Published in final edited form as:

J Neurochem. 2011 June ; 117(6): 937–948. doi:10.1111/j.1471-4159.2011.07258.x.

Amphetamine Augments Action Potential-Dependent Dopaminergic Signaling in the Striatum *in Vivo*

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Abstract

Amphetamine (AMPH) is thought to disrupt normal patterns of action potential-dependent dopaminergic signaling by depleting dopamine (DA) vesicular stores and promoting non-exocytotic DA efflux. Voltammetry in brain slices concurrently demonstrates these key drug effects, along with competitive inhibition of neuronal DA uptake. Here we perform comparable kinetic and voltammetric analyses *in vivo* to determine whether AMPH acts qualitatively and quantitatively similar in the intact brain. Fast-scan cyclic voltammetry measured extracellular DA in dorsal and ventral striata of urethane-anesthetized rats. Electrically evoked recordings were analyzed to determine K_m and V_{max} for DA uptake and vesicular DA release, while background voltammetric current indexed basal DA concentration. AMPH (0.5, 3, and 10 mg/kg i.p.) robustly increased evoked DA responses in both striatal subregions. The predominant contributor to these elevated levels was competitive uptake inhibition, as exocytotic release was unchanged in the ventral striatum and only modestly decreased in the dorsal striatum. Increases in basal DA levels were not detected. These results are consistent with AMPH augmenting action potential-dependent dopaminergic signaling *in vivo* across a wide, behaviorally relevant dose range. Future work should be directed at possible causes for the distinct *in vitro* and *in vivo* pharmacology of AMPH.

INTRODUCTION

Because of prominent clinical and recreational uses, the effects of amphetamine (AMPH) on behavior and brain function have been extensively studied. In the rat, AMPH increases locomotion at low doses and stereotypic movements at high doses (Gaytan et al. 1998). These drug-elicited behaviors are due, at least in part, to AMPH acting on midbrain dopaminergic neurons, with the projections to the nucleus accumbens (ventral striatum) involved in locomotor activation and the caudate-putamen (dorsal striatum) in stereotypy (Kelly et al. 1975). Not unexpectedly, AMPH robustly increases basal extracellular dopamine (DA) levels in both striatal subregions as measured by microdialysis (Kuczenski et al. 1991; Kuczenski et al. 1997). Comparatively greater increases in dialysate DA suggest that AMPH acts distinctly from other psychostimulants, such as cocaine, whose primary mechanism is inhibition of the dopamine transporter (DAT) (Kuczenski et al. 1991).

A large body of work indicates that AMPH elevates striatal extracellular levels of DA through a concerted action on multiple sites of dopaminergic neurotransmission (Sulzer et al. 2005; Fleckenstein et al. 2007). Early studies demonstrated DA synthesis activation (Kuczenski 1975), monoamine oxidase inhibition (Miller et al. 1980), competitive blockade of DA uptake (Krueger 1990), depletion of vesicular DA stores (Floor and Meng 1996), and

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The authors declare no conflict of interest.

increased non-exocytotic DA release or DA efflux via reversal of DAT (Sulzer et al. 1995). More recent investigations have extended these initial findings by resolving the effects of AMPH on DA uptake blockade from DAT reversal and from exocytotic DA release (Jones et al. 1998;Schmitz et al. 2001), by examining the effects of AMPH on cytosolic DA pools directly (Mosharov et al. 2003), and by demonstrating new actions of AMPH on DA uptake including DAT insertion (Johnson et al. 2005) and internalization (Saunders et al. 2000), and activation of DAT channel-like activity (Kahlig et al. 2005).

By compromising vesicular DA release and promoting DA efflux, AMPH should disrupt normal patterns of action potential-dependent dopaminergic neurotransmission. Indeed, it is these effects that may distinguish AMPH and its analogs from other addictive substances, which are thought to augment phasic DA signals elicited by burst firing of dopaminergic neurons (Stuber et al. 2005;Cheer et al. 2007;Robinson et al. 2009) and to cause an overlearning of cues predicting drug availability (Hyman 2005;Wanat et al. 2009). However, because most mechanistic investigations have utilized *in vitro* preparations, there is a great need to establish whether AMPH acts qualitatively and quantitatively similar *in vivo*. Several lines of evidence suggest important differences between the two preparations. For example, deafferentation of inputs to midbrain dopaminergic neurons potentiates AMPH-induced increases in extracellular levels of striatal DA (Miller et al. 2002), indicating that intact circuitry plays an important role in drug action. While brain slice measurements demonstrate robust decreases in electrically evoked DA levels with AMPH (Jones et al. 1998;Schmitz et al. 2001), some, but not all, studies in the anesthetized rat actually show an augmentation (Kuhr et al. 1986;May et al. 1988;Dugast et al. 1994). Finally, when monitored by fast-scan cyclic voltammetry (FSCV) at a carbon-fiber microelectrode (CFM), AMPH elicits a large, micromolar increase in basal DA levels in the brain slice attributed to DA efflux (Jones et al. 1998;Schmitz et al. 2001), but no detectable signal *in vivo* (Wiedemann et al. 1990).

