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Chimeric dopamine–norepinephrine transporters delineate structural domains influencing selectivity for catecholamines and 1-methyl-4-phenylpyridinium

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ABSTRACT **The dopamine (DA) and norephinephrine (NE) transporters demonstrate important differences in their selectivity for catecholamines and the parkinsonism-inducing neurotoxin 1-methyl-4-phenylpyridinium (MPP^+), yet their primary sequences and predicted topology are strikingly similar. To delineate discrete structural domains contributing to pharmacologic and kinetic differences between the DA and NE transporters, a series of recombinant chimeras was generated by a restriction site-independent method and expressed in mammalian cells. Functional analyses of the chimeras delineate two discrete regions spanning the first through the third transmembrane domains (TM1–3) and TM10–11 that contribute to differences in their apparent affinities for DA, NE, and MPP^+. These studies also suggest that TM2–3 of the DA transporter have a role in selectively increasing the rate of DA uptake as compared with NE. TM4–8 of the DA transporter may influence the relative rate with which MPP^+ is taken up into cells and could contribute to its selective toxicity in neurons expressing the DA transporter. These structure–function studies using chimeras of members of the superfamily of Na^+- and Cl^--dependent transporters provide a framework for identifying the specific structural or regulatory determinants contributing to substrate recognition and translocation by the DA and NE transporters.**

Reuptake of dopamine (DA) and norepinephrine (NE) into presynaptic terminals is mediated by distinct plasma membrane transporter proteins which regulate the effective synaptic and extracellular levels of these neurotransmitters and limit the availability of DA and NE for activation of pre- and postsynaptic receptors. The NE and DA transporters (NET and DAT) are members of a family of Na^+- and Cl^--dependent carriers (1). Although these two transporters have highly similar sequences, they have distinct substrate selectivities. DAT mediates uptake of DA but is an inefficient carrier of NE and other biogenic amines (2–6). 1-Methyl-4-phenylpyridinium (MPP^+), the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is also a substrate of catecholamine transporters. This compound causes a selective and irreversible loss of nigrostriatal dopaminergic neurons and provides the basis for an experimental model of Parkinson disease (7–9).

The structural domains responsible for the functional properties of NET and DAT—e.g., substrate and inhibitor selectivity—and conserved mechanistic features such as ion coupling and substrate translocation have yet to be determined. NET and DAT cDNAs predict protein sequences of 617 and 618 amino acids, and hydropathy analyses of these sequences indicate 12 hydrophobic regions proposed to represent membrane-spanning domains (1). The two transporters are most similar in putative transmembrane domains (TM) and least conserved in the amino and carboxyl termini and in a large extracellular domain between TM3 and TM4. In order to assign specific properties of NET and DAT to defined structural domains, we generated and expressed a series of recombinant chimeric transporters. Analyses of the properties of functional chimeras provide an assayable phenotype and allow positive inferences to be drawn from the pharmacologic and kinetic properties associated with specific domains of DAT and NET. In the present studies, a series of functional chimeras delineate structural domains which influence the apparent affinities and relative translocation efficacy for uptake of DA, NE, and MPP^+.

MATERIALS AND METHODS

Generation of Transporter Chimeras. Chimeras were constructed by an *in vivo* method that generates chimeras within bacteria transformed with linear plasmid DNA containing a single copy of each parental cDNA in a tail-to-head configuration. Chimera formation is believed to involve partial exonuclease digestion of the linear DNA and base pairing between exposed ends of complementary DNA, followed by bacterial repair to a single sequence and ligation to recircularize the chimera plasmids. Using this method, we have generated a series of chimeras that junction in regions of sequence conservation between DAT and NET. Our data indicate that most junctions within conserved regions of DAT and NET are not disruptive of transporter function.

