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Dopamine transporter site-directed mutations differentially alter substrate transport and cocaine binding

(1-methyl-4-phenylpyridinum/reuptake/ligand recognition)

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ABSTRACT **Polar amino acids lying within three hydrophobic regions of the dopamine transporter (DAT) are analogous to those important for ligand recognition by catecholamine receptors. Possible functional significance of these amino acids was examined by expressing DAT cDNAs mutated in these polar residues. Replacement of aspartate at position 79 with alanine, glycine, or glutamate dramatically reduced uptake of [^{3}H]dopamine and the tritium-labeled Parkinsonism-inducing neurotoxin 1-methyl-4-phenylpyridinium (MPP$^+$) and reduced the mutants' affinity for the tritium-labeled cocaine analog (−)-2β-carbomethoxy-3β-(4-fluorophenyl)tropane (CFT) without affecting B_{max}. Replacement of the serine residues at positions 356 and 359 in the seventh hydrophobic region by alanine or glycine caused reductions in [^{3}H]dopamine and [^{3}H]MPP$^+$ uptake, whereas [^{3}H]CFT binding was less affected. Substitution of two serines in the eighth hydrophobic region yielded wild-type values for [^{3}H]dopamine and [^{3}H]MPP$^+$ uptake and [^{3}H]CFT binding. These results demonstrate that aspartate and serine residues lying within the first and seventh hydrophobic putative transmembrane regions are crucial for DAT function and provide identification of residues differentially important for cocaine binding and for dopamine uptake.**

The dopamine transporter (DAT) aids in terminating dopaminergic neurotransmission by sodium-dependent reaccumulation of released dopamine into presynaptic neurons (1, 2) and is key for actions of cocaine and dopamine-specific neurotoxins such as 1-methyl-4-phenylpyridinium (MPP$^+$) (3, 4). cDNA cloning studies have recently elucidated the primary structures of DATs and other homologous members of the sodium-dependent neurotransmitter (plus) transporter family that are expressed in brain or kidney (5–12). However, the transporter sites recognizing neurotransmitters, sodium, neurotoxins, and drugs, including cocaine, are unknown. Clues as to DAT regions that could participate in dopamine recognition could come from studies of catecholamine binding to mutant adrenergic receptors (13, 14), which implicate aspartic acid and serine residues lying in hydrophobic regions in catecholamine binding. DAT contains a single aspartic acid residue in its first hydrophobic domain and serine residues in its seventh and eighth hydrophobic domains. We now report selective effects of mutations of these residues on DAT dopamine uptake and cocaine analog recognition. To more directly compare influences on these two end points, we have examined both binding and uptake in intact cell preparations.

MATERIALS AND METHODS

Single-stranded template for mutagenesis was derived from pcDNADAT1 (5); confirmed by DNA sequencing; and mutated by annealing oligonucleotides corresponding to the mutant sequences (Fig. 1), *in vitro* synthesis and ligation of the mutant strand, nicking of the nonmutant strand, digestion of the nonmutant strand, and repolymerization and ligation of the gapped DNA as described (ref. 15; Amersham kit no. RPN.1523). Mutations were confirmed by DNA sequencing; mutation-containing regions were isolated in 460-base-pair *Not* I–*Bgl* II (1D-G, 1D-A, and 1D-E; see the legend to Fig. 1 for mutation designations) or 800-base-pair *Bgl* II–*Pvu* I (7S-G, 8S-G, and 7S-A) DNA fragments and reintroduced into wild-type nonmutagenized pcDNADAT1 vector by enzymatic digestion, DNA fragment isolation, and ligation to avoid double mutations. DNA sequence analysis of the final six vectors confirmed that the shuttled fragment and the mutations were intact after these manipulations.

