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A MULTISUBSTRATE MECHANISM OF STRIATAL DOPAMINE UPTAKE AND ITS INHIBITION BY COCAINE

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Abstract—A study of Na^+ and Cl^- as co-substrates in dopamine uptake into striatal suspensions and inhibition of dopamine uptake by cocaine was made by monitoring the initial velocity of the uptake of exogenously added non-radioactively labeled dopamine using a rotating disk electroanalytical technique with 50 msec resolution. Dopamine, in the concentration range of 0.025 to 4.00 μM, was found to be taken up rapidly into the tissue phase of striatal suspensions following the apparent zero order rate law for the first 25 sec. The observed, dopamine concentration-dependent, initial velocity data were first analyzed graphically using the Eadie–Hofstee transformation of the Michaelis–Menten kinetic equation and, subsequently, using all of the velocity data and the results of the graphical analyses, by non-linear curve fitting. Dopamine uptake was found to be first order in dopamine with a V_{max} of 582 pmol/sec/g wet weight and a K_m of 1.2 μM. The results of experiments in which choline and isethionate were substituted for Na^+ and Cl^-, respectively, suggested that the uptake process is second order in Na^+ and first order in Cl^-. Multisubstrate analyses of the initial velocities of uptake over the concentration range of 0.025 to 1.5 μM dopamine suggested that the mechanism of binding of dopamine to the uptake carrier is a partially random, sequential mechanism where dopamine or Na^+ binds first with the uptake carrier and Cl^- binds last. Cocaine was found to uncompetitively inhibit dopamine uptake and competitively inhibit both Na^+ and Cl^- binding (apparent K_m values: 131 and 51 mM, respectively), suggesting that the mechanism of cocaine inhibition may be to bind to the dopamine occupied uptake carrier complex at the Na^+ binding site.

Cocaine [3-(benzoyloxy)-8-methyl-8-azabicyclo-[3.2.1] octane-2-carboxylic acid methyl ester], a naturally occurring alkaloid in the coca shrubs, *Erythroxylon coca* and *Erythroxylon novogranatense*, has been found to be a local anesthetic and psychomotor stimulant having significant potential for abuse [1]. Its mechanism of action in relation to its psychomotor stimulant and abuse potential is thought to be related, at least in part, to its ability to inhibit the neuronal reuptake of dopamine in the central nervous system [1–3]. The uptake process for dopamine is known to require involvement of Na^+ and Cl^- and although a number of reports have appeared in which the inorganic ion dependency of dopamine uptake has been studied [4–8] and dopamine uptake inhibition by cocaine has been demonstrated [1, 8, 9], to our knowledge, no study of the multisubstrate transport mechanism of dopamine uptake and its blockade by cocaine has appeared. Applying this type of analysis and its results to the dopamine uptake system in the central nervous system may be particularly important in the elucidation of mechanistic differences that may define pharmacological actions of different dopamine uptake inhibitors. For example, it has been shown that the dopamine uptake inhibitor, mazindol, may have some utility in the treatment of cocaine addiction [10]; its behavioral profile in humans does not appear to include the potential for abuse [10, 11] and its effects are not additive with cocaine [11]. In addition, it does not appear to bind at the same binding site as cocaine although differences in binding of these two ligands to different affinity states of the recognition site could exist [12]. Thus, a basic biochemical mechanism of dopamine uptake inhibition, based on an actively transporting system, would be useful as a model in studies of mechanistic and functional differences between structurally and behaviorally dissimilar dopaminergic neuronal uptake inhibitors.

Herein we describe the results of our recent work using subsecond to second monitoring of the initial velocities of non-radioactively labeled dopamine uptake into rat striatal suspensions [13–15] to define the multisubstrate mechanism of dopamine uptake. In this current study our goals were to: test initial, time resolved velocity data of non-radioactively labeled dopamine uptake into striatal suspensions for adherence to Michaelis–Menten kinetics; confirm that the rapid components of uptake of non-radioactively labeled dopamine into striatal suspensions are dependent on extracellular Na^+ and Cl^-; define the apparent uptake inhibition mechanism of cocaine in this tissue preparation; propose a multisubstrate mechanism of the dopamine uptake process; and define how the multisubstrate uptake mechanism is altered by cocaine. A preliminary report of some of the results of this work was made at a recent meeting of the New York Academy of Sciences [14].

MATERIALS AND METHODS

The uptake model. Dopaminergic chemical neuro-

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transmission is thought to occur initially by the release of dopamine from a presynaptic terminal into the synaptic area. Following release, dopamine interacts with receptors on follower cells to exert its influence(s). This action is believed to be terminated by uptake of the released, extracellular dopamine into cells and diffusion from the local area [16, 17]. The kinetic conditions chosen for the studies and the methods of analysis of the resulting data were designed to take these simple physical features into account. First, a rotating disk electrode (RDE*) system was chosen in order to continuously measure, in the extracellular compartment, the uptake of dopamine with kinetics unperturbed by the timing of extracellular diffusion [13–15]. Second, dopamine was added as an "instantaneous" pulse to mimic the release process. Finally, the amount of dopamine added was chosen so that it could be quantitatively taken up into the tissue phase.

The amount of dopamine added was chosen by first noting that Cunnane [18] has suggested that terminals can release either 1–5% or all of their contents following an action potential. Since the total striatal tissue concentration of dopamine is *ca.* 69 nmol/g wet wt [19], then the amount of dopamine that should be added to be consistent with the model of Cunnane is 28 pmol to 2.8 nmol of dopamine to *ca.* 40 mg of striatal tissue in the 500-μL incubation chamber (see below). Thus, the resulting "instantaneous" extracellular dopamine concentration could range between 56 and 5600 nM. As will be shown, this experimental design resulted in experimental conditions in which the physical features of uptake were met, kinetic results amenable to Michaelis–Menten analyses were obtained, and cocaine inhibition of the uptake process confirmed that the uptake observed was pharmacologically relevant. Furthermore, the multisubstrate involvement of Na^+ and Cl^- could be kinetically demonstrated and a kinetics based multisubstrate mechanism of dopamine uptake and its inhibition by cocaine could be defined.

Rapid detection of dopamine uptake. Time resolved uptake of dopamine was measured in 500-μL striatal suspensions using an RDE voltammetric method as previously described [13, 15]. Briefly, the temperature of the striatal suspension in the glass incubation chamber, of the same design as illustrated previously [13], was controlled by a recirculating water bath (Lauda RM6, Brinkmann, Westbury, NY). The RDE was purchased from Pine Instruments Inc. (Grove City, PA), the detector electrode was a 3-mm diameter glassy carbon disk mounted in a 5-mm diameter Teflon shield, and the potentiostat was a Bioanalytical Systems (West Lafayette, IN) LC-4A amperometric detector (response time = 20 msec). The potential of the electrode was set at +450 mV vs Ag/AgCl in order to oxidatively detect dopamine as a limiting current, i_L [13]. The dopamine uptake profile was recorded as voltage output (proportional to i_L) from the potentiostat vs time (t) and stored onto a Nicolet (Madison, WI) 2090 digital storage oscilloscope. The uptake profiles were analyzed from tracings obtained from hard copy printed from the oscilloscope floppy disk storage system onto paper with a Houston Instruments (Austin, TX) Omnigraphic X-Y recorder. The voltage–t output values were converted to i_L–t values, and i_L values were converted to extracellular dopamine concentrations, $[DA]_o$, using a calibration factor. The calibration factor was obtained by measuring the i_L as a function of the $[DA]_o$, ranging from 0.025 to 4.00 μM. The recorded DA uptake profiles were analyzed as $[DA]_o$ vs t. The temporal resolution of $[DA]_o$ measurements by the RDE was *ca.* 40 msec [13] and uptake $[DA]_o$ vs t data were recorded with 50 msec resolution.