The human NET and rat DAT cDNAs were previously cloned in our laboratory (2, 10). The NET cDNA was cloned into the *Xho* I site of pBluescript SKII(−) (pBSK; Stratagene). The DAT cDNA was cloned into the *Kpn* I and *Xba* I sites. To generate DAT/NET (DN) chimeras, the 1.9-kb NET cDNA was subcloned into the *Not* I site of DAT/pBSK, leaving unique *Eco*RI and *Xba* I sites between the DAT and NET cDNAs. To generate NET/DAT (ND) chimeras, the 1.9-kb DAT cDNA was subcloned into the *Xba* I site of NET/pBSK, leaving unique *Eco*RV and *Xba* I sites. The DN and ND tandem plasmids (2 μg) were linearized by digestion at these unique sites. The DNA was gel purified with QIAEX resin (Qiagen, Chatsworth, CA), and 50–100 ng of linear DNA was transformed by heat shock (42°C, 60 sec) into 10 μl of competent *Escherichia coli* DH5α (GIBCO/BRL). About 300 resulting carbenicillin (50 μg/ml)-resistant colonies were subjected to alkaline lysis and electrophoresed in 1% agarose gels. Bacteria carrying monomer-sized inserts (possibly chimeric) were grown overnight in 250 ml of LB broth containing carbenicillin (50 μg/ml) and their plasmid DNA was purified by the Qiagen plasmid maxi purification procedure. Each plasmid was subjected to diagnostic digests to ascertain the approximate location of the chimera junction. Dideoxy se-

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Abbreviations: DA, dopamine; NE, norepinephrine; DAT, DA transporter; NET, NE transporter; DN, DAT/NET chimera; ND, NET/DAT chimera; MPP^+, 1-methyl-4-phenylpyridinium; TM, transmembrane domain(s).

quencing (Sequenase version 2.0; United States Biochemical) of this region identified the precise location of the chimera junction and confirmed that the junction was in frame.

Substrate Uptake in Transfected Mammalian Cells. Wild-type and chimeric transporter cDNAs were expressed in HeLa cells using the vaccinia/T7 transient expression system. The method uses a recombinant vaccinia virus strain (VTF_{7-3}) which expresses a T7 RNA polymerase. In host cells, VTF_{7-3} replicates in the cytoplasm, resulting in high-level and rapid expression of proteins encoded by plasmids bearing T7 promoters (11). Cells were plated at 2×10^5 per well in 24-well tissue culture plates and transfected the following day. Thirty minutes prior to transfection, cells were infected at 10 plaque-forming units per cell in 100 μl of Dulbecco's modified Eagle's medium. Plasmids were added in liposome suspension (1 μg of DNA and 3 μg of Lipofectin; GIBCO/BRL) in a total volume of 350 μl per well. After 16 hr, the cells were washed with 1 ml of KRTH (120 mM NaCl/4.7 mM KCl/2.2 mM $CaCl_2$/1.2 mM $MgSO_4$/1.2 mM KH_2PO_4/5 mM Tris/10 mM Hepes, pH 7.4), and preincubated for 15 min at 37°C in 500 μl of KRTH. Uptake was initiated by the addition of [^{3}H]NE, [^{3}H]DA, or [^{3}H]MPP^+ (10 nM) (DuPont/NEN) with or without various concentrations of unlabeled substrate (Research Biochemicals, Natick, MA) diluted in KRTH containing L-ascorbate (100 μM). Each concentration was tested in quadruplicate, and well-to-well variability was typically <10% of the mean. Uptake was terminated after 20 min at 37°C, the cells washed with 1 ml of cold KRTH and solubilized in 0.5 M NaOH, and the accumulated radioactivity was determined by scintillation spectrometry. Nonspecific transport was determined in pBSK-transfected cells and subtracted from the data. Kinetic parameters were determined by nonlinear least-squares fits of substrate/velocity profiles from three to six independent concentration saturation experiments, using the data analysis program INPLOT. DAT and four DN chimeras were best fit by a two-component model (unpublished data) and the predominant component, representing about 90% of total transport, is reported. Six chimeras and NET were fit by assuming a single population of noninteracting sites obeying Michaelis–Menton kinetics.

