Cationic Modulation of Human Dopamine Transporter: Dopamine Uptake and Inhibition of Uptake

NIANHANG CHEN, CLARENCE G. TROWBRIDGE, and JOSEPH B. JUSTICE, JR.

Department of Pharmacology, Nanjing Medical University, Nanjing, Peoples Republic of China (N.C.); and Department of Chemistry, Emory University, Atlanta, Georgia (N.C., C.G.T., J.B.J.)

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ABSTRACT

Effects of cations on dopamine (DA) uptake into cells expressing the human dopamine transporter and on inhibition of DA uptake by various substrates and inhibitors were investigated by using rotating disk electrode voltammetry. The Na⁺ dependence of DA uptake varied with Na⁺ substitutes, hyperbolic with Li⁺, almost linear at 1 μM DA but hyperbolic at 8 μM DA with choline, and sigmoidal with K⁺. With Na⁺ substituted by Li⁺, K_{max} decreased and V_{app} remained constant with increasing [Na⁺], whereas K_{Na+} decreased and V_{app} increased with increasing [DA], suggesting an ordered sequence with Na⁺ binding before DA. Similar trends for the Na⁺-DA interactions were observed in the presence of cocaine. Cocaine inhibited DA uptake solely by increasing K_{Na+} with its Kᵦ not significantly different at 55 and 155 mM [Na⁺], whereas it inhibited Na⁺ stimulation by reducing V_{app} more than K_{Na+} at 1 μM DA, and V_{app} only and less potently at 8 μM DA. Thus, cocaine may compete with DA, not with Na⁺, for the transporter, and might not follow a strictly ordered reaction with Na⁺. With Na⁺ substituted by K⁺, K_{DA} or K_{Na+} became insensitive to Na⁺ or DA. K⁺ impaired the DA uptake mainly by reducing V_{app}, but affected cocaine inhibition by elevating Kᵦ. Despite their different patterns for inhibiting DA uptake, nontransportable inhibitors cocaine, methylphenidate, mazindol, and 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenyl-2-propyl)piperazine (GBR12909) showed similarly modest Na⁺ dependence in their Kᵦ values. In contrast, substrates DA, m-tyramine, and amphetamine displayed a similarly stronger Na⁺ requirement for their apparent affinities.

The dopamine (DA) transporter (DAT) mediates uptake of synaptic DA into neurons (Amara and Kuhar, 1993; Uhl and Johnson, 1994). It also takes up structurally similar substrates, including tyramine and amphetamine derivatives (Sitte et al., 1998). This uptake process is dependent on external Na⁺ and Cl⁻, with K⁺ being primarily inhibitory (Krueger, 1990; Amejdkí-Chab et al., 1992; McElvain and Schenk, 1992; Gu et al., 1994). The DAT is also a target for nontransportable compounds such as cocaine, mazindol, methylphenidate, and 1-[2-[bis(4-fluorophenyl) methoxy]ethyl]-4-(3-phenyl-2-propyl)piperazine (GBR12909; Andersen, 1989; Tatsumi et al., 1997). The reinforcing effects of cocaine are best correlated with its DAT-blocking property (Ritz et al., 1987; Giros et al., 1996), whereas the other three compounds are currently under investigation for potential pharmacotherapeutic application to treat cocaine abuse (for reviews, see Grabowski et al., 1997; Wu et al., 1997; Zhang, 1998).

Binding studies on the DAT have led to the conclusion that the DA and cocaine sites are different but overlapped; Na⁺ stimulates the binding of DA and cocaine analogs allosterically, whereas K⁺ blocks their binding by recognizing a cation site included in both DA and cocaine sites (Reith et al., 1992; Chen et al., 1997a,b; Li and Reith, 1999). In those studies, the binding affinity of DA was assessed indirectly through inhibition of binding of radiolabeled cocaine analogs, 2β-carboxymethoxy-3β-(4-fluorophenyl)tropone ([3H]WIN 35,428) and [125I]3β-(4-iodophenyl)tropone-2β-carboxylic acid isopropyl ester (RTI-121), to the DAT. Similar suggestions for cations to modulate the binding of a GBR analog, [3H]1-[2-

ABBRIVATIONS: DA, dopamine; DAT, dopamine transporter; hDAT, human DAT; RDE, rotating disk electrode; K_{DA}, concentration of DA to produce half-maximal initial rate of DA uptake at a given concentration of Na⁺; K_{Na+}, dissociation constant of DA at saturating concentrations of Na⁺; K_{Na+} at 0 concentration of DA; V_{app}, apparent maximal initial rate of DA uptake at a given concentration of DA and saturating concentrations of Na⁺ or at saturating concentrations of DA and a given concentration of Na⁺; V_{max}, maximal initial rate of DA uptake at saturating concentrations of DA and Na⁺; GBR12909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenyl-2-propyl)piperazine; GBR12783, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenyl-2-propyl)piperazine; RTI-121, 3β-(4-iodophenyl)tropone-2β-carboxylic acid isopropyl ester; WIN 35,428, 2β-carboxymethoxy-3β-(4-fluorophenyl)tropone.
dressed for the apparent affinity and the action patterns of analogues and mathematical modeling. This provides a basis for impacts the entire transport process. In contrast, the K1-transportation of the hDAT occurs mainly at the binding level, which facilitates, the binding sequence of DA and Na+ was characterized by complementary kinetic analyses and mathematical modeling. This provides a basis for comparing Na+ modulation between substrates and inhibitors. Third, interactions of cocaine with DA, Na+, and K+ during the entire transport process were tested and compared with those at the binding step (Chen et al., 1997a; Li and Reith, 1999). Finally, the Na+ requirement was addressed for the apparent affinity and the action patterns of other DAT ligands. A difference between substrates and inhibitors or between cocaine and the potential agents to ameliorate cocaine abuse would suggest the involvement of mechanisms differently linked to the Na+ site. We used rotating disk electrode (RDE) voltammetry to monitor the rapid rate of DA clearance by the recombinant human DAT (hDAT) stably expressed in heterogeneous cells. This approach facilitates the measurement of the DA uptake by circumventing problems encountered in previous DA uptake assays, such as the release of endogenous/accumulated DA and metabolism of accumulated DA. Our findings suggest that the Na+ modulation of the hDAT occurs mainly at the binding level, which impacts the entire transport process. In contrast, the K+ modulation may occur at both binding and translocation levels, and the modulation after binding steps seems rate-limiting.

