaling generalized across psychostimulants and striatal sub-regions. Our results thus support a model of AMPH differentially targeting vesicular stores to reconcile its paradoxical effects on dopamine neurons and identify regionally distinct actions of this psychostimulant in the striatum that may relate to its addictive and therapeutic properties.

Methods

Experimental Design

The experimental design is shown in Figure 1. Three durations of stimulus trains, short (0.4 s), intermediate (2 s), and long (10 s), were applied to each animal and repeated after administration of the saline control or drug treatment. A frequency of 60 Hz was used for all stimulations. Stimulus current was ± 300 μA for long and intermediate trains, and ± 125 μA for the short train. The lower current intensity was selected for the short train to elicit evoked responses mirroring the amplitude and dynamics of naturally occurring phasic dopamine transients [26]. As such, we refer to these responses as "phasic-like". This short train is also reinforcing in the operant paradigm of intracranial self-stimulation [27]. Sufficient time was allowed between trains for evoked responses to recover (5 s per pulse; [28]). Extracellular dopamine was measured in urethane-anesthetized rats by fast-scan cyclic voltammetry (FSCV) at a carbon fiber microelectrode (CFM) implanted in the dorsal and ventral striatum, as described previously [12]. Vesicular dopamine release was resolved from dopamine uptake for all evoked responses [28,29]. A low (1 mg/kg, i.p.) and high (10 mg/kg, i.p.) dose of AMPH was evaluated to assess dose-dependent effects. A high dose of cocaine (40 mg/kg i.p.) was evaluated for comparison.

Animals

Adult male Sprague-Dawley rats (~350–400 g), purchased from Harlan (Indianapolis, IN, USA), were housed under standard conditions of lighting and temperature. Food and water were provided *ad libitum*. Protocols were approved by the Institutional Animal Care and Use Committee of Illinois State University. Care was in accordance with NIH guidelines (publication 86–23).

Surgery

Rats were anesthetized with urethane (1.6 g/kg, i.p.) and immobilized in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Deltaphase Isothermal Pads (Braintree Scientific, Braintree, MA, USA) maintained core temperature throughout surgery. Burr holes were drilled overlying targeted regions, dura was removed, and electrodes lowered along a vertical trajectory using stereotactic coordinates obtained from a brain atlas based on a flat-skull position [30] and utilizing bregma and dura as reference points. All coordinates, anteroposterior (AP), mediolateral (ML) and dorsoventral (DV) are given in mm. The stimulating electrode targeted the medial forebrain bundle (AP: −4.6, ML: +1.4, DV: −7.0), and a CFM targeted the dorsal (AP: +1.2, ML: +3.0, DV: −4.5 to 5.0) and ventral (AP: +1.2, ML: +2.0, DV: −6.5 to 7.5) striatum. The reference electrode, a chloridized silver wire, was placed in the contralateral superficial cortex.

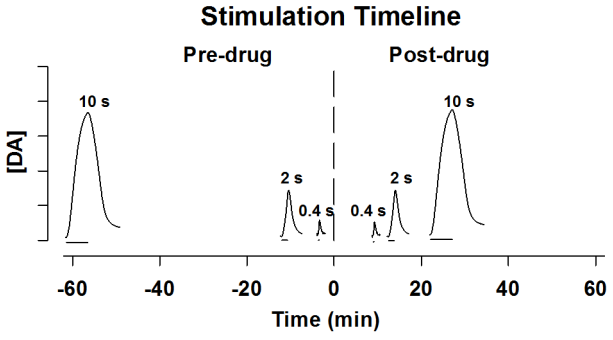

Figure 1. Experimental timeline. Three stimulation trains with different durations (0.4 s, 2 s, and 10 s), indicated by the horizontal line under each evoked response, were applied before and after psychostimulant administration at time 0 min. Note that evoked responses are on a second timescale, while the overall design is shown in minutes.
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Electrochemistry

FSCV was performed by a Universal Electrochemistry Instrument (UEI; Department of Chemistry Electronic Shop, University of North Carolina, Chapel Hill, NC, USA), which was computer controlled by commercially available software (ESA Bioscience, Chelmsford, MA, USA). The potential of the CFM was linearly scanned at 10 Hz from a resting value of −0.4 V to 1.3 V (versus the reference electrode) and back again at a rate of 400 V/s. The peak oxidation current for dopamine recorded during each scan was converted to a concentration based on post-calibration of the CFM using flow-injection analysis in a buffer consisting of 150 mM sodium chloride with 15 mM TRIS and adjusted to a pH of 7.4 [31]. Dopamine was identified from the background subtracted voltammogram [32].

Electrical Stimulation

Electrical stimulation was computer generated and consisted of biphasic pulses (2 ms each phase). Stimulus trains were applied to a twisted bipolar stimulating electrode (Plastics One, Roanoke, VA, USA) through a constant-current generator and optical isolator (NL 80, Neurolog, Medical Systems, Great Neck, NY, USA).

Data Analysis

Dopamine responses electrically evoked by short and medium stimulations were analyzed for maximal concentration ($[DA]_{max}$) and parameters described vesicular dopamine release and dopamine uptake according to [28]:

$$d[DA]/dt = [DA]_p \times f - k \times [DA] \quad (1)$$

where $[DA]_p$ is the concentration of dopamine released per stimulus pulse, f is the frequency of stimulation, and k is the first-order term describing dopamine uptake. Data were best fit to Equation 1 using non-linear regression with a simplex algorithm [29]. First-order, as opposed to Michaelis-Menten, kinetics was selected to characterize dopamine uptake because of concern that AMPH alters both K_m and V_{max}, which is difficult to resolve with *in vivo* voltammetry [13,14]. However, similar AMPH-induced changes in $[DA]_p$, the focus of the present study, have been reported using both kinetic models [13]. Dopamine responses evoked by long trains were analyzed for vesicular dopamine release using single curve analysis [29]. The reason is that Equation 1 assumes that vesicular dopamine release is constant, and AMPH clearly caused time-dependent changes in recordings evoked by long trains as evident by the pronounced slowing of the upward slope during the train, especially in the dorsal striatum. In single curve analysis, which does not assume a kinetic mechanism

for dopamine uptake, the slope of the downward portion of the evoked signal (i.e., uptake) is subtracted from the upward portion (i.e., release - uptake) to calculate vesicular dopamine release:

$$\{d[\mathrm{DA}]/dt\}_{\mathrm{upward}}\text{-}\{d[\mathrm{DA}]/dt\}_{\mathrm{downward}} = [\mathrm{DA}]_{\mathrm{p}} \times f \qquad (2)$$

