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Cocaine Affinity Decreased by Mutations of Aromatic Residue Phenylalanine 105 in the Transmembrane Domain 2 of Dopamine Transporter

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ABSTRACT

Dopamine transporter (DAT) is a major target of cocaine, one of the most abused drugs. Major efforts have been focused on defining residues in DAT involved in cocaine binding. We have isolated the *Drosophila melanogaster* DAT (dDAT) cDNA, which is 10-fold less sensitive to cocaine than the mammalian DATs. Replacing transmembrane domain 2 (TM2) of mouse DAT (mDAT) with dDAT sequence reduced cocaine sensitivity. The reciprocal construct exhibited increased cocaine sensitivity. Switching residue 105 in TM2, a phenylalanine conserved in all mammalian DATs, to methionine, the corresponding residue in dDAT, resulted in a functional transporter with cocaine sensitivity 4-fold lower. Replacing F105 with alanine, leucine, isoleucine, serine, threonine, asparagine, or glutamine resulted in transporters with low transport activity. In contrast, changing F105 to the other aromatic residues tyrosine or tryptophan retained more than 75% transport activity and high cocaine sensitivity. Most significantly, the reciprocal construct, switching the methionine in dDAT at the corresponding residue to phenylalanine, increased cocaine sensitivity 3-fold. Finally, the mDAT mutant with a cysteine at this position had normal transport activity but exhibited cocaine sensitivity that was 15-fold lower. These results suggest that F105 in mDAT contributes to high-affinity cocaine binding. The functional cocaine-insensitive mutants provide tools for the study of the mechanism of cocaine addiction.

Nerve cells communicate with each other and with other cells by releasing neurotransmitters which bind to their respective receptors on target cells, thus transmitting the signals. Neurotransmitter transporters terminate neurotransmission by the reuptake of the released neurotransmitters from synaptic clefts and surrounding areas (Iversen, 1971; Kanner and Schuldiner, 1987). The cloning of the rat γ-aminobutyric acid-1 transporter (Guastella et al., 1990), the human norepinephrine transporter (Pacholczyk et al., 1991), and later other transporters established the presence of a gene family of Na^+-and Cl^--dependent neurotransmitter transporters (Amara and Arriza, 1993; Rudnick and Clark, 1993).

Transporters for the biogenic amines dopamine (DA), 5-hydroxytryptamine, and norepinephrine are high-affinity targets for cocaine and amphetamines (Ritz et al., 1987; Giros and Caron, 1993; Gu et al., 1994). These stimulants are highly addictive and thus are major drugs of abuse worldwide. The biogenic amine transporters are also molecular targets for therapeutic agents such as bupropion, methylphenidate, imipramine, desipramine, fluoxetine, and sertraline, which are used in the treatment of neurological and mood disorders such as attention-deficit/hyperactivity disorder, minimal brain dysfunction, depression, and others (Koe, 1990; Goodnick, 1991; Barr et al., 1992; Boyer and Feighner, 1992; Ascher et al., 1995; Klein, 1995; Seeman and Madras, 1998; Smith et al., 1998). These agents bind to the biogenic amine transporters, disrupt transport function, and thereby prolong transmitter presence in the extracellular spaces of the brain. This leads to the profound psychiatric effects.

A wealth of evidence suggests that dopamine pathways are involved in the addiction process of cocaine. It has been proposed that the dopamine transporter plays the most important role in the mechanism of cocaine addiction among the three biogenic amine transporters (Ritz et al., 1987). To identify regions and residues that may be involved in cocaine binding directly or indirectly, several groups of investigators have made numerous DAT mutants. They made chimeric constructs between transporters with distinct properties or mutated selected Phe, Tyr, Trp, Pro, and other residues and

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ABBREVIATIONS: DA, dopamine; DAT, dopamine transporter; PCR, polymerase chain reaction; RE, restriction enzyme; SERT, serotonin transporter; mDAT, mouse dopamine transporter; dDAT, *Drosophila melanogaster* dopamine transporter; TM, transmembrane domain; IL, internal loop; wt, wild type; hDAT, human dopamine transporter; CFT, 2β-carbomethoxy-3β-(4-fluorophenyl)tropane.

tested the effects of these mutations on the binding affinity of cocaine analogs and/or sensitivity to cocaine inhibition of transport (Kitayama et al., 1992; Giros et al., 1994; Buck and Amara, 1995; Lin et al., 1999, 2000; Chen et al., 2001). These efforts provided some information about specific regions or single amino acid residues in DAT that might be involved in cocaine binding.