**Experimental Procedures**

**Materials.** Dopamine hydrochloride, cocaine hydrochloride (COC), and the buffer salts were from Sigma Chemical Co. (St. Louis, MO). m-Tyramine hydrochloride, d-amphetamine sulfate, mazindol, methylphenidate hydrochloride, and GBR12909 dihydrochloride were from Research Biochemicals International (Natick, MA). The pcdNA3-hDAT was a generous gift from Dr. Amy J. Eschleman (Department of Physiology and Pharmacology, Oregon Health Science University, Portland, OR).

**Stable Expression of hDAT in Human Embryonic Kidney Cells.** Human embryonic kidney cells (HEK-293; CRL 1573; American Type Culture Collection, Manassas, VA) were transfected by Lipofectin (Life Technologies, Grand Island, NY) with the pcdNA3-hDAT construct at 60% confluence. After 3 days, the cells were dissociated with versene (Sigma Chemical Co.) and split at a 1:80–100 ratio into the growth medium containing 600 μg/ml geneticin. The resistant colonies were isolated 2 weeks later with sterile clone rings. Lines stably expressing hDAT (HEK-hDAT cells) were identified by Na+-dependent and cocaine-sensitive DA uptake with RDE voltammetry.

**Cell Culture.** The parental HEK-293 cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM l-glutamine at 37°C and 5% CO2. The transfected cell lines were maintained in the same medium except that gentamicin was added at a concentration of 200 μg/ml.

**Transport Assays.** DA transport assays were performed as described previously (Burnette et al., 1996; Chen and Justice, 1998; Chen et al., 1998). Briefly, when cells had grown to confluence in 150-mm cell culture dishes, they were harvested by scraping and centrifugation. The harvested cells were then resuspended in 300 μl of 37°C assay buffer and used for RDE measurement. After the cell suspension was placed into the electrochemical cell, the working electrode was introduced just below the surface of the solution and rotated with an AFM/SK Analytical Rotator System (Pine Instrument Company, Grove City, PA) at 4000 rpm. A potential of 400 mV relative to a Ag/AgCl reference electrode was applied to the RDE with a potentiosstat (EI-400; Einsman Instrumentation, Bloomington, IN), and the output of the signal was amplified and recorded. All experiments were performed at 37°C. Origin software (version 4.0; MicroCal Software, Northampton, MA) was used for data acquisition.

For uptake assays, cells were preincubated for 3 min, and then a 1-min baseline was collected. Subsequently, DA was added to the cell suspension and the decrease in the DA signal was recorded. In studies involving inhibition of DA uptake by various compounds, m-tyramine and d-amphetamine, whose inhibition decreases with time, were added simultaneously with DA; cocaine, methylphenidate, mazindol, and GBR12909, whose inhibition increases within a certain time period, were added 3 min earlier than DA. Increasing the pretreatment time from 0 to 3 min had no effect on the Na+ dependence of the inhibitors. A total of 100 μM cocaine was used to define nonspecific uptake. Initial transport rates were obtained from linear regression analysis of the decrease in medium DA concentration versus time over the first 10–15 s after an addition of DA (Burnette et al., 1996; Chen and Justice, 1998; Chen et al., 1998).

The assay buffer contained 150 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 10 mM HEPES, and 10 mM glucose. The pH of the assay buffer was adjusted to 7.4 with either NaOH (resulting in approximately 5 mM additional Na+) in the assay buffer) or LiOH. For Na+ dependence of DA uptake, Na+ was substituted with isosmolar quantities of Li+, choline, or K+ in chloride salt form. The cell protein was determined with a Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA).

**Data Analysis and Statistics.** The Hill coefficients of the uptake curves were estimated from curve-fitting of the initial rate data with the expression \( v = \left( V_{app} \left[ S \right] / K_{app}^n + [S]^n \right) \), in which \( v \) is the initial uptake rate, \( V_{app} \) is the apparent maximal initial rate of DA uptake, \( S \) is substrate (Na+ or DA), \( K_{app}^n \) is the concentration for a substrate to produce half-maximal initial rate of DA uptake \( K_{[Na+]}, K_{[DA]} \), and \( n \) is the Hill coefficient. The resulting Hill values were then rounded up to the next integer values as an estimated value of the true Hill number (1 or 2) and used for further kinetic analysis. Multiple models for the binding sequence of DA and Na+ were examined by both linear (Stein, 1986; Segel, 1993) and nonlinear (see Results for details) regressions of the experimental rate data. \( K_{[Na+]}, K_{[DA]}, V_{app} \) were calculated with Lineweaver-Burk transforma-
tion of the Michaelis-Menten equation. The maximal initial rate of 
DA uptake at saturating concentration of Na\(^+\) \(V_{\text{max}}\), dissociation constant for DA at saturating concentration of Na\(^+\) \(K_{\text{DA}}\), and dissociation constant for Na\(^+\) at 0 concentration of DA \(K_{\text{Na+}}\) were calculated with replots of the results obtained from Lineweaver-Burk analysis (Stein, 1986; Segel, 1993). The half-saturation inhibition constant \((K_i)\) of various compounds was estimated with the Michaelis-Menten expressions modified for competitive, noncompetitive, and mixed inhibitions (Segel, 1993).

