

# Dopamine Transporter: Transmembrane Phenylalanine Mutations Can Selectively Influence Dopamine Uptake and Cocaine Analog Recognition

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## ABSTRACT

Cocaine blocks the normal role of the dopamine transporter (DAT) in terminating dopamine signaling through molecular interactions that are only partially understood. Cocaine analog structure-activity studies have suggested roles for both cationic and aromatic interactions among DAT, dopamine, and cocaine. We hypothesized that phenylalanine residues lying in putative DAT transmembrane (TM) domains were good candidates to contribute to aromatic and/or cationic interactions among DAT, dopamine, and cocaine. To test this idea, we characterized the influences of alanine substitution for each of 29 phenylalanine residues lying in or near a putative DAT TM domain. Cells express 22 mutants at near wild-type levels, manifest by DAT immunohistochemistry and binding of the radiolabeled cocaine analog [ $^3\text{H}$ ](–)-2- $\beta$ -carbomethoxy-3- $\beta$ -(4-fluorophenyl)tropane (CFT). Seven mutants fail to express at normal levels. Four

mutations selectively reduce cocaine analog affinities. Alanine substitutions at Phe<sup>76</sup>, Phe<sup>98</sup>, Phe<sup>390</sup>, and Phe<sup>361</sup> located in TM domains 1 and 2, the fourth extracellular loop near TM 4 and in TM 7, displayed normal affinities for dopamine but 3- to 8-fold reductions in affinities for CFT. One TM 3 mutation, F<sup>155</sup>A, selectively decreased dopamine affinity to less than 3% of wild-type levels while reducing CFT affinity less than 3-fold. In a current DAT structural model, each of the residues at which alanine substitution selectively reduces cocaine analog or dopamine affinities faces a central transporter cavity, whereas mutations that influence expression levels are more likely to lie at potential helix/helix interfaces. Specific, overlapping sets of phenylalanine residues contribute selectively to DAT recognition of dopamine and cocaine.

The dopamine transporter (DAT) normally provides a principal determinant of the spatial distribution and time course of action of released dopamine, a major neurotransmitter involved in movement and behavioral reward (Kitayama et al., 1992a; Self and Nestler, 1995). Much literature attributes rewarding effects of cocaine to its blockade of DAT and transient enhancement of synaptic dopamine concentrations in brain pathways, including those linked to euphoria and behavioral reward (Ritz et al., 1987; Bergman et al., 1989). However, little detailed information about the ways in which dopamine or cocaine interact with the DAT protein is available.

Much of the current information about the ways in which DAT interacts with dopamine and cocaine was derived from analyses of structure-activity relationships. Cocaine analog structure-activity studies suggest roles for both cationic and

aromatic interactions among DAT, dopamine, and cocaine (Carroll et al., 1992). The phenyl ring of cocaine is especially necessary for normal cocaine recognition by DAT; only limited substitutions on this ring can be tolerated without significant losses of affinity (Lieske et al., 1998).

These data about small molecule structure-activity relationships have been superimposed on models of the DAT protein topology (Fig. 1A) that are currently supported by several lines of evidence (Kilty et al., 1991; Shimada et al., 1991; Vandenberg et al., 1992; Uhl and Johnson, 1994; Donovan et al., 1995; Nirenberg et al., 1997a,b). One possible site for cationic interactions between small molecules and DAT suggested by initial mutagenesis studies involved a transmembrane domain (TM) 1 aspartic acid, for example (Kitayama et al., 1992b). The emerging body of structure-function data (see also Buck and Amara, 1994, 1995; Giros et al., 1994) must be superimposed with some caution on TM topologies, such as those described in Fig. 1A, however, be-

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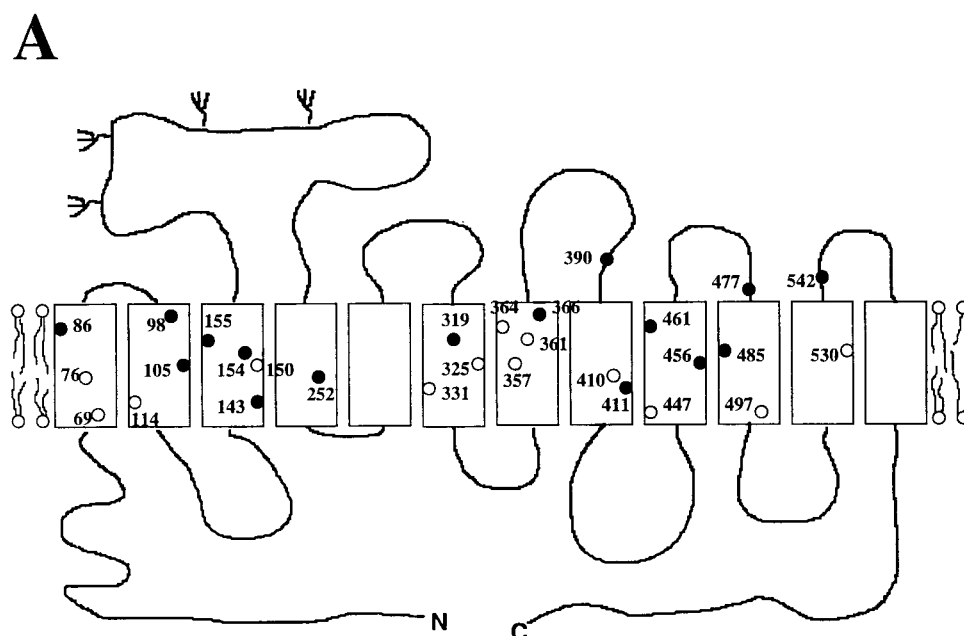
**ABBREVIATIONS:** DAT, dopamine transporter; CFT, (–)-2- $\beta$ -carbomethoxy-3- $\beta$ -(4-fluorophenyl)tropane; TBS, Tris-buffered saline; rDAT, rat dopamine transporter; NET, norepinephrine transporter; SERT, serotonin transporter; TM, transmembrane (domain); A, alanine; D, aspartic acid; F, phenylalanine; G, glycine; M, methionine; I, isoleucine; L, leucine; N, asparagine; P, proline; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; WT, wild-type.

cause no transporter with this or related topology has been successfully analyzed by X-ray crystallography.

Improved understanding of DAT structure-activity relationships, especially improved elucidation of DAT interactions with its substrates and inhibitors, could have substantial practical importance. The identification of transporter amino acids selectively involved in dopamine uptake or cocaine recognition could aid definition of targets for selective "dopamine-sparing" cocaine antagonist compounds (Uhl et al., 1998). A blocker of the recognition by cocaine of DAT that left dopamine uptake intact could even display clinical use as a cocaine antiaddiction and/or overdose therapeutic agent. Many of the most promising candidate sites for such selective blockade could lie within TM domains. Analysis of the effects of mutations on the DAT properties in recognizing ligands

and substrates and in transporting substrates represents one approach to developing an increasingly clear picture of the molecular details of these important processes.

DAT phenylalanine residues, especially those in TM domains, are attractive targets for mutagenesis. The important aromatic ring of cocaine could contribute to ligand and substrate recognition through aromatic or  $\pi$ - $\pi$  interactions with DAT TM domain phenyl rings (Burley and Petsko, 1985; Baldock et al., 1996). DAT TM aromatic residues could also contribute to recognition of the positive charges or polarities that can be found in virtually all DAT substrates and inhibitors, because cation binding to the  $\pi$  faces of aromatic rings can produce noncovalent cation- $\pi$  interactions (Dougherty, 1996). DAT sequences display 29 phenylalanine (F) residues located within or adjacent to 10 of its 12 putative TM do-



**B**

Conservation of phenylalanines among 12-transmembrane domain monoamine transporters.

Transporters							Transporters						
rDAT	hDAT	bDAT	hNET	rSERT	hSERT	dmSERT	rDAT	hDAT	bDAT	hNET	rSERT	hSERT	dmSERT
1F69							7F361						
1F76				Y	Y		7F364						
1F86							7F366			I	V	V	V
2F98							F390						
2F105				M	M		8F410						
2F114							8F411						
3F143		Y	Y	Y	Y	Y	9F447						
3F150	L	L	L			I	9F456				V		
3F154				S	S	M	9F461				L	V	P
3F155			Y	Y	Y	Y	F477				Y	Y	Y
4F252							10F485				T	T	
6F319							10F497						
6F325		L					11F530						
6F331						L	F542				P	P	Y
7F357													

**Fig. 1.** Distribution and conservation of 29 phenylalanine residues tested in this study throughout the DAT protein, with a current depiction of the protein topology. A, distribution of phenylalanine residues in putative DAT topology (Kilty et al., 1991; Shimada et al., 1991). The 12 boxes represent 12 TM helices; at the two ends are membrane lipid molecules. The upper side represents the extracellular side, and the lower side represents the cytoplasmic side. N, NH<sub>2</sub> terminal; C, COOH terminal of the DAT; four potential glycosylation sites are indicated on the putative extracellular loop 2 of the protein. ●, those on the (arbitrarily assumed) visible sides of TM helices; ○, those on the opposite sides. B, conservation of phenylalanine residues in members of Na<sup>+</sup>- and Cl<sup>-</sup>-dependent monoamine transporter gene family (Uhl and Johnson, 1994). The prefix number of each phenylalanine residue indicates the TM number. The conserved phenylalanine residues in transporters other than rDAT are not marked; the altered residues are shown.

mains. Many of these phenylalanines are conserved in DAT sequences from several species and in the norepinephrine transporter (NET) and serotonin transporter (SERT; Uhl and Johnson, 1994).

Mutagenesis studies that produce altered patterns of dopamine uptake or cocaine binding need to be interpreted with important caveats. Functional alterations observed in mutants in which single phenyl side chains are deleted could arise from the direct consequences of the removal of this side chain on DAT interactions with small molecule substrates and inhibitors but also from more gross structural rearrangements in the DAT protein. Dopamine transport could be influenced by mutations in residues important for any of several steps in transport, including ion recognition, substrate translocation, release of substrate and ions into cytoplasm, and transporter reorientation steps that might not directly affect affinities for ligand or substrate (Rudnick, 1997).

