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The interaction of methylphenidate and benztropine with the dopamine transporter is different than other substrates and ligands

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Abstract

A substantial body of evidence suggests that the dopamine transporter (DAT) is the principal site for cocaine-induced reward and euphoria. Interactions between the DAT and its substrates and ligands may therefore be of clinical relevance. The pharmacological characteristics of DAT compounds were compared in wild type (WT) and mutant DATs. The DAT mutants chosen for study were those with reduced binding and uptake activities (aspartic acid 79 mutated to alanine, termed D79A), reduced binding but normal uptake (tyrosine 251 mutated to alanine, termed Y251A; tyrosine 273 mutated to alanine, termed, Y273A), and normal binding but reduced uptake (a double mutation: serines 356 and 359 mutated to alanine, termed S356,359A). The WT and mutant DATs were transfected into COS-7 cells, and their pharmacological activities were examined 3 days later. Different patterns of pharmacological activity emerged. GBR 12909, cocaine, and mazindol each showed reduced affinity for the Y251A and the Y273A mutants, but their affinity for the S356,359A mutant was similar to that of the WT DAT. *d*-Amphetamine, MPP+, and dopamine each showed reduced affinity for the S356,359A mutant. Benztropine and methylphenidate had a different effect. Relative to the WT DAT, they both showed reduced affinity for the S356,359A mutant when displacing radioactive carboxyfluorotropane (CFT) binding, but similar affinity when inhibiting radioactive dopamine uptake. These results indicate that methylphenidate and benztropine may interact with the DAT in a different fashion then other substrates and ligands.

Keywords: Dopamine; Cocaine; Transporter; Uptake; Substrates; Ligands

1. Introduction

Cocaine abuse and addiction is a serious health and social concern worldwide. Over the past two decades, research efforts have been directed towards understanding the neurochemical mechanisms underlying the addictive properties of cocaine. Those studies showed that the euphoric and reinforcing effects of cocaine are primarily due to its binding to the DAT with consequent inhibition of dopamine uptake [1,2]. The DAT is a member of a family of sodium- and chloride-dependent neurotransmitter transporters, which terminates neurotransmission by reuptake of dopamine into the presynaptic terminal from which it was released [3–6].

In addition to serving as the site of dopamine uptake and cocaine inhibition, the DAT is also the binding site for a variety of other substrates and ligands. Substrates such as dopamine, *d*-amphetamine, MPP+, and 6-hydroxydopamine are believed to gain access to the intracellular side of the plasma by translocation into the cell [7–10]. On the other hand, ligands such as mazindol, GBR 12909, methylphenidate and benztropine are thought to block transport

Abbreviations: D79A, aspartic acid 79 mutated to alanine; Y251A, tyrosine 251 mutated to alanine; Y273A, tyrosine 273 mutated to alanine; S356,359A, serines 356 and 359 mutated to alanine; DAT, dopamine transporter; WT, wild-type; CFT, carboxyfluorotropane (Win 35,428); TM, transmembrane; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum

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without the ability to translocate [11–18]. It is not known whether the various DAT substrates and ligands act on the same or different domains of the transporter, and the exact nature of the interactions of these small molecules within the DAT primary sequence has yet to be elucidated [19–22]. However, studies using site directed mutagenesis [8], molecular modeling [23], binding [24,25] and thermodynamic approaches [26] have each suggested that there maybe separate or overlapping, but not identical, cocaine and dopamine recognition sites on the DAT.

The cloning of the DAT cDNA [5] has made it possible to study the fine interactions within the DAT molecule. Studies employing site-directed mutagenesis have indicated the importance of certain amino acids for the uptake of dopamine or the binding of cocaine and its analog (CFT). Mutation of DAT's aspartic acid 79, located within the first hydrophobic domain (Fig. 1), reduced the affinity of both dopamine and cocaine for their respective sites [8]. Mutations of DAT serines 356 and 359 located at the 7th transmembrane (TM) domain (Fig. 1) resulted in reduced efficacy of dopamine uptake through the transporter with no effect on cocaine binding [8]. In contrast, mutations of DAT tyrosines 251 and 273 located within the 4th and 5th TM domains respectively (Fig. 1), reduced the affinity of cocaine for its binding site with no effect on the K_m for dopamine uptake [27].

The different psychomotor-stimulant effect of DAT compounds may partially stem from their different molecular action on the DAT. We were interested in studying the functions of small molecules of several different structural classes in DAT specific amino acids mutants. This type of information may indicate whether some amino acids are more important then others for the activity of certain compounds. We, therefore, characterized the interactions of DAT substrates and ligands (Fig. 2) with the above mentioned alanine-based mutants (Fig. 1). The observed patterns of interaction between these small molecules and the DAT are described and discussed below. Thus, the results of this study, further characterize the complex drug interactions with the DAT at the molecular level.