The only assumption of single curve analysis regarding uptake is that rates governing up- and downward portions are identical at the same dopamine concentration, which is also the same assumption as in Equation 1. It should be emphasized that because of DAT reversal, uptake measured in the presence of AMPH more faithfully represents net dopamine clearance, i.e., the difference between extracellular removal by uptake and addition by efflux [12,33]. Nevertheless, the combination of these effects is accounted for in the analysis, which permits a direct determination of vesicular dopamine release (i.e., $[\mathrm{DA}]_{\mathrm{p}}$).

Non-electrically evoked changes in extracellular dopamine representing tonic and phasic dopamine signaling were chemically resolved from the FSCV recordings with principal component regression (PCR) using dopamine, pH and background drift as analytes [34,35]. For training sets, dopamine and pH changes were obtained from the electrically evoked responses, whereas background drift was obtained during baseline recording in the time between stimulations. PCR was performed sequentially on 5-min epochs. Spontaneously occurring dopamine transients were identified and characterized with peak-finding software (Mini-Analysis, Synaptosoft, Decatur, GA, USA).

Statistical Analysis

When appropriate, data are presented as the mean ± SEM. $[\mathrm{DA}]_{\mathrm{max}}$ and $[\mathrm{DA}]_{\mathrm{p}}$ were statistically analyzed using a two-way ANOVA with drug treatment and stimulus duration as independent variables, followed by sequential Bonferroni *post hoc* tests. Effects of drug treatment on k were analyzed using a one-way ANOVA with a Tukey's *post hoc* test. Tonic dopamine levels were statistically analyzed using a one-way ANOVA with repeated measures. Statistical analysis was performed using SPSS Version 18 for Windows (SPSS). Significance was set at $p<0.05$.

Drugs

Urethane, cocaine hydrochloride, and d-amphetamine sulfate were purchased from Sigma (St. Louis, MO, USA). All drugs were dissolved in 150 mM NaCl prior to injection. *d*-amphetamine and cocaine doses were determined by base weight.

Results

Psychostimulant effects on evoked dopamine levels

Individual recordings of electrically evoked dopamine levels collected during the four treatments are shown in Figure 2 for the dorsal striatum and Figure 3 for the ventral striatum. Average results for $[\mathrm{DA}]_{\mathrm{max}}$, the maximal concentration of the evoked signal, and obtained from these recordings are shown in Figure 4A (left, dorsal striatum; right, ventral striatum). Both individual responses and averaged results demonstrate drug-, dose-, stimulus-, and region-dependent effects, and four general observations can be made. First, psychostimulant effects were inversely related to stimulus duration in both striatal regions. Second, AMPH but not cocaine decreased $[\mathrm{DA}]_{\mathrm{max}}$ evoked by the long train, and this only occurred in the dorsal striatum. Third, AMPH was more proficient in increasing $[\mathrm{DA}]_{\mathrm{max}}$ evoked by the short train in the ventral striatum, whereas cocaine elicited greater effects in the dorsal striatum. And fourth, the high dose of AMPH was more proficient at increasing $[\mathrm{DA}]_{\mathrm{max}}$ during short trains in both striatal regions compared to the low dose. Statistical analysis of $[\mathrm{DA}]_{\mathrm{max}}$ revealed a significant effect of drug treatment in the dorsal ($F_{3,75} = 13.45$, $p = <0.001$) and ventral ($F_{3,74} = 8.81$, $p<0.001$) striatum, a significant effect of stimulus duration in the dorsal ($F_{2,75} = 47.94$, $p<0.001$) and ventral ($F_{2,74} = 13.96$, $p<0.001$) striatum, and a significant interaction in the dorsal ($F_{6,75} = 8.45$, $p<0.001$) and ventral ($F_{6,74} = 3.08$, $p<0.01$) striatum. In the dorsal striatum, 10 mg/kg AMPH and 40 mg/kg cocaine significantly ($p<0.002$) increased $[\mathrm{DA}]_{\mathrm{max}}$ evoked by the short train, but only cocaine was effective at the intermediate train ($p<0.001$). Both doses of AMPH (1 and 10 mg/kg) significantly ($p<0.001$) decreased $[\mathrm{DA}]_{\mathrm{max}}$ evoked by the long train, whereas cocaine was without effect. In the ventral striatum, both doses of AMPH and cocaine significantly ($p<0.01$) increased $[\mathrm{DA}]_{\mathrm{max}}$ evoked by short and intermediate trains, but were without effect with the long train.

Psychostimulant effects on vesicular dopamine release and dopamine uptake

Observed psychostimulant-induced changes in $[\mathrm{DA}]_{\mathrm{max}}$ could arise from altered vesicular dopamine release and/or dopamine uptake, because both mechanisms regulating extracellular dopamine in the striatum operate concurrently during the stimulus train [28]. Evoked responses were therefore analyzed to determine

