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Dopamine Transporter Mutants with Cocaine Resistance and Normal Dopamine Uptake Provide Targets for Cocaine Antagonism

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ABSTRACT

Cocaine's blockade of dopamine reuptake by brain dopamine transporters (DAT) is a central feature of current understanding of cocaine reward and addiction. Empirical screening of small-molecule chemical libraries has thus far failed to provide successful cocaine blockers that allow dopamine reuptake in the presence of cocaine and provide cocaine "antagonism". We have approached this problem by assessing expression, dopamine uptake, and cocaine analog affinities of 56 DAT mutants in residues located in or near transmembrane domains likely to play significant roles in cocaine recognition and dopamine uptake. A phenylalanine-to-alanine mutant in putative DAT transmembrane domain 3, F154A, retains normal dopamine uptake, lowers cocaine affinity 10-fold, and reduces cocaine stereospecificity. Such mutants provide windows into DAT structures that could serve as targets for selective cocaine blockers and document how combined strategies of mutagenesis and small molecule screening may improve our abilities to identify and design compounds with such selective properties.

The dopamine transporter (DAT) is a putative 12-transmembrane domain (TM) protein that takes up dopamine into neurons of brain pathways that contribute to behavioral reward (Ranaldi et al., 1999; Redgrave et al., 1999). Cocaine blockade of dopamine uptake by the DAT expressed by these neurons has been identified as a crucial component for cocaine reward, suggesting that selective blockade of cocaine recognition in this pathway could have therapeutic importance for development of anticocaine medication (Uhl et al., 1998; Villemagne et al., 1999).

To improve understanding of the ways in which DAT recognizes cocaine and dopamine, hundreds of cocaine analogs have been synthesized and hundreds of DAT mutant and chimeric molecules constructed and characterized (Kitayama et al., 1992; Giros et al., 1994; Buck and Amara, 1994, 1995; Mitsuhata et al., 1998; Lin et al., 1999, 2000; Itokawa et al., 2000). Cocaine analogs and chemical libraries of more than 300,000 compounds have been screened for differential activities in inhibiting cocaine analog binding and in blocking dopamine uptake (Chalon et al., 1999; Javanmard et al., 1999; Hoepping et al., 2000). Studies with these small molecules have identified the importance of many features of cocaine's structure for its ability to block dopamine uptake or to inhibit cocaine analog binding by DAT. Polar, cationic, and aromatic interactions between DAT, dopamine, and cocaine (Carroll et al., 1992) are important, as is cocaine's phenyl ring (Lieske et al., 1998). However, no small-molecule blocker of cocaine recognition by DAT that substantially spares dopamine uptake, one major goal of anticocaine medication development, has yet been identified.

Mutagenesis data support the importance of amino acids located in or near DAT transmembrane domains (TMs) 1, 3, 4, 6, 9, 11, and 12 for dopamine affinity in uptake assays. Residues in or near TMs 1, 2, 4 to 6, and 8 to 11 seem to be important for recognizing the widely-used cocaine analog (−)-2-β-carbomethoxy-3-β-(4-fluorophenyl) tropane (CFT; Kitayama et al., 1992; Lin et al., 1999, 2000a,b; Mitsuhata et al., 1998; Itokawa et al., 2000). However, no DAT amino acid that is so selectively involved with cocaine recognition that it could provide a target for a selective dopamine sparing-cocaine antagonist has been fully identified.

Studies with small molecules and mutants that are available currently can suffer from limits in pharmacologic and methodologic precision. Compound affinities measured at 37°C, a condition typically used for uptake assays, may differ substantially from affinities assessed under the 4°C condition typically used for cocaine analog binding. Structural differences between cocaine and the cocaine analogs typically used for binding studies, usually CFT, could render results

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ABBREVIATIONS: DAT, dopamine transporter; TM, transmembrane domain; CFT, (−)-2-β-carbomethoxy-3-β-(4-fluorophenyl) tropane; MPP^+, 1-methyl-4-phenylpyridinium; 5-HT, serotonin; KRH, Krebs-Ringer-Henseleit; WT, wild-type.

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from studies of cocaine analogs different from those of cocaine itself.

We now report detailed assessments of the recognition of dopamine, cocaine analogs, and cocaine itself by a series of DAT mutants in single amino acids lying in or near putative DAT TM domains. These results support subtle differences between cocaine and cocaine analog recognition processes. They identify an aromatic amino acid target for development of anticocaine medication. They support the idea that improved knowledge about cocaine selective target(s) in DAT could help the search for substrate-sparing anticocaine compounds acting selectively at DAT and/or at other monoamine transporters (Slusher et al., 1997; Smith et al., 1999).