2. Material and methods

2.1. Materials

[^{3}H]CFT (84.5 ci/mmol) was from Perkin-Elmer Life Science (Boston, MA, USA). [^{3}H]dopamine (46 ci/mmol) and GBR12909 were from Pharmacia Amersham (Amersham, England). Mazindol, MPP^+ methylphenidate and benztropine were from Sigma-RBI (Natic, MA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and trypsin/EDTA were from Life Technologies (Gaithersburg, MD, USA). Fish sperm DNA was from Hoffmann-La Roche Inc. (Nutley, NJ, USA). Cocaine hydrochloride and *d*-amphetamine were supplied by the National Institute on Drug Abuse.

2.2. Preparation of DAT mutants

Mutagenesis of aspartic acid 79 to alanine (D79A), tyrosine 251 to alanine (Y251A), tyrosine 273 to alanine (Y273A) and serines 356,359 to alanine (S356,359A) was carried out by Kitayama et al. [8,27].

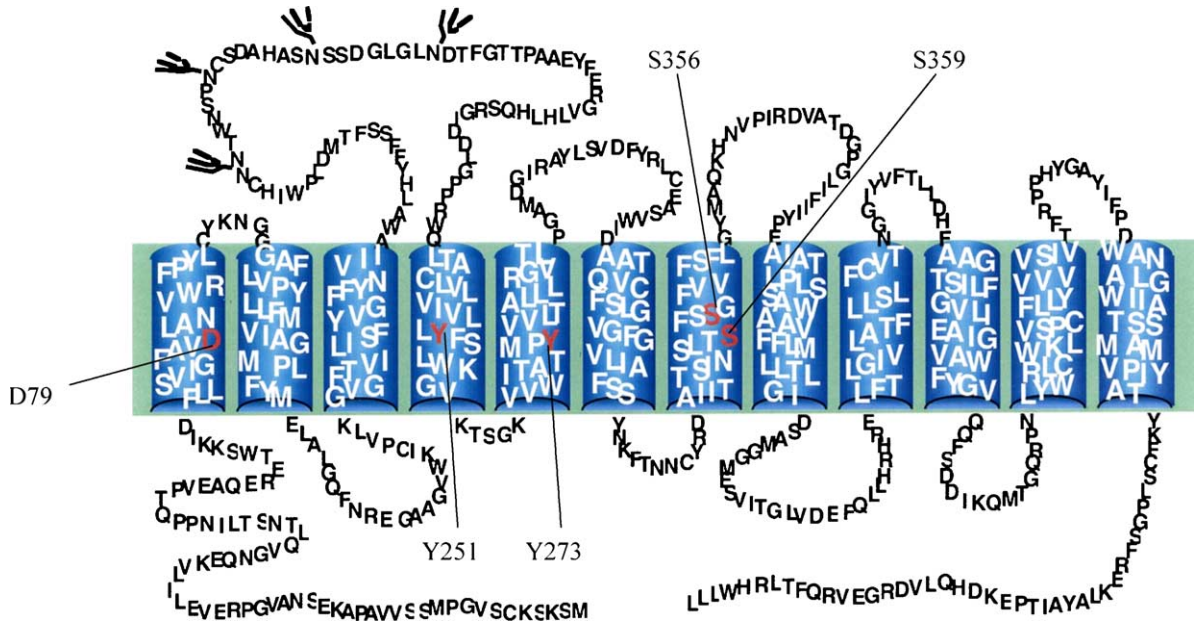

Fig. 1. Schematic model of the rat dopamine transporter containing 12 transmembrane domains. Dopamine transporter mutants with one (or two in the case of the double mutant) of the red amino acids within a transmembrane domain (aspartic acid 79, tyrosine 251, tyrosine 273, and serines 356, 359) mutated to alanine were used in the present study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

