

# Dopamine Transporter Tryptophan Mutants Highlight Candidate Dopamine- and Cocaine-Selective Domains

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## ABSTRACT

Cocaine blocks the normal role of the dopamine transporter (DAT) in terminating dopamine signaling and in restricting its spatial spread through molecular interactions that remain largely obscure. Cocaine analog structure-activity studies suggest roles for cationic and hydrophobic interactions between DAT, dopamine, cocaine, and the sodium and chloride ions whose gradients power uptake processes. Tryptophan residues lying in putative DAT transmembrane domains could contribute to both aromatic and cationic interactions between DAT and dopamine or cocaine. We thus produced mutant DATs with alanine substitutions for tryptophans lying in or near putative DAT transmembrane domains. We have focused analyses on mutations that exert selective influences on affinities for dopamine or the cocaine analog CFT [(–)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane]. Substitutions

W162A, W255A, and W310A reduced dopamine uptake affinities. 5W266A, 12W555A, and 12W561A each reduced dopamine superficial recognition affinities by more than 3-fold and all retained affinity for CFT. W406A, W496A and W523A each reduced CFT affinity, and W84A increased CFT affinity. None of these four mutations decreased dopamine uptake affinity. These data, current provisional DAT structural models, and results from parallel studies of other mutants identify candidate dopamine-selective DAT domains for transmembrane dopamine permeation and regions in which mutations selectively lower CFT affinities. Tryptophan residues may contribute more extensively to these selective domains than other previously studied DAT amino acids. These sites provide tempting targets for selective blockers of cocaine recognition by DAT.

The dopamine transporter (DAT) is an important constituent of brain pathways that contribute to movement and behavioral reward (Ranaldi et al., 1999; Redgrave et al., 1999; Robbins and Everitt, 1999). Cocaine recognition by the DAT expressed by neurons in brain reward pathways has been especially linked to cocaine euphoria, suggesting that selective blockade of cocaine recognition in this pathway could have therapeutic importance for anticocaine medications (Volkow et al., 1997; Uhl et al., 1998; Villemagne et al., 1999). However, only limited information about the ways in which dopamine and cocaine interact with DAT is currently available.

Much of the current information about the ways in which DAT interacts with dopamine and cocaine comes from small molecule structure-activity relationships. Such studies imply both cationic and aromatic interactions between DAT, dopamine, and cocaine (Carroll et al., 1992). Cocaine's phenyl is important; many substitutions on this ring produce significant losses of affinity (Lieske et al., 1998). However, these studies could underestimate the complexities of DAT/dopa-

mine interactions, because initial dopamine recognition is likely to be followed by a series of molecular events required for normal dopamine and ionic translocation by this complex protein.

Data from small molecule structure-activity relationships can be superimposed on DAT topologic models, current models of DAT function, and data from initial studies of chimeras and mutants, although caution must be applied because only one transporter has been successfully analyzed by X-ray crystallography (Fig. 1A) (Kilty et al., 1991; Shimada et al., 1991; Vandenberg et al., 1992; Uhl and Johnson, 1994; Donovan et al., 1995; Javitch, 1998; Williams, 2000). Cationic interactions between small molecules and DAT were suggested by initial mutagenesis of the TM1 aspartic acid D79, for example (Kitayama et al., 1992; see also Buck and Amara, 1994, 1995; Giros et al., 1994). Continued improvements in understanding DAT interactions with its substrates and inhibitors are likely to derive from continued efforts to analyze the effects of mutations on DAT's properties in recognizing ligands and substrates and in transporting substrates. Such information is important for several purposes, including identification of transporter amino acids selectively involved in dopamine uptake or cocaine recognition that could provide

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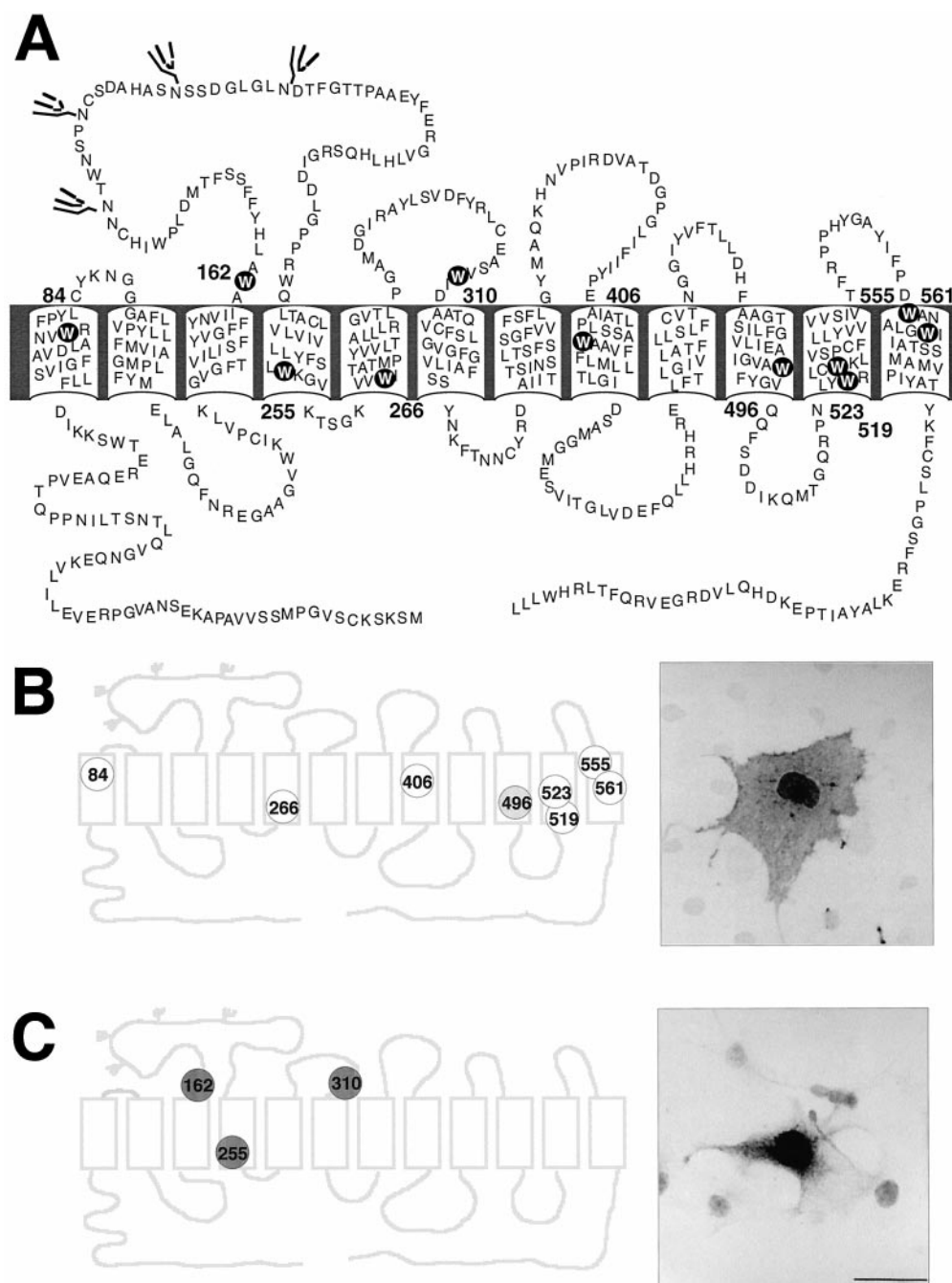
**ABBREVIATIONS:** DAT, dopamine transporter; TM, transmembrane (domain); NET, norepinephrine transporter; SERT, serotonin transporter; rDAT, rat dopamine transporter; WT, wild-type; CFT, (–)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane; ECL, extracellular loop.

targets for “dopamine sparing” cocaine antagonist compounds (Uhl et al., 1998).