In this study, we isolated a cDNA encoding the *Drosophila melanogaster* dopamine transporter (dDAT). This transporter is 10-fold less sensitive to cocaine inhibition than the mouse dopamine transporter (mDAT). Comparison studies between dDAT and mDAT revealed that TM2 and specifically Phe 105 in TM2 are involved in cocaine binding.

Materials and Methods

Isolation of the *D. melanogaster* Dopamine Transporter cDNA. A tube of approximately 100 live fruit flies was chilled in a freezer at −80°C for 1 min and poured into cell-lysing buffer from the RNeasy Total RNA Kit (QIAGEN, Valencia, CA). They were homogenized using three 10-s bursts with a polytron homogenizer (model PT 10/35, Brinkmann Instruments, Westbury, NY). Total RNA was purified according to the kit protocol. Poly(A) RNA was isolated using the Oligotex mRNA Kit (QIAGEN). The RNA preparations were stored in a freezer at −80°C until use. cDNA was synthesized using a specially designed oligonucleotide, Q_T, as the primer. Q_T contains 22 Ts at the 3′ end that anneal to mRNA poly(A) tails and a stretch of 30 nucleotides that can be annealed to by two anchor primers (Q_{in} and Q_{out}) (Wu and Gu, 1999). Reverse transcription was performed using avian myeloblastosis virus reverse transcriptase (Roche Diagnostics, Mannheim, Germany) at 48°C and Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Beverly, MA) at 42°C following manufacturer's protocols. The products from the two reactions were pooled. The mRNA was then degraded with Ribonuclease H (Invitrogen, Carlsbad, CA) treatment, and the cDNA was cleaned with the QIAquick DNA purification kit (QIAGEN) and stored at −80°C. Numerous oligonucleotide primers were synthesized according to the DNA sequences from the *D. melanogaster* database, including forward primers FP1 (GAGCGCGAAACATGGAGC), FP2 (TTTTATTATCGGTTATTGGATTCGC), and FP3 (AATTGGTACCGGCGGCGGGATCTCCAC), and reverse primer RP1 (ACCTAAGCTTTATAGATGGCACTAAAG). Polymerase chain reactions (PCRs) were performed using outer primers FP1 and Q_{out} for the first round and inner primers FP2 and Q_{in} for the second round, yielding the 3′ portion of the dDAT cDNA. The 5′ portion of the cDNA was PCR-amplified using primers Fp3 and RP1. The full-length coding region was then amplified with primers annealing to regions just outside the coding sequence. The final PCR reactions were performed using Pfu polymerase (Stratagene, La Jolla, CA), which has a much lower error rate than *Taq* polymerase. The cDNA clones were sequenced in both directions by the sequencing facility at Yale University (New Haven, CT).

Chimeric and Mutant Transporter Construction. There are numerous unique restriction enzyme (RE) sites within the mDAT cDNA. Additional RE sites were also introduced by silent mutations. Chimeric transporters were constructed by replacing a region of mDAT between two RE sites with a PCR fragment of dDAT cDNA amplified with primers incorporating the proper RE sites. Site-specific mutagenesis was performed using a method similar to that described by Nelson and Long (1989). The sequences of the chimeric and mutant constructs were confirmed by sequencing.

Functional Analysis. We subcloned the dDAT and mDAT cDNA into a bluescript vector (Strategene) with a T7 promoter. The cDNAs were transiently expressed in monkey intestine 407 cells (CCL-6, American Type Culture Collection, Manassas, VA) and characterized as described previously (Blakely et al., 1991; Gu et al., 1994; Wu and Gu, 1999). Briefly, cells were plated in 48-well or 96-well plates, transfected with plasmid DNA using Lipofectin (Invitrogen), and infected with a recombinant vaccinia virus VTF-7 which carries the T7 polymerase gene. After overnight incubation, the cells were washed once with assay buffer (phosphate-buffered saline solution supplemented with 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 50 μM L-ascorbic acid) and incubated in assay buffer containing ^{3}H-labeled dopamine and/or other reagents (as described in each figure legend) for a predetermined length of time at 22°C. At the end of the incubation, cells were washed three times with the same buffer and then dissolved in 0.1 M NaOH. The amounts of substrates accumulated in the cells were determined by counting in scintillation fluid (OPTIFLUOR, PerkinElmer Life Sciences, Boston, MA) in a liquid scintillation counter. All experiments were performed in triplicate. The K_M and IC_{50} values were determined by nonlinear regression fits of experimental data using the computer program Origin (OriginLab-Corp, Northampton, MA).