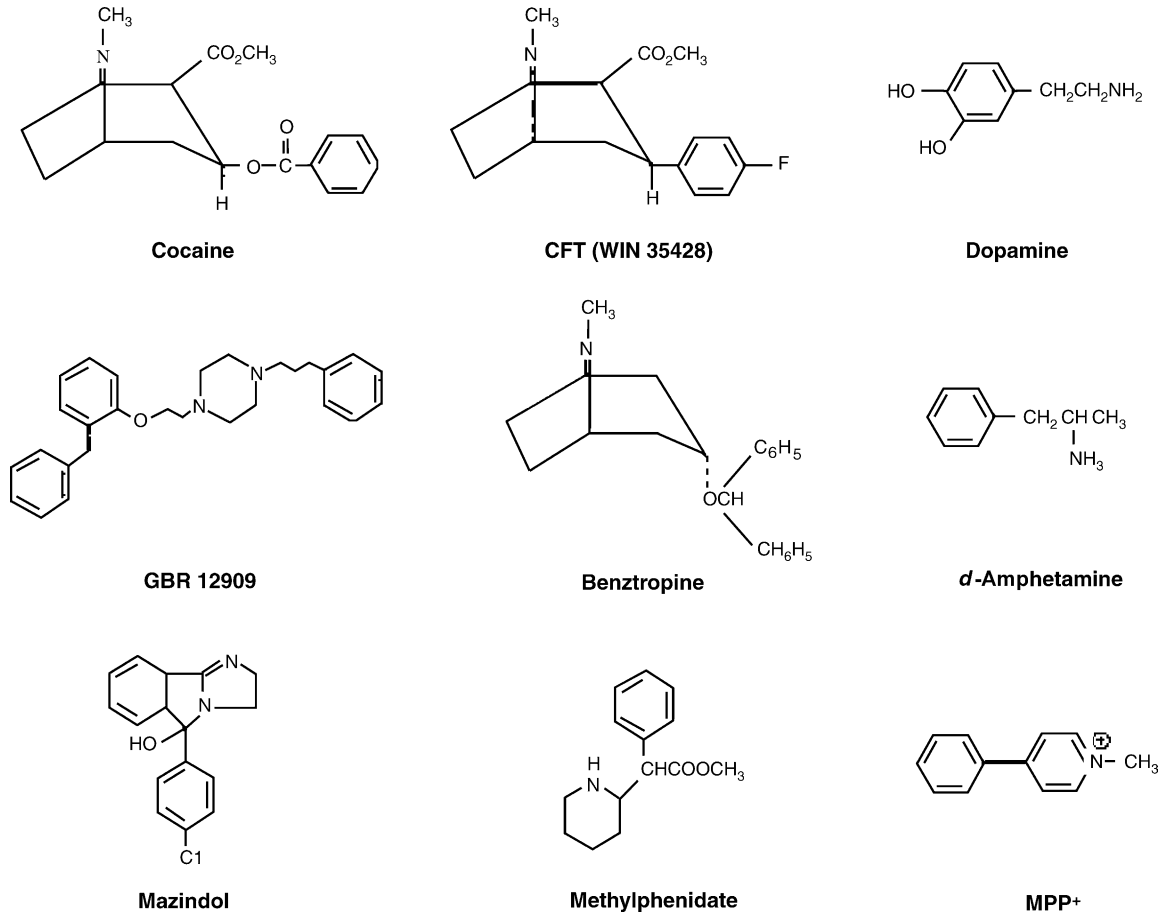

Fig. 2. Structures of the dopaminergic agents used in this study. Dopaminergic agents include cocaine, CFT, dopamine, GBR12909, benztropine, *d*-amphetamine, mazindol, methylphenidate, and MPP+.

2.3. Cell transfection

COS-7 cells, cultured in DMEM supplemented with 10% FCS and 5% CO_2 at 37 °C were grown to confluence and then split to 2. The next day, cells were harvested using trypsin/EDTA, centrifuged (200 × *g*) for 10 min at 4 °C, and washed with cold sterile HEBS buffer (in mM: NaHepes 20, NaCl 130, KCl 5, $Na_2HPO_4$0.7, glucose 6). The cells were resuspended in HEBS buffer at 2×10^7 cells/ml. 0.9 ml of suspension was transfected with 20 μg of pcDNA1 containing WT or mutant DAT in the presence of 500 μg of fish sperm DNA using geneZAPPER 450/2500 (IBI, New Haven, Conn, USA). Electroporation was carried out at 1100 μF and 300 V as described by Schaeffer et al. [28]. Transfected cells were suspended in DMEM and transfered to 12-well plates. After 3 days cells were assayed for their pharmacological function. All measurements were performed using intact cells.

2.4. [^{3}H]Dopamine uptake

[^{3}H]Dopamine uptake was performed in COS-7 cells transfected with WT or mutant rat DAT. The medium was removed and wells were rinsed three times with 1 ml of Krebs–Ringer–Henseleit (KRH) buffer (in mM: HEPES 25, NaCl 125, KCl 4.8, $CaCl_2$ 1.3, Mg_2SO_4 1.2, KH2PO4 1.2, glucose 5.6 pH 7.4, supplemented with 10 μM ascorbic acid) using the Nunc-Immuno Wash 12 vacuum system (Nunc, International, Rochester, NY, USA). Cells were incubated in triplicate with 20 nM [^{3}H]dopamine and 10^{-7} to 3×10^{-5} M of dopamine in total volume of 0.5 ml for 5 min in 37 °C. The wells and their contents were washed and supplemented with radioligand at 15 s intervals to allow strict control of substrate exposure to the cells. The wells were then rinsed three times with KRH buffer and their contents were solubilized in 0.25 ml of 1% SDS.

2.5. [^{3}H]CFT binding

Binding of [^{3}H]CFT to COS-7 cells was measured under conditions similar to those described above for [^{3}H]dopamine uptake. Briefly, 3 days after transfection, the medium was removed and the wells were rinsed three times with 1 ml of KRH buffer (without ascorbic acid). Cells were incubated in triplicate with ice-cold buffer containing increasing concentrations of [^{3}H]CFT (from 0.234 to 100 nM) in a total volume of 0.5 ml. Following incubation for 2 h at 4 °C, the

cells were washed three times with 0.5 ml of ice-cold KRH buffer and solubilized in 1% SDS (0.25 ml).