Preparation of striatal suspensions and protocol for measuring dopamine uptake. The preparation of striatal suspensions for use in the RDE system has been described previously [15]. Briefly, striatal tissues were obtained from 275–350 g male Sprague–Dawley albino rats by rapid decapitation and dissection on an ice-cold watch glass. The tissue was weighed on a millibalance and chopped by hand on a clean ice-cold watch glass using a razor blade. The wet weight of a typical striatal tissue sample from a single striatum was in the 35–40 mg range. (The average weights and numbers of striata used are given in the description of each series of experiments.) The dissected and weighed tissue was then placed into 500 μL of oxygenated 37° physiological buffer and washed with the aid of a Hamilton (Reno, NV) Microlab M programmable diluter/dispenser equipped with a 1000-μL gas tight syringe. Each step of the washing sequence was programmed with an operating speed of 12 sec. A single washing sequence consisted of the following steeps: (1) removal of 250 μL of buffer, (2) addition of 500 μL of fresh oxygenated buffer, (3) removal of five sequential 100-μL volumes of buffer, and finally (4) the addition of 250 μL of physiological buffer to bring the total volume in the chamber back to 500 μL. This sequence of steps was repeated five times. After the last wash the RDE was placed into the 37° thermostatted incubation chamber, a stream of a 95% O_2/5% CO_2 gas mixture was gently directed across the surface of the buffer/tissue mixture, electrode rotation was begun at 2000 rotations per min, and the potentiostat was activated. The rotation of the electrode disrupts tissue organization to produce a striatal whole tissue suspension [15]. The suspension was allowed to incubate under these conditions for *ca.* 3 min prior to experimentation. At the end of the preincubation period a small quantity of dopamine dissolved in H_2O was added to the incubate and the rate of disappearance of $[DA]_o$ was monitored by the potentiostat. The output was stored onto the digital oscilloscope. In studies of the effects of cocaine on dopamine uptake the drug was added 30 sec prior to experimentation.

Preparation of striatal synaptosomes. Striatal synaptosomes were prepared with minor modifications to the procedure described by Booth and Clark [20]. Striata from six rats were pooled and

* Abbreviations: ANOVA, analysis of variance; cc, correlation coefficient; DA, dopamine; i_L, limiting current; int, intercept; k, rate constant; K_m, Michaelis–Menten constant; K_i, inhibition constant; K_s, substrate dissociation constant; m, slope; RDE, rotating disk electrode; t, time; and x, order of reaction.

minced by hand with a razor blade, added to 10 mL of isolation buffer at pH 7.4 (composition, in mM: 320 sucrose, 1.0 EDTA, and 10 Tris–HCl and washed with the isolation buffer as described above. The washed minces were homogenized by hand in a Pyrex 7725 tissue grinder (Corning Inc., Corning, NY) and diluted to 80 mL with isolation buffer. The homogenate was divided into four fractions and spun at 1300 *g* for 3 min at 4° in a Sorvall RC-5B centrifuge equipped with a SA 600 fixed angle rotor (Du Pont, Wilmington, DE). The supernatant was collected and centrifuged at 17,000 *g* for 10 min at 4° in the centrifuge. The resulting pellet was resuspended in 5 mL of isolation buffer and diluted to 30 mL with a 12% Ficoll-containing solution [composition: 12% (w/w) Ficoll, 320 mM sucrose, and 50 μM EDTA, pH 7.4]. Then the supernatant was collected, manually homogenized in a Teflon Potter–Elvehjem tissue grinder (Wheaton, Millville, NJ) and added to 8 mL of the 12% Ficoll solution. The homogenized tissue was divided into two 19-mL fractions, placed into centrifuge tubes, and layered with 9.5 mL of 7.5% (w/w) Ficoll-containing solution [composition: 7.5% (w/w) Ficoll, 320 mM sucrose, and 50 μM EDTA, pH 7.4] and 9.5 mL of isolation buffer. The tubes were centrifuged at 99,000 *g* for 30 min at 4° in a Beckman (Fullerton, CA) L5-65 preparative ultracentrifuge equipped with an SW 27 swinging bucket rotor. The synaptosomes were collected, diluted to 30 mL in isolation buffer, and manually homogenized with the Teflon tissue grinder. The resulting homogenate was diluted to 60 mL and centrifuged at 5500 *g* for 10 min at 4° in the Sorvall RC-5B centrifuge. The resulting pellet was resuspended in 4 mL of physiological buffer to achieve a final protein concentration of 11.4 mg protein/mL. The protein assays were made by the dye binding method of Bradford [21] using 1 mg/mL bovine serum albumin (Sigma, St. Louis, MO) as a standard. Spectrophotometric measurements were made with a Perkin–Elmer 320 ultraviolet/visible spectrometer.

Measurement and analysis of rate data. Once a steady baseline had been obtained in either the striatal whole tissue suspension or synaptosomes, 10 μL of H_2O (for a control) or cocaine solution in H_2O (to give a final concentration of 0.5, 1.0, 2.0, 4.0, and 8.0 μM) was added to the preparation. Increasing amounts of exogenous non-radioactively labeled dopamine was then introduced into the suspension using a Hamilton CR-700 constant flow rate syringe, to give final concentrations of 25, 50, 100, 250, 500, 1000, 1500, 2000, 2500, 3000, or 4000 nM. The resulting dopamine uptake rates were measured as initial apparent zero order velocities from approximately the first 25 sec of the uptake profile (*vide infra*).

The average dopamine uptake velocities were first analyzed graphically using the Eadie–Hofstee transformation of the Michaelis–Menten equation [22],

$$v = V_{max} - \{K_m(v)/[DA]^x\} \quad (1)$$

where v is the transport velocity, V_{max} is the maximal transport velocity, K_m is the Michaelis–Menten constant, and x is the order of the reaction. The resulting kinetic parameters, V_{max}, K_m and x, were used as initial "guesses" in non-linear regression analyses of the raw initial velocity data (using a Quasi-Newton based fitting routine, SYSTAT, Evanston, IL [23]) in which Eq. 1 was employed as the model. Inhibition constants, K_i, were estimated by non-linear curve fitting using the form of the Michaelis–Menten equation modified for the type of inhibition observed [22, 24]. Unless noted otherwise, the errors indicated for the rate parameters are standard errors of regression. Other statistical evaluations are identified where applied.

In the studies of the inorganic ion dependencies of dopamine uptake, Na^+ was substituted with choline [4, 8], Cl^- was substituted with isethionate [4, 8], and NaCl was substituted with sucrose to maintain osmolality [4]. The resulting initial velocity data were analyzed as described above for dopamine.