Results are expressed as mean ± S.E. of \(n\) experiments, unless indicated otherwise. The \(F\)-test was used to determine whether the goodness of fits obtained with different models was statistically different (Munson and Rodbard, 1980). In brief, the "extra sum of squares" principle is applied: \(F = (\sum_{i=1}^{n} y_i - y_i^*)/df_1/(\sum_{i=1}^{n} y_i-y_i^*)(SS_2)\), in which SS is the residual sum of squares of the deviations of the experimental points to the fitted curve and \(df_i\) is the degrees of freedom, going from the original model 1 to the more complex model 2 with added parameters. Other statistics included one-way ANOVA followed by the Dunnett’s test, and paired or unpaired Student’s \(t\) test, as appropriate. The accepted level of significance was .05.

Results
Effects of Substituted Cations on Na\(^+\)-Dependent DA Uptake. When the uptake was determined as a function of Na\(^+\) concentration (5–155 mM) in the presence of different cation substitutes, the shape of the curve varied with the substitute used (Fig. 1). At 1 \(\mu\)M DA, the Na\(^+\) curve was hyperbolic for Li\(^+\) substitution (Fig. 1A), almost linear for choline substitution (Fig. 1B), and sigmoid for K\(^+\) substitution (Fig. 1C). The fitted Hill value for the Na\(^+\) curve was close to 1 with Li\(^+\) substitution and close to 2 with K\(^+\) substitution (Table 1). At this DA concentration, we failed to get a reliable Hill value for the choline-substituted Na\(^+\) curve because of its linear trend. At 8 \(\mu\)M DA, the Na\(^+\) curve was hyperbolic for both Li\(^+\) and choline substitutions (Fig. 1, A and B), and sigmoid for K\(^+\) substitution (Fig. 1C). This concentration of DA did not significantly change the Hill value for the Li\(^+\)- or K\(^+\)-substituted Na\(^+\) curve, but it improved the fitting of the Hill coefficient for the choline-substituted Na\(^+\) curve, giving a value close to 1 (Table 1).

With Li\(^+\) or choline substitution, the uptake rate of 8 \(\mu\)M DA at the lowest [Na\(^+\)] was generally close to the uptake rate of 1 \(\mu\)M DA at the highest [Na\(^+\)] (Fig. 2). This result was not attributable to a nonspecific uptake at higher [DA], because 100 \(\mu\)M cocaine reduced this uptake to a rate similar to that measured in parent HEK-293 cells (Fig. 2).

The \(K_{\text{Na+}}\) and \(V_{\text{app}}\) for the Na\(^+\) curves at each fixed [DA] were obtained by fitting the rate data to the Lineweaver-Burk transformation of Michaelis-Menten equation with the Hill coefficient fixed at 1 for Li\(^+\) and choline substitutions, and at 2 for K\(^+\) substitution. Although we failed to assess the Hill coefficient for the choline-substituted Na\(^+\) curve at 1 \(\mu\)M DA, we chose a value of unity because this number allowed a reasonable estimation of \(V_{\text{app}}\) and was close to the Hill value obtained from the choline-substituted Na\(^+\) curve at 8 \(\mu\)M DA. At 1 \(\mu\)M DA, the rank order of \(K_{\text{Na+}}\) was K\(^+\) > choline > Li\(^+\) substitution (Table 1). At 8 \(\mu\)M DA, the \(K_{\text{Na+}}\) with Li\(^+\) and choline substitution were greatly reduced to a similar level, whereas that with K\(^+\) substitution was not significantly changed (Table 1).

When the DA uptake was determined as a function of DA concentration (1–8 \(\mu\)M) with Na\(^+\) concentration fixed at either 155 mM or 55 mM, the Hill values for all curves were close to 1 (Table 2). At lower [Na\(^+\)] (55 mM), the change in parameters varies with substituted cations. The \(K_{\text{DA}}\) was markedly raised, whereas the \(V_{\text{app}}\) was unchanged under the Li\(^+\)- or choline-substituted condition. In contrast, the \(V_{\text{app}}\) was prominently reduced, whereas the \(K_{\text{DA}}\) was not significantly changed under the K\(^+\)-substituted condition (Table 2).

Interactions between DA and Na\(^+\) with Li\(^+\) as a Substitute. Initially, we measured the kinetic parameters of DA uptake over an extended [DA] range (0.5–16 \(\mu\)M) either at a

<table>
<thead>
<tr>
<th>[DA] Substituted Cations</th>
<th>Hill Coefficient</th>
<th>(K_{\text{Na+}}) mM</th>
<th>(V_{\text{app}}) pmol/s/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Li(^+)</td>
<td>0.89 ± 0.05</td>
<td>66.0 ± 3.5</td>
<td>19.8 ± 0.1</td>
</tr>
<tr>
<td>Choline</td>
<td>Failed to fit</td>
<td>86.4 ± 6.1</td>
<td>19.0 ± 0.8</td>
</tr>
<tr>
<td>K(^+)</td>
<td>1.90 ± 0.19</td>
<td>126.7 ± 5.8</td>
<td>22.7 ± 1.3</td>
</tr>
<tr>
<td>8 Li(^+)</td>
<td>0.78 ± 0.09</td>
<td>14.4 ± 2.6(a)</td>
<td>36.0 ± 1.2(a)</td>
</tr>
<tr>
<td>Choline</td>
<td>0.69 ± 0.05</td>
<td>11.8 ± 1.6(a)</td>
<td>36.0 ± 1.7(a)</td>
</tr>
<tr>
<td>K(^+)</td>
<td>1.66 ± 0.03</td>
<td>115.0 ± 1.7</td>
<td>43.8 ± 5.1(a)</td>
</tr>
</tbody>
</table>

