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Structural Domains of Catecholamine Transporter Chimeras Involved in Selective Inhibition by Antidepressants and Psychomotor Stimulants

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SUMMARY

Reuptake systems for monoamines are the initial sites of action for a wide range of therapeutic antidepressants and drugs of abuse, such as cocaine. To delineate structural domains of the dopamine and norepinephrine transporters that contribute to differential interaction with reuptake inhibitors with antidepressant or reinforcing properties, a series of recombinant transporter chimeras were generated and transiently expressed in HeLa cells. The inhibition constants (K_i values) for cocaine and a variety of selective transport inhibitors were determined for each chimera. Analyses of functional chimeras delineate a segment spanning transmembrane domains 5–7 of the norepinephrine transporter of primary importance for high affinity binding of tricyclic and nontricyclic antidepressants (e.g., K_i <20 nM desipramine or nisoxetine). In contrast, all chimeras containing dopamine transporter sequences from this region resemble the dopamine transporter, which demonstrates higher affinity for psychomotor stimulants compared with antidepressants (e.g., K_i = 391 ± 39 nM cocaine compared with 9365 ± 1260 nM desipramine). A region including transmembrane domains 1–3 of the norepinephrine transporter also contributes to the interaction of desipramine and nisoxetine, whereas the analogous region of the dopamine transporter influences the affinity for piperazine derivatives (e.g., GBR12909 and LR1111) that are selective for the dopamine transporter. These analyses provide a framework for identifying the precise structural determinants of monoamine transporters involved in selective interactions with antidepressant and psychomotor stimulant reuptake inhibitors.

NET and DAT mediate reuptake of catecholamines into presynaptic terminals, thus limiting the extracellular content of norepinephrine and dopamine and the availability of these neurotransmitters for receptor activation. Monoamine transporters are important targets for a number of classic tricyclic antidepressants and second-generation antidepressants (1). Clinical and behavioral studies indicate that blockade of norepinephrine reuptake by tricyclic antidepressants is correlated with antidepressant activity (2). These studies suggest that blockade of norepinephrine uptake contributes, at least initially, to antidepressant activity. In contrast, drugs such as cocaine, amphetamine, and methylphenidate, which nonselectively inhibit NET and DAT, are poor antidepressants in severe depression, despite demonstrating stimulant and euphoric effects in some individuals (2). In self-administration studies, the potencies of cocaine and related drugs for inhibiting [^{3}H]mazindol binding to DAT but not their potencies for ligand binding to NET or the serotonin transporters are correlated with their reinforcing properties (3). These studies indicate that inhibition of dopamine reuptake is the primary mechanism mediating self-administration of cocaine and related psychomotor stimulants.

The structural domains responsible for differences in the interactions of DAT or NET with reuptake antagonists (e.g., tricyclic and nontricyclic antidepressants, cocaine and related reinforcing drugs, and therapeutics that may reduce cocaine craving) and the mechanisms by which these drugs block substrate translocation have yet to be elucidated. DAT and NET are members of a family of Na^+- and Cl^--dependent carriers that are predicted by hydrophathy analyses to have 12 hydrophobic TMDs (4). The primary sequence of DAT and NET are most similar in the putative membrane-spanning domains and least conserved in the amino and carboxyl termini thought to be oriented toward the cytoplasm and the large extracellular domain between the third and fourth TMDs. One approach to identify the structural domains responsible for the differences in functional and pharmacolog-

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ABBREVIATIONS: DAT, dopamine transporter; NET, norepinephrine transporter; GBR12909, 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine; GBR12935, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine; LR1111, 1-[2-diphenylmethoxy]ethyl)-4-(3-phenylpropyl)homopiperazine; K_i, uptake inhibition constant; TMD, transmembrane domain.

ical properties between transporters has been to generate functional chimeras between closely related family members (5–7). These chimeras provide an assayable phenotype that allows particular properties to be associated with general protein domains, unlike other methods of mapping functional domains, e.g., use of site-directed or deletion mutants in which the function of interest is frequently impaired or destroyed.

