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TITLE PAGE

Modafinil activates phasic dopamine signaling in dorsal and ventral striata

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MOD, modafinil; DA, dopamine; DAT, dopamine transporter; ADHD, attention deficit hyperactivity disorder; FSCV, fast-scan cyclic voltammetry; CFM, carbon-fiber microelectrode; PCR, principle component regression; AP, anteroposterior; ML, mediolateral; DV, dorsoventral; $[DA]_{max,}$ maximal concentration of dopamine evoked by electrical stimulation; k, first-order rate constant for dopamine uptake; $[DA]_{p,}$ concentration of dopamine release per stimulus pulse; MFB, medial forebrain bundle; L-DOPA, L-3, 4-dihydroxyphenylalanine

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ABSTRACT

Modafinil (MOD) exhibits therapeutic efficacy for treating sleep and psychiatric disorders, but its mechanism is incompletely understood. Compared to other psychostimulants inhibiting dopamine (DA) uptake, MOD weakly interacts with the dopamine transporter (DAT) and modestly elevates striatal dialysate DA, suggesting additional targets besides DAT. However, the ability of MOD to induce wakefulness is abolished with DAT knockout, conversely suggesting that DAT is necessary for MOD action. Another psychostimulant target, but one not established for MOD, is activation of phasic DA signaling. This communication mode, during which burst firing of DA neurons generates rapid changes in extracellular DA, the so-called DA transients, is critically implicated in reward learning. Here we investigate MOD effects on phasic DA signaling in the striatum of urethane-anesthetized rats with fast-scan cyclic voltammetry. We found that MOD (30-300 mg/kg i.p.) robustly increases the amplitude of electrically evoked phasic-like DA signals in a time- and dose-dependent fashion, with greater effects in dorsal versus ventral striata. MOD-induced enhancement of these electrically evoked amplitudes was mediated preferentially by increased DA release compared to decreased DA uptake. Principal component regression of non-electrically evoked recordings revealed negligible changes in basal DA with high-dose MOD (300 mg/kg i.p.). Lastly, in the presence of the D2 DA antagonist, raclopride, low-dose MOD (30 mg/kg i.p.) robustly elicited DA transients in dorsal and ventral striata. Taken together, these results suggest that activation of phasic DA signaling is an important mechanism underlying the clinical efficacy of MOD.

INTRODUCTION

Modafinil (MOD; Provigil®) exhibits therapeutic efficacy for treating a variety of neuropathologies, including sleep-related disorders such as narcolepsy (Wise, et al., 2007), obstructive sleep apnea syndrome (Pack, et al., 2001), and shift-work sleep disorder (Czeisler, et al., 2005), attention deficit hyperactivity disorder (ADHD) (Swanson, et al., 2006), and drug addiction (Anderson, et al., 2009; Shearer, et al., 2009; but see Anderson, et al., 2012). Similar to other psychostimulants used therapeutically, such as amphetamine (Adderall®) and methylphenidate (Ritalin®), MOD enhances locomotor activity (Kuczenski, et al., 1991; Edgar and Seidel, 1997; Kuczenski and Segal, 2001), wakefulness (Wisor, et al., 2001; Ishizuka, et al., 2008), and cognitive ability (Barch and Carter, 2005; Kumar, 2008; Repantis, et al., 2010). Indeed, a recent meta-analysis study has concluded that MOD can be safely used as a cognitive enhancer in healthy subjects (Battleday and Brem, 2015). Moreover, unlike other therapeutic psychostimulants, MOD exhibits limited potential for abuse (Deroche-Gamonet, et al., 2002). These attractive psychostimulant characteristics have thus generated considerable interest in establishing the neuropharmacologic mechanism of MOD action.

Although MOD has been found to alter various neurotransmitter systems in the brain, including those for histamine, hypocretin (orexin), GABA, glutamate, norepinephrine, and serotonin, its effects on midbrain dopamine (DA) systems have received the greatest attention (Tanganelli, et al., 1992; Ferraro, et al., 1997; Chemelli, et al., 1999; de Saint, et al., 2001; Ishizuka, et al., 2003). This atypical psychostimulant preferentially interacts with the dopamine transporter (DAT), compared to transporters for other monoamines such as norepinephrine and serotonin, and shows little affinity for receptors of monoamines and other neurotransmitters (Mignot, et al., 1994; Madras, et al., 2006; Zolkowska, et al., 2009). However, whether MOD

acts directly through DAT remains highly controversial. On the one hand, MOD exhibits weak affinity for DAT (Mignot, et al., 1994; Madras, et al., 2006; Zolkowska, et al., 2009) and elicits only relatively modest increases in striatal dialysate DA (Ferraro, et al., 1997; Loland, et al., 2012). On the other hand, MOD's effects appear to rely on DAT, as MOD-induced wakefulness is abolished in DAT-knockout mice (Wisor, et al., 2001).

Based on work investigating actions of other DAT-inhibiting psychostimulants, another potential target for MOD is phasic DA signaling. DA neurons signal in two distinct modes, with slow, irregular firing of DA neurons generating a basal level of extracellular DA called tone during tonic DA signaling and burst firing of DA neurons generating rapid increases in extracellular DA called transients during phasic DA signaling (Grace and Bunney, 1984). Substantive evidence implicates a critical role played by phasic DA signaling in reward learning (Schultz, et al., 1997; Day, et al., 2007) and seeking (Phillips, et al., 2003), and alterations in phasic DA signaling are hypothesized to contribute to ADHD (Tripp and Wickens, 2008) and drug abuse (Covey, et al., 2014). DAT-inhibiting psychostimulants have also been shown to activate phasic DA signaling by increasing burst firing of DA neurons (Shi, et al., 2000; Shi, et al., 2004; Koulchitsky, et al., 2012) and the frequency of DA transients in the striatum (Venton and Wightman, 2007; Covey, et al., 2013; Daberkow, et al., 2013), and by presynaptically enhancing DA release, in addition to inhibiting DA uptake (Wu, et al., 2001a; Venton, et al., 2006; Chadchankar, et al., 2012). Whether MOD acts similarly to activate phasic DA signaling has not been examined.

Here we use fast-scan cyclic voltammetry (FSCV) at a carbon-fiber microelectrode (CFM) to investigate the effects of MOD on phasic DA signaling in urethane-anesthetized rats. The effects of MOD were examined in dorsal and ventral striata across a wide behaviorally

relevant range of doses (30 - 300 mg/kg i.p.), based on effects on cognitive function, locomotion, and wakefulness (Edgar and Seidel, 1997; Beracochea, et al., 2001; Ward, et al., 2004). Two measures of phasic DA signaling were assessed: the amplitude of electrically evoked phasic-like DA signals (Avelar, et al., 2013) and the frequency of DA transients elicited in the presence of the D2 DA antagonist, raclopride, (Venton and Wightman, 2007). DA transients were determined from non-electrically evoked DA traces processed by principal component regression (PCR)(Keithley, et al., 2009). In addition, the effects of MOD on the presynaptic mechanisms of DA release and uptake (Wu, et al., 2001b) and on basal DA levels processed by PCR were examined. Taken together, our results suggest that activation of phasic DA signaling is a novel mechanism contributing to the therapeutic efficacy of MOD.

METHODS

Animals. Male Sprague-Dawley rats (300-400 g) were purchased from Harlan (Indianapolis, IN, USA) and housed in a temperature-controlled vivarium on a diurnal light cycle (12h light/dark) with food and water provided *ad libitum*. Animal care conformed to the NIH *Guide for the Care and Use of Laboratory Animals*, and experimental procedures were approved by the Institutional Animal Use and Care Committee at Illinois State University.

Surgery. Rats were anesthetized with urethane (1.6 g/kg, i.p.) and immobilized in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Holes for reference, stimulating, and two CFMs were drilled. All coordinates, anteroposterior (AP), mediolateral (ML) and dorsoventral (DV), are given in mm and are referenced to bregma (Paxinos and Watson, 1986). The stimulating electrode targeted the medial forebrain bundle (MFB; -4.6 AP, +1.3 ML, -7.5 DV). CFMs targeted ipsilateral dorsal (+1.2 AP, +3.0 ML, -4.5 DV) and ventral (+1.2 AP, +1.5 ML, -6.5 DV) striata. The Ag/AgCl reference electrode was placed in contralateral cortex. Final coordinates for CFMs and stimulating electrode were based on optimizing the electrically evoked DA signal and were not changed for the duration of the experiment.