Analysis of the multisubstrate mechanism. The multisubstrate studies of the involvement of Na^+ and Cl^- in dopamine uptake were conducted using the methods for multisubstrate studies as outlined by Fromm [24]. Briefly, the ratio of $[Na^+]$ to $[Cl^-]$ was held constant at 1.15 to 1.00 while the initial, instantaneous $[DA]_o$ was varied from 50 to 1500 nM in separate experiments. The initial dopamine uptake velocities were measured and recorded as described above. The extracellular concentrations of Na^+ and Cl^- in the trials studied were (in mM): 150.0 and 130.8, 126.0 and 109.4, 106.0 and 91.9, 86.0 and 75.0, 66.0 and 57.6, and 46.0 and 40.1, respectively. The resulting data was plotted as $1/v$ versus $1/[DA]_o$. The slopes and/or intercepts of these plots were replotted with respect to $1/[Cl^-]_o$ and the resulting shape of the replot was used to identify the multisubstrate mechanism as described by Fromm [24].

Chemicals and solutions. Solutions were made in high purity water from a Barnstead Nanopure (Dubuque, IA) water purification system. The chemicals were reagent grade and used as received. Dopamine, choline chloride, and Ficoll 400-DL were purchased from the Sigma Chemical Co., sodium isethionate was obtained from Pfaltz & Bauer (Waterbury, CT), and cocaine hydrochloride was a gift from Dr. Peter Kalivas (Department of VCAPP, Washington State University). The uptake studies were conducted at pH 7.4 in a 95% O_2/5% CO_2 saturated physiological buffer composed of (in mM): 124 NaCl, 3.00 KCl, 1.24 KH_2PO_4, 1.30 $MgSO_4$, 2.50 $CaCl_2$, 26.0 $NaHCO_3$, and 10.0 glucose [15].

RESULTS

Figure 1 shows examples of $[DA]_o$ vs time profiles of the uptake of 1.00 μM of dopamine alone and in the presence of increasing concentrations of cocaine. Under control conditions, dopamine was found to be taken up rapidly by the striatal suspension with an apparent half-time of clearance of approximately 43 sec. Complete clearance, defined as removal of 98% of the added dopamine, occurred within 150 sec (Fig. 1A).

Kinetic analyses suggested that the clearance profile followed mixed order kinetics where the

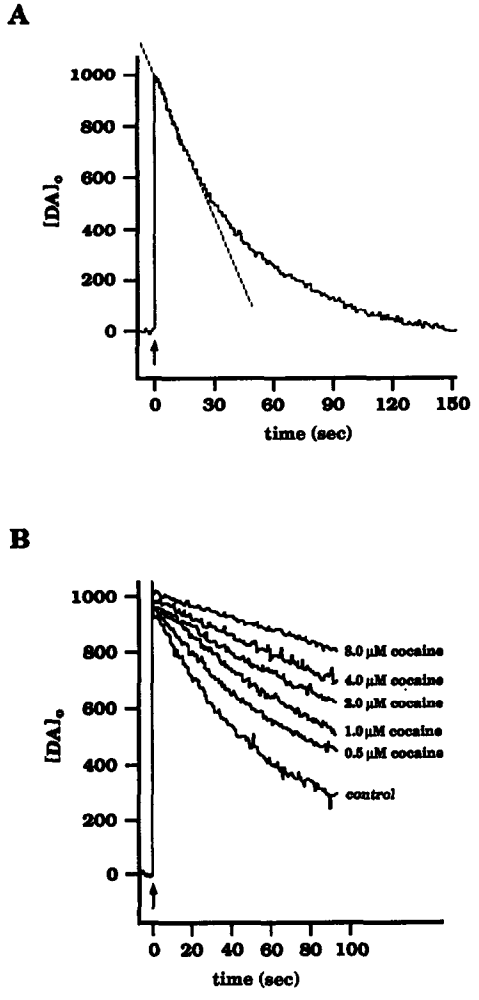

Fig. 1. Time course of uptake of exogenously added dopamine into striatal suspensions and its inhibition by cocaine. Panel A shows a typical profile of the clearance of $[DA]_o$ after addition (arrow) of an amount of dopamine required to achieve an instantaneous concentration of 1.0 μM. The profile was found to follow mixed order kinetics with an average overall half-time of approximately 45 sec. The initial 25 sec of the time course (dotted line) was found to follow apparent zero order kinetics ($[DA]_o$ vs t was linear, slope (m) = 18.0 ± 0.4 nM/sec (or 225 pmol/sec/g wet wt), intercept (int) = 1011 nM, and correlation coefficient (cc) = 0.995). The remainder of the profile followed apparent first order kinetics (ln $[DA]_o$ vs t was linear, m = 0.0264 ± 0.002 sec^{-1}, int = −13.61 ± 0.23, and cc = 0.989) with a half-time of 25 sec. Complete clearance (98%) was observed by *ca.* 150 sec. In Panel B separate striatal suspensions were treated (one dose per suspension) with increasing concentrations of cocaine approximately 30 sec prior to the introduction (arrow) of enough dopamine to obtain an instantaneous 1.0 μM pulse of dopamine. At each concentration of cocaine, the uptake process was found to be mixed order as described above and the clearance time was observed to increase in both kinetic regions in a cocaine concentration-dependent manner. In subsequent experimentation, the initial velocity of dopamine uptake, expressed as pmol/sec/g wet wt, was estimated from the apparent zero order region of the profile.

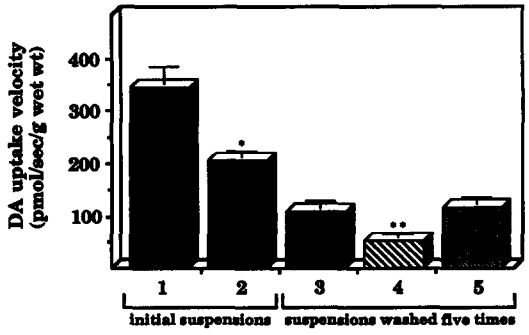

Fig. 2. Reversal of cocaine's inhibition of dopamine uptake by washing with physiological buffer. Individual striatal suspensions were incubated with either no cocaine or 1.0 μM cocaine prior to the introduction of 1.5 μM dopamine. The dopamine uptake velocities were recorded, and then the suspensions were washed with five washing sequences as described in Materials and Methods. After the final washing sequence either no cocaine or 1.0 μM cocaine was added to the suspensions just before a second 1.5 μM pulse of dopamine. Key: (1) control dopamine uptake velocity observed in unwashed striatal suspensions; (2) dopamine uptake velocity observed in unwashed striatal suspensions after introduction of 1.0 μM cocaine; (3) dopamine uptake velocity observed in control suspensions after five washes with fresh physiological buffer; (4) dopamine uptake velocity observed in washed suspensions after introduction of 1.0 μM cocaine; and (5) dopamine uptake velocity observed in suspensions to which 1.0 μM cocaine had been added prior to washing with physiological buffer; (*) indicates a statistical difference relative to 1 (via t-test, $P \leq 0.01$); and (**) indicates statistical differences (tested using ANOVA followed by Scheffe's F-test ($P \leq 0.010$)) for 4 relative to 3 and 5. Values are means ± SD, N = 4 for each bar, The average wet tissue weight was 40.3 ± 0.9 mg (mean ± SD, N = 20).

initial $[DA]_o$ vs t profile from zero to *ca.* 25 sec was found to be linear, thereby having the characteristics of an apparent zero order initial velocity process. The kinetics of the clearance profile changed from apparent zero order kinetics to apparent first order kinetics at an $[DA]_o$ value of *ca.* 600 nM (see Fig. 1A). All of the results of subsequent experimentation were based on rate measurements of $[DA]_o$ vs t profiles measured in the temporal region defined by zero order kinetics. The clearance of dopamine was inhibited by cocaine in a concentration-dependent manner (Fig. 1B) and was found to be reversed by washing cocaine-treated suspensions with fresh cocaine-free physiological buffer (Fig. 2).

