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Dopamine Transporter Transmembrane Domain Polar Mutants: ΔG and $\Delta\Delta G$ Values Implicate Regions Important for Transporter Functions

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ABSTRACT

Polar residues in dopamine transporter (DAT) transmembrane domains (TMs) are likely to act individually and even interactively in recognizing cocaine and dopamine. We initially evaluated the effects of alanine substitution mutants that remove the polar side chains from residues in each of the 12 putative DAT TMs on the recognition of dopamine and the cocaine analog CFT. Eleven combination mutants with multiple substitutions in DAT TMs 4, 5, 7, or 11 were then selected as candidates for more detailed evaluation based on mutation effects on dopamine and cocaine analog affinities. An evaluation of Gibbs free energy changes displayed by single and combined TM mutants ($\Delta G°$ and $\Delta\Delta G°_{int}$) reveals three categories of potential interactions among mutants: 1) independent, noncooperative interactions (five influenced CFT and two influenced dopamine affinities), 2) synergistic influences (two for CFT and four for dopamine), and 3) complementation of influences on CFT recognition (four mutants) or on dopamine affinity (five). Combined mutations in TMs 4 and 5 yield the largest $\Delta\Delta G°_{int}$ values for dopamine uptake. TMs 4 and 11 mutants provide the largest $\Delta\Delta G°_{int}$ for CFT binding. Interactions between residues lying in DAT TMs 4 and 5 support current DAT structural models that suggest the juxtaposition of these two TMs. These data also support contributions of TM 4 and 11 residues to a polar pocket important for cocaine recognition. These candidate interactive DAT polar domains provide larger target sites for compounds that could modulate specific DAT functions than those provided by single mutations alone.

The Na^+/Cl^--dependent 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 locomotor modulation and behavioral reward (Boja et al., 1944; Kitayama et al., 1992b; Woolverton and Johnson, 1992; Pifl et al., 1993; Witkin, 1994; Self and Nestler, 1995). Rewarding effects of cocaine have been attributed at least in part to DAT blockade by the drug 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).

Cloning of DAT cDNAs and genes from several species has elucidated the primary structure of the transporter and its relationship to other members of the neurotransmitter transporter gene family, including the norepinephrine and serotonin transporters that also serve as cocaine recognition sites. However, little is known about the molecular details of major DAT functions: the ways in which DAT interacts with substrates and ligands and the mechanisms by which it translocates dopamine. An understanding of how DAT works could benefit from information about the tertiary structure of DAT. Unfortunately, no 12-transmembrane domain (TM) transporter has been subjected to successful crystallographic structural determination. Current DAT topological models are thus largely based on data from hydrophobicity analyses, sequence comparisons among gene family members, analyses of DAT post-translational changes, and results of mutagenesis studies. Although TM structural assignments such as those shown in Fig. 1 represent some of the best current understanding of possible DAT topologies, such topological hypotheses must be considered in light of the limited supporting evidence currently available.

Models for interactions between dopamine and DAT have been influenced by studies of initial DAT mutants and chi-

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ABBREVIATIONS: DAT, dopamine transporter; CFT, (−)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane; rDAT, rat dopamine transporter; TM, transmembrane domain; WT, wild type.

meras. Polar residues have represented especially attractive targets for mutagenesis studies that seek to provide a better understanding of DAT function. Initial data suggested that mutations of DAT TM polar and charged residues could display selective or nonselective influences on transporter affinities for dopamine and cocaine analogs. These data suggested that dopamine recognition might occur through at least some mechanisms analogous to those used in catecholamine recognition by their seven-TM G protein-linked receptors. The catechol of dopamine could interact with paired serine residues disposed in putative DAT TM 7, for example (Kitayama et al., 1992b).

Polar residues in TMs could make contributions to the structure of DAT without ligands or substrates. They could contribute to DAT "pockets" important for ligand recognition. They could even participate in the dynamic changes in DAT structures required for translocation of dopamine, sodium, and chloride. Side chains of polar residues could contribute to such functions in several ways. First, they could contribute through direct interactions with ligands, ions, or substrates. Both positively and negatively polar or charged amino acids could participate in the multiple interactions with anionic and cationic components required for recognition and/or translocation of sodium, dopamine, chloride, and cocaine. Second, they could contribute through their differential ability to interact with the microenvironments surrounding DAT. Polar residues could provide the more hydrophilic faces for DAT TM amphipathic helices that turn away from plasma membrane phospholipids but turn toward pockets necessary for interactions among DAT, dopamine, cocaine, and ions. Third, polar residues could participate in helix/helix interactions important for proper DAT assembly and function. Polar residue pairs that lie near each other, residing at approximately the same distances from the cytoplasmic borders of neighboring TMs or even at different "depths" of the same TM, could interact with each other. Evidence supporting such interactions in wild type (WT) DAT could come from observations of interactions between the effects of mutations in one TM and the effects of mutations in another TM.

Evidence for intramolecular interaction has been obtained from mutagenesis studies of other proteins with multiple transmembrane domains, including rhodopsin (Farrens et al., 1996; Han et al., 1998; Vishnivetskiy et al., 1999) and the β_2-adrenergic, glutamate, κ-opioid, angiotensin I, and brady-