Experimental design. DA was recorded in sequential 5-minute epochs and for most recordings, FSCV was performed simultaneously at separate CFMs implanted in dorsal and ventral striata of a single animal. In the first experimental design, four pre-drug and 30 post-drug epochs were collected, and electrical stimulation of the MFB was applied 5 seconds into each epoch. The effects of (2-hydroxypropyl)-β-cyclodextrin (vehicle) or MOD (30, 60, 100 or 300 mg/kg) were examined on electrically evoked phasic-like DA signals for the entire duration of DA measurements (15 min pre-drug and 160 min post-drug). The effects of vehicle or MOD across the same dose range as above on DA release and uptake were examined pre-drug and at 30 and

60 min post-drug. The effects of vehicle or high-dose MOD (300 mg/kg) on changes in basal DA were examined pre-drug and for 40 min post-drug. In the second experimental design, electrical stimulation was applied pre-drug and every 30 min post-drug to assess the veracity of the CFM. After pre-drug recordings, raclopride (2 mg/kg) was co-administered with low-dose MOD (30 mg/kg), and DA transients were analyzed during 5-min epochs pre-drug and post-drug at 15, 30, 60 and 120 min. All vehicle and drugs were administered intraperitoneally (i.p.) in a total volume of 2 ml. n = 4-7 each in the dorsal and ventral striatum.

Electrochemistry. DA measurements were recorded with FSCV by applying a triangular waveform (-0.4 to +1.3V and back) to the CFM at a rate of 400V/s every 100 ms. CFMs were fabricated by aspirating a single carbon fiber (r=3.55 µm; HexTow AS4, HexCel Corp., Stamford, CT, USA) into a borosilicate capillary tube (1.2mm o.d.; Sutter Instrument, Novato, CA, USA) and pulling to a taper using a micropipette puller (Narishige, Tokyo, Japan). The carbon fiber was then cut to ~100 µm distal to the glass seal. FSCV was performed by a Universal Electrochemistry Instrument (UEI; Department of Chemistry Electronic Shop, University of North Carolina, Chapel Hill, NC, USA) and commercially available software (ESA Bioscience, Chelmsford, MA, USA). Current recorded at peak oxidative potential for DA (~+0.6 V) was converted to DA concentration based on post-calibration of the CFM using flow-injection analysis in a modified TRIS buffer (Kume-Kick and Rice, 1998; Wu, et al., 2001b). DA was identified by the background subtracted voltammogram (Michael, et al., 1998; Heien, et al., 2004). In experiments assessing effects of MOD on basal DA and DA transients, DA was additionally identified using PCR (see below).

Electrical stimulation. Electrical stimulation was computer generated and passed through an optical isolator and constant-current generator (Neurolog NL800; Digitimer Limited, Letchworth

Garden City, UK). Biphasic stimulation pulses were applied to a twisted bipolar electrode (Plastics One, Roanoke, VA, USA), with tips separated ~1 mm. Stimulus parameters were an intensity of ±300 μA and a duration of the biphasic pulse of 4 ms (2 ms each phase), with trains applied at a frequency of 60 Hz for 0.4 s (i.e., total of 24 pulses).

Analysis of DA release and uptake. Electrically evoked phasic-like DA signals were analyzed to determine maximal amplitude ($[DA]_{max}$) and parameters for presynaptic DA release and uptake according to (Wightman, et al., 1988; Wu, et al., 2001b):

$$[1] \qquad d[DA]/dt = [DA]_p * f - k[DA]$$

where $[DA]_p$ is the concentration of DA elicited per stimulus pulse and is used to index DA release, k is the first-order rate constant describing DA uptake, and *f* is the frequency of electrical stimulation. $[DA]_p$ and k were determined by fitting electrically evoked DA signals to Equation 1 using nonlinear regression with a simplex-minimization algorithm (Wu, et al., 2001b). Temporal distortion in measured DA responses was accounted for using a diffusion gap model, with the gap width held constant for each CFM across pre- and post-drug measurements (Wu, et al., 2001b).

Analysis of basal DA and DA transients. Changes in DA during non-electrically evoked recording were assessed using PCR to resolve DA, pH, and background drift from raw FSCV recordings (Hermans, et al., 2008; Keithley, et al., 2009). In select files, PCR additionally resolved a repetitive background noise component. PCR analysis was accepted if any current in the recordings not accounted for by the retained principal components of the training sets, or residual (Q), was less than the 95% confidence threshold (Q_α). Epochs where Q exceeded Q_α were not used for analysis. Changes in basal (i.e., non-electrically evoked) DA (Δ[DA]) per 5 min-epoch were determined by averaging all data points post-stimulation of PCR-resolved

traces. Δ[DA] is independently presented for each five-minute epoch, not as a contiguous concatenation, to avoid resetting Q with a new background subtraction at the start of each epoch. DA transients were identified in PCR-resolved traces as peaks greater than 5-times the root-mean-square-noise using peak-finding software (MINI ANALYSIS; Synaptosoft, Decatur, GA, USA).

Statistical analysis. Where appropriate, data are expressed as the mean±SEM. Unless noted below, statistical analyses were performed with SAS/STAT software version 9.3 (SAS Institute Inc. 2011). Time courses for $[DA]_{max}$, $[DA]_p$, and k were analyzed using a three-way ANOVA with repeated measures with time, drug dose, and striatal region as factors. Path analysis (Mitchell 2001) was conducted to assess the dose-dependent direct effects of MOD on $[DA]_p$ and k, and the indirect effects of MOD on $[DA]_{max}$ via $[DA]_p$ and k. Alternative, reduced models were compared to the full model using Akaike Information Criteria (AIC)(Anderson, 2008). A two-way ANOVA with repeated measures assessed differences in DA-transient frequency with time and dose as factors. Correlations were performed with Sigma Plot 12.0 (Systat Software Inc., San Jose, CA, USA). Significance was set at $p < 0.05$.

Drugs. Urethane, (2-hydroxypropyl)-β-cyclodextrin and raclopride were purchased from Sigma (St. Louis, MO, USA). MOD was provided by Research Triangle Institute-National Institute on Drug Abuse, Raleigh, NC. Urethane and raclopride were dissolved in 150 mM NaCl prior to injection. MOD was dissolved in a mixture of 50% (2-hydroxypropyl)-β-cyclodextrin and nanopure w/v.

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RESULTS

MOD robustly increases the amplitude of electrically evoked phasic-like DA signals

Figure 1 shows representative effects of MOD on electrically evoked phasic-like DA signals collected in dorsal (Fig. 1A) and ventral (Fig. 1B) striata. Recordings in the top of each panel show electrical stimulation of MFB increasing DA as measured by FSCV at a CFM. Pseudo-color plots below, which serially display all voltammograms collected during the recording, and individual voltammograms (inset) identify DA as the primary analyte recorded. MOD (100 mg/kg) increased $[DA]_{max}$ in both dorsal and ventral striata 60 min post-drug administration.

Figure 2 shows averaged time courses of MOD effects on $[DA]_{max}$ expressed as a percent change from pre-drug in dorsal (Fig. 2A) and ventral (Fig. 2B) striata. All four doses of MOD, 30, 60, 100, 300 mg/kg, appeared to elevate $[DA]_{max}$ compared to vehicle control for more than 2.5 h post-drug administration. Increases elicited by the highest MOD dose tested (300 mg/kg) were particularly robust, at approximately 3-fold pre-drug levels. Consistent with representative recordings shown in Figure 1, MOD appeared to elevate $[DA]_{max}$ to a greater extent in the dorsal than in the ventral striatum. A three-way repeated measures ANOVA revealed significant effects of time ($F_{33,1419} = 30.83$, $p < 0.0001$), dose ($F_{4,43} = 15.47$, $p < 0.0001$), and region ($F_{1,43} = 5.85$, $p = 0.0198$). There were also significant time-by-dose ($F_{132,1419} = 9.04$, $p < 0.0001$) and time-by-region ($F_{132,1419} = 4.17$, $p = 0.0207$) interactions, but no time-by-dose-by-region ($F_{132,1419} = 0.68$, $p = 0.7031$) and region-by-dose ($F_{4, 43} = 0.70$, $p = 0.5970$) interactions. Thus, MOD increased $[DA]_{max}$ in a time- and dose-dependent manner, with a greater relative effect in the dorsal striatum, different time courses in the two striatal regions, and different time courses for drug doses.