2.6. Competition studies

Cells were transfected, plated and washed as described above. Inhibition of [^{3}H]dopamine uptake was determined by incubating 320 nM of [^{3}H]dopamine (3Ci/mmol) and competing compound (10^{-10} to 10^{-3} M) for 5 min at 37 °C. The compounds to be tested were preincubated with the cells for 5 min in 0.4 ml of KRH buffer supplemented with 10 μM ascorbic acid, after which 100 μl of KRH solution containing the radioligand was added to the cells for 5 min. The addition of compounds and radioligands to the wells was done at intervals of 15 s to ensure strict control of exposure time. Displacement of [^{3}H]CFT binding was determined by incubating 2 nM [^{3}H]CFT for 2 h at 4 °C with competing compounds in the same concentration range that was used for inhibition of [^{3}H]dopamine uptake. Both binding and uptake assays were terminated by three washes with KRH buffer and cells were then dissolved in 0.25 ml of 1% SDS solution.

For all pharmacological assessments, including [^{3}H]dopamine uptake, [^{3}H]CFT binding and competition studies, non-specific binding was estimated by the addition of 10^{-4} M of benztropine. Radioactivity was determined using a Beckman Instrument liquid scintillation counter LS 6000 (Fullerton, CA, USA) at approximately 50% efficiency. Cells that had undergone similar growing and treatment conditions to those used for experiments were solubilized in 0.5 ml of 1 N NaOH, and the protein level was determined using Bio-Rad (Hercules, CA, USA) protein assay solution.

2.7. Data analysis

GraphPad Prizm Software (v2.01) was used to determine K_m, K_d and K_i values, Hill coefficient value and to compare goodness of fit to one- or two- site competition by sum of squares. K_i values were calculated using the Cheng–Prusoff equation [29]. Hill coefficient values were calculated as described by Bylund and Yamamura [30]. The relative binding to the high and low affinity binding sites was calculated using GraphPad Prizm two-site competition fit. Fraction 1 equals to the fraction of the high affinity sites from the total amount of sites.

Statistical analysis, including un-paired *t*-test or one way ANOVAs with Tukey's post hoc tests, were applied using GraphPad Instat 3 for Windows 95.

3. Results

3.1. Measurements of CFT and dopamine affinities to the wild type and mutated dopamine transporter

Selected DAT mutants were expressed in cultured COS-7 cells as explained in the methods. Alanine-based mutations were chosen, because alanine's methyl side chain is not expected to create polar or hydrophobic interactions. K_m and K_d measurements for DAT mutants are presented in Table 1. [^{3}H]CFT affinity to D79A was reduced compared to its affinity to the WT (although the difference was not statistically significant, $p > 0.05$), and there was no accumulation of radioactive dopamine in that mutation. The affinity of [^{3}H]CFT to the tyrosine mutations was slightly (Y251A) or very significantly (Y273A) ($p < 0.001$) reduced compared to the WT DAT, however, both mutants displayed normal [^{3}H]dopamine uptake. In the S356,359A mutant, the K_m value for dopamine uptake was significantly higher than that of the WT ($p < 0.001$), but [^{3}H]CFT binding was close to normal.

Table 1
[^{3}H]dopamine uptake and [^{3}H]CFT binding in COS-7 cells expressing wild type or mutant rat dopamine transporters

Transporter	K_m (nM)	K_d (nM)
WT DAT	2,936 ± 238.9	20.7 ± 2.58
D79A	n.d.	47.9 ± 6.8
Y251A	1,923 ± 235	28.91 ± 5.5
Y273A	2,340 ± 137	69.3 ± 14.5*
S356,359A	12,786 ± 1,547*	25.64 ± 5.3

Cells were transfected with 20 μg of pcDNA1 containing WT or mutated DAT and assayed for [^{3}H]dopamine transport and [^{3}H]CFT binding. Values of K_m and K_d are presented as the mean ± S.E.M. from 4 to 7 independent experiments performed in triplicate. n.d. = not detectable.
* $p < 0.001$ vs. wild type (Tukey test).

3.2. Affinity of the dopamine transporter substrates and ligands as measured by inhibition of [^{3}H] dopamine uptake

Next, the ability of DAT substrates and ligands to inhibit [^{3}H]dopamine uptake was examined (Fig. 3, Table 2). With the exception of *d*-amphetamine and MPP$^+$, each compound tested for dopamine uptake inhibition displayed affinity to the S356,359A mutant that was similar to its affinity to the WT, affinity to the Y251A mutant that was lower than its affinity to both the WT and the S356,359A mutants, and affinity to the Y273A mutant that was lower than its affinity to all other tested transporters. The relative alterations in K_m value was different for each compound (Table 2). *d*-Amphetamine and MPP+ displayed a very significant reduction in uptake affinity to the S356,359A mutant in comparison to their affinities to the WT DAT ($p < 0.001$). Surprisingly, MPP$^+$ displayed a significantly enhanced uptake affinity to the Y251A and Y273A mutants. *d*-Amphetamine displayed a slightly enhanced affinity at the Y251A mutant and reduction in affinity to the Y273A mutant, although neither difference was statistically significant. The effect of compounds on [^{3}H]dopamine uptake by the D79A mutant could not be evaluated as no uptake was observed by that mutant.