The present study assessed the mechanism of AMPH action on dopaminergic signaling *in vivo*. FSCV at a CFM was used, in the urethane-anesthetized rat, to monitor extracellular DA levels in the dorsal and ventral striatum elicited by electrical stimulation of the medial forebrain bundle (MFB). Parameters for exocytotic DA release and neuronal DA uptake were determined by kinetic evaluation of these evoked responses. Background voltammetric current was used to index changes in basal DA concentration. This experimental design, which is comparable to previous work in brain slices (Jones et al. 1998;Schmitz et al. 2001), fosters comparisons between AMPH actions *in vitro* and *in vivo* on the basis of similar voltammetric and kinetic analyses. We found that AMPH robustly increased electrically evoked DA levels in both dorsal and ventral striata across a wide dose range (0.5, 3, and 10 mg/kg i.p.). Consistent with the brain-slice work, AMPH competitively inhibited DA uptake in a dose-dependent manner. Unlike *in vitro* studies, however, vesicular DA release was either unchanged in the ventral striatum or only modestly decreased in the dorsal striatum, while increases in basal DA levels were not detected in either striatal subregion. Taken together with previous work, these results suggest that AMPH acts substantively different in urethane-anesthetized rats compared to brain slices, with competitive uptake inhibition predominating over vesicular depletion and DA efflux. These results additionally demonstrate that AMPH robustly augments action potential-dependent dopaminergic signaling *in vivo*.

METHODS

Animals

Male Sprague-Dawley rats (300–500 g) were purchased from Harlan Industries (Indianapolis, IN, U.S.A.). Food and water were provided *ad libitum*. Animals were housed

in temperature-controlled facility with a 12 hour light/dark cycle, and care was in accordance with NIH guidelines (Institute of Laboratory Animal Resources 1996) and approved by the Institutional Animal Care and Use Committee of Illinois State University.

Surgical Procedure

Rats were anesthetized using urethane (1.5 g/kg, i.p.) and immobilized in a stereotaxic frame (Kopf Instrumentation, Tujunga, CA, USA). Skin and connective tissue were removed, exposing the skull. Holes were drilled through the skull, and electrodes were positioned according to flat-skull stereotaxic coordinates (Paxinos G and Watson C 1986) using bregma and top of brain as landmarks. Core body temperature was maintained at approximately 37°C with a Deltaphase Isothermal Pad (Braintree Scientific, Braintree, MA, USA). The stimulating electrode was placed just dorsal to the MFB (−4.6 AP, +1.4 ML, −6.5 DV). Recording electrodes were positioned in the dorsal (+1.2 AP, +2.0 or 3.0 ML, −4.5 DV) or ventral striatum (+1.2 AP, +2.0 or 3.0 ML, −6.5 DV). Electrodes positioned at +3.0 ML were angled at 6°. A Ag/AgCl reference electrode (i.e., chloridized silver wire) was placed contralaterally in superficial cortex. The stimulating electrode was incrementally lowered until robust DA release was achieved in both striatal regions. Recording electrodes were then lowered until a fast, high rate of evoked DA release was observed. After optimization, the positions of the stimulating and recording electrodes were not changed for the duration of the experiment.

Fast-Scan Cyclic Voltammetry

FSCV was recorded at a glass-sealed CFM. Individual carbon fibers (3.5 μm-radius, Cytec Engineering Materials, West Patterson, NJ, USA) were aspirated into borosilicate capillary tubes (1.0 or 1.2 mm o.d., Sutter Instrument, Novato, CA, USA) and pulled in a micropipette puller (Narishige, Tokyo, Japan). The carbon fiber was cut so that approximately 100 μm extended beyond the glass/carbon-fiber seal. Electrical connection between the carbon fiber and 26-gauge lead wire (Newark, Palatine, IL, USA) was provided by a low-melting point bismuth alloy (Small Parts, Inc., Miramar, FL, USA), which was melted with a heat gun (Master Appliance Corp., Racine, WI, USA) and allowed to cool. A triangle waveform (−0.4 V to 1.0 V, 300 V/s) was applied at 10 Hz, with a bias potential of −0.4 V. Electrochemistry was performed by a EI400 (Ensmann Instrumentation, Bloomington, IN, USA) or UEI (University of North Carolina at Chapel Hill, Department of Chemistry Electronics Facility, Chapel Hill, NC, USA) bipotentiostat and controlled by TH-1 software (ESA, Chelmsford, MA, USA). Background subtracted cyclic voltammograms were used for electrochemical identification. Evoked DA dynamics were obtained by monitoring current at the DA oxidation peak (~+0.6 V) and converting to concentration based on postcalibration, as previously described (Wu et al. 2001b).

Electrical Stimulation

Stimulus pulses were applied to a twisted bipolar electrode (Plastics One, Roanoke, VA, USA), with tips separated approximately 1 mm. Stimulus parameters were ±300 – 350 μA, 4 ms biphasic pulses (2 ms each phase), either 20 Hz or 60 Hz, and 2 s trains. Electrical stimulation was computer generated and passed through an optical isolator and constant-current generator (Neurolog NL800, Digitimer Limited, Letchworth Garden City, UK).

Experimental Design

After evoked DA responses stabilized, a time course using 20-Hz trains was collected. For the predrug responses, 4 trains were applied every 5 minutes. For the first 20 min after the injection of AMPH (0, 0.5, 3, or 10 mg/kg i.p.; n = 5 to 9 and 4 to 6 for dorsal and ventral striatum, respectively), trains were applied every 5 min. Thereafter, trains were applied

every 15 min. Two 60-Hz trains were also applied. The first occurred immediately after response stabilization and 10 min before collecting the 20 Hz predrug trains. This predrug 60 Hz response and the average of the four predrug 20-Hz responses were used to obtain predrug kinetic parameters for DA release and uptake. The second 60-Hz train was applied 25 min postdrug. This train and the 20-Hz train applied at 35 min were used to obtain postdrug kinetic parameters for DA release and uptake. The different dynamics elicited by these two stimulation frequencies improve the accuracy of curve fitting (Wu et al. 2001b). Background current at the DA-oxidation potential, an index of basal DA levels (Jones et al. 1998; Schmitz et al. 2001), was also determined from the pre-stimulation portion of each evoked recording during the time course. With the exception of Figure 5, all averaged postdrug effects were expressed as a percent of predrug responses.