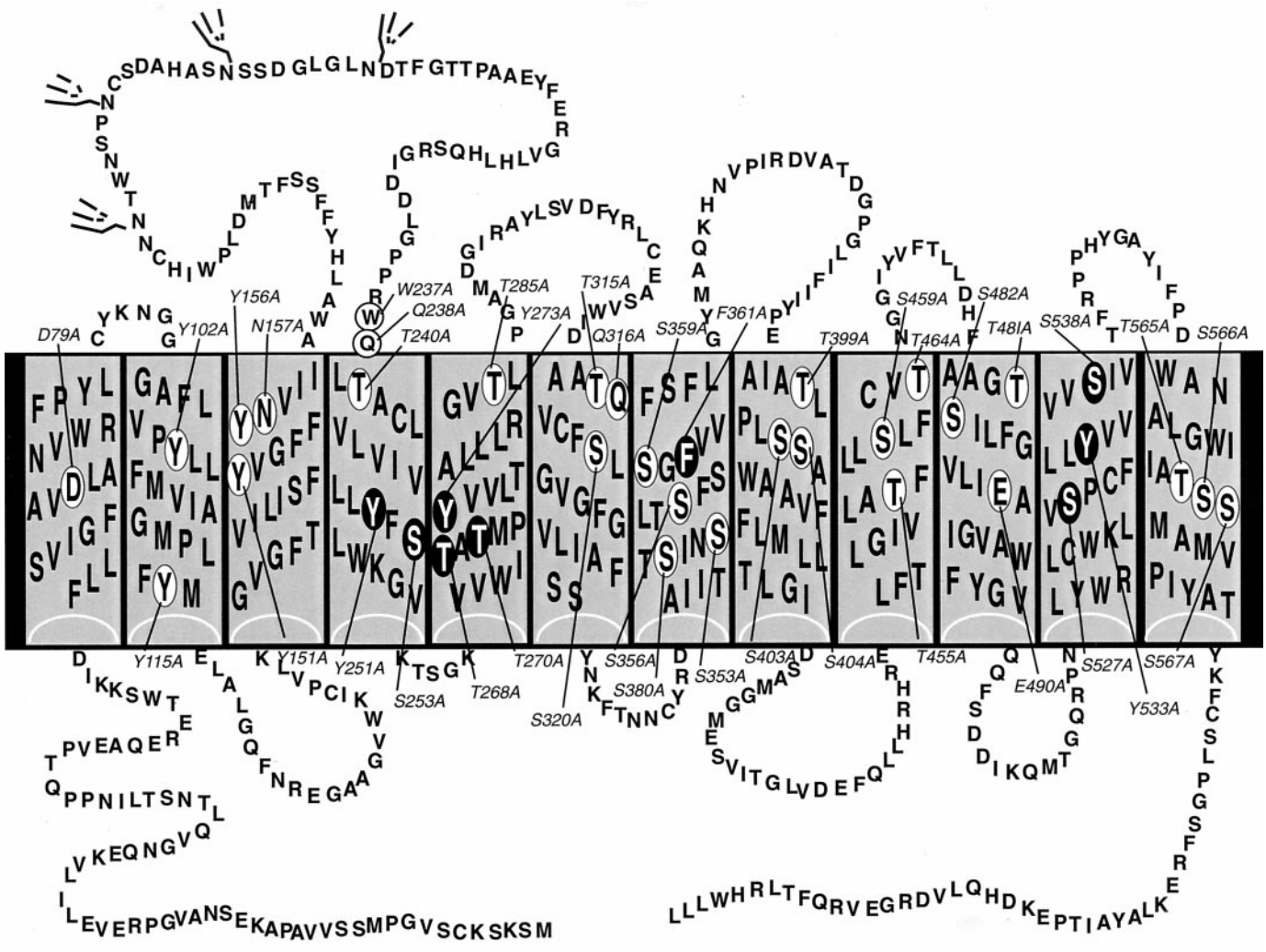

Fig. 1. Depiction of the distribution of the 38 polar residues studied here in a current depiction of DAT topology. The 12 boxes represent putative TM helices (TM 1 left, TM 12 right), up represents the extracellular face, and down represents the cytoplasmic face. The NH_2 terminal is marked by N, the COOH terminal is marked by C, and the four potential N-linked glycosylation sites in the second extracellular loop are indicated by forked structures. Residues substituted with an alanine are circled and labeled. ●, residues used for combined TM mutations.

kinin 2 receptors (Paas et al., 1996; Balmforth et al., 1997; Gether et al., 1997; Paterlini et al., 1997; Marie et al., 1999). A possible interaction between vesicular monoamine transporter TMs 2 and 11 has been suggested by mutagenesis results (Merickel et al., 1997). Each of these studies has used thermodynamic analyses of binding energy changes when individual amino acids are mutated and compared these values with binding energy changes produced by multiple mutations. The observation of additive, supra-additive, and complementary influences of the combined mutations has been used to infer different sorts of interactions between the residues under study. Additive influences, for example, are thought to provide little evidence for interdependence of the residues under study, whereas interactions such as complementation provide suggestive evidence for substantial direct or indirect interactions between the influences of the mutations studied. However, we are aware of no corresponding examination of possible intramolecular interactions for DAT or any other member of the plasma membrane monoamine transporter subfamily to which it belongs.

DAT displays acidic amino acid side chains in putative TMs 1 and 10 and basic or polar amino acids in each of its other putative TMs. We therefore initially evaluated the effects of alanine substitution mutations by altering polar or charged residues in each of the 12 putative DAT TMs. Mutants with substitutions in DAT TMs 4, 5, 7, or 11 were then selected as candidates for more detailed evaluation based on the effects of initial mutations on dopamine and cocaine analog affinities. We also assessed interactions with one of the most interesting DAT aromatic residue mutants, as identified in recent concurrent studies of DAT TM aromatic residues, and we assessed changes in Gibbs free energies associated with interactions between combined TM mutants. These data provide evidence for independence of interactions between some TM mutation combinations, synergistic influences between other mutation combinations, and complementation of influences on cocaine analog or dopamine affinities. These data combine with data from DAT structural modeling to provide a novel approach to assessment of structure/functional relationships of especial importance for DAT functions of recognizing cocaine and accumulating dopamine into dopaminergic neurons.

Experimental Procedures

Plasmid pcDNA 3.1/ZL-Rat DAT (rDAT) Construction. pcDNA 3.1/ZL-rDAT is a DAT-expressing mammalian vector based on pcDNA3.1$^+$ (InVitrogen, San Diego, CA). pcDNA3.1$^+$ has three single restriction sites outside the multiple cloning site: *Bgl*II in a nonessential region, *Pvu*I in its ampicillin resistance gene, and *Pst*I in its neomycin resistance gene. Simply, before shuttling the 3.4-kb DAT cDNA fragment from a pBluescript/rDAT cDNA (Shimada et al., 1991) into pcDNA3.1$^+$, site *Bgl*II was removed by digestion, fill-in reaction, and religation, and sites *Pvu*I and *Pst*I were removed using site-directed mutagenesis (same procedures as later). Thus, pcDNA 3.1/ZL-rDAT carries DAT cDNA under the control of CMV promoter, two origins for replication in bacteria and mammalian cells, respectively, ampicillin and neomycin resistance genes.

Mutagenesis. 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. Uracil-containing single-stranded template for mutagenesis was derived from a pBluescript/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 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 position number and the substituted amino acid. A prefix number represents the putative transmembrane domain in which the mutation is located. Mutations in TMs 1 and 2 were isolated in *Not*I-*Bgl*II fragments; mutations in TMs 3 to 7 were isolated in *Bgl*II-*Pvu*I fragments, and mutations in TMs 8 to 12 were isolated in *Pvu*I-*Pst*I fragments of the plasmid (Shimada et al., 1991). Each mutation was confirmed by DNA sequencing, isolated restriction fragments were shuttled into an rDAT-expressing mammalian plasmid pcDNA 3.1/ZL-rDAT, and correct sequences were reconfirmed.

Thirty-four DAT TM residues (Fig. 1) were substituted with alanine, singly or in groups. Multiple polar residues found in several TMs were mutated. In TMs 4 and 7, where multiple polar residues are scattered from the cytoplasmic to the intracellular borders, polar residues were mutated in two groups: an outer group and an inner group. Residues in TMs 4, 5, 6, 7, and 9 were mutated both singly and in groups.

Functional Analyses. COS cells (10^7) were grown in 6-well plates, transfected with 20 μg of pcDNA 3.1/ZL-rDAT or mutant DNAs using electroporation (300 V, 1100 μF, Gene Zapper 450/2500; IBI, New Haven, Conn), allowed to express the plasmid for 3 days, and then assayed for their abilities to accumulate [^{3}H]dopamine (49 Ci/mmol; New England Nuclear, Boston, MA) or to bind the cocaine analog [^{3}H]CFT [(−)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane, 83.5 Ci/mmol; New England Nuclear] by incubation in Krebs-Ringer HEPES-buffered solution (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). Kinetic and saturation analyses were used to determined K_M, V_{max}, or K_D and B_{max} values, respectively, as described previously (Kitayama et al., 1992b). For uptake assays, 10 nM [^{3}H]=dopamine and 0.1, 1, 5, 10, 20, 30, and 50 μM unlabeled dopamine concentrations were used. For initial binding assays, 2 nM [^{3}H]CFT was adjusted to 1.5, 3, 5, 15, 30, and 60 nM concentrations using unlabeled CFT. Cells transfected with WT pcDNA 3.1/ZL-rDAT served as controls. Parallel incubations with 30 μM unlabeled (−)-cocaine allowed an estimation of nonspecific binding and uptake. Uptake assays were carried out for 5 min at 37°C, followed by two complete washes with 2 ml of KRH with 50 μM ascorbic acid. Binding assays were carried out for 2 h at 4°C followed by three washes with 2 ml of 4°C KRH buffer. Assay temperatures and times differed to provide optimal conditions for each. Cells were solubilized in 0.5 ml of 1% SDS solution, and radioactivity was determined using a Beckman LS 6000 liquid scintillation counter at ~50% efficiency. Studies of dopamine inhibition of 2 nM [^{3}H]CFT binding used several concentrations of dopamine in 50 μM ascorbic acid. 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.

