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EFFECTS OF COCAINE AND REPEATED COCAINE FOLLOWED BY WITHDRAWAL

ALTERATIONS OF DOPAMINERGIC TRANSPORTER TURNOVER WITH NO CHANGES IN KINETICS OF SUBSTRATE RECOGNITION

SUSAN M. MEIERGERD,* JAMES S. MCELVAIN†‡ and JAMES O. SCHENK*†§‖

Departments of *Chemistry and †Biochemistry and Biophysics, and §Programs in Pharmacology/Toxicology and Neuroscience, Washington State University, Pullman, WA 99164, U.S.A.

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Abstract—The turnover of the transporter for dopamine (*ca.* 1.5 sec^{-1}) and the apparent second order rate constant of association of dopamine with the outward facing form of the transporter protein (*ca.* 10^6 $M^{-1}sec^{-1}$) were estimated using kinetic data from rotating disk voltammetric measures of the inward transport of dopamine in striatal suspensions, standard treatments of the kinetics of transport, and values in the literature for density of striatal transporter sites. Under apparent steady-state conditions of transporter functioning, inhibition of the transport of dopamine by cocaine following its addition to the incubation buffer was found to decrease the turnover of the transporter and not affect the kinetics of substrate recognition. The kinetics of binding of dopamine to the transporter were estimated also by apparent pre-steady-state kinetics. These experiments confirmed the second order nature of the binding of dopamine to the transporter and the numerical value of the rate constant estimated under steady-state conditions; they also demonstrated that the binding of dopamine has an absolute dependence on Na^+, and that the second order rate constant of association of dopamine with its transporter is not influenced by cocaine. In separate studies, similar experiments were conducted in tissues from animals that had been treated with cocaine for 3 days and withdrawn for 1 day or 2 weeks. Repeated treatments with cocaine followed by either a 24-hr or 2-week period of withdrawal resulted in increases in the V_{max} and turnover of the transporter with no apparent changes in the kinetics of association of substrate. No differences between the K_i for cocaine observed in direct inhibitions of the transport of dopamine and the K_i for cocaine observed in tissues obtained from animals treated repeatedly with cocaine were observed. Taken together, these data suggest that cocaine exerts its effects by altering an intramembrane translocation step for the movement of dopamine and not by changing the recognition of dopamine by the externally facing binding site or the apparent K_i for cocaine. Finally, repeated treatments with cocaine followed by a period of withdrawal appear to kinetically activate the transporter for dopamine.

Key words: dopamine transporter; repeated cocaine treatments; rotating disk electrode voltammetry

Repeated administration of cocaine, an inhibitor of the neuronal transport of dopamine, has been shown to produce a long-lasting sensitization of dopamine-mediated behaviors [1, 2]. In addition, it has been shown that the addictive property of drugs of abuse in general, and cocaine in particular, is correlated with a direct interaction between the drug and the transporter for dopamine [3]. While most investigators agree that cocaine inhibits the transport of dopamine, recent work attempting to determine what biochemical changes in the release and reuptake of dopamine occur due to repeated administration of cocaine following various periods of withdrawal have produced variable results. Some investigators have reported increases in the maximal velocity of transport (V_{max}) [4, 5] or data consistent with this kinetic change [6], whereas others have reported decreases in [7] or no effect on [8] this kinetic parameter. Further assessment of what effects repeated cocaine has on the transport of dopamine is further complicated by the findings of Peris *et al.* [9], suggesting that handling in the protocols used to treat animals with cocaine may alter the kinetics of the inward transport of dopamine and that the amphetamine-induced outward transport of dopamine via the transporter is increased due to repeated cocaine treatments [10, 11]. Recently, this laboratory developed a method to measure the apparent time-resolved inward transport of dopamine into suspensions of the striatum [12, 13], showed that evaluations of the resulting kinetic information fit a pre-existing kinetically-defined multisubstrate mechanistic model for transport and its inhibition by cocaine [13], and demonstrated that the rate constant of association of dopamine with its transporter (k_{ass})¶ and transporter turnover (TN) could be resolved using the rotating disk voltammetric technique [14]. The relationship between the

‡ Current address: Marion Merrill Dow, Kansas City, MO, U.S.A.

‖ Corresponding author: James O. Schenk, Ph.D., Department of Chemistry, Washington State University, Pullman, WA 99164-4630. Tel. (509) 335-7517 or 335-4300 FAX (509) 335-8867.