COS cells were transfected with pcDNADAT1 or mutants by electroporation as described (16), allowed to express the plasmid for 2 days, and then assayed for their abilities to accumulate [^{3}H]dopamine or [^{3}H]MPP$^+$ or for their membranes to bind the tritium-labeled cocaine analog (−)-2β-carbomethoxy-3β-(4-fluorophenyl)tropane (CFT) as described (17, 18). Briefly, COS cells transfected with pcDNAI carrying wild-type or mutant DAT cDNAs were diluted with Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum and plated on either 35-mm diameter Petri dishes or 24-well plates. Cells were cultured for 2 or 3 days, yielding 0.5–1.5 × 10^5 cells per dish at harvest day. Uptake was assessed by using 10 nM [^{3}H]dopamine (30.3 Ci/mmol; 1 Ci = 37 GBq; NEN) or [^{3}H]MPP$^+$ (83 Ci/mmol; NEN) for 5 min at 37°C. [^{3}H]CFT binding was determined by using intact cells incubated with 1 nM [^{3}H]CFT (87 Ci/mmol; NEN) for 120 min at 4°C. Thirty micromolar (−)-cocaine was added to parallel incubations to provide estimates of nonspecific binding and uptake. In some experiments, the ability of unlabeled dopamine (10^{-7}–10^{-4} M) to inhibit binding of [^{3}H]CFT was also assessed. Three washes with ice-cold buffer terminated both binding and uptake assays. Saturation analyses were performed (*i*) in cells incubated with 20 nM [^{3}H]dopamine and unlabeled dopamine (30 nM–30 μM) or with 2 nM [^{3}H]CFT and unlabeled CFT (3 nM–10 μM), or (*ii*) in cells incubated with 0.3–100 μM [^{3}H]dopamine or 5–500 nM [^{3}H]CFT for some experiments using 1D-G and 7S-G.

Analyses used Student's *t* test and EBDA and LIGAND binding programs (5).

RESULTS

Expression of wild-type DAT in the expression plasmid pcDNADAT1 conferred avid uptake of dopamine and MPP$^+$

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Abbreviations: DAT, dopamine transporter; CFT, (−)-2β-carbomethoxy-3β-(4-fluorophenyl)tropane; MPP$^+$, 1-methyl-4-phenylpyridinium.

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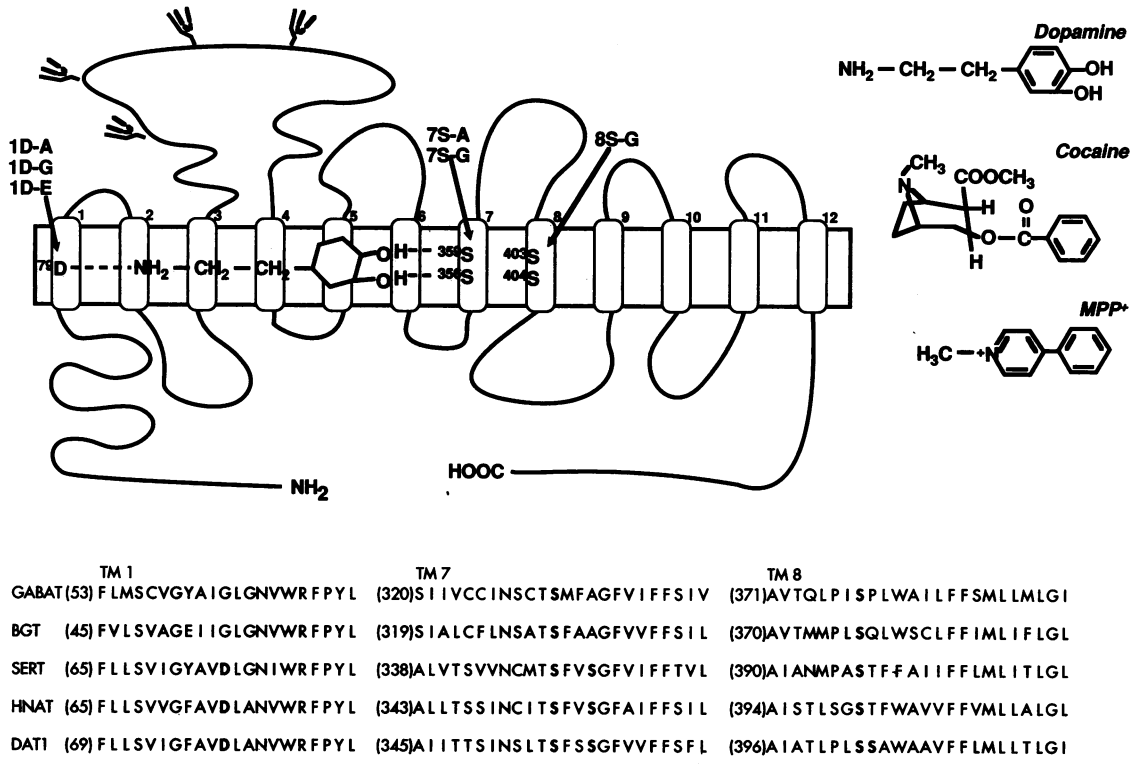