To test the idea that some catechol- $\pi$  interactions could allow DAT to recognize dopamine while distinct phenyl- $\pi$  interactions could contribute to the affinity of DAT for cocaine, we now report characterization of the influences of mutations in each of the 29 phenylalanines distributed in or near 10 of the 12 putative TM domains of DAT (Fig. 1A) on DAT expression, cocaine analog affinity, and dopamine transport. The influences exerted by these mutations, especially the five mutations that selectively affect cocaine analog or dopamine recognition, help sharpen our understanding of the structure-function relationships for this important brain protein and provide sites for the design of possible cocaine antagonists.

## Materials and Methods

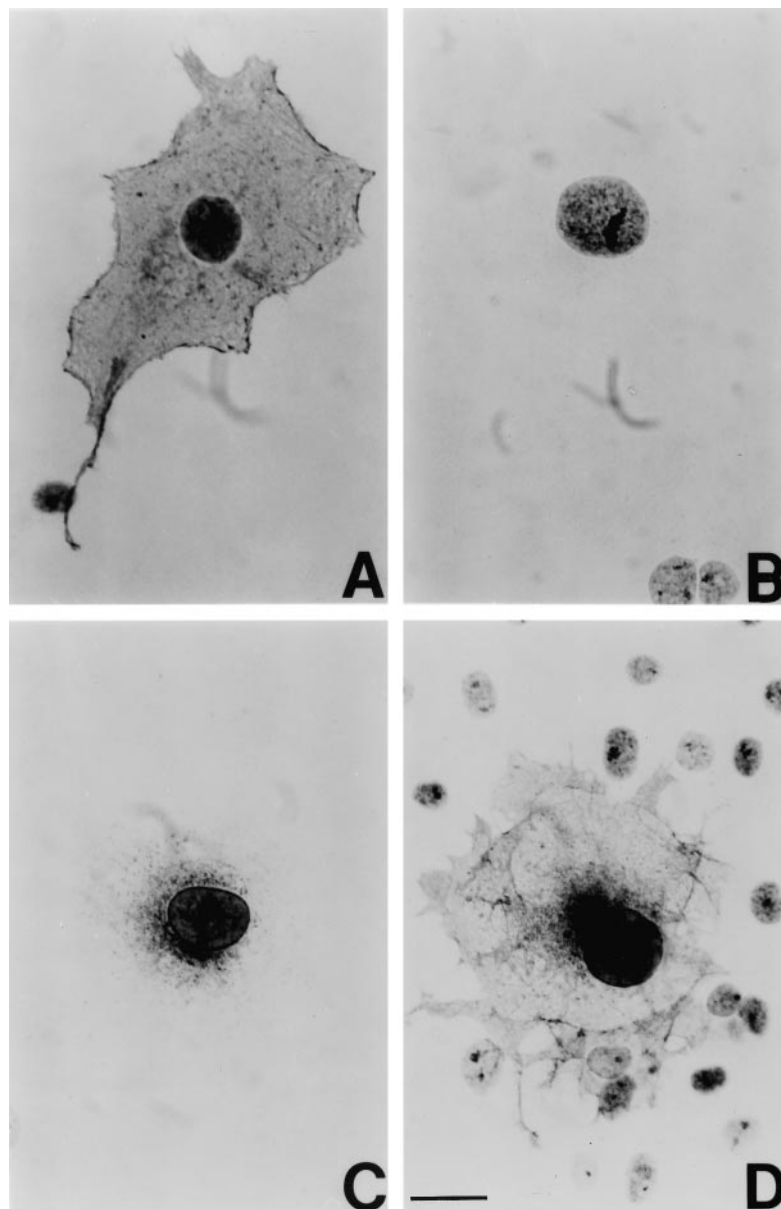
**Preparation of Wild-Type (WT) and Mutant DNAs.** Oligonucleotides corresponding to the sequences for mutations were synthesized using an Applied Biosystems (Foster City, CA) synthesizer and purified by electrophoresis using 12% polyacrylamide gels. Mutagenic oligonucleotides used GCC and GCT as the codons for alanine because these codons are used in DAT with frequencies of 0.46 and 0.33, respectively. Uracil-containing single-stranded template for mutagenesis was derived from a pBluescript/rat DAT (rDAT) cDNA (Shimada et al., 1991), as described (Muta-Gene Phagemid In Vitro Mutagenesis Version 2; Bio-Rad, Hercules, CA). Mutagenesis was undertaken by annealing the oligonucleotides to the single-stranded WT DAT template, in vitro synthesis and ligation of the mutant strand, nicking and digestion of nonmutant strand, and repolymerization and ligation of the gapped DNA as described by the manufacturer. Mutations are defined using a single letter for the WT amino acid's position number and the substituted amino acid. A prefix number represents the putative TM domain in which the mutation is located. Mutations in TMs 1 and 2 were isolated in *NotI/BglII* fragments; mutations in TMs 3 to 7 and F<sup>390</sup>A were isolated in *BglII/PvuI* fragments, and mutations in TMs 8 to 12 were isolated in *PvuI/PstI* fragments of the plasmid pBluescript/rDAT. Each mutation was confirmed by DNA sequencing, isolated restriction fragments were shuttled into a WT rDAT-expressing mammalian plasmid pcDNADAT1 (Shimada et al., 1991), and the correct sequences were reconfirmed. Transfections used DNA preparations with  $A_{260}/A_{280}$  ratios of  $\geq 1.65$ .

**Functional Analyses.** COS cells ( $10^7$ ) grown to confluence in flasks in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD) containing 10% FCS (Life Technologies) were split 1:2, harvested the next day using trypsin/EDTA, centrifuged (200g) for 10 min at 4°C, washed with sterile HEBS (20 mM HEPES,

137 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, and 6 mM dextrose), recentrifuged, and resuspended at  $10^7$  cells/ml in 4°C HEBS. Then, 0.9 ml of suspended cells was transfected by electroporation at 300 V and 1100  $\mu$ F in 400-mm Gene Pulser cuvettes (Bio-Rad) containing 20  $\mu$ g of plasmid DNA and 500  $\mu$ g of fish sperm DNA (Boehringer-Mannheim, Mannheim, Germany) using a geneZAPPER 450/2500 (IBI, New Haven, CT). The transfected cells were suspended in Dulbecco's modified Eagle's medium, followed by distribution into 6-well plates. Cells were grown for 3 days and then assayed for their abilities to accumulate [<sup>3</sup>H]dopamine (49 Ci/mmol; New England Nuclear Research Products, Boston, MA) or to bind the cocaine analog [<sup>3</sup>H](–)-2- $\beta$ -carbomethoxy- $\beta$ -3-(4-fluorophenyl)tropane (CFT; 83.5 Ci/mmol; New England Nuclear Research Products).

Kinetic and saturation analyses were used to determine  $K_m$ ,  $V_{max}$ ,  $K_D$ , and  $B_{max}$  values as described (Pfenning and Richelson, 1990). For uptake assays, 10 nM [<sup>3</sup>H]dopamine and 0.1, 1, 5, 10, 20, and 30  $\mu$ M unlabeled dopamine concentrations were used. For initial binding assays, 2 nM [<sup>3</sup>H]CFT was adjusted to 3.5, 5, 7, 17, 32, and 62 nM concentrations using unlabeled CFT. When little CFT binding was displayed in preliminary studies, Scatchard analyses with increasing concentrations of [<sup>3</sup>H]CFT were used. COS cells transfected with pcDNADAT1 and a truncated, promoter-deleted version of pcDNADAT1, named pcDEDAT, served as positive and negative controls, respectively. Parallel incubations with 30  $\mu$ M unlabeled cocaine allowed estimation of nonspecific uptake and binding. Uptake assays were carried out for 5 min at 37°C, followed by two washes each with 2 ml of Krebs-Ringer-Henseleit buffer containing 50  $\mu$ M ascorbic acid. Most binding assays were carried out for 2 h at 4°C followed by three washes each with 2 ml of Krebs-Ringer-Henseleit buffer; some experiments were also conducted at 37°C for 30 min. Cells were solubilized in 0.5 ml of 1% SDS, and radioactivity was determined using a Beckman Instruments (Palo Alto, CA) LS 6000 liquid scintillation counter at approximately 50% efficiency. Cells from parallel wells were solubilized in 0.5 ml of 1 N NaOH for protein amount measurements using a Bio-Rad Protein Assay solution (Bio-Rad). Studies of dopamine inhibition of 2 to 5 nM [<sup>3</sup>H]CFT binding used several concentrations of unlabeled dopamine in 50  $\mu$ M ascorbic acid.