The initial velocity of dopamine uptake was found to be dependent on the instantaneous $[DA]_o$ value, saturable and described by the Michaelis–Menten expression (Eq. 1) with a first order dependence on $[DA]_o$ (Fig. 3A). The K_m for dopamine uptake, obtained by non-linear curve fitting, was found to be 1.24 ± 0.06 μM. The V_{max} was found to be 582 ± 11 pmol/sec/g striatal wet wt, and the reaction order with respect to dopamine was 0.99 ± 0.02. When characterized in synaptosomal preparations, the K_m and V_{max} values were found to be

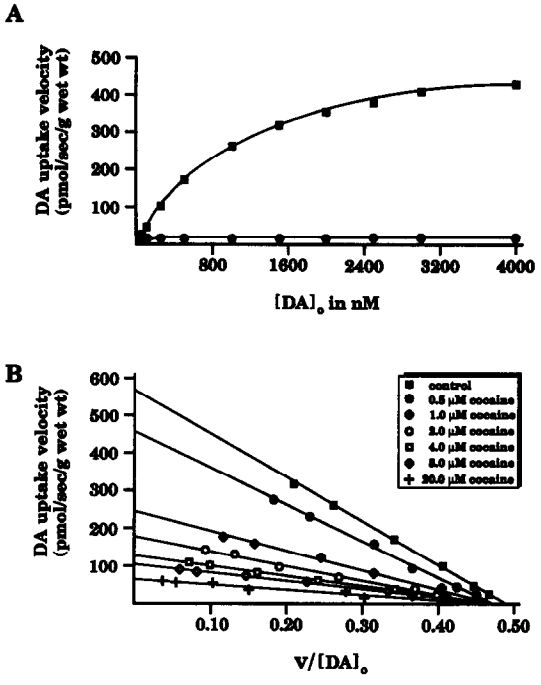

Fig. 3. Velocity of the uptake of dopamine as a function of $[DA]_o$ and its inhibition by cocaine. Panel A shows the initial velocity of the uptake of dopamine as a function of $[DA]_o$. Iterative non-linear curve fitting of the rate data (closed squares) to the Michaelis–Menten equation is illustrated by the line of the upper curve. Because of the scaling of the plot, the uptake velocity of 25 nM dopamine, 11.7 ± 2.5 pmol/sec/g wet wt, is not shown. Equation 1 and the rate parameters (see Results) predict a value of 11.5 ± 0.6 pmol/sec/g wet wt at this $[DA]_o$ value. The closed circles show the apparent velocity of the disappearance of dopamine due to oxidation by dissolved O_2 plus the RDE in the absence of striatal tissue. These latter data were normalized by the average striatal tissue weight [39.9 ± 0.4 mg (mean ± SD, N = 16)] used in this series of experiments. Each datum point is the mean ± SD of eight replicate trials. In this and subsequent studies the error bars were found to fall within the dimensions of the symbols. Panel B shows Eadie–Hofstee plots of the rate data of dopamine uptake in the presence of cocaine. Individual striatal suspensions were incubated with 0.0, 0.5, 1.0, 2.0, 4.0, 8.0, or 20 μM cocaine and increasing $[DA]_o$ 50 to 1500 nM, was added to each suspension. The values of the x-intercepts were statistically indistinguishable and the value of V_{max}/K_m was constant (average value, 462 ± 16 pmol/sec/g wet wt/μM, N = 48). The experimentally defined Michaelis–Menten equation modified for uncompetitive inhibition predicts 98 ± 8% inhibition of dopamine uptake at 100 μM cocaine (data not shown). Each datum point is the mean ± SD of eight separate experiments. The average weight of striatal tissue used was 38.9 ± 1.6 mg (mean ± SD, N = 48).

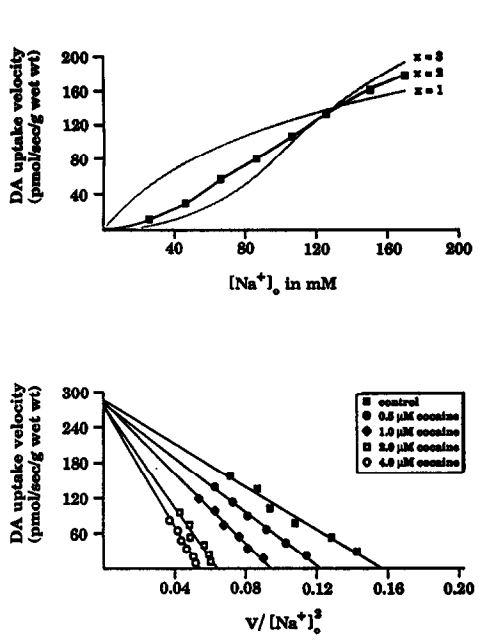

Fig. 4. Velocity of the uptake of dopamine as a function of $[Na^+]_o$ and the effect of cocaine on the involvement of Na^+ in the uptake process. Panel A shows the rate of uptake of 500 nM dopamine as a function of $[Na^+]_o$ (substituted by choline). The iterative fit of the rate data to the Michaelis–Menten equation (solid line) suggests that the best fit apparent reaction order of the Na^+ effect is 2. The dashed lines show the expected uptake rate vs $[Na^+]_o$ lines for reaction orders 1 and 3. At $[Na^+]_o$ = 0.5 mM the v value was 0.004 ± 0.002 pmol/sec/g wet wt, a value indistinguishable from zero. Each datum point is the mean ± SD of eight separate experiments. The average wet weight of the striatal tissue used was 39.5 ± 1.2 mg (mean ± SD, N = 64). Panel B shows Eadie–Hofstee plots of the uptake of 500 nM dopamine in the presence of 0.0, 0.5, 1.0, 2.0, or 4.0 μM cocaine in separate striatal suspensions in which $[Na]_o$ had been reduced also by replacement with choline. The y-intercepts (V_{max}) of the six lines were statistically indistinguishable (average value, V_{max} = 293 ± 11 pmol/sec/g wet wt). The average striatal tissue weight used in these studies was 39.7 ± 1.4 mg (mean ± SD, N = 96).

1.36 ± 0.06 μM and 418 ± 8 pmol/sec/g protein, respectively. The K_m for DA uptake in the striatal suspension was found to be statistically indistinguishable from the K_m value observed in the synaptosomal preparation. The V_{max} obtained in the suspension was approximately 9.3 times larger than the value observed in the synaptosomal preparation when normalized on the basis of protein content (0.15 g protein/g wet wt). The results of analyses of Eadie–Hofstee plots suggested that cocaine is an uncompetitive inhibitor of dopamine uptake (Fig. 3B). When this set of dopamine uptake data was fit to the Michaelis–Menten equation for uncompetitive inhibition [22], it was found that the V_{max} for dopamine uptake, the K_m for dopamine, and the K_i for cocaine were 587 ± 8 pmol/sec/g striatal wet wt, 1.27 ± 0.04 μM, and 1.05 ± 0.03 μM, respectively.