	TM 1	TM 7	TM 8
GABAT	(53) F L M S C V G Y A I G L G N V W R F P Y L	(320) S I I V C C I N S C T S M F A G F V I F F S I V	(371) A V T Q L P I S P L W A I L F F S M L L M L G I
BGT	(45) F V L S V A G E I I G L G N V W R F P Y L	(319) S I A L C F L N S A T S F A A G F V V F F S I L	(370) A V T M M P L S Q L W S C L F F I M L I F L G L
SERT	(65) F L L S V I G Y A V D L G N I W R F P Y L	(338) A L V T S V V N C M T S F V S G F V I F F T V L	(390) A I A N M P A S T F F A I I F F L M L I T L G L
HNAT	(65) F L L S V V G F A V D L A N V W R F P Y L	(343) A L L T S S I N C I T S F V S G F A I F F S I L	(394) A I S T L S G S T F W A V V F F V M L L A L G L
DAT1	(69) F L L S V I G F A V D L A N V W R F P Y L	(345) A I I T T S I N S L T S F S S G F V V F F S F L	(396) A I A T L P L S S A W A A V F F L M L L T L G I

FIG. 1. (*Upper Left*) Depiction of sites of DAT mutations. Assignment of putative transmembrane domains is based on hydrophobicity analyses (5). Structures of compounds (*Upper Right*) and comparisons of transporter family member sequences [*Lower*; for GABA (GABAT), betaine (BGT), serotonin (SERT), norepinephrine (HNAT), and dopamine (DAT1)] in the first (TM1), seventh (TM7), and eighth (TM8) putative transmembrane regions are made for convenience. Aspartic acid residue 79 was mutated to alanine (1D-A), glycine (1D-G), or glutamate (1D-E). Serine residues 356 and 359 were mutated to alanine (7S-A) or to glycine (7S-G). Serine residues 403 and 404 were mutated to glycine (8S-G). All structures are depicted out of scale; the alignment of dopamine depicted is speculative and for illustrative purposes only.

and high-affinity binding of the cocaine analog [^{3}H]CFT on expressing COS cells, as described (5, 17). Each of the mutations in the aspartic acid residue in hydrophobic domain 1 [replacement by alanine (1D-A), glycine (1D-G), or glutamic acid (1D-E); Fig. 1] substantially reduced [^{3}H]CFT binding (Fig. 2 and Table 1). The 1D-A and 1D-G mutations reduced the affinity to 10–15% of wild-type values with no reductions in B_{max}. Uptake was dramatically impacted. V_{max} values were <7% of wild-type values, while K_m values for dopamine uptake revealed 16–36% of the affinity of the wild-type