Materials. [^{3}H]dopamine was purchased from PerkinElmer Life Sciences. Cocaine was obtained from Sigma Chemical Co. (St. Louis, MO). Oligonucleotides were synthesized by the Pathology Department DNA Synthesis Lab at Yale University. All other reagents were from commercial sources.

Results

Isolation of dDAT cDNA. We searched the genomic DNA database of *D. melanogaster* and found many segments of sequenced DNA that encoded proteins or portions of proteins with sequences similar to the mammalian biogenic amine transporters. One of these deposited DNA sequences in GenBank (accession number AC005647) contains the gene exhibiting the highest homology to mammalian dopamine transporters. We designed two forward primers, 51006 and 51007, which anneal to the region around the first transmembrane domain. Two rounds of nested PCR amplification yielded a DNA fragment of 1.9 kilobases using these two forward primers and two reverse anchor primers (Q_{in} and Q_{out}, see *Materials and Methods*). Next, we searched the GenBank expressed sequence tags database and found a 5′ end partial mRNA sequence (accession number AI403478) that overlapped with the sequence of our cDNA fragment. We synthesized forward primers according to this partial mRNA sequence and reverse primers annealing to the cDNA fragment. We then PCR-amplified the 5′ portion of the cDNA and ligated it with the 3′ portion at an overlapping restriction site (EcoN1) to yield the full-length cDNA (accession number AF439752). We further confirmed the presence of this full-length cDNA by amplifying the entire coding region with primers annealing at the start and stop codons. The encoded transporter protein has 51% residues identical with mouse DAT, 52% with mouse norepinephrine transporter, 48% with mouse serotonin transporter (SERT), and 47% with *D. melanogaster* SERT. When expressed in cultured cells, this transporter takes up dopamine and norepinephrine as substrates but not serotonin, suggesting that this transporter is a catecholamine transporter. We searched the nearly complete *D. melanogaster* genome and other *D. melanogaster* databases but failed to find any other genes or cDNAs that are likely to be a catecholamine transporter. Recently, Porzgen et al. (2001) described the isolation and characterization of the same transporter cDNA and deposited the sequence of the coding region in GenBank (AF260833). They determined that this cDNA encodes a dopamine transporter from its restricted expression in dopami-

nergic cells. Among the three independently cloned dDAT sequences, there are 11 discrepancies within the coding region at the nucleotide level. Ten of these discrepancies are at the third position of codons and do not result in changes in protein sequence. In our dDAT sequence, the genomic sequence, and the partial 5′ mRNA sequence, nucleotides 46 to 48 (with the start codon as +1) are CAC, encoding amino acid residue number 16 as histidine, whereas the same nucleotides in the other dDAT mRNA sequence (AF260833) are CGC encoding an arginine.

Comparison of dDAT, mDAT, and Their Chimeras. We expressed the cDNAs in intestine 407 cells using the vaccinia-T7 transient expression system. Figure 1 shows that dDAT has similar DA uptake kinetics but is approximately 10-fold less sensitive to cocaine inhibition compared with mDAT (IC_{50} = 10.8 μM versus 0.95 μM). To identify the regions that are responsible for the difference in cocaine sensitivity between dDAT and mDAT, we made 10 chimeric constructs of the two transporters (dmDAT1 to dmDAT10) (Fig. 2). Most of these chimeric transporters have very low or no DA transport activity, except for dmDAT1 and dmDAT6. Chimera dmDAT1 is mostly dDAT with only a short mDAT N terminus before the first transmembrane domain (TM1), and it has virtually the same properties as dDAT. In contrast, switching the intracellular C-terminal tails of the two transporters (dmDAT2 and dmDAT3) resulted in chimeras with low transport activity (Fig. 3A). Generally, replacing one or more TMs from one transporter with that from the other transporter resulted in chimeras (dmDAT4, -5, -7, -8, -9, and -10) (Fig. 2B) with low or no transport activity (data not shown). There were two exceptions: the TM1 sequences from mDAT and dDAT are identical, and switching the TM2 sequences between mDAT and dDAT was well tolerated (dmDAT6) (Figs. 2A and 3A). Residue 121 in dDAT is an aspartate among hydrophobic residues of putative TM3. In contrast, all mammalian transporters for dopamine and norepinephrine have a glycine at the corresponding position, and an alanine is conserved at this position in all mammalian SERTs. To test whether this potentially disruptive acidic residue in the transmembrane domain was the reason that dmDAT8 was not functional, we changed this residue to Gly in dmDAT8. The resulting mutant remained nonfunctional (data not shown), suggesting that other dDAT residues in dmDAT8 besides Asp 121 also contribute to the inactivation of the chimeric transporter.