The eleven DAT tryptophan (Trp or W) residues located in or near 9 of its 12 putative TM domains are attractive targets for such mutagenesis studies. Aromatic rings could contribute to ligand and substrate recognition through aromatic interactions that could include  $\pi$ - $\pi$  interactions between cocaine’s phenyl and tryptophan’s indole rings (Burley and Petsko, 1985; Baldock et al., 1996). DAT TM tryptophan residues could also contribute to cation- $\pi$  interactions important for recognition of the positive charges or polarities that can be found in virtually all DAT substrates and inhibitors. This is because tryptophans can mediate the strongest cation binding to the  $\pi$  faces of tryptophan indole rings (Dougherty, 1996). The conservation of many DAT TM tryptophans in

DATs from several species in the norepinephrine transporter (NET) and the serotonin transporter (SERT) is also consistent with these residues’ biological importance (Uhl and Johnson, 1994). Studying TM tryptophans also avoids several possible less specific effects likely with non-TM tryptophan side-chain removals. Two tryptophan residues in DAT’s putative large, second extracellular loop abut candidate sites for DAT N-linked glycosylation that are strongly conserved among the monoamine transporters.

To test the idea that interactions with tryptophans could allow DAT to recognize dopamine and cocaine, we now report characterization of mutation effects in each of these 11 tryptophans distributed in or near a DAT TM domain (Fig. 1A). We have examined mutation influences on DAT expression, cocaine analog affinity, and features of dopamine transport that include



**Fig. 1.** Distribution of the 11 tryptophan residues studied and patterns of DAT immunoreactivity in COS cells expressing wild-type and these Trp-to-Ala mutant DATs. A, Distribution of the tryptophan residues studied (dark outlined circles) in a diagram of current DAT topologic assignments (Kilty et al., 1991; Shimada et al., 1991). The 12 TM helices, extracellular loops (up) with potential N-linked glycosylation sites (forked structures in the second extracellular loop) and intracellular loops (down) are indicated. All tryptophan residues are conserved in monoamine transporters from different species except 8W406, which is phenylalanine, and 12W561, which is tyrosine, in both rat and human SERTs. B, wild-type DAT and the eight mutants listed (left side, clear circles) express DAT immunoreactivity relatively evenly in association with their plasma membranes with less than one third of immunoreactivity found in perinuclear regions (photomicrograph at right). 10W496 displayed mild disruption of DAT expression with approximately half of the protein associated perinuclear regions (light gray circle). C, three mutations significantly disrupted plasma membrane expression (left side, dark circles), leaving more than two thirds of the immunoreactivity in perinuclear regions (photomicrograph at right). Negative control COS cells expressing pcDEDAT lacked DAT immunostaining (data not shown). Scale bar, 30  $\mu$ m.

its ion dependence. Mutagenesis studies that produce altered patterns of dopamine uptake or cocaine binding need to be interpreted with important caveats, including the possibility of indirect effects through DAT structural alterations (see *Discussion*). However, the results obtained here parallel those found in studying DAT TM domain phenylalanine, proline, tyrosine, charged, and polar amino acids. We can thus compare the results of tryptophan mutagenesis with results from alanine substitution of other amino acids, seek patterns of selective mutagenesis influences that point toward selective involvement of specific DAT domains in specific DAT functions, and use these data to test features of emerging structural and functional models for DAT.

## Materials and Methods

**Preparation of Wild-Type (WT) Alanine-Substitution Mutant and Promoter-Deleted Control DNAs.** Oligonucleotides corresponding to the sequences for mutations of the 11 tryptophans, as indicated in Fig. 1A, 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/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 wild-type 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 wild-type amino acid's position number and the substituted amino acid. A prefix number represents the putative transmembrane domain in which the mutation is located. Mutation 1W84A was isolated in a *NotI*-*BglII* fragment; mutations W162A, 4W255A, 5W266A, and W310A were isolated in *BglII*-*PvuI* fragments; and mutations 8W406A, 10W496A, 11W519A, 11W523A, 12W555A and 12W561A were isolated in *PvuI*-*PstI* fragments of pBluescript/rDAT. Each mutation was confirmed by DNA sequencing.

**Subcloning into a Modified Expression Vector, pTracer-CMV/LIN-rDAT.** Mutation-bearing restriction fragments were shuttled into the rDAT-expressing mammalian plasmid pTracer-CMV/LIN-rDAT and correct sequences reconfirmed. pTracer-CMV/LIN-rDAT was derived from pTracer-CMV (Invitrogen Corporation, San Diego, CA). The pTracer-CMV *BglII* site was removed by digestion, fill-in reactions, and religation. A *PvuI* site outside the multiple cloning site was removed using site-directed mutagenesis as described above. Subcloning the 3.4-kb rat DAT cDNA fragment from the pBluescript/rDAT cDNA into the *EcoRI*-*XbaI* sites of the modified pTracer-CMV, designated pTracer-CMV/LIN, produced pTracer-CMV/LIN-rDAT that displayed single sites for shuttling of the *NotI*-*BglII*, *BglII*-*PvuI* and *PvuI*-*PstI* pBluescript/rDAT cDNA fragments carrying the DAT mutations studied here.

**Expression.** COS cells ( $10^7$ ) grown to confluence in flasks in Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD) containing 10% fetal calf serum (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  $\text{Na}_2\text{HPO}_4$  and 6 mM dextrose), recentrifuged, and resuspended at  $10^7$  cells/ml in 4°C HEBS. Suspended cells (0.9 ml) were transfected by electroporation at 300 V/1100  $\mu\text{F}$  in 400-mm gene pulser cuvettes (Bio-Rad) containing 20  $\mu\text{g}$  of plasmid DNA and 500  $\mu\text{g}$  of fish sperm DNA (Roche Diagnostics, Mannheim, Germany) using a geneZAPPER 450/2500 (IBI, New Haven, CT). Transfections employed DNA preparations with  $A_{260}/A_{280}$  ratios  $\geq 1.65$ . The transfected cells were

then suspended in Dulbecco's modified Eagle's medium, followed by distribution into six-well plates.

**Immunostaining Transfected COS Cells.** Cells transfected with pcDEDAT (Lin et al., 1999) carrying a truncated and promoterless DAT cDNA provided a negative control. Cells were grown to 80% confluence in six-well plates and cellular patterns of DAT immunoreactivity were assessed by immunohistochemistry using specific polyclonal rabbit anti-DAT sera, as described previously (Lin et al., 1999). Stained cells were washed 3 times with Tris-buffered saline, dehydrated, mounted on microscope slides, and examined for semiquantitative assessments of the patterns of DAT immunoreactivity by an observer unaware of the mutations. Expression was also monitored using assays for  $\beta$ -galactosidase expressed by a cotransfected plasmid. Transfection efficiencies varied between 8 and 12% for each DAT. Patterns of DAT immunoreactivity were defined as three different types of subcellular locations (Fig. 1, B and C).

**Functional Assays.** COS cells were grown for 3 days and then assayed for their abilities to accumulate [ $^3\text{H}$ ]dopamine (49 Ci/mmol; NEN, Boston, MA) or to bind the tritium-labeled cocaine analog [ $^3\text{H}$ ]CFT (83.5 Ci/mmol; NEN). Kinetic and saturation analyses determined  $K_M$ ,  $V_{\text{max}}$ ,  $K_D$ , and  $B_{\text{max}}$  values as described previously (Pfenning and Richelson, 1990). For uptake assays, 10 nM [ $^3\text{H}$ ]dopamine and 0.1, 1, 5, 10, 20, 30, and 50  $\mu\text{M}$  unlabeled dopamine concentrations were used. For experiments demonstrating the  $\text{Na}^+$  and  $\text{Cl}^-$ -dependence of uptake, dopamine at a concentration of the  $K_M$  value for wild-type or each mutant was prepared to contain 99.5% unlabeled and 0.5% [ $^3\text{H}$ ]dopamine in different concentrations of  $\text{Na}^+$  or  $\text{Cl}^-$  in which lithium substituted for  $\text{Na}^+$  and acetate for  $\text{Cl}^-$  to maintain osmolarity. Fifty micromolar pargyline and 1  $\mu\text{M}$  RO 41-0960 (catechol-*O*-methyl transferase inhibitor; RBI, Natick, MA) were included in assay buffers. For initial binding assays, 2 nM [ $^3\text{H}$ ]CFT was adjusted to 3.5, 5, 7, 17, 32, and 62 nM concentrations using unlabeled CFT. COS cells transfected with plasmids carrying pTracer-CMV/LIN-rDAT or pcDEDAT served as positive and negative controls, respectively. Parallel incubations with 50  $\mu\text{M}$  unlabeled cocaine allowed estimation of nonspecific uptake and binding. Uptake assays were carried out for 5 min at 37°C, followed by 2 washes each with 2 ml of Krebs-Ringer-Henseleit buffer containing 50  $\mu\text{M}$  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 LS 6000 liquid scintillation counter (Beckman Instruments, Columbia, MD) at approximately 50% efficiency. Cells in parallel wells were solubilized in 0.5 ml of 1 N NaOH for protein amount measurements using a Bio-Rad Protein Assay solution. Studies of dopamine inhibition of 6 nM [ $^3\text{H}$ ]CFT binding used several concentrations of unlabeled dopamine in 50  $\mu\text{M}$  ascorbic acid.