¶ Abbreviations: DA, dopamine; k_{ass}, rate constant of association; NHC, non-handled controls; RC, repeated treatments with cocaine; RDE, rotating disk electrode; SER, standard error of regression; T, transporter; t, time; T_d, tissue density of the transporter for dopamine; and TN, turnover.

dopamine and Na^+ binding sites was found to be allosteric, and in the studies of inhibition of transport by cocaine an *un*competitive mechanism of inhibition of transport was observed. The relationship between the binding sites for cocaine and Na^+ was found to be competitive. This result suggests that the kinetics of recognition of dopamine by the transporter are not influenced by cocaine but the kinetics of translocation are inhibited. However, the kinetics of association of dopamine and transporter turnover were not resolved, and the effects of repeated administration of cocaine on these processes were not evaluated. Herein, further kinetic evaluations were made to obtain information on changes in substrate recognition and turnover, both under apparent steady-state and apparent pre-steady-state conditions, resulting from direct inhibitions by cocaine and repeated treatments with cocaine followed by withdrawal.

MATERIALS AND METHODS

Rationale. Previously, this laboratory reported that cocaine, added to the extracellular phase of an incubating striatal suspension, inhibited the inward transport of dopamine from the same phase by an *un*competitive mechanism [13]. In those studies, an experimental model was chosen in which an instantaneous increase in extracellular dopamine concentration, $[DA]_o$, was used to initiate transport, and initial rates of disappearance of dopamine from the extracellular phase were measured. The justification of this approach was to present the transporter with substrate in a manner similar to that expected to be operational in chemical neurotransmission [13], a process that can be envisaged as first involving rapid presentation of substrate to the transporter followed by recognition and binding of substrate at the outer face of the membrane with subsequent translocation of substrate into or across the membrane to remove it from the extracellular compartment. Mathematical treatments of the data to resolve kinetic parameters to determine whether or not cocaine influences substrate recognition at the outer facing form of the transporter or the translocation of substrate into or across the membrane were not made. Determining the V_{max} and the Michaelis constant (K_m) values for transport provide only part of the story, since V_{max} is a constant defined by both the kinetic facility of catalysis by the transporter protein for movement of substrate within the membrane and the density of transporter sites [15, 16]. The K_m is also a kinetic constant which involves both the catalytic constant of translocation of substrate within the membrane as well as the binding affinity of the transporter with the substrate [15, 16]. Thus, these variables must be resolved before the question of whether alterations in substrate recognition, translocation, or both result from treatments with cocaine. Previously, we showed that it was possible to resolve the kinetics of association of dopamine with its transporter from transporter turnover [14]. The series of steps for accomplishing this goal are outlined below. They are based on standard kinetic treatments of enzymic [15] and transporter function [16] and an apparent agreement in the literature, concerning the density of the striatal transporter for dopamine, between laboratories working to define the binding of ligands to the striatal transporter for dopamine and how it is altered by drugs of abuse. In the work described here, we used estimates of k_{ass}, the second order rate constant of association of dopamine with its transporter, and turnover (TN) of the transporter to evaluate how cocaine inhibits the transport of dopamine *in vitro* and how transporter function is altered following withdrawal from repeated treatments with cocaine. Initially, these evaluations were made from data obtained under conditions of steady state defined by the kinetic condition where $d[\text{dopamine bound transporter}]/dt = 0$. In separate experiments, the values of k_{ass} obtained from the steady-state estimates were confirmed by apparent pre-steady-state measurements.

Treatments of animals, preparation of tissue, and measurement of the inward transport of dopamine. Male Sprague–Dawley rats, weighing 275–325 g, were not treated at all or were treated with cocaine or saline following the general protocol described by Kalivas *et al.* [2]. Briefly, animals received 15 mg/kg cocaine, intraperitoneally, or saline (referred to as sham-treated controls) for 3 days. Striatal tissue for experimentation was obtained from animals that had not been treated or handled (referred to as non-handled controls, NHC) as well as sham-treated controls and animals treated with cocaine followed by a withdrawal period of 24 hr or 2 weeks. The inward transport of dopamine in striatal suspensions obtained from animals in the various treatment groups was measured with rotating disk electrode (RDE) voltammetry as described previously [12–14]. The RDE was calibrated over the dopamine concentration range of 25 to 4000 nM. The initial apparent zero order velocity of dopamine transport was measured following the addition of 100, 250, 500, 1000, and 1500 nM dopamine, one concentration value per striatal suspension, as described previously, and the resulting values for the velocities obtained were expressed in picomoles per second per gram wet weight [13]. Four striata from four separate rats were used for each concentration point. Thus, twenty rats were used to establish the parameters of transport in each treatment group.