**Analyses of Expression in COS Cells.** Cellular patterns of expressed DAT immunoreactivity were examined by immunohistochemistry using specific polyclonal rabbit anti-DAT sera. COS cells transfected with DAT or DAT mutant plasmids were grown on coverslips in 6-well plates for 3 days. Cells transfected with pcDEDAT provided a negative control. Cells grown to approximately 80% confluence in 6-well plates were quickly washed twice with 2 ml of PBS, fixed by adding 1 ml/well of 4% paraformaldehyde in PBS, and incubated at 4°C for 1 h. After four or five washes with PBS, endogenous peroxidase was inactivated by incubations with 1 ml of 10% methanol and 0.6% H<sub>2</sub>O<sub>2</sub> in PBS for 10 min at 22°C. After three washes with PBS and two washes with Tris-buffered saline (TBS; 50 mM, pH 7.6), nonspecific protein binding was blocked by incubation in 1 ml of augmented TBS [TBS with 2% skim milk powder (Fluka Chemical Corp., Ronkonkoma, NY), 0.2% Triton X-100, 0.01% Na azide] for 1 h. The primary antisera used were a rabbit serum raised against the N-terminal peptide of rDAT from amino acids 42 to 59, termed 16B, and an antibody raised against the C-terminal amino acids 580 to 608, termed 18A (Vaughan et al., 1993). Sera were diluted with augmented TBS at 1:20,000 and 1:1500, respectively; cells were incubated overnight at room temperature and then washed three times with 5 ml of augmented TBS. Cells were incubated for 1 h with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), diluted 1:333 in TBS, washed three times with TBS, and incubated for 1 h with avidin-biotin complex solution prepared 30 min before use (Vectastain Elite Peroxidase ABC Kit; Vector Laboratories). After three washes with TBS, labeling was visualized by 1-min reaction with a freshly prepared solution containing 1.4 mM diaminobenzidine tetrahydrochloride, 1.6 mM NiCl<sub>2</sub>, and 2.2 mM H<sub>2</sub>O<sub>2</sub> in TBS. Stained cells on coverslips were washed three times with TBS, dehydrated, mounted on microscope slides,



**Fig. 2.** Photomicrographs of the immunohistochemical staining with anti-DAT sera of COS cells expressing different patterns of DAT mutants. A, the WT DAT, showing even distribution of DAT in the plasma membrane. This group included, besides the WT, all the 22 mutants that are not included in groups C and D (see below). B, a negative control for DAT expression. The COS cells were transfected with pcDNA1 carrying a promoterless DAT cDNA. C and D, two different patterns of mutant expression, showing different degrees of plasma membrane expression due to intracellular trafficking disruptions. Group C included 1F86A, 2F98A, 7F364A, F390A, and 8F411A; group D included 7F357A and 7F366A. Scale bar, 20  $\mu$ M.

and examined for semiquantitative assessments of the patterns of DAT immunoreactivity. Expression was also monitored using assays for  $\beta$ -galactosidase expressed by a cotransfected plasmid. Transfection efficiencies ranged from 6 to 8% for each DAT. Patterns of DAT immunoreactivity were judged independently by an observer unaware of the genotype, and defined on a scale of staining intensity (+ to +++++; see Table 2).

**Analyses and Definitions.**  $K_m$  and  $V_{max}$  values for [ $^3$ H]dopamine uptake,  $K_D$  and  $B_{max}$  values for [ $^3$ H]CFT binding activities,  $IC_{50}$  values, calculation of Hill coefficients, curves fit to data using sigmoidal curve models for binding competition data, calculation of data fitting, and  $t$  tests or ANOVAs followed by Tukey's multiple comparison tests were all carried out using Prism Version 2 programs (GraphPAD Software, San Diego, CA).  $K_i$  values were calculated as described by Cheng and Prusoff (1973). Transporter modeling was based on coordinates kindly provided by Dr. Edvardsen (Edvardsen and Dahl, 1994). Localizations of phenylalanine residues in the photosynthetic reaction center (icode, 4RCR) and bacteriorhodopsin (icode, 2BRD) was based on their published coordinates in the PDB database using Sybyl 6.4 programs (Tripos, Inc., St. Louis, MO).

We arbitrarily defined two criteria for possible biological signifi-

cance of mutation effects. To meet the first criterion, the mutants needed to display  $K_m$ ,  $V_{max}$ ,  $K_D$ , or  $B_{max}$  values more than 3-fold different from WT values. The second criterion required that the  $t$  test statistical comparisons were at  $P \leq .05$ . We list results from mutants that did not meet the first criterion but achieve statistical significance separately (see Fig. 5). Transporter turnover rate reflected the number of dopamine molecules transported/s/site, defined as  $[V_{max} \text{ for dopamine uptake in fmol}/\mu\text{g}/\text{min}]/[B_{max} \text{ for CFT binding in fmol}/\mu\text{g} \times 60 \text{ (s/min)}]$ . A ratio, termed  $K_D/K_m$  ratio, between mutation influences on cocaine and dopamine recognition was calculated as  $[K_D \text{ for CFT binding}_{mutant}/K_D \text{ WT}]/[K_m \text{ for dopamine uptake}_{mutant}/K_m \text{ WT}]$ . The prefix number before the location of a phenylalanine residue indicated the TM domain in which the residue was located.

## Results

### Most Alanine Substitutions Allow WT Expression Patterns

**Immunohistochemical Determinations of DAT Immunoreactivity.** COS cells expressing the WT DAT dis-



played a pattern of DAT immunoreactivity characterized by relatively dense plasma membrane immunostaining and modest immunoreactivity associated with nuclear or perinuclear regions (Fig. 2A). Twenty-two of the 29 phenylalanine-to-alanine mutants displayed patterns of DAT immunostaining similar to those of WT DAT (Table 1), including substitutions in putative TMs 1, 2, 3, 4, 6, 7, 8, 9, 10, and 11. Interestingly, staining intensities for six of the mutants, 1F76A, 2F105A, 3F150A, 4F252A, 6F319A, and 7F361A, were occasionally stronger than those for WT DAT (data not shown).

Seven mutations did alter this pattern of expression. Cells expressing 7F357A and 7F366A displayed about half of their DAT immunoreactivity on plasma membranes and half in perinuclear regions (cited as ++ or + staining intensities; Table 1 and Fig. 2D). For the mutants 1F86A, 2F98A, F390A, and 8F411A, more than two thirds of DAT immunoreactivity appeared to be perinuclear, whereas less than one third was distributed, often unevenly, at the plasma membrane (Table 1 and Fig. 2C). 1F86A displayed only small amounts of DAT plasma membrane immunostaining, about 1/10th of WT levels.

**$B_{\max}$  Values from [ $^3$ H]CFT Radioligand Binding.** Most of the data from studies of DAT immunoreactivity were supported by expression data derived from Scatchard analyses of [ $^3$ H]CFT radioligand binding to whole-cell preparations. [ $^3$ H]CFT  $B_{\max}$  values for each of the mutants ranged between 2 and 4 fmol/ $\mu$ g, comparable with the 4.0 fmol/ $\mu$ g values observed for the WT transporter (see Fig. 5D). Reductions in  $B_{\max}$  values to roughly half of WT levels were observed for mutants 1F86A, 7F357A, 7F366A, F390A, and 8F411A, consistent with immunohistochemical analyses suggesting reduced plasma membrane expression levels (see Fig. 5D). Despite the severe disruption of plasma membrane expression noted in immunohistochemical experiments, mutant 2F98A displayed only a modest reduction in apparent  $B_{\max}$ . The modestly increased  $B_{\max}$  values for mutants 1F76A, 6F319A, and 7F361A were also consistent with the data from studies of plasma membrane expression by immunohistochemistry (see above). Transient transfection experi-

ments provided levels of expression that can vary from experiment to experiment. When the results of repeated saturation radioligand binding studies were analyzed, scatter in the Scatchard analyses suggested caution in interpreting these modest differences as true discrepancies. However, analyses of the data from up to eight experiments expressing 1F69A, 7F357A, 8F411A, and 10F485A alongside the WT DAT documented that the  $B_{\max}$  values of these mutants differed statistically from the WT values ( $P < .05$  by  $t$  test; see Fig. 5D).

### Selected Alanine Substitutions Alter [ $^3$ H]Dopamine Uptake Properties

Cells expressing the WT DAT (Fig. 3A) displayed saturable uptake of [ $^3$ H]dopamine. Eadie-Hofstee analyses (Fig. 4) allowed  $K_m$  determinations of dopamine affinity ( $K_m$ ) and  $V_{\max}$  determinations of uptake rates for each of the 29 mutants.

Twenty-four mutants retained normal or near-normal dopamine affinities, as manifest by  $K_m$  values close to those of the WT transporter (see data from mutant 7F361A; Figs. 3A, 4A, and 5A). However, five mutations significantly altered the affinity of DAT for dopamine. Mutants 1F69A, 6F331A, and 11F530A significantly increased dopamine affinity, decreasing  $K_m$  values by 3-, 3-, and 5-fold, respectively. Mutants 3F155A and 7F364A decreased dopamine affinities by 30- and 10-fold. The 3F155A mutant displayed strikingly reduced dopamine affinity, although it still retained radioligand binding sufficient to allow kinetic analyses (Figs. 3A, 4A, and 5A).

Eadie-Hofstee analyses revealed that 15 of the mutants displayed normal dopamine uptake rates, as evidenced by  $V_{\max}$  values within 3-fold of WT levels (Fig. 5B). However, 14 mutants displayed dopamine uptake  $V_{\max}$  values reduced to levels less than one third of WT levels; these mutants included 1F69A, 1F76A, 1F86A, 2F98A, 2F105A, 2F114A, 3F155A, 6F319A, 6F331A, 7F364A, 7F366A, F390A, 8F410A, and 11F530A.

$V_{\max}$  reductions observed in cells expressing 1F86A, 2F98A, 7F364A, 7F366A, and F390A could be consistent, at least in part, with the reduced plasma membrane expression

TABLE 1

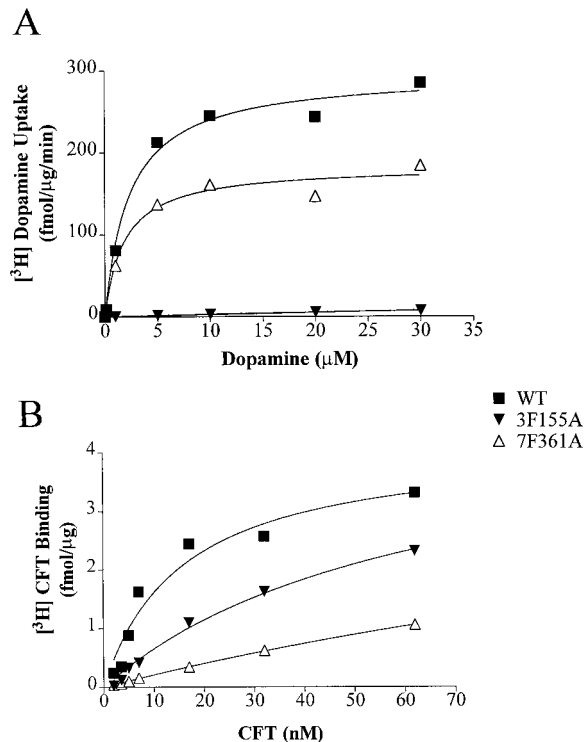
Semiquantitative description<sup>a</sup> of cellular distribution of DAT immunoreactivity in COS cells transiently expressing each of the 29 Phe→Ala mutants

Apparent perinuclear and plasma membrane staining was rated by an observer unaware of the mutants using a quantitative 1 to 4 "+" scale. Number of plus signs represents a fraction of the DAT protein. For example, the WT has three plus signs; there is one in *Perinuclear* and two in *Plasma Membrane*. This means that approximately one third of the protein is at perinuclear location and two thirds of the protein are in the plasma membrane. Total number of plus signs for each mutant represents relative intensity of immunostaining, compared with WT: the greater the number, the more intense the immunostaining.