\(a\) Unpaired Student’s \(t\) test, \(p < .01\) versus 1 \(\mu\)M DA.
the reversed Na⁺ external Na⁺ remained measurable. However, it is difficult to maintain a constant Na⁺ concentration by replacing NaCl with isomolar quantities of LiCl, choline chloride, or KCl. Even with a Na⁺-free, Li⁺-fully substituted buffer (LiOH was used to adjust the pH of the buffer), the uptake rate was further reduced by a Na⁺-free, Li⁺-free buffer. Even with a Na⁺-free buffer, the uptake remained measurable. However, it is difficult to maintain a zero external Na⁺ condition, because under the influence of the reversed Na⁺ gradient, intracellular Na⁺ unavoidably leaves the cells, which makes the Na⁺ concentration in the external medium nonzero.

By keeping the concentration of Cl⁻ constant and saturating (Gu et al., 1994; Pavlock and Schenk, 1997), we can consider the hDAT as a bireactant system, in which all binding and dissociation steps are very rapid compared with the translocation of the ternary complex. The assumption of rapid equilibrium is often used in modeling transporters (Stein, 1986). Two binding models are proposed in such a system: random binding and ordered binding (Stein, 1986; Segel, 1993). To test the binding sequence of DA and Na⁺, we performed complementary experiments in which initial rates of DA uptake were determined at various concentrations of Na⁺ and DA. The data were then analyzed by both linear and nonlinear regression (Stein, 1986; Segel, 1993). In accordance, as the [Na⁺] increased from 5 mM to 155 mM NaCl, the [Na⁺] remained the same (Table 3). A replot of the slope versus 1/[DA] gave an intercept on the abscissa at a value lower than 155 mM NaCl. Notably, the replot of the slope versus 1/[DA] gave an intercept on the ordinate at a value higher than 155 mM NaCl. In low [Na⁺] medium, 150 mM LiCl was added to a basal medium containing approximately 5 mM Na⁺ delivered from HEPES/NaOH buffer. In high [Na⁺] medium, 150 mM NaCl was added to the basal medium. Eadie-Hofstee plot was used to determine the values for $V_{app}$ and $K_{app}$. The straight solid line represents the result of the least-squares linear regression. The calculated parameters for this experiment were as follows: at 5 mM [Na⁺], $V_{app} = 39.2 \pm 4.8 \text{ pmol/s/mg}$, and $K_{app} = 18.7 \pm 2.4 \mu M$; at 155 mM [Na⁺], $V_{app} = 41.5 \pm 1.6 \text{ pmol/sec/mg}$, and $K_{app} = 1.98 \pm 0.1 \mu M$. Inset shows the fit of data to the Michaelis-Menten expression $v = \frac{(V_{app})([DA])}{(K_{app}[DA]+[DA])}$, which gave similar values for $V_{app}$ and $K_{app}$. Shown are means ± S.E. of three experiments.
estimated from the two sets of analyses were extremely similar (Tables 3 and 4).

In the nonlinear regression approach, the same data were fit by mathematical expressions describing various possible models for the binding sequence of DA and Na⁺. Different translocation complexes were also considered. The scheme and the equation are as follows:

\[
\begin{align*}
T & \xrightarrow{K_{DA1}} \text{T-DA} \xrightarrow{V_1} \text{Translocation} \\
\text{T-Na}^+ & \xrightarrow{K_{Na1}} \text{T-Na}^+ - \text{DA} \xrightarrow{V_2} \text{Translocation} \\
\text{[Na]} & \xrightarrow{K_{DA2}} \text{T-Na}^+ - \text{DA} \\
\end{align*}
\]

Here, \( K_{DA1} \) and \( K_{DA2} \) are the dissociation constants for the binding of DA to the free hDAT (T) and Na⁺-bound hDAT (T-Na⁺); and \( K_{Na1} \) and \( K_{Na2} \) are the dissociation constants for the binding of Na⁺ to the free hDAT and DA-bound hDAT (T-DA); \( V_1 \) and \( V_2 \) are the maximal translocation rate for the T-DA and T-Na⁺-DA complex, respectively. Three models were tested: 1) ordered binding, Na⁺ binds before DA and only the T-Na⁺-DA form translocates (fixing \( 1/K_{DA1} \) and \( V_1 \) at 0); 2) random binding, both Na⁺ and DA bind the free transporter but only the form of T-Na⁺-DA translocates (fixing \( V_1 \) at 0); and 3) random binding, both Na⁺ and DA bind the free transporter and both the T-Na⁺-DA and the T-DA form translocate (allowing all parameters to be free).

The fitted parameter values corresponding to each model and the equation are as follows:

\[
V = \frac{V_1[DAG] + V_2[DA][Na^+]}{1 + \frac{[DA]}{K_{DA1}} + \frac{[Na^+]}{K_{Na1}} + \frac{[DA][Na^+]}{K_{DA1}K_{Na1}}}
\]

(1)

Fig. 4. Initial rate of DA uptake as a function of DA concentrations at several fixed Na⁺ concentrations. The Na⁺ concentration was altered by replacing NaCl with isomolar quantities of LiCl. Lineweaver-Burk plot of the data was used to analyze the reaction sequence. The straight solid line represents the result of the least-squares linear regression. Shown is a representative experiment with five concentrations (0.5–8 mM) of DA and indicated concentrations of Na⁺ on the same generation of cells. Each experiment was performed five times with similar results. Top inset shows a replot of the slope of the Lineweaver-Burk plot as a function of \( 1/[Na^+] \). Bottom inset shows a replot of the \( K_{DA1} \) obtained from the Lineweaver-Burk plot as a function of \( 1/[Na^+] \). The true \( V_{max}, K_{DA1} \) and \( K_{Na1} \) were estimated from these replots (see Table 3).