MOD increases DA release and decreases DA uptake

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MOD-induced increases in $[DA]_{max}$ could be mediated by enhanced DA release and/or inhibited DA uptake. To initially assess whether MOD decreased DA uptake, electrically-evoked decay curves were overlaid beginning at the same concentration (Fig. 3, inset), and the slopes were visually inspected between pre- and post-drug traces. The downward slope of the evoked trace is thought to reflect DA uptake and not DA release (Wu, et al., 2001b). Thus, the flatter post-drug traces after MOD indicate slower DA extracellular clearance (Fig. 3). This qualitative approach suggests that MOD decreases DA uptake and that the increase in $[DA]_{max}$ may be due to DA uptake inhibition, at least in part. However, MOD-induced increases in DA release may also play a role, because the upward slope of the evoked trace reflects the balance of both DA release and uptake (Wu, et al., 2001b).

To quantitatively resolve MOD's effect on DA release and uptake on $[DA]_{max}$, electrically evoked responses were fit to Equation 1. Figure 4 compares the effects of MOD on $[DA]_{max}$ (left), DA release as indexed by $[DA]_p$ (middle), and DA uptake as indexed by k (right) for dorsal (Fig. 4A) and ventral (Fig. 4B) striata. Three time points were assessed: pre-drug, 30-min and 60-min post drug to canvass the initial MOD-induced increase in $[DA]_{max}$. Three-way repeated measures ANOVA was used for statistical analysis of each parameter. Statistical analysis of $[DA]_{max}$ revealed significant effects of time ($F_{2,88} = 43.27$, $p < 0.0001$) and dose ($F_{4,44} = 13.14$, $p = 0.0151$), and significant time-by-dose ($F_{8,88} = 11.64$, $p < 0.0001$) and time-by-region ($F_{8,88} = 3.59$, $p = 0.0316$) interactions, but no significant time-by-dose-by-region ($F_{8,88} = 0.50$, $p = 0.8555$) and region-by-dose ($F_{4,44} = .041$, $p = 0.7981$) interactions. However, there was a trend for an effect of region ($F_{1,44} = 3.24$, $p = 0.0782$). Compared to the complete time course for $[DA]_{max}$ in Figure 2, which found a significant effect of region, the strong, but non-significant, trend for a region effect of MOD on $[DA]_{max}$ in Figure 4 could be attributed to the reduced

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number of time points examined at maximal drug effect (> 60 min). Analysis of $[DA]_p$ revealed significant effects of time ($F_{2,88} = 30.65$, $p < 0.0001$), dose ($F_{4,44} = 9.96$, $p < 0.0001$), and region ($F_{1,44} = 4.32$, $p = 0.0436$) and significant time-by-dose ($F_{8,88} = 8.56$, $p < 0.0001$) and time-by-region ($F_{8,88} = 4.32$, $p = 0.0162$) interactions, but no significant time-by-dose-by-region ($F_{8,88} = 0.80$, $p = 0.6082$) and region-by-dose ($F_{4,88} = 0.71$, $p = 0.5925$) interactions. Finally, analysis of k revealed significant effects of time ($F_{2,88} = 132.04$, $p < 0.0001$) and dose ($F_{1,44} = 12.50$, $p < 0.0001$), as well as a significant time-by-dose interaction ($F_{8,88} = 8.95$, $p < 0.0001$), but no significant time-by-dose-by-region ($F_{8,88} = 0.81$, $p = 0.5890$), time-by-region ($F_{2,88} = 2.90$, $p = 0.0603$), and region-by-dose ($F_{4,88} = 0.47$, $p = 0.7588$) interactions. However, there was a trend for an effect of region ($F_{1,44} = 3.69$, $p = 0.0611$). Taken together, these results demonstrate that MOD increases DA release and decreases DA uptake in a time- and dose-dependent fashion and preferentially increases DA release in the dorsal striatum; MOD may additionally inhibit DA uptake preferentially in the ventral striatum.

In theory, both an increase in DA release and a decrease in DA uptake could mediate an increase in $[DA]_{max}$ (Wu, et al., 2001b). We therefore used path analysis to directly evaluate the respective contribution of these two presynaptic mechanisms to the dose-dependent effects of MOD on $[DA]_{max}$. Path analysis (Mitchell RJ, 1998) is a statistical technique that tests effects of multiple independent variables on a dependent variable, much like multiple regression; however, path analysis allows for the possibility that variables can be both dependent and independent (i.e., variables can be both affected by MOD and affect other variables; Fig. 5). The output of path analysis, path coefficients, are standardized regression coefficients that indicate the strength (i.e., maximum of 1) and direction (i.e., positive or negative) of the causal relationships between the variables. To increase statistical power, data in dorsal and ventral striata were combined. This

was justified, because both regions show a similar direction for the effects of MOD on the parameters analyzed in path analysis: increased DA release, decreased DA uptake, and increased $[DA]_{max}$.

Figure 5 shows the full path analysis model, with arrows demarcating direct relationships between variables and the path coefficient given above each arrow, for 60-min data. Path analysis of this complete model suggests that MOD exerts almost equal, but opposite, direct effects on DA release (+0.6504; $p < 0.0001$) and uptake (-0.6710; $p < 0.0001$). Based on 95% confidence intervals, the effects of MOD on DA release (95% confidence interval, 0.49-0.81) and DA uptake (95% confidence interval, 0.52-0.82) were not significantly different; thus, MOD increases DA release and decreases DA uptake to a similar magnitude. However, DA release exerted a greater direct effect on $[DA]_{max}$ as compared to DA uptake, +0.8563 ($p < 0.0001$) and -0.1623 ($p = 0.003$), respectively, and the effect of DA release (95% confidence interval, 0.78-0.93) on $[DA]_{max}$ was significantly greater than that of DA uptake (95% confidence interval, 0.05-0.27). The relative contribution of the two paths through DA release or uptake to the $[DA]_{max}$ increase are the indirect effects of MOD on $[DA]_{max}$. These can be estimated by the products of the direct path coefficients on each path (Fig. 5). For DA release this product is 0.5570, whereas for DA uptake this product is 0.1089. Thus, the indirect effect via DA release is more than 5 times greater than that via DA uptake, indicating that MOD-induced increases in $[DA]_{max}$ are primarily mediated via increased DA release.

Alternative path models (i.e., omitting either effects of DA release or uptake on $[DA]_{max}$ from the full model) were conducted, and AIC values, an indicator of information lost by using models to describe data (Anderson, 2008), were compared to determine which model was most appropriate. Omitting effects of DA release or uptake resulted in increased AIC (109.5 and 23.4

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respectively), as compared to the full model (AIC = 16.4; Fig. 5), which included both parameters, suggesting that both DA release and uptake together best explain MOD effects on $[DA]_{max}$. Additionally, the larger AIC calculated after omission of DA release, as compared to DA uptake, suggests that the model omitting the DA release effect is a poorer description of the data, which is consistent with the analysis of path coefficients derived from the full model and additionally indicated that MOD primarily increases DA release to increase $[DA]_{max}$.

MOD and basal DA

MOD effects on basal DA were assessed by applying a chemometrics analysis called PCR (Hermans, et al., 2008; Keithley, et al., 2009) to the non-electrically evoked portion of the raw FSCV recording. Figure 6A shows representative FSCV and PCR recordings for pre-drug and 60 min post-300 mg/kg MOD, the highest dose tested. The raw FSCV recording (top; black trace) shows a steady increase in current for both pre- and post-drug conditions (left and right, respectively). However, the current cannot be attributed solely to DA, as the color plot below shows additional electrochemical changes not attributed to DA. Furthermore, individual voltammograms (inset) contain other analytes (blue) that would mask changes in DA (black) if present. PCR resolves DA from these interferents, and the representative traces resolved by PCR (red) suggest negligible changes in basal DA with MOD.