Immunostaining of Transfected COS Cells. Cellular patterns of expressed DAT immunoreactivity were assessed by immunohistochemistry using specific polyclonal rabbit anti-DAT sera, as described previously (Lin et al., 1999). COS cells transfected with DAT or DAT mutant plasmids were grown on coverslips in 6-well plates for 3 days. Cells transfected with a truncated, promoterless version of pcDNA 3.1/ZL-rDAT, 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 the addition of 1 ml/well of 4% paraformaldehyde in PBS, and incubated at 4°C for 1 h. After four or five timed washes with PBS, endogenous peroxidase was inactivated by incubation of the cells with 1 ml of 10% methanol, 0.6% H_2O_2 in PBS for 10 min at room temperature. After several washes with PBS and two brief washes with Tris-buffered saline (TBS, 50 mM, pH 7.6), proteins were blocked by incubation in 1 ml of augmented TBS* [TBS with 2% skim milk power (Fluka,

Ronkonkoma, NY), 0.2% Triton X-100, 0.01% Na Azide) for 1 h. The primary antiserum used was a rabbit serum raised against the N-terminal peptide of rDAT, termed 16 B. An antibody raised against the C-terminal peptide, 18A, was used for some confirmatory studies of several mutants, producing results identical with those of 16B. Sera were diluted with augmented TBS* at 1:20,000 and 1:1500, respectively; incubated with cells overnight at room temperature; and washed from cells three times with 5 ml of augmented TBS*. Cells were incubated for 1 h with 10 ml of augmented TBS* plus 30 μl of biotinylated goat anti-rabbit IgG (BA-1000; Vector Laboratories, Burlingame, CA). The wells were washed several times with TBS to remove secondary antibodies and then incubated for 1 h with avidin-biotin complex solution prepared 30 min before use (PK-6100, Vectastain Elite; Vector Laboratories). After three washes with TBS, labeling was visualized by 1-min reaction with freshly prepared solution containing 20 mg of diaminobenzidine tetrahydrochloride, 388 μl of 4% NiCl, and 100 μl of 3% H_2O_2 in 40 ml of TBS. The stained cells on coverslips were washed three times with TBS, dehydrated, mounted onto microscope slides, and examined for semiquantitative assessments of the patterns of DAT immunoreactivity by an observer who was unaware of the mutations.

Analyses and Calculations of Gibbs Free Energies. K_M and V_{max} values for [^{3}H]dopamine uptake, K_D and B_{max} values for [^{3}H]CFT binding activity, and K_i and IC_{50} values for dopamine against [^{3}H]CFT were calculated with Prism Version 2 (GraphPad Software, San Diego, CA). Values for Gibbs free energy of uptake and binding were derived from the equation $\Delta G^o = -RT \ln K$, where r = 1.987 cal/°/mol, T = 277.18 K for [^{3}H]CFT binding and dopamine inhibition reactions performed at 4°C, and T = 310.18 K for [^{3}H]dopamine uptake assays performed at 37°C. K is an equilibrium constant: K_D for CFT affinities determined from Scatchard analyses of [^{3}H]CFT binding data or K_i for dopamine potencies in inhibiting [^{3}H]CFT binding [$\Delta\Delta G^o = \Delta G^o_{WT} - \Delta G^o_{MT}$, where MT is the mutant DAT; $\Delta\Delta G^o_{int} = \Delta\Delta G^o_{AB} - (\Delta\Delta G^o_A + \Delta\Delta G^o_B)$ for double mutants, where A is mutant A, B is mutant B, and AB is the combined mutant A + B; and $\Delta\Delta G^o_{int} = \Delta\Delta G^o_{ABC} - (\Delta\Delta G^o_A + \Delta\Delta G^o_B + \Delta\Delta G^o_C)$ for triple mutants, where A is mutant A, B is mutant B, C is mutant C, and ABC is the combined mutant A + B + C]. Statistical analyses were made with Student's t tests.

Results

Initial Studies of DATs with Mutations in Single TMs

Affinity and ΔG^o Estimates from Screening Studies of Initial Polar Residue Alanine Substitution Mutants with Varying Levels of Expression. The results from initial screening studies of alanine substitution mutations in individual TMs fell into several patterns, based on changes in expression levels, affinities for dopamine, and affinities for the cocaine analog CFT (Figs. 2 and 3).

First, two mutations produced little alteration of any of these parameters (Figs. 2 and 3). Alanine-substitution mutations in the inner aspect of TM 2 (Y115A) and in TM 4 (T240A, plus adjacent W237 + Q238) each resulted in normal patterns of expression as detected by DAT immunohistochemistry, dopamine uptake affinity, CFT binding affinity, and maximal binding (B_{max}).

A second pattern was displayed by mutants that revealed such sharp reductions in dopamine uptake and such prominent disruptions of DAT immunostaining patterns that they were poor candidates for further analyses as combination mutants (Fig. 2). These included mutants Y102A in TM 2, Y151A + Y156A + N157A in TM 3, T315A + Q316A + S320A in TM 6, S320A in TM 6, T455A + S459A + T464A in TM 9, and E490A in TM 10. Interestingly, the S320A mutant retains 20% of WT CFT affinity (Fig. 3) and at least the B_{max} values displayed by WT DAT.

The 26 remaining DAT single or multiple polar or charged amino acid mutants displayed altered affinities for dopamine or CFT in the face of reasonable expression levels. Some of these influences were relatively selective, and some were less selective.

Third, mutants that altered affinities for both dopamine and CFT by more than 3-fold in the face of near-normal expression patterns included D79A, Y251S, Y251W + Y273W, and T455A (Fig. 3). Interestingly, dopamine affinities were increased in Y251S and Y251W + Y273W, whereas CFT affinities are decreased more than 3-fold for both of these mutants. Q316A enhanced dopamine affinities by more than 3-fold while reducing CFT affinity to 36% of WT values.

Fourth, mutants that reduced affinities for CFT with no sizable reduction, or even enhancement, in affinity for dopamine were perhaps the most interesting mutations in this group (Fig. 3). This pattern of selective effects on dopamine uptake was initially displayed by mutations in TM regions 4 Y251A + S253A, the single mutant Y251A, T315A, T399A + S403A + S404A in TM 8, as well as S459A and T464A in TM 9.

Overall, one fourth of the mutations displayed near WT dopamine affinities, 39% yielded greater than 20% decreases in dopamine affinity, and 35% of the mutants increased dopamine affinity by more than 20%. Eighty-six percent of the mutant DATs displayed decreased CFT affinity (Fig. 3). The ratio of affinity for CFT to affinity for dopamine thus changed more than 2-fold for approximately half the mutants in which multiple residues were substituted in single TMs. TM 4, 5, 8, and 11 mutants displayed at least 20% changes in this ratio, with selective decreases in CFT affinities. The TM 10 mutant displayed a more than 20% change in this ratio due to a predominant loss of dopamine affinity. The remainder of the mutants, those in TMs 4, 7, and 12, perturbed recognition of both dopamine and cocaine analogs without selectivity.

Dopamine Transport V_{max} Estimates from Screening Studies of Initial Polar Residue Alanine Substitution Mutants. Characterization of dopamine transport V_{max} values enhanced the picture obtained from the classification of transporter mutants based on effects on expression, dopamine affinity, and CFT affinity (Fig. 3). Dopamine transport V_{max} values were selectively reduced in the TM 1 D79A, in the TM 4 Y251A and Y251S substitutions, and in each of the TM 5 Y273A, Y273F, and Y273S substitutions. Phenylalanine replacement of Y251, however, restored virtually WT dopamine transport V_{max} values. More modestly reduced V_{max} values were noted for transporter mutants T285A in TM 5, for S356A + S359A in TM 7, for T399A + S403A + S404A in TM 8, and for S527A + Y533A + S538A in TM 11.