Fig. 5. Initial rate of DA uptake as a function of Na⁺ concentrations at several fixed DA concentrations. The Na⁺ concentration was altered by replacing NaCl with isomolar quantities of LiCl. Lineweaver-Burk plot of the data was used to analyze the reaction sequence. The straight solid line represents the result of the least-squares linear regression. Shown is a representative experiment with seven concentrations (15–155 mM) of Na⁺ and indicated concentrations of DA on the same generation of cells. Each experiment was performed five times with similar results. Top inset shows a replot of the intercept of the Lineweaver-Burk plot as a function of \( 1/[DA] \). Bottom inset shows a replot of the slope of the Lineweaver-Burk plot as a function of \( 1/[DA] \). The true \( V_{max}, K_{DA1} \) and \( K_{Na1} \) were estimated from these replots (see Table 4).

<table>
<thead>
<tr>
<th>[Na⁺] (mM)</th>
<th>( H ) Coefficient</th>
<th>( K_{DA1} ) (μM)</th>
<th>( V_{max} ) (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>1.08 ± 0.08</td>
<td>11.0 ± 0.90</td>
<td>46.1 ± 2.2</td>
</tr>
<tr>
<td>30</td>
<td>1.08 ± 0.12</td>
<td>7.1 ± 1.30</td>
<td>48.2 ± 3.6</td>
</tr>
<tr>
<td>55</td>
<td>1.26 ± 0.08</td>
<td>4.2 ± 0.60</td>
<td>45.2 ± 2.6</td>
</tr>
<tr>
<td>80</td>
<td>1.24 ± 0.13</td>
<td>3.7 ± 0.4</td>
<td>47.6 ± 2.2</td>
</tr>
<tr>
<td>105</td>
<td>1.37 ± 0.14</td>
<td>3.2 ± 0.5</td>
<td>47.0 ± 3.5</td>
</tr>
<tr>
<td>130</td>
<td>1.35 ± 0.14</td>
<td>2.8 ± 0.7</td>
<td>44.4 ± 2.3</td>
</tr>
<tr>
<td>155</td>
<td>1.35 ± 0.11</td>
<td>2.3 ± 0.3</td>
<td>45.3 ± 2.6</td>
</tr>
</tbody>
</table>

* Dunnett’s test, \( p < 0.01 \) versus [Na⁺] = 155 mM.
TABLE 4
Kinetic parameters for DA uptake at various concentrations of DA

<table>
<thead>
<tr>
<th>[DA] (M)</th>
<th>H Coefficient</th>
<th>$K_{D{\text{a}}-1}$ (mM)</th>
<th>$V_{\text{app}}$ (pmmol/s/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.87 ± 0.04</td>
<td>70.0 ± 9.4$^*$</td>
<td>11.5 ± 1.7$^*$</td>
</tr>
<tr>
<td>1.0</td>
<td>0.89 ± 0.05</td>
<td>57.4 ± 5.6$^*$</td>
<td>19.5 ± 2.4$^*$</td>
</tr>
<tr>
<td>2.0</td>
<td>0.91 ± 0.13</td>
<td>41.4 ± 4.2$^*$</td>
<td>27.6 ± 3.4$^*$</td>
</tr>
<tr>
<td>4.0</td>
<td>0.86 ± 0.04</td>
<td>24.4 ± 1.0$^*$</td>
<td>32.0 ± 1.7$^*$</td>
</tr>
<tr>
<td>8.0</td>
<td>0.78 ± 0.09</td>
<td>12.8 ± 1.2</td>
<td>34.0 ± 2.8$^*$</td>
</tr>
</tbody>
</table>

$^*$ Dunnett’s test, $p < 0.01$ versus $[\text{DA}] = 8 \mu\text{M}$.

TABLE 5
Nonlinear curve fitting of the uptake rate data with various models for interactions between DA and Na$^+$.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Na$^+$ Binds before DA</th>
<th>DA and Na$^+$ Bind Randomly</th>
<th>Na$^+$-DA Translocates$^a$</th>
<th>DA and Na$^+$ Bind Randomly</th>
<th>Na$^+$-DA Translocates$^b$</th>
<th>Na$^+$-DA Translocates$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_0$ (pmmol/s/mg)</td>
<td>39.0 ± 0.49</td>
<td>39.5 ± 0.85</td>
<td>39.8 ± 0.93</td>
<td>89.5 ± 111</td>
<td>39.7 ± 35.5</td>
<td>97.0 ± 12.0</td>
</tr>
<tr>
<td>$K_{D{\text{a}}}$ (M)</td>
<td>0.94 ± 0.065</td>
<td>1.0 ± 0.11</td>
<td>0.97 ± 0.12</td>
<td>129 ± 11</td>
<td>116 ± 7</td>
<td>130 ± 28</td>
</tr>
<tr>
<td>$K_{v{\text{c}}}$ (M)</td>
<td>1.20 ± 1.25</td>
<td>3.18 ± 2.95</td>
<td>3.18 ± 2.95</td>
<td>3.2 ± 1.25</td>
<td>3.18 ± 2.95</td>
<td>3.18 ± 2.95</td>
</tr>
</tbody>
</table>

$^a$ $V_0$ and $K_{D{\text{a}}}$ were fixed at zero.
$^b$ $V_0$ was fixed at zero.
$^c$ All parameters were allowed to be free.
$^d$ $V_0$ was significantly lower than the first model.
$^e$ Introduction of additional parameters did not improve the fit compared with the first model.

In the later two models, the relative S.D. values of the added parameter, $K_{D{\text{a}}}$ or $V_0$, was greater than 80$.%$. Therefore, there was no strong evidence either for the binding of DA before Na$^+$ or for the translocation of the hDAT-DA complex.