**Analyses and Definitions.**  $K_M$  and  $V_{\text{max}}$  values for [ $^3\text{H}$ ]dopamine uptake,  $K_D$  and  $B_{\text{max}}$  values for [ $^3\text{H}$ ]CFT binding activities,  $\text{IC}_{50}$  values, curve fit to data using sigmoidal curve models for binding competition data, calculation of data fitting, and *t* tests or ANOVA analysis followed by Tukey's multiple comparison tests were all carried out using Prism (ver. 3; GraphPad Software, Inc., San Diego, CA).  $K_i$  values were calculated as described previously (Cheng and Prusoff, 1973). Transporter modeling was based on coordinates kindly provided by Dr. Edvardsen (Edvardsen and Dahl, 1994), using Sybyl 6.4 programs (Tripos, Inc., St. Louis, MO).

We arbitrarily defined two criteria for possible biological significance of mutation effects. To meet the first criterion, the mutants needed to display  $K_M$ ,  $V_{\text{max}}$ ,  $K_D$ , or  $B_{\text{max}}$  values more than 3-fold different from wild-type values. The second criterion required that the *t* test statistical comparisons be at  $P < .05$ . We list results from mutants that did not meet the first criterion but achieved statistical significance separately in Fig. 2. Transporter turnover rate reflected the number of dopamine molecules transported per second per site, defined as [ $V_{\text{max}}$  for dopamine uptake (fmol/ $\mu\text{g}/\text{min}$ )] / [ $B_{\text{max}}$  for CFT binding (fmol/ $\mu\text{g}/\text{min}$ )]. Ratios, termed  $K_D/K_M$  and  $K_D/K_i$ , between



mutation influences on cocaine and dopamine recognition were calculated as:  $[K_D \text{ for CFT binding mutant} / K_D \text{ WT}] / [K_M \text{ for dopamine uptake mutant} / K_M \text{ WT}]$ .

## Results

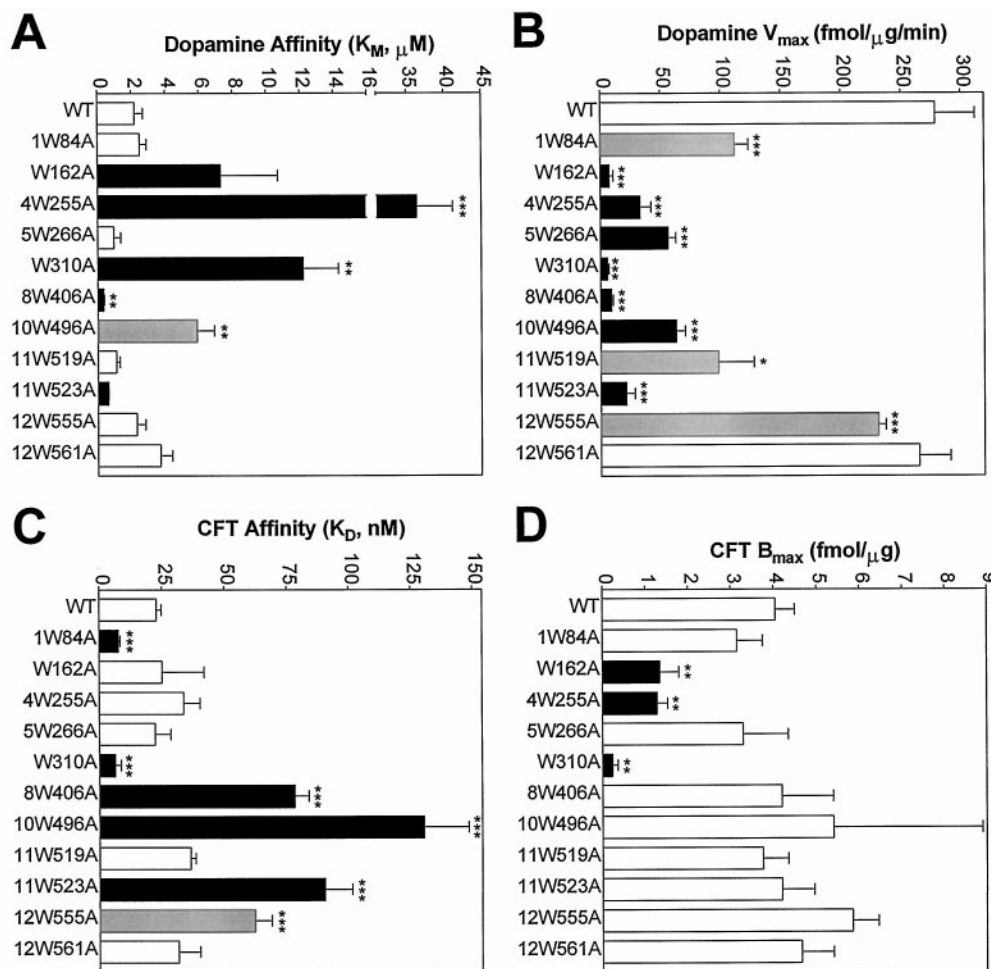
### Most Alanine Substitutions Allow Wild-Type Expression Patterns

**Immunohistochemical Determinations of DAT immunoreactivity.** COS cells expressing wild-type DAT displayed a pattern of DAT immunoreactivity characterized by relatively dense plasma membrane immunostaining and modest immunoreactivity associated with nuclear or perinuclear regions. This pattern was not found in mock transfected COS cells or in COS cells transfected with the promoter-deleted pcDEDAT construct (data not shown). Seven of the

11 Trp-to-Ala mutants displayed patterns of DAT immunostaining similar to those of wild-type DAT (Fig. 1B), including substitutions in putative TMs 1, 5, 8, 10, 11, and 12.

Four mutations did alter this pattern of expression. Cells expressing 10W496A displayed about half of their DAT immunoreactivity associated with plasma membranes and half in perinuclear regions. For the mutants W162A, 4W255A, and W310A, more than two thirds of the DAT immunoreactivity seemed to be perinuclear, whereas less than one third was distributed, often unevenly, in the plasma membranes (Fig. 1C). W310A displayed only small amounts of DAT plasma membrane immunostaining, about 10% of wild-type levels.