Mutant <sup>b</sup>	Perinuclear	Plasma Membrane	Mutant	Perinuclear	Plasma Membrane
WT	+	++	7F357A	++	++
1F69A	+	+++	7F361A	+	+++
1F76A	+	+++	7F364A	++	+
1F86A	+++	+	7F366A	+	+
2F98A	++	+	F390A	++	+
2F105A	+	+++	8F410A	+	++
2F114A	+	++	8F411A	++	+
3F143A	+	++	9F447A	+	++
3F150A	+	+++	9F456A	+	++
3F154A	+	++	9F461A	+	++
3F155A	+	++	F477A	+	++
4F252A	+	+++	10F485A	+	++
6F319A	+	+++	10F497A	+	++
6F325A	+	++	11F530A	+	++
6F331A	+	++	F542A	+	++

<sup>a</sup> This description mainly shows expression pattern differences of the mutants relative to WT.

<sup>b</sup> Italic, F conserved in other Na<sup>+</sup>/Cl<sup>-</sup>-dependent neurotransmitter transporters.



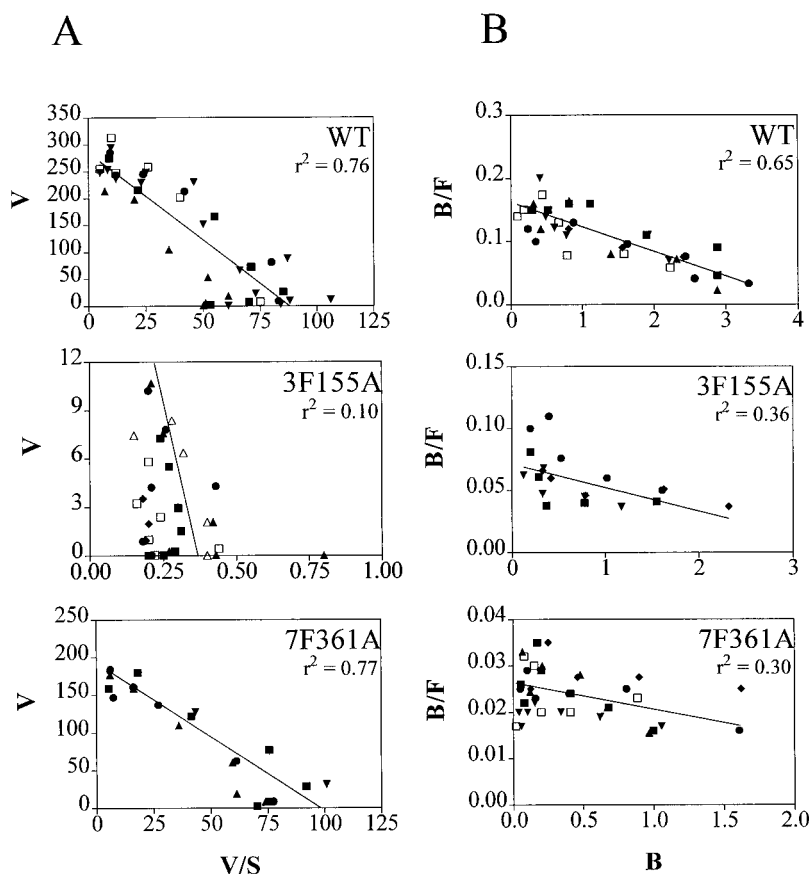
**Fig. 3.** Saturation assay of  $[^3\text{H}]$ dopamine uptake and  $[^3\text{H}]$ CFT binding activities with the WT DAT and two representative DAT mutants (3F155A and 7F361A). 3F155A and 7F361A are chosen here because they displayed selective mutation influences on dopamine uptake and CFT binding affinities, respectively. A, dopamine uptake. B, CFT binding. Data represent the mean values of four to six independent experiments.

of these mutants noted above. However, 1F76A, 1F69A, 2F105A, 2F114A, 6F319A, 6F331A, 8F410A, and 11F530A each displayed normal plasma membrane immunostaining, and each displayed reduced  $V_{\text{max}}$  values. These mutants  $V_{\text{max}}$  values ranged between 0.4 and 5.9 fmol/ $\mu\text{g}/\text{min}$ ; small fractions of the 292 fmol/ $\mu\text{g}/\text{min}$  displayed by cells expressing the WT DAT. Alanine substitution for 3F155, which reduced dopamine affinity in the context of normal patterns of plasma membrane immunostaining, also reduced  $V_{\text{max}}$  values. The mutation 9F461A yielded  $V_{\text{max}}$  values almost one and a half times higher than the WT levels, a difference at the margin of statistical significance ( $P = .06$ , by  $t$  test; Fig. 5B).

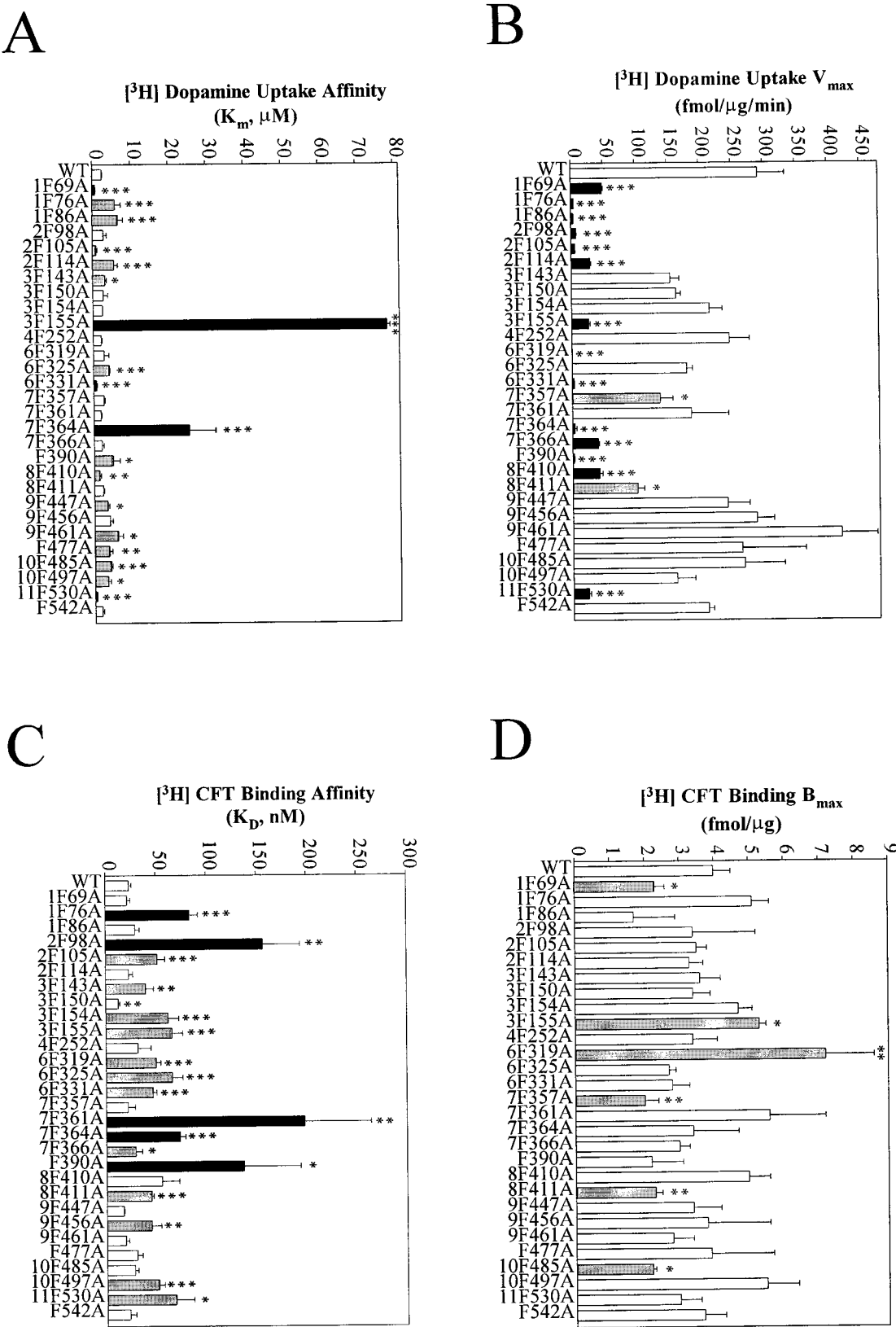
#### Certain Alanine Substitutions Alter $[^3\text{H}]$ CFT Binding Affinity

We assessed the ability of the WT DAT and the 29 mutants to recognize CFT using saturation  $[^3\text{H}]$ CFT binding (Figs. 3B and 4B). Mutation-induced changes in affinity for CFT could be compared with mutation-induced changes in dopamine affinities assessed in two manners:  $K_m$  values from uptake studies (see above) and  $K_i$  values obtained in studies of the competition of unlabeled dopamine for  $[^3\text{H}]$ CFT binding (see below).