Data Analysis

Evoked DA responses were mathematically described as a balance between DA release and uptake (Wightman et al. 1988):

$$d[DA]/dt = [DA]_p * f - V_{max} / (K_m / [DA] + 1) \quad (1)$$

where $[DA]_p$ is the concentration of DA released per stimulus pulse, f is the frequency of stimulation, V_{max} is the Michaelis-Menten uptake term related to DAT number and turnover rate, and K_m is the Michaelis-Menten uptake term inversely related to the affinity of DA for DAT. Curve fitting, to determine $[DA]_p$, V_{max} , and K_m , employed a simplex-minimization algorithm (Wu et al. 2001b). Two recordings, evoked by a 20- and 60-Hz train, were analyzed simultaneously for each animal during both predrug and postdrug conditions. One set of DA release and uptake parameters were obtained for each pair of recordings.

Statistical Analysis

When appropriate, data are presented as the mean $\pm$ SEM. Statistical analysis was performed using SAS version 9.1.3 (SAS Institute Inc. 2004). Specifically, a repeated-measures MANOVA with pairwise multivariate contrasts was used to analyze time courses in Figure 2 and Figure 6. Grouped data in Figure 3 and Figure 4 and in Sup. Table S1 were compared using a one-way ANOVA followed by Ryan-Einot-Gabriel-Welsch Multiple Range Test (REGWQ) as a post-hoc test. Student's t-test was used to compare linear-regression slopes in Figure 5 and the combined groups in Sup. Table S1. Significance was set at $p < 0.05$.

Drugs

Urethane and *d*-amphetamine sulfate were purchased from Sigma (St. Louis, MO, USA). Both drugs were dissolved in 150 mM saline prepared with nanopure water (Barnstead/Thermolyne Corporation, Dubuque, IA, USA) prior to injection. *d*-AMPH dose was determined as base weight.

RESULTS

Effects of AMPH on evoked DA levels

Figure 1 shows representative effects of AMPH on extracellular DA dynamics electrically evoked in the dorsal (Fig. 1A) and ventral (Fig. 1B) striatum by MFB stimulation. These responses were collected 35 minutes after drug administration. In each recording, the stimulus pulse train (20 Hz, 2 s) was delivered at 5 s, as demarcated by the solid line underneath. In both striatal subregions, AMPH elicited robust increases in evoked DA levels at all doses examined. The slowed extracellular clearance of released DA indicated that uptake inhibition contributed to observed increases. Changes in exocytotic DA release

cannot be ruled out, however, because the amplitude of the evoked response is determined by the balance of release and uptake processes (Wightman et al. 1988; Wu et al. 2001b). Interestingly, the effects of AMPH on signal amplitude were greatest in the ventral striatum at the highest dose (10 mg/kg), but plateaued in the dorsal striatum going at 3 mg/kg. In the dorsal striatum, the further slowing of extracellular DA clearance without a change in signal amplitude as AMPH dose is increased from 3 to 10 mg/kg is consistent with a decrease in exocytotic DA release. Voltammograms (Insets) indicate that DA was the origin of these evoked responses.

Figure 2 shows the time course of AMPH effects on the maximal DA concentration ($[DA]_{max}$) elicited by 20-Hz stimulation. Both the amplitude and duration of the effects of AMPH on $[DA]_{max}$ in the dorsal (Fig. 2A) and ventral (Fig. 2B) striatum were dose related. Similar to representative recordings, averaged $[DA]_{max}$ responses did not further increase in the dorsal striatum from 3 to 10 mg/kg AMPH. Also similar to the recordings shown in Figure 1, $[DA]_{max}$ appear to increase in a dose-dependent fashion in the ventral striatum. Dissimilar from other doses, which elicited more transient elevations, 10 mg/kg AMPH caused $[DA]_{max}$ to plateau for the duration of the time course. A repeated-measures MANOVA revealed a significant effect of time in the dorsal ($F_{14,6} = 5.95$, $p = 0.0187$) but not ventral ($F_{14,2} = 9.37$, $p = 0.1005$) striatum, and no significant interaction ($F_{42,24} = 1.75$, $p = 0.0727$ and $F_{42,12} = 1.45$, $p = 0.2473$, respectively). There was a main effect of drug treatment in both the dorsal and ventral striatum ($F_{3,19} = 3.84$, $p = 0.0264$ and $F_{3,15} = 8.30$, $p = 0.0017$, respectively), and post-hoc tests determined significant differences between 10 mg/kg and all other doses in both subregions ($p < 0.05$).

Effects of AMPH on DA release and uptake

Real-time recordings evoked by 20 and 60 Hz stimulation (Sup. Fig. S1 and Sup. Fig. S2) were kinetically analyzed to resolve the relative contributions of DA release and uptake mechanisms to the AMPH-induced alterations in DA dynamics and $[DA]_{max}$. Pre-drug values for all measures were similar across group and consistent with previous descriptions (Sup. Table S1). The effects of AMPH on best-fit parameters for DA release and uptake, expressed as a percent of the predrug response, are found in Figure 3. As shown in the top panel, ANOVA revealed no significant main effect of AMPH on $[DA]_p$ in either the dorsal (Fig. 4A) or ventral (Fig. 4B) striatum ($F_{3,21} = 2.76$, $p = 0.0678$ and $F_{3,19} = 1.41$, $p = 0.2711$, respectively). Because the p value (0.0678) approached significance (0.05), the modest decrease in exocytotic DA release in the dorsal subregion at 3 and 10 mg/kg AMPH, with the highest dose eliciting the greatest reduction, is considered in this study a statistical trend. In contrast, the p value (0.2711) in the ventral striatum is considerably higher, and trend is not used to describe these data.