RESULTS

Generation and Screening of Functional Transporter Chimeras. An *in vivo* method was used to construct a series of chimeric transporters. From 300 resulting plasmids, diagnostic restriction enzyme digests and dideoxy sequencing identified 59 in-frame chimeric cDNAs with junctions positioned throughout much of the transporter molecule in regions of sequence homology between DAT and NET. In all cases, chimeras junctioned at single sites, and no deletions or insertions of nucleotide sequence were observed. Forty-six chimeras catalyzed the transport of DA, a substrate efficiently carried by both NET and DAT. The functional activity of these chimeras suggests that the encoded proteins were not grossly misfolded and were expressed and inserted into the plasma membrane in a conformation recognized by substrates. Chimeras showing translocation of DA comparable to DAT were selected for detailed analysis of their pharmacologic and kinetic properties (Fig. 1*B*). Interestingly, 13 chimeras demonstrating much less translocation of catecholamine substrates (and therefore excluded from the present kinetic studies) all junction in a region spanning TM5–8, suggesting that this domain may be important for substrate translocation or for appropriate processing and insertion of transporters into the plasma membrane (Fig. 1*C*). Chimeras are referred to as ND or DN to reflect their relative orientations and are numbered to indicate a TM near their junction.

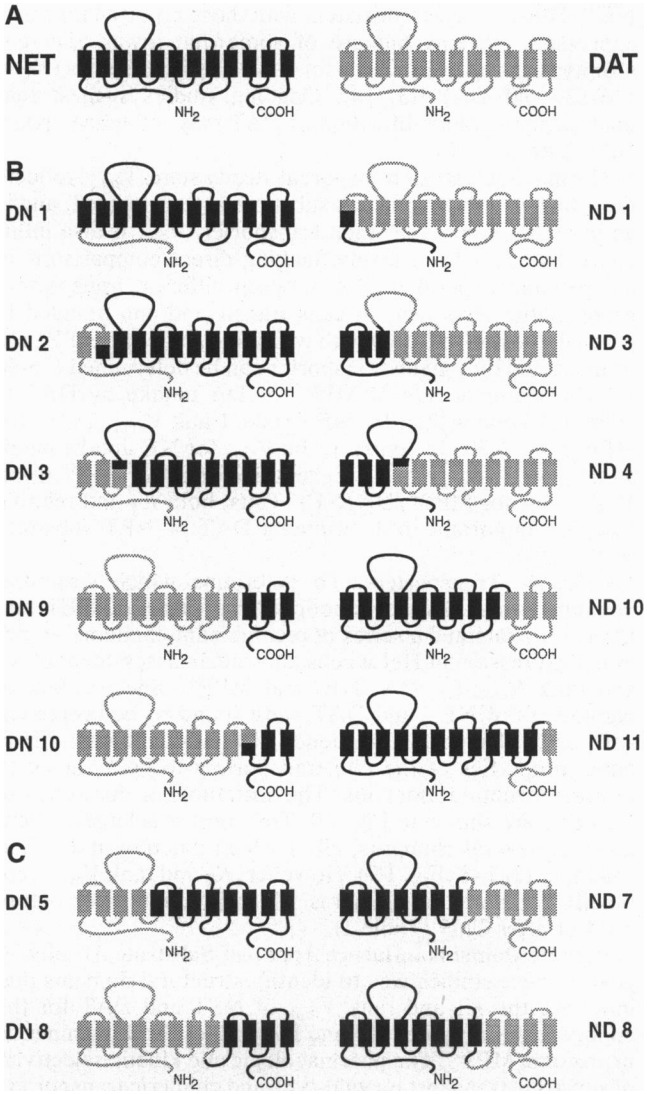

FIG. 1. Wild-type and chimeric catecholamine transporters. (*A*) Schematic representation of NET and DAT. Twelve hydrophobic domains are modeled as membrane-spanning domains. (*B*) Ten functional chimeras made from combinations of NET (black) and DAT (gray) are shown. Each chimera is designated as DN or ND in orientation and numbered to indicate a TM near its junction. Most demonstrate DA uptake (V_{max}) > 90% that observed for DAT. Chimeras ND3, DN1, and DN3 show DA uptake that is 60–80% that of DAT. (*C*) Four chimeras, each junctioning in a region spanning TM5–8, show substantially attenuated uptake of substrates when compared with DAT or NET.