**$B_{\max}$  Values from  $[^3\text{H}]\text{CFT}$  Radioligand Binding.** The data from studies of DAT immunoreactivity were supported



**Fig. 2.** Pharmacological characterization of COS cells expressing wild-type DAT and the 11 alanine substitution mutants for wild-type tryptophans in putative TM domains and amino acid numbers shown. Data are means  $\pm$  S.E.M. from 3 to 14 separate determinations (see Table 1 for details). ■,  $> 3$ -fold from wild-type value; □, no significant change from wild-type; ▨, statistically significant, but less than 3-fold change. Levels of significance by  $t$  test indicated next to bars: \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ ; results of 2-way ANOVAs listed below. A, dopamine uptake affinity, derived from Eadie-Hoosier analyses of uptake experiments performed with eight concentrations of  $[^3\text{H}]\text{dopamine}$ . ANOVA:  $P < .01$  for W310A,  $P < .001$  for 4W255A, and  $P > .05$  for other mutants compared with WT. B, dopamine uptake  $V_{\max}$  derived from Eadie-Hoosier analyses of uptake experiments performed with eight concentrations of  $[^3\text{H}]\text{dopamine}$ . ANOVA:  $P \geq .05$  for 12W555A and 12W561A but  $P < .001$  for all other mutants compared with WT. C, CFT binding affinity, derived from Scatchard analyses of binding assays using seven concentrations of  $[^3\text{H}]\text{CFT}$ . ANOVA significance:  $P < .001$  for 8W406A, 10W496A and 11W523A,  $P \geq .05$  for all other mutants compared with WT. D, CFT binding  $B_{\max}$  derived from Scatchard analyses of binding assays using seven concentrations of  $[^3\text{H}]\text{CFT}$ . No significant differences were measured by ANOVA.  $[^3\text{H}]\text{dopamine}$  uptake and  $[^3\text{H}]\text{CFT}$  binding assays and determinations of  $K_M$ ,  $V_{\max}$ ,  $K_D$ , and  $B_{\max}$  values are described under *Materials and Methods*. Data for negative controls are not shown; both  $V_{\max}$  and  $B_{\max}$  values were  $< 1$  for these controls. Functional alterations observed in mutants in which single indole tryptophan side-chains are removed could arise from the direct consequences of this side-chain's replacement 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 impact affinities for ligand or substrate (Rudnick, 1997).

by expression data derived from Scatchard analyses of [ $^3\text{H}$ ]CFT radioligand binding to whole-cell preparations. [ $^3\text{H}$ ]CFT  $B_{\text{max}}$  values for each of the mutants ranged between 0.2 and 5.9 fmol/ $\mu\text{g}$ , comparable with the 4.1 fmol/ $\mu\text{g}$  values observed for the wild-type transporter (Fig. 2D). Reductions of  $B_{\text{max}}$  values to roughly a third of wild-type levels were observed for mutants W162A and 4W255A; reduction to 6% of wild-type levels were observed for W310A. These pharmacological data were consistent with immunohistochemical analyses that suggested reduced plasma membrane expression levels (Fig. 1C). Despite the disruption of plasma membrane expression noted in immunohistochemical experiments, mutant 10W496A did not display any reduction in apparent  $B_{\text{max}}$ . When the results of repeated saturation radioligand binding studies were subject to Scatchard analyses, the standard error was 67% of mean  $B_{\text{max}}$  values for 10W496A. Thus the immunohistochemical data was a better indication of 10W496A expression than the  $B_{\text{max}}$  values. Although this feature suggests caution in interpreting these modest differences as true discrepancies, differences in expression levels from wild-type DAT were documented for the  $B_{\text{max}}$  values for W162A, 4W255A, and W310A ( $P < .01$  by  $t$  test for each, see Fig. 2D).

**Selected Alanine Substitutions Alter [ $^3\text{H}$ ]Dopamine Uptake Properties.** Six mutants retained normal or near-normal dopamine uptake affinities, as manifested by  $K_{\text{M}}$  values near those of the wild-type transporter (Fig. 2A). However, five mutations significantly altered DAT's affinity for dopamine. Mutations 8W406A and 11W523A significantly increased dopamine uptake affinity, decreasing  $K_{\text{M}}$  values by 6.1- and 3.5-fold, respectively. Mutations W162, 4W255A, and W310A decreased dopamine uptake affinities by 3.3-, 16.5-, and 5.5-fold, respectively.

Four of the mutants displayed normal dopamine uptake rates, as evidenced by  $V_{\text{max}}$  values within 3-fold of wild-type levels (Fig. 2B). By contrast, the other seven mutants displayed dopamine uptake  $V_{\text{max}}$  values reduced to levels less than one third of wild-type levels. These mutants included W162A, 4W255A, 5W266A, W310A, 8W406A, 10W496A, and 11W523A.

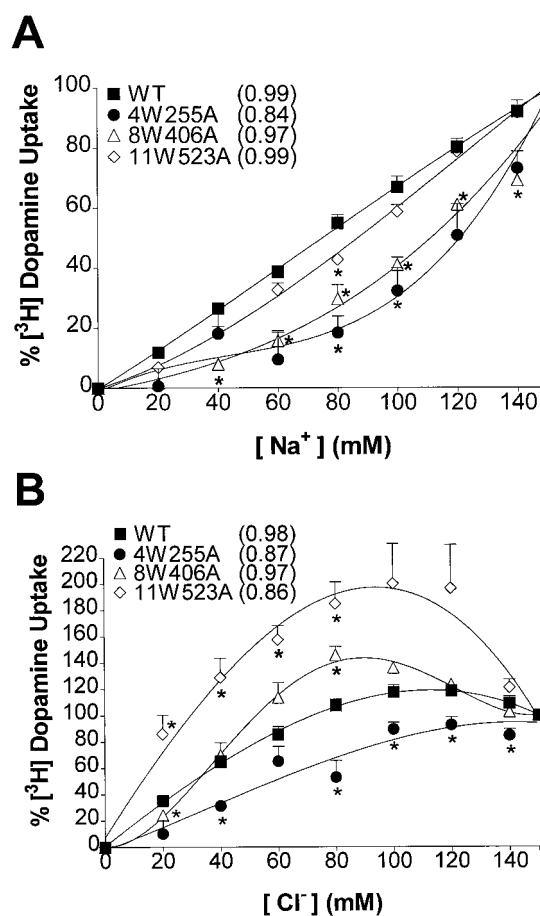
Low plasma membrane expression seemed to make the major contribution to the  $V_{\text{max}}$  reductions noted for W162A, 4W255A, and W310A. 5W266A, 8W406A, and 11W523A each displayed normal plasma membrane immunostaining but displayed  $V_{\text{max}}$  values ranging from 9.2 to 56.6 fmol/ $\mu\text{g}/\text{min}$ , small fractions of the 278.9 fmol/ $\mu\text{g}/\text{min}$  displayed by cells expressing the wild-type DAT. Alanine substitution for 8W406 increased dopamine uptake affinity, and provided normal patterns of plasma membrane immunostaining, but reduced  $V_{\text{max}}$  values by greatest extent (Fig. 2B).

**Ion Dependence of Dopamine Uptake in Selected Mutants.**  $\text{Na}^+$  and  $\text{Cl}^-$ -dependence of dopamine uptake was examined in mutants 4W255A, 8W406A, and 11W523A because these mutants displayed the largest changes in dopamine uptake affinity (4W255A) or  $V_{\text{max}}$  values (8W406A and 11W523A) (Fig. 2, A and B). It was noticed that the  $\text{Na}^+$ -dependence curve did not show any saturation with the experimental conditions used in this study. Repeated experiments, performed for this study as well as other studies in this laboratory (Lin et al., 2000), did accumulate good confidence about the  $\text{Na}^+$ -dependence data. The presence of 50  $\mu\text{M}$  cocaine was not able to decrease [ $^3\text{H}$ ]dopamine uptake

when  $\text{Li}^+$  was substituted for all of the  $\text{Na}^+$  ion in the uptake assay buffer (data not shown). Studies of 4W255A, 8W406A, and 11W523A reveal only modest effects of 11W523A on the  $\text{Cl}^-$  concentrations necessary for uptake (Fig. 3). However, mutation 8W406A increased the concentration of  $\text{Na}^+$  required to achieve the same percentage of dopamine uptake as the wild-type DAT. Mutation 4W255A increased the concentrations of both  $\text{Na}^+$  and  $\text{Cl}^-$  that were required to achieve the same percentage of dopamine uptake as the wild-type.