Estimation of kinetic parameters: K_m, V_{max}, K_i, *turnover number (TN), the rate constant of association of dopamine with the transporter* (k_{ass}), *and statistical treatment of the data.* Values for K_m and V_{max} were obtained by fitting the observed velocities of transport at various $[DA]_o$ values to the Michaelis–Menten expression using a Quasi-Newton based non-linear regression analysis subroutine of the commercially available software SYSTAT (Systat, Inc., Evanston, IL) as described previously [13]. Unless otherwise noted, the indicated values of precision are standard errors of regression (SER). The quality of the fit of the data to the Michaelis–Menten expression was determined by comparing the predicted values of v (using the estimated values of K_m and V_{max}, and the numerical values of $[DA]_o$) to the experimentally observed values and expressing the difference as a percentage relative to the experimentally observed value of v. Inhibition constants for cocaine were

estimated in separate experiments by measuring the inhibition of the transport of dopamine over the range of concentration values indicated previously in the presence of 1.0 μM cocaine and calculating the apparent K_i value from the Michaelis–Menten equation modified for the *un*competitive mechanism as described previously [13]. The TN and k_{ass} values were estimated following the procedures outlined by Meiergerd and Schenk [14] (which are based on the work of Fersht [15] and Stein [16]) using

$$TN = V_{max}/T_d \quad (1)$$

where TN is expressed in sec^{-1}, T_d is the density of transporter sites expressed in pmol/g wet weight, and

$$k_{ass} = TN/K_m \quad (2)$$

where the variables have been defined previously and k_{ass} is expressed in $M^{-1}sec^{-1}$. The value used for T_d was an average calculated from published values in the literature from three different laboratories [9, 17–19]. The conversion factor, 0.15 mg protein/g striatal wet tissue weight, for converting from values normalized on the basis of protein to those normalized on the basis of wet tissue weight was obtained from McElvain and Schenk [13]. The average calculated value for T_d was 416 pmol/g wet wt. This value is within 14% of a similar estimate made by Madras *et al.* [20]. Data for estimating what effects acute administration of cocaine has on the TN of the transporter and k_{ass} values for substrate binding were calculated from the V_{max} and K_m data that were obtained under steady-state conditions and appear in Table 1 of McElvain and Schenk [13]. Repeated administration of cocaine was assumed to not alter the striatal T_d used in the estimations here, since it has been shown that the B_{max} of binding of ligands to the striatal transporter for dopamine is not altered by repeated treatments with cocaine [8, 9, 21]. Statistical differences at the 95% confidence level were identified using a statistical test for detecting differences between sets of data produced by linear regression analyses [22, 23].

Kinetic treatments of the apparent pre-steady-state kinetic data. To confirm the k_{ass} values obtained from estimates made under steady-state conditions, measurements were made under apparent pre-steady-state conditions. The kinetic condition of pre-steady-state kinetics of the transport of dopamine is depicted by

$$DA + T \underset{k_{diss}}{\overset{k_{ass}}{\rightleftarrows}} DA\text{-}T$$

where DA represents dopamine, T is the outward facing form of the transporter, DA-T is the dopamine–transport complex, k_{diss} is the rate constant of dissociation, and turnover of the transporter is ignored because of the concentration of dopamine and the time domain chosen for experimentation [15, 24]. The binding reaction is second order. At time (t) = 0, the DA and T concentrations are designated $[DA]_{o,t=0}$ and $[T]_{o,t=0}$, respectively. At some elapsed t, the concentrations are defined as $[DA]_{o,t=0} - x$ and $[T]_{o,t=0} - x$, where $[DA\text{-}T] = x$. The rate of reaction is given by [25].

$$dx/dt = k_{ass}([DA]_{o,t=0} - x)([T]_{o,t=0} - x) \quad (3)$$

which integrates to

$$k_{ass}t = \{1/\{[DA]_{o,t=0} - [T]_{o,t=0}\}\} \ln \{[T]_{o,t=0}([DA]_{o,t=0} - x)/[DA]_{o,t=0}([T]_{o,t=0} - x)\} \quad (4)$$