Experimental Procedures

Transient Expression in COS Cells. DAT mutants were assessed in transiently-expressing COS cells as described previously (Lin et al., 1999). Transfection employed DNA preparations with OD_{260}/OD_{280} ratios ≥ 1.75, and transfection efficiencies examined by immunostaining of the transfected COS cells as described previously (Freed et al., 1995; Lin et al., 1999).

Functional Assays. COS cells expressing DATs were grown for 3 days and then assayed for their abilities to accumulate [^{3}H]dopamine (25.9 Ci/mmol) or to bind tritium-labeled levo-(−)-[benzoyl-3,4-^{3}H(N),] cocaine (83.5 Ci/mmol). Kinetic and saturation analyses determined K_M, V_{max}, K_D, and B_{max} values, including those for [^{3}H]dopamine uptake, as described previously (Pfenning and Richelson, 1990; Lin et al., 1999). For [^{3}H]MPP$^+$ (20 nM, 77.5 Ci/mmol), [^{3}H]serotonin (5-HT) (190 nM, 24.0 Ci/mmol), [^{3}H]norepinephrine (50 nM, 54.7 Ci/mmol), or [^{3}H]epinphrine (30 nM, 61.7 Ci/mmol) uptake, tritium-labeled substrate concentrations were adjusted to 1, 5, 10, 20, 40, 60, and 100 μM with the corresponding unlabeled chemicals. Fifty micromolar pargyline, 1 μM RO 41-0960 (a catechol-*O*-methyltransferase inhibitor; RBI/Sigma, Natick, MA) and 50 μM ascorbic acid were included in all uptake assay buffers. Binding assays were performed with scraped cell suspensions. Cells in two dishes were washed twice each with 10 ml of Krebs-Ringer-Henseleit buffer (KRH, 125 mM NaCl, 4.8 KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.6 mM glucose, and 25.0 mM HEPES) buffer, harvested by scraping, suspended in 10 ml of ice-cold KRH buffer, mixed for 5 s, and then distributed so that each 12- × 75-mm Kimble borosilicate glass culture tube (MG Scientific, Pleasant Prairie, WI) contained approximately 5×10^5 cells. For [^{3}H]cocaine binding assays, 20 nM [^{3}H]cocaine was adjusted to 40, 60, 80, 120, 220, 420, 620, or 1020 nM with unlabeled cocaine. One micromolar 1-(α-diethylaminopropionyl)-phenothiazine (a butyrylcholinesterase inhibitor; RBI/Sigma), and 0.35 mM lidocaine (a cocaine binding competitor; RBI/Sigma) were included in the binding assay buffers. 30 min 37°C incubations were terminated by filtration and three 5-ml washes with KRH buffer using Whatman GF/B filters pretreated with 0.05% polyethylenimine and a Brandel filter apparatus (Biomedical Research and Development Laboratories, Inc., Gaithersburg, MD). Membrane fragments were released from the filter paper by gentle shaking in scintillation liquid for at least 6 h before radioactivity was assessed. COS cells transfected with the plasmids pcDNA1/rDAT and pcDEDAT, served as positive and negative (MOCK) controls, respectively (Shimada et al., 1991; Lin et al., 1999). To assess cocaine inhibition of the binding of 3 nM [^{3}H]mazindol, cocaine was added at nine concentrations ranging from 0 to 10 mM and incubated at 37°C for 30 min in buffers containing 1 μM 1-(α-diethylaminopropionyl)-phenothiazine to prevent degradation of cocaine.

Uptake competition experiments were carried out at 37°C for 5 min using 20 nM [^{3}H]dopamine, 250 nM [^{3}H]5-HT, 30 nM [^{3}H]MPP$^+$, 30 nM [^{3}H]norepinephrine, or 30 nM [^{3}H]epinephrine. Unlabeled compounds were added at nine concentrations ranging from 0 to 1 mM in buffers containing ascorbate to prevent degradation of monoamines and cocaine. Each experiment was carried out in duplicate. Tritium-labeled substrates and cocaine were from PerkinElmer Life Sciences (Boston, MA); unlabeled substrates, dopamine, 5-HT, norepinephrine, and ligands were from RBI/Sigma, and other chemicals, including MPP$^+$, were from Sigma (St Louis, MO).