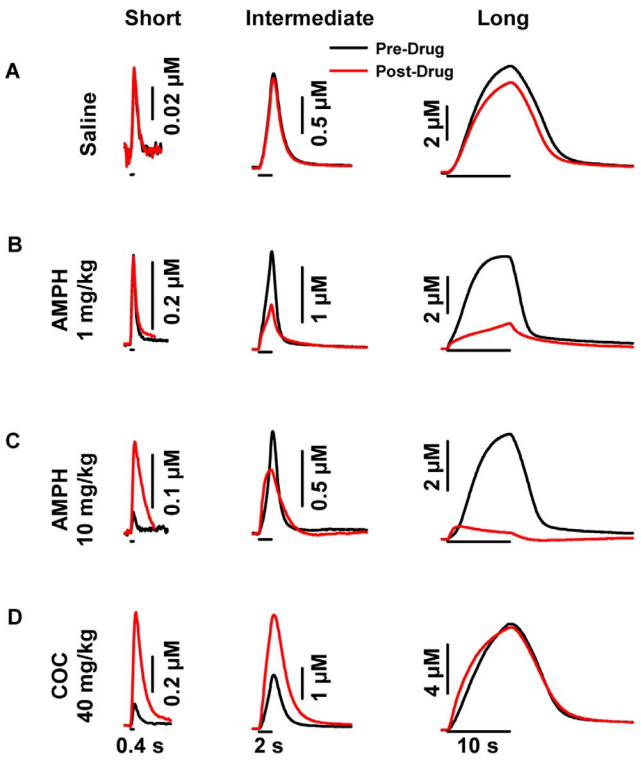

Figure 2. Representative psychostimulant- and stimulation-dependent effects on evoked dopamine dynamics in the dorsal striatum. A. Saline. **B.** 1 mg/kg AMPH. **C.** 10 mg/kg AMPH. **D.** 40 mg/kg cocaine (COC). AMPH and cocaine altered the amplitude of evoked dopamine signals in the dorsal striatum, while saline had no effect. In contrast to cocaine, there was an inverse relationship between stimulus duration and evoked dopamine amplitude following AMPH. Application of the stimulus train is indicated by the solid line underneath each representative response for short (left), intermediate (middle) and long (right) durations.
doi:10.1371/journal.pone.0060763.g002

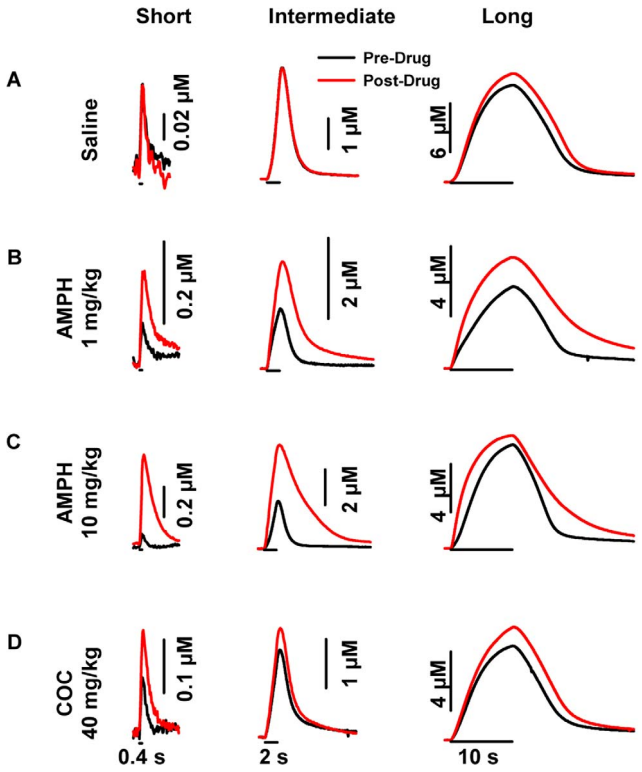

Figure 3. Representative psychostimulant- and stimulation-dependent effects on evoked dopamine dynamics in the ventral striatum. A. Saline. **B.** 1 mg/kg AMPH. **C.** 10 mg/kg AMPH. **D.** 40 mg/kg cocaine (COC). AMPH and cocaine increased evoked dopamine amplitude for at stimulus durations in the ventral striatum, while saline had no effect. Application of the stimulus train is indicated by the solid line underneath each representative response for short (left), intermediate (middle) and long (right) durations.
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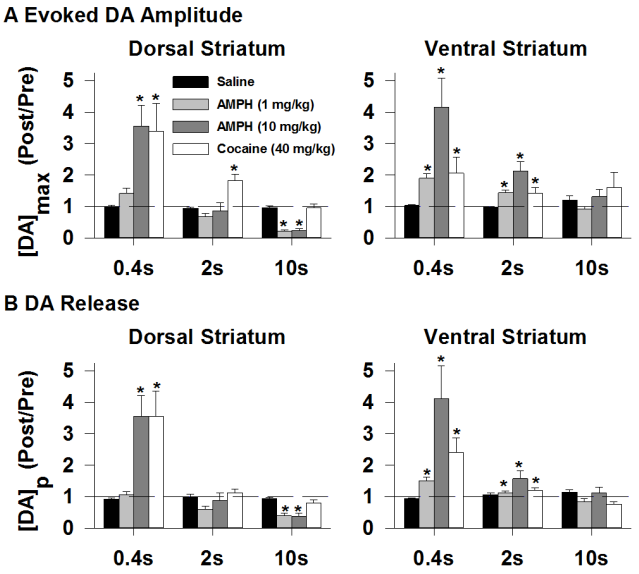

Figure 4. Averaged psychostimulant- and stimulation-dependent effects. A. The maximal concentration of electrically evoked dopamine ($[DA]_{max}$). **B.** Vesicular release ($[DA]_p$). Stimulus duration is shown along the x axis. Psychostimulants differentially elicited stimulus-dependent effects on $[DA]_{max}$ and $[DA]_p$. Data are the ratio of post-drug over pre-drug response (Post/Pre) for the dorsal (left) and ventral (right) striatum and are expressed as mean ± SEM. *, significantly different from saline ($p<0.05$).
doi:10.1371/journal.pone.0060763.g004

the respective contributions of these presynaptic mechanisms to $[DA]_{max}$. Figure 4B shows vesicular dopamine release ($[DA]_p$). Overall, $[DA]_p$ and $[DA]_{max}$ (Fig. 4A) tracked each other well. Statistical analysis of $[DA]_p$ revealed a significant effect of drug treatment in the dorsal ($F_{3,73} = 8.36$, $p<0.001$) and ventral ($F_{3,72} = 6.79$, $p<0.001$) striatum, a significant effect of train duration in the dorsal ($F_{2,73} = 30.45$, $p<0.001$) and ventral ($F_{2,72} = 19.53$, $p<0.001$) striatum, and a significant interaction in the dorsal ($F_{6,73} = 6.33$, $p<0.001$) and ventral ($F_{6,72} = 4.26$, $p<0.001$) striatum. In the dorsal striatum, 10 mg/kg AMPH and cocaine significantly increased $[DA]_p$ for the short train ($p<0.02$). 1 mg/kg AMPH was without effect, and no treatment had significant effects for the intermediate train. Both doses of AMPH significantly decreased $[DA]_p$ for the long train ($p<0.01$), while cocaine had no effect. All drug treatments significantly increased $[DA]_p$ in the ventral striatum for both short and intermediate trains ($p<0.03$) but were without effect for the long stimulation.