### [ $^3\text{H}$ ]CFT Binding

**Properties of [ $^3\text{H}$ ]CFT Binding to Expressing COS Cells.** [ $^3\text{H}$ ]CFT binding to COS cells expressing wild-type DAT and cells expressing the negative control plasmid pcDE-DAT carrying the promoterless DAT cDNA was evaluated at both 4°C and 37°C in initial studies. For the wild-type DAT, the observed average  $K_{\text{D}}$  value was  $25.0 \pm 1.4$  nM (mean  $\pm$  S.E.M.) and the  $B_{\text{max}}$  value was  $4.2 \pm 0.1$  fmol/ $\mu\text{g}$  at 4°C. At 37°C,  $K_{\text{D}}$  values were  $14.3 \pm 3.1$  nM and  $B_{\text{max}}$  values  $4.7 \pm$



**Fig. 3.**  $\text{Na}^+$ -dependence (A) and  $\text{Cl}^-$ -dependence (B) of uptake of [ $^3\text{H}$ ]dopamine at a concentration of the corresponding  $K_{\text{M}}$  value by wild-type and three mutated DATs. Curves were fit to experimental data and  $R^2$  goodness of fit values calculated (parentheses) using GraphPad Prism. Data represent mean  $\pm$  S.E.M. values from five independent experiments for 4W255A in A or three independent experiments for the others in both A and B, each experiment in duplicate; \*  $P < .05$  and \*,  $P < .01$  by  $t$  test comparisons between individual mutants and wild-type at the same ion concentrations. ANOVA:  $P < .05$  for 8W406A at 80 mM;  $P < .01$  for 4W255A at both 80 and 100 mM in A.  $P < .05$  for 4W255A at 80 mM; 11W523A at 20, 100, and 120 mM;  $P < .01$  for 4W255A at 140 mM; 11W523A at 40, 60, and 80 mM in B; otherwise  $P > .05$  compared with WT.

*t* tests: \*,  $P < .05$ ; \*\*,  $P < .01$ ; \*\*\*,  $P < .001$ ; otherwise  $P > .05$  compared with WT. One-way ANOVA:  $P < .05$  for W266A;  $P < .01$  for 12W561A compared with WT.



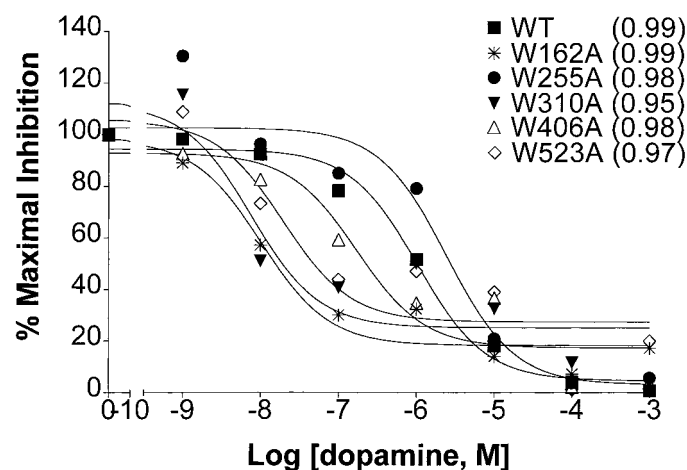
respectively (Table 1). Increasing values for these ratios suggest more negative impacts of the mutation on cocaine recognition than on dopamine recognition or features of the translocation process that determine its  $K_M$ . The  $K_D/K_M$  and  $K_D/K_i$  ratios are altered in at least generally parallel fashions in eight of the mutants studied (Figs. 2 and 5; Table 1). They were decreased in parallel in studies of 1W84A, 4W255A, 8W406A, 11W519A, 11W523A, and 12W561A. Values for these two indices were within 10-fold for 5W266A and 12W555A. However, differences that are much more striking were noted for W162A (366-fold) and W310A (1292-fold). Each of these large discrepancies seems to be caused by the markedly greater increases in dopamine's ability to compete

for CFT binding, and each is accompanied by the largest reductions in  $V_{max}$  values noted in these mutants (Fig. 2; Table 1).

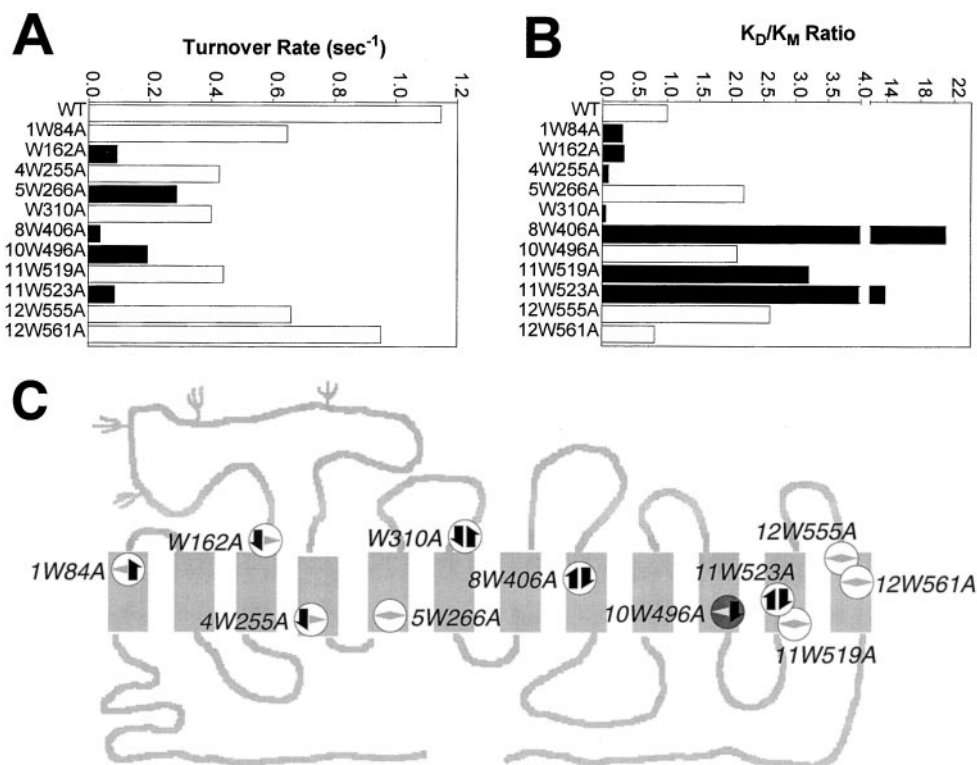
## Discussion

Alanine substitutions for DAT tryptophans reveal evidence for contributions of DAT tryptophan sidechains to recognition of cocaine, recognition of dopamine, translocation of dopamine, and DAT's ability to attain configurations necessary for its physiological and pharmacological functions. We focus on evidence for the selective contributions, positive and negative, that some of these tryptophans seem to make to cocaine or to dopamine recognition. Identification of selective contributions of tryptophans allows interesting comparisons with data that have identified similarly selective contributions of other DAT TM aromatic, polar, or charged residues. Several features of these data also accord with several of the current working hypotheses concerning DAT structure/function relationships. Each of these comparisons, however, also needs to be made in light of the possibility that some mutation effects on these functions are likely to be indirect and to reflect larger alterations in DAT conformation.

**DAT Tryptophans and Hydrophobic Features of Dopamine and Cocaine Recognition.** Tryptophan mutagenesis results add to data about structure-activity relationships of cocaine and dopamine congeners and support DAT models that postulate hydrophobic interactions between dopamine, cocaine, and DAT. Such hydrophobic interactions are supported by losses of much cocaine and dopamine potency after removal of their respective phenyl or catechol rings (Horn, 1973; Horn, 1978; Ritz et al., 1990). Several of the tryptophans mutated in the current study could contribute to the presumably overlapping cocaine and dopamine recognition sites on DAT. Interestingly, however, none seems to contrib-



**Fig. 4.** Dopamine potency in inhibiting binding of 6 nM [ $^3$ H]CFT to wild-type DAT and five alanine substitution mutants. Data are normalized average values from 5 to 14 independent experiments,  $R^2$  goodness of fit values, calculated as described in the legend to Fig. 3, are in parentheses.