Figure 3, middle and bottom panels, shows the effects of AMPH on the Michaelis-Menten parameters for neuronal DA uptake. Because curve fitting of 10 mg/kg responses was not stable when all three parameters ($[DA]_p$, V_{max} and K_m) were permitted to float, V_{max} was fixed at the predrug level for analysis of postdrug responses measured with the highest AMPH dose. This procedure assumes that AMPH competitively inhibits DA uptake at the highest dose, which is consistent with drug action observed at the two lower doses (see below). As a result, statistical analysis of V_{max} only used the AMPH doses of 0, 0.5 and 3 mg/kg. As shown in the middle panel, there were no significant main effects of AMPH on V_{max} in the dorsal (Fig. 4A; $F_{2,17} = 1.30$, $p = 0.2983$) or ventral (Fig. 4B; $F_{2,14} = 3.19$, $p = 0.0724$) striatum. The effects of AMPH on K_m are shown in the bottom panel. Overall, AMPH appeared to inhibit DA uptake to a greater extent in the dorsal (Fig. 3A) compared to the ventral (Fig. 3B) striatum. Moreover, in contrast to V_{max} , AMPH elicited highly significant main effects on K_m in both the dorsal ($F_{3,21} = 26.39$, $p < 0.0001$) and ventral ($F_{3,19} = 17.31$, $p < 0.0001$) striatum. In the dorsal striatum, post-hoc tests revealed a

significant difference between all AMPH doses and saline and a significant difference between 0.5 mg/kg and the higher doses ($p < 0.05$). In the ventral striatum, 3 and 10 mg/kg AMPH were significantly different from saline and 0.5 mg/kg ($p < 0.05$). Overall, these results suggest that AMPH competitively inhibits DA uptake in both striatal subregions.

Determination of a K_i for the Effects of AMPH on DA uptake

To further assess the regional potency of AMPH on DA uptake, a dissociation constant (K_i) for the binding of the competitive inhibitor to DAT was determined by plotting apparent K_m ($(K_m)_{app}$ or postdrug K_m) versus dose. In this plot, the slope of the linear regression line is K_m/K_i (Jones et al. 1995). Instead of mg/kg, drug dose is expressed as micromoles (μmol)/kg (i.e., 0.5, 3.0, and 10 mg/kg equals 4.1, 24.6, and 74.0 $\mu\text{mol/kg}$, respectively). This conversion permits comparisons between AMPH and two other psychostimulants, cocaine and nomifensine, on a mole-drug dose basis (see below). Figure 4 shows a significant linear relationship between $(K_m)_{app}$ and AMPH dose for both the dorsal (solid circles, DS; $r = 0.9985$, $p = 0.0015$) and ventral (open circles, VS; $r = 0.9863$, $p = 0.0137$) striatum. The slope of the linear regression is steeper for the dorsal ($0.101 \pm 0.0039 \mu\text{M}/\mu\text{mol/kg}$) compared to the ventral ($0.0487 \pm 0.0058 \mu\text{M}/\mu\text{mol/kg}$) striatum, and statistical analysis demonstrated a significance difference ($t = 7.483$, $p < 0.001$). Based on these slopes, K_i for AMPH inhibiting DA uptake was calculated to be 3.123 and 5.996 $\mu\text{mol/kg}$ in the dorsal and ventral striatum, respectively. Thus, AMPH is ~2 times more potent for competitively inhibiting DA uptake in the dorsal striatal subregion.

Also shown in Figure 4 is the apparent K_m for two other psychostimulants that inhibit DA uptake, nomifensine (NOM, squares) and cocaine (COC, diamonds). These data were previously, but comparably determined *in vivo* (Wu et al. 2001a). AMPH appears substantively more efficacious than cocaine for inhibiting DA uptake in both the dorsal (solid circles, DS) and ventral (open circles, VS) striatum, but more similar to nomifensine (solid squares, DB; open squares, VS). Taken together, these results suggest that AMPH is inhibiting neuronal DA uptake quantitatively and qualitatively similar *in vivo* and *in vitro*. AMPH also appears more efficacious in inhibiting DA uptake *in vivo* than cocaine.

Effects of AMPH on background voltammetric current

Similar to previous brain slice studies using FSCV at a CFM (Jones et al., 1998; Schmitz et al., 2001), AMPH-induced changes in basal DA levels were indexed by background voltammetric current measured at the peak oxidation potential for DA. As shown in Figure 5, only the highest dose of AMPH elicited an increase in background current in the dorsal striatum, and this increase (~5 %) was relatively small (Fig. 5A). ANOVA demonstrated significant main effects of drug treatment ($F_{3,19} = 4.36$, $p = 0.0169$) and time ($F_{14,6} = 5.97$, $p = 0.0186$), but no significant interaction between time and treatment ($F_{42,24} = 1.69$, $p = 0.0862$). Post-hoc tests indicated a significant difference between 10 mg/kg and both saline and 3 mg/kg ($p < 0.5$). No effects of AMPH on background current were apparent in the ventral striatum (Fig. 5B), and no significant main effects of drug ($F_{3,15} = 3.02$, $p = 0.0629$) or time ($F_{14,2} = 11.81$, $p = 0.0807$), and no significant interaction ($F_{42,12} = 1.52$, $p = 0.22$) were determined.