Analyses and Modeling. Data analyses were carried out as described previously (Lin et al., 1999). Molecular modeling was carried with the use of Sybyl 6.6 programs (Tripos, Inc., St. Louis, MO). Weighted root mean square distances between modeled side chain and backbone positions in wild-type and mutant DATs were calculated using the program "Fit Monomers". Electrostatic potential differences between wild-type and mutant DATs were calculated using the program "vdW Dot Surface". Values for the unit of V were calculated at 1.4-Å distances from molecules' van der Waals' surfaces as described previously (Weiner et al., 1982).

Results

Fifteen of 56 Characterized DAT Mutants Spare [^{3}H]Dopamine Uptake Activity

The 56 DAT alanine-substitution mutants assessed for the characteristics of the dopamine uptake that they could confer on transiently expressing COS cells included 29 phenylalanine,16 proline, and 11 tryptophan mutants (Kitayama et al., 1992; Lin et al., 1999, 2000a,b). Fifteen of these mutants (F154A, P234A, P235A, F252A, L400A, F447A, F456A, F461A, F477A, F485A, F542A, P544A, P545A, W555A, and W561A) displayed both normal patterns of expression, assessed immunohistochemically, and near–wild-type dopamine affinities. K_M assessments of dopamine affinity were within 2-fold of wild-type values and V_{max} estimates of transport velocity at least 70% of wild-type values. Dopamine K_M values for mutants P235A, F485A, and F477A were lower than wild-type values, reaching statistical significance.

Eleven of 15 DAT Mutants with Nearly Normal Dopamine Uptake Display Reduced Affinity for [^{3}H]CFT

CFT affinities were within 3-fold of wild-type values for mutants P234A, L400A, F447A, and F461A when assessed under binding conditions with our modified 37°C 30-min incubation conditions that provide parallels with incubation conditions for uptake assays (see below). Affinities were reduced 3-fold by P545A, 4-fold by P235A, 5-fold by W561A, 7-fold by W555A, 11-fold by P544A, 12-fold by F477A, and 693-fold by F252A. Mutants F154A, F456A, F485A, and F542A displayed such low CFT affinities that their binding could not be accurately assessed. We thus estimate that each displays less than 1/1000th the affinities of the wild-type transporter.

One of the Eight DAT Mutants with Selectively Reduced [^{3}H]CFT Affinity Displays Detectable [^{3}H]Cocaine Binding

Of the eight DAT mutants that displayed no statistical difference from wild-type dopamine uptake K_M values and displayed reduced [^{3}H]CFT affinities, only W555A displayed clearly-detectable [^{3}H]cocaine binding above background levels (Fig. 1, A-D). These binding experiments used [^{3}H]cocaine concentrations up to 1020 nM and unlabeled cocaine concentrations of 0.1 mM to determine nonspecific binding (Table 1). Interestingly, W555A is a mutant for which cocaine affinities in inhibiting dopamine uptake were also close to wild-type values (see below). No detectable [^{3}H]cocaine binding

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could be achieved even for the six other mutants for which cocaine was an effective inhibitor of dopamine uptake (see below).

Three of the Eight DAT Mutants with Selectively Reduced [^{3}H]CFT Affinity Display Significantly Reduced Affinity for Cocaine in Dopamine Uptake Inhibition Experiments

In contrast to the data for cocaine binding, cocaine inhibition of dopamine uptake could be determined for each of the eight mutants noted in Table 1. Reductions in cocaine's affinity for DAT, assessed in this fashion, were statistically significant for F456A, P545A, and F154A. The 10-fold reduction in affinity for cocaine manifest by F154A was especially impressive (Fig. 2) because this represented the single amino acid substitution mutant with one of the most selective effects on cocaine recognition of any mutation or small molecule reported to date.