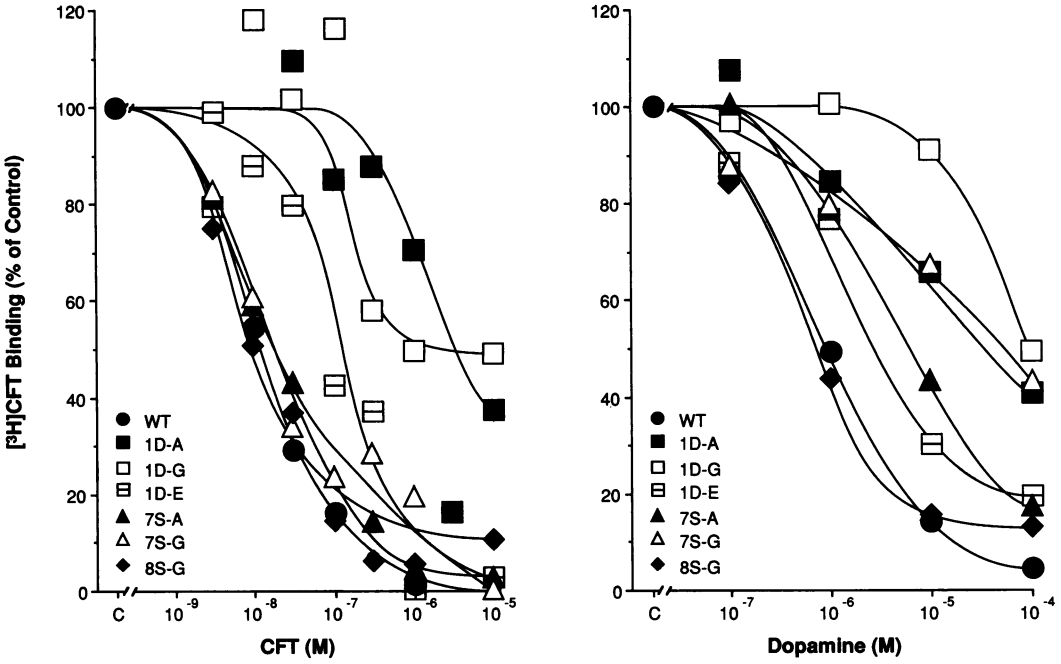

FIG. 2. Displacement of [^{3}H]CFT binding to wild type and mutant DATs by unlabeled CFT (*Left*) and unlabeled dopamine (*Right*). Results are means of triplicate determinations and are representative of one to three other experiments. See Table 1 for more details.

Table 1. [^{3}H]CFT binding in COS cells transfected with wild-type and mutant DATs

DAT	[^{3}H]CFT binding		Dopamine competition	
	K_D, nM	B_{max}, % wild type	IC_{50}, μM	K_i, μM
Wild type	20.8 ± 2.7	100	1.73 ± 0.5	1.50 ± 0.42
1D-A	193 ± 42*	131 ± 16‡	55.7	55.2
1D-G	148 ± 63*	114 ± 34‡	>100	>100
1D-E	102 ± 26*	168 ± 19‡	5.35 ± 1.35	5.25 ± 1.25*
7S-A	38 ± 6.3*	119 ± 15‡	3.9 ± 0.9	3.62 ± 0.79*
7S-G	43.9 ± 18.2†	117 ± 18‡	38.0 ± 28.6	37 ± 28.2†
8S-G	18 ± 3.9‡	99.9 ± 7.3‡	1.07 ± 0.39	0.97 ± 0.38‡

Values are the mean ± SEM derived from 3 to 10 experiments, except for 1D-A studies of dopamine competition for which 2 experiments were performed. B_{max} values were normalized to those in cells expressing wild-type DAT (320 ± 71 fmol per 10^5 cells; n = 10).
*$P < 0.01$ vs. wild type.
†$P < 0.05$ vs. wild type.
‡Not significantly different from wild type.

transporter. MPP^+ uptake in these mutants was also <3% of wild-type values; the very low levels of transport observed precluded accurate saturation analysis (Fig. 3 and Table 2). 1D-E mutants produced less sizable effects on either binding or uptake (Tables 1 and 2).

Mutations in serine residues in hydrophobic domains 7 and 8 had different effects on DAT function. Substitution of glycine for the two serine residues in hydrophobic region 8 (8S-G) resulted in [^{3}H]CFT binding, [^{3}H]dopamine uptake, and [^{3}H]MPP^+ uptake indistinguishable from wild-type values (Tables 1 and 2). When serine residues in hydrophobic domain 7 were mutated to glycine (7S-G) or alanine (7S-A), however, dopamine and MPP^+ transport were both dramatically reduced (Fig. 3 and Table 2). K_m values for dopamine uptake for the 7S-A and 7S-G mutants were more than twice wild-type values. V_{max} values for the 7S-A mutant decreased to less than one-third of wild-type values, while those for 7S-G decreased to one-eighth of wild-type levels. Effects on [^{3}H]CFT binding were smaller. Both the 7S-A and 7S-G mutants showed only modest reductions in [^{3}H]CFT binding affinity without changes in B_{max} (Fig. 2 and Table 1).