Figure 6 describes an analysis of voltammograms to establish the electrochemical origin of the increase in background current elicited in the dorsal striatum by 10 mg/kg AMPH. Figure 6A shows individual time-course responses for the entire high-dose group. Three out five responses appeared to show no increase with AMPH. The individual time-course response that demonstrated the greatest increase was further analyzed. Shown in Figure 6B are the four evoked responses at the time points demarcated by the closed circles and Roman Numerals in 6A. Notice the increase in the amplitude of the evoked response, consistent

with Figure 1 and Figure 2. Stimulus delivery is demarcated by the line underneath each recording. Also notice the increase in background current (i.e., pre-stimulus signal) with time, consistent with 6A. In contrast to recordings shown in Figure 1, background current is not subtracted and is rather reported as an absolute value in the y-axis. The effects of stimulation are still observed on top of this rising background, which changes more slowly than the evoked DA signal. Figure 6C shows the individual cyclic voltammogram ("Subtracted CV") determined by subtracting the background response (i.e., pre-stimulation) in recording I from the corresponding background responses in recordings II through IV. All voltammograms showed non-specific changes in the electrochemical signal, perhaps due to electrode drift, but no evidence for DA.

Figure 6D shows additional cyclic voltammograms calculated to determine whether a change in DA, if present, could contribute to these non-specific changes. The solid line describes the characteristic voltammogram for DA, with an upward peak at $\sim +0.6$ V, due to the oxidation of DA to a quinone, and a downward peak at ~ -0.2 V, due to the reduction of the electroformed quinone. This voltammogram, calculated by subtracting the background response in IV from the peak of the evoked response in this same recording, identified as V, demonstrates that DA is the source of the evoked signal, consistent with Figure 1 (Insets). The long-dashed line is the same voltammogram shown for "IV - I" in 6C, which describes the change in background electrochemistry between these two time points and whose origin appears to be non-specific drift of the CFM.

The dotted line in Figure 6D is the voltammogram calculated by subtracting the background response in I from the peak of the evoked response V. This voltammogram clearly shows the non-specific change in background signal, as well as the characteristic redox peaks for DA. In terms of absolute current, the non-specific background change (~ 30 nA) is about two-times the amplitude of the evoked signal (~ 15 nA). Thus, if present at this concentration, approximately $1 \mu\text{M}$ after postcalibration conversion, DA should also appear on top of the non-specific voltammogram. Based on an estimated detection limit for the present *in vivo* measurements of ~ 150 nM (i.e., three times the standard deviation of baseline noise or a signal-to-noise ratio of three), even lower DA concentrations would appear in the voltammograms as well. Taken together, these results suggest that AMPH-induced increases in basal levels of extracellular DA were not detected in either the dorsal or ventral striatum.

DISCUSSION

We examined the mechanism of AMPH action *in vivo*. Voltammetric and kinetic analyses were comparable to those used previously in brain slices demonstrating key drug effects acting concurrently on presynaptic dopaminergic signaling: competitive inhibition of DA uptake, depletion of vesicular DA stores, as indexed by exocytotic DA release, and increased DA efflux, as indexed by increases in basal DA levels. We were able to replicate robust competitive inhibition of DA uptake. However, no effect of AMPH on vesicular DA release was observed in the ventral striatum and only modest decreases were found in the dorsal striatum. Moreover, increases in basal DA levels were not detected in either striatal subregion. Taken together, these results suggest distinct *in vivo* and *in vitro* pharmacology of AMPH.

Effects of AMPH on electrically evoked DA levels are complex

While voltammetry in brain slices consistently demonstrates a decrease in electrically evoked DA levels with AMPH (Jones et al. 1998; Schmitz et al. 2001; Patel et al. 2003), results with *in vivo* voltammetry are mixed. Robust ($\sim 70\%$) decreases have been observed in the dorsal striatum with very long (10 s), high-frequency (60 Hz) trains (Ewing et al. 1983; Kuhr et al. 1985; Kuhr et al. 1986), but with shorter trains (0.25 to 2 s) an increase has

been reported (May et al. 1988). In the ventral striatum, the same long, high-frequency trains elicited no change (Kuhr et al. 1986), and increases have been demonstrated with short, low-frequency trains (20 Hz, 0.3 s; Dugast et al. 1994). We report here that AMPH increases DA levels evoked by 20-Hz stimulation in both the dorsal and ventral striatum across a wide range of doses (Fig. 2). It thus appears that *in vivo*, the use of long-duration, high-frequency trains is associated with AMPH-induced decreases in evoked DA levels in the dorsal striatum, while shorter-duration, low-frequency stimulation supports augmented responses in both striatal subregions. The ventral striatum also appears more resistant to AMPH-induced decreases in evoked DA levels.