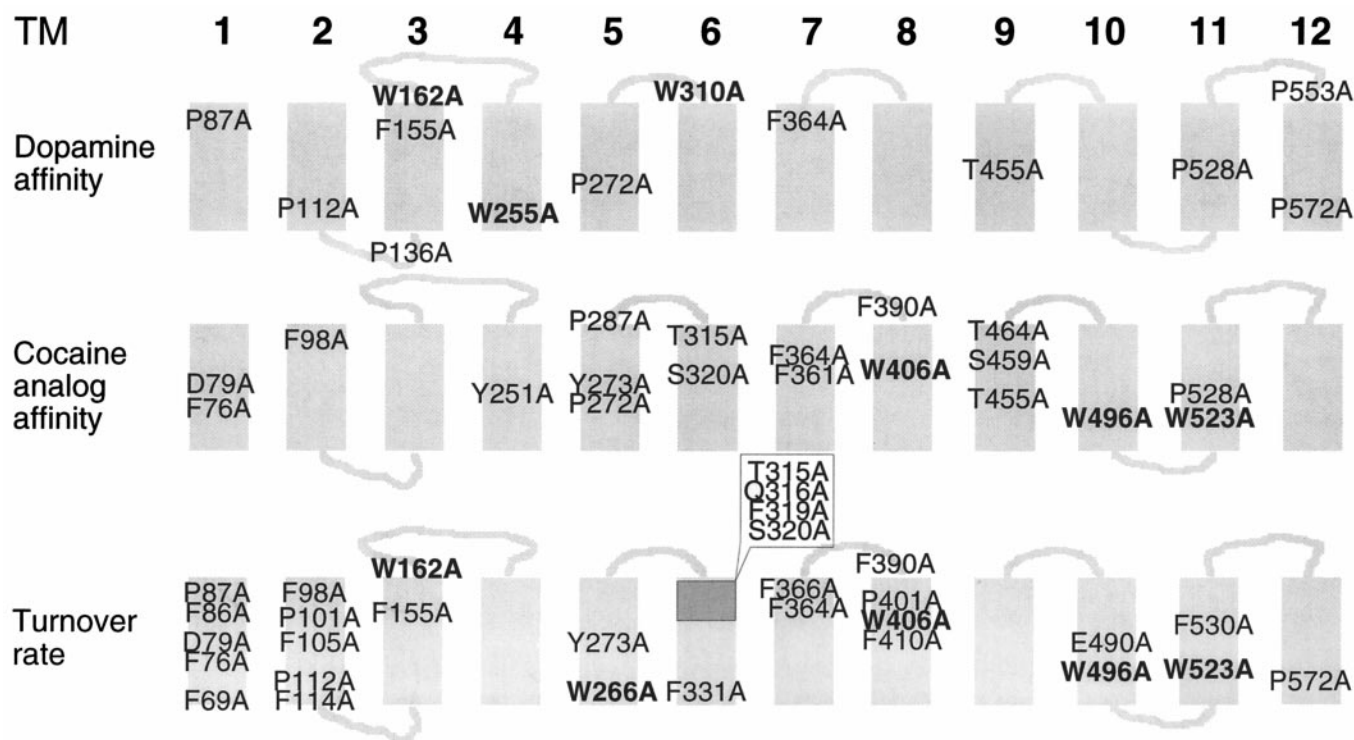
**Fig. 5.** Changes in dopamine uptake turnover rates (A) and in the ratio of CFT to dopamine uptake affinities (B) in the 11 alanine substitution mutants. ■, > 3-fold change from wild-type values; □, < 3-fold change. C, diagram of the location of tryptophan residues for which alanine substitution alters dopamine uptake affinities (left arrow) and CFT affinities (right arrow). Increases are indicated by upward arrows, decreases by downward arrows, and no change by gray double-headed arrows.

ute equally to recognition of each of these ligands (Fig. 5C). There are also substantial gains in dopamine's affinity in CFT binding competition assays after several mutations. Such data might even indicate that tryptophan sidechains could block dopamine interactions with DAT that could otherwise confer higher affinity interactions.

**Mutations Reducing Cocaine or Dopamine Affinities.** Tryptophan residues that are likely to contribute to the recognition of either cocaine or dopamine are found in several sites in DAT. Increases in CFT  $K_D$  values of more than 3-fold are found in 8W406A, 10W496A, and 11W523A. The large effects of mutation 10W496 on cocaine analog recognition seem to be relatively selective. They highlight roles for this residue, conserved among the other members of the cocaine-recognizing monoamine transporter subfamily, and for TM

10, not previously identified in studies of transporter chimeras or other mutants as providing such selective contributions (Fig. 6B, Giros et al., 1994; Buck and Amara, 1994, 1995; Lin et al., 1999). It is interesting that each of these amino acids lies in inner aspects of their respective DAT TM domains, in current DAT models, and reduces dopamine transport  $V_{max}$  values. Mutation-induced subtle alterations in DAT structure would most readily explain each of these observations.

Mutants W162A, 4W255A, and W310A reduce dopamine  $K_M$  measures of affinity assessed in uptake assays. Substitutions for the TM3 phenylalanine 3F155 located two helical turns below W162 also selectively reduce dopamine uptake affinity (Lin et al., 1999). W162 and W310 are located just outside the extracellular borders of ECLs 2 and 3, respec-



**Fig. 6.** Comparisons of localizations of tryptophan (W) residues at which alanine substitutions produce significant changes in DAT functions with the localizations of phenylalanine (F), proline (P), tyrosine (Y), threonine (T), serine (S), aspartic acid (D), glutamic acid (E), and glutamine (Q) residues at which previously described mutations produce changes assessed in similar fashion (see references below). Here are shown the residues of which mutations produce changes  $\geq 3$ -fold of the wild-type values; tryptophans are in boldface. The provisional nature of the DAT models used here is emphasized in the text. A, Top, localizations of amino acids at which alanine substitutions alter dopamine uptake affinity, as assessed by  $K_M$  values determined using [ $^3$ H]dopamine uptake assays; middle, localizations of amino acids at which alanine substitutions alter CFT affinity, as assessed by  $K_D$  values determined using [ $^3$ H]CFT binding assays; bottom, localizations of amino acids at which alanine substitutions alter dopamine turnover, as assessed by the ratio of the  $V_{max}$  values determined using [ $^3$ H]dopamine uptake assays to the  $B_{max}$  values determined using [ $^3$ H]CFT binding assays. B, space-filling models of the location of the side-chains of residues for which alanine substitutions selectively reduced dopamine uptake affinity by more than 3-fold (left side) or selectively reduced CFT affinity by more than 3-fold (right side) in a current energy-minimized model of DAT protein (Edwards and Dahl, 1994). Scale bar, 10 Å. Top, view from extracellular side, looking down the putative central DAT pore. Bottom, side view, with DAT TM domains indicated as largely  $\alpha$ -helical structures. Tryptophan (red), phenylalanine (orange), tyrosine (dark orange), proline (light blue), serine (violet), threonine (magenta), and aspartic acid (green) are indicated. Mutations displaying normal dopamine uptake affinity in uptake assays included: 1F76A, 1F86A, 1W84A, 2F98A, 2F105A, 2F114A, 3F150A, 3F143A, 3F154A, P234A, P235A, 4F252A, 5W266A, 6F319A, 6F325A, 7F357A, 7F361A, 7F366A, F390A, P394A, 8P401A, 8F410A, 8F411A, 9F447A, 9F456A, 9F461A, 10F477A, 10F485A, 10W496A, 10F497A, P515A, 11W519A, 11F523A, F542A, P544A, P545A, 12W555A, and 12W561A. Affinity increased in 1F69A, 2P101A, 6F331A, **8W406A**, and 11F530A. Normal CFT affinity was found in: 1F69A, 1F86A, 2P101A, 2P105A, 2P112A, 2F114A, P136A, 3F143A, 3F150A, 3F154A, 3F155A, W162A, P234A, P235A, 4F252A, 4W255A, 5W266A, 6F319A, 6F325A, 6F331A, 7F357A, 7F366A, P394A, 8P401A, 8F410A, 8F411A, 9F447A, 9F456A, 9F461A, F477A, 10F485A, 10F497A, P515A, 11W519A, 11F530A, F542A, P544A, P545A, P553A, 12W555A, 12W561A, and 12P572A. Affinity for CFT increased in **1W84A** and **W310A**. Normal turnover rates were found in 1W84A, P136A, 3F143A, 3F150A, 3F154A, P234A, P235A, 4F252A, 4W255A, 5P272A, P287A, W310A, 6F325A, 7F357A, 7F361A, P394A, 8F411A, 9F447A, 9F456A, F477A, 10F485A, 10F497A, P515A, 11W519A, 11P528A, F542A, P544A, P545A, P553A, 12W555A, and 12W561A, and turnover rate increased in 9F461A. Mutations that disrupted plasma membrane expression included 1F86A, 1P87A, 2F98A, 2P112A, P136A in intracellular loop (ECL) 1, W162 in ECL 2, W310A in ECL 3, 7F364A, F390A in ECL 4, P553A in ECL 6, and 12P572, whereas mild disruption was noted for P287A in ECL 3, 7F357A, 7F366A, 8P401A, P515A in intracellular loop 5, and 11P528A. Data describing polar mutants (threonine, serine, tyrosine, glutamine, and glutamic acid) are derived from Itokawa et al. (2000); data for phenylalanine (Lin et al., 1999), proline (Lin et al., 2000), and aspartic acid (Kitayama et al., 1992) mutants have been described previously.