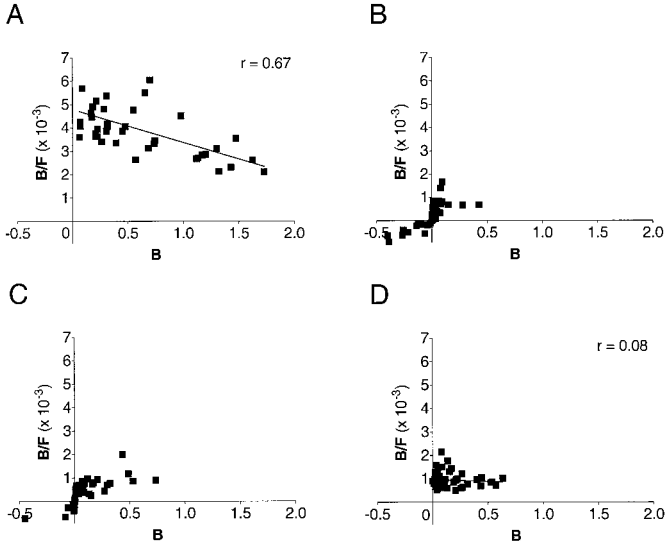

Fig. 1. Scatchard analyses of [^{3}H]cocaine binding activities for the WT DAT (A), negative control MOCK (B), and the DAT mutants F154A (C) and W555A (D). Those mutants are chosen to show that DAT mutants may (D) or may not (C) display detectable cocaine binding activities. C also represents six other mutants (Table 1, U.D.). Different symbols represent data from different independent experiments. Correlation coefficient (r) is a fraction between 0 and 1. When $r = 0$, there is no linear relationship between B and B/F; $r = 1$, all points lie exactly on a straight line with no scatter. B and C do not show any cocaine binding activities and no r values are listed.

Detailed Characterization of F154A DAT

Dopamine Uptake Activity with Normalization for Transfection Efficiency. To improve estimates of possible differences between wild-type V_{max} values and those for F154A, we calculated the dopamine uptake activity in this mutant after normalization for transfection efficiencies using counts of DAT-immunopositive COS cells (Fig. 3). F154A uptake V_{max} values were even closer to wild-type values after normalization. This mutant displayed V_{max} values of 189.8 ± 6.6 fmol/μg/min, 78% of wild-type values of 243.5 ± 6.5 fmol/μg/min. F154A K_M values of 2.1 ± 0.1 μM were indistinguishable from wild-type values of 2.0 ± 0.1 μM ($p > 0.05$, $n = 4$) and close to values reported previously (Lin et al., 1999). Each of these results supported the intactness of dopamine uptake in this mutant.

Uptake of Other Substrates by the F154A Mutant and Inhibition by Cocaine. F154A displayed uptake affinities similar to wild-type values for [^{3}H]5-HT, [^{3}H]epinephrine, [^{3}H]norepinephrine and [^{3}H]*N*-methyl-4- phenylpyridinium (MPP$^+$) *(data not shown)*. Cocaine was less potent at inhibiting uptake of each of these alternative DAT substrates at the F154A mutant than at the wild-type transporter. Cocaine was more than 19-fold less potent in inhibiting 5-HT uptake by the F154A mutant than the wild-type transporter. It was 2.2- to 3.5-fold less potent in inhibiting epinephrine, norepinephrine and MPP$^+$ uptake at F154A than wild-type DAT (Table 2, *Cocaine Inhibition of Substrate Uptake*). Other substrates were also less potent in inhibiting [^{3}H]5-HT uptake by F154A than the wild-type transporter (Table 2, *Substrate Inhibition of 5-HT Uptake*).

Inhibition of Dopamine Uptake by the F154A Mutant Using Other Compounds.

Substrates. Dopamine inhibition of dopamine uptake was similar in wild-type and F154A DATs. 5-HT, epinephrine, norepinephrine, and MPP$^+$ displayed affinities within 1.3- to 1.5-fold of wild-type values in inhibiting dopamine uptake by the F154A transporter (Table 2, *Substrate Inhibition of Dopamine Uptake*).

Inhibitors. The abilities of other DAT ligands to inhibit dopamine uptake by F154A were also examined. Whereas

TABLE 1

DAT mutants' affinities for cocaine

Experiments were performed at 37°C (see *Experimental Procedures*). Each of the *t* test results was confirmed by analyses of variance except for F456A ($p > 0.05$).

	Binding Affinity			Inhibition of Dopamine Uptake	
Genotype	K_D	B_{max}	*n*	K_I	*n*
	μM	*fmol / μg*		*μM*	
WT	0.71 ± 0.10	3.36 ± 0.44	7	0.30 ± 0.07	8
MOCK	U.D.		5	N.D.	
F154A	U.D.		7	3.0 ± 0.6**	6
P234A	U.D.		6	0.41 ± 0.08	4
F252A	U.D.		6	0.34 ± 0.07	4
F456A	U.D.		6	0.53 ± 0.09*	4
F542A	U.D.		6	0.39 ± 0.09	4
F544A	U.D.		6	0.40 ± 0.08	4
P545A	N.D.			0.88 ± 0.02**	4
W555A	3.42 ± 2.37	3.10 ± 2.14	7	0.36 ± 0.07	4
W561A	U.D.		5	0.37 ± 0.07	4

U.D., undetectable; N.D., not determined.
*, $p < 0.05$ compared with WT, as measured by Student's *t* test.
**, $p < 0.001$ compared with WT, as measured by Student's *t* test.