To assess MOD-induced changes in basal DA within individual 5 min-epochs (Δ[DA]), all data in PCR-resolved DA traces after the electrically evoked response returned to baseline were averaged pre-drug and for the first 40 min of drug response. This time period was selected to examine the initial effects of MOD on basal DA corresponding to the initial robust increase in the amplitude of electrical evoked phasic-like DA signals (Fig. 2). Time 0 min was excluded because of noise introduced during drug administration. MOD exerted negligible effects on

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Δ[DA] (Fig. 6B) in either dorsal (top) or ventral (bottom) striata. The three-way repeated measures ANOVA yielded no significant effect of time ($F_{10,90}$ = 1.80, p = 0.1764), dose ($F_{1,9}$ = 0.11, p = 0.7433), and region ($F_{1,9}$ = 0.47, p = 0.5118) and no significant time-by-dose-region ($F_{10,90}$ = 1.57, p = 0.1287), region-by-dose ($F_{1,9}$ = 0.24, p = 0.6384), time-by-dose ($F_{10,90}$ = 0.52, p = 0.8748), and time-by-region ($F_{10,90}$ = 0.56, p = 0.8392) interactions, indicating that there were no significant effects of MOD on basal DA.

The lack of significant effect of MOD on basal DA assessed by PCR opposes previous findings demonstrating increases in dialysate DA at the same dose (Ferraro, et al., 1997; Loland, et al., 2012). To address the concern that PCR assigned a portion of the DA signal to a non-DA principal component and that this misplaced DA led to the inability to detect an increase in basal DA with MOD, a linear regression was performed between $[DA]_{max}$ of the electrically evoked response determined from raw FSCV recordings ($[DA]_{FSCV}$) and from PCR-resolved data ($[DA]_{PCR}$). The current attributed to the electrically-evoked response measured by FSCV over short time scales (i.e., a few seconds) in the anesthetized animal has previously been determined to be primarily due to DA (Wightman, et al., 1986). There was a tight association of data to the trend line, as indicated by the significant correlation between $[DA]_{FSCV}$ and $[DA]_{PCR}$, in both dorsal (Fig. 6C left; r = 0.8901, p < 0.0001) and ventral (Fig. 6C right; r = 0.9639, p < 0.0001). Slopes of the trend line were also significantly different from zero in both dorsal (Fig. 6C left; b = 1.1012, t = 30.9139, p < 0.0001) and ventral (Fig. 6C right; b = 0.9890, t = 52.9325, p < 0.0001) striata, indicating significant relationships between $[DA]_{FSCV}$ and $[DA]_{PCR}$. Thus, this evidence suggests that PCR accurately resolves DA from the mixed analyte signal recorded by FSCV.

MOD activates DA transients

Co-administration of a DA D2 receptor antagonist with a DAT-inhibiting psychostimulant elicits DA transients in urethane-anesthetized rats, without affecting these phasic signals when administered alone (Venton and Wightman, 2007; Park, et al., 2010). Presumably, the DA D2 receptor antagonist in the anesthetized preparation prevents the psychostimulant-induced autoinhibition of DA neurons but reveals the psychostimulant-induced activation of burst firing by DA neurons (Shi, et al., 2000; Shi, et al., 2004). In contrast, psychostimulant-induced activation of DA cell burst firing (Koulchitsky, et al., 2012) and DA transients (Stuber, et al., 2005; Aragona, et al., 2008; Daberkow, et al., 2013) in awake animals does not require administration of a DA D2 receptor antagonist. Similar to other psychostimulants, co-administration of raclopride (2 mg/kg), a DA D2 receptor antagonist, with MOD (30 mg/kg) elicited DA transients in both dorsal (Fig. 7A left) and ventral (Fig. 7A right) striata. Asterisks demarcate transients on the FSCV current trace taken at the peak DA oxidative potential. Transients were confirmed to be DA by the electrochemical profile in the pseudo-color plot and comparison of the individual transient voltammogram (inset, red) to the electrically evoked DA voltammogram (inset, black). Prior to assessing transient frequency at select time points, DA in the raw FSCV traces was resolved with PCR. As shown in Figure 7B, while no transients were recorded pre-drug, there was a robust increase in transient frequency 15 min post-drug administration and thereafter. A two-way repeated measures ANOVA revealed a significant effect of time on transient frequency ($F_{3,27} = 11.85$, $p < 0.0001$). However, there was neither a significant effect of region ($F_{1,9} = 0.18$, $p = 0.6835$) nor a significant time-by-region interaction ($F_{3,27} = 1.72$, $p = 0.1932$).

DISCUSSION

Here we demonstrate that MOD activates phasic DA signaling in dorsal and ventral striata. Activation was indicated by increased amplitude of electrically evoked phasic-like DA signals, enhanced DA release, inhibited DA uptake, and increased frequency of DA transients. Taken together, these results suggest that activation of phasic DA signaling is a novel mechanism contributing to the therapeutic efficacy of MOD.

MOD and basal DA

PCR was used to investigate the effects of MOD on basal DA. This approach revealed no significant changes in basal DA in either dorsal or ventral striata with the highest dose of MOD tested (300 mg/kg). In contrast, an ≈3-fold elevation in striatal dialysate DA has been reported for the same dose (Ferraro, et al., 1997; Loland, et al., 2012). The determination of basal DA is analytically difficult (Sandberg and Garris, 2010), and this discrepancy could be attributed to differences in the two monitoring techniques, which are not fully understood. The use of FSCV coupled to PCR for monitoring basal DA is also an emerging approach. While we demonstrated that PCR was not incorrectly assigning DA to a non-DA principal component in electrically evoked DA signals, the DA concentrations analyzed were much greater than the non-significant changes detected in basal DA and recent estimates of basal DA of ≈100 nM (Atcherley, et al., 2015). However, PCR has previously detected both increases and decreases in DA levels within these non-significant concentration changes and well below 100 nM (≈5 to 40 nM; Hart, et al., 2014; Roitman et al., 2008). Thus, although PCR appears to have the requisite sensitivity to detect a change in basal DA, a definitive determination of whether MOD acts on basal DA requires further study.

MOD activates phasic DA signaling via effects at DA terminals

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Consistent with other DAT-inhibiting psychostimulants, such as cocaine, amphetamine, and methylphenidate (Venton, et al., 2006; Ramsson, et al., 2011b; Chadchankar, et al., 2012; Covey, et al., 2013; Avelar, et al., 2013; Daberkow, et al., 2013), we show that MOD increases DA release and inhibits DA uptake. Thus, our results support the notion that DAT-inhibiting psychostimulants share a common action of altering both presynaptic mechanisms (Covey, et al., 2014). How MOD increases DA release is not known. Cocaine and methylphenidate increase DA release via actions on synaptic proteins such as synapsin and α-synuclein, respectively (Chadchankar, et al., 2012; Venton, et al., 2006), whereas amphetamine increases DA release by inhibiting DA degradation and increasing DA synthesis (Avelar, et al., 2013). Further work is needed to determine whether MOD increases DA release by these or other mechanisms.

Because the upward slope of the electrically evoked DA signal reflects the balance between DA release and uptake (Wightman, et al., 1988), both presynaptic mechanisms could mediate the observed MOD-induced increases in $[DA]_{max}$. While indirect evidence suggests that enhanced DA release, as compared to inhibited DA uptake, is more responsible for increases in $[DA]_{max}$ elicited by other psychostimulants (Venton, et al., 2006; Avelar, et al., 2013; Covey, et al., 2013; Daberkow, et al., 2013), this hypothesis has never been directly tested as we do here. Indeed, path analysis indicated that MOD-induced increases in $[DA]_{max}$ are more strongly mediated by enhanced DA release. The relative contributions of DA release and uptake to $[DA]_{max}$ may inform alterations of DA transients by DAT-inhibiting psychostimulants. For example, while it is thought that increased burst firing of DA neurons drives increased transient frequency and inhibited DA uptake drives increased transient duration, the mechanism underlying increased transient amplitude is debated (Covey, et al., 2014). Our results suggest that enhanced DA release, not inhibited DA uptake, is primarily responsible for the increased

transient amplitude with DAT-inhibiting psychostimulants. However, caution is urged because this conclusion assumes that the parameters for DA release and uptake obtained from electrically evoked phasic-liked DA signals relate to DA transients, and this assumption has been difficult to test.