Substrate Selectivity of Catecholamine Transporters. Substrate selectivity was examined in HeLa cells expressing DAT or NET cDNAs using a vaccinia/T7 expression system. In kinetic analyses of substrate transport, the Michaelis constant (K_T) reflects substrate affinity for the kinetically relevant site(s) but is also influenced by rate constants for events which occur subsequent to recognition (e.g., translocation and dissociation). K_T therefore represents an "apparent" affinity constant which is inversely related to uptake affinity. NET has high apparent affinity for both NE and DA transport (K_T = 410 ± 98 and 165 ± 30 nM), which is consistent with reported values for inhibition of [^{3}H]NE uptake in rat cortical synaptosomes by NE and DA (K_T = 140 and 100 nM) (12). DAT expresses lower apparent affinity for DA and NE uptake (K_T = 3.2 ± 0.4 and 5.7 ± 2.7 μM) than

NET. These data are consistent with those reported for other expression systems but are of somewhat lower apparent affinity than reported values for striatal synaptosomes (K_T = 110–537 nM DA) (13, 14). Ongoing studies suggest that posttranslational modification of DAT may influence apparent substrate affinity.

The maximal rate of transport at steady state (V_{max}) reflects the efficacy with which each substrate is translocated, and is an intrinsic property of each transporter. V_{max} is also influenced by expression levels, making direct comparisons in independent experiments or between different transporters problematic. However, in cells transfected and assayed in parallel, the rank efficacy with which DA, NE, and MPP^+ are translocated by a given transporter can be determined (Table 1). The maximal rate of MPP^+ or DA uptake by DAT is markedly greater than for NE uptake (rank V_{max}, 16:9:1 for MPP^+/DA/NE). In contrast, the V_{max} for NE uptake mediated by NET is only 2-fold higher than for DA or MPP^+ (rank V_{max}, 1:1:2 for MPP^+/DA/NE). Thus, both K_T and relative V_{max} are important in determining DAT or NET substrate selectivity.

Chimeric Transporters. To determine which structural domains confer distinct functional properties of NET and DAT, we examined a series of recombinant chimeras. Functional expression in HeLa cells allowed an assessment of K_T and rank V_{max} for DA, NE, and MPP^+. Specific kinetic parameters of NET and DAT were found to be correlated with particular protein sequence elements, allowing differential properties of the two transporters to be assigned to discrete structural domains. The structures of this series of chimeras are shown in Fig. 1*B*. Transport was largely attenuated in several chimeras, all of which junction in a region spanning TM5–8 (Fig. 1*C*). However, K_T and rank V_{max} were readily compared in chimeras which junction in or before TM4 or after TM9 (Table 1).

Discrete Domains Influence Apparent Substrate Affinity. A goal of these studies was to identify structural domains that influence the K_T and rank V_{max} of NET and DAT for the endogenous substrates NE and DA and for the dopaminergic neurotoxin MPP^+. The pharmacologic and kinetic selectivity of substrate transport by wild-type and chimeric transporters is summarized in Table 1. Comparison of the K_T values for the complementary chimeras DN3 and ND3 to those for NET and DAT indicates the importance of a region spanning TM1–3 in specifying differences in K_T for NE, DA, and MPP^+. The apparent affinities of DAT and ND3 (which differs from DAT in a region extending from the amino terminus through TM3) differ 3-fold for DA, 4-fold for NE, and 17-fold for MPP^+. Similarly, the apparent affinities of NET and DN3 (which differs from NET in an analogous region extending from the amino terminus through TM3) differ 5-fold for DA and NE and 7-fold for MPP^+. The even greater differences in K_T observed between chimeras DN1 and DN2 may indicate that determinants within TM1–2 are particularly important in specifying K_T. The chimera DN1 (in which the amino terminus of NET is replaced with that of DAT) does not differ from NET in its K_T for DA, NE, or MPP^+, indicating that the amino terminus does not contribute to differences between NET and DAT in their apparent affinities for a variety of substrates.

A region spanning TM10–11 also contributes to differences in K_T between NET and DAT for catecholamines and particularly for MPP^+. Chimera ND10 has nearly 4-fold lower apparent affinity for MPP^+ uptake than ND11 and about 2-fold lower apparent affinity for NE and DA than ND11 (Table 1). Furthermore, chimera DN10 (which differs from DAT in a region spanning TM10–12) has about 3-fold higher apparent affinity for MPP^+ than DAT and about 2-fold higher apparent affinity for NE, supporting a role for TM10–12 in influencing the K_T for NE and MPP^+. Chimera ND11 (which differs from NET in TM12 and the carboxyl terminus) has a K_T for MPP^+ and catecholamines virtually identical to that of NET, indicating that TM12 and the carboxyl terminus do not contribute to differences between NET and DAT in their apparent affinities for MPP^+, DA, or NE.