Interaction between Cocaine and Cations at the hDAT. First, the DA uptake was determined as a function of Na$^+$ in the presence of 0.5 $\mu$M cocaine. To facilitate comparison, we performed these experiments in parallel with the experiments in Table 1. At 1 $\mu$M [DA], cocaine reduced both $K_{D{\text{a}}}$ and $V_{\text{app}}$. The relative reduction in magnitude in $K_{D{\text{a}}}$ was similar regardless of the substituted cation used (Table 6), with the ratio of $K_{D{\text{a}}}$ at the three substitutions (1:1.3:1.8 for Li$^+$, choline, and K$^+$, respectively) similar to that in the absence of cocaine (1:1.3:1.9). With DA concentration elevated to 8 $\mu$M, the effect of cocaine on the $K_{D{\text{a}}}$ was abolished, and its effect on the $V_{\text{app}}$ was significantly reduced (Table 6). The data also were evaluated as a percentage of inhibition as a function of Na$^+$ concentration. Cocaine-induced inhibition of DA uptake (1 $\mu$M) was significantly less at 30 $\mu$M Na$^+$ (for Li$^+$ and choline substitution) or 55 $\mu$M Na$^+$ (for K$^+$ substitution) than at 155 $\mu$M Na$^+$. 

TABLE 6
Na$^+$ dependence of DA uptake in the presence of cocaine

<table>
<thead>
<tr>
<th>[DA] (M)</th>
<th>Substituted Cations</th>
<th>$K_{D{\text{a}}}$ (M)</th>
<th>$V_{\text{app}}$ (pmmol/s/mg)</th>
<th>$K_i$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>155</td>
<td>5</td>
<td>11.1 ± 0.5$^a$</td>
<td>39.4 ± 1.7</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>55</td>
<td>7</td>
<td>19.1 ± 1.9$^b$</td>
<td>43.9 ± 2.3</td>
<td>0.40 ± 0.05</td>
</tr>
<tr>
<td>Choline</td>
<td>5</td>
<td>16.6 ± 1.3$^b$</td>
<td>39.9 ± 1.0</td>
<td>0.63 ± 0.08$^b$</td>
</tr>
<tr>
<td>K$^+$</td>
<td>7</td>
<td>8.3 ± 0.7$^b$</td>
<td>14.0 ± 1.0$^b$</td>
<td>0.78 ± 0.11$^b$</td>
</tr>
</tbody>
</table>

$^a$ Paired Student’s $t$ test, $p < 0.01$ versus control data in Table 2.
$^b$ Dunnett’s test, $p < 0.01$ versus 155 mM Na$^+$.

As shown in Tables 6 and 7 under Li$^+$ substitution condition, with cocaine, the changes in $K_{D{\text{a}}}$, $V_{\text{app}}$, and $K_{N{\text{a}}}$ were in the same direction as those without cocaine, indicating that cocaine may not modify the binding sequence.
Inhibition of DA Uptake by Other Substrates and Inhibitors and Their Na⁺ Requirement. The inhibition pattern and potency of other substrates and nontransportable inhibitors were tested at two levels of the Na⁺, 155 mM and 55 mM (with Li⁺ as a substitute). Two substrates, m-tyramine and d-amphetamine, inhibited the DA uptake solely by elevating $K_{i}$ regardless of the Na⁺ concentration (Table 8). Among the nontransportable inhibitors, methylphenidate only increased $K_{i}$, regardless of the Na⁺ concentration; mazindol affected both $K_{i}$ and $V_{max}$ at 155 mM [Na⁺], but mainly affected $K_{i}$ at 55 mM [Na⁺]; and GBR12909 mainly reduced $V_{max}$, regardless of the Na⁺ concentration (Table 8).

The apparent noncompetitive inhibition by GBR12909 of DA uptake in the present study, although agreeing with a recent study on another GBR analog, GBR12783 (Do-Régo et al., 1998), differs from previous studies showing that GBR12909 exerted a competitive inhibition on [³H]DA uptake in synaptosomal preparations (Andersen, 1989). As the binding of a [³H]GBR analog to membranes prepared from the transfected cells exhibits a pharmacological profile indicative of the DAT (Eshleman et al., 1995), it seems impossible that GBR12909 inhibits DA uptake by acting at a protein distinct from the hDAT. One possibility is that the slow, tight-binding feature of GBR12909 may not allow DA to fully displace it during a short time (10 s in the present study). Other mechanisms may also be involved (Pristupa et al., 1994; Do-Régo et al., 1998).

Compared with those at 155 mM [Na⁺], the $K_{i}$ values of m-tyramine and d-amphetamine were more than doubled, whereas the $K_{i}$ values of the nontransportable inhibitors were raised by less than 50% at 55 mM [Na⁺] (Table 8). The ratios of the $K_{i}$ ($K_{i}$) value at 155 mM [Na⁺] to the $K_{i}$ ($K_{i}$) value at 55 mM [Na⁺] ($K_{i,155/55}$) for all tested substrates and nontransportable inhibitors were calculated with data measured in pairs (Fig. 6). The results showed that the sensitivity of the two categories of compounds to Na⁺ was different, with the $K_{i,155/55}$ for the substrates significantly higher than for inhibitors ($p < .01$ on pooled data between substrates and inhibitors).