MOD activates DA transients

We investigated the ability of MOD to elicit DA transients in urethane-anesthetized rats when co-administered with the DA D2 antagonist, raclopride. Our findings show that MOD, a low-affinity DAT inhibitor, elicited DA transients in both dorsal and ventral striata at the lowest dose tested (30 mg/kg) when co-administered with raclopride. While MOD increasing the frequency of DA transients is consistent with eliciting burst firing of DA neurons (Covey, et al., 2014), the combination of MOD and raclopride could additionally have increased the amplitude of spontaneously occurring (i.e., ongoing) transients above the FSCV detection limit, which may also have contributed to the observed frequency increase. Interestingly, the frequency of DA transients elicited by MOD was similar to that elicited by a high-affinity DAT inhibitor, nomifensine, under similar conditions (Venton and Wightman, 2007), suggesting that the MOD-induced activation is robust. Unfortunately, quantitatively comparing this MOD effect to the psychostimulant-induced activation of DA transients observed in awake rats (Venton and Wightman, 2007; Covey, et al., 2013; Daberkow, et al., 2013) is tenuous, because the use of ralcopride in the present experiment, particularly its blockade of somatodendritic DA autoreceptors, is confounding in isolating the specific effects of MOD. Another potential concern in interpreting the observed MOD-induced activation of DA transients is the profound effects of anesthetics on DA neuron firing (Chiodo, 1988; Kelland, et al., 1990). Thus, there is a great need

to establish the MOD-induced activation of DA transients in awake animals and in the absence of raclopride.

Addictive nature of psychostimulants

A long-held view in addiction research is that, despite diverse cellular actions, all abused drugs increase brain extracellular DA, with a preferential action in ventral compared to dorsal striata (Di Chiara and Imperato, 1988). More recent work has refined this view by hypothesizing that abused drugs excessively activate phasic DA signaling (Covey, et al., 2014), leading to the hijacking of reward circuits and aberrant reward learning (Hyman, et al., 2006). While cocaine and amphetamine conform to this hypothesis (Venton and Wightman, 2007; Covey, et al., 2013; Daberkow, et al., 2013), other mechanisms have been proposed to explain differences in abuse potential for DAT-inhibiting psychostimulants, including DAT affinity (Ritz, et al., 1987), speed of brain drug action (Yorgason, et al., 2011), and actions via DAT mimicking G protein-coupled receptors, i.e., via the so-called "transceptor" (Schmitt, et al., 2013). Because MOD increases electrically evoked DA levels in the dorsal striatum to a greater extent than in the ventral striatum, it is interesting to speculate that MOD targeting DA signaling in the dorsal striatum contributes to its limited abuse potential (Deroche-Gamonet, et al., 2002).

The basis for differential effects of DAT-inhibiting psychostimulants in striatal sub-regions is not known. Heterogeneity of DA neurons innervating the dorsal and ventral striatum (Doucet, et al., 1986; Marshall, et al., 1990; Lammel, et al., 2008) favors a similar sub-regional specificity in drug effects, which is not the case. Thus, other factors must be involved. DAT is a potential mediator, and different classes of DAT-inhibitors bind to different sites on DAT (Loland, et al., 2012; Schmitt, et al., 2013). Not unexpectedly, DAT-inhibiting psychostimulants differentially inhibit DA uptake in the striatal sub-regions, but these effects appear unrelated to

abuse potential (present study; Jones, et al., 1995; Wu, et al., 2001a; Ramsson, et al., 2011a). DA release is another potential mediator, but much less is known about the effects of psychostimulants on this presynaptic mechanism. Clearly, more work is needed to identify the cellular mechanisms distinguishing the differential effects of DAT-inhibiting psychostimulants in the striatum and whether this differential activation involves DA transients.

Clinical efficacy of MOD

It is interesting to speculate that activation of phasic DA signaling as demonstrated herein contributes to the clinical efficacy of MOD. For example, therapeutic for ADHD (Swanson, et al., 2006), MOD may be targeting the insufficient phasic DA signaling proposed to underlie deficits in reward learning observed with this neurodevelopmental pathology (Tripp and Wickens, 2008). A similar activation of phasic DA signaling, albeit from normal levels, may mediate MOD-enhanced cognitive ability in healthy subjects (Muller, et al., 2013). Moreover, L-DOPA has been shown to restore the amplitude of DA transients reduced by long-access cocaine self-administration (Willuhn, et al., 2014), and MOD may be acting similarly in psychostimulant abusers (Anderson, et al., 2009; Shearer, et al., 2009). Finally, while roles for serotonin, norepinephrine, and acetylcholine are well established in sleep-wakefulness (Pace-Schott and Hobson, 2002), more recent evidence implicates DA (Wisor, et al., 2001; Dahan, et al., 2007) and perhaps phasic DA signaling (Dahan, et al., 2007). Consistent with activation of phasic DA signaling as reported herein, MOD-induced wakefulness is dependent on DA receptors (Qu, et al., 2008) and an intact striatum (Qiu, et al., 2012).

Conclusions

We found that MOD increases the frequency of DA transients, enhances DA release, and inhibits DA uptake in dorsal and ventral striata. Based on these measurements, we propose a mechanism

for MOD of activating phasic DA signaling, whereby burst firing of DA neurons and the duration, amplitude, and frequency of DA transients are increased. Further investigation is required to identify the role of these actions in the therapeutic efficacy of MOD.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Garris

Conducted experiments: Bobak, Weber, Doellman

Performed data analysis: Bobak, Weber, Juliano, Schuweiler, Athens, Garris

Wrote or contributed to the writing of the manuscript: Bobak, Weber, Garris, Schuweiler, Juliano

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FOOTNOTES

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M. J. B. and M. W. W. contributed equally to this work and should be considered as co-first authors.

LEGENDS FOR FIGURES

Figure 1. MOD (100 mg/kg i.p.) effects on electrically evoked phasic-like DA signals in (A) dorsal and (B) ventral striata measured by FSCV. (Top) Evoked DA signals elicited by electrical stimulation (demarcated by black line at time 0 s) pre-drug (left) and 60 min post-MOD (right). **INSET.** Individual background subtracted cyclic voltammogram taken from the peak signal (white vertical line) identifies the analyte as DA. (Bottom) Pseudo-color plot serially displaying all background-subtracted cyclic voltammograms (x-axis: time; y-axis: applied potential; z-axis: current). White horizontal line identifies the DA peak oxidative potential where the evoked DA trace was collected.

Figure 2. MOD elicits time- and dose-dependent effects on the maximal concentration of the electrically evoked phasic-like DA signal ($[DA]_{max}$) in dorsal (A) and ventral (B) striata. Data are expressed as a percent of pre-drug and are the mean±SEM. Arrow demarcates MOD administration at time 0 min. Data were analyzed for significance using three-way repeated measures ANOVA (n = 4-7).

Figure 3. Representative time- and dose-dependent effects of MOD on the extracellular clearance of electrically evoked DA in (A) dorsal and (B) ventral striata. FSCV traces of the electrically evoked DA signal (stimulus demarcated by short black line) are shown for 100 mg/kg MOD (left) and 300 mg/kg MOD (right) at select time points. **INSET.** Pre- and post-drug clearance curves are overlaid beginning at the same DA concentration and illustrate DA uptake inhibition.

Figure 4. Effects of MOD on presynaptic DA release and uptake. Increases in the maximal concentration of the electrically evoked phasic-like DA signal ($[DA]_{max}$) (left) are associated with an increase in DA release or $[DA]_p$ (middle) and a decrease in DA uptake or k (right) in

dorsal (**A**) and ventral (**B**) striata. Data are expressed as a percent of pre-drug and are the mean±SEM. Data were analyzed for significance using three-way repeated measures ANOVA (n = 4-7).

Figure 5. Path analysis model demonstrating the direct relationships between dose, DA release ($[DA]_p$), DA uptake (k), and $[DA]_{max}$. Values given above each arrow are standardized path coefficients describing each direct effect. Two indirect effects of MOD on $[DA]_{max}$ are described by the two paths from MOD to $[DA]_{max}$ through DA release and DA uptake.

Figure 6. MOD effects on changes in basal DA in dorsal and ventral striata. (**A**) The red line displays PCR resolved DA changes from the black FSCV trace (taken at the white horizontal line) for pre-drug and 60 min post-drug (300 mg/kg). A pseudo-color plot beneath displays all background subtracted cyclic voltammograms. **INSET.** Representative voltammogram (blue) collected at 285 s (white vertical line) overlaid with a voltammogram taken at peak electrically evoked signal (black). Y-axis is normalized current. (**B**) PCR reveals no significant effect of MOD on basal DA in dorsal (top) and ventral (bottom) striata. Data were analyzed for significance using three-way repeated measures ANOVA (n = 4). (**C**) Verification of PCR selectivity for the DA component in FSCV recordings. There was a strong correlation between $[DA]_{max}$ measured with FSCV ($[DA]_{FSCV}$) and PCR ($[DA]_{PCR}$) in both dorsal (left) and ventral (right) striata.