The chimeras ND3 and ND4 do not differ in their apparent affinities for NE and MPP^+, suggesting that TM4 and the large putative extracellular loop between TM3 and TM4 do not contribute to K_T differences between DAT and NET for MPP^+ or NE. However, ND3 and ND4 show a 2-fold difference in K_T for DA uptake, indicating that TM4 or the extracellular loop between TM3 and TM4 may have some influence on apparent affinity for DA.

Structural Domains Influencing Transport Efficacy. In cells transfected and assayed in parallel, the maximal rate of uptake by DAT for MPP^+ or DA is substantially greater than for NE (rank V_{max}, 1:9:16 for NE/DA/MPP^+). In contrast for NET, the V_{max} for NE uptake is 2-fold higher than for DA or

Table 1. Apparent substrate affinity and relative uptake efficacy of chimeric and wild-type transporters

Transporter	Junction*	Apparent affinity constant (K_T), μM			Uptake efficacy (V_{max})	
		NE	DA	MPP^+	Rank order (NE/DA/MPP^+)	DA uptake,† % of DAT
NET	Wild-type	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	2:1:1	160
ND11	NET(F550)	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	1:1:1	130
ND10	NET(F474)	0.9 ± 0.4	0.6 ± 0.3	1.5 ± 0.3	1:1:1	110
ND4	NET(L237)	1.6 ± 0.1	1.5 ± 0.5	1.8 ± 0.4	1:1:2	130
ND3	NET(L163)	1.7 ± 0.5	0.8 ± 0.2	1.6 ± 0.3	1:1:1	60
ND1	NET(N78)	2.6 ± 0.8	2.6 ± 0.7	5.4 ± 1.7	1:4:4	100
DAT	Wild-type	5.7 ± 2.7	3.2 ± 0.4	27.6 ± 5.0	1:9:16	100
DN10	DAT(F484)	2.4 ± 0.8	2.2 ± 0.8	8.6 ± 1.3	1:20:17	180
DN9	DAT(V469)	2.3 ± 0.6	2.1 ± 0.5	7.8 ± 1.2	1:17:10	170
DN3	DAT(F154)	2.0 ± 0.6	1.1 ± 0.4	2.6 ± 0.6	1:5:2	80
DN2	DAT(L113)	2.1 ± 0.3	1.8 ± 0.2	3.7 ± 0.6	4:5:1	90
DN1	DAT(W63)	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	1:2:2	70

The kinetics of NE, DA, and MPP^+ transport were assessed in HeLa cells expressing wild-type or chimeric transporters. The parameters K_T and V_{max} were determined by nonlinear least-squares fits of substrate/velocity profiles for each substrate. K_T (apparent affinity constant for transport) values are reported as the mean ± SEM as determined from three to six independent experiments performed in quadruplicate. Rank V_{max} is reported for NE/DA/MPP^+ uptake into cells transfected and assayed in parallel for uptake of NE, DA, and MPP^+.

*Chimera junctions are identified by the amino acid residue immediately preceding the junction (standard one-letter code and its position in NET or DAT).

†V_{max} for DA uptake, expressed relative to DAT (as % of DAT V_{max} for DA).

MPP^+ (V_{max}, 2:1:1 for NE/DA/MPP^+). These data indicate that relative translocation efficacy can also influence substrate selectivity of DAT and NET. Chimeras DN3 and ND1 translocate DA with greater efficacy than NE (V_{max}, ≥4:1 for DA/NE), whereas ND3 and DN2 show no such difference (V_{max}, 1:1 and 5:4 for DA/NE), indicating that a domain spanning TM2–3 of DAT contributes to selective translocation of DA relative to NE (Table 1). Thus, TM2–3 also appear to play an important role in contributing to differences between DAT and NET in relative V_{max}. However, DN9 and DN10 even more closely resemble DAT in their capacity to translocate DA and MPP^+ at a faster rate than NE, suggesting that additional determinants within a central domain spanning TM4–8 may also contribute to differences in rank V_{max} (Table 1). However, the marked reduction in substrate translocation observed in chimeras that junction in TM5–8 does not allow us to directly test the possible contribution of this region on relative V_{max}.