Further Mutagenesis of TM2. Chimera dmDAT6 is mostly mDAT sequence with TM2 and part of internal loop 1 (IL1) replaced by dDAT sequence (Fig. 2A). This chimera exhibited DA transport activity similar to that of mDAT but significantly lower sensitivity to cocaine inhibition (IC_{50} = 3.2 μM), suggesting that the region replaced by dDAT sequence may contribute to cocaine binding in the dopamine transporter. To narrow down the region as a potential cocaine binding site, five more chimeric transporters were constructed (Fig. 2A). All of these chimeras were functional and exhibited differing sensitivities to cocaine inhibition (Figs. 2A and 3A and Table 1). Chimeras having mostly mDAT sequence with only the partial IL1 (dmDAT6a), all of TM2 (dmDAT6b), or only the upper portion of TM2 (dmDAT6c) from dDAT exhibited lower sensi-

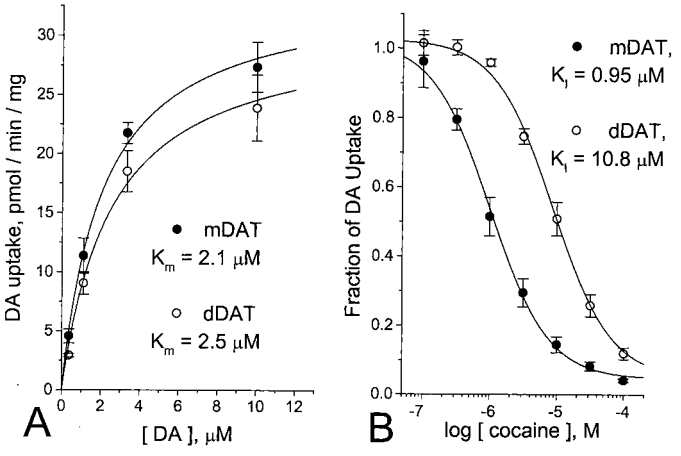

Fig. 1. Comparison between dDAT and mDAT. Intestine 407 cells growing in 48-well plates transiently transfected with dDAT or mDAT cDNA were incubated for 5 min in buffer containing 0.01 μM ^{3}H-labeled DA plus cold DA or cocaine at the concentrations indicated on the horizontal axes. The cells were then washed, and the amount of DA uptake was measured by scintillation counting. ○, data for mDAT; ●, data for dDAT. Transport rates are expressed as picomoles of DA accumulated per minute per milligram of protein, corrected for background by subtracting rates of no DNA controls. Each point is the average of triplicate experiments, and the error bars show S.D. The K_m and IC_{50} (for cocaine inhibition) values were calculated with use of a computer program (Origin) using nonlinear regression analysis. Solid lines represent the best fitting curves of the experimental data. The results shown here are representative of three or more experiments that gave similar K_m or IC_{50} values. A, saturation kinetics of DA uptake. B, cocaine inhibition of dopamine uptake.