Studies on Mutants in Multiple TMs

Selection of DAT TM Mutants for Studies of TM Interactions. Several criteria influenced our choices of DAT TM mutants to study for possible interactive influences on dopamine and cocaine analog recognition. Chief among these were the abilities of the mutants in individual TMs 1) to express nearly normally and 2) to produce selective influences on affinities for cocaine analogs compared with influences on dopamine recognition. TM 4, 5, and 11 mutants were chosen on the basis of these criteria. Because one of the

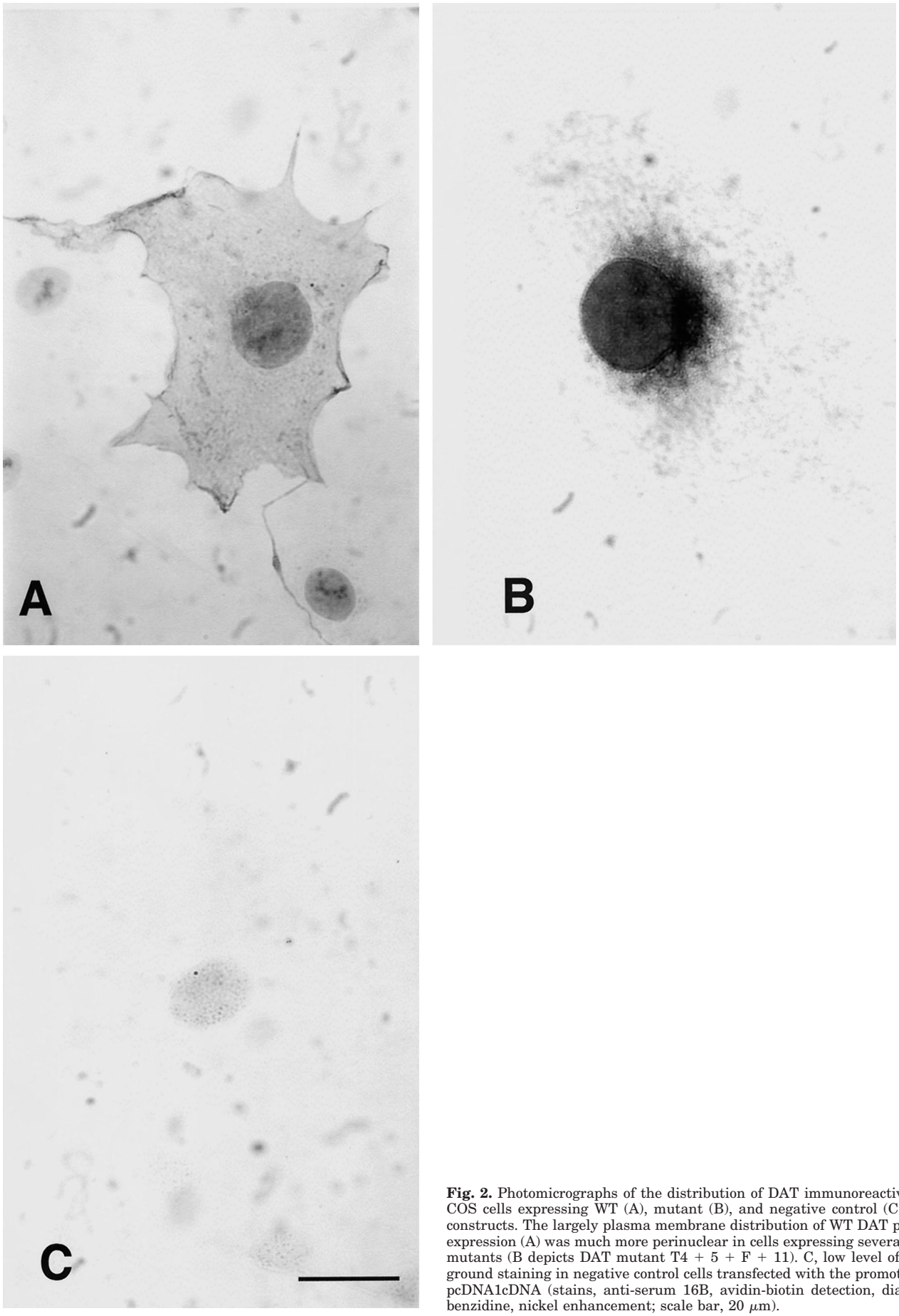

Fig. 2. Photomicrographs of the distribution of DAT immunoreactivity in COS cells expressing WT (A), mutant (B), and negative control (C) DAT constructs. The largely plasma membrane distribution of WT DAT protein expression (A) was much more perinuclear in cells expressing several DAT mutants (B depicts DAT mutant T4 + 5 + F + 11). C, low level of background staining in negative control cells transfected with the promoterless pcDNA1cDNA (stains, anti-serum 16B, avidin-biotin detection, diaminobenzidine, nickel enhancement; scale bar, 20 μm).

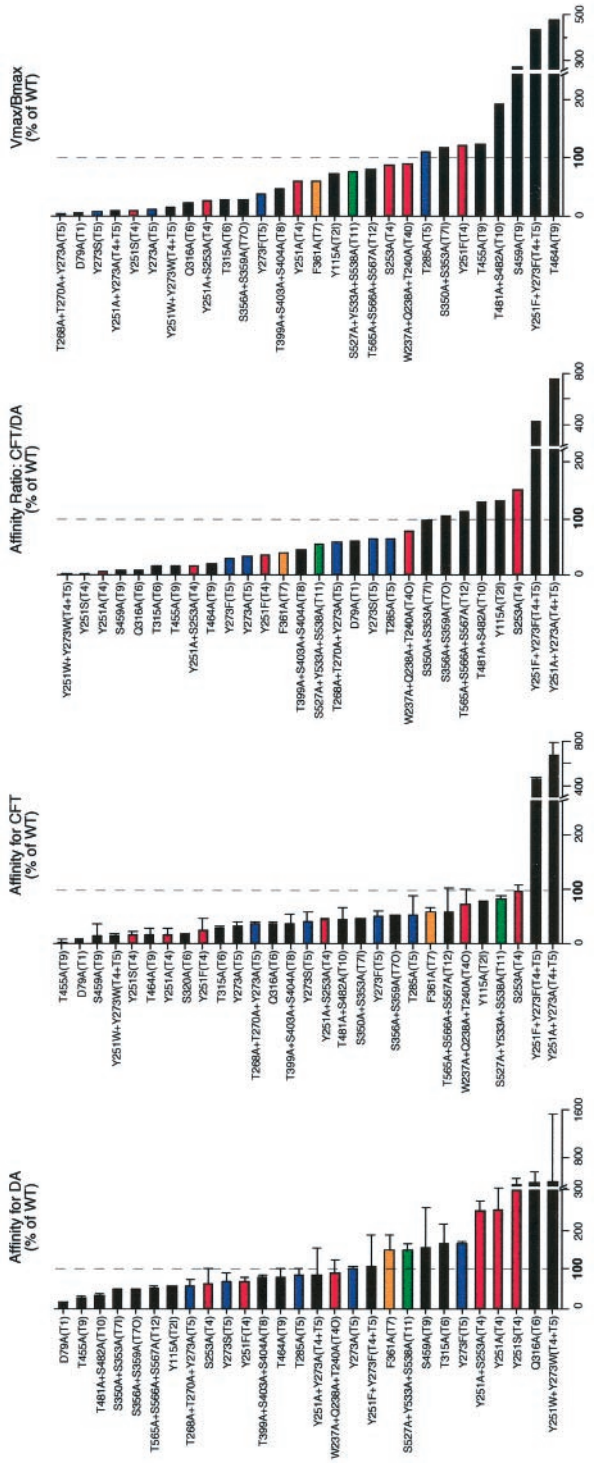

Fig. 3. A, rank-order lists of the characteristics of single-TM DAT mutants. Values from screening assays using these mutants are derived from the mean ± S.D. of parameters from Scatchard and Eadie-Hofstee analyses from one to four independent experiments. Affinities were normalized to WT values ($K_{M\ MT}^{-1}/K_{M\ WT}^{-1} \times 100$ for dopamine and $K_{D\ MT}^{-1}/K_{D\ WT}^{-1} \times 100$ for CFT). Colored columns show the single-TM mutations chosen for more detailed evaluations in subsequent multiple mutant DAT constructions (red, TM 4; blue, TM 5; yellow, TM 7; and green, TM 11). B, rank-order lists of the characteristics of single-TM DAT mutants. Values from screening assays using these mutants are derived from the mean ± S.D. of parameters from Scatchard and Eadie-Hofstee analyses with one to four independent experiments. Affinities were normalized to WT values ($K_{M\ MT}^{-1}/K_{M\ WT}^{-1} \times 100$ for dopamine and $K_{D\ MT}^{-1}/K_{D\ WT}^{-1} \times 100$ for CFT).

mutants from a series of aromatic mutations, F361A, also shared this property (Lin et al., 1999), we also studied its combined influences with TM 4, 5, and 11 mutants. We thus studied a total of 11 mutants each with mutations in multiple TMs, and for 4, we compared results from these mutants with those of the corresponding single-TM mutants.