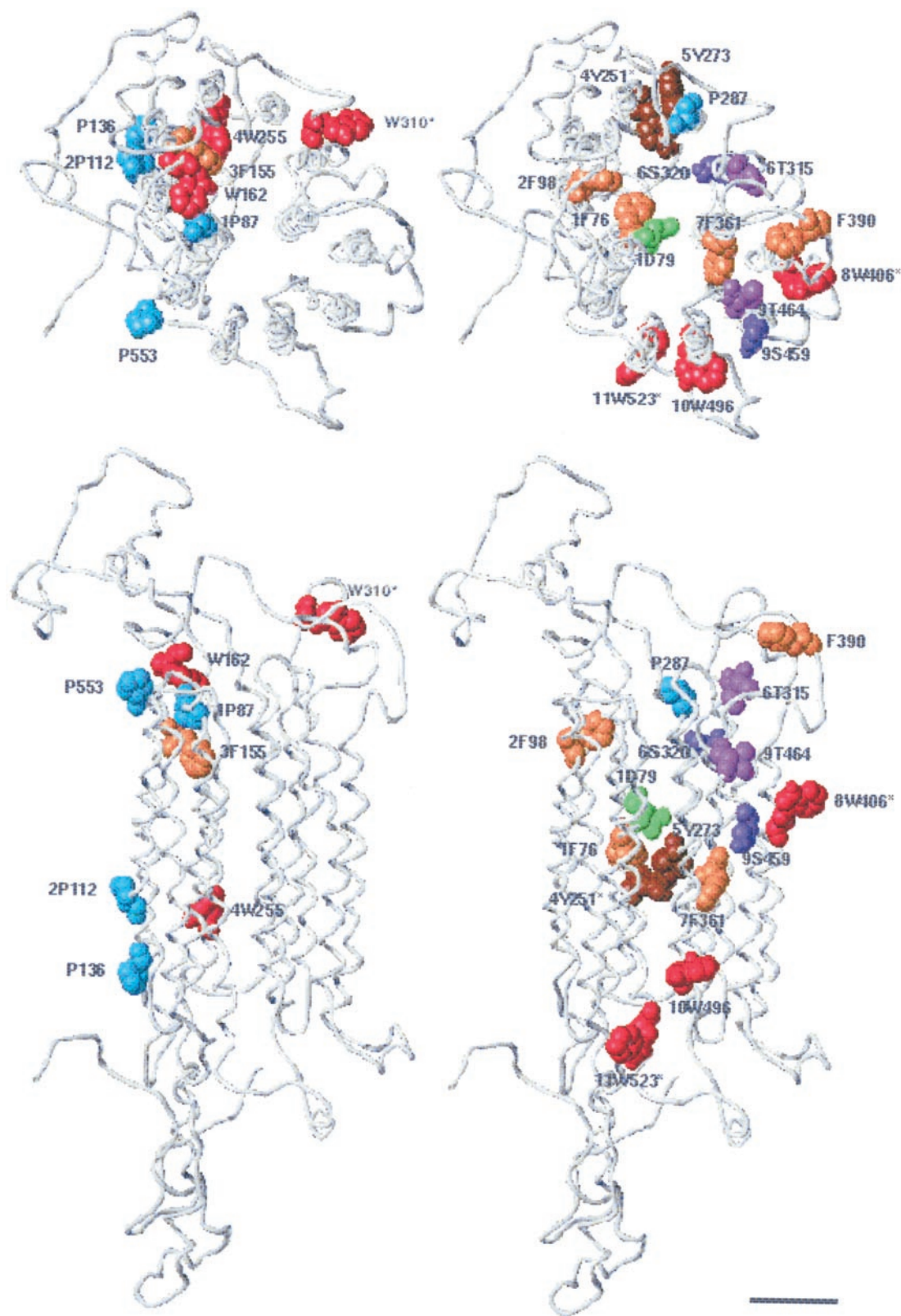


Fig. 6b.

tively. Conceivably, they could even contribute to aspects of the same recognition site important for transport, although the great length of the DAT ECL 2 reduces certainty about its location relative to ECL3. 4W255 is located one helical turn

below the middle of TM 4 in current models. Because the 4W255A substitution mutation alters several DAT properties, including expression and ion dependence, this residue could thus conceivably play an important role in DAT config-

urations that could influence dopamine uptake affinity more indirectly.

These influences on  $K_D$  values display differences from influences on dopamine  $K_i$  values in competition for [ $^3\text{H}$ ]CFT binding. 4W255 effects on  $K_M$  values (3-fold decrease) parallel the 2.2-fold decrease in  $K_i$  values, suggesting similar mutational influences on sites important for dopamine recognition and those important for dopamine translocation. The substantially larger  $K_M$  values in the face of smaller  $K_i$  values noted for the W162A and W310A mutants, conversely, clearly reveal these mutational effects on the DAT aspects important for dopamine recognition differ sharply from those important for dopamine translocation. The superficial location of these residues makes it tempting to ascribe these differences to greater participation of superficial DAT aspects in dopamine recognition by DAT sites that do not determine its  $K_M$  values. Conceivably, these sites could even be partially or totally different from those involved in dopamine translocation.

**Mutations Selectively Increasing Cocaine Analog or Dopamine Affinities.** Several mutations selectively increase CFT affinities. 1W84A selectively increases CFT affinity by 3-fold without influencing dopamine uptake affinity. W310A increases cocaine analog binding affinity by 3-fold, although it decreases dopamine uptake affinity by 5-fold. 1W84A and W310A are the only DAT mutants that display such heightened affinities for cocaine analogs, to our knowledge (Lin et al., 1999, 2000). The bulky indole ring sidechains of 1W84 and W310 could possibly provide steric hindrance and limit the affinity of CFT for DAT. Phenylalanine mutations in TMs 1 (1F76A) and 7 (7F361A) selectively influence CFT affinities (Lin et al., 1999). Interestingly, 1W84 is located two helical turns above 1F76A, whereas W310 is situated two turns above 7F361A in current models. 1W84A and W310 could thus contribute to a cocaine recognition pocket, although the disruption of DAT expression found in W310A (Fig. 1) suggests that this residue could also play an important role in DAT configuration.

Two mutations, 8W406A and 11W523A, increased dopamine uptake affinity  $K_M$  values by 3- and 6-fold, respectively. Interestingly, each decreased cocaine analog affinity (3-fold and 4-fold, respectively; Fig. 2). None of the mutants substituting alanines for 29 DAT phenylalanines or 16 DAT prolines had such effects (Lin et al., 1999, 2000). It is possible that bulky tryptophan sidechains present in the wild-type DAT prevent the high-affinity dopamine binding that can be observed in these mutant DATs. A phenylalanine is found in the position of 8W406 in SERT. 11W523 is conserved in each transporter studied. 8W406 is located two helical turns below 7F364 in the neighboring TM 7, a residue at which mutation (7F364A) decreased both dopamine uptake and cocaine analog binding affinities. 11W523 is located two helical turns below 11F530, at which alanine substitution selectively increased dopamine uptake affinity (Lin et al., 1999).