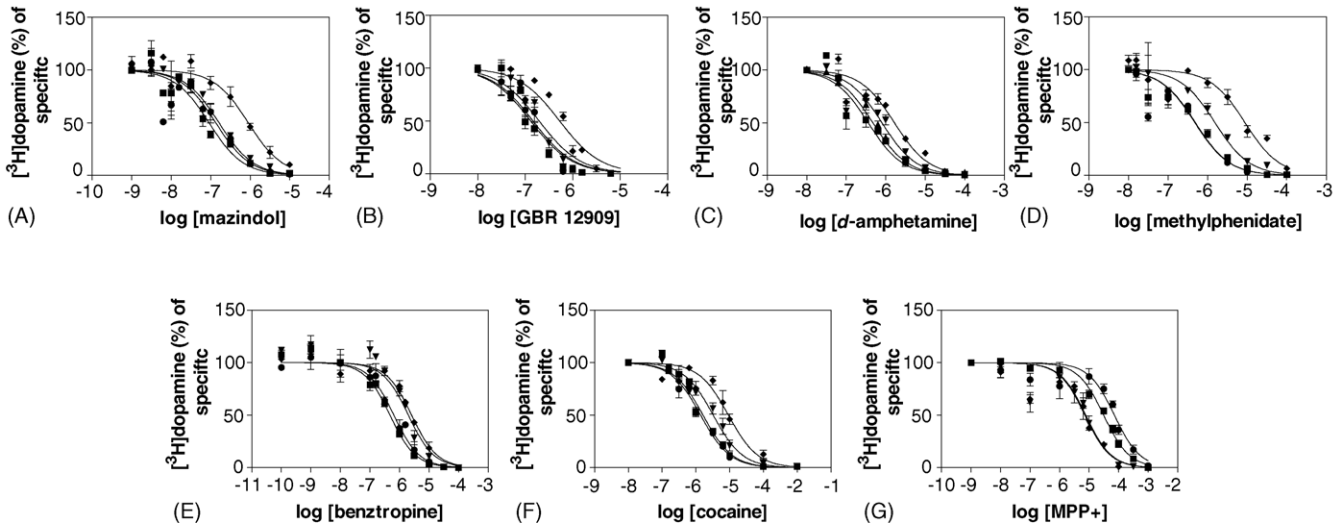

Fig. 3. Inhibition of radiolabeled dopamine uptake by mazindol (A), GBR 12909 (B), *d*-amphetamine (C), methylphenidate (D), benztropine (E), cocaine (F), and MPP+ (G) in wild type (■) and mutated Y251A (▼), Y273A (◆) and S356,359A (●) rat dopamine transporters. K_i values are presented in Table 2.

3.3. Affinity of dopamine transporter substrates and ligands as measured by displacement of [^{3}H]CFT binding

The ability of DAT substrates and ligands to inhibit [^{3}H]CFT binding was examined (Fig. 4, Table 3). In the WT, all tested compounds gave competition data that best fit a two-site model. Hill coefficients for WT data were lower than unity (0.4–0.62), indicating no interaction between the sites (Table 4). In contrast, analysis of many of the competition data taken from experiments using mutants best fit a one-site model. The Hill coefficient calculated for those curves was close to unity (Table 4). In general, there was no statistically significant difference in competition data for each specific compound between the low affinity-binding site in WT DAT and the low affinity (or only binding site, if a single site was identified) binding site in mutant DAT. That was not the case only for *d*-amphetamine displacement of [^{3}H]CFT binding to the S356,359A mutant, which displayed a K_i value significantly different from that of the WT's low-affinity binding site ($p < 0.05$). [^{3}H]CFT competition experiments using mock-transfected cells were conducted (data not shown), and a site similar to the WT low affinity site was observed. Therefore, it is likely that the described low affinity site does not result from DAT interaction, and our discussion will consider the high affinity site to be the CFT/cocaine site.

Mazindol and GBR 12909 were unable to bind to the D79A, Y251A and Y273A mutants, but had only a small (but significant for GBR 12909; $p < 0.05$) reduction in affinity for the S356,359A mutant (Table 3). Cocaine displayed a similar binding pattern to mazindol and GBR 12909, although binding to the tyrosine mutants was significantly lowered instead of completely lost ($p < 0.05$). Methylphenidate were unable to bind to the D79A, Y251A and Y273A mutants, but it had a very large (10-fold), although not statistically significant, reduction in S356,359A mutant binding compared to the WT. Benztropine and *d*-amphetamine could not bind to all of the tested mutants.

Table 2
Estimated K_i values for the inhibition of [^{3}H]dopamine uptake in COS-7 cells expressing wild type or mutant rat dopamine transporters by several dopamine transporter substrates and ligands

Compound	WT	Y251A	Y273A	S356,359A
Mazindol	78.64 ± 7.0	137.7 ± 13.4	830.5 ± 128.4**	91.7 ± 8.1
GBR12909	110.8 ± 16.4	161.1 ± 30.8	488.3 ± 41.1**	119.5 ± 16.1
d-Amphetamine	424.9 ± 48.5	402.9 ± 37.9	703.7 ± 8.6	1,638.95 ± 203.3**
Methylphenidate	460.8 ± 88.8	1,310 ± 153.4	7,860 ±1413**	479.4 ± 37.6
Benztropine	477.5 ± 40.9	1,727.3 ± 211.1**	2,247.8 ± 271**	579.2 ± 51.5
Cocaine	1,473 ± 111.8	2,835.3 ± 235.9	8,229.8 ± 2,117*	1,344 ± 55.6
MPP+	28,398 ± 3,633	6,738 ± 265*	6,291 ± 789.8*	70,684 ± 8,227**

Cells were transfected with 20 μg of pcDNA1 containing WT or mutated DAT and assayed for [^{3}H]dopamine uptake after treatment with DAT substrates and ligands. Data are presented as the mean ± S.E.M. from 3 to 6 independent experiments performed in triplicate.
* $p < 0.05$ vs. wild type (Tukey test).
** $p < 0.001$ vs. wild type (Tukey test).