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active (−)-cocaine's ability to inhibit dopamine uptake by the F154A was reduced by 10-fold, the potency of inhibition by the less-active (+)-cocaine was reduced by only 3.0-fold. Affinity of (+)-cocaine was 219-fold lower than (−)-cocaine for the wild-type DAT, but only 65-fold lower with the F154A mutant DAT. The F154A mutant thus loses a large amount of its stereoselectivity for cocaine isomers. Potencies of unlabeled benztropine, GBR 12909, methamphetamine, mazindol, and nomifensine were also reduced by 1.5- to 2.6-fold in this DAT mutant. CFT lost affinity for this mutant by 2.6-fold. Desipramine and both *d*- and *l*-amphetamine isomers inhibited dopamine uptake by F154A mutant DAT with potencies similar to those displayed for wild-type DAT (Table 2, *Ligand Inhibition of Dopamine Uptake*). However, none of the other compounds displayed the 10-fold potency loss noted for cocaine. The structure-activity relationships of the F154A mutant thus differ significantly from those of wild-type DAT.

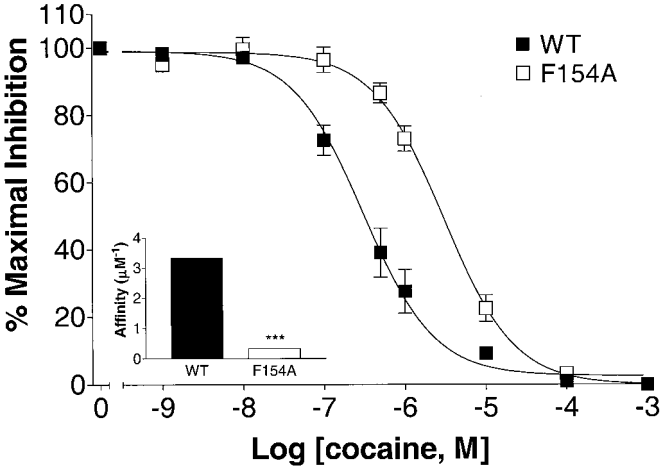

Fig. 2. Cocaine inhibition of [^{3}H]dopamine uptake displayed by the WT DAT and mutant F154A. The [^{3}H]dopamine concentrations used were 20 nM. Data were from eight and six independent experiments for WT and F154A, respectively. The goodness of fit (R^2) is 0.9996 for WT and 0.9997 for F154A. R^2 is a fraction between 0 and 1. When $R^2 = 0$, the best-fit curve fits the data no better than a horizontal line going through the mean of all Y values; $R^2 = 1$, all points lie exactly on the curve with no scatter. *Insert:* Comparison of cocaine affinity ($1/K_i$) between WT and F154A. The Student's t test for statistical significance was based on the K_i values (Table 1).

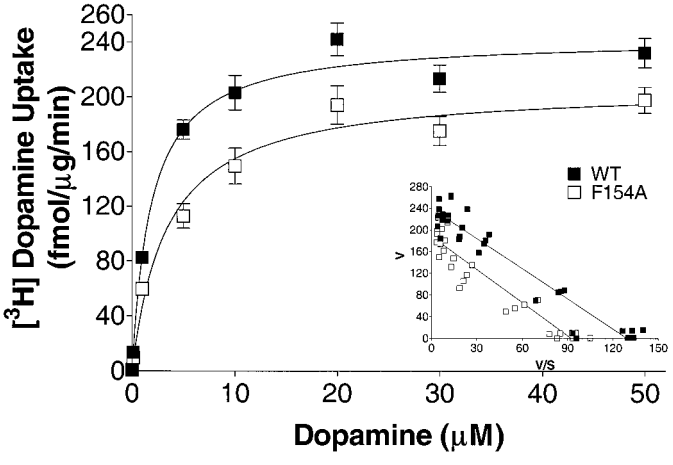

Fig. 3. Saturation assays of [^{3}H]dopamine uptake activity with the WT DAT and mutant F154A. Data represent the mean values of four independent experiments. The goodness of fit (R^2) is 0.97 for WT and 0.94 for F154A. Inset, Scatchard analyses of the same [^{3}H]dopamine uptake activities. Different symbols represent data from different independent experiments; solid symbols, WT; open symbols, F154A. The uptake K_M values are 2.0 ± 0.1 μM for WT and 2.1 ± 0.1 μM for F154A; the V_{max} values are 243.5 ± 6.5 fmol/μg/min for WT and 189.8 ± 6.6 fmol/μg/min for F154A, according to these Scatchard analyses. There are no significant differences ($p \geq 0.05$) of the K_M values but there are significant differences ($p < 0.01$) of the V_{max} values between WT and F154A, by Student's t tests. These V_{max} values were all normalized to a transfection efficiency level of 20%, among different transient expression experiments.