Twenty-four of the 29 mutants retained affinities for  $[^3\text{H}]$ CFT similar to those of WT DAT (Fig. 5C). Five mutations significantly reduced CFT affinities. Affinities decreased more than 3-fold for 1F76A and 7F364A, 6-fold for 2F98A, 8-fold for 7F361A, and almost 6-fold for F390A. Although some of these values were derived from saturation experiments with modest signal-to-noise ratios (e.g., data from 7F361A in Figs. 3B and 4B), they did provide evidence



**Fig. 4.** Eadie-Hofstee analysis of  $[^3\text{H}]$ dopamine uptake activity (A) and Scatchard analysis of  $[^3\text{H}]$ CFT binding activity (B) with the WT DAT (top) and two representative DAT mutants 3F155A (middle) and 7F361A (bottom). Those mutants are chosen for the same reasons as in Fig. 3. Different symbols represent data from different independent experiments ( $n = 5$  for the WT DAT and 3F155A, 4 for 7F361A in A; 6 for the WT DAT and 7F361A, 4 for 3F155A in B). Correlation coefficient ( $r^2$ ) is a fraction between 0 and 1. When  $r^2 = 0$ , there is no linear relationship between  $V/S$  and  $V$  (A) or  $B$  and  $B/F$  (B);  $r^2 = 1$ , all points lie exactly on a straight line with no scatter.



**Fig. 5.** Pharmacological characterization of the WT DAT and the 29 Phe→Ala DAT mutants. A, [ $^3$ H]dopamine uptake affinity (ANOVA,  $P < .001$  for 3F155A and 7F364A). B, [ $^3$ H]dopamine uptake  $V_{max}$  [ANOVA,  $P < .001$  for all the mutants that showed significance by  $t$  tests (asterisks)]. C, [ $^3$ H]CFT binding affinity (ANOVA,  $P < .05$  for F390A,  $P < .01$  for 2F98A,  $P < .001$  for 7F361A). D, [ $^3$ H]CFT binding  $B_{max}$  (ANOVA, no significant difference). Labels on the left side of each panel are the WT and mutant names. [ $^3$ H]Dopamine uptake or [ $^3$ H]CFT binding assays and determinations of  $K_m$ ,  $V_{max}$ ,  $K_D$ , and  $B_{max}$  values are described in *Materials and Methods*. Data are mean  $\pm$  S.E. ( $n = 3-14$ ). Data for negative controls are not shown here because either  $V_{max}$  or  $B_{max}$  values were less than 1 unit. Open bar, no significant change; gray bar,  $t$  test statistically significant: \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ ; black bar, significant change by more than 3-fold of WT. Both  $t$  tests and ANOVA are for comparisons between each mutant and WT.

that phenylalanine residues in TMs 1, 2, and 7 and the putative fourth extracellular loop are required for WT cocaine analog affinities.

### Dopamine Competition for [ $^3$ H]CFT Binding

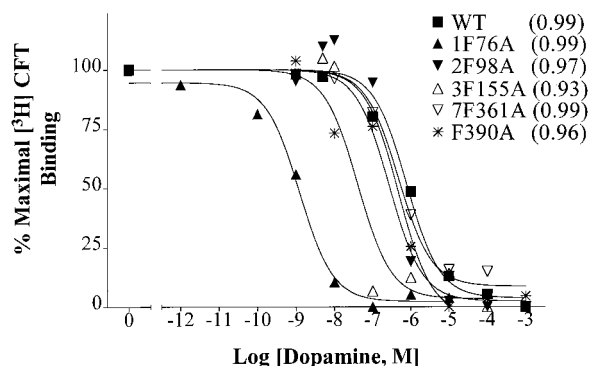
We assessed the ability of dopamine to compete for [ $^3$ H]CFT binding to cells expressing the five mutants that selectively affected dopamine uptake or CFT affinities (Fig. 6). Mutation 3F155A reduced dopamine affinity values in uptake assays without significant effects on cocaine analog binding (see above). This mutation also increased the potency of dopamine in inhibiting [ $^3$ H]CFT binding more than 30-fold (Table 2). The potencies of dopamine in competing for [ $^3$ H]CFT binding were enhanced considerably for the four mutants 1F76A, 2F98A, 7F361A, and F390A that reduced cocaine analog affinities while retaining normal or near-normal dopamine  $K_m$  values in uptake assays.

### $n_H$ Determinations

Hill coefficients determined for the WT DAT were indistinguishable from 1, as described previously (Kitayama et al., 1992b).  $n_H$  values were also  $\geq 0.7$  for mutants 3F155A, 7F361A, and F390A (Table 2). For mutants 1F76A and 2F98A, however, Hill coefficients were reduced to 0.244 and 0.350, respectively. These TM 1 and TM 2 mutations thus appear to alter aspects of the interaction between [ $^3$ H]CFT and DAT that extend beyond just altering affinities.

### Values Derived from Comparisons between Results of [ $^3$ H]CFT Binding and [ $^3$ H]Dopamine Uptake

**Turnover Rates.** Information about  $B_{max}$  and  $V_{max}$  values allowed calculations of DAT turnover rates for dopamine. The WT DAT showed a turnover rate of 1.2/s, in the range of previous assessments (Meiergerd et al., 1994). Fifteen mutants displayed turnover rates within 3-fold of the values found for the WT DAT (Fig. 7). Fourteen mutants displayed greater than 3-fold alterations in turnover rates. These ranged from 0.001 ( $s^{-1}$ ) for 6F319A to 2.5 ( $s^{-1}$ ) for 9F461A (Fig. 7). Mutation 6F319A decreased the turnover rate by 1000-fold. 1F76A, 2F105A, 6F331A, 7F364A, and F390A decreased rates by 30- to 100-fold. 1F86A, 2F98A, 2F114A, 8F410A, and 11F530A decreased turnover rates by 10- to



**Fig. 6.** Dopamine inhibition of CFT binding onto DAT displayed by the WT DAT and five of the Phe→Ala DAT mutants. The [ $^3$ H]CFT concentrations used were 2 to 5 nM. Data were from 5 to 14 independent experiments. The number in parentheses is  $R^2$ , which represents the goodness of fit and 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.

30-fold, whereas 1F69A and 7F366A decreased the rates by 3- to 5-fold. With its elevated  $V_{max}$  value, 420 fmol/ $\mu$ g/min, 9F461A doubled the turnover rate of the WT transporter.

**Selective Influences on [ $^3$ H]Dopamine or [ $^3$ H]CFT Affinities.** The mutants that displayed altered affinities for dopamine uptake were not identical with those that displayed altered affinities for CFT binding (Fig. 5, A and C). The ratios between mutation effects on these two parameters thus were significantly different from unity for 5 of the 29 mutants examined (Table 3). To facilitate comparisons between mutation effects on dopamine affinity and CFT affinity, we have calculated a  $K_D/K_m$  ratio for each mutant (Fig. 8). The  $K_D/K_m$  ratio assesses changes from WT values for the  $K_D$  value for CFT divided by changes for the  $K_m$  value for dopamine from WT values (see *Materials and Methods*). Increasing values for this ratio suggest more negative impacts of the mutation on cocaine recognition than on dopamine recognition. The  $K_D/K_m$  ratio was greater than 3 for seven mutants. For 2F98A it was 5.7; for 2F105A, 6.0; for 6F331A, 6.1; for 7F361A, 11.0; for F390A, 3.0; for 8F410A, 3.9; and for 11F530A, 14.4 (Fig. 8). These alterations were largely due to enhanced dopamine affinities for 2F105A, 6F331A, and 11F530A and to decreased CFT affinities for 2F98A, 7F361A, and F390A. The alteration for 8F410A was due to a modest increase in dopamine affinity and a modest decrease in CFT affinity (Fig. 5, A and C). Ratios were  $\leq 0.3$  for 3F155A (0.1), 7F364A (0.3), and 9F461A (0.3). These low ratios were due largely to the lower affinities of the mutants for dopamine, with relatively modest contributions from altered CFT  $K_D$  values (Fig. 5, A and C).

## Discussion

Alanine substitutions for DAT phenylalanines provide novel structure-function information for this important brain protein. These results elucidate contributions of DAT phenylalanine side chains to recognition of cocaine, recognition of dopamine, translocation of dopamine, and/or to DAT assembly. The new information that these studies provide can be interpreted in light of information about the conservation of DAT sequences with other transporters, structure-function relationships of cocaine and dopamine, functions of other DAT mutants and transporter chimeras, and current models for the structure of DAT and possibly related TM domain proteins.

We focus on evidence for the selective contributions that some of these phenylalanines can make to cocaine or dopamine recognition. We discuss the implications of this selectivity of specific DAT functions regarding the development of selective cocaine-blocking antagonists.

### Contributions of DAT Phenylalanine Phenyl Rings to Hydrophobic Components of Dopamine and Cocaine Recognition Sites

Current data support DAT models that postulate hydrophobic interactions among dopamine, cocaine, and DAT. They add to data about structure-activity relationships from cocaine and dopamine congeners. Cocaine analogs that lack phenyl rings display less than 1/300th the affinity for DAT displayed by their pharmacologically active 3- $\beta$ -phenyl homologs, whereas dopamine also requires a catechol group for reasonable potency at DAT (Horn, 1973, 1978; Ritz et al.,



1990). Taken together, current information fits well with the idea that several of the phenylalanines mutated in the current study could contribute to overlapping binding pockets that could constitute cocaine and dopamine recognition sites on DAT.