Psychostimulant effects on dopamine uptake are shown in Table 1. Low- and high-dose AMPH and cocaine robustly decreased dopamine uptake (k) to a similar degree in both striatal regions. Statistical analysis of k revealed a significant effect of drug treatment in both the dorsal ($F_{3,54} = 10.53$, $p<0.001$) and ventral ($F_{3,54} = 15.80$, $p<0.001$) striatum. Each drug treatment significantly decreased dopamine uptake compared to saline control in both striatal regions ($p<0.01$). AMPH- and cocaine-mediated uptake inhibition is consistent with our previous work using Michaelis-Menten kinetics [12,29,31], and the degree of inhibition was similar to our previous work using first-order kinetics [14], as is used here. This result, indicating no distinct effects of drug treatment or striatal region on dopamine uptake, and the excellent correspondence between $[DA]_{max}$ and $[DA]_p$ shown in Figure 3, suggest that psychostimulant-induced changes in $[DA]_{max}$ evoked by the trains used in this study are dominated by changes in vesicular dopamine release. The one overt exception is the intermediate train in the dorsal striatum, where cocaine increased $[DA]_{max}$ without a corresponding change in $[DA]_p$. In this case, reduced dopamine uptake dominates the increase in $[DA]_{max}$. Overall, these results demonstrate that AMPH and cocaine increase vesicular dopamine release in both striatal regions with the short train but that AMPH decreases vesicular dopamine release in the dorsal striatum with the long train.

Psychostimulant effects on tonic dopamine signaling

Figure 5 shows a representative background-subtracted FSCV recording (black) collected immediately surrounding the time of injecting high-dose AMPH. This non-electrically evoked trace, representing current measured at the peak oxidative potential for dopamine (i.e., along the horizontal white line of the pseudocolor plot below), gradually increases across the 5-min epoch. Individual voltammograms collected along the two vertical white lines of the pseudocolor plot (blue) are overlaid with a dopamine voltammogram collected during electrical stimulation (black) earlier in this recording (data not shown). While there is evidence for dopamine in the individual voltammograms and in the sequential voltammograms displayed in the pseudocolor plot for this non-electrically evoked trace, other analytes obscure its selective measurement with FSCV alone. However, PCR (red) resolves the dopamine component of this FSCV recording, demonstrating an activation of tonic dopamine signaling by AMPH.

Table 1. Psychostimulant effects on dopamine uptake.

	Saline	AMPH (1 mg/kg)	AMPH (10 mg/kg)	Cocaine (40 mg/kg)
Dorsal	0.96±0.05	0.57±0.04**	0.53±0.08**	0.66±0.06**
Ventral	0.95±0.04	0.62±0.05**	0.50±0.08**	0.60±0.05**

Data are the mean ± SEM.
**, significantly different from saline ($p<0.01$).
doi:10.1371/journal.pone.0060763.t001

Figure 6 shows the average effects of the four treatments on tonic dopamine signaling as determined by PCR analysis for the first 10 min of the FSCV recording after drug injection, which is just prior to the first stimulation of the post-drug period (see Fig. 1). This initial recording period was selected for analysis to avoid interactions between stimulation, psychostimulants, and tonic dopamine signaling. In the dorsal striatum (Fig. 6A), AMPH (10 mg/kg) elicited the fastest and largest increase in tonic dopamine levels. Statistical analysis revealed a significant effect of treatment ($F_{3,22} = 3.38$, $p = 0.04$), time ($F_{4,22} = 11.99$, $p<0.001$), and interaction ($F_{12,22} = 2.13$, $p = 0.03$). A *post hoc* comparison of the average change across the last two minutes of the time course (INSET) revealed that only 10 mg/kg AMPH significantly increased tonic dopamine levels compared to saline ($p<0.01$). In the ventral striatum (Fig. 6B) region, the effects of each psychostimulant were largely indistinguishable from each other and only slightly different than the saline control. Statistical analysis revealed a significant effect of only time ($F_{4,22} = 3.90$, $p = 0.02$). Overall, these results suggested that AMPH is more effective at increasing tonic dopamine signaling than cocaine and in the dorsal compared to the ventral striatum initially after drug injection.