Expression of DATs with Mutations in Multiple TMs. Three of the 11 combined TM mutants displayed patterns of DAT immunostaining that differed modestly from these WT expression patterns (Table 1, Fig. 2). Each of these mutants displayed a modestly to moderately reduced CFT binding B_{max} value (Table 2) but expressed at levels sufficient for further analyses.

[^{3}H]Dopamine and [^{3}H]CFT Affinities of Combined TM Mutants. Each of the 11 combined TM mutants decreased CFT affinities (Table 2). One third also reduced dopamine affinities, as manifested by more than 3-fold increases in K_M values for dopamine uptake compared with the corresponding single-TM mutants. These losses of dopamine affinity assessed by K_M values from uptake experiments correlated well with dopamine potencies obtained from studies of the ability of dopamine to compete for CFT binding to these mutant DATs (Pearson correlation coefficient for K_M and K_i values, $r = 0.783$, $P < .0001$). Losses of dopamine affinities in the T4 + 5 combined mutant were most striking, whether assessed through values for K_M or values for potency in competition for binding of radiolabeled CFT. Losses of CFT affinity, on the other hand, were more striking for the T5 + F + 11 and T4 + 5 + F combination mutants.

Gibbs Free Energy Calculations for Single and Combined TM Mutants. The affinity changes from WT values conferred by single-TM mutants allowed calculation of the Gibbs free energy changes (Table 3). The ΔG^o values for CFT and dopamine recognition by the WT transporter, approximately 10 and 12 kcal/mmol, respectively, were altered only modestly by the single-TM mutations studied in the combination mutants studied subsequently.

Calculations of $\Delta\Delta G^o$ values and comparisons with $\Delta\Delta G^o_{int}$ values provided a quantitative yardstick for evaluating the possible interactions between mutation effects in different TMs. In the combination mutants, the range of $\Delta\Delta G^o$ values for CFT recognition was smaller (0.13–1.11 kcal/mol) than the range of $\Delta\Delta G^o$ values for dopamine recognition (−1.52 to 1.54 kcal/mol; Table 3). This difference reflects our selection of single-TM mutants that gave the largest differences between dopamine and CFT potencies, enriching the sample for mutants that enhanced dopamine affinities as well as those that reduced CFT potencies.

Analyses of $\Delta\Delta G^o_{int}$ values suggested the possibility that these TMs might interact more actively in recognition of dopamine and relatively more independently in recognition of CFT (Table 4). Eighty-two percent of the combination mutants provided significant $\Delta\Delta G^o_{int}$ values for dopamine recognition, whereas only 54% provided significant $\Delta\Delta G^o_{int}$ values for CFT binding. The magnitude of $\Delta\Delta G^o_{int}$ values was greater for dopamine than for CFT affinities. The totals of positive and negative $\Delta\Delta G^o_{int}$ values for dopamine and CFT were 5.61 versus 0.61 and −4.03 versus −1.94 kcal/mol, respectively.

Careful analyses of $\Delta\Delta G^o_{int}$ values also revealed evidence for three categories of interactions among the amino acid side chains in different DAT TMs evaluated here:

1) Apparently independent, noncooperative interactions were found in mutants with $\Delta\Delta G^{o}_{int}$ values close to zero. The relative independence of the T5 and T4 mutants from the TM 7 F361 mutant was maintained for effects on both dopamine and CFT, whereas the independent effects of the T4 + 5, T5 + 11, and T5 + F + 11 combinations on CFT recognition were not found for dopamine recognition (Table 4).

2) Positively cooperative or synergistic interactions were found between different mutants when evaluated based on mutation-induced changes in CFT affinity (T4 + 11 and F + T11) or in dopamine affinity (T4 + F + 11, T4 + 5, T4 + 5 + F, and T4 + 5 + F + 11). However, the presence of T4, T11, and F in both groups of combined mutations with cooperative interactions does hint at the possibility of some sharing of the

TABLE 1
Transporter immunoreactivity in COS cells transiently expressing each of the mutants
Appeared perinuclear and plasma membrane staining were rated by an observer unaware of the mutants with a quantitative scale of 1 to 3 +.

	Perinulcear	Plasma Membrane		Perinulcear	Plasma Membrane
WT	+	+++	T5 + F	+	+++
T4	+	+++	T5 + 11	+	+++
T5	+	+++	F + T11	+	+++
F361A	+	+++	T4 + 5 + F	+	+++
T11	+	+++	T4 + 5 + 11	++	++
T4 + 5	+	+++	T5 + F + 11	+	+++
T4 + F	+	+++	T4 + F + 11	++	++
T4 + 11	+	+++	T4 + 5 + F + 11	++	++

TABLE 2
Pharmacological characterization of dopamine transporter with multiple mutations

	[^{3}H]Dopamine Uptake		Dopamine Competition		[^{3}H]CFT Binding		[^{3}H]Dopamine Uptake V_{max}	[^{3}H]CFT Binding B_{max}
	K_m	% of WT	K_i	% of WT	K_D	% of WT		
	μM		*μM*		*nM*		*fmol/μg/min*	*fmol/μg*
WT	2.2 ± 0.6	100	0.37 ± 0.14	100	20.9 ± 7.4	100	255.3 ± 9.0	6.3 ± 0.6
T4	0.9 ± 0.1*	41	0.23 ± 0.01	62	46.9 ± 3.7**	224	62.3 ± 27.8***	6.6 ± 1.0
T5	3.8 ± 0.8*	173	0.78 ± 0.47	211	60.7 ± 12.6**	290	3.9 ± 2.0***	5.8 ± 0.7
F361A	1.5 ± 0.4	68	0.39 ± 0.12	105	36.8 ± 7.0	176	145.1 ± 44.7***	6.2 ± 0.6
T11	1.1 ± 0.1*	50	0.16 ± 0.01	43	25.2 ± 2.4	121	136 ± 15.2***	6.0 ± 0.2
T4 + 5	44 ± 11.8***	2000	6.0 ± 1.9**	1622	100.0 ± 30.6**	478	5.5 ± 4.4***	4.8 ± 0.5*
T4 + F	1.1 ± 0.2*	50	0.34 ± 0.31	92	62.0 ± 6.6***	297	48.2 ± 34.8***	6.9 ± 0.2
T4 + 11	0.5 ± 0.1**	23	0.036 ± 0.023*	10	120.3 ± 35.9***	576	19.9 ± 12.7***	5.9 ± 0.3
T5 + F	3.5 ± 0.6*	159	0.59 ± 0.26	159	101.2 ± 19.7***	484	4.1 ± 0.8***	5.4 ± 0.1*
T5 + 11	0.4 ± 0.1**	18	0.025 ± 0.016**	7	80.6 ± 17.4***	386	0.6 ± 0.4***	6.1 ± 0.4
F + T11	0.7 ± 0.1**	32	0.048 ± 0.023*	13	70.7 ± 3.2***	338	46.8 ± 34.4***	6.3 ± 0.1
T4 + 5 + F	13 ± 1.3***	591	4.80 ± 3.3*	1297	146.1 ± 15.3***	699	68.7 ± 3.5***	5.9 ± 0.1
T4 + 5 + 11	0.5 ± 0.1**	23	0.054 ± 0.02*	15	62.4 ± 11.2***	299	0.1 ± 0.5***	4.0 ± 0.9*
T5 + F + 11	0.7 ± 0.1**	32	0.080 ± 0.011*	22	157.0 ± 57**	751	1.1 ± 0.1***	6.2 ± 0.3
T4 + F + 11	5.9 ± 1.4**	268	2.6 ± 1.6*	703	64.5 ± 11.4***	309	9.0 ± 2.0***	4.9 ± 0.6*
T4 + 5 + F + 11	8.6 ± 1.1***	391	2.6 ± 1.0**	703	85.5 ± 3.9***	409	5.4 ± 2.4***	3.5 ± 1.3*

Values are the mean ± S.D. of three independent experiments, each performed in duplicate determinations.
* $P < .5$ versus WT. ** $P < .01$ versus WT. *** $P < .001$ versus WT.