In discussing comparative mutation effects on dopamine and CFT affinities, we compare radioligand binding data resulting from studies performed at 4°C with results of uptake experiments performed at 37°C. In preliminary [<sup>3</sup>H]CFT-binding studies with 37°C, 30-min incubations, background nonspecific binding more than tripled at low CFT concentrations and increased even more at higher CFT concentrations. The 37°C incubations were thus ineffective for accurately characterizing cocaine analog affinities in these mutants (data not shown). Similarly, uptake studies performed at 4°C display less than 20% of the uptake found at 37°C. The different conditions used for assays of uptake and binding in the current studies thus provide technically tractable data, but the differences in conditions do provide a caveat to the discussions below.

**A Mutation Reducing Both Cocaine and Dopamine Affinities.** 7F364 could contribute to recognition of both cocaine and dopamine. This TM 7 residue is conserved in each of the monoamine transporters that recognize cocaine; DAT, SERT and NET (Fig. 1B). Alanine substitution at this site reduces affinity for both dopamine and cocaine (Fig. 5, A and C). 7F364 can be modeled close to the extracellular border of DAT TM 7 (Edvardsen and Dahl, 1994). Its phenyl side chain can easily be modeled as directed toward a putative central binding cavity. Interestingly, TM 7 serines that are modeled at the middle of this TM domain are selectively implicated in dopamine recognition, suggesting TM 7 involvement in interactions with dopamine (Kitayama et al., 1992b). The more extracellularly directed 7F364 thus is a good candidate to interact with both dopamine and the less-permeable cocaine. Interactions with both serines and hydrophobic pockets are characteristic of ligand recognition by seven-TM domain G protein-linked receptors, including receptors for catecholamines (Strader et al., 1995; Javitch et al., 1998; Wang et al., 1998).

**Mutations Selectively Reducing Cocaine or Dopamine Affinities.** Phenylalanine residues that selectively contribute to the recognition of either cocaine or dopamine are found in several DAT TM domains. Mutation in 1F76, a position at which the other members of the cocaine-recognizing monoamine transporter subfamily display a tyrosine, selectively reduces cocaine affinity. This residue is one helical turn below the TM 1 aspartic acid 79 residue. When this aspartic acid side chain is replaced by a hydrogen or a methyl

group, DAT affinities for both dopamine and cocaine analogs are reduced (Kitayama et al., 1992b).

The phenyl side chain of 1F76 could be a necessary component of a cocaine recognition pocket. It could also position the TM 1 domain appropriately for optimal contacts with cocaine (see below). In SERT, the corresponding residue is either a phenylalanine or tyrosine (Fig. 1B). The homologous residue has recently been suggested to participate in SERT contacts with antidepressant SERT blockers (Baker et al., 1998). Chimeric transporters with TMs 1 and 2 from DAT or from NET and the remainder of their sequences from the other transporter display catecholamine and cocaine recognition properties appropriate for the transporter that provides their TM 1 + 2 domains (Giros et al., 1994). These observations are consistent with roles of TM 1 or 2 residues in dopamine and cocaine recognition.

Mutant 2F98A selectively reduces cocaine analog affinities. This amino acid is embedded in a motif that is among the most conserved across members of the Na<sup>+</sup>- and Cl<sup>-</sup>-dependent neurotransmitter transporter gene family (Uhl and Johnson, 1994). The short extracellular loop connecting TMs 1 and 2 is likely to constrain these TM domains to lie next to each other. 2F98 could thus lie one helical turn above the level of the TM 1 D79 residue, which is also implicated in cocaine recognition. It is tempting to speculate that this phenyl side chain could contribute to a wall of a hydrophobic or even cation-recognizing (see below) cocaine recognition pocket. This interpretation of these observations is also consistent with data from chimeric transporters (Giros et al., 1994).

Selective reduction in dopamine affinity noted with the 3F155A mutation is interesting. This 3F155 residue could contribute to substrate specificity because tyrosines are found at this position in the NET and SERT sequences (Fig. 1B). Its location within less than two helical turns of the extracellular border in current models is also suggestive. Conceivably, the presence of the larger, more polar tyrosine side chain in NET could contribute to charge or steric features that allow this transporter to selectively recognize the  $\beta$ -hydroxyl of norepinephrine.

The 7F361A substitution selectively decreases cocaine analog affinity. This mutation effect contrasts with the results of nearby but more extracellularly disposed mutants. 7F366A has no influence on cocaine analog or dopamine affinities, whereas the 7F364A mutation influences both cocaine analog and dopamine affinities. It also contrasts with the results of other TM 7 serine mutations (Kitayama et al., 1992b). Each of these results points to the importance of residues in and near TM 7 for cocaine and dopamine recognition.

TABLE 2

Characterization of selective Phe → Ala mutation effects on dopamine's ability to compete for CFT binding site or sites on the DAT protein. From 2 to 5 nM of [<sup>3</sup>H]CFT was used for competition experiments, and  $K_i$  values were calculated according to Cheng and Prusoff (1973). Data are presented in mean ± S.E.

Mutant	$K_i$ $\mu\text{M}$	$n$	Hill Coefficient ( $n_H$ )	$n$
WT	1.02 ± 0.06	(7)	0.983 ± 0.045	(5)
1F76A	0.00353 ± 0.00066 <sup>c</sup>	(14)	0.244 ± 0.037 <sup>b</sup>	(13)
2F98A	0.350 ± 0.072 <sup>c</sup>	(5)	0.350 ± 0.119 <sup>b</sup>	(4)
3F155A	0.0364 ± 0.0090 <sup>c</sup>	(9)	0.711 ± 0.071 <sup>a</sup>	(7)
7F361A	0.391 ± 0.0625 <sup>c</sup>	(5)	0.917 ± 0.147	(5)
F390A	0.509 ± 0.091 <sup>c</sup>	(7)	0.742 ± 0.240	(7)

Both *t* tests and ANOVA comparisons of each mutant with WT: <sup>a</sup>*P* < .01, <sup>b</sup>*P* < .001, <sup>c</sup>*P* < .0001; otherwise, *P* > .05.

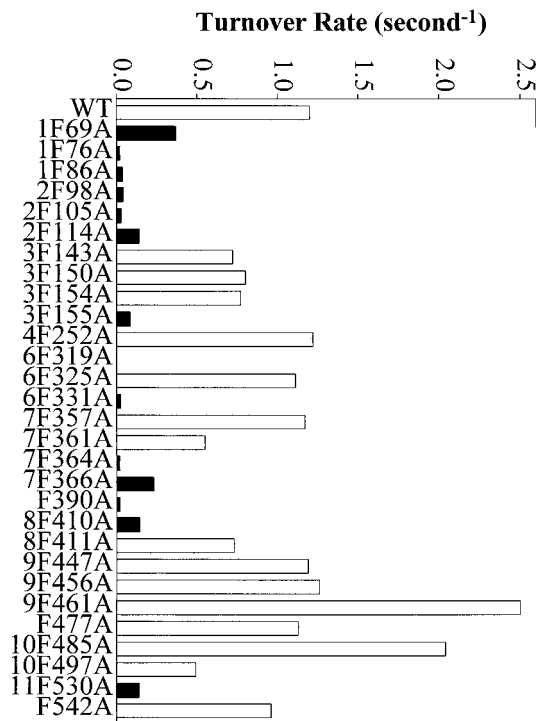
Mutation of the highly conserved 11F530 enhances dopamine  $K_m$ ; this mutation site lies in the midportion of this TM domain in current DAT models. Other mutations that enhance DAT affinity for its substrate 1-methyl-4-phenylpyridinium also lie in TM 11 (Mitsuhashi et al., 1998). Both of these lines of evidence point to the possible importance of TM 11 in transport processes.

A hydrophobic nine-amino acid sequence of the putative fourth extracellular loop PGLIFIYP is flanked by prolines

and contains the highly conserved residue F390. Mutation at the F390 site produced some of the most remarkable selective decreases in affinity for cocaine analogs noted in this study. This sequence is contained in a putatively extracellular domain whose flexibility makes its exact topology difficult to model. Conceivably, however, the side chain of this phenylalanine could participate in a small molecule recognition pocket along with residues in TM 7, an adjacent domain that forms one of the anchoring segments to which the fourth extracellular loop is connected.

#### Possible Contributions of DAT Phenylalanine Phenyl Rings to Cationic Interactions with Dopamine or Cocaine

Aromatic ring interactions with cations can contribute to molecular interactions and to ligand affinities (Dougherty, 1996). If cocaine recognition does indeed require cationic interactions with DAT phenyl ring  $\pi$  electron clouds, they could involve: 1) cocaine's tropane nitrogen, the most impressive cationic portion of cocaine and 2) a phenylalanine side chain that is located close to the extracellular face of DAT TM domains, conserved across each of the monoamine transporters that serve as cocaine recognition sites, and lowers cocaine affinities when mutated to alanine. 1F76, 1F86, 2F98, 6F319, 7F357, 7F361, 7F364, 8F410, and 11F530 are each candidates to contribute to cationic interactions based on their location and conservation. 3F155 is also a candidate because it is altered only to tyrosine in other monoamine transporters. Of these residues, alanine substitutions for 1F76, 2F98, 7F357A, 7F361, and 7F364 each also decreases cocaine ana-



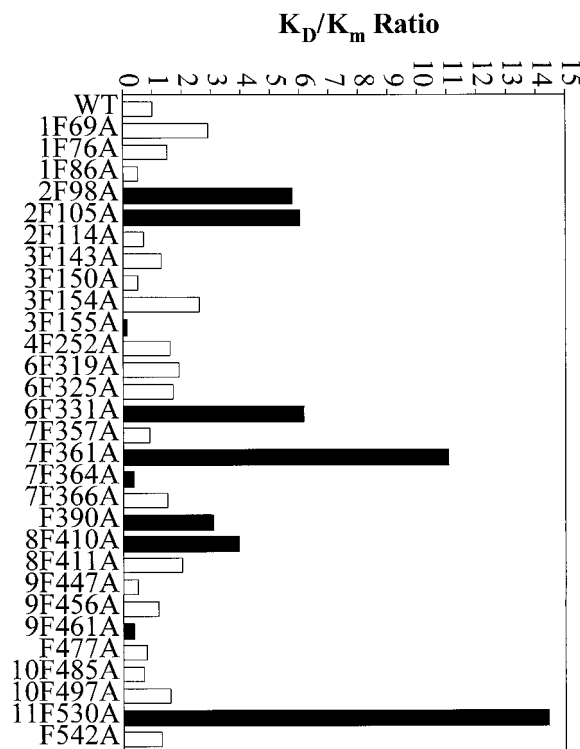
**Fig. 7.** Turnover rates of dopamine uptake by the WT DAT and the 29 Phe→Ala DAT mutants. Labels on the left side of each panel are WT and mutant names. Calculation of a turnover rate used the mean values of  $V_{max}$  and  $B_{max}$  displayed by each mutant, as presented in Fig. 5, B and D, respectively; therefore, the turnover rate numbers did not have any standard deviations. The method of calculation is described in *Materials and Methods*. Open bar, no apparent change; black bar, change by more than 3-fold of WT.