The structural domains responsible for differential selectivity of NET and DAT for a variety of substrates (dopamine, norepinephrine, 1-methyl-4-phenylpyridinium) have been examined in two studies (5, 6). Several general conclusions arise from this work. A region spanning TMDs 5–8 appears to be most important for determining the maximal transport rate (V_{max}) for substrates selectively transported by each of the two carriers (5). Transport is attenuated in all chimeras that have junctions in this region providing support for its involvement in substrate translocation (5, 6). This same region also appears to be important for conferring sensitivity to transport inhibitors including the norepinephrine-selective uptake inhibitors nortryptyline and desipramine (6). In the present study, functional analyses of a series of NET/DAT chimeras have been used to delineate specific structural domains involved in the interaction of catecholamine transporters with tricyclic and nontricyclic antidepressants (e.g., desipramine and nisoxetine) and psychomotor stimulants (e.g., cocaine, GBR12909, GBR12935, LR1111, and mazindol).

Materials and Methods

Transporter chimeras. A series of chimeras between the human NET (8) and rat DAT (9) were recently constructed using a restriction site-independent method (5, 10). The method generates chimeras *in vivo* in bacteria transformed with linear plasmid DNA containing a single copy of each parental cDNA in a tandem tail-to-head configuration. Chimera formation is believed to involve partial exonuclease digestion of the linear DNA within bacterium and base pairing between exposed ends of cDNA within regions of high homology, followed by bacterial repair and ligation to recircularize the chimera plasmids. Each chimera was subjected to diagnostic digests to determine the approximate location of the chimera junction. Dideoxynucleotide sequencing (Sequenase Version 2.0; United States Biochemicals) was used to determine the precise location of the chimera junction and confirm that the junction was in-frame. Of the 59 chimeras identified, chimeras junctioning in or carboxyl-terminal to all except two putative TMDs (TMDs 5 and 6) were obtained. Chimeric transporters are referred to as ND (NET/DAT) or DN (DAT/NET) to reflect their relative orientations and are numbered to indicate a transmembrane domain near their junction.

Catecholamine uptake assay. Wild-type and chimeric transporter genes were expressed in mammalian cells using the vaccinia-T7 RNA polymerase expression system as previously described (5). This method uses a recombinant vaccinia virus strain that encodes a bacteriophage T7 RNA polymerase and allows rapid high-level expression of proteins encoded by plasmids bearing T7 promoters (11, 12). Briefly, HeLa cells were plated (2×10^5/well) into 24-well tissue culture plates and transfected the following day. The recombinant vaccinia virus strain VTF-7 was used to infect cells at 10 plaque-forming units/cell in 100 μl of growth medium. T7 promoter-driven plasmids with cDNA inserts encoding wild-type NET or DAT or chimeric transporters were added 30 min later as liposome suspensions (1 μg DNA and 3 μg Lipofectin; GIBCO-BRL) in a total volume of 350 μl/well. Sixteen hours after transfection, the virus/liposome suspension was removed by aspiration, and the cells were washed once with 37° KRTH medium (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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4). Cells were preincubated for 20 min at 37° in 500 μl KRTH with various concentrations of uptake inhibitors. Uptake was initiated by the addition of 20 nM [^{3}H]dopamine in KRTH containing L-ascorbate (100 μM final). Cells expressing DN5, ND7, ND8, or DN8 showed less transport (<10%) than DAT or NET and were analyzed using 200 nM [^{3}H]dopamine. Uptake was terminated after 20 min at 37° by washing twice with 1 ml ice-cold KRTH medium, cells were solubilized with 0.5 N NaOH, and the accumulated radioactivity was determined by scintillation spectrometry. Nonspecific transport was determined by assays of cells transfected with the plasmid vector (pBluescript SKII$^-$) on the same plate and subtracted from the data. The inhibition constant (K_i) for each inhibitor was obtained by nonlinear least-squares fit of uptake-velocity profiles using the program INPLOT from four to six independent experiments performed in triplicate.

Chemicals. [^{3}H]Dopamine was obtained from (DuPont-NEN, Boston, MA). Desipramine, mazindol, and nisoxetine were obtained from Research Biochemical (Natick, MA). (−)-Cocaine was purchased from the National Institute on Drug Abuse (Bethesda, MD). LR1111 (13) was provided by Drs. Agu Pert and Kenner Rice (National Institutes of Health, Bethesda, MD). GBR12909 and GBR12935 were kindly provided by Dr. Aaron Janowsky (Oregon Health Sciences University, Portland, OR).