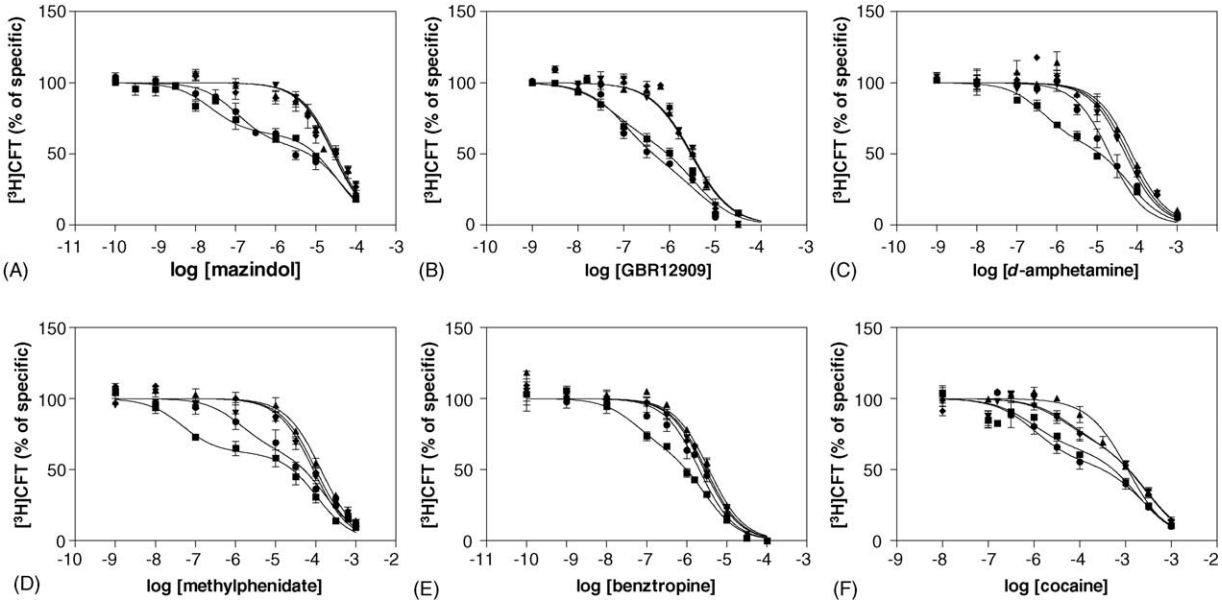

Fig. 4. Inhibition of radiolabeled carboxyfluorotropane binding by mazindol (A), methylphenidate (B), benztropine (C), GBR 12909 (D), *d*-amphetamine (E) and cocaine (F) in wild type (■) and mutated D79A (▲), Y251A (▼), Y273A (◆) and S356,359A (●) rat dopamine transporter. K_i and IC_{50} values are presented in Table 3.

3.4. The relationship between the affinities of dopaminergic compounds to the CFT/cocaine site and their affinities to the dopamine site in the wild type dopamine transporter

K_i values obtained by the inhibition of [^{3}H]dopamine uptake were similar to those obtained by the displacement of [^{3}H]CFT binding in WT when mazindol, GBR 12909 and cocaine were used (WT data from Tables 2 and 3). In contrast, methylphenidate and benztropine displayed about an order of magnitude higher affinity to the CFT/cocaine site than to the dopamine site. This effect was also observed when LLCPK cells stably transfected with the WT rat DAT were used. In that system, benztropine and

Table 3
Estimated K_i and IC_{50} values (nM) for the inhibition of [^{3}H]CFT binding to COS-7 cells expressing wild type or mutant rat dopamine transporter by several dopamine transporter substrates and ligands