TABLE 3
Ligand recognition energistics of single and combined TM mutants
Gibbs free energies (ΔG^o), its change ($\Delta\Delta G^o$), and interaction energy ($\Delta\Delta G^o_{int}$) are calculated as described in *Materials and Methods*.

	CFT Recognition			Dopamine Recognition		
	ΔG^o	$\Delta\Delta G^o$	$\Delta\Delta G^o_{int}$	ΔG^o	$\Delta\Delta G^o$	$\Delta\Delta G^o_{int}$
			kcal/mol			
WT	9.77 ± 0.21			11.99 ± 0.21		
T4	9.30 ± 0.04	0.47 ± 0.10		12.22 ± 0.03	−0.23 ± 0.11	
T5	9.16 ± 0.12	0.61 ± 0.12		11.62 ± 0.35	0.37 ± 0.22	
F361A	9.44 ± 0.11	0.33 ± 0.11		11.96 ± 0.23	0.03 ± 0.14	
T11	9.64 ± 0.05	0.13 ± 0.10		12.42 ± 0.05	−0.43 ± 0.11	
T4 + 5	8.90 ± 0.18	0.87 ± 0.14	−0.21 ± 0.12	10.45 ± 0.20	1.54 ± 0.15	1.40 ± 0.17**
T4 + F	9.14 ± 0.06	0.63 ± 0.10	−0.18 ± 0.10	12.16 ± 0.60	−0.17 ± 0.44	0.04 ± 0.32
T4 + 11	8.79 ± 0.15	0.98 ± 0.13	0.37 ± 0.11*	13.36 ± 0.43	−1.37 ± 0.24	−0.70 ± 0.16*
T5 + F	8.88 ± 0.10	0.89 ± 0.11	−0.05 ± 0.11	11.75 ± 0.29	0.24 ± 0.20	−0.15 ± 0.18
T5 + 11	9.00 ± 0.12	0.76 ± 0.11	0.02 ± 0.11	13.51 ± 0.33	−1.52 ± 0.22	−1.45 ± 0.20**
F + T11	9.07 ± 0.02	0.70 ± 0.09	0.24 ± 0.10*	13.14 ± 0.30	−1.15 ± 0.20	−0.74 ± 0.15*
T4 + 5 + F	8.67 ± 0.06	1.10 ± 0.10	−0.32 ± 0.12*	10.64 ± 0.36	1.35 ± 0.23	1.19 ± 0.21**
T4 + 5 + 11	9.14 ± 0.11	0.63 ± 0.11	−0.59 ± 0.12**	13.04 ± 0.19	−1.05 ± 0.14	−0.76 ± 0.18**
T5 + F + 11	8.66 ± 0.20	1.11 ± 0.15	−0.18 ± 0.13	12.81 ± 0.07	−0.82 ± 0.12	−0.37 ± 0.18*
T4 + F + 11	9.12 ± 0.10	0.64 ± 0.11	−0.29 ± 0.12*	10.96 ± 0.38	1.03 ± 0.29	1.67 ± 0.24*
T4 + 5 + F + 11	8.96 ± 0.03	0.81 ± 0.09	−0.74 ± 0.13**	10.91 ± 0.21	1.08 ± 0.18	1.35 ± 0.22*

* $P < .5$, ** $P < .1$, $\Delta\Delta G^o_{AB}$ versus $(\Delta\Delta G^o_A + \Delta\Delta G^o_B)$.

difficulties that combined losses of these amino acid side chains provides for recognition of the two ligand classes.

3) Negative cooperativity, or complementarity of effects, on CFT recognition or dopamine affinity was found in a surprisingly large fraction of mutant combinations. T4 + 5 + 11 provides negative cooperativity of influences on recognition of both substances, whereas three combination mutants provide such influences for CFT and four provide them for dopamine recognition.

It is interesting, overall, that only the two mutants that combine TM 4 or 5 with a TM 7 aromatic mutation (termed F) provide evidence for no interactions in recognizing either ligand, based on these analyses. Polar or charged residues in several DAT domains thus function interactively in the transporter activities examined here.

Dopamine Transport V_{max} Estimates from Screening Studies of Combined Polar Residue Mutants. Each of the combination mutants displayed greater than 3-fold changes in dopamine transport V_{max} rates (Table 2). Eighty percent of the combination mutants displayed evidence for interactions between the influences of changes in one TM and the influences of changes in other TMs on V_{max}. Interestingly, six of these interactions were greater than 3-fold and displayed synergy, such that the losses of V_{max} velocities from the combined mutants were greater than the sum of the effects of single-TM mutants. The T4 + 5 + 11 mutant had an almost 2500-fold greater impact on V_{max} rate than the sum of the influences of the T 4, 5, and 11 when tested separately, for example. Three combination mutants, T4 + 5, T4 + 5 + F, and T4 + 5 + F + 11, also displayed evidence for significant complementation of influences on dopamine transport V_{max} compared with the values for single-TM mutants.

Specificity of effects is also evident in a comparison of the influences on V_{max} and the influences on $\Delta\Delta G^{o}_{int}$ (Table 4). T4 + 5 + 11, T5 + 11, and T5 + F + 11 displayed complementary effects on $\Delta\Delta G^{o}_{int}$ yet yielded apparently synergistic effects in V_{max} studies. Combined mutants T4 + 5, T4 + 5 + F + 11, and T4 + 5 + F demonstrated synergistic $\Delta\Delta G^{o}_{int}$ interactions but complementary effects on V_{max}.

Discussion

The present results support individual and interactive roles of DAT polar residues in recognition of cocaine and dopamine and in translocation of dopamine. We discuss these data in light of 1) the influences of single-TM mutations, 2) plausible interpretations of the synergistic and complementary results of studies of combined TM mutations, 3) the limitations of the inferences that can be derived from this sort of approach, and 4) implications for DAT modeling and for designing agents that could selectively antagonize cocaine recognition by this multidomain protein.

Influences of Mutations in Polar Amino Acids Lying in Single TMs. Near WT functions are retained by DAT mutants in amino acids located in several TMs. Many of these residues are poorly conserved among members of the 12-TM, sodium- and chloride-dependent neurotransmitter transporter family, including T240, W237, and Q238 in the TM 4 domain. Although it is conceivable that transporter functions not tested in detail here, such as the subtleties of ion-dependent translocation mechanisms, might not be altered in assays using only single concentrations of sodium and chloride, it seems likely that these side chains play little role in substantial DAT functions.

Other single-TM mutations disrupted DAT expression. These mutations appeared to be poor candidates for subsequent studies of their effects in combined TM mutants and were not included in these studies. Disruption of expression of the TM 3, 6, 9, and 10 multiple mutants appeared to restrict our abilities to assess contributions from amino acids in these TMs to combined TM mutants. We cannot rule out the possibility, however, that combining some of these mutants with other mutants could have complemented the effects of the initial mutations and allowed expression.