DISCUSSION

To date, little information has been available regarding the structural domains which determine substrate selectivity of catecholamine transporters. Furthermore, the positions of these domains relative to one another are largely unknown. In order to define the structural domains influencing the functional properties of DAT and NET, we have constructed and expressed a series of recombinant chimeras in which similar sequence domains, and distinct functional properties, of DAT and NET are exchanged. These structure–function analyses using chimeras representing two members of the superfamily of Na^+- and Cl^--dependent carriers represent the first step in identifying the underlying structural determinants of transporter function. These analyses have allowed us to identify specific domains of DAT and NET which influence their selectivity for NE, DA, and MPP^+. Substrate recognition and translocation are likely to involve some shared determinants of NET and DAT. However, the present studies focus on structural domains which distinguish NET and DAT and contribute to differences in their relative translocation efficacies and apparent substrate affinities.

Structural Determinants of Catecholamine Selectivity. Structure–activity studies indicate that a protonated amine group is a critical feature of transported catecholamines (15, 16). The terminal ammonium of monoamines is likely to associate with a negatively charged residue of the transporters, whereas planar aromatic moieties of these compounds are believed to associate with an analogous surface by hydrophobic and/or van der Waals bonding. Our studies delineate TM1–3 as having a pronounced effect on K_T. Chimeras that include NET sequence in TM1–3 have at least 3-fold higher apparent affinity for DA than DAT or chimeras that include DAT sequence elements within this domain. Within TM1–3, negatively charged aspartate and glutamate residues at positions DAT_{D68}/NET_{D64}, DAT_{D79}/NET_{D75}, and DAT_{E117}/NET_{E113} are conserved in monoamine transporters and may recognize the terminal ammonium of catecholamines. Mutation of aspartate residue DAT_{D79} to glutamate or neutral residues has been shown to reduce apparent affinity for DA by a factor of 3–6 but also dramatically impairs DA uptake (17). DAT_{D79} is conserved in NET, indicating that this residue is not directly responsible for differences between the two carriers. However, our results indicate that determinants in close proximity to conserved acidic residues in TM1–3 may contribute to differences in K_T between DAT and NET for catecholamine substrates (Fig. 2). In addition, our data indicate that a domain spanning TM10–11 also influences the K_T of DAT and NET for catecholamines and suggest that determinants within the TM10–11 and TM1–3 domains may interact.

Catecholamine selectivity is also influenced by the relative V_{max} with which DAT or NET transport DA as compared with NE. NET efficiently translocates both NE and DA, whereas DAT translocates DA with greater efficacy than NE (V_{max}, 9:1 for DA/NE). A region spanning TM2–3 appears to contribute to differences in DAT and NET in relative uptake of DA and NE. Chimeras that possess DAT sequence elements within this region transport DA with greater efficacy than NE (rank V_{max}, ≥4:1 for DA/NE), whereas ND3, which