Dose-dependent, AMPH-induced increases in evoked DA signals were observed in both striatal regions, but to different degrees. In contrast to the ventral striatum, no further increase in signal was observed going from 3 to 10 mg/kg (Fig. 2). A similar response plateau has been reported in the olfactory tubercle, also with low-frequency stimulation (Suaud-Chagny et al. 1989). We additionally show in both dorsal and ventral striata that 10 mg/kg elevated evoked DA levels for the entire 2-h time course, in contrast to lower doses that increased signals more transiently. This effect, which has not been reported previously to our knowledge, could be due to the combination of high AMPH dose (10 mg/kg) and low-frequency (20 Hz) stimulation. It thus appears that multiple aspects of AMPH-induced changes in evoked DA levels are dose dependent. Interestingly, this extended time course closely parallels the stereotypy observed in awake rats, which remains high for >2 h and is elicited by high doses of AMPH (Kelley et al., 1975). In contrast, low doses of AMPH have more transient effects on both locomotor activity (Clausing et al., 1995) and electrically evoked DA levels. Thus, these voltammetric measurements may have implications for AMPH-induced changes in motor behavior.

Effects of AMPH on exocytotic DA release

To our knowledge, this is the first study to isolate AMPH effects on dopaminergic signaling *in vivo* to a parameter for vesicular DA release. We show that AMPH does not alter exocytotic DA release in the ventral striatum, and only a trend for a modest decrease was observed in the dorsal striatum (Fig. 3). The further reduction in exocytotic DA release from 3 to 10 mg/kg AMPH could mediate the similar $[DA]_{max}$ observed for the two doses (Fig. 2), however, because this small decrease in release is additive with each stimulus pulse across the entire train. Our *in vivo* results are in sharp contrast to those obtained with voltammetry in brain slices, which demonstrate that AMPH robustly (~75 to 100 %) decreases exocytotic DA release in the dorsal striatum (Jones et al. 1998; Schmitz et al. 2001; Patel et al. 2003). The origin of this decrease in the brain slice is debated. Schmitz et al. (2001) argue that 47 % is due to vesicular DA depletion, with the remaining 35 % stemming from autoreceptor activation, while Jones et al. (1998) and Patel et al. (2003) found no evidence for presynaptic DA inhibition.

While there is a precedent for discrepant effects of drugs on DA release between brain-slice and whole-animal preparations (e.g., Wu et al 2000; Kulagina et al 2001), it is difficult to fully reconcile the marked decrease in exocytotic DA release observed *in vitro* with the minimal to modest effects of AMPH *in vivo*. AMPH dose does not appear to be the origin of this discrepancy. A similar inhibition of DA uptake was achieved in the two preparations, and brain AMPH concentrations sufficient to decrease exocytotic DA release in the slice, are detected after systemic administration of similar doses used in the present study (see Results; Clausing et al. 1995; Kuczenski et al. 1997). This discrepancy could be due to differences in the responsiveness of isolated terminals versus intact neurons to AMPH. Circuit-level modulation may play a role as well (Miller et al. 2002). Another potential origin for this discrepancy is electrical stimulation. A single pulse was applied directly to slice dopaminergic terminals, as opposed to stimulus trains activating action potentials in

ascending dopaminergic axons *in vivo*. Perhaps as a result, per-stimulus-pulse DA release differs as well, reaching $\sim 2.5 \mu\text{M}$ (Schmitz et al. 2001) in the slice, but only $\sim 0.05 \mu\text{M}$ *in vivo* (Sup. Table S1). How this ~ 50 -fold difference in eliciting exocytotic DA release might alter AMPH action, however, is not established. It is known that intense stimulation recruits vesicles from the reserve pool (Rizzoli and Betz 2005) and that AMPH differentially depletes different classes of DA vesicles (Anderson et al. 1998). Thus, the slice stimulation may be preferentially interrogating AMPH-sensitive DA pools.

AMPH competitively inhibits DA uptake

Consistent with classical uptake studies (Harris and Baldessarini 1973; Richelson and Pfenning 1984; Krueger 1990) and voltammetry in brain slices (Jones et al. 1998; Schmitz et al. 2001; John and Jones 2007), AMPH competitively inhibits DA uptake *in vivo* (Fig. 3C). Taken together with the analysis of exocytotic DA release (Fig. 3A), these results indicate that inhibition of neuronal DA uptake is the predominant mechanism underlying AMPH-induced increases in electrically evoked DA levels in the ventral striatum *in vivo*. The situation is more complex in the dorsal striatum, where both decreased exocytotic DA release and DA uptake inhibition contribute. Although reduced exocytotic DA release was modest, inhibited DA uptake was robust and significant at all AMPH doses, suggesting that the latter mechanism also predominates in the dorsal striatum.

One important caveat is that other AMPH effects besides competitive inhibition may cause an apparent change in K_m . Indeed, AMPH has been shown to reverse DAT action (Sulzer et al., 1995) and induce channel-like activity in DAT (Kahlig et al., 2005). Both of these phenomena could conceivably cause an apparent slowing of DA uptake. Because neither is accounted for by the kinetic model used in the present study to analyze evoked DA signals, we cannot rule out a contribution of non-exocytotic DA release to our measurements of DA uptake. However, it would also seem that all studies using simple Michaelis-Menten kinetics, as we do here, would suffer from the same criticism. Thus, we argue that there still is value comparing AMPH-induced changes K_m across studies, even though the exact mechanism by which this drug alters DA uptake is not fully established.

A related issue is that for responses recorded after administration of 10 mg/kg AMPH, curve fitting by permitting all three parameters, $[\text{DA}]_p$, V_{max} , and K_m to float independently was not possible. Instead, competitive inhibition was assumed by fixing V_{max} to predrug levels. We justify this manipulation on the following grounds. First, previous studies using voltammetry in brain slices did not demonstrate a change in V_{max} with AMPH (Jones et al. 1999; Schmitz et al. 2001). Second, competitive inhibition has similarly been assumed for analyzing AMPH and related drugs that elicit non-exocytotic DA release also using slice voltammetry (John and Jones 2007). And third, all values for K_m , whether determined independently or by fixing V_{max} , are linearly related to AMPH dose in both the dorsal and ventral striatum. This linear relationship is a hallmark of competitive inhibition. Although AMPH has been shown to alter V_{max} *in vitro* (Johnson et al. 2005) and *ex vivo* (Fleckenstein et al. 1999), further study is required to determine whether AMPH acts as a uncompetitive inhibitor *in vivo*.