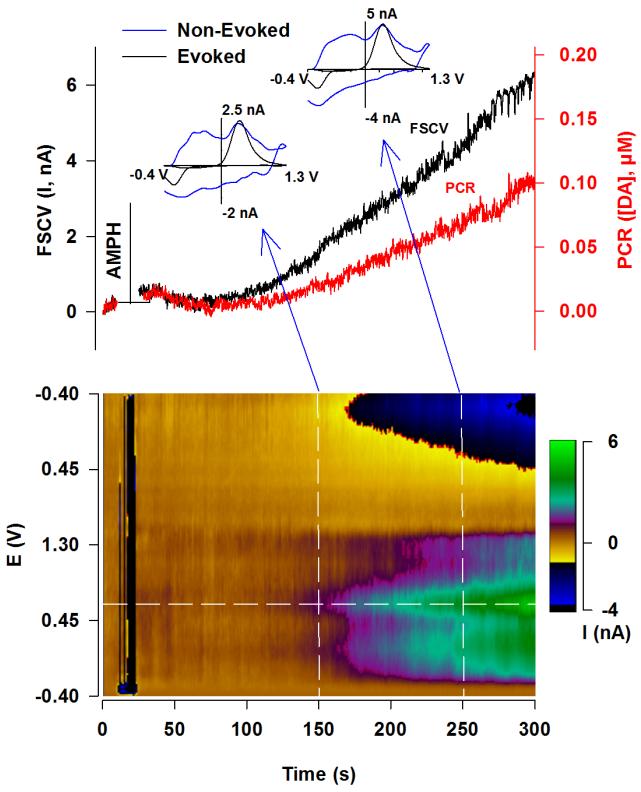

Figure 5. Representative effects of AMPH on tonic dopamine levels in the dorsal striatum. The black line (left y axis) in the top panel shows background-subtracted current, and pseudocolor plot underneath displays all background-subtracted cyclic voltammograms immediately following administration of the high dose (10 mg/kg) of AMPH. Current, which was measured at the peak oxidative potential for dopamine (horizontal white line on the pseudocolor plot), was converted to dopamine concentration (red line, right y axis) using PCR. **INSET.** Background-subtracted cyclic voltammograms taken at 150 s and 250 s (blue arrows, blue line) and from the post-drug electrically evoked (60 Hz, 0.4 s) dopamine signal (black line).
doi:10.1371/journal.pone.0060763.g005

Psychostimulant effects on phasic dopamine signaling

Increased $[DA]_{max}$ of phasic-like dopamine responses evoked by the short train (Figs. 2, 3, 4) suggests that both amphetamine and cocaine activate phasic dopamine signaling. These results are thus consistent with the two psychostimulants augmenting naturally occurring dopamine transients in awake, freely behaving animals [13,36,37]. While psychostimulant-induced burst firing of dopamine neurons is typically blunted under anesthesia [38] unless revealed by D2 antagonists [9,10], dopamine transients are elicited by AMPH in a subset of animals in this preparation [14]. An example of this activation is shown in Figure 7. Before drug injection, the dopamine response evoked by the short train was small and no dopamine transients were observed (Fig. 7A). In sharp contrast, high-dose AMPH dramatically increased this evoked phasic-like signal, mediated by augmented vesicular dopamine release and inhibited dopamine uptake (Fig. 4 and Table 1), and transient frequency (Fig. 7B). To better view the presence or absence of dopamine transients, FSCV recordings are expanded in the INSET. These short-lived, non-electrically evoked deflections were identified as dopamine by the sequential voltammograms displayed in the pseudocolor plot below each trace and by the overlay of the individual voltammogram for the transients (black) with that obtained from the evoked signal established to be dopamine (red) to the left in the INSET.

To complement evoked phasic-like responses, we thus analyzed these dopamine transients to obtain a more physiological assessment of psychostimulant effects on phasic dopamine signaling. Figure 8 shows the time course of dopamine transients for high-dose AMPH (Panel A) and cocaine (Panel B) in the dorsal and ventral striatum (top and bottom, respectively) in the subset of animals where this phasic activity was observed (see legend for details). Transients were analyzed for frequency (left), amplitude (middle), and duration (right). Time 0 min is drug injection. The time when short, intermediate and long trains were applied during the post-drug period is demarcated by vertical dashed lines at 10, 12 and 22 min, respectively. High-dose AMPH and cocaine activated dopamine transients in both striatal subregions. Transients were rarely observed during pre-drug recording and were not observed after saline or low-dose AMPH. Both psychostimulants increased the frequency of dopamine transients to a greater extent in the ventral compared to the dorsal striatum, and AMPH was more effective than cocaine in both striatal subregions. The