Note that for this integration $[DA]_{o,t=0} \neq [T]_{o,t=0}$ [25]. The *ca.* 40 mg of striatal tissue used in the transport experiments should be able to take up 16.6 pmol of dopamine by the binding event alone given the density of the striatal transporter (calculation: 0.04 g wet weight · 416 pmol/g wet weight= 16.6 pmol). Thus, in the 500 μL incubation volume, the binding event should decrease the concentration of dopamine by *ca.* 33 nM, suggesting that the kinetics of the binding of dopamine to the transporter (pre-steady-state kinetics) should be measurable by the RDE system because it can measure concentration values at this level. Furthermore, if the value of k_{ass} is in the 10^6 to $10^7\ M^{-1}\ sec^{-1}$ range, as suggested by the results of our previous work [14], then the half-time of association of 50 nM dopamine with the transporter should be in the 1.7 to 17 sec range, timing easily measured by the RDE. Furthermore, given the K_m and V_{max} values for striatal transport reported by McElvain and Schenk [13], the predicted transporter v at 34 nM should be 16 pmol/sec/g wet wt. This represents a TN value of 0.038 sec^{-1} with a first order half-time of 18 sec. Thus, the experimental protocol in these experiments was to add 50 nM dopamine to the striatal suspension and monitor the reduction in $[DA]_o$ value to 17 nM as a function of t. The data was then tested for a fit to Equation 4 by plotting the right-hand side of the equation versus t at times <18 sec, checking for linearity, and obtaining the numerical value of k_{ass} from the slope. Given the previous rationale, this protocol should provide apparent k_{ass} values of the binding of dopamine to its transporter with timing less than that required for a single turnover of the dopamine bound transporter. As a check on the validity of the procedure, the k_{ass} was substituted back into Equation 4, and the theoretical $[DA]_o$ versus t curve was calculated and compared with that observed

Table 1. Summary of the effect of cocaine (COC) on the kinetic parameters of inward transport of dopamine in striatal suspensions from non-treated animals*

Treatment†	TN (sec^{-1})	k_{ass} 10^{-6}
Non-handled controls	1.4 ± 0.03	1.2 ± 0.10
Acute COC (0.50)	1.1 ± 0.04‡	1.0 ± 0.08
Acute COC (1.0)	0.58 ± 0.01‡	1.2 ± 0.05
Acute COC (2.0)	0.44 ± 0.01‡	1.0 ± 0.07
Acute COC (4.0)	0.31 ± 0.005‡	1.0 ± 0.04
Acute COC (8.0)	0.24 ± 0.005‡	1.2 ± 0.06

* TN and k_{ass} were calculated from Equations 1 and 2 using data from Table 1 in McElvain and Schenk [13] and a T_d of 416 pmol/g wet wt (see text). The precision indicated represents the propagated standard error of regression due to regression analyses and subsequent mathematical operations according to standard treatments [26]; each datum point was obtained from a minimum of 4 datum points, and values are means ± SER.

† The micromolar concentration of cocaine that was added to the suspension is indicated by the numbers in parentheses.

‡ Significantly different from non-handled controls at $P \leq 0.05$, using a z-test [22, 23].

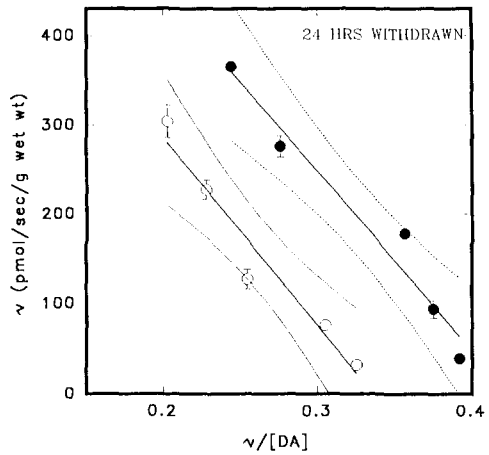

Fig. 1. Eadie–Hofstee (EH) analyses of the transport of dopamine into striatal suspensions prepared from rats treated repeatedly with saline (○) or cocaine (●) and withdrawn from treatments for 24 hr. The EH plot was shifted to the right along the x-axis in experiments conducted with cocaine-treated animals, relative to saline-treated animals, and the slope was unchanged, resulting in an increased intercept (V_{max}). The lines are different at $P \leq 0.05$. The solid lines through the data points represent the linear regression lines, and the curved lines represent the 95% confidence intervals. Numerical values of K_m and V_{max} are listed in Table 2. The average tissue weight used in these experiments was 39.5 ± 0.7 mg, N = 40, from 4 different striata from 4 different animals per datum point. The bars represent SEM. Points with no bars have error values within the dimensions of the symbol.