### Table 8

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$K_{i}$ (µM)</th>
<th>$V_{max}$ (pmol/s/mg)</th>
<th>$K_{i}$ (µM)</th>
<th>$V_{max}$ (pmol/s/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA alone</td>
<td>1.65 ± 0.11</td>
<td>36.5 ± 1.8</td>
<td>3.60 ± 0.15a</td>
<td>39.7 ± 0.6</td>
</tr>
<tr>
<td>+ m-Tyramine (5 µM)</td>
<td>5.83 ± 0.29b</td>
<td>36.3 ± 2.7</td>
<td>7.43 ± 0.62ab</td>
<td>38.4 ± 3.5</td>
</tr>
<tr>
<td>+ d-Amphetamine (1 µM)</td>
<td>6.30 ± 0.80b</td>
<td>37.5 ± 2.9</td>
<td>8.08 ± 0.55ab</td>
<td>41.3 ± 2.3</td>
</tr>
<tr>
<td>+ Methylphenidate (0.3 µM)</td>
<td>6.74 ± 0.21b</td>
<td>34.5 ± 4.2</td>
<td>11.4 ± 0.78ab</td>
<td>39.2 ± 3.7</td>
</tr>
<tr>
<td>+ Mazindol (0.1 µM)</td>
<td>4.22 ± 0.07b</td>
<td>24.9 ± 2.3b</td>
<td>9.75 ± 1.60ac</td>
<td>35.4 ± 4.4</td>
</tr>
<tr>
<td>+ GBR12909 (0.1 µM)</td>
<td>1.43 ± 0.16</td>
<td>14.4 ± 0.77b</td>
<td>3.33 ± 0.30abc</td>
<td>18.9 ± 0.60</td>
</tr>
</tbody>
</table>

**a** Paired Student’s t-test, $p < .01$ versus 155 mM Na⁺ for corresponding compounds.

**b** Dunnett’s test, $p < .05$ versus DA alone at the same Na⁺ concentration.

**c** Paired Student’s t-test, $p < .05$ versus 155 mM Na⁺ for corresponding compounds.

**d** Dunnett’s test, $p < .05$ versus DA alone at the same Na⁺ concentration.
Discussion

Profile of Na\(^+\) Dependence. With Li\(^+\) as a Na\(^+\) substitute, we observed a hyperbolic Na\(^+\) dependence of the DA uptake, which is different from the sigmoid Na\(^+\) dependence reported by previous studies. In those studies, some measured \(^{3}H\)DA accumulation in synaptosomes (Krueger, 1990) or nonisotopic DA clearance by brain tissues or transfected cells (McElvain and Schenk, 1992; Povlock and Schenk, 1997; Earles and Schenk, 1999) with choline as a Na\(^+\) substitute, and others measured \(^{3}H\)DA accumulation in transfected cells with Li\(^+\) as a Na\(^+\) substitute (Gu et al., 1994; Piñol et al., 1997). Choline has been found inhibitory in both tissues and transfected cells (Shank et al., 1987; Amejdki-Chab et al., 1992, Gu et al., 1994; the present study). With an inhibitory Na\(^+\) substitute, a sigmoid shape of the Na\(^+\) curve is expected to be readily observed because dual effects occur along the Na\(^+\)-substituted cation curve: reduced stimulation by Na\(^+\) and enhanced inhibition by substituted cation of DA uptake. Indeed, we failed to observe a hyperbolic Na\(^+\) dependence with choline substitution at 1 μM [DA], a concentration close to that used by the previous studies. Additionally, low Na\(^+\)-induced release of endogenous DA or accumulated \(^{3}H\)DA (Levi and Raiteri, 1993; Piñol et al., 1997) and metabolism by catechol-O-methyltransferase of accumulated \(^{3}H\)DA (Eshleman et al., 1997) may confound the measurement of DA clearance from tissues or \(^{3}H\)DA accumulation into transfected cells.

Although Li\(^+\) also appears to be a potent inhibitor of DA uptake in synaptosomes (Shank et al., 1987; Amejdki-Chab et al., 1992), it acts like a relatively inert Na\(^+\) substitute in recent uptake and binding studies on hDAT-expressing intact cells (Gu et al., 1994; Wu et al., 1997). This further confirmed by the present study. Thus, Li\(^+\) was used as a Na\(^+\) substitute for more detailed kinetic studies.

The Hill value is commonly used to reflect cotransport stoichiometry. However, this value technically varies with the Na\(^+\) substitutes used, and, theoretically, is only valid if the affinities for the different Na\(^+\) binding sites are similar (Rudnick, 1998). Under our experimental condition, it is possible that more than one Na\(^+\) ion is involved in DA binding/translocation, but that the initial transport rate is dependent on only one low-affinity Na\(^+\) binding site. It is also possible that one Na\(^+\) ion is required for DA binding/translocation, but that more than one Na\(^+\) ion is actually cotransported.

Kinetic Binding Sequence of DA and Na\(^+\). The current study suggests a fixed binding order of DA and Na\(^+\) at the hDAT, with Na\(^+\) binding before DA. Furthermore, translocation occurs only after the binding of both Na\(^+\) and DA. The reaction scheme could be shown as follows:

\[
\text{hDAT} + \text{Na}^+ \rightleftharpoons \text{hDAT-Na}^+ \\
\text{DA} \rightleftharpoons \text{hDAT-Na}^+-\text{DA} \rightarrow \text{translocation}
\]

The presence of DA drives the reaction to the right, thereby the apparent affinity of the hDAT for Na\(^+\) increases with DA. Because a concentration of Na\(^+\) far below the \(K_{N_a}^+\) may still be much higher than the concentration of the hDAT, it would be expected that a saturating concentration of DA would shift all the hDAT to the hDAT-Na\(^+\)-DA form. This finding agrees with our observation that appreciable DA uptake remained at extremely low [Na\(^+\)] but saturating [DA]. In electrophysiological studies with cloned hDAT, DA readily blocks leak conductance carried by Li\(^+\) or K\(^+\) in the absence of external Na\(^+\), questioning the requirement of Na\(^+\) for the binding of external DA (Sonders et al., 1997). The difference in cationic selectivity of the DA transport and leak conductance indicates that DA might bind to different sites or states of the hDAT to block leak conductance. However, such an effect is not measurable in the present study monitoring the specific Na\(^+\)-coupled DA transport.