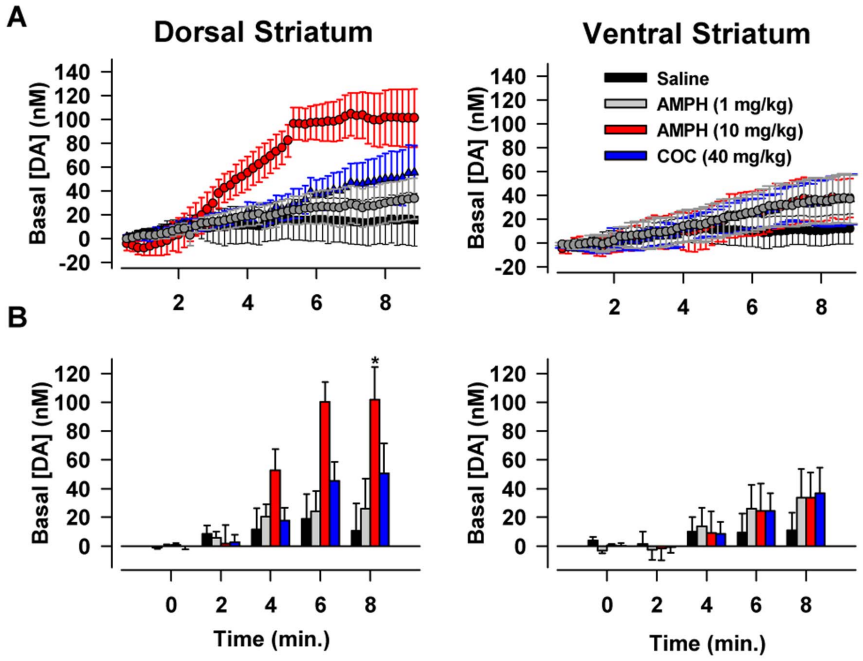

Figure 6. Averaged psychostimulant-induced increases in tonic dopamine levels. A. Time course of the effects of AMPH and cocaine (COC) on tonic dopamine levels. Dopamine concentrations were determined using PCR and averaged across 10-s bins. The time period is the epoch immediately following drug injection and prior to the first post-drug. **B.** Dopamine levels from **A.** above but only shown at two-minute intervals. These data were used for statistical analysis. Data in the dorsal (left) and ventral (right) striatum are expressed as mean ± SEM. *, significantly different from other treatments ($p<0.05$).
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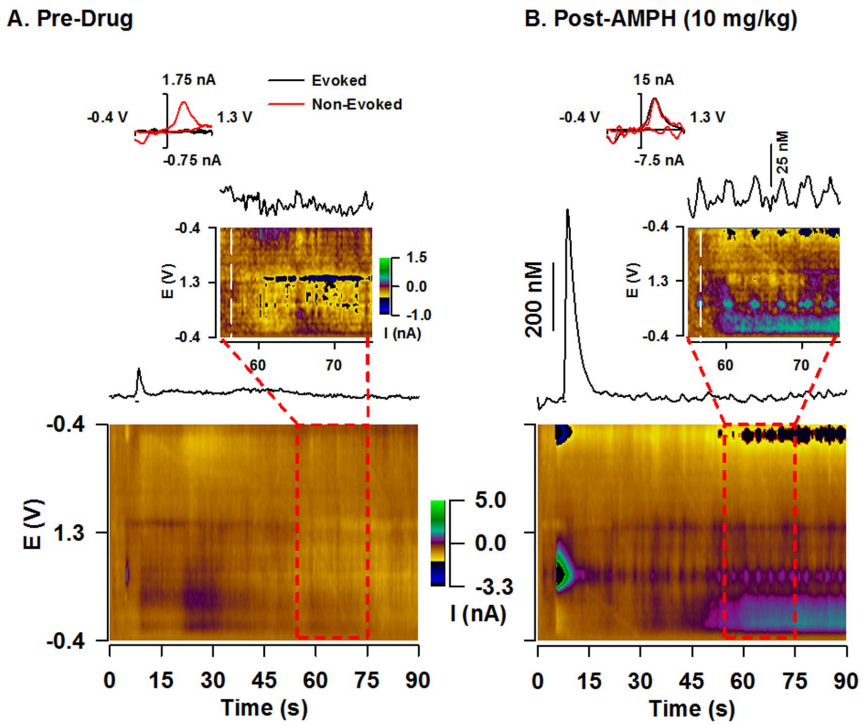

Figure 7. Representative effects of AMPH on phasic dopamine signaling in the ventral striatum. A. Pre-drug. **B.** Post-AMPH. Traces show 90 s of a recording with a short-duration (0.4 s) stimulation applied at 5 s (see line underneath). The color plot serially displaying all background-subtracted cyclic voltammograms is shown underneath. **INSET.** Time-expanded view. Individual background-subtracted voltammograms are shown at the top left and compare dopamine collected during the evoked phasic-like response (black line) to pre-drug baseline (**A.**) or a dopamine transient collected post-drug (**B.**) as indicated by vertical white line in the pseudocolot plot (red line).
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onset of dopamine transient activation was also slower for cocaine. A clear inhibition and rebound in transient frequency was observed following the long train in both the dorsal and ventral striatum after AMPH. This effect is most likely related to feedback inhibition by released dopamine [39], with the additional combination of AMPH and the long train depleting vesicular dopamine release in the dorsal striatum (Fig. 4). Overall, results for dopamine transients are consistent with those for evoked phasic-like responses and suggest that AMPH and cocaine activate phasic dopamine signaling.

Discussion

The goal of the present study was to reconcile the paradoxical effects of AMPH on dopamine neurons. To this end, we tested the novel hypothesis that AMPH depletes the reserve pool but up-regulates the readily releasable pool. This hypothesis was formulated based on three key observations reported in the literature. First, dopamine neurons contain distinct vesicular storage pools. Second, different train durations interrogate different vesicular storage pools. And third, AMPH effects on electrically evoked dopamine levels in the dorsal striatum appear inversely related to train duration. We tested this hypothesis using a novel experimental design. When taken together, our results support a model of AMPH activating tonic dopamine signaling by depleting the reserve pool to drive non-exocytotic efflux, but activating phasic dopamine signaling by up-regulating the readily releasable pool to drive vesicular dopamine release.

Experimental Design

Four features highlight the utility of the experimental design. First, different train durations, selected to demonstrate stimulus-dependent AMPH effects, were applied to the same animal. Although this strategy fosters inter-animal comparisons, it also risks train interactions because dopamine release depends upon stimulation history [40]. However, stability of the saline control and replicating stimulus-dependent AMPH effects demonstrated previously in separate animals indicated that judicial spacing of trains was sufficient to minimize interaction. Second, evoked dopamine dynamics were resolved into the respective contributions of vesicular release and uptake. Most previous studies examining stimulus-dependent AMPH effects report dopamine levels only and therefore do not directly assess release. Third, the status of dopamine storage pools was related to tonic and phasic dopamine signaling. Such an integrated view of AMPH action has not been available. And fourth, we compared AMPH to cocaine, which is recognized to inhibit DAT and increase vesicular release, but not to deplete vesicular stores *in vivo*.