Substitution of $[Na^+]_o$ with choline diminished the velocity of dopamine uptake in a concentration-dependent manner with an apparent reaction order in Na^+ of 1.96 ± 0.05 (Fig. 4A). The K_m was found to be 131 ± 5 mM, a value very close to the $[Na^+]_o$ of the physiological buffer, and the V_{max} was 282 ± 11 pmol/sec/g wet wt. Na^+ was found to non-

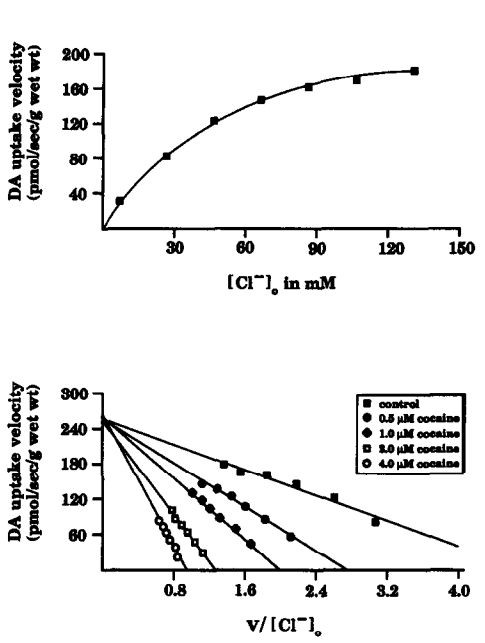

Fig. 5. Velocity of the uptake of dopamine as a function of $[Cl^-]_o$ and the effect of cocaine on the involvement of Cl^- in the dopamine uptake process. Panel A shows the rate of uptake of 500 nM dopamine as a function of $[Cl^-]_o$ (substituted by isethionate). The iterative fit (solid line) of the rate data (closed squares) to the Michaelis–Menten equation suggests that the apparent order of the Cl^- effect is 1. Each datum point is the mean ± SD of eight separate experiments. The average wet weight of striatal tissue used was 40.2 ± 1.0 mg (mean ± SD, N = 56). Panel B is an Eadie–Hofstee plot of the uptake rate of 500 nM dopamine in the presence of 0.0, 0.50, 1.0, 2.0, or 4.0 μM cocaine incubated in physiological buffer in which the $[Cl^-]_o$ was reduced also by replacement with isethionate. The y-intercepts (V_{max}) of the six lines were statistically indistinguishable (average value, 250 ± 6 pmol/sec/g wet wt). The average weight of striatal tissue used was 40.0 ± 1.0 mg (mean ± SD, N = 96).

competitively inhibit dopamine uptake since the V_{max} changed while the K_m was observed to remain constant at 1.11 ± 0.07 μM. Eadie–Hofstee analyses of dopamine uptake velocities obtained in the presence of increasing concentrations of cocaine added to striatal suspensions in which Na^+ had been replaced with choline suggested that cocaine competitively inhibits the involvement of Na^+ in the uptake process (Fig. 4B). The K_i for cocaine was found to be 1.67 ± 0.08 μM, by non-linear fitting of the raw velocity data to the Michaelis–Menten competitive inhibition model [22].

Figure 5A shows that Cl^- substitution by isethionate was also found to diminish the velocity of dopamine uptake in a concentration-dependent manner; however, the apparent reaction order was found to be unity (0.99 ± 0.02). The V_{max} and K_m were 251 ± 8 pmol/sec/g wet wt and 51 ± 4 mM, respectively. Cl^- was found to uncompetitively influence dopamine uptake since the V_{max}/K_m value was constant with an average value of 478 ± 16 pmol/sec/g wet wt/μM. Eadie–Hofstee analyses (Fig. 5B) suggested that cocaine competitively inhibits Cl^- involvement in the dopamine uptake process and the K_i (via non-linear regression analyses) was found to be 0.82 ± 0.04 μM. Table 1 summarizes the numerical results of the analyses of the inhibition of dopamine uptake by cocaine.

The results of multisubstrate analyses, shown in Fig. 6, in combination with the observations that cocaine reversibly inhibited the uptake of dopamine uncompetitively, Na^+ competitively, and Cl^- competitively as well as the findings that Na^+ influenced dopamine uptake noncompetitively whereas the influence of Cl^- was uncompetitive suggest that: (1) the uptake mechanism of dopamine is a sequential, partially random process where Na^+ and dopamine can bind randomly to the uptake carrier, and Cl^- binds last prior to dopamine transport; and (2) cocaine inhibits the uptake of dopamine by binding at the Na^+ binding site on the carrier following the binding of dopamine.

DISCUSSION

The studies described here were intended to model *in vitro* the uptake condition(s) throught to be operational in dopaminergic chemical neurotransmission *in vivo* where the neurotransmitter is instantaneously released by the nerve terminal (mimicked by exogenous addition of dopamine) and then is *quantitatively* recaptured by uptake processes in the tissue in local proximity to the neuron or neurons which released the neurotransmitter. This entire process was monitored with 50 msec resolution and any extracellular diffusional influence on the time course of clearance is assumed to be eliminated by the rapid (*ca.* 40 msec mixing time) forced convective property of the RDE in the 500 μL incubation chamber [13, 15]. In addition, under the conditions employed, the $[DA]_o$ on the outer membrane surface in close proximity to the uptake site is maintained at a more constant value than occurs under more static incubation conditions in which diffusive mass transport to the uptake sites contributes to the kinetics of the overall process. Two other conditions should be noted that distinguish this experimental approach. The first is that the clearance of $[DA]_o$ was measured as an apparent unidirectional flux from the extracellular compartment (as opposed to measurements of accumulation into tissue) under non-steady state conditions, and the second is that the intracellular metabolic and sequestration pathways were intact.

Comments on the kinetic analyses of dopamine uptake. The results of these studies suggest that non-radioactively labeled dopamine is taken up avidly into striatal suspensions (with half-time ≤ 45 sec) by a saturable, mixed order process (initially zero order with a change to apparent first order) which was described by Michaelis–Menten kinetics. Complete clearance of a concentration pulse of dopamine equivalent to the apparent K_m value was found to occur within 150 sec. Analyses of the mixed order profile of the uptake of $[DA]_o$ can provide an estimate of the apparent density of dopamine up-

Table 1. Summary of the kinetic parameters of dopamine uptake in rat striatal suspensions measured by the RDE*

Conditions of uptake of exogenous dopamine	[Cocaine] (μM)	V_{max} (pmol/sec/g wet wt)	K_m (μM)	V_{max}/K_m (pmol/sec/g wet wt/μM)	Number of trials performed
Physiological buffer	Control	582 ± 11	1.24 ± 0.06	470 ± 9	8
Physiological buffer	0.5	471 ± 15	1.06 ± 0.07	444 ± 33	8
Physiological buffer	1.0	240 ± 4	0.50 ± 0.02	480 ± 21	8
Physiological buffer	2.0	183 ± 5	0.42 ± 0.03	436 ± 33	8
Physiological buffer	4.0	131 ± 2	0.30 ± 0.01	437 ± 16	8
Physiological buffer	8.0	101 ± 2	0.20 ± 0.01	505 ± 27	8
Buffer with choline†	Control	282 ± 11	(131 ± 5) × 10^3	(2.15 ± 0.12) × 10^{-3}	8
Buffer with choline	0.5	321 ± 24	(169 ± 10) × 10^3	(1.90 ± 0.18) × 10^{-3}	8
Buffer with choline	1.0	313 ± 19	(187 ± 8) × 10^3	(1.67 ± 0.12) × 10^{-3}	8
Buffer with choline	2.0	280 ± 35	(206 ± 18) × 10^3	(1.36 ± 0.21) × 10^{-3}	8
Buffer with choline	4.0	303 ± 44	(240 ± 22) × 10^3	(1.26 ± 0.22) × 10^{-3}	8
Buffer with isethionate‡	Control	251 ± 8	(51 ± 4) × 10^3	(4.92 ± 0.42) × 10^{-3}	8
Buffer with isethionate	0.5	283 ± 16	(109 ± 11) × 10^3	(2.60 ± 0.30) × 10^{-3}	8
Buffer with isethionate	1.0	224 ± 15	(102 ± 13) × 10^3	(2.20 ± 0.32) × 10^{-3}	8
Buffer with isethionate	2.0	214 ± 21	(154 ± 24) × 10^3	(1.39 ± 0.26) × 10^{-3}	8
Buffer with isethionate	4.0	252 ± 31	(260 ± 44) × 10^3	(0.97 ± 0.17) × 10^{-3}	8

* Precision is expressed as standard error of regression.
† Sodium was isosmotically replaced with choline chloride.
‡ Chloride was isosmotically replaced with sodium isethionate.