Figure 7. DA transients are elicited in both dorsal and ventral striata by co-administration of MOD (30 mg/kg) and raclopride (2 mg/kg). (**A**) Representative recording of DA transients in dorsal (left) and ventral (right) striata. A pseudo-color plot underneath serially displays all background-subtracted cyclic voltammograms. Transients (denoted by red asterisks) are displayed in the FSCV current trace collected at the peak oxidative potential of DA (white

horizontal line). **INSET.** Normalized background subtracted cyclic voltammograms taken from the electrically-evoked response (black line) and a DA transient (red line) collected at the white vertical line in the pseudo-color plot. (**B**) Average transient frequency per 5 minute epoch for pre- and post-drug administration expressed as mean±SEM. Data were analyzed for significance using two-way repeated measures ANOVA.

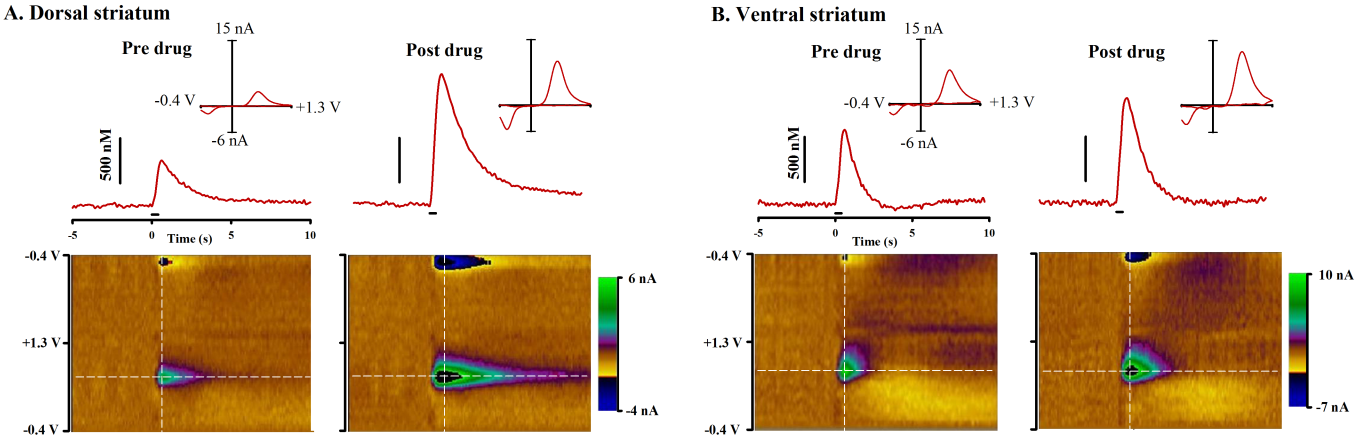
A. Dorsal striatum
Pre drug
15 nA
-0.4 V
+1.3 V
-6 nA
Post drug
500 nM
-5
0
Time (s)
5
10
-0.4 V
+1.3 V
-0.4 V
6 nA
-4 nA
B. Ventral striatum
Pre drug
15 nA
-0.4 V
+1.3 V
-6 nA
Post drug
500 nM
-5
0
Time (s)
5
10
-0.4 V
+1.3 V
-0.4 V
10 nA
-7 nA

Figure 1

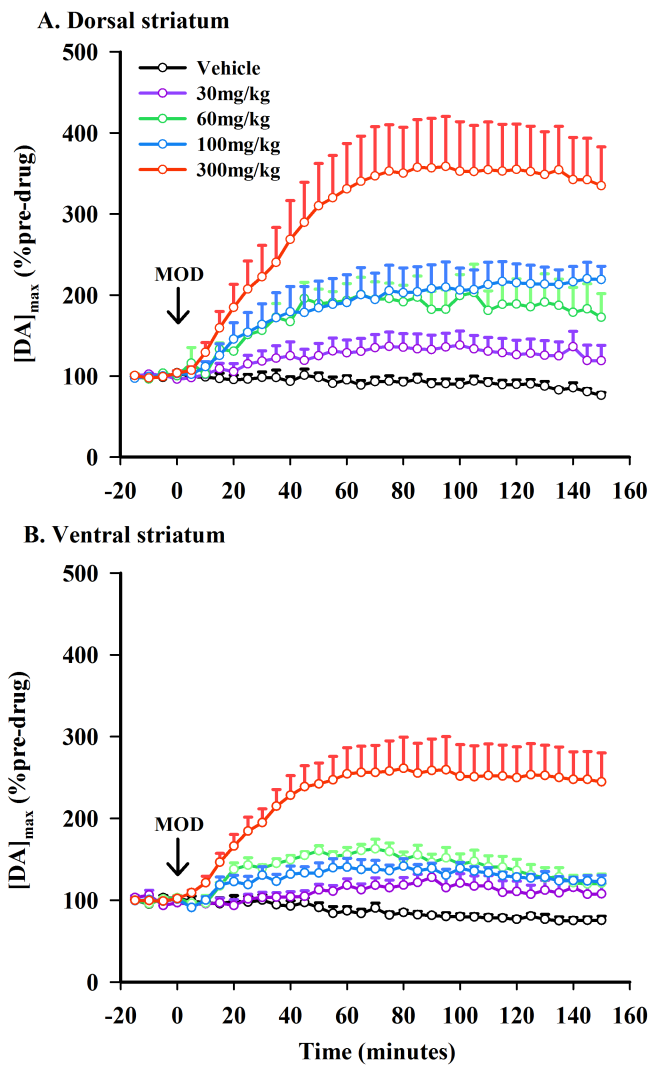
A. Dorsal striatum
Vehicle
30mg/kg
60mg/kg
100mg/kg
300mg/kg
MOD
[DA]max (%pre-drug)
500
400
300
200
100
0
-20
0
20
40
60
80
100
120
140
160
B. Ventral striatum
MOD
[DA]max (%pre-drug)
Time (minutes)

Figure 2

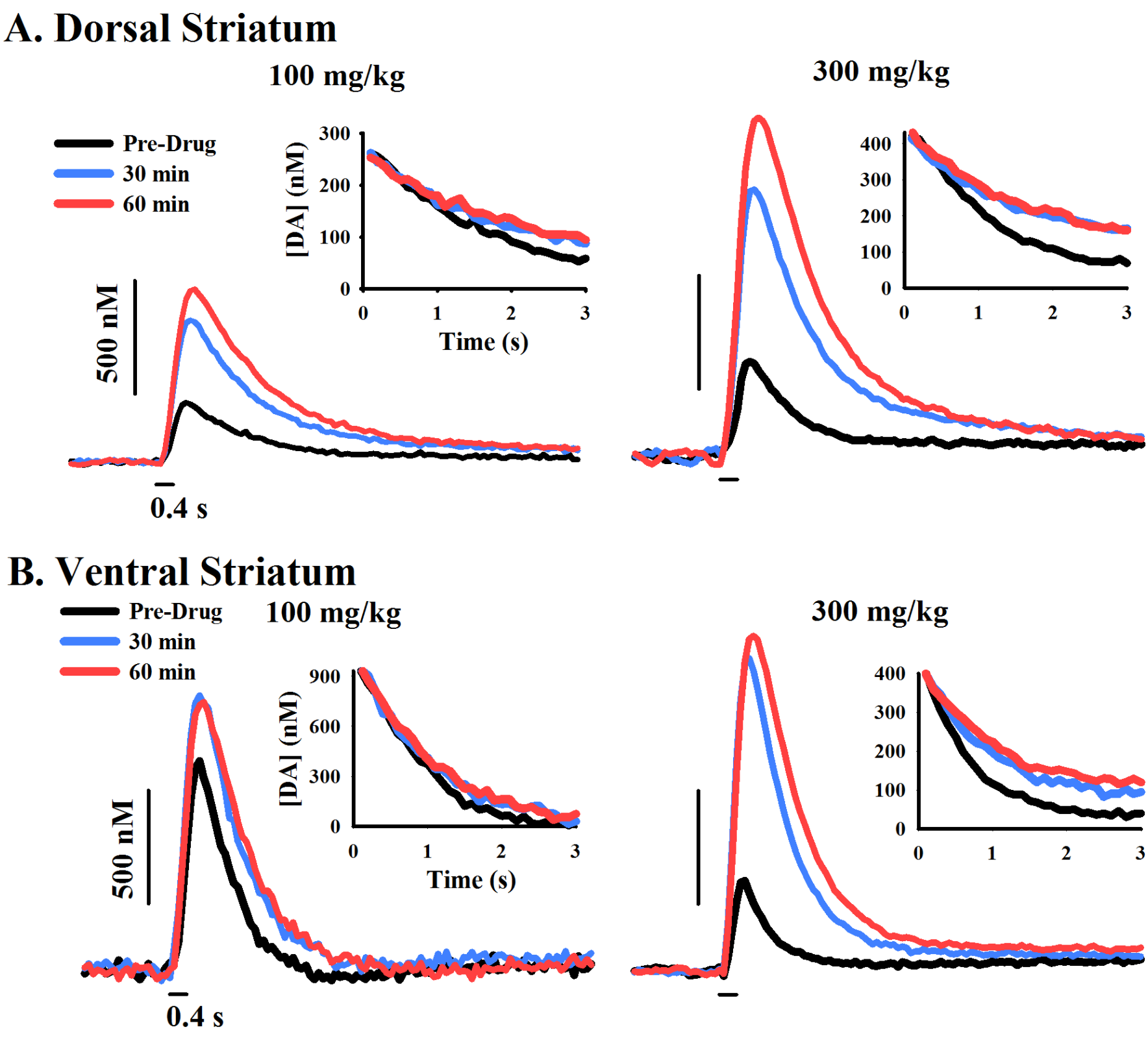
A. Dorsal Striatum
100 mg/kg
300 mg/kg
Pre-Drug
30 min
60 min
[DA] (nM)
Time (s)
500 nM
0.4 s
B. Ventral Striatum
100 mg/kg
300 mg/kg
Pre-Drug
30 min
60 min
[DA] (nM)
Time (s)
500 nM
0.4 s