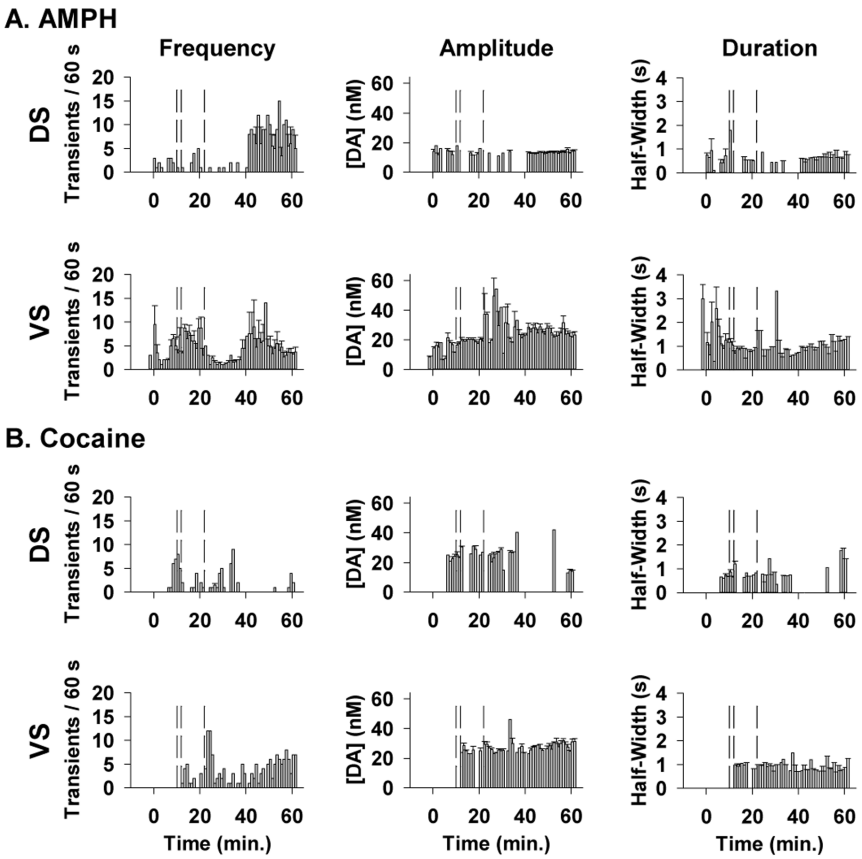

Figure 8. Averaged effects of psychostimulants on dopamine transients. A. AMPH. **B.** Cocaine. Dopamine transients were analyzed in terms of frequency (left), amplitude (middle) and duration (right) in both the dorsal and ventral striatum (DS and VS, respectively). Data are transient characteristics compiled into 60-s bins and express as the mean ± SEM. Each histogram shows transient characteristics for the 10 minutes before, and the 65 minutes after drug injection (at time 0 min). Phasic dopamine transients were observed following AMPH in 3 of 7 animals in the dorsal striatum and 5 of 7 in the ventral striatum and following cocaine in 3 of 7 animals in the dorsal striatum and 1 of 7 animals in the ventral striatum.
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AMPH enhances tonic and phasic dopamine signaling

Tonic dopamine signaling, which is characterized by a steady-state basal level of dopamine and controlled by slow irregular firing of dopamine neurons and presynaptic input [41], enables movement, cognition and motivation [15]. AMPH robustly increases tonic dopamine levels measured by microdialysis [6], but comparatively greater elevations in dialysate dopamine relative to other DAT-inhibiting psychostimulants such as cocaine [5] are attributed to the unique action of AMPH eliciting non-exocytotic efflux [7,8]. We show here that only high-dose AMPH increased tonic dopamine levels and this only occurred in the dorsal striatum. Analytical differences between measurement techniques may have contributed to discrepancies between the present measures with FSCV and microdialysis studies [42]. While FSCV excels at fast measurements with a small probe, inherent limitations in selectivity require the use of statistical methods such as PCR to resolve the dopamine component of tonic changes [34]. Microdialysis exhibits superior selectivity but suffers from implantation damage due to the considerably larger probe that overestimates the increase in tonic dopamine levels with dopamine uptake inhibitors [42]. Thus, measurements of tonic dopamine levels using both approaches should be carefully scrutinized. We should also emphasize that a conservative approach with FSCV was used to minimize error, by only characterizing the first 10-min post-drug epoch and by incorporating background drift as a PCR component [35]. Increases in tonic dopamine levels may thus have occurred after this time. Another consideration when comparing the present and microdialysis studies is anesthesia, which inhibits dopamine neuron firing [43]. However, observed effects of saline and low- and high-dose AMPH on tonic dopamine levels are consistent with un-anesthetized recordings [13]. The contribution of efflux to AMPH-induced increases in tonic dopamine levels measured by FSCV and observed here and elsewhere [13,14] has not been determined. However, efflux is implicated using the present experimental design because increased tonic levels are associated exclusively with vesicular depletion.

Phasic dopamine signaling, in which burst firing of dopamine neurons generates sub-second changes in extracellular dopamine called transients [44], is important for goal-directed behavior and reinforcement learning [15]. Cocaine activates burst firing [38], the amplitude, frequency and duration of naturally occurring dopamine transients [36,37], and evoked phasic-like dopamine responses [14,45]. AMPH has also been shown to augment evoked phasic-like dopamine responses, as well as spontaneously occurring and cue-evoked dopamine transients [13,14]. Consistent with these previous studies, we show here that both AMPH and cocaine activated evoked phasic-like dopamine responses and dopamine transients. Anesthesia likely attenuated these effects by inhibiting burst firing [43] and phasic activation by psychostimulants [13,14,37,38,46]. However, awake, freely behaving animals do not tolerate intermediate and long stimulus trains, so anesthesia is required to assess recycling and reserve pools.

Stimulus-dependent effects of AMPH on $[DA]_{max}$

The present results, obtained by applying different train durations to the same animal, are consistent with previous work applying these same trains individually in separate animals. For example, in the presence of AMPH and in the dorsal striatum, the long train decreased $[DA]_{max}$ [25,47,48], the intermediate train elicited minimal to no effect [11,12], and the short train increased $[DA]_{max}$ [13,14]. Similar results were obtained in the ventral striatum, except that the long train did not decrease $[DA]_{max}$, which is also consistent with previous work [48]. We additionally extend these studies by comparing AMPH effects to cocaine, which only elicited increases or no change in $[DA]_{max}$, and by determining the underlying change in vesicular dopamine release, which permits analysis of storage pools. Indeed, because both AMPH and cocaine robustly inhibit dopamine uptake (Table 1, [12–14,31,33,49]), observed alterations in $[DA]_{max}$ have a complex origin.

AMPH elicits opposing actions on readily releasable and reserve pools for dopamine

Work with model synapses indicates that readily releasable, recycling, and reserve pools of neurotransmitters are interrogated by short, intermediate, and long duration trains, respectively [16]. We used this approach to investigate the effects of AMPH on dopamine stores. In the dorsal striatum, each stimulus train elicited a distinct action on vesicular dopamine release in the presence of high-dose AMPH: increase, no change, and decrease for short, intermediate, and long trains, respectively. Taken together, these results suggest that AMPH augments the readily releasable pool, exerts no effect on the recycling pool, and depletes the reserve pool in the dorsal striatum. By contrast, the readily releasable pool and to a lesser extent the recycling pool were up-regulated without depletion of the reserve pool by AMPH in the ventral striatum and cocaine in both striatal sub-regions. As a psychostimulant with multiple actions, AMPH could augment vesicular dopamine release by several mechanisms, such as: (1) inhibiting monoamine oxidase [50] and activating tyrosine hydroxylase [51], leading to greater cytosolic dopamine levels, vesicular packaging, and ultimately quantal size; (2) increasing membrane excitability as a DAT substrate [52]; and (3) enhancing exocytosis by liberating vesicular Ca^{2+} stores [53]. Depleting the reserve pool suggests another mechanism, re-distributed cytosolic dopamine being re-packaged by the readily releasable pool. This latter postulate is supported by the greater capacity of this vesicle population to sequester cytosolic dopamine [54,55]. Moreover, robust depletion of vesicular dopamine stores by AMPH, well established using reduced preparations [21,22,53,56–61], appears to occur independently in separate classes of dopamine vesicles [21,22]. Depletion involves AMPH acting as a weak base to destabilize the proton gradient across vesicles and as a substrate of the vesicular monoamine transporter to inhibit and/or reverse its action [7,8]. How these mechanisms might differ across dopamine storage pools, as our results would suggest, remains to be determined.