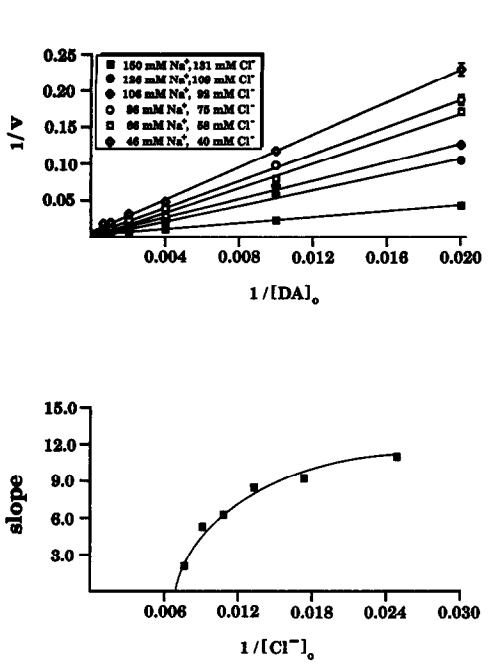

Fig. 6. Multisubstrate mechanistic studies of dopamine uptake. Panel A shows a Lineweaver–Burk plot of the uptake rate of dopamine at increasing $[DA]_o$ values in individual striatal suspensions incubated in physiological buffer in which the $[Na^+]_o$ to $[Cl^-]_o$ ratio was held constant at 1.15. The data represented show that these lines intersect. Panel B shows replots of the slopes from the lines in Panel A against $1/[Cl^-]_o$ in the buffer and were observed to be non-linear with non-zero intercepts. The average striatal wet tissue weight used in this set of studies was 40.0 ± 1.0 mg (mean ± SD, N = 48).

take sites (for comparison to binding data) since the $[DA]_o$ at which the zero order uptake profile changes to apparent first order is in the region where the $[DA]_o$ is close or equal to the concentration of uptake sites in the tissue phase of the suspension [25]. When normalized for the protein concentration in the suspension, the density of dopamine uptake sites was found to be 15 pmol/mg protein, a value that agrees with literature values of the number of high affinity uptake sites as assayed by [^{3}H]cocaine binding (average B_{max} = 10 pmol/mg protein, range 5.56 to 18.3 pmol/mg protein [26–28]).

The K_m values obtained from studies in striatal suspensions and synaptosomes were found to be within the 0.1 to 2.3 μM range of previously reported literature values for the K_m of dopamine uptake [29–32] and matches almost exactly the K_m values, 1.2 and 0.9 μM, recently reported for tritiated dopamine uptake in expressed clones of dopamine transporters [33, 34]. However, it should be noted that the reported K_m in our studies is considered a "formal" K_m strictly defined as the $[DA]_o$ at which the uptake process was half-maximal. The relevent kinetics of the disappearance of dopamine from the extracellular milieu due to transmembrane flux can be described by the model [35, 36],

$$DA + Carrier \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} DA\text{–}Carrier \xrightarrow{k_2} Carrier\text{–}DA$$

where k_1 and k_{-1} are rate constants of association and dissociation, respectively, of dopamine with the uptake carrier at the outside of the membrane, k_2 is the rate constant of translocation of the dopamine–carrier complex, and the dissociation of dopamine from the carrier is ignored [36]. The K_s, the affinity for dopamine with the carrier, is given by k_{-1}/k_1 and the K_m, the measured quantity, by

$$K_m = K_s + (k_2/k_1). \quad (2)$$

Thus, the K_m value obtained should be larger than the K_s [29]. Furthermore, intracellular metabolism could increase by mass action the apparent k_2 value, thereby increasing the apparent K_m as observed by Near *et al.* [32]. Finally, other investigators have used longer incubation times (minutes), thereby increasing the likelihood that equilibrium at the internal surface of the membrane is operational and the carrier could be transporting dopamine toward the outside as well as internally (bidirectional transport). The apparent K_m under this condition would be approximated by $(k_{-1} + k_2)/(k_1 + k_{-2})$, where k_{-2} is the rate constant for movement of the carrier from inside to outside. This condition should result in different K_m values than those observed under initial rate conditions [36]. Thus, it is reasonable to find a range of apparent or "formal" K_m values which depend on experimental conditions [36, 37].

The value of V_{max} in the striatal suspensions of whole tissue was found to be approximately a factor of 2.5 times larger than previously reported values obtained in synaptosomal preparations in which data was normalized also on the basis of wet tissue weight [7]. However, the value obtained from experiments in synaptosomal preparations in this study (0.42 pmol/sec/mg protein) was found to be in relatively close agreement to that reported by Yi and Johnson [38] (0.6 pmol/sec/mg protein).

The findings that dopamine uptake is dependent on Na^+ and Cl^- having reaction orders of 2 and 1, respectively, are in agreement with the results of previously published studies [4–8]. In addition, it should be noted that the 1.0 μM value for the K_i of inhibition of dopamine uptake by cocaine also agrees with results of previous studies [8, 9].

Comments on the multisubstrate mechanism of dopamine uptake and its inhibition by cocaine. The results of the multisubstrate studies suggest that dopamine is taken up by a partially random sequential (as opposed to a ping pong) mechanism where dopamine or Na^+ binds to the carrier first in random order followed by the binding of Cl^- last. This series of steps is schematically illustrated in Fig. 7 and is different from the multisubstrate scheme of up-take of norepinephrine in the peripheral nervous system [37, 39–42] in several respects. First, the norepinephrine system is thought to involve a sequential, ordered mechanism with regard to norepinephrine and Na^+ binding with a first, rather than a second order, dependence on Na^+. Although