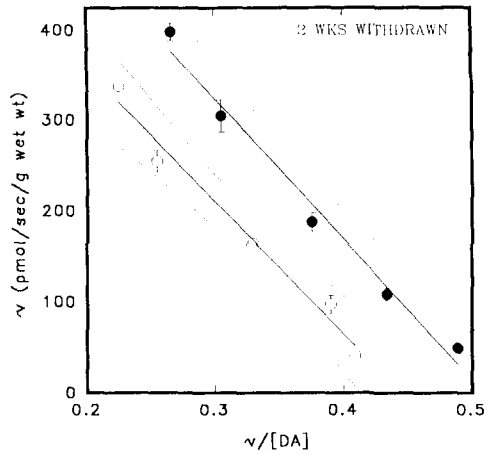

Fig. 2. Eadie–Hofstee analyses of the transport of dopamine into striatal suspensions prepared from rats treated repeatedly with saline (○) or cocaine (●) and withdrawn from treatments for 2 weeks. The EH plot was shifted to the right along the x-axis in experiments conducted with cocaine-treated animals, relative to saline-treated animals, and the slope was unchanged, but the intercept (V_{max}) was increased. The lines are different at $P \leq 0.05$. The solid lines through the data points represent the linear regression lines, and the curved lines represent the 95% confidence intervals. Numerical values of K_m and V_{max} are listed in Table 2. The average tissue weight used in these experiments was 40.2 ± 0.8 mg, N = 40, from 4 different striata from 4 different animals per datum point. The bars represent SEM. Points with no bars have error values within the dimensions of the symbol.

experimentally. The overall fit was expressed as per cent deviation obtained by comparing the theoretical values relative to the experimental ones. In one set of experiments, the extracellular [Na^+] was lowered by replacement with choline chloride as described previously [13].

Drugs, chemicals, and solutions. Dopamine and choline chloride were purchased from the Sigma Chemical Co. (St. Louis, MO), and cocaine was provided by the Washington Alcohol and Drug Abuse Program (Professor Peter Kalivas, Director). The common buffer salts were obtained from the Baker Chemical Co. (Phillipsburg, NJ).

RESULTS

Kinetic evaluations under apparent steady-state conditions. The transporter TN and k_{ass} values for substrate binding were estimated from data obtained under apparent steady-state conditions in order to obtain mechanistic insights into how cocaine influences the transport of dopamine. The results of these analyses are listed in Table 1. The data show that cocaine, when present in the incubation buffer, inhibited the inward transport of dopamine by reducing the TN values, with no statistically significant alterations in the values of k_{ass}. These data were obtained by calculations using Equations 1 and 2 and the raw data published previously by McElvain and Schenk [13]. Eadie–Hofstee plots of the dependence of the inwardly directed velocity of transport of dopamine on the initial values of $[DA]_o$ into striatal suspensions prepared from rats treated with saline or cocaine and withdrawn from treatments for 24 hr and 2 weeks are shown in Figs. 1 and 2, respectively. The lines obtained in striatal suspensions from sham-treated animals were different at the 95% confidence level from those obtained in striatal suspensions prepared from cocaine-treated rats. Table 2 lists the kinetic parameters observed. The significance of linear regression of the Eadie–Hofstee analyses was at the ≥99.4% confidence level, and the overall relative deviation of experimental data from the theoretical values predicted from the Michaelis–Menten model was ±1.4%, range: 0.80 to 2.5%. No differences between the values of K_m were observed in suspensions prepared from sham-treated animals relative to studies conducted in suspensions from animals withdrawn from cocaine treatments. In contrast, the values of V_{max} were increased by 20–22% following cocaine treatments and withdrawal, relative to sham-treated controls. The apparent K_i of cocaine in non-handled controls, 1.1 ± 0.03 μM [13], was not statistically different from the apparent K_i in animals treated repeatedly with cocaine followed by a 24-hr or 2-week period of withdrawal (1.2 + 0.21 μM, N = 4 and 0.85 ± 0.13 μM, N = 4, respectively). Cocaine-treated rats that had been withdrawn from treatments for 24 hr or 2 weeks exhibited increased values of TN but no changes in the values of k_{ass}, relative to sham-treated controls (Table 2).

Kinetic evaluations under apparent pre-steady-state conditions. The lack of statistically detectable changes in the k_{ass} estimated from steady-state data

Table 2. Effect of repeated treatments of saline or cocaine on the kinetic parameters of the striatal transport of dopamine

Treatment*	K_m (μM)	V_{max} (pmol/sec/g wet wt)	TN (sec^{-1})	k_{ass} (M^{-1} sec^{-1})
Sham 24 hr WD	2.1 ± 0.2	710 ± 40	1.7 ± 0.14	0.81 ± 0.079 × 10^6
RC 24 hr WD	2.0 ± 0.2	849 ± 79†	2.0 ± 0.19†	1.0 ± 0.14 × 10^6
Sham 2 week WD	1.5 ± 0.1	648 ± 41	1.6 ± 0.10	1.1 ± 0.10 × 10^6
RC 2 week WD	1.5 ± 0.1	789 ± 44‡	1.9 ± 0.10‡	1.3 ± 0.11 × 10^6

* Key: Values are means ±SER. RC, repeated treatments with cocaine; Sham, repeated treatments with saline; and WD, withdrawn.