Although the binding sequence proposed by the present study differs from previous studies in which a random binding of one DA and two Na\(^+\) ions was proposed (McElvain and Schenk, 1992; Wheeler et al., 1993; Povlock and Schenk, 1997), a similar trend for the ordered binding with choline substitution has recently been observed (Earles and Schenk, 1999). This trend is also reflected in our experiments using choline to replace Na\(^+\), even though choline is not a satisfactory Na\(^+\) substitute at low [DA]. With K\(^+\) as a substitute, \(K_{[DA]}\) or \(K_{[Na]}\) became insensitive to Na\(^+\) or DA as if the hDAT system were a random system with one ligand having no effect on the binding of the other. Such a pattern possibly is due to the fact that K\(^+\) is not the right substitute for analysis of the binding order.

Distinct Modulation by Na\(^+\) and K\(^+\) of Cocaine Inhibition and DA Uptake. The results in Tables 6 and 7 indicate that the presence of cocaine may not modify the binding sequence of DA and Na\(^+\). In this scenario, an apparently competitive inhibition of DA uptake by cocaine could arise from interactions of cocaine with either DA or Na\(^+\) sites. Our data are in favor of interactions of cocaine with DA sites because of the failure of cocaine to raise \(K_{[Na]}\) and vice versa.

Consistent with its stimulatory effect on the binding of cocaine analogs (Chen et al., 1997; Li and Reith, 1999), Na\(^+\) enhances the inhibition by cocaine of DA uptake (1 μM). This is revealed by the reduced \(K_{[Na]}\) in the presence of cocaine. Noticeably, the reduction in magnitude is independent of the substituted cations, excluding the possibility that the reduced \(K_{[Na]}\) is caused by interactions between cocaine and substituted cations. However, cocaine affects the Na\(^+\) stimulation by reducing \(V_{app}\) more than \(K_{[Na]}\), suggesting that cocaine inhibition is not entirely dependent on the presence of Na\(^+\). This raises the question whether Na\(^+\) and cocaine bind in a strictly ordered sequence. Indeed, both stimulatory and inhibitory effects of Na\(^+\) have been observed on the binding affinity of cocaine analogs (Reith and Coffey, 1993; Chen et al., 1997a,b; Wu et al., 1997). Furthermore, Na\(^+\) accelerates the dissociation of a cocaine analog (Chen et al., 1997a) and can therefore bind to the cocaine analog-DAT complex. This complication remains to be investigated further.

At the binding level, K\(^+\) reduces the binding affinity of DA and cocaine analogs in a similar fashion (Li and Reith, 1999). In the present transport study, K\(^+\) affects the effect of cocaine again by reducing the affinity of cocaine, as deduced from the increased \(K_{c}\), whereas it affects the DA uptake mainly by reducing the \(V_{app}\). However, our results do not rule out a possible effect of K\(^+\) on the binding affinity of external DA. According to the simplest kinetic scheme (Bönsch, 1998), \(K_{[DA]}\) is proportional to both the binding affinity of DA
and \( k_2 \), a rate constant reflecting multiple steps associated with DA translocation, whereas \( V_{\text{app}} \) is proportional to both the total concentration of hDAT and \( k_2 \). The reduced \( V_{\text{app}} \) observed with \( K^+ \) most probably results from a reduced \( k_2 \) caused by \( K^+ \)-induced membrane depolarization (Krueger, 1990; Sonders et al., 1997). Accordingly, a decrease in \( k_2 \) should have reduced the \( K_{\text{IDA}} \) if the binding affinity did not change. In contrast, we observed an almost unchanged \( K_{\text{IDA}} \) in the presence of \( K^+ \), which may actually indicate that both binding and translocation of DA are impaired. However, it remains possible that the rate-limiting step in DA transport occurs subsequent to substrate binding and depends on the membrane potential. This may explain why cocaine, despite competing with \( K^+ \) for the DA binding at the binding level (Chen et al., 1997; Li and Reith, 1999), failed to antagonize the inhibitory effect of \( K^+ \) on \( K_{\text{IDA}} \).

**Different Na\(^+\) Dependence Between Substrates and Nontransportable Inhibitors.** The present study reveals that one property distinguishing between substrates and inhibitors may be the different Na\(^+\) dependence. Thus, raising [NaCl] reduces the binding affinity and its \( k \) of a substrate more than that of an inhibitor. Interestingly, despite their diverse chemical structures and different inhibition patterns, the inhibitory potency of all nontransportable inhibitors tested showed a similarly modest response to Na\(^+\). This finding suggests that there is a similarity among the interactions of cocaine, mazindol, and GBR12909 with Na\(^+\), albeit, some differences between their interactions with DA.

In transport assays, the \( k_1 \) of a substrate depends on both its binding affinity and its \( k_2 \); whereas the \( k_1 \) of an inhibitor is identical with its binding affinity (Bönisch, 1998). Could the higher Na\(^+\) sensitivity of the substrates represent a Na\(^+\)-induced change in their translocation rate? This is unlikely because we have demonstrated that Na\(^+\) changes only the \( K_{\text{IDA}} \), not the \( V_{\text{app}} \) of DA uptake. It is more likely that Na\(^+\) differentially influences the binding affinity and its \( k_2 \) of DA uptake and its \( k_1 \) of an inhibitor is identical with its binding affinity (Bönisch, 1998).

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Send reprint requests to: Dr. Nianhang Chen, Department of Chemistry, 1515 Pierce Dr., Emory University, Atlanta, GA 30322-2210. E-mail: nchen@emory.edu