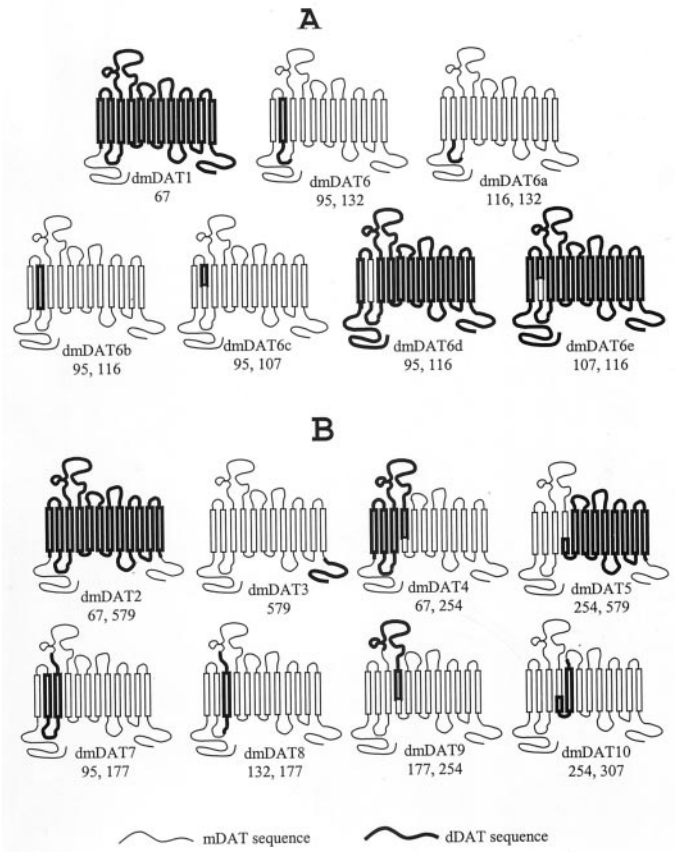

Fig. 2. Chimeric constructs between dDAT and mDAT. The chimeric constructs are illustrated according to the proposed 12 transmembrane span topology. The thick and thin lines represent the sequences from dDAT and mDAT, respectively. The numbers below each diagram represent the junction points according to the mDAT sequence. A, constructs with nearly full transport activity. B, constructs with low or no activity.

tivity to cocaine than wt mDAT (IC_{50} = 1.8, 2.1, and 4.54 μM, respectively), suggesting that both IL1 and TM2 regions affect cocaine binding. More significantly, the reciprocal construct (dmDAT6d) (Fig. 2), which contains mostly dDAT sequence with only TM2 from mDAT, exhibited increased cocaine sensitivity (IC_{50} = 4.3 versus 10.8 μM for dDAT), which is a shift from dDAT toward mDAT. When only the lower portion of mDAT TM2 was inserted into dDAT (dmDAT6d), the resulting construct (dmDAT6e) exhibited lower cocaine sensitivity than dDAT (IC_{50} = 17.9 μM). We also measured cocaine sensitivity for dmDAT2, -3, and -9, which have 20 to 30% wt transport activity. The IC_{50} values for these mutants are 6.2, 0.9, and 2.4 μM, respectively.

Point Mutations at Residue 105. These results suggest that the upper portion of TM2 may be involved in cocaine binding. There are four residues in this region that differ between mDAT and dDAT. Residue 105 is a methionine in dDAT but a phenylalanine in mDAT, which is conserved among all mammalian DATs and norepinephrine transporter. We substituted Phe 105 in mDAT with a Met. The point mutation (mDAT-F105M) caused a significant decrease in cocaine sensitivity (IC_{50} = 3.1 μM versus 0.95 μM). Next, we made the reciprocal construct, replacing the Met in dDAT at the corresponding position with Phe. The resulting mutant dDAT-M72F exhibited increased sensitivity to cocaine inhibition of transport (IC_{50} = 3.5 versus 10.8 μM for dDAT). We then focused on F105 and substituted the residue with amino acids with varying side chain properties. Substitutions with Ala, Ser, Thr, Leu, Ile, Asn, and Gln resulted in mutants with low or no transport activity (Fig. 3B). Replacing Phe 105 with the other two aromatic amino acids tyrosine or tryptophan not only retained transport function (Fig. 3B), but also high sensitivity to cocaine inhibition (IC_{50} = 0.66 and 0.53 μM, respectively) (Fig. 4 and Table 1). Substitution of F105 with cysteine resulted in a

TABLE 1

Comparison of the cocaine potency of transport inhibition of the mutant DAT constructs with wt mDAT and dDAT

The IC_{50} values for cocaine inhibition were determined as described in the legend to Fig. 1. Data represent means ± S.E. of results from three or more experiments. The IC_{50} values for each mutant were compared with those for mDAT and/or dDAT using a two-sided Student's *t* test, and the *P* values are listed.