Interestingly, we demonstrate that AMPH inhibition of DA uptake is ~ 2 -fold more potent in the dorsal compared to ventral striatum (Fig. 4). The regional potency of AMPH parallels another DA uptake inhibitor, nomifensine, which has been shown using voltammetry *in vivo* (Wu et al., 2001) and in brain slices (Jones et al., 1995) to increase K_m to a greater extent in the dorsal than ventral striatum, but not cocaine, which has similar effects. Different effects of cocaine and nomifensine on DA uptake have been suggested to be mediated by different binding sites on DAT (Meiergerd and Schenk, 1994). One likely explanation for the regional effects of DA uptake inhibitors is that DAT may differ in the dorsal and ventral striatum. For

example, DAT in the ventral striatum exhibits a higher molecular weight than in the dorsal striatum due to greater glycosylation (Lew et al., 1992). Another possibility is that with a higher $V_{\max}$ (Sup. Table S1; Wu et al., 2001), the reverse transport of DA, if present, would have a more prominent effect on slowing DA uptake in the dorsal compared to the ventral striatum.

By itself, more potent inhibition by AMPH leads to greater increases in evoked DA levels. However, because of decreased exocytotic DA release in the dorsal subregion, greater increases were observed in the ventral subregion (Fig. 1 and Fig. 2). This result is consistent with AMPH-induced increases in dialysate DA (Kuczenski and Segal, 1992), suggesting convergent measurements by these two different analytical techniques. AMPH also appears more efficacious than cocaine in inhibiting DA uptake (Fig. 4). While AMPH is a poorer binder to DAT relative to cocaine (Ritz et al. 1987), *in vitro* studies are equivocal with regards to which is a more potent inhibitor of DA uptake (Harris and Baldessarini 1973; Richelson and Pfenning 1984; Krueger 1990; John and Jones 2007). It is important to consider that, with systemic injections, pharmacokinetics and brain access also factor into potency.

It may be possible to quantitatively relate the K_i for AMPH inhibiting DA uptake determined in the present study *in vivo* to previously reported values obtained in brain slices by using comparable voltammetric measurements in the two preparations to equate doses. For example, Jones et al. (1999) demonstrated that 10 μM AMPH increased K_m from 0.2 μM to 8 μM , which is nearly identical to the 40-fold increase at 10 mg/kg from 0.2 μM to 7.98 μM that we report here. Also, there is a similar ~25-fold increase in K_m for DA uptake measured by Schmitz et al. (2001) from 0.89 to 24 μM . Assuming that a similar effect on K_m reflects similar drug concentrations acting on DAT, then the *in vivo* and *in vitro* AMPH doses are equivalent. This equivalence seems reasonable, because systemic AMPH doses in the range administered in the present study result in low micromolar drug levels measured in striatal extracellular fluid (Clausing et al. 1995; Kuczenski et al. 1997). Accordingly, the K_i of 3.123 $\mu\text{mol/kg}$ measured in the dorsal striatum *in vivo* thus converts to 0.425 μM , which is quite similar to values determined *in vitro* with voltammetry (0.42 μM , John and Jones 2007) and classical uptake measurements using ^3H -dopamine (0.22 μM , Krueger 1990); 0.28 μM , Lin and Uhl 2002).

AMPH-induced increases in basal DA levels

Consistent with previous work using FSCV at a CFM in the urethane-anesthetized rat (Wiedemann et al. 1990), AMPH-induced increases in basal DA levels were not detected in the striatum (Fig. 5 and Fig. 6). These results are clearly at variance with studies using slice voltammetry and microdialysis in awake animals. Slice studies report a ~4 μM increase in basal DA levels after 10 μM AMPH (Jones et al. 1998; Schmitz et al. 2001). Because identical FSCV parameters were used, discrepancies are most likely not due to differences in DA sensitivity. Indeed, the estimated detection limit for the present *in vivo* measurements (~150 nM) is more than sufficient to measure the large DA efflux recorded in the slice. Basal DA levels measured in the brain slice preparation appear to be a good index of AMPH-induced DA efflux, because, without input from cell bodies, action potential-dependent DA release would not occur at the excised dopaminergic terminals. Interestingly, with ongoing dopaminergic activity in the urethane-anesthetized rat (Tepper et al. 1995), one might expect a greater increase in basal DA levels *in vivo*, due to the combined actions of AMPH eliciting DA efflux and inhibiting DA uptake, which indirectly augments exocytotic DA release.

Our result of no increase in basal DA levels is also surprising given that the robust effects of AMPH on dialysate DA levels are well documented (Kuczenski et al. 1991; Kuczenski et al.

1997). However, quantitative comparisons between these results with microdialysis and the present *in vivo* voltammetry study are more challenging. The critical issue is that determination of an absolute basal concentration for DA is made difficult by interactions between the implanted probe and surrounding brain tissue (Bungay et al. 2003). If, according to the no-net-flux method, basal DA levels are ~5 nM (Justice, Jr. 1993), however, then the ~30-fold increase in dialysate DA recorded in awake animals with moderate to high AMPH doses (Kuczenski et al. 1991; Kuczenski et al. 1997) might fall below the present *in vivo* detection limit. This postulate can be tested with more sensitive FSCV (Heien et al. 2003). Anesthesia may also play a role, but AMPH robustly increases dialysate DA in the striatum under urethane as well (Yamamoto and Pehek 1990).