TABLE 3

Pharmacological categorization of the 29 Phe → Ala DAT mutants based on [ $^3$ H]dopamine uptake and [ $^3$ H]CFT binding affinities. This categorization is a summary of data and analyses presented in Figs. 5, A and C, and 8.

Cocaine Analog Affinity <sup>a</sup>	Dopamine Affinity <sup>a</sup>		
	Decrease	Increase	Normal
Decrease	7F364A		1F76A 2F98A 7F361A F390A
Increase		1F69A 6F331A 11F530A	
Normal	3F155A		8F410A and 19 others

<sup>a</sup> A change in  $K_D$  or  $K_m$  value of at least 3-fold magnitude compared with the WT. Bold indicates mutation selectively affects cocaine analog binding affinity and/or the  $K_D/K_m$  ratio is  $\geq 3$ .



**Fig. 8.**  $K_D/K_m$  ratios displayed by the WT DAT and the 29 Phe→Ala DAT mutants. Labels on the left side of each panel are the WT and mutant names. Calculation of a  $K_D/K_m$  ratio used the mean values of  $K_m$  and  $K_D$  displayed by each mutant, as presented in Fig. 5, A and C, respectively; therefore, the  $K_D/K_m$  ratio numbers did not have any standard deviations. The method of calculation is described in *Materials and Methods*. Open bar, no apparent change; black bar, change by more than 3-fold of WT.

log affinity. This list of reasonable candidate residues is significantly shortened if we postulate that interactions between cocaine and DAT must also involve cationic interactions with the TM 1 D79 aspartic acid side chain, as previously suggested (Kitayama et al., 1992b). 1F76 and 2F98 might thus be the best candidates to lie close enough to the TM 1 D79 to contribute to a single cationic pocket important for cocaine recognition.

Aromatic-cation interactions could also conceivably contribute to dopamine affinity. Dopamine affinities, as reflected in  $K_m$  values from uptake studies, could be influenced by phenyl rings disposed at extracellular faces, midpositions, or even intracellular faces of DAT TM regions. The molecular context of dopamine's amine nitrogen is similar to that of norepinephrine but differs more substantially from the indole nitrogen of serotonin. Conceivably, residues that are conserved among the NET and DAT and at which mutations reduce dopamine affinities, regardless of whether they are also conserved in SERT, are the most plausible candidates for interactions with catecholamines. On this basis, 7F364, which is conserved in the NET and SERT, is one of the most plausible candidates for catecholamine-selective interactions. However, this residue, like 3F155, cannot be conveniently modeled to lie near the TM 1 D79 at which mutations reduce dopamine affinity.

#### Possible Contributions of DAT Phenylalanine Phenyl Rings to Proper DAT Assembly and Structural Elements

Several of the current mutants join the growing number of DAT sequence variants that disrupt expression of this complex multidomain protein. Because DAT immunoreactivity is found for each of the expressed DAT mutants, DAT protein is likely to be translated from the products of each mutant plasmid. The current data fit best with the idea that the mutations interfere with processes required for plasma membrane targeting. Strikingly, removal of even the single amino acid side chains at 1F86, 2F98, 7F357, 7F364, F390, or 8F411 is sufficient to disrupt appropriate plasma membrane expression. These data are in accord with data from DAT/NET transporter chimeras. Chimeric transporters with most of the junctional sites selected to date, even between these closely related gene subfamily members, do not express properly (Giros et al., 1994).

**Helix/Helix Interactions.** What could these phenyl side chains normally contribute to DAT assembly? Contributions to helix/helix interactions between TM domains could aid transporter assembly by providing oriented hydrophobic interactions. Such hydrophobic interactions could be important for the disposition of putative helices within the mature DAT TM topology. In the Dahl-Edwardsen model, 7F357, 8F411, and 11F530 are each positioned to readily form helix/helix interactions (Edwardsen and Dahl, 1994). 1F86, 2F98, and 7F364 could also contribute to interhelical interactions but lie at the borders of more hydrophilic domains. The fact that each of these mutations disrupts plasma membrane expression could indicate that each of these six amino acids does contribute significantly to the interactions required for proper DAT assembly, including proper helix/helix assembly.

Each amino acid whose mutation disrupts expression does not necessarily play a key role in attaining the WT tertiary structure only. Alterations in secondary structure also inter-

rupt proper expression patterns. Removal of more than two of the four sites for N-linked glycosylation or either of the sites for possible disulfide bond formation in the second extracellular loop of DAT dramatically reduces appropriate plasma membrane expression (Wang et al., 1995; J. B. Wang and G.R.U., unpublished observations). Introducing cysteine residues into the SERT TM 3 also decreases cell surface expression of this transporter (Chen et al., 1997).

**Nonhelix/Helix Interactions.** Disruption of DAT structure and expression through nonhelix/helix interactions is also possible. The reduced cell surface expression characteristic of the F390 mutation, based on current DAT modeling, could be consistent with the possibility that this residue's side chain could interact with other TM domain segments or even extracellular segments important for the ultimate assembly of this complex protein.

#### Implications for Dopamine Transport

Dopamine transport can be modeled as involving: 1) sodium and then 2) dopamine and chloride recognition by an "outwardly facing" transporter state, 3) dopamine and ion translocation, 4) intracellular unloading of dopamine and ions, and 5) return of the unloaded carrier to its extracellularly facing state (Povlock and Schenk, 1997). One measure of these processes, taken together, can be found in assessments of DAT turnover rates for dopamine. In the current study, turnover rates were altered by more of the normally expressed DAT mutants than any of their other properties.

Mutations in TMs 9 and 10, likely to be adjacent due to the short extracellular loop that connects these TM domains, enhanced uptake rates. 9F461A and 10F485A increased turnover rates by 2.1- and 1.7-fold, respectively (Fig. 7), although these are accompanied by 2.5- and 1.7-fold reductions in dopamine affinity (Fig. 5A).

Nine of the 22 normally expressing mutants displayed reductions in turnover rates for dopamine to less than one third of WT values (Fig. 7). The largest effect on turnover rate, 1200-fold, was induced by mutations in 6F319, a highly conserved phenylalanine lying in the second helical turn of the DAT TM 6 in current DAT models. 6F319 is situated near the TM 7 domain serines implicated in dopamine translocation in several mutagenesis studies. Alanine substitution for the less conserved 6F331 residue reduced the turnover rate by 67-fold. 7F364A mutates another highly conserved mid-TM 7 phenylalanine. This mutation decreased dopamine turnover rate more than 67-fold. Fivefold effects on turnover were also observed for mutations of the less-conserved 7F366, which is also located near Phe<sup>364</sup> and Phe<sup>319</sup> in current DAT models.

Another cluster of phenylalanine-to-alanine substitutions that yielded specific reductions in DAT dopamine turnover are found in TMs 1 and 2. 1F76A, a mutation in an amino acid modeled as lying two thirds of the way through the TM region, provided an 86-fold reduction in DAT turnover. Aromatic residues are found at corresponding positions in each monoamine transporter (Fig. 1B). Smaller reductions in turnover were also observed for each other TM 1 and 2 mutant. 2F105A led to a 43-fold reduction, 1F86A and 2F98A caused ~31-fold reductions, 2F114A yields a 9-fold reduction, and 1F69A reduced turnover rate by 3-fold.

TM helical pairings involving both TM 1 + 2 and TM 6 + 7 are likely due to the structural constraints exerted by the



sizes of the loops that connect them. Each of these pairs contains the highest densities of TM DAT phenylalanines. These are also the DAT zones at which phenylalanine-to-alanine mutations most robustly reduce the ability of DAT to move dopamine. These domains may thus represent zones to be avoided in design of "dopamine-sparing" cocaine antagonists (see below).