Constructs	IC_{50}	*P* < (Compared with)	
		mDAT	dDAT
	μM		
mDAT	0.95 ± 0.14		0.01
dDAT	10.8 ± 2.7	0.01	
dmDAT1	9.6 ± 2.5	0.01	
dmDAT6	3.2 ± 0.2	0.01	
dmDAT6a	1.8 ± 0.5	0.1	
dmDAT6b	2.1 ± 0.4	0.05	
dmDAT6c	4.5 ± 1.2	0.01	
dmDAT6d	4.3 ± 0.7	0.01	0.05
dmDAT6e	17.9 ± 1.5	0.01	0.05
mDAT-F105M	3.5 ± 1.4	0.05	0.05
mDAT-F105C	13.9 ± 1.1	0.01	0.05
mDAT-F105Y	0.66 ± 0.3		
mDAT-F105W	0.53 ± 0.05		
dDAT-M72F	3.1 ± 0.4	0.01	0.01

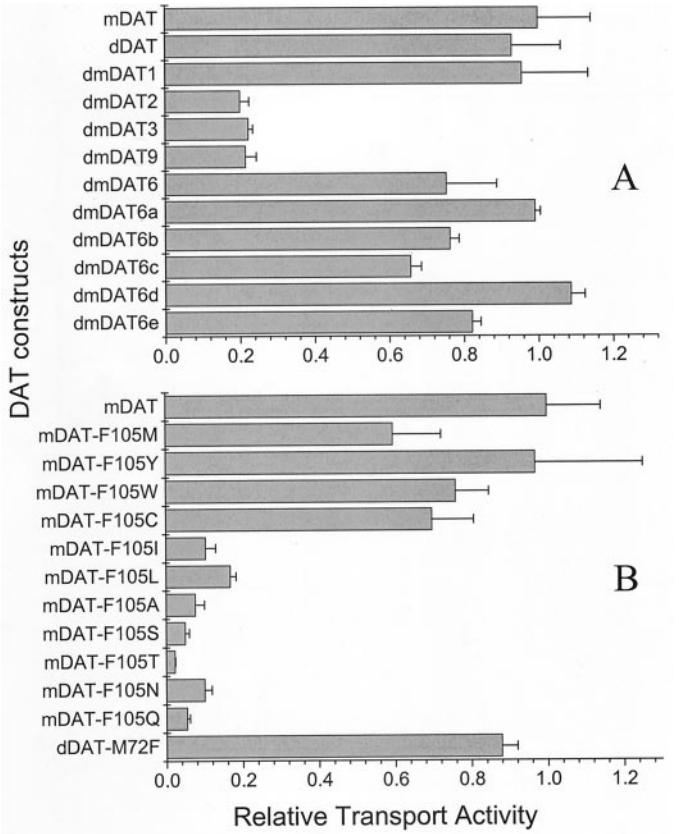

Fig. 3. Relative dopamine uptake activities of the wild-type mDAT, dDAT, chimeric, and site-directed mutant transporters. The transporter constructs as labeled on the vertical axis were transfected into intestine 407 cells and infected with vaccinia virus VTF-7, as described under *Materials and Methods*. Cells were incubated for 5 min in assay buffer containing 0.01 μM ^{3}H-labeled dopamine. The amounts of accumulated radioactivity are presented as relative transport activities, with that for mDAT considered as 1. At lease three separate experiments were performed for each construct, and every experiment included mDAT, dDAT, and no DNA controls. Error bars show S.D. Backgrounds measured by no DNA controls were subtracted. A, results for the DAT constructs illustrated in Fig. 2. B, results for DAT mutants with point mutations.

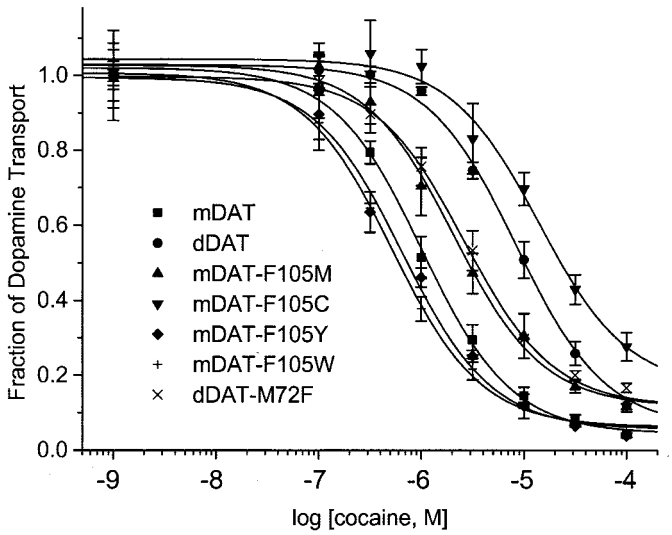

Fig. 4. Sensitivity to cocaine inhibition of transport by mDAT, dDAT, and mutant transporters at residue 105. Dopamine transport activities were measured, as described under *Materials and Methods,* in the presence of cocaine at the concentrations indicated by the horizontal axis. Each data point represents the average of triplicate measurements with S.D. shown as error bars. The data are representative of three or more separate experiments and are presented as the fraction of transport activity when no cocaine is added. The solid lines are the best fitted curve determined by nonlinear regression fits of experimental data with use of the computer program Origin.