The reduced dopamine uptake in mutants could result from their reduced ability to recognize dopamine or from reductions in mutant abilities to transport dopamine after recognition. Dopamine's IC_{50} or K_i for [^{3}H]CFT binding inhibition was consistent with direct mutation effects on catecholamine recognition in each case (Table 2 and Fig. 2).

DISCUSSION

The present studies demonstrate that the aspartic acid residue lying within hydrophobic, putative transmembrane region 1 is crucial for DAT function. The most attractive explanation for this observation is that the carboxylic acid of aspartic acid 79 interacts with dopamine's amine to play a crucial role in dopamine transport (Fig. 1); this amino acid may even recognize cocaine's tropane nitrogen to participate in cocaine binding. Studies of DATs with mutations in serine residues in hydrophobic domain 7 suggest that these residues are selectively and highly important for dopamine and MPP^+ transport but make smaller contributions to cocaine binding.

The current work provides evidence against, but cannot exclude, roles for substantial mutation-induced alterations in transporter secondary and tertiary structure in the effects on dopamine and MPP^+ transport and cocaine analog binding observed here. Less polar alanine and glycine substitutions for more polar aspartic acid or serine residues found within hydrophobic regions are unlikely to dramatically alter the membrane embedding of DAT tertiary structure. Mutation of hydrophobic region 8 serines to glycines was without effect. Lack of large mutation-induced changes in the [^{3}H]CFT

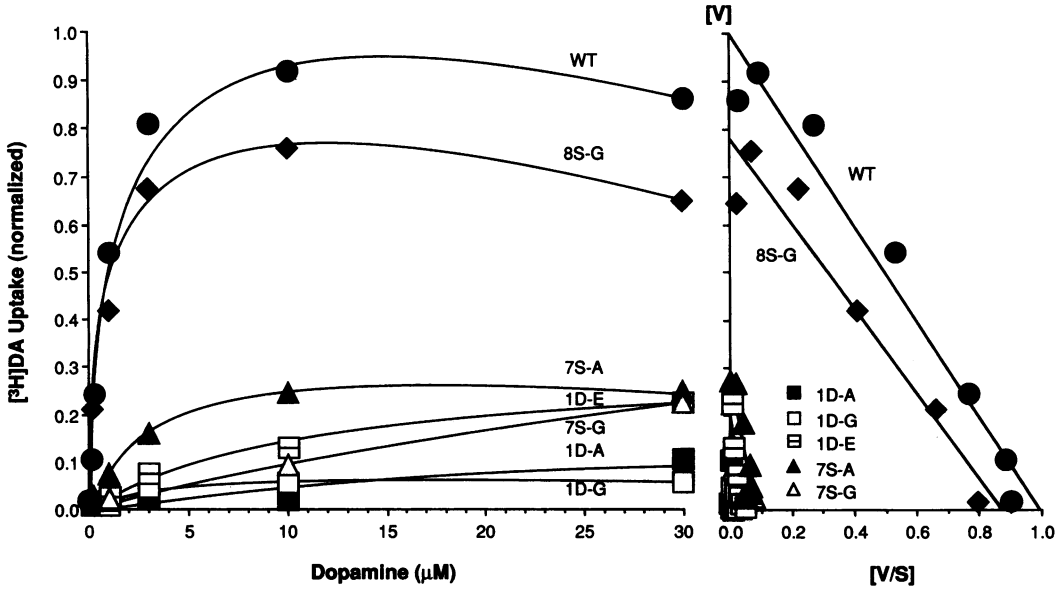

FIG. 3. [^{3}H]Dopamine uptake in wild-type (WT) and mutant DATs. (*Left*) Saturation analysis. (*Right*) Eadie–Hofstee plot of data shown in *Left*. Results are the means of triplicate determinations and are representative of at least two other experiments. See Table 2 for more details.