Most of the remaining single-TM mutations, however, do express sufficiently well that their distinct influences on specific binding or transport properties can be characterized. Many of these mutants alter dopamine transport, often with separable alterations in dopamine affinities and transport V_{max} rates. If transport is a complex process, then changes in many DAT features, including substrate affinity, ion affinity, substrate/transporter conformational changes during transport, ion/transporter conformational changes during transport, and cytoplasmic release rates for ions and substrates, could each alter the transport properties assessed here. Interestingly, when multiple different amino acid substitutions at the same position were tested in screening studies, not all substitutions for the WT amino acid exerted the same influences. The ability of the Y251F substitution to virtually normalize the dopamine transport V_{max} lost with alanine sub-

TABLE 4

Categories of interaction among TMs ($\Delta\Delta G_{int}$)

Values are presented by rank order. Statistical significance was calculated and presented in Table 3. Categories are defined in which synergistic means $\Delta\Delta G^{o}_{AB}$ is significantly larger than ($\Delta\Delta G^{o}_{A}$ + $\Delta\Delta G^{o}_{B}$), Complementive means $\Delta\Delta G^{o}$ for combined TM mutants is significantly smaller than that of the sum of each individual TM, and there is no significant difference between $\Delta\Delta G^{o}_{AB}$ and ($\Delta\Delta G^{o}_{A}$ + $\Delta\Delta G^{o}_{B}$) on independent interaction.

	Category	CFT Recognition			Category	Dopamine Recognition	
		kcal/mol	*% of* ΔG_{WT}			*kcal/mol*	*% of* ΔG_{WT}
T4 + 11	Synergistic	0.37	3.8*	T4 + F + 11	Synergistic	1.67	13.9*
F + T11		0.24	2.4*	T4 + 5		1.40	11.7**
T5 + 11	Independent	0.02	0.2	T4 + 5 + F + 11		1.35	11.3*
T5 + F		−0.05	−0.5	T4 + 5 + F		1.19	9.9**
T5 + F + 11		−0.18	−1.8	T4 + F	Independent	0.04	0.3
T4 + F		−0.18	−1.9	T5 + F		−0.15	−1.3
T4 + 5		−0.21	−2.1	T5 + F + 11	Complementive	−0.37	−3.1*
T4 + F + 11	Complementive	−0.29	−3.0*	T4 + 11		−0.70	−5.9*
T4 + 5 + F		−0.32	−3.3*	F + T11		−0.74	−6.2*
T4 + 5 + 11		−0.59	−6.0**	T4 + 5 + 11		−0.76	−6.3**
T4 + 5 + F + 11		−0.74	−7.6**	T5 + 11		−1.45	−12.1**

stitutions for the WT tyrosine at this position is consistent, for example, with a crucial aromatic side chain role in transport function.

CFT affinities are also influenced by mutations in several TMs. Affinities are altered by more than 50% after mutations in TMs 1, 4, 5, 6, 8, 9, and 10. Selective reductions in CFT affinity in mutants that spare or even enhance dopamine affinities to produce CFT/DA affinity of ratios less than 0.5 were found in TMs 4, 5, 6, 8, 9, and 11. Although not all of the combination mutants expressed well, mutants that combined the TM 4, 5, and 11 mutations showed expression patterns near enough to WT that in combinations with the previously described TM 7 phenylalanine mutation, they formed the bases for further detailed analyses. Conceivably, studies in which multiple mutants in one TM were added to single mutants in other TMs could provide one approach to this problem. It is interesting to note that even in the current work, we were able to observe the full range of types of interactions by studying combinations of the TM mutants that did express and for which mutagenesis provided evidence of relatively CFT-selective effects: TMs 4, 5, 11, and the single phenylalanine mutant in TM 7, termed F.

Combined Influences of Mutations in Polar Amino Acids in Multiple TMs. Studies of combined TM DAT mutants and comparisons of these results with data from individual TM mutants provide indirect evidence of independent effects of mutations in several TMs. When $\Delta\Delta G^{o}$ values for a combined mutant that incorporates mutations at two sites are equal to the sum of the ΔG^{o} values from DATs mutated in the two sites separately, $\Delta\Delta G^{o}_{int}$ values are near zero. Although these data do not directly identify separate locations for the two sites, they provide a relatively strong kinetic argument against substantial interactions between them. If, on the other hand, $\Delta\Delta G^{o}_{int}$ values are much different from zero, there is prima facia kinetic evidence for interactions between mutation effects. Such kinetic evidence again does not always indicate juxtaposition of the two domains studied, but it does make them candidates for such direct interactions, as well as interactions at a distance.

Interestingly, for most TM combination mutants, kinetic interactions identified in transporter recognition of dopamine were larger than those identified for CFT binding. Those observations are consistent with the idea that dynamic conformational motions could be involved in dopamine recognition processes that must serve as the first steps in complex transport process (Clackson et al., 1998). In this regard, it is interesting that the sum of all of the interactive energy for DAT dopamine recognition by these mutants was almost 4-fold higher than that for CFT (9.6 versus 2.5 kcal/mol). For mutants with synergistic $\Delta\Delta G^{o}_{int}$ values, effects on dopamine recognition were almost 10-fold higher than effects on CFT recognition (5.61 versus 0.61 kcal/mol).

Independent Effects of Combined TM Mutations. Several combination mutants, even among TM mutants that were individually able to exert large effects on dopamine or CFT affinities, displayed little evidence for interactions. Seventy percent of the double-TM mutations produced independent effects on CFT affinity, whereas 30% displayed independent effects on dopamine recognition. The degree of interaction differed from mutation combination to mutation combination, underscoring the specificity of the observations. The striking interactions found in the T4 + 5 mutant could be due to the proximity of TMs 4 and 5 mandated by the short length of the cytoplasmic loop that connects them. T4 + F and T5 + F yielded independent interactions. This failure of T4 or T5 to interact with the TM 7 F mutant could bespeak greater distances between TM 4 or 5 and 7, although mutation effects may be more likely to be independent at greater separations (Serrano et al., 1990; Schreiber and Fersht, 1995), thermodynamic estimates for interaction do not always correlate well with the distances estimated between two potentially interactive partners (Cunningham and Wells, 1993; Schreiber and Fersht, 1995).

Synergistic Effects of Combined TM Mutations. $\Delta\Delta G^{o}$ of T4 + 5 is larger than that of sum of two ΔG^{o} values for single-TM mutants. These helices appear to bind dopamine better when functionally coupled. These synergistic influences on dopamine recognition contrast with the virtually independent effects on CFT recognition from these combined mutations. TM 4 mutants are involved in each of the combined TM mutants that display synergy for dopamine recognition and in one of the two combinations that display synergy for CFT binding. Studies of combined mutants in other proteins have suggested that synergistic influences could arise from combinations of parental mutants that individually contribute different parts or steps of the same function (Uze et al., 1994); conceivably, the synergistic effects on affinity noted here could come from contributions to different parts of recognition sites for these two molecules.

Complentarity among Effects of Combined TM Mutations. Among the most informative genetic lesions are the second lesions that functionally reverse, or complement, the effects of a first mutation. Changes in free energy for complementary double mutants should be less than the sum of the effects of the two single mutants (Schreiber and Fersht, 1995). Evidence for this sort of interaction between TM 4 and 11, or TM 5 and 11, is found in the observation that $\Delta\Delta G^{o}$ values for TMs 4 + 11 or TMs 5 + 11 on dopamine recognition are smaller than that of sum of the ΔG^{o} values for the single-TM mutants assembled to make these combined mutants (Table 3). It is interesting that all of the complementary influences on apparent dopamine affinities (K_M) and all except one of the complementary effects on CFT recognition involve TM 11 (Table 4). TM 11 mutations can thus complement effects of other mutants in TM 4, 5, or 7.

Differential Effects of Combined TM Mutations on Dopamine Recognition, CFT Recognition, and Dopamine Uptake. Several of the helix combinations at which combined mutations provided complementary or synergistic influences on dopamine recognition differed from those that provided such influences on CFT recognition. Synergistic interactions of TM 4 + 11 and of F + T 11 mutants on CFT recognition contrast with the complementary effects of each of these mutant pairs on dopamine recognition. Synergistic interactions between T4 + 5 + F, T4 + F + 11, and T4 + 5 + F + 11 for dopamine recognition contrast with complementary interactions between these mutants for CFT recognition. Each of these differences underscores the basic findings from the single-TM mutation analyses: CFT and dopamine recognition not only depend on different portions of DAT but also respond differently to changes in the interactions between different DAT domains.