Cocaine Inhibition of [^{3}H]Mazindol Binding. Cocaine displayed modest potency in competing for mazindol binding to DAT. K_i values for cocaine inhibition of [^{3}H]mazindol binding were similar for wild-type and F154A DAT variants (113.7 ± 11.6 versus 116.3 ± 15.4 μM, respectively; $n = 4$).

Discussion

Many small molecule ligands for DAT and many DAT mutations influence both cocaine recognition and dopamine uptake. Identifying small molecules or mutants that are able to block cocaine affinities for DAT while perfectly sparing other transporter properties has proven much more difficult. Currently available DAT mutants most often affect transport V_{max} rate. This may not be surprising if large parts of DAT are implicated in at least one aspect of complex transport processes that can include dopamine and ion recognition, translocation, and unloading at the intracellular side of the protein. We thus began by identifying mutants that had little effect on dopamine uptake rates or dopamine affinities.

Recent reports from the National Institute on Drug Abuse-sponsored screening programs suggest that a number of compounds in several chemical libraries can block cocaine analog recognition by DAT under conditions optimized for CFT binding. A number of these candidate structures fail to inhibit dopamine uptake using assays performed under different conditions favorable for uptake. However, when conditions are adjusted so that uptake and cocaine analog binding are performed under similar conditions, all of the candidate compounds identified to date are reported to fail to retain their differential potencies, and thus lose their promise as therapeutic leads (N. M. Appel, personal communication, 2000). In the present and in previous experiments, we have also identified different behaviors of DAT mutants in cocaine analog recognition and in cocaine recognition, as well as different behaviors in the 4°C assays characteristic of binding and in the 37°C assays used for uptake assessments. Our observations that these mutants display different profiles of interaction with CFT in [^{3}H]CFT binding experiments, with cocaine in [^{3}H]cocaine binding experiments, and with cocaine in [^{3}H]dopamine uptake competition experiments underscore the complexities of these interactions. They point to the need to evaluate test compounds and mutants using a number of different paradigms. Current data also point to a major weakness of cocaine binding studies performed at 37°C. Although each of the 15 studied mutants except F154A displayed significant cocaine K_i values in blocking dopamine uptake, most showed little cocaine binding above background levels. The relatively low sensitivity of cocaine binding assays is also reflected in the high background values for in these experiments. Use of several approaches may be necessary to reliably obtain biologically relevant cocaine binding parameters.

The data for F154A are thus particularly interesting. F154

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is predicted to lie in the N-terminal, extracellular side DAT TM3. This mutant is expressed normally, retains its affinity for dopamine, and displays near–wild-type dopamine transport V_{max} values. However, it loses affinity for cocaine and reduces the stereospecificity of cocaine's ability to block dopamine uptake (Table 2). This mutant reduces CFT binding affinities measured at either 37°C or 4°C. It also reduces cocaine's abilities to block uptake of several substrates in addition to dopamine. It reduces cocaine's ability to inhibit 5-HT uptake by 19-fold and other substrates' abilities to inhibit 5-HT uptake by 3- to 8-fold, compared with wild-type DAT (Table 2). Residue 154 could thus contribute to substrate specificity. This idea is consistent with the fact that serine in serotonin transporters from human, rat and mouse occupies this equivalent position. F154A lies in the putative TM3 domain that contains residues important for cocaine recognition or substrate uptake by DAT and by other monoamine transporters (Chen et al., 1997; Redgrave et al., 1999). Loss of DAT TM3 phenylalanine 155 and valine 152 side chains substantially reduce dopamine uptake and moderately reduce CFT binding (Lin et al., 1999; Lee et al., 2000). Because current DAT models place these two residues on the same side of the TM3 helical domain (Fig. 2), this face of the TM3 helix has been especially implicated in uptake. Mutations in the serotonin transporter isoleucine 172, the equivalent of DAT valine 152, also dramatically reduce serotonin uptake (Chen and Rudnick, 2000). TM domain models place phenylalanine 154 in the side of the TM3 helix opposite that containing the above-mentioned two DAT residues 152 and 155. This position is thus consistent with our current data documenting the sparing of dopamine uptake in the F154A mutation. Mutants F456A and P545A also displayed statistically significant reductions in cocaine's affinity for DAT (Table 1). F456 and P545 cannot be placed in the same cocaine-binding pocket as F154 because of their distal locations according to the current DAT models. Combination of the mutations in these three residues could conceivably result in a DAT mutant with even lower sensitivity to cocaine.