Compound	WT	D79A	Y251A	Y273A	S356,359A
Mazindol	40.32 ± 16.9 (f_1 = 0.39)				94.3 ± 27.9 (f_1 = 0.41)
	40,277 ± 4,330	*29,445 ± 3,391*	*31,243 ± 3,405*	*28,760 ± 9,016*	*29,603 ± 7,109*
GBR 12909	56.6 ± 7.1 (f_1 = 0.43)				93.85 ± 17.23* (f_1 = 0.56)
	3,490 ± 169	*3,040 ± 311*	*3,122 ± 176*	*3,093 ± 488*	*3,750 ± 272*
d-Amphetamine	442 ± 80.24 (f_1 = 0.43)				
	66,006 ± 1,117	*70,523 ± 10,052*	*47,517 ± 9,436*	*62,430 ± 8,096*	*20,663 ± 6,232**
Methylphenidate	41.3 ± 16.1 (f_1 = 0.4)				434.1 ± 150.2 (f_1 = 0.33)
	113,153 ± 26,378	*134,233 ± 9,379*	*83,010 ± 16,855*	*101,683 ± 5,324*	*146,883 ± 34,843*
Benztropine	56.3 ± 4.1 (f_1 = 0.39)				
	3,217 ± 289	*3,640 ± 245*	*2,718 ± 200*	*2,999 ± 295*	*2,093 ± 424*
Cocaine	1,186 ± 398 (f_1 = 0.38)		6,163 ± 1,462* (f_1 = 0.32)	6,314 ± 2,154* (f_1 = 0.34)	1,423 ± 832 (f_1 = 0.45)
	213,000 ± 44,015	*85,873 ± 8,572*	*309,333 ± 55,757*	*322,333 ± 43,056*	*265,333 ± 52,454*

Dopaminergic compounds were tested for their ability to inhibit 2 nM [^{3}H]CFT in COS-7 cells transfected with pcDNA1 containing WT or mutant DAT. K_i values of the high affinity site (upper number), IC_{50} value of the low affinity site (in italics) and the fraction of the high affinity component (f_1) are presented. Data are presented as the mean ± S.E.M. from 3 to 6 independent experiments performed in triplicate.

* $p < 0.05$ vs. wild type (GBR 12909, Student's *t*-test; *d*-amphetamine and cocaine, Tukey test).

Table 4
Hill coefficient values calculated from inhibition curves of [^{3}H]CFT by various dopamine transporter substrates and ligands in COS-7 cells expressing wild-type or mutated rat dopamine transporter (mean ± S.E.M.)

Compound	WT	D79A	Y251A	Y273A	S356,359A
Mazindol	0.4 ± 0.04	0.9 ± 0.04***	0.92 ± 0.02***	0.78 ± 0.13**	0.42 ± 0.03
GBR 12909	0.55 ± 0.03	1.09 ± 0.05***	1.06 ± 0.05***	1.16 ± 0.05***	0.38 ± 0.02*
d-Amphetamine	0.51 ± 0.03	0.8 ± 0.02**	0.87 ± 0.06***	0.82 ± 0.03**	0.8 ± 0.04**
Methylphenidate	0.5 ± 0.133	0.89 ± 0.07*	0.8 ± 0.015	0.83 ± 0.05	0.52 ± 0.084
Benztropine	0.62 ± 0.02	1.11 ± 0.03**	1 ± 0.06*	1 ± 0.13*	0.95 ± 0.02*
Cocaine	0.54 ± 0.01	0.73 ± 0.07	0.67 ± 0.05	0.64 ± 0.08	0.43 ± 0.06

* $p < 0.05$ vs. wild type (Tukey test).
** $p < 0.01$ vs. wild type (Tukey test).
*** $p < 0.001$ vs. wild type (Tukey test).

methylphenidate inhibition of [^{3}H]CFT binding was 9.2 and 10.25 times more potent than their inhibition of [^{3}H]dopamine uptake ($n = 1$, data not shown).

4. Discussion

The mechanism of interaction of the DAT with its substrates and ligands and the specific DAT residues that contribute to its function are not well understood. Our strategy in this study was to use DAT mutants in an attempt to gain insight into the interaction of various dopaminergic agents with the transporter. The rat DAT mutants used for this purpose were D79A, Y251A, Y273A and S356,359A, all of which have altered binding or uptake activities [8,27]. Although each of the tested compounds showed a unique mode of interaction with the DAT, it was possible to discern three main patterns of interactions between the small molecules and the DAT.

In the WT rat DAT, the affinities of mazindol, GBR 12909 and cocaine (all considered to be non-transported inhibitory ligands of DAT) for the CFT/cocaine-binding site were similar to, but slightly higher than, their affinities for the dopamine uptake site (K_i for [^{3}H]dopamine uptake/ K_i for [^{3}H]CFT binding: mazindol, 1.95; GBR12909,1.95; cocaine, 1.24). When examined for inhibition of [^{3}H]CFT binding or [^{3}H]dopamine uptake, these three compounds showed complete or partial loss of affinity in the D79A, Y251A, and Y273A mutants, but their affinities in the S356,359A mutant were similar to those obtained in the WT DAT. These findings indicate that mazindol, GBR12909 and cocaine interact with aspartic acid 79 and tyrosines 251 and 273, but probably not with serines 356 and 359. This might be related to the way in which they cause the inhibitory effect at the DAT. These results strongly support the findings of Reith and Selmeci [21] that cocaine, mazindol, and GBR12909 compete for the same site on the mouse DAT. They, however, do not support recent data indicating that GBR 12909 and benztropine have similar mode of binding interaction, different then that of cocaine with the human DAT [31].

CFT is a phenyltropane derivative of cocaine in which the esteric link of cocaine is eliminated by attaching the benzene ring directly to the tropane ring (Fig. 2). In previous studies, using various in vivo and in vitro preparations, the potency of CFT was 3- to 60-fold greater than that of cocaine [32–34]. In our study, CFT (Table 1) and cocaine (Tables 2 and 3) showed different affinity values, but demonstrated similar profiles of activity. Compared to the WT DAT, both showed reduced affinities in the Y251A and Y273A mutants, but similar affinities in the S356,359A mutant (Tables 1–3). Binding to the D79A mutant could not be detected with cocaine (Table 3) and was reduced with CFT.