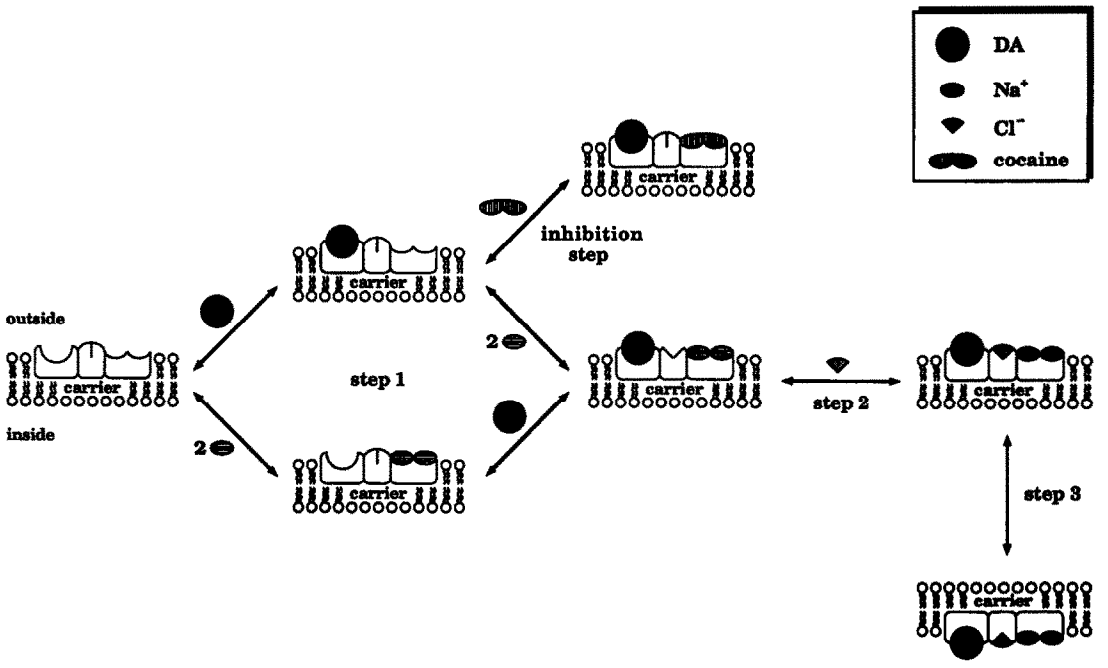

Fig. 7. Schematic illustration of the partially random, sequential multisubstrate mechanism of dopamine uptake in striatal tissues and how it may be influenced by cocaine. The sequence proceeds from left to right. In Step 1 dopamine or Na^+ binds to the uptake carrier in random order followed by Cl^- binding in Step 2. In Step 3 the carrier transports dopamine across the membrane. If cocaine is present, the process is inhibited by cocaine binding at the Na^+ binding site as illustrated in the *inhibition step*. Note: the illustration is highly schematic and is not intended to represent the structure of the carrier protein and its mechanism of translocation.

Na^+ effects are first order in the peripheral norepinephrine system, the finding of second order in the striatal dopamine uptake system agrees with that reported by Krueger [8] and has been observed in uptake processes for other neurotransmitter systems [43].

In this current study, cocaine was shown to inhibit dopamine uptake uncompetitively, whereas it competitively inhibited the Na^+ and Cl^- dependencies. Thus, the inhibition reaction appears to involve the binding of cocaine with the dopamine bound carrier by competition with a Na^+ binding site (Fig. 7). The involvement of the Na^+ binding site in the mechanism of inhibition of the active dopamine transporter by cocaine is intriguing since it has been shown that the binding of [^{3}H]cocaine to the cocaine binding site in the striatum is Na^+ dependent and is thought to bind at the transporter site [27]. In addition, the finding that cocaine competitively inhibited Na^+ in the multisubstrate mechanism for dopamine is in agreement with findings of Zeitner and Graefe [40] in their studies of cocaine inhibition of norepinephrine uptake in the peripheral nervous system.

The finding that cocaine is also competitive with Cl^- may be a consequence of the inhibition of Na^+ for its binding site since the Cl^- binding to the uptake carrier appears to occur in a step following Na^+ binding [44]. However, this assertion cannot be proved in this model. Further analyses based on literature information is difficult at the present time since it has been reported by Ungell *et al.* [42] that cocaine inhibition of norepinephrine uptake is Cl^- dependent and appears to be competitive with a presumed Cl^- binding site, as reported here for the dopamine system, whereas Diliberto *et al.* [5] have suggested that uptake inhibitors do not require Cl^- for inhibiting dopamine uptake. An alternate explanation may be that the Na^+ and Cl^- binding sites are physically close to one another and thus cocaine acts as a bisubstrate analog in the actively transporting dopamine uptake system. However, these hypothetical possibilities cannot be confirmed or rejected at the present time.

The literature findings that cocaine binding requires Na^+ [28] and competes with Na^+ in the multisubstrate model developed here provide intuitive appeal to the finding that cocaine inhibits dopamine uptake uncompetitively. However, it should be noted also that these findings contrast with the results of Krueger [8] and Missale *et al.* [3] who showed competitive and non-competitive inhibition of striatal dopamine uptake by cocaine, respectively. There is no simple explanation to account for these discrepancies. However, the kinetic conditions used in the measurement of dopamine uptake in the studies described here were different from those used in the cited work. First, they are based on a system that measures changes in the total analytical concentration of dopamine in the

extracellular medium as opposed to radiotracer concentrations only. Second, the measurements are based on analysis of time resolved continuously measured kinetics of a transient extracellular pulse of dopamine in a system in which metabolism was intact. Finally, the experimental conditions allowed for the measurement of the kinetics of the quantitative uptake of dopamine. These experimental conditions may better mimic functional conditions in chemical neurotransmission than the approach to steady-state equilibrium conditions [45] and measurements of intracellular steady-state accumulation of dopamine. Thus, the results obtained by the RDE voltammetric method should not be perturbed by competing multiple equilibria [45].

In summary, time resolved initial velocity data of quantitative uptake of non-radioactively labeled dopamine into dopaminergic tissues were found to be amenable to biochemical mechanistic evaluations. The initial velocity of dopamine uptake was found to be dependent on extracellular Na^+ as well as Cl^- and inhibited by cocaine. Finally, a multisubstrate mechanism of striatal dopamine uptake and its inhibition by cocaine have been suggested as a basis for future work. Studies to compare mechanistic differences between various catecholamine uptake inhibitors are currently underway.