More detailed comparisons of molecular models of the wild-type and F154A DAT TM3 domains also indicate that mutation-induced DAT structural differences are likely to be small as indicated by weighted root-mean-square distance assessments. Negligible average distances (~0.0001 Å) are found between the modeled positions of wild-type and mutant TM3 amino acid side chains or backbones. Removing the F154 aromatic side chain reduces the modeled net electrostatic potentials of the side chains of nine nearby amino acids, especially that of F155. It also increases the modeled electrostatic potentials in the side chains of two residues (Fig. 4). More than two thirds of the residues for which electrostatic potentials may be modestly reduced by the F154A mutation are located toward the N-terminal, cytoplasmically directed side of F154. The intensity of this net mutation influence on electrostatic potentials seems higher on the helical side than seems to be important for dopamine recognition. The subtle changes observed in DAT modeling data are also consistent with experimental observations that this mutant displays normal dopamine uptake affinities and only modest reductions in the V_{max} values. These data contribute to the set of results indicating that DAT may not use

TABLE 2
Inhibition affinities of mutant F154A
Experiments were all performed at 37°C, as described under *Experimental Procedures*.

Substrate/Ligand	K_i		n	Reduction
	WT	F154A		
	μM			
Cocaine Inhibition of Substrate Uptake				
5-HT	7.9 ± 1.3	152.8 ± 32.7*	3	19.3×
Epinephrine	0.25 ± 0.02	0.54 ± 0.10*	3	2.2×
Norepinephrine	0.29 ± 0.01	0.64 ± 0.03***	3	2.2×
MPP^+	0.38 ± 0.01	1.32 ± 0.09***	3	3.5×
Substrate Inhibition of 5-HT Uptake				
5-HT	0.27 ± 0.03	1.40 ± 0.32*	3	5.2×
Epinephrine	0.42 ± 0.05	1.30 ± 0.10**	3	3.1×
Norepinephrine	0.26 ± 0.02	2.07 ± 0.29**	3	8.0×
MPP^+	0.36 ± 0.04	1.52 ± 0.17**	3	4.2×
Substrate Inhibition of Dopamine Uptake				
5-HT	99.5 ± 6.7	129.7 ± 10.8	4	1.3×
Epinephrine	69.2 ± 4.0	88.1 ± 2.7**	4	1.3×
Norepinephrine	21.3 ± 1.6	32.6 ± 1.8**	4	1.5×
MPP^+	14.7 ± 1.4	18.8 ± 0.8*	4	1.3×
Dopamine	2.2 ± 0.2	1.7 ± 0.1	4	0.8×
Ligand Inhibition of Dopamine Uptake				
(−)-Cocaine	0.30 ± 0.07	3.0 ± 0.6***	8,6	10.0×
(+)-Cocaine	65.7 ± 4.8	195.0 ± 23.8**	4	3.0×
CFT	0.05 ± 0.003	0.13 ± 0.004***	4	2.6×
Benztropine	0.21 ± 0.01	0.55 ± 0.04***	4	2.6×
GBR 12909	0.016 ± 0.002	0.039 ± 0.005**	4	2.5×
Methamphetamine	0.33 ± 0.02	0.79 ± 0.01***	4	2.4×
Mazindol	0.042 ± 0.004	0.062 ± 0.002**	4	1.5×
Nomifensine	0.11 ± 0.01	0.26 ± 0.01***	4	2.4×
Desipramine	22.5 ± 4.4	31.9 ± 4.5	4	1.4×
d-Amphetamine	0.28 ± 0.005	0.32 ± 0.02	4	1.1×
l-Amphetamine	1.8 ± 0.2	2.0 ± 0.3	4	1.1×

*, $p < 0.05$ compared with WT, as measured by Student's t test.
**, $p < 0.01$ compared with WT, as measured by Student's t test.
***, $p < 0.001$ compared with WT, as measured by Student's t test.