We also do not know why AMPH depleted vesicular dopamine stores in the dorsal but not ventral striatum. One possible origin is regional differences in DAT. For example, DAT binding and V_{max} for dopamine uptake are higher in the dorsal striatum [62,63], and DAT is more glycosylated with a higher molecular weight in the ventral striatum [64]. Although K_m for dopamine uptake is similar in the two regions [31,63], AMPH is a more potent competitive inhibitor of dopamine uptake in the dorsal compared to the ventral straitum [12]. We are not aware of comparable regional differences in the vesicular monoamine transporter. Another possible origin is regional differences in vesicular dopamine stores. As mentioned above, different classes of dopamine vesicles exhibit different sensitivities to the depleting actions of AMPH [21,22]. Consistent with region-specific actions of AMPH on vesicular dopamine stores, we have recently shown that AMPH may up-regulate vesicular dopamine release in the ventral striatum by mobilizing the reserve pool but by activating dopamine synthesis and inhibiting dopamine degradation in the dorsal striatum [65]. Different distributions of small, clear and large, dense-core vesicles in the two striatal sub-regions [66] may also contribute to the differential response to AMPH. Clearly, more work needs to be

done to resolve the differential depleting effects of AMPH on dopamine vesicles in the dorsal and ventral striatum.

New model of amphetamine action

We propose a new model of AMPH action: activating tonic dopamine signaling by depleting the reserve pool, which elevates cytosolic dopamine and drives reverse transport through DAT, while concurrently activating phasic dopamine signaling by up-regulating the readily releasable pool, which drives vesicular dopamine release. This model is supported here by the first report of a selective coupling between tonic activation and vesicular depletion coincident with phasic activation and up-regulated vesicular release. Revealing this unique combination of AMPH effects underscores the utility of the experimental design employed. Indeed, slice voltammetry has demonstrated a parallel between robust vesicular depletion and micromolar dopamine efflux, but no measures of phasic signaling or its release component were examined [58,60,61]. Moreover, *in vivo* voltammetry has demonstrated concurrent activation of tonic and phasic dopamine signaling and up-regulated vesicular release, but effects on the reserve pool were not assessed [13,14]. Further supporting our proposed model is that, in contrast to AMPH in the dorsal striatum, AMPH in the ventral striatum and cocaine in both striatal sub-regions did not deplete vesicular stores or elevate tonic dopamine levels, despite phasic activation and up-regulated vesicular release.

Two confounds need addressing. First, coupling between tonic activation and vesicular depletion was not observed for low-dose AMPH in the dorsal striatum. It could be that, while cytosolic dopamine increased as a result of vesicular depletion, low-dose AMPH was insufficient to inhibit monoamine oxidase and prevent its intracellular degradation and/or to reverse DAT direction and cause efflux. Both AMPH effects are dose-dependent [50,67]. Also consistent with this interpretation is that vesicular depletion alone does not elicit efflux [58] and that both vesicular depletion and blockade of monoamine oxidase are required for cytosolic levels to increase [59]. In contrast, there are other reports demonstrating that increases in cytosolic dopamine alone are sufficient to induce efflux [44,68]. Second, low-dose AMPH also did not activate phasic dopamine signaling or vesicular dopamine release in the dorsal striatum. However, this lack of response is an anesthesia artifact, because both are enhanced in awake, freely behaving animals [13].

Implications for psychostimulant neurobiology

We demonstrate fundamentally similar and distinct mechanisms for two major classes of psychostimulants, AMPH representing the so-called dopamine "releasers" (i.e., eliciting non-exocytotic efflux) and cocaine representing the DAT "inhibitors" [33]. While AMPH and cocaine share phasic activation through augmented vesicular dopamine release (Fig. 4, [13,14,45,49,69–71]) and enhanced burst firing [9,10,38], they differ in tonic activation. In particular, cocaine requires action potential-dependent mechanisms whereas AMPH does not [72–74]. Inhibition of dopamine uptake (Table 1, [12–14,31,33,49]) would contribute to augmented tonic and phasic signaling by both psychostimulants. However, activation of vesicular dopamine release may be more important than uptake inhibition, especially for phasic signaling, because release better tracks $[DA]_{max}$ (Fig. 4, [13]).

The neurobiological implications of these psychostimulant actions are not presently known, but they could be profound. Several drugs of abuse have now been demonstrated to augment dopamine transients, including amphetamine, cocaine, nicotine and ethanol [13,37,46,75,76]. The greater activation of phasic dopamine signaling by abused drugs compared to natural rewards and the subsequent usurpation of normal reward processing to promote addiction [77] may thus represent a unifying mechanism. While both classes of psychostimulants would promote reinforcement learning by activating the direct ("Go") pathway in the basal ganglia via enhanced phasic signaling and D1 receptor binding, AMPH would more robustly inhibit the indirect ("No Go") pathway (i.e., disinhibition of behavior) via enhanced tonic signaling and D2 receptor binding [78], because of the added contribution of non-exocytotic efflux. Future directions should also investigate how intrastriatal differences in AMPH action relate to the diverse roles of dopamine signaling in this region for promoting drug reinforcement and addiction [79,80].

Author Contributions

Conceived and designed the experiments: PAG DPC. Performed the experiments: DPC. Analyzed the data: DPC. Wrote the paper: PAG DPC SAJ. Assisted with statistical analysis: SAJ.

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