Differential effects on dopamine uptake V_{max} rates can also be observed. The apparent complementary changes in T4 + 5,

T4 + 5 + F, and T4 + 5 + F + 11 V_{max} rates contrast with the synergistic alterations in dopamine affinities, determined in the same uptake assays, for these combination mutants. Synergistic changes in V_{max} rates noted in T5 + 11, T5 + F + 11, and T4 + 5 + 11 each contrast with the complementary influences of these mutation combinations on dopamine affinities. Conceivably, interactions that reduce DAT affinities for its substrate during some portions of the transport processes could actually speed these processes, and vice versa.

Limitations of Analysis of Single and Multiple Mutations for $\Delta\Delta G^{o}_{int}$ Terms. Analysis of single mutations is a frequent approach to elucidating contributions of a single protein site to interactions with ligands, even though altered affinities that result from a single mutation can result when it changes not only direct contacts with the ligand but also structural features required for normal affinity (Clackson et al., 1998). DAT/ligand and DAT/substrate interactions are unlikely to directly involve all of the amino acids whose changes alter affinities. In current DAT models, only a minority of the residues at which mutations change affinities can even come close to dopamine or CFT (G.R. Uhl and Z. Lin, unpublished observations).

Such considerations apply even more forcefully in the current study. We seek interactive aspects of multiple mutations among members of a mutant series that contains some single- and combined-TM mutants whose expression is so severely disrupted that they are not even suitable candidates for further study. Nevertheless, several factors do suggest that gross structural rearrangements cannot provide the basis for each of the observations made on the normally expressing multiple TM mutants:

1) Analyses of multiple mutants have provided substantial insights into functions of other important proteins, including transporters (Sullivan et al., 1991; Himanen et al., 1996; Chen et al., 1997; Merickel et al., 1997; Lo and Silverman, 1998; Norregaard et al., 1998; Nguyen et al., 1998; Penado et al., 1998; Romero et al., 1998).

2) Alanine substitution minimally disrupts structures compared with many other mutation, chimera, and molecular probe strategies (Wells, 1991).

3) We have not studied mutant DATs whose cell surface expression is grossly abnormal, as frequently observed in mutants with presumed gross structural rearrangements

4) The sum of the $\Delta\Delta G^{o}$ values produced by the multiple TM mutants (10.67 for CFT and 12.38 kcal/mol for dopamine) does not dramatically exceed the total free energies of ligand recognition (9.77 for CFT and 11.99 kcal/mol for dopamine of ΔG^{o} in WT), as might be anticipated if most of the changes produced gross structural rearrangements.

None of these features, however, totally excludes the possibility that occult rearrangements of DAT structure could contribute to the interactive terms that we tentatively interpret in terms of normal TM/TM interactions. Although it seems unlikely that large rearrangements are compatible with the normal expression of the combinations studied here, it seems equally unlikely that our current results totally lack more subtle DAT molecular rearrangements when mutations in as many as nine amino acids (e.g., in T4 + 5 + F + 11) are combined.

Observed Interactions and Current DAT Structural and Functional Models. Edvardsen and Dahl (1994) modeled the DAT as composed of 12 TM spanning helices arranged with respect to several considerations, including the lengths of interconnecting segments and analogies with features of other proteins with multiple TMs. These workers tentatively placed TMs in 1-to-12 order in a figure-eight configuration, with a narrower waist formed by closer proximity of TMs 1 and 7. This model generally implied a pathway for dopamine mobility that was relatively perpendicular to the plane of the membrane.

Our observations document $\Delta\Delta G^{o}$ terms for apparent interactions between effects of mutations in several TMs that lie close to each other in the Edvardsen and Dahl model, especially TMs 4 and 5. These data support this model. However, the observed interactions among TMs 4, 5, 7 (which contains the F361A F mutant), and 11 are not as readily explained on the basis of the close approximation of each of these helices to a single recognition site domain for dopamine or cocaine in this model. If the model is correct in all of its features, which is seemingly unlikely, then interactions such as those among TMs 4, 5, 7, and 11 may be more readily understood in light of likely normal contributions of the polar amino acids in these TMs to recognition of different aspects of dopamine or cocaine. The interactions between these mutants could also signal the normal participation of residues in these TMs in serial steps of the multistep processes likely to be involved with dopamine and ion transport or recognition, as modeled for G protein-coupled receptors (Spain and Coscia, 1987; Moro et al., 1999; Raman et al., 1999). Alternatively, the failure of these data to readily fit the model could be due to imperfections in this initial model. It is interesting, for example, that this model differs significantly from the recently elucidated structural motif of the 12-TM prokaryotic sodium/proton exchanger, NhaA transporter, which displays a more triangular helical arrangement (Williams, 2000).

The detailed mechanisms of dopamine transport by DAT remain unknown. However, the current results can add support to speculative models for this process. In current DAT models, dopamine recognition pockets could be found in either or both of two portions of a putatively "figure eight-shaped" pocket (Edvardsen and Dahl, 1994). Each of these figure of eight halves measures 13 to 15 Å, which is larger than the 6.5-Å dopamine molecule. Conceivably, DAT helices could move toward dopamine during its initial recognition and subsequent translocation steps, provide transiently more snug fits between DAT and dopamine and facilitate the specificity of DAT/substrate interactions. Many of the mutations that alter affinities of cocaine analogs and dopamine are found in the extracellular third to half of putative DAT TMs. Conceivably, the influences of at least some of these mutations could arise from alterations in the ability of their helices to optimize affinities for dopamine or CFT by moving closer to the small molecules.

Current data and other recent observations also point to the possibility that dopamine may not translocate through a pathway perpendicular to the plane of the membrane. Many of the tryptophan, phenylalanine, and proline DAT mutations that alter dopamine uptake rates in the C-terminal DAT TMs (especially TMs 10 and 11) lie in the cytoplasmic half to third of these TMs. This distribution contrasts with the influence of residues distributed chiefly toward the extracellular half to third of the N-terminal and mid-TMs of DAT (especially TMs 1, 2, and 6–9) in altering uptake rate. Conceivably, earlier steps in dopamine translocation could involve the N-terminal portion of DAT to greater extent, and

later steps in dopamine uptake could preferentially involve more C-terminal TMs, especially TM 11. Such interactions could imply an "inward and sideways" path for dopamine that was not just perpendicular to the plane of the membrane. Dopamine could even rotate while in transit, as suggested for molecular interactions between steroids and a bacterial P-450 (Moro et al., 1999).

Implications for Development of Cocaine Antagonists. The current results provide a substantial advance in thinking about the selective features of the DAT, a key member of this neurotransmitter transporter gene family due to its central role in cocaine reward. Small molecules that recognize these transporter domains could provide selective interference with cocaine recognition by the transporter, allow it to exert its normal function in dopamine uptake, and provide cocaine resistance in a fashion that may have therapeutic benefits. The observations show here that a number of single-TM polar mutants can provide selective influences on cocaine analog affinities and suggest that a dopamine-sparing cocaine antagonist could conceivably gain potency and selectivity for blocking cocaine recognition by DAT through interactions with DAT polar domains, especially with those in TMs 4, 5, and 11, as well as with the previously reported TM7 aromatic residue.

Many more of the combined TM mutants showed relatively independent influences on cocaine analog recognition (five combinations) than on dopamine affinity (two combinations). These observations again point to the possibility that a dopamine-sparing cocaine antagonist could recognize portions of DAT while providing interactions that were sufficiently modest to display a lower likelihood of altering other important DAT functions. The advances in the knowledge of regions of the DAT contained in the current work that are selectively involved in its different functional properties, expression, cocaine analog recognition, and dopamine uptake, may thus have substantial practical implications for the development of medication to treat cocaine addiction.

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