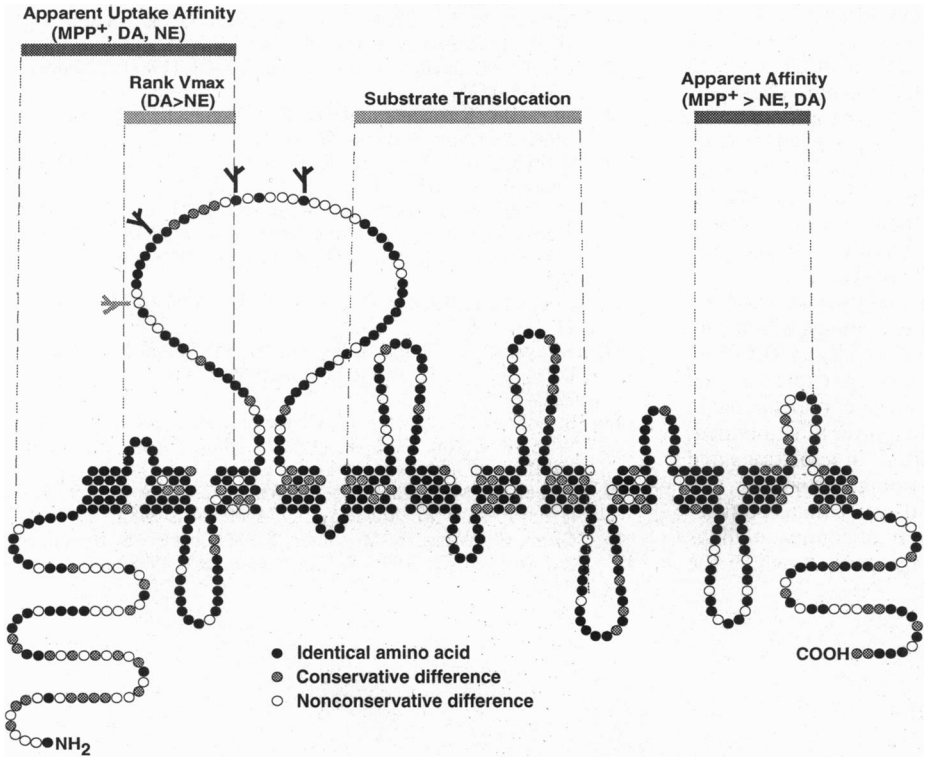

FIG. 2. Summary of the structural domains influencing catecholamine transporter kinetics and pharmacology. Structural domains which influence the different kinetic properties and pharmacological selectivity of NET and DAT are illustrated in this schematic representation of a catecholamine transporter. Domains which influence the apparent substrate affinity or translocation efficacy for catecholamines or MPP^+ are identified. The positions of membrane-spanning domains relative to one another are largely unknown, although the primary sequence predicts that TM1 and TM2 may interact with one another, as could TM4 and TM5. Conservative and nonconservative amino acid differences between NET and DAT are indicated.

includes a complementary region of NET, transports NE as efficiently as DA. These data suggest that determinants within TM1–3 and TM2–3 play an important role in specifying differences in catecholamine K_T and rank V_{max} between DAT and NET (Fig. 2).

Studies of catecholamine binding to mutant adrenergic receptors indicate that serine residues within the transmembrane domains are important in catecholamine recognition (18, 19). Similarly, serine residues of DAT and NET may interact with the hydroxyl moieties of substrate catecholamines. In DAT, replacement of serine residues at positions DAT_{S356} and DAT_{S359} in TM7 by alanine or glycine has been shown to reduce DA uptake (17). Serine residues S356 and S359 are conserved in NET, indicating that these amino acids are not directly responsible for differences in the transport properties of the two carriers. However, DAT_{S358} in TM7 is not conserved in NET and could influence the ability of DAT to selectivity translocate DA. Interestingly, chimeras that junction within TM5–8 show a lower capacity to transport substrates than wild-type or other chimeric transporters, suggesting that this domain may be involved in substrate translocation.

Domains Involved in MPP^+ Transport. MPP^+, a potent neurotoxin which causes a selective loss of dopaminergic neurons, provides the best available experimental model of a parkinsonian syndrome. The basis of the selective toxicity of MPP^+ remains to be elucidated but most likely involves both the vesicular and plasma membrane transporters. The present studies using cloned transporters indicate that DAT translocates MPP^+ at a greater velocity than DA (rank V_{max}, ≈9:16 for DA/MPP^+). In contrast, NET transports MPP^+ at a slower rate than NE (V_{max}, 2:1 for NE/MPP^+). This suggests that the relatively high V_{max} of DAT for MPP^+ uptake into cells may contribute to the selective neurotoxic effects of MPP^+ on dopaminergic neurons. Nigrostriatal neurons may also express DAT at higher levels than mesolimbic DA neurons, which are less vulnerable to MPP^+ toxicity.

Analyses of MPP^+ uptake by chimeric transporters clearly show that K_T and V_{max} can very independently and are influenced by different domains. An amino-terminal domain spanning TM1–3 influences the apparent affinity of chimeric transporters of MPP^+. The K_T of chimeras that include NET sequence in TM1–3 is about 6-fold lower than for DN3, which has DAT sequence elements within this domain. Thus, residues within a region spanning TM1–3 may influence the apparent affinity of NET and DAT for the toxin MPP^+ (Fig. 2). In addition, TM10–11 also appear to influence K_T. Recognition or translocation of the cationic substrate MPP^+, which possesses a pyridinium rather than a terminal ammonium, may involve electronic interaction of its charged moiety with residues within TM1–3 or TM10–11.