mutant that is even less sensitive to cocaine than dDAT (IC_{50} = 13.9 μM). To determine whether the loss of transport activity by mutants at residue 105 were caused by intrinsic defects on uptake or poor expression at the plasma membrane, we added a Flag tag (peptide sequence: DYKDDDDK) to the C termini of wt mDAT and 9 mutants with substitutions at residue 105. We then measured the surface expression levels of these DAT constructs by expressing them in cultured cells, biotinylating surface proteins, isolating the biotinylated proteins with streptavidin-agarose beads, and Western blotting using antibodies against the Flag tag (Gu et al., 1996). The constructs with low or no transport activity had lower but detectable expression on the cell surface (data not shown), suggesting that both intrinsic defects and poor surface expression contribute to low transport activity by the mutants.

Discussion

Through evolution, nature has provided a large number of variant forms of the same protein from diverse species. These protein variants often exhibit substantial differences in their functional properties and/or binding to drugs, which provide clues of regions or residues responsible for the differences. Comparison studies of protein variants from diverse species could reveal the structural bases of functional properties. This strategy has been successfully used to localize domains and residues critical for receptor-ligand interaction in G protein-coupled receptors (Fong et al., 1992; Oksenberg et al., 1992; Hall et al., 1993) and to identify residues important for drug binding in biogenic amine transporters (Barker et al., 1998; Lee et al., 2000). In this study, we cloned the full-length cDNA encoding the dopamine transporter from *D. melanogaster*. This transporter exhibits significant differences from mammalian DATs in binding cocaine and other drugs, such as amphetamine, bupropion, methylphenidate (Ritalin), fluoxetine, desipramine, and paroxetine (data not shown). The cloning of dDAT provides us and other investigators with a tool to identify the residues responsible for the differences in functional properties between dDAT and the mammalian DATs. Through comparison studies of mDAT and dDAT, we were able to determine that the phenylalanine residue at position 105 in mDAT is involved in cocaine binding, either by interacting directly with cocaine or by keeping the transporter in the conformation required for high-affinity cocaine binding.

Three independently cloned dDAT sequences have been deposited in GenBank. Of the 11 discrepancies in the coding regions of these dDAT sequences, 10 are at the third positions of codons and thus do not result in changes in their predicted amino acid sequences. This phenomenon has been observed in several other transporters cloned from other insects in our laboratory (data not shown). Porzgen et al. (2001) also cloned dDAT and determined that the IC_{50} value of cocaine inhibition of transport for dDAT was approximately 10-fold higher than that for hDAT, which is in agreement with our results.

The dopamine transporters from the two diverse species, mouse and *D. melanogaster,* have only 51% of amino acid residues that are identical. Despite similar transport properties for the two parent transporters, most chimeric constructs between mDAT and dDAT, even constructs with a single TM region replaced, are not functional. This result suggests that the interactions between different parts of the protein are critical to form a functional transporter. When the N terminus of dDAT was replaced with that from mDAT (dmDAT1), the chimera had the same properties as the wild-type dDAT, suggesting that the N terminus is not essential in the transport function of the protein expressed in cultured cells. This is consistent with our earlier result showing that the N terminus-deleted DAT mutant still exhibited full transport activity (Gu et al., 1996). In contrast, chimeras with switched C termini between mDAT and dDAT (dmDAT2 and dmDAT3) had significantly reduced transport activity. Because the C termini from both species function well in the wt mDAT and dDAT, this result suggests that the C terminus from one species and the rest of DAT from the other species are not compatible with certain mechanism(s), possibly because of interactions with other proteins necessary for correct trafficking and surface expression.