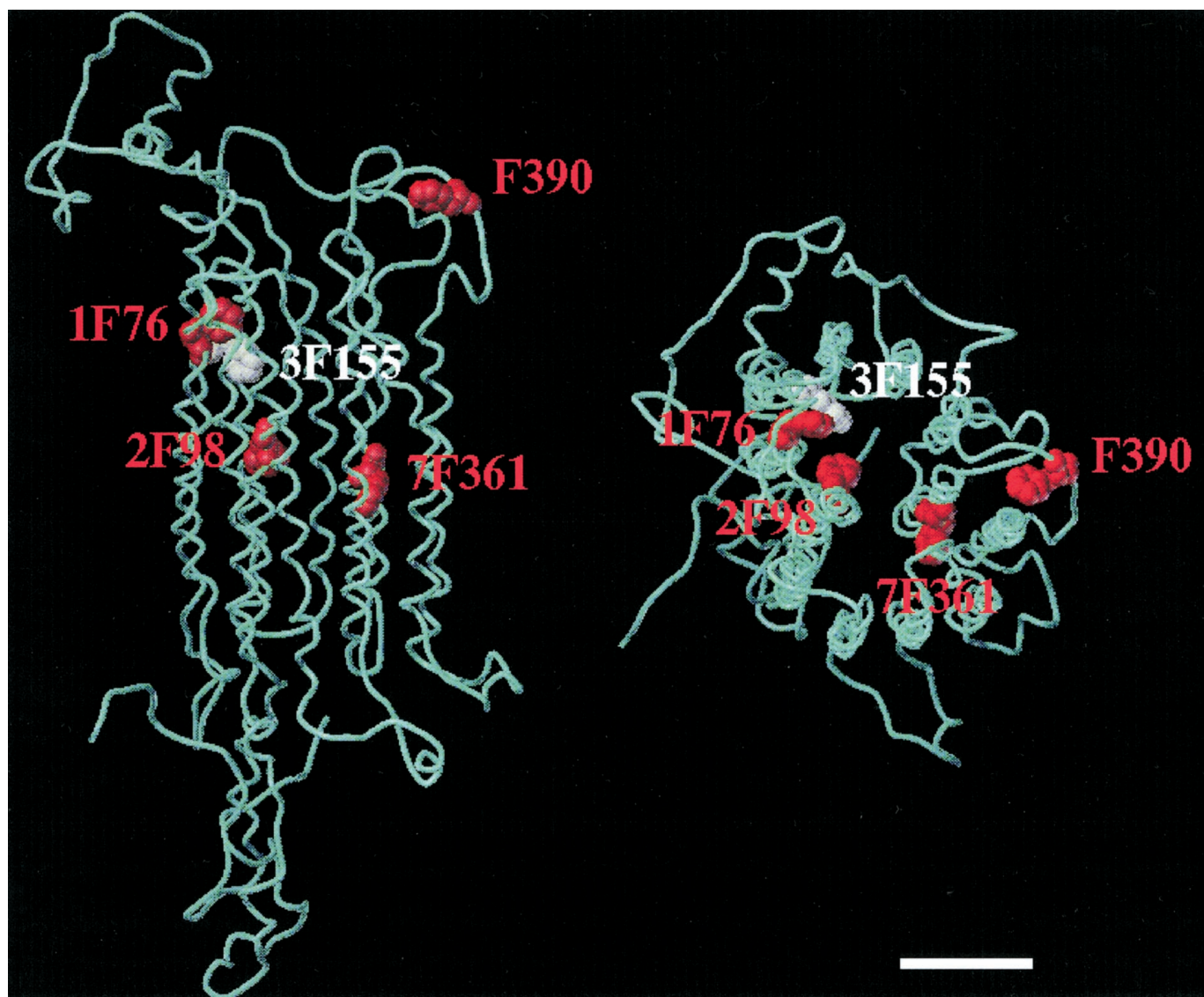
The 3F155A mutation exerted a 14-fold and the 8F410A mutation caused a 9-fold influence on dopamine uptake rates. These amino acids could interact with the TM 1 + 2 and 6 + 7 clusters to which their TM domains are apposed in current DAT models. 8F410A mutates a highly conserved amino acid and reduces turnover rates by 8.6-fold, suggesting an important role in translocation processes.

It is conceivable that the mutations that increase dopamine affinity also reduce dopamine turnover rates. Muta-

tions with these properties, 1F76A, 2F105A, 6F331A, and 11F530A, could initially bind so tightly to dopamine that its movement to the next steps important for transport processes could be impaired.

#### Implications for Current DAT Models

All current DAT models are assembled with no data from crystallographic or other direct structural determinations. Nevertheless, these current DAT models provide a heuristic image from which experimental ideas can flow and onto which experimental data can be superimposed. The most detailed current model of the DAT (Fig. 9) is based on analyses of the primary DAT sequence information (Kilty et al., 1991; Shimada et al., 1991), the results of initial DAT mutagenesis, and calculations for energy minimization, electrostatic potentials, and helical hydrophobic moments. Infer-



**Fig. 9.** Accessibility of phenylalanine side chains whose mutations selectively affected cocaine analog binding affinity (1F76, 2F98, 7F361, and F390, in red) or selectively influenced dopamine uptake affinity (3F155, in white), predicted by a preexisting model of DAT (Edvardsen and Dahl, 1994). The left is a cross-membrane view of DAT, where the top represents the extracellular side and the bottom represents the cytoplasmic side. The right is a membrane-surface view of DAT, where a channel-like cavity in the protein can be clearly seen from the extracellular side. Phenylalanine side chains of interest are shown in a space-filling model. Scale bar, 10 Å. The approximate distance (Å) between two phenyl rings is 17 for 1F76/2F98, 14 for 1F76/3F155 and 1F76/7F361, 38 for 1F76/F390, 4 for 2F98/3F155, 27 for 2F98/7F361, 29 for 2F98/F390, 25 for 3F155/7F361, 30 for 3F155/F390, and 37 for 7F361/F390.



ences from the studies of other proteins, including the TM helix arrangements of a prokaryotic photosynthetic reaction center, have allowed educated estimates of likely TM domain helical content, helical packing patterns, and steric helix-helix interactions (Edwardsen and Dahl, 1994).

A surprising amount of the data from the current study fit with this current model. Modeling the relative positions of the TM phenylalanines at which mutations yielded selective effects on dopamine or cocaine analog affinities is especially instructive. Using mutations that provide such selective influences could reduce the likelihood of gross mutation-induced disruption of DAT structural features. Losses of the phenyl side chains of Phe<sup>76</sup>, Phe<sup>98</sup>, Phe<sup>155</sup>, Phe<sup>361</sup>, or Phe<sup>390</sup> selectively reduces dopamine (3F155) or CFT affinities (1F76, 2F98, 7F361, and F390). Each of these side chains is oriented toward a central DAT recognition pocket domain in the current DAT model, consistent with their contributions to substrate and cocaine recognition (Fig. 9). The mutation 6F331A, which almost eliminated dopamine uptake activity (Fig. 5B), can also be aligned with a central transporter dopamine recognition and/or translocation domain. Only 20% of the TM DAT phenylalanine residues are situated facing a central pocket in a fashion that could readily allow interactions with substrates or ligands. This percentage is similar to the 18 and 20% of TM domain phenylalanines oriented toward central cavities of bacteriorhodopsin (Grigorieff et al., 1996) and the bacterial photosynthetic reaction center (Yeates et al., 1988), respectively.

The removal of phenyl side chains from seven DAT TM phenylalanines disrupts appropriate expression (Fig. 2, Table 1), likely due to interference with proper transporter assembly. Five of these seven, amino acids 86, 357, 364, 411, and 530, can be readily modeled as contributing to interhelical interactions (data not shown). These helix/helix interactions could well make essential contributions to DAT assembly.

Examination of the 20 mutants that display little effect on affinities for either dopamine or CFT reveals that 8 are modeled as facing toward the lipid bilayer and 10 are modeled as potentially involved with intrahelical interactions (data not shown). Only two of the side chains whose removal leaves dopamine and CFT affinities intact are modeled as facing toward a central DAT cocaine/dopamine recognition pocket domain (data not shown). Comparison of these fractions with the effects of mutations provides a reasonable correlation and is consistent with general validity of the current DAT model.

Current DAT models place Phe<sup>390</sup> in the extracellular space in the fourth extracellular loop. This loop is modeled as within striking distance of possible interactions with several other DAT segments. The largely helical content of this loop is broken by proline residues that flank a hydrophobic nine amino acid sequence (PGLIFIYP) that contains Phe<sup>390</sup> in its center. This sequence could be associated with plasma membrane and/or with DAT hydrophobic TM domain residues. Because mutation of Phe<sup>390</sup> produced some of the most remarkably selective decreases in affinity for cocaine analogs, it is conceivable that this residue could make contributions to the small molecule interactions with DAT. It could also interact with residues in TM 7, which forms one of the anchoring segments to which the this loop is connected. Phe<sup>390</sup> thus could contribute to formation of the walls of a putative co-

caine recognition pocket segment. 7F364A displays a perinuclear expression pattern. The phenylalanine side chain normally found at this position thus could lie next to that of Phe<sup>390</sup> and contribute to helix-helix stabilization or hydrophobic pocket formation.

The current results continue to encourage the possibility that a selective small molecule cocaine antagonist might be able to occlude DAT domains selectively involved in cocaine recognition, allow dopamine recognition and translocation, and provide a useful cocaine antagonism. Clustering of the phenylalanines at which alanine substitutions yield selective effects on cocaine affinities toward the extracellular borders of TMs 1, 2, and TMs 7 and the "extracellular" domain adjacent to TM 7 suggests two DAT regions that could contribute to aromatic and/or cationic interactions with cocaine but appears less important for interactions with dopamine. Could small molecules of the size range of dopamine ( $\sim 3 \times 8$  Å) or cocaine ( $\sim 6 \times 12$  Å) fit only into the portions of DAT in which mutations produce selective reductions in cocaine analog affinities? As noted in Fig. 9, sufficient space remains in the cocaine-selective domain centered around 7F361 ( $\sim 13$ – $20$  Å) to accommodate a cocaine antagonist of the size of cocaine. A cocaine antagonist need not be restricted to this size range, however. As long as such a small molecule antagonist obstructed cocaine-selective DAT domains, it could prevent cocaine binding while gaining affinity for DAT by additional interactions with DAT regions distant from the DAT regions that recognize dopamine.

We and others have identified small molecule "partially selective" cocaine antagonist lead compounds. We have shown that a trihexyphenidyl derivative compound with these properties in vitro can partially antagonize the locomotor stimulation of cocaine in vivo (Uhl et al., 1998; D. Dar and G.R.U., in preparation). Improved DAT modeling and better assignment of DAT features that are responsible for cocaine and dopamine recognition should spur efforts to design better and better cocaine antagonists. Such efforts could even identify compounds selective enough for human clinical use. As improved structural information about DAT and related family members becomes available from biophysical studies, the present mutagenesis results can be more properly placed into direct physical context.

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