The enhanced dopamine uptake affinity or reduced  $K_M$  value found in 8W406 is accompanied by a 5-fold reduction in  $K_i$  value for this mutant. Conceivably, this mid-TM tryptophan could make similar contributions to dopamine recognition and translocation. Three mutations reveal substantial dissociations between effects on  $K_M$  and  $K_i$  values, however. The reduced  $K_M$  value for 11W523A is accompanied by a  $K_i$  value not different from wild-type. Even more striking are

the marked reductions in  $K_i$  values noted for W162A and W310A. These  $K_i$  values are 100-fold or greater than those of the wild-type DAT, despite opposite increases in  $K_M$  values. The superficial or nearly superficial residues are again sites for large differences between mutation effects on measures of dopamine uptake affinity and dopamine translocation.

**DAT Tryptophans and Cationic Interactions with Dopamine and Cocaine.** Aromatic ring interactions with cations could also contribute to ligand affinities (Dougherty, 1996). Cocaine recognition could conceivably require cationic interactions with DAT indole ring  $\pi$  electron clouds. It could even involve interactions between cocaine's cationic tropane nitrogen and a tryptophan side-chain. The best candidate tryptophans are those that are conserved as aromatic residues in each of the monoamine transporters that serve as cocaine recognition sites and those at whose positions alanine substitutions lower cocaine affinities. 8W406, 10W496, and 11W523 each fulfills these criteria and candidate contributors to cationic DAT cocaine interactions.

Aromatic-cation interactions could also contribute to dopamine uptake affinity. Because molecular features surrounding dopamine's amine nitrogen are similar to those of norepinephrine's but different from those that surround serotonin's indole nitrogen, residues conserved in DAT and NET, but not necessarily in SERT, become plausible candidates for interactions with catecholamines. W162 and W310 may be among the best candidates for interactions with cationic dopamine features, because they meet this criterion and are also disposed toward the extracellular half of DAT that is likely to contribute most to dopamine uptake affinity.

**DAT Tryptophans and Proper DAT Assembly and Structure.** Expression studies indicate that removal of even the single amino acid side-chain of W162, 4W255, W310, or 10W496 is sufficient to disrupt appropriate plasma membrane expression in COS cells, although abundant immunoreactivity is expressed by each. These data are in accord with data from DAT/NET transporter chimeras, for which chimeras at most of the junctional sites selected to date do not express properly (Giros et al., 1994; Buck and Amara, 1995). Other DAT TM sequence variants that disrupt expression, 1F86A, 1P87A, 2F98A, 2P112A, P136A, 7F357A, 7F364A, F390A, 8P401A, 8F411A, 11P528A, and P515A (Lin et al., 1999, 2000) implicate residues in TM domains 1, 2, 4, 7, 8, 10 and 11 as critical for correct DAT assembly. Mutations in extracellular domains 2, 3, 4, and 6 also disrupt DAT expression (Wang et al., 1995; Lin et al., 1999, 2000).

**Dopamine Transport.** Dopamine transport has been modeled as involving a number of discrete steps likely to involve many different domains of the DAT protein. These could include sodium, dopamine, and chloride recognition by an "outward facing" transporter state, dopamine and ion translocation, intracellular unloading of dopamine and ions, and return of the unloaded carrier to its extracellular-facing state (Povlock and Schenk, 1997). DAT's turnover rate for dopamine could be influenced by mutations that altered any of these processes. Interestingly, dopamine turnover was altered by a higher fraction of the normally expressed DAT tryptophan mutants, 43%, than the fraction at which mutations alter affinities for either dopamine or CFT. Proline, phenylalanine, and aspartic acid mutant series also displayed prominent alterations in turnover number: 41% of phenylalanine substitutions display turnover number

changes, although the maximal magnitude of the change is larger for the phenylalanine mutants (Kitayama et al., 1992; Lin et al., 1999, 2000). Because the tryptophan mutations that most prominently alter turnover number, 5W266, 8W406, and 11W523, can be modeled as located in mid- to cytoplasmic-side TM domains, these residues could conceivably help form a pocket that orients dopamine for its translocation (Fig. 6A).

**Cocaine and Dopamine Recognition Pockets: Current Models.** In the absence of crystallographic or other direct structural determinations, current DAT models (Fig. 6) are based on analyses of DAT sequence (Kilty et al., 1991; Shimada et al., 1991), initial mutagenesis results, energy minimization calculations, and inferences from studies of other proteins (Edwardsen and Dahl, 1994). These models are thus entirely provisional, yet help to provide a tentative three dimensional focus for the discussion of mutagenesis results.

Modeling the positions at which mutations yield selective effects on dopamine uptake affinities after substituting for tryptophans or for other residues suggests that the side-chains of these residues (tryptophans 162, 255, and 310; phenylalanine 155; and prolines 87, 112, 136, and 553) are largely oriented toward a central DAT recognition pocket and, in all cases except W310A, oriented toward a possible wall of a potential dopamine translocation corridor that could extend along TMs 1 to 4 (Fig. 6B, left). Such contributions of TM 3 and 4 residues to dopamine uptake affinities were not identified in studies of chimeric transporters (Buck and Amara, 1994, 1995; Giros et al., 1994).

Cocaine analog affinities are selectively altered by removal of the sidechains of tryptophans 406, 496, and 523; phenylalanines 76, 98, 361, and 390; proline 287; threonines 315 and 464; serines 320 and 459; tyrosines 251 and 273; and aspartic acid 79. These residues are currently modeled as lying in several DAT regions (Fig. 6B, right). Dopamine's small size ( $\sim 3 \times 8 \text{ \AA}$ ) relative to cocaine ( $\sim 6 \times 12 \text{ \AA}$ ) makes it plausible that dopamine-specific DAT components could well be smaller than cocaine-selective recognition features.

**DAT Structure/Function Relationships in Cocaine, Ion and Dopamine Recognition, and Translocation.** Several features of the current data provide surprisingly good support to the structure/function relationship hypotheses. Mutations in DAT TM tryptophans produce decreases in dopamine uptake affinity in the absence of grossly disordered expression, suggesting roles for at least several of these in DAT recognition and translocation of dopamine. Findings that affinities for cocaine analogs are altered by mutations in an overlapping but nonidentical set of TM domain tryptophans support the idea that the cocaine and dopamine recognition sites are overlapping but not identical.

None of the current data clearly indicates that the tryptophans mutated here are selectively involved in recognizing basic, as opposed to aromatic, features of dopamine or cocaine analogs. Although we were initially attracted to the possible use of non-nitrogen-containing CFT analogs to help make this distinction, recent data clearly indicating that these nontropane, oxygen-substituted cocaine analogs are recognized by DAT in a fashion substantially different from its cocaine recognition make this a less valuable approach (Miller et al., 1999). We have thus not clearly identified whether DAT TM domain tryptophan residues are important for recognizing basic elements of DA and cocaine.

The large variety of tryptophan mutations that influence dopamine uptake  $V_{\max}$  values provide substantial evidence consistent with the ideas that DAT is likely to interact with dopamine, ions or both in many ways, at many different "depths" and via more conformations and more conformational changes than its interactions with cocaine. The current data do not clearly indicate which mutations selectively alter ion versus dopamine interactions, although the mutations that alter the ion-dependence of uptake provide hints of possible selectivity in this regard. These data also cannot distinguish how DAT conformational changes likely to be involved in translocation could change its TM axial rotations, tilts, interhelical distances, or TM borders.

Many of these mutations alter expression, consistent with substantial roles for TM tryptophans in proper DAT assembly or trafficking. We cannot distinguish, based on the current data, which TM domain tryptophan side-chains may contribute to overall static and dynamic DAT structures by facing and interacting with membrane lipids, hydrophobic side-chains from other TM domains, or more interiorly disposed recognition pockets for DA and cocaine, however.

Despite the above limitations, our current data and previous work substantially enhance our understanding of the fit between DAT and current provisional physical/spatial (Fig. 6B) models for its structure and structure/function relationships. They allow better assignment of DAT features likely to be responsible for cocaine and dopamine recognition, providing special emphasis on selectively important roles of TM 3 and 4 residues for dopamine uptake affinity and of residues in TMs 8 and 11 for selectivity of cocaine recognition. This sort of information should spur efforts to design better compounds for possible use as dopamine-sparing cocaine antagonists. As improved structural information about DAT and related family members becomes available from biophysical studies, the present mutagenesis results can be placed ever more precisely onto ever fewer provisional DAT models.

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