Conclusion

We show using voltammetry in the urethane-anesthetized rat that AMPH augments action potential-dependent presynaptic dopaminergic signaling. This activation occurred across a wide dose range that elicits behaviors spanning the psychostimulant spectrum from locomotion at the low end to stereotypy at the high end (Kelly et al. 1975; Gaytan et al. 1998). This result also starkly contrasts the near-complete disruption of normal patterns of action potential-dependent signaling observed with slice voltammetry and mediated by depletion of vesicular DA stores and robust DA efflux (Jones et al. 1998; Schmitz et al. 2001). Future work should be directed at determining the origin of the discrepant results obtained in the two preparations. Possible contributing factors are anesthesia, different responsiveness of intact versus severed neurons, and dissimilar stimulation characteristics.

Due to the combined actions of robust inhibition of neuronal DA uptake and minimal to modest reductions of exocytotic DA release, AMPH may thus function like other drugs of abuse and potentiate phasic DA signaling (Stuber et al. 2005; Cheer et al. 2007; Robinson et al. 2009). These transient elevations of brain extracellular DA are evoked by burst firing of dopaminergic neurons in response to primary rewards and cues predictive of these rewards (Carelli and Wightman 2004; Schultz 2007). A DA-mediated overlearning of cues predicting drug availability could thus be a general mechanism of abused drugs in the addiction process (Hyman 2005; Wanat et al. 2009).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

DA	dopamine
DAT	dopamine transporter
AMPH	amphetamine
FSCV	fast-scan cyclic voltammetry
CFM	carbon-fiber microelectrode
[DA]_p	concentration of dopamine released per stimulus pulse
[DA]_{max}	maximal evoked concentration of dopamine

Acknowledgments

This work was supported by the National Institute on Drug Abuse [DA 021770 and DA 024036] and ISU POENB.

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Figure 1. Effects of AMPH on evoked DA signals

AMPH alters the amplitude and dynamics of extracellular DA levels in the dorsal (A) and ventral (B) striatum measured by FSCV at a CFM and elicited by electrical stimulation of the MFB. The stimulus train (20 Hz, 2 s) was applied at 5 seconds, as indicated by the solid line underneath these representative responses. Recordings were collected 35 minutes after drug injection (i.p.). **Insets:** Background-subtracted cyclic voltammograms with voltage (E) as the abscissa.

Figure 2. Time course of the effects of AMPH on evoked DA levels
 AMPH elicits time- and dose-dependent effects on [DA]_{max} in the dorsal (A) and ventral (B) striatum. [DA]_{max} is the maximal concentration of DA evoked by MFB stimulation with a 20-Hz train. Data are expressed as a percent of the predrug value (% predrug).

Figure 3. Effects of AMPH on best-fit parameters for DA release and uptake

The 20- and 60-Hz responses represented in Figure 1 and Figure S1 were kinetically analyzed to determine parameters for exocytotic DA release ([DA]_p, top) and neuronal DA uptake (V_{max}, middle; K_m, bottom). These values, determined for both the dorsal (A) and ventral (B) striatum, were expressed as a percent of the predrug value (% predrug).

*significantly different than saline values ($p < 0.05$). +significantly different than 0.5 mg/kg ($p < 0.05$).

Figure 4. In vivo determination of a K_i for competitive inhibition of DA uptake by AMPH

The x axis describes AMPH dose converted from mg/kg to $\mu\text{mol/kg}$. The y axis is apparent K_m ($(K_m)_{\text{app}}$ or postdrug K_m) determined from the kinetic analysis shown in Figure 4. r is the coefficient for the linear regression. $(K_m)_{\text{app}}$ values for cocaine and nomifensine were taken from Wu et al. (2001a) after converting to $\mu\text{mol/kg}$ (original drug dose: 40 and 10 mg/kg i.p., respectively). Abbreviations: AMPH, amphetamine; NOM, nomifensine; COC, cocaine; DS, dorsal striatum; VS, ventral striatum.

Figure 5. Time course of the effects of AMPH on background voltammetric current
Background currents were measured during the pre-stimulation (i.e., baseline) portion of evoked recordings collected in the dorsal (**A**) and ventral (**B**) striatum and used to generate the time courses in Figure 2. Background Current is expressed as a percent of the predrug value (% predrug).

Figure 6. FSCV analysis of the change in background current elicited by AMPH

A. Data are all responses from the 10 mg/kg-AMPH group collected in the dorsal striatum and averaged in Figure 6. **B.** Data are the evoked responses designated with the closed circles and Roman Numerals in **A**. Absolute current (i.e., not background subtracted) is plotted (I). **C.** Data are background-cyclic voltammograms ("Subtracted CV") with current (I) as the ordinate, and voltage (E) as the abscissa. The pre-stimulation background current from time point I was subtracted from the corresponding region in time points II, III, and IV, and designated "II - I", "III - I", and "IV - I", respectively. **D.** Data are background-cyclic voltammograms ("Subtracted CV"). The "IV - I" voltammogram from **B** is replicated, along with two additional voltammograms. The first, "V - IV", was calculated by subtracting the pre-stimulation background current in IV from the peak of the evoked response also in IV, labeled in **B** as V. The second, "V - I", was calculated by subtracting the pre-stimulation background current in I from the peak of the evoked response, V, in recording IV.