In the present studies, functional analyses of chimeric transporters delineate discrete structural domains which contribute to pharmacologic and kinetic selectivity of DAT and NET. Notably, the chimeras reported here are expressed and inserted into the plasma membrane in a conformation in which the structural integrity of the transporter is maintained, allowing robust translocation of a variety of substrates and high-affinity interaction with uptake antagonists—e.g., cocaine and desipramine (unpublished data). Unlike conventional methods of mapping functional domains—such as analyses of site-directed or deletion mutants, in which the function of interest is frequently destroyed—these chimeras provide an assayable phenotype which allows positive inferences to be drawn from functions associated with discrete protein domains. Our results provide a framework for examining the specific structural and regulatory determinants of transporter function in greater detail. As more information becomes available on the higher-order structure of members of the family of Na^+/Cl^--dependent transporters, precise interactions between domains and residues identified in these studies, their role in the mechanism of transport, and their contribution to pharmacologic selectivity of DAT and NET will become apparent.

Note. While this manuscript was under review, a communication on DAT and NET transporter chimeras was published (20).

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1. Amara, S. G. & Kuhar, M. J. (1993) *Annu. Rev. Neurosci.* **16,** 73–93.
2. Kilty, J. E., Lorang, D. & Amara, S. G. (1991) *Science* **254,** 578–579.
3. Shimada, S., Kitayama, S., Lin, C.-L., Patel, A., Nanthakumar, E., Gregor, P., Kuhar, M. & Uhl, G. (1991) *Science* **254,** 576–578.
4. Usdin, T. B., Mezey, E., Chen, C., Brownstein, M. J. & Hoffman, B. J. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 11168–11171.
5. Giros, B., El Mestikawy, S., Bertrand, L. & Caron, M. G. (1991) *FEBS Lett.* **295,** 149–154.
6. Giros, B., El Mestikawy, S., Godinot, N., Zheng, K., Han, H., Yang-Feng, T. & Caron, M. G. (1992) *Mol. Pharmacol.* **42,** 383–390.
7. Snyder, S. H. & D'Amato, R. J. (1986) *Neurology* **36,** 250–258.
8. Boyson, S. J. (1991) *Ann. Neurol.* **30,** 330–331.
9. Shoffner, J. M., Watts, R. L. & Juncos, J. L. (1991) *Ann. Neurol.* **30,** 332–339.
10. Pacholczyk, T., Blakely, R. D. & Amara, S. G. (1991) *Nature (London)* **350,** 350–354.
11. Blakely, R. D., Clark, J. A., Rudnick, G. & Amara, S. G. (1991) *Anal. Biochem.* **194,** 302–308.
12. Lee, C. M., Javitch, J. A. & Snyder, S. H. (1982) *J. Neurosci.* **2,** 1515–1525.
13. Reith, M. E. A., Meisler, B. E., Sershen, H. & Lajtha, A. (1986) *Biochem. Pharmacol.* **35,** 1123–1129.
14. Javitch, J. A., Blaustein, R. O. & Snyder, S. H. (1984) *Mol. Pharmacol.* **26,** 35–44.
15. Maxwell, R. A., Ferris, R. M. & Burscu, J. E. (1976) in *The Mechanism of Neuronal and Extraneuronal Transport of Catecholamines*, ed. Paton, D. M. (Raven, New York), pp. 95–153.
16. DePaulis, T., Kelder, D. & Ross, S. B. (1978) *Mol. Pharmacol.* **14,** 596–606.
17. Kitayama, S., Shimada, S., Xu, H., Markham, L., Donovan, D. M. & Uhl, G. R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7782–7785.
18. Strader, C. D., Sigal, I. S., Candelore, M. R., Rands, E., Hill, W. S. & Dixon, R. A. F. (1988) *J. Biol. Chem.* **263,** 10267–10271.
19. Fraser, C. M., Chung, F.-A., Wang, C.-D. & Venter, J. C. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5478–5482.
20. Giros, B., Wang, Y.-M., Suter, S., McLeskey, S. B., Pifl, C. & Caron, M. G. (1994) *J. Biol. Chem.* **269,** 15985–15988.