The sequences of TM1 from mDAT and dDAT are identical, and switching TM2 between the two transporters is well tolerated. Substitution of TM2 or only the upper part of TM2 in mDAT with corresponding regions of dDAT (dmDAT6b and -6c) decreased cocaine potency to inhibit transport. More importantly, the reverse construct, dDAT with mDAT TM2 (dmDAT6d) exhibited higher potency to cocaine inhibition than did wt dDAT. Replacing only the lower part of TM2 from dDAT with mDAT sequence did not result in increased cocaine sensitivity. This result suggests that certain residue(s) in the upper part of mDAT TM2 that is absent in dDAT TM2 may be important in cocaine binding. Substitution of the Phe residue at position 105 within this region of mDAT with a Met, the corresponding residue in dDAT (mDAT-F105M), reduced cocaine potency, whereas switching the corresponding Met in dDAT to Phe (dDAT-M72F) increased cocaine sensitivity (IC_{50} = 3.1 versus 10.8 μM for dDAT). Switching residues at many positions between mDAT and dDAT may alter their cocaine binding pockets, resulting in decreased cocaine sensitivity, whereas only very limited number of switches at specific residues from dDAT to mDAT will change the cocaine binding pocket in dDAT toward that of mDAT resulting in increased cocaine sensitivity. These specific residues are likely to contribute to cocaine binding.

Substitutions of Phe 105 in mDAT with nonaromatic amino acids resulted in mutants with low or no transport activity or in mutants with decreased cocaine binding affinity. This result suggests that this residue is in a region that is important for transport function and cocaine binding. Replacing F105 with either of the other two aromatic residues Tyr and Trp not only retained transport function, but also retained high sensitivity to cocaine inhibition. This result suggests that an aromatic residue at this position contributes to high-affinity cocaine binding by either a direct aromatic interaction with cocaine or an aromatic interaction with other residues to maintain the cocaine binding pocket in the required conformation for high-affinity cocaine binding. To examine the pharmacological features of the DAT mutants at physiological temperature, we also measured the cocaine inhibition IC_{50} values at 37°C for mDAT, dDAT, mDAT-105M, and mDAT-105C. The IC_{50} values at 37°C were not significantly different from those at 22°C (data not shown).

There are two types of conceivable mutations that could decrease ligand binding: 1) a mutation that takes away one of several specific interactions between the ligand and its target protein; and 2) a mutation that introduces a disruption, mak-

ing other specific interactions weaker. At position 105 in mDAT, all aromatic residues (Phe, Tyr, or Trp) allowed strong cocaine binding, suggesting a possible specific aromatic interaction. When this interaction was taken away by inserting a Met at that position, the cocaine affinity decreased. Moreover, when Cys was placed at that position, cocaine sensitivity decreased even more, lower than that of dDAT, suggesting that switching to Cys not only removes a possible specific aromatic interaction, it also introduces a disruption or a change in the binding pocket that weakens other interactions between DAT and cocaine. At the equivalent position of mDAT-105F, serotonin transporters have a methionine residue, but they serve as good binding sites for cocaine. The residue three positions down (one helical turn) is a phenylalanine in the SERTs, whereas it is an isoleucine in the DATs. It is possible that this Phe residue in the SERTs plays a role similar to that of F105 in the DATs. It is also possible that cocaine binding in the SERTs does not require an aromatic residue at this position.

Previous reports have identified residues involved in cocaine binding. Ritz et al. (1987), Kozak (1991), and Ritz and Kuhar (1993) mutated a large number of aromatic resides and prolines located in or near the putative transmembrane domains and found that some of the mutations decreased binding affinity to cocaine analog CFT without significantly affecting dopamine uptake. More recently they reported that changing F154 in TM3 to alanine retains normal DA uptake but lowers cocaine affinity by 10-fold (Lin and Uhl, 2002). Using a similar approach, Chen et al. (2001) found that mutant D68N in hDAT exhibited transport activity that was nearly that of wt hDAT and 4-fold lowered sensitivity to cocaine. They also showed that mutant D345N lost the ability to bind CFT completely but was still inhibited by cocaine, demonstrating that CFT binding affinity may not always be a good indication of cocaine sensitivity. Reith et al. (2001) showed that modification of C90 in hDAT by methanethiosulfonate reagents increased CFT affinity. Barker et al. (1998) identified residues in SERT that were important for the binding of drugs by species-scanning mutagenesis between hSERT and dSERT. They reported that Y95F in hSERT retained normal transport function but had 5-fold increased cocaine sensitivity. These studies and current work contribute to defining where and how cocaine binds to the transporters, which will help in designing new drugs for the treatment of cocaine addiction.

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