Figure 3

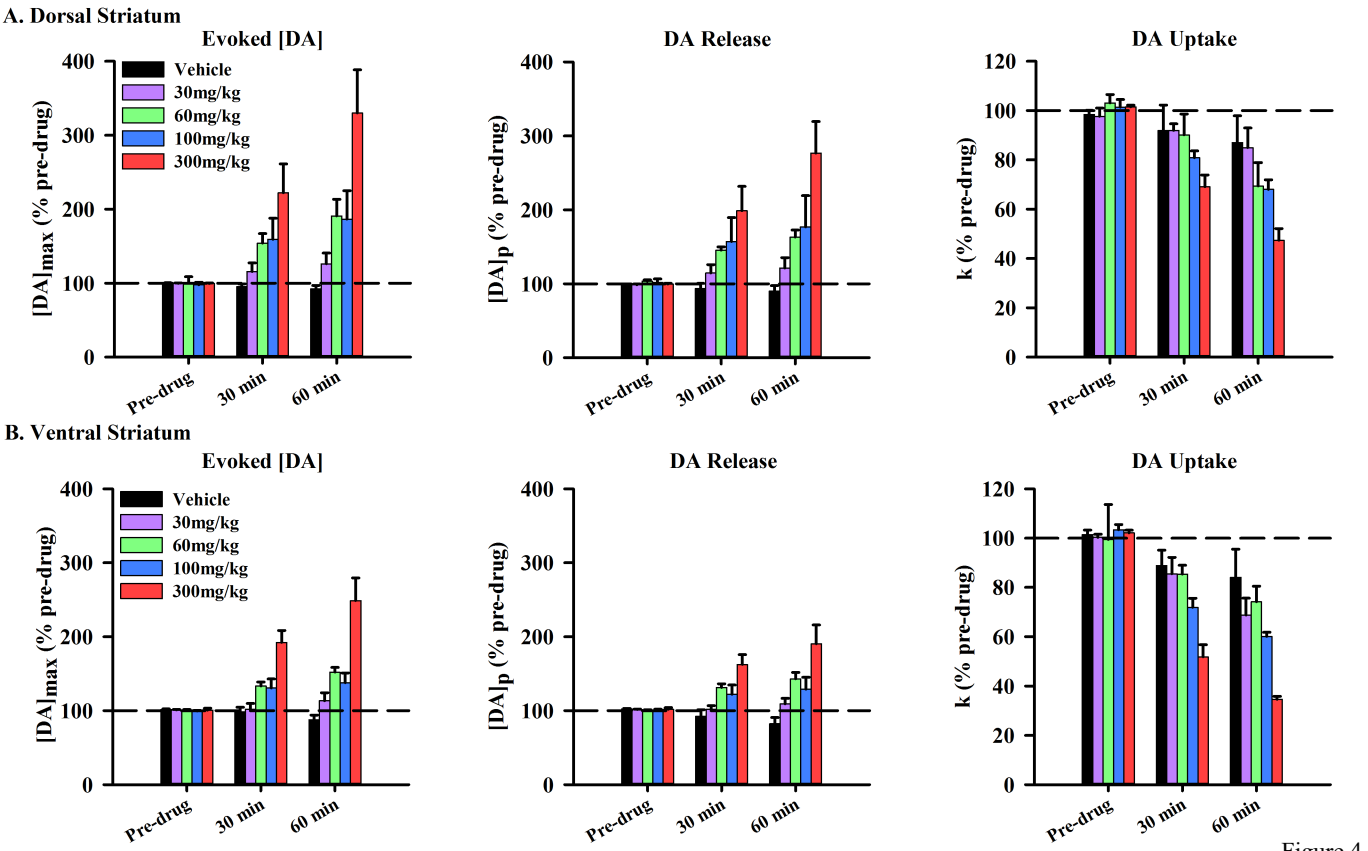
A. Dorsal Striatum
Evoked [DA]
DA Release
DA Uptake
Vehicle
30mg/kg
60mg/kg
100mg/kg
300mg/kg
[DA]max (% pre-drug)
[DA]p (% pre-drug)
k (% pre-drug)
Pre-drug
30 min
60 min
B. Ventral Striatum
Evoked [DA]
DA Release
DA Uptake
Vehicle
30mg/kg
60mg/kg
100mg/kg
300mg/kg
[DA]max (% pre-drug)
[DA]p (% pre-drug)
k (% pre-drug)
Pre-drug
30 min
60 min