Table 2. [^{3}H]Dopamine and [^{3}H]MPP$^+$ uptake by wild-type and mutant DATs

DAT	[^{3}H]Dopamine uptake		[^{3}H]MPP$^+$ uptake, % wild type
	K_m, μM	V_{max}, % wild type	
Wild type	1.63 ± 0.26	100	100
1D-A	4.47 ± 0.85*	6.9 ± 4.9*	2.8 ± 0.6†
1D-G	10.2 ± 7.2*	4.0 ± 3.8*	1.1 ± 1.4†
1D-E	6.19 ± 2.48*	23.7 ± 3.3*	14.2 ± 3.4†
7S-A	3.84 ± 0.45*	34.9 ± 16.2†	14.2 ± 0.9†
7S-G	3.62 ± 0.89*	12.5 ± 7.6*	2.7 ± 1.0†
8S-G	1.01 ± 0.12‡	110.0 ± 33‡	98.5 ± 6.3‡

Values are the means ± SEM (n = 3–12). V_{max} values for [^{3}H]dopamine uptake were normalized to those for wild-type DAT (9.51 ± 1.65 pmol per 10^5 cells per min; n = 12). The [^{3}H]MPP$^+$ uptake values are the percent of the wild-type value at 10 nM [^{3}H]MPP$^+$ (16.8 ± 3.5 fmol per 10^5 cells per min; n = 3).
*$P < 0.01$ vs. wild type.
†$P < 0.05$ vs. wild type.
‡Not significantly different from wild type.

binding B_{max} in each case provide evidence against DAT structural alterations sufficiently drastic to significantly inhibit translation or membrane insertion of the resultant protein. Substitutions of both alanine and glycine at most positions yielded similar effects on DAT mutant function, although the different potential influences on α-helical secondary structure that these two amino acids can exert (19) could possibly be reflected in the modest differences between [^{3}H]CFT binding to 7S-G and 7S-A mutants. The influences of the 1D-E mutation are relatively large for a conservative amino acid substitution, although an aspartate-to-glutamate change has also been reported to exert a substantial impact on dopamine recognition by dopamine receptors (20). No direct data on the tertiary structure of any member of the sodium-dependent neurotransmitter transporter family has been obtained to date, however. Without such evidence, a gross structural change cannot be excluded. This caveat is common to interpretation of studies using site-directed mutation to aid understanding of molecules whose hydrophobic nature and size makes crystallization and analyses such as x-ray diffraction difficult.

Similarities between amino acids important for catecholamine recognition in DAT and the dopamine D2 and β-adrenergic receptors, as well as conservation of these amino acids in corresponding positions of the norepinephrine and serotonin transporters (Fig. 1; refs. 8–10, 21), suggest two evolutionary processes at work. Evolutionary convergence must account for the DAT/receptor similarities, since the transporters show no overall sequence conservation with guanine nucleotide-binding regulatory-protein-linked receptors. However, conservation of the aspartic acid and serine residues in the putative transmembrane regions of dopamine, norepinephrine, and/or serotonin transporters could indicate that similar residues might play similar roles in transport of several monoamines due to evolutionary conservation.

The current results do not provide any clear distinction between amino acids necessary for dopamine transport and those key for MPP$^+$ uptake. Since MPP$^+$ lacks catechol hydroxyls, its interactions with the transporter are likely to be significantly different from dopamine's, but the current studies fail to define any such differences. No changes in the dramatic sodium dependence of uptake were noted in preliminary studies of these mutants; current studies provide no evidence concerning areas important for sodium dependence (S.K., unpublished observations). By contrast, these data highlight differences between mutation effects on cocaine analog binding and dopamine transport. They provide evidence that some amino acids are of likely importance for both cocaine binding and dopamine transport while other cocaine binding residues might be dissected from those more important for dopamine transport. Structure–activity analyses of cocaine analog binding potencies are also consistent with the idea that some regions of cocaine that are homologous to features of dopamine are important for binding but that other features necessary for full cocaine analog potency do not have obvious homologs in the structure of dopamine (22–24). Characterization of the activities of structurally modified cocaine analogs at both wild-type and mutant DAT molecules could guide further development of agents with selective affinity for DAT sites important for cocaine binding and less involved in dopamine transport. Such cocaine antagonists that might spare dopamine transport would provide powerful tools in treating cocaine overdose and, possibly, cocaine abuse.

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