† Significantly different at $P < 0.05$ relative to Sham 24 hr WD via the non-overlap of the Eadie–Hofstee plots of Fig. 1.

‡ Significantly different at $P < 0.05$ relative to Sham 2 week WD via the non-overlap of the Eadie–Hofstee plots of Fig. 2.

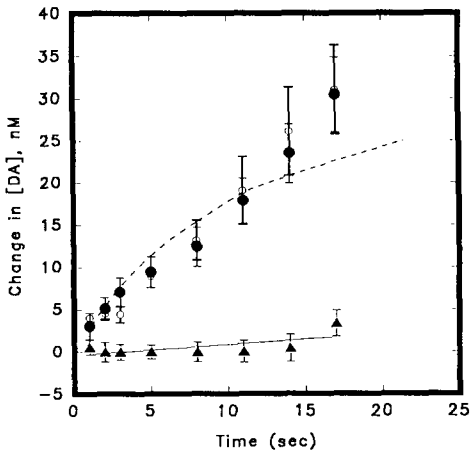

Fig. 3. $[DA]_o$ versus t profiles of uptake into striatal tissue under apparent pre-steady-state conditions. The open circles indicate profiles observed in non-treated animals, the closed circles are profiles observed in the presence of 1.0 μM cocaine, the triangles represent profiles observed in buffer with a lowered $[Na^+]$ (from 150 to 26 mM, substituted with choline), and the dashed line is the theoretically expected profile according to Equation 4. The value of $[DA]_{o,t=0}$ was 50 nM, the value for $[T]_o$ was taken as 34 nM (see text), and values of $[DA]_{o,t}$ were measured by the RDE. Values are means ±SEM, N = 4.

could be a result of propagated error of the mathematical procedures used in its calculation. Thus, the apparent pre-steady-state kinetics of the association of dopamine with its transporter were monitored in order to confirm the value of k_{ass}. Figure 3 shows that the experimental $[DA]_o$ versus t profile for the reduction in extracellular dopamine at 50 nM followed second order kinetics (fit to Equation 4, a process first order in dopamine, first order in apparent concentration of transporter, and second order overall) up to about the 15- to 18-sec range. Deviations at longer times are assumed to be due to the kinetic TN of the transporter since the half-time of TN was estimated to be *ca.* 18 sec at 50 nM dopamine (*vide ante*). In addition, it was found that the apparent binding of dopamine to the externally facing form of its transporter was not affected by 1.0 μM cocaine, but the binding of dopamine was almost quantitatively inhibited (≥98%) by reducing the extracellular $[Na^+]$. Table 3 lists the numerical values for pre-steady-state k_{ass} under various treatment conditions. First, the upper section shows that the k_{ass} values, obtained by similar treatment of the time course of the reduction of the $[DA]_o$, were not different over the initial concentration range of 34 to 250 nM (lower and higher concentrations were not tested), and agreed with an overall relative standard error of 14%. The average value from apparent pre-steady-state assessments agrees exactly with values derived by treatments of kinetic data obtained under apparent steady-state conditions here (see Tables 1 and 2), in our previous studies (see Table 2 in Ref. 14), and with similar calculated values from previously published data from other laboratories (see Table 2 in Ref. 14). No alterations in the values of k_{ass} were observed in the presence of 1.0 μM cocaine or following repeated treatments with cocaine and withdrawal. However, reduction of extracellular Na^+ to 26 mM quantitatively abolished binding.

DISCUSSION

Most investigators studying the effects of cocaine on the transport of dopamine agree that cocaine presented directly to tissue preparations that transport dopamine results in inhibition of the function of the transporter. (For an alternative view, see Stamford *et al.* [27].) In the studies presented here, the inhibition appeared to occur by a reduction in the TN of the transporter with no apparent change in the kinetics of binding of dopamine to the outward facing form of the transporter protein. The relative magnitudes of the two kinetic parameters suggest that the rate-limiting step in transport is the TN of the transporter and not substrate association with the transporter binding site. The TN was found to be increased with no changes in the apparent k_{ass} in animals withdrawn from repeated treatments with cocaine. Thus, it appears that modulation of transporter function following withdrawal from repeated treatments with cocaine may occur via alterations at intramembrane sites. Comments and cautions concerning the kinetic measurements, apparent mechanism of inhibition by cocaine, and the effects of repeated treatments with cocaine follow.