A different pattern of activity was observed for the uptake of *d*-amphetamine and MPP^+ by the transporter. MPP^+ was not tested for [^{3}H]CFT displacement, as our preliminary experiments showed that this compound exhibits only slight competitive activity at that site (data not shown). When tested for inhibition of [^{3}H]dopamine uptake, the affinity of *d*-amphetamine and MPP^+ was very significantly reduced in the S356,359A mutant compared to the WT DAT ($p < 0.001$; Table 2) by 3.9- and 2.5-fold, respectively. These results resemble the reduction in affinity for dopamine uptake by this mutant ($p < 0.001$; Table 1) and indicate that serines 356 and 359 are important for the uptake mechanism. Because these two serines are important for the uptake of MPP^+ and *d*-amphetamine as well as of dopamine, it is unlikely that they create a hydrogen bond with the dopamine hydroxyl groups as suggested elsewhere [8]. It seems likely that they have a more general role in uptake, such as ion translocation. When examined for [^{3}H]dopamine uptake inhibition, the affinity of MPP^+ ($p < 0.05$) and to a lesser extent the affinity of *d*-amphetamine (not significant) for the Y251A mutant were higher then that of the WT (Table 2). The affinity of MPP^+ (but not of *d*-amphetamine) for the Y273A mutant was also significantly higher than for the WT in that assay ($p < 0.05$, Table 2). In a previous study, using a double mutation at the 11th TM domain in which serines 507 and 518 were replaced by alanine, [^{3}H]MPP^+ uptake was found to be similarly increased [35]. As mentioned in that study, this phenomenon might be indicative of a mutant-induced alteration in the secondary and tertiary structure of the transporter.

For benztropine and methylphenidate, yet another pattern was observed. When examined in the WT DAT, both showed significantly higher affinity for the CFT/cocaine site than for the dopamine site (K_i [^{3}H]dopamine uptake/K_i [^{3}H]CFT binding: benztropine 8.5; methylphenidate 11; Tables 2 and 3). When displacement of [^{3}H]CFT binding in the S356,359A mutant was examined, the affinity of methylphenidate for this site was reduced relative to the WT (by 10.5-fold) and that of benztropine was completely lost. Surprisingly, this reduction or loss of affinity for the S356,359A mutant seen with [^{3}H]CFT binding displacement was not reciprocal with [^{3}H]dopamine uptake. Thus, in the S356,359A mutant, the affinity of methylphenidate and benztropine for the CFT/cocaine binding site were lower than those of the WT (Table 3) but were similar to those of the WT for the dopamine uptake site (Table 2). This differential inhibition of [^{3}H]CFT binding or [^{3}H]dopamine uptake by benztropine and methylphenidate suggests that these two ligands do not overlap with either non-transported inhibitors or with substrates. This might indicate that serines 356 and 359 are located in a unique position that enables them to interact both with compounds that are taken up by the DAT and with compounds that bind to it in separate ways that have yet to be elucidated.

It was suggested that methylphenidate and benztropine might act by blocking the inward transport of dopamine into the presynaptic terminal, resulting in a prolonged dopamine stimulus [36]. However two studies have shown an unusual type of action. Wayment et al. [37], using rotating disk electrode volumetry, found that methylphenidate inhibits the striatal transport of dopamine by a competitive mechanism that can be distinguished from that of cocaine, but not from that of *d*-amphetamine. Methylphenidate was not found, however, to induce dopamine release, suggesting that it is not a substrate analog, a notion that agrees with our findings. In the second study, in which the DAT was photoaffinity-labeled with the benztropine analog [^{125}I]GII34, this compound bound to a different part of the DAT than the one to which the cocaine analog binds [38]. In contrast to our findings, however, it did overlap the presumed site of GBR as tested with the GBR analog, [^{3}I]DEEP [38]. In our study, methylphenidate and benztropine behaved like transported ligands when tested for inhibition of radioactive CFT binding and like non-transported ligands when tested for inhibition of radioactive dopamine uptake. The results of the present and previous studies suggest that these two compounds do not act either as classical non-transported inhibitors or as classical substrates. Their overall affinities are higher at the cocaine site than at the dopamine site, but their interaction with the CFT/cocaine site more closely resemble the way in which transported substrates interact.

In summary, we have examined the effects of DAT ligands and substrates in four DAT mutants. We found that the binding of mazindol and GBR 12909 overlaps with that of cocaine, and they can therefore be considered to be non-transported inhibitors. The activity of MPP+ and *d*-amphetamine overlapped with that of dopamine, and these compounds are therefore considered to be transported substrates. Methylphenidate and benztropine interacted with the transporter in a way that may indicate an interaction with a domain on the transporter that is distinct from those of the two previous groups. These results highlight the need for a model that can account for these findings and might contribute to the eventual development of an agent that can serve as a cocaine antagonist.

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