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REFERENCES

1. Pitts DK and Marwah J, Neuropharmacology of cocaine: Role of monoaminergic systems. In: *Neurobiology of Drug Abuse, Monographs in Neural Sciences* (Ed. Marwah J), Vol. 13, pp. 34–54. Karger, Basel, 1987.
2. Kalivas PW, Duffy P, DuMars LA and Skinner C, Behavioral and neurochemical effects of acute and daily cocaine administration in rats. *J Pharmacol Exp Ther* **245**: 485–492, 1988.
3. Missale C, Castelletti L, Govoni S, Spano PF, Trabucchi M and Hanbauer I, Dopamine uptake is differentially regulated in rat striatum and nucleus accumbens. *J Neurochem* **45**: 51–56, 1985.
4. Shank RP, Schneider CR and Tighe JJ, Ion dependency of neurotransmitter uptake: Inhibitory effects of ion substitutes. *J Neurochem* **49**: 381–388, 1987.
5. Diliberto PA, Jeffs RA and Cubeddu LX, Effects of low extracellular chloride on dopamine release and the dopamine transporter. *J Pharmacol Exp Ther* **248**: 644–653, 1989.
6. Holz RW and Coyle JT, The effects of various salts, temperature, and the alkaloids veratridine and batrachotoxin on the uptake of [^{3}H]dopamine into synaptosomes from rat striatum. *Mol Pharmacol* **10**: 746–758, 1974.
7. Harris JE and Baldessarini RJ, The uptake of [^{3}H]-dopamine by homogenates of rat corpus striatum: Effects of cations. *Life Sci* **13**: 303–312, 1973.
8. Krueger BK, Kinetics and block of dopamine uptake in synaptosomes from rat caudate nucleus. *J Neurochem* **55**: 260–267, 1990.
9. Hyttel J, Inhibition of [^{3}H]dopamine accumulation in rat striatal synaptosomes by psychotropic drugs. *Biochem Pharmacol* **27**: 1063–1068, 1978.
10. Berger P, Gawin F and Kosten TR, Treatment of cocaine abuse with mazindol. *Lancet* **I**: 283, 1989.
11. Chait LD, Uhlenhuth EH and Johanson CE, Reinforcing and subjective effects of several anorectics in normal human volunteers. *J Pharmacol Exp Ther* **242**: 777–783, 1987.
12. Berger P, Elsworth JD, Reith MEA, Tanen D and Roth RH, Complex interaction of cocaine with the dopamine uptake carrier. *Eur J Pharmacol* **176**: 251–252, 1990.
13. Schenk JO, Patterson TA and McElvain JS, Rotating disk voltammetric measurements in neurobiology and neuropharmacology. *Trends Anal Chem* **9**: 325–330, 1990.
14. McElvain JS and Schenk JO, Studies of the mechanism of inhibition of the dopamine uptake carrier by cocaine *in vitro* using rotating disk electrode voltammetry. *Ann NY Acad Sci*, in press.
15. McElvain JS and Schenk JO, Dopamine autoreceptor blockade by haloperidol and the apparent dynamics of endogenous dopamine release from and reuptake into rat striatal suspensions. *Neuropharmacology*, in press.
16. Eccles JC and Jaeger JC, The relationship between the mode of operation and the dimensions of the junctional regions at synapses and motor-end organs. *Proc R Soc Lond [Biol]* **148**: 38–56, 1956.
17. Schenk JO and Bunney BS, Quantitative interpretations of *in vivo* chronoamperometry: Clues toward understanding the spatial and temporal influences of dopamine. In: *Voltammetry in the Neurosciences* (Ed. Justice JB), pp. 139–160. Humana Press, Clifton, NJ, 1987.
18. Cunnane TC, The mechanism of neurotransmitter release from sympathetic nerves. *Trends Neurosci* **7**: 247–253, 1984.
19. Patterson TA and Schenk JO, Effects of acute and chronic systemic administration of some typical antipsychotic drugs on turnover of dopamine and potassium ion-induced release of dopamine in the striatum of the rat *in vivo*. *Neuropharmacology* **30**: 943–952, 1991.
20. Booth RF and Clark JB, A rapid method for the preparation of relatively pure metabolically competent synaptosomes from rat brain. *Biochem J* **176**: 365–370, 1978.
21. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
22. Fersht A, *Enzyme Structure and Mechanism*, 2nd edn. W. H. Freeman & Co., New York, 1985.
23. Wilkinson L, *SYSTAT: The System for Statistics*. SYSTAT Inc., Evanston, 1990.
24. Fromm HJ, *Initial Rate Enzyme Kinetics*. Springer, New York, 1975.
25. Cappelos C and Bielski BHJ, *Kinetic Systems: Mathematical Description of Chemical Kinetics in Solution*, pp. 7–8. Wiley-Interscience, New York, 1972.
26. Reith MEA, Meisler BE, Sershen H and Lajtha A, Structural requirements for cocaine congeners to interact with dopamine and serotonin uptake sites in mouse brain and to induce stereotyped behavior. *Biochem Pharmacol* **35**: 1123–1129, 1986.
27. Calligaro DO and Eldefrawi ME, High affinity stereospecific binding of [^{3}H]cocaine and striatum and its relationship to the dopamine transporter. *Membr Biochem* **7**: 87–106, 1988.
28. Schoemaker H, Pimoule C, Arbilla S, Scatton B, Javoy-Agid F and Langer SZ, Sodium dependent [^{3}H]cocaine binding associated with dopamine uptake sites in the rat striatum and human putamen decrease after dopaminergic denervation and in Parkinsons disease.

Naunyn Schmiedebergs Arch Pharmacol **329**: 227–235, 1985.

29. Paton DM, Neuronal transport of noradrenaline and dopamine. *Pharmacology* **21**: 85–92, 1980.
30. Graefe K-H and Bonisch H, The transport of amines across the axonal membranes of noradrenergic and dopaminergic neurons. In: *Handbook of Experimental Pharmacology* (Eds. Trendlenberg U and Weiner N), Vol. 90/I, pp. 193–244. Springer, Berlin, 1988.
31. Cao C-J, Eldefrawi AT and Eldefrawi ME, ATP-regulated neuronal catecholamine uptake. *Life Sci* **47**: 655–667, 1990.
32. Near JA, Bigelow JC and Wightman RM, Comparison of uptake of dopamine in rat striatal chopped tissue and synaptosomes. *J Pharmacol Exp Ther* **245**: 921–927, 1988.
33. Shimada S, Kitayama S, Lin C-L, Patel A, Nanthakumar E, Gregor P, Kuhar M and Uhl G, Cloning and expression of a cocaine-sensitive dopamine transporter complementary DNA. *Science* **254**: 576–578, 1991.
34. Kilty JE, Lorang D and Amara SG, Cloning and expression of a cocaine-sensitive rat dopamine transporter. *Science* **254**: 578–579, 1991.
35. Parker EM and Cubeddu LX, Comparative effects of amphetamine, phenethylamine, and related drugs on dopamine efflux, dopamine uptake, and mazindol binding. *J Pharmacol Exp Ther* **245**: 199–210, 1988.
36. Stein WD, *The Movement of Molecules across Cell Membranes*, pp. 126–206. Academic Press, New York, 1967.
37. White TD, Models for noradrenaline uptake. In: *The Mechanism of Neuronal and Extraneuronal Transport of Catecholamines* (Ed. Paton DM) pp. 175–193. Raven Press, New York, 1976.
38. Yi S-J and Johnson KM, Effects of acute and chronic administration of cocaine on striatal uptake, compartmentalization, and release of [^{3}H]dopamine. *Neuropharmacology* **29**: 475–486, 1990.
39. Sammet S and Graefe KH, Kinetic analysis of the interaction between noradrenaline and Na^+ in neuronal uptake: Kinetic evidence for co-transport. *Naunyn Schmiedebergs Arch Pharmacol* **309**: 99–107, 1979.
40. Zeitner C-J and Graefe K-H, Sodium-dependence of the potency of inhibitors of the neuronal noradrenaline carrier in the rat vas deferens. *Naunyn Schmiedebergs Arch Pharmacol* **334**: 397–402, 1986.
41. Graefe K-H, Ion dependency of neuronal amine transport. In: *Progress in Catecholamines Research Part A: Basic Aspects and Peripheral Mechanisms* (Eds. Dahlstrom A, Belamker RH and Sandler M), pp. 131–134. Alan R. Liss, New York, 1988.
42. Ungell A-L, Oberleithner H and Graefe K-H, Chloride-dependence of the potency of inhibitors of the neuronal noradrenaline carrier in the rat vas deferens. *Naunyn Schmiedebergs Arch Pharmacol* **339**: 65–70, 1989.
43. Kanner BI, Bioenergetics of neurotransmitter transport. *Biochim Biophys Acta* **726**: 293–316, 1983.
44. Monod J, Changeux J-P and Jacob F, Allosteric protein and cellular control systems. *J Mol Biol* **6**: 306–329, 1963.
45. Cleland WW, The statistical analysis of enzyme kinetic data. *Adv Enzymol Relat Areas Mol Biol* **29**: 1–32, 1967.