Comments on the kinetics of dopamine–transporter interactions and the apparent mechanism of inhibition of dopamine transport by cocaine. The apparent pre-steady-state kinetics of association of dopamine with

Table 3. Estimated second order rate constants (k_{ass}) for the association of dopamine with its striatal transporter

Initial conditions	k_{ass}(M^{-1} sec^{-1}) (N)
Effects of varying the initial values of $[DA]_o$	
$[T]_{o,t=0}$ = 34 nM; $[DA]_{o,t=0}$ = 34 nM	1.8 (±0.3) × 10^6 (5)
$[T]_{o,t=0}$ = 34 nM; $[DA]_{o,t=0}$ = 50 nM	1.5 (±0.3) × 10^6 (4)
$[T]_{o,t=0}$ = 34 nM; $[DA]_{o,t=0}$ = 100 nM	1.4 (±0.5) × 10^6 (6)
$[T]_{o,t=0}$ = 34 nM; $[DA]_{o,t=0}$ = 250 nM	8.4 (±1.8) × 10^5* (7)
	Average = 1.4 (±0.2) × 10^6
Effects of a reduction in $[Na^+]_o$ and addition of cocaine (COC)	
$[T]_{o,t=0}$ = 34 nM; $[DA]_{o,t=0}$ = 50 nM; $[Na^+]_o$ = 26 mM	Not different from zero (7)
$[T]_{o,t=0}$ = 34 nM; $[DA]_{o,t=0}$ = 50 nM, +1.0 μM COC	1.6 (±0.3) × 10^6 (8)

* From the pseudo first order rate constant (k_{p1}) obtained from 34×10^{-9}-$[DA]_{o,t} = 34 \times 10^{-9}\exp(k_{p1}t)$ where $k_{p1} = 250 \times 10^{-9} k_{ass}$.

its striatal transporter suggests that the interaction is first order in dopamine, first order in transporter density, and second order overall. This is in agreement with the results of Krueger [28] and McElvain and Schenk [13], who showed by curve-fitting experimental velocities to the Michaelis–Menten equation that the transport of dopamine is first order in dopamine. The numerical value of k_{ass} obtained under apparent pre-steady-state conditions agrees with the value obtained by analyses of kinetics in the steady-state condition. The relative values of k_{ass} and TN suggest that the rate of movement of dopamine mediated by conformational changes of the transporter is rate-limiting rather than the binding of dopamine to the external facing form of the transporter. Indeed, the value observed for k_{ass} is within a factor of 100 of diffusion control. Note that diffusion-controlled enzymes, such as acetylcholinesterase, have k_{ass} values of approximately 10^8 $M^{-1}sec^{-1}$ [15]. Given the differences between molecular diffusion (with reference to enzymes in solution) and movement of biological structures containing transporters (with reference to supramolecular structures such as synaptosomes and membranes), a value of 10^6 $M^{-1}sec^{-1}$ may represent diffusion control. It is interesting to note that the k_{ass} value for the association of catechol substrates with a variety of enzymes using catechols as substrates falls in the range of 10^6 to 10^7 M^{-1} sec^{-1} (see Table 5.2 in Ref. 24) and that the k_{ass} values reported here are in the higher range for values reported for the binding of transported substrates to other transporters (see Table 4.22 in Ref. 16).

The apparent binding of dopamine to its striatal transporter was found to be abolished by lowering the $[Na^+]$ from physiological levels to 26 mM by substitution with choline. The Na^+-dependent binding observed here is not thought to be related to dopamine binding to dopamine receptors because agonist binding to the dopamine receptors is inhibited by increased $[Na^+]$ [29] and the density of dopamine receptors, 17 pmol/g wet wt [30], represents only about 4% of the binding capacity relative to the transporter for dopamine. As a note of caution, choline, used commonly in the study of Na^+ dependent transport of dopamine [13, 28], as well as other ionic substitutes for Na^+, can have inhibitory effects of their own [31]. These effects are difficult to control with buffer conditions requiring constant ionic strength.