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identical sites to recognize the substituted phenyl rings of dopamine and of cocaine.

The current work that identifies selectively altered cocaine affinity with a TM3 DAT mutation contrasts with the failure of empirical screening approaches to develop small molecules that would selectively drop cocaine affinities for DAT in the manner displayed by the F154A mutant. Comparison of the location of F154 with the nearby locations of residues at which mutations lead to less specific effects on cocaine affinities is sobering. These comparisons suggest that a quite carefully crafted molecular epitope may be required to subserve a function similar to that of the mutation in reducing cocaine affinity for DAT. Availability of this mutant provides an excellent opportunity to add to the power of small molecule screening approaches to identifying lead compounds based on their selective interactions with this site. Our findings might suggest that small molecule features interacting with a phenyl ring, for example, could aid the chance of identifying a cocaine-selective DAT antagonist (Wang et al., 2000). Screening other DAT mutants (Itokawa et al., 2000; Chen et al., 2001) or other monoamine transporter mutants (Chen et al., 1997; Penado et al., 1998) for some of the features expressed by F154A could lead to identification of additional cocaine-selective residues. Subsequent efforts to improve designs and to add features that could interact with additional selective DAT sites, identified through further mutagenesis studies, could improve affinity and selectivity for cocaine blocker lead compounds. Such combined bootstrapping strategies for mutagenesis and selective small molecule screening could be applicable to the broad range of pharmacological targets that demand increasingly-selective small molecule design and selection to make increasingly-selective interactions with specific domains of important cellular proteins which may have numerous closely-related family members. Screens comparing small molecule interactions with wild-type and carefully selected mutants could thus become a much more widely used strategy to identify novel therapeutics. F154A is a strong candidate mutant for use in screening compounds for selective cocaine antagonism.

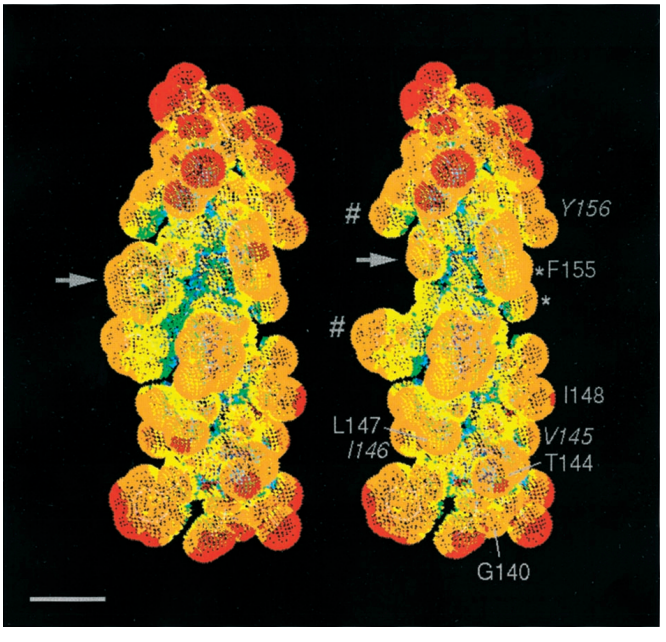

Fig. 4. DAT TM3 electrostatic potential models. A, dot surface displays of electrostatic potentials in WT (left) and F154A (right) TM3 helices. Each TM3 was built as an α-helix and energy-minimized. The 21 amino acids of TM3 cross the plasma membrane from the lower (N-terminal, cytoplasmic) side to the upper (C-terminal, extracellular) side. F154A is indicated by arrows. Dot surfaces were displayed using Sybyl 6.6 "vdW Dot Surface" with red indicating electrostatic potential measures of "V" > 24.9; orange, yellow or green $3.3 > V > 0$, cyan $0 > V > -3.3$ and blue, purple or white $V < -24.9$. "V" is defined as described by Weiner et al. (1982) at an arbitrary distance of 1.4 Å. At the helical side important for cocaine binding, there are seven residues; three (42%) are decreased and another three increased in electrostatic potentials. At the side for dopamine uptake, there are eight residues; seven of them (87%) are decreased and none of them is increased in electrostatic potentials. Indicated are amino acid residues visible on this graph to the reader and whose electrostatic potentials are reduced by the F154A substitution. Italic labels denote residues hiding from the reader (located at the opposite side of the domain). #, increased electrostatic potentials for asparagine 157 (above the arrow) and phenylalanine 150 (below the arrow). Scale bar, 5 Å.

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