Figure 4

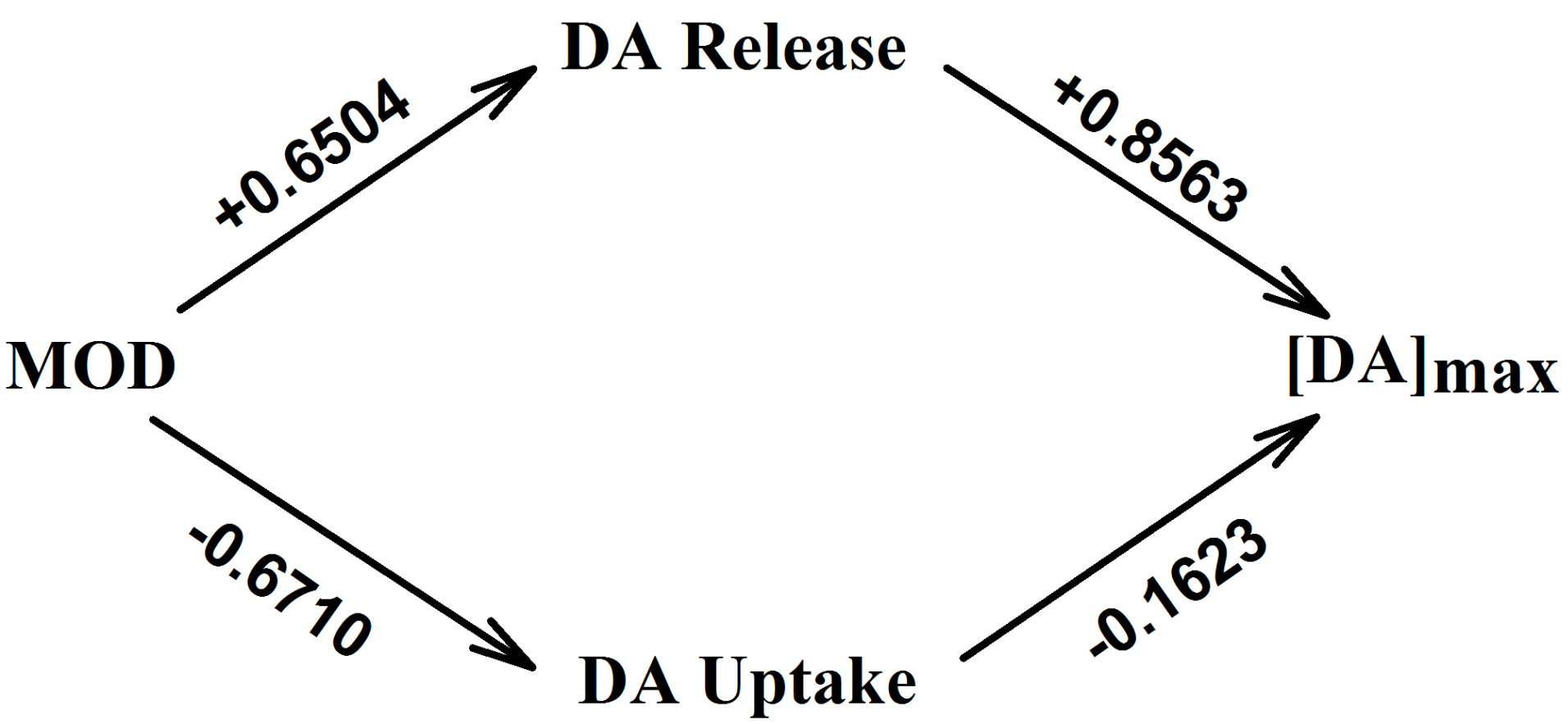

Figure 5

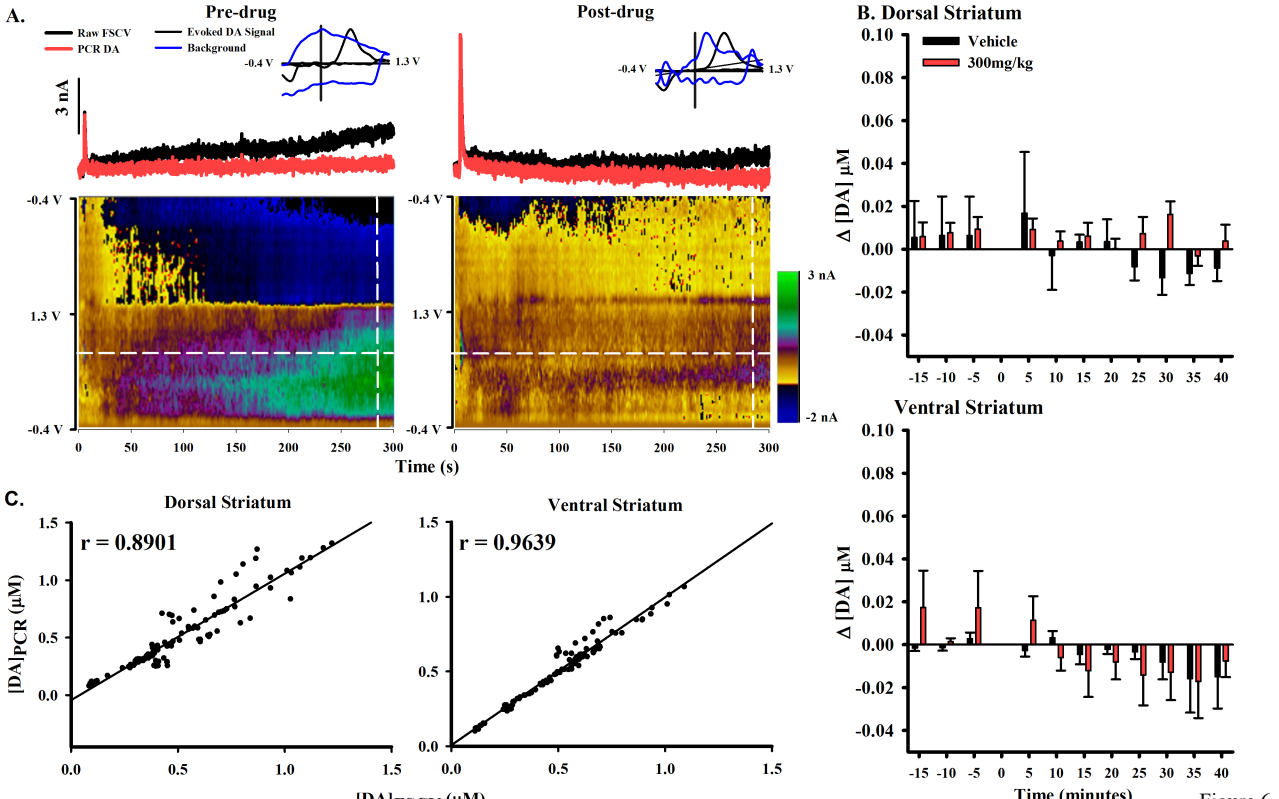
A.
Pre-drug
Post-drug
Raw FSCV
PCR DA
Evoked DA Signal
Background
-0.4 V
1.3 V
3 nA
-0.4 V
1.3 V
-0.4 V
0
50
100
150
200
250
300
Time (s)
3 nA
-2 nA
B. Dorsal Striatum
Vehicle
300mg/kg
Δ [DA] μM
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-15 -10 -5 0 5 10 15 20 25 30 35 40
Ventral Striatum
Time (minutes)
C.
Dorsal Striatum
r = 0.8901
Ventral Striatum
r = 0.9639
[DA]PCR (μM)
[DA]FSCV (μM)
1.5
1.0
0.5
0.0

Figure 6

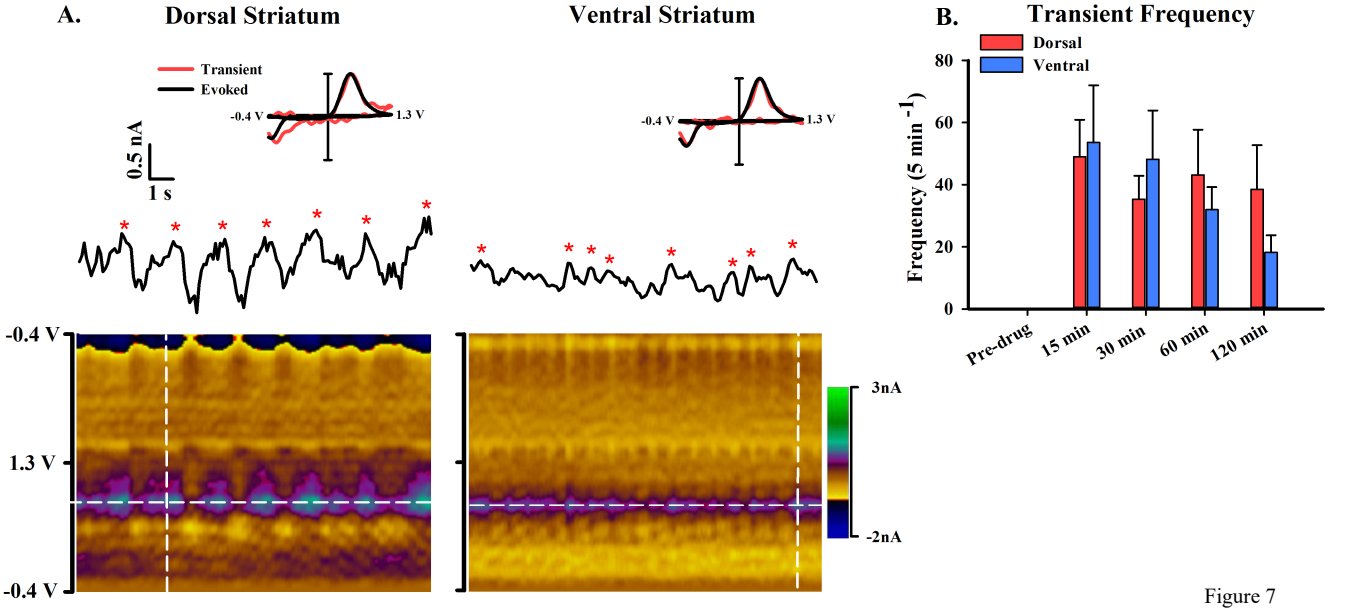
A.
Dorsal Striatum
Ventral Striatum
Transient
Evoked
-0.4 V
1.3 V
0.5 nA
1 s
-0.4 V
1.3 V
-0.4 V
3nA
-2nA
B.
Transient Frequency
Dorsal
Ventral
Frequency (5 min -1)
80
60
40
20
0
Pre-drug
15 min
30 min
60 min
120 min

Figure 7