The results of a series of deductions, using data from this and other laboratories, suggest that an *un*competitive relationship between dopamine and cocaine, as reported previously by this laboratory [13], is reasonable. First, the observation here is that 1.0 μM cocaine does not affect the value of k_{ass} at physiological values of $[Na^+]$. Second, both Holz and Coyle [32] and McElvain and Schenk [13] reported that lowering the $[Na^+]$ resulted in decreased V_{max} with no changes in K_m values for dopamine transport, suggesting that an allosteric relationship may exist between the binding site for dopamine and that for Na^+. Third, Kennedy and Hanbauer [33], Reith *et al.* [19], and Calligaro and Eldefrawi [18] have shown that, although Na^+ is required for cocaine binding at subphysiological levels, it inhibits the binding of cocaine at $[Na^+]$ values in excess of *ca.* 30 mM and up to and exceeding physiological levels. This observation supports the previously published model [13] of a competitive relationship between the Na^+ and cocaine binding sites. In general support of the findings obtained in binding studies and our observation of the diminution of cocaine effects on transport kinetics by increased $[Na^+]$, Wheeler *et al.* [34] have shown that cocaine inhibitions (at a fixed concentration) of the transport of dopamine are more efficacious at lower levels of $[Na^+]$. However, Wheeler *et al.* [34] have noted also that an exact correlation between the Na^+ requirement of cocaine binding and the Na^+ requirement of transport cannot be made easily at the present time. Thus, a competitive relationship between cocaine and Na^+ along with an allosteric relationship between dopamine and Na^+ leads to the expectation of a *non*competitive or *un*competitive relationship between dopamine and cocaine, suggesting separate sites for binding to the transporter. Furthermore, it has been shown that dopamine is more potent in inhibiting the transport of [^{3}H]dopamine than cocaine but is less potent than cocaine in displacing [^{3}H]cocaine binding [20, 35]. Finally, evidence for different molecular sites for the interaction of substrate and cocaine has been obtained in studies of site-directed mutagenesis of the cloned transporter [36], although others have not found binding data supportive of this result [37].

The *un*competitive mechanism operational under the conditions used in this actively transporting

preparation implies that dopamine binding externally is required for the binding of cocaine. Thus, reasonable hypotheses relevant to this finding include: (a) that the reduced TN produced by cocaine is related to an alteration in the inward movement of substrate-loaded transporter rather than the reorientation of the unloaded transporter from its inward to outward facing form, (b) that cocaine only inhibits the reorientation of unloaded transporter to outward facing form, and (c) that dopamine transport results in recruitment of cocaine binding sites to the externally exposed transporter [38]. Investigations of these possibilities are currently underway. As a final note of caution, it should be pointed out that the transport experiment described here and in our previous work [13] approximates a *zero trans* entry experiment, only one of six different types of experiments that can be designed for the study of transport processes [16, 38–40]. The apparent mechanism of inhibition in a transport experiment can differ depending on experimental conditions, and only a systematic evaluation of inhibitions observed under the various experimental conditions possible for studying transport can reveal the *molecular* mechanism of inhibition [38–40].

Comments on the effects of repeated cocaine treatments on the transport kinetics of striatal dopamine. The results reported here on the effects of repeated treatments with cocaine followed by withdrawal support previously published observations and provide additional insights into the mechanism of how cocaine affects transporter function following its repeated use. Alteration of the function of the dopaminergic transporter by cocaine following a 24-hr or a 2-week period of withdrawal was observed. Repeated cocaine administration followed by withdrawal increased the TN of the transporter with *no* change in the kinetics of association of dopamine with the transporter.

There are four currently accepted mechanisms by which the function of transporters can be regulated [39]: (a) a new transporter can be synthesized and/or inserted into the membrane; (b) transporters that preexist in the cytoplasm can be recruited into function by insertion into the membrane following appropriate signalling; (c) transporters already in the membrane can be activated or inhibited by chemical modification via kinases and phosphatases; and (d) transporters can be up-regulated by the level of their substrates. To a first approximation, mechanisms related to increasing the amount of transporter in the membrane can be eliminated as an explanation of how the observed up-regulation of transporter function occurs following repeated treatments with cocaine. Striatal transporter density does not appear to be altered by cocaine (*vide ante*). However, the effects of the presence of substrate on the density of the dopamine transporter have not been examined. It should be noted also that these results are predicated on the current observation of the field that the density of the transporter for dopamine is not altered following repeated treatments with cocaine. A recently published paper [21] reported a numerical decrease in the density of the striatal transporter for dopamine following withdrawal from repeated treatments with cocaine. The authors reported that the reduced values were not statistically different from controls. However, if this trend were shown to be valid in the future, it would enhance the results obtained here.

In conclusion, the results of this work suggest that it is possible to resolve kinetics relevant to substrate recognition from those related to the movement of substrate across the striatal dopaminergic neuronal membrane. It appears that the rate-limiting step in striatal dopaminergic transport may be mediated by the conformational change or changes of the transporter to effect movement of dopamine into or across the membrane. This may include the rate of internal movement, reorientation, or both. Direct or repeated treatments with cocaine followed by a withdrawal period of 24 hr or 2 weeks differentially affects the kinetics of turnover without changing the kinetics of substrate recognition. Direct presentation of cocaine to the transporter externally reduces substrate turnover, whereas repeated treatments followed by a period of withdrawal results in increased turnover. Thus, it seems likely, given data in the literature suggesting a lack of effect of cocaine on the density of the striatal transporter for dopamine [8, 9, 21], that withdrawal from repeated use of cocaine results in kinetic activation of the transporter for dopamine at an intramembrane translocation step.

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