Results

Functional analyses of transporter chimeras. As described in a previous report (5), a restriction enzyme independent approach was used to generate a series of chimeric transporters between DAT and NET. Diagnostic restriction enzyme digests identified 59 chimeric cDNAs with junctions positioned throughout much of the transporter molecule in regions of sequence homology between DAT and NET. DNA sequence analysis of the chimeras demonstrated that in all cases, chimeras junctioned at single sites, and no deletions or insertions of nucleotide sequence were observed. Functional screening identified 46 chimeras that efficiently catalyzed the transport of dopamine, a substrate carried by both DAT and NET. The functional activity and pharmacological profile of these chimeras demonstrates that the encoded proteins are expressed and inserted into the plasma membrane in a conformation recognized by substrates and antagonists. Nine chimeras demonstrating translocation of dopamine comparable with DAT (5) were selected for analysis of their interaction with uptake inhibitors (Table 1). Four chimeras junctioning in a region spanning TMDs 5–8, which show attenuated uptake, were also selected for analysis of their pharmacological properties. Chimeras are referred to as ND (NET/DAT) or DN (DAT/NET) to reflect their relative orientations and are numbered to indicate the transmembrane domain near their junction.

Inhibitor selectivity of catecholamine transporters. Inhibitor selectivity of wild-type catecholamine transporters was examined in HeLa cells expressing NET or DAT cDNAs using a T7/vaccinia virus-based transient expression system. Each of the inhibitors examined are uptake antagonists rather than competitive substrates for NET or DAT. Thus, the inhibition constants (K_i) directly reflect the affinity of the kinetically relevant site(s) for the uptake antagonists. Inhibition constants for DAT and NET reported in Table 1 are in agreement with reported values for blockade of [^{3}H]dopamine or [^{3}H]norepinephrine uptake in homogenates of striatum or frontal cortex, respectively (14–18). NET exhibits

TABLE 1
Analysis of the interaction of 13 chimeras with uptake inhibitors

		Junction[a]	DA uptake[b]	Desipramine	Nisoxetine	Mazindol	Cocaine	GBR 12909	GBR 12935	LR1111
			% of DAT							
DAT		Wild-type	100	9365 ± 1260	1945 ± 144	60 ± 5	391 ± 39	1 ± 2	1 ± 1	3 ± 2
NET		Wild-type	160	4 ± 2	6 ± 2	2 ± 1	612 ± 53	20 ± 2	21 ± 2	51 ± 5
DN1		DAT (W63)	70	6 ± 2	9 ± 2	2 ± 2	1119 ± 93	19 ± 2	20 ± 2	53 ± 5
DN2		DAT (L113)	90	35 ± 12	38 ± 15	5 ± 2	535 ± 42	11 ± 2	12 ± 2	46 ± 6
DN3		DAT (F154)	80	178 ± 35	237 ± 46	2 ± 2	488 ± 35	10 ± 2	8 ± 2	40 ± 4
DN5		DAT (G261)	<10	189 ± 44	277 ± 112	3 ± 4	507 ± 191	9 ± 4	8 ± 4	38 ± 7
DN8		DAT (E426)	<10	9426 ± 2078	2226 ± 246	58 ± 9	498 ± 163	2 ± 3	2 ± 4	5 ± 4
DN9		DAT (V469)	170	9133 ± 1385	2102 ± 98	50 ± 6	403 ± 36	1 ± 2	1 ± 2	2 ± 3
DN10		DAT (F484)	180	9606 ± 1039	2141 ± 152	61 ± 4	519 ± 47	2 ± 2	2 ± 2	4 ± 3
ND1		NET (N78)	100	9995 ± 1384	1762 ± 196	56 ± 4	1101 ± 107	1 ± 1	3 ± 2	4 ± 3
ND3		NET (L163)	60	2724 ± 558	1191 ± 88	10 ± 3	272 ± 42	3 ± 2	4 ± 2	5 ± 4
ND7		NET (G383)	<10	15 ± 8	18 ± 10	3 ± 5	446 ± 107	17 ± 4	18 ± 4	49 ± 8
ND8		NET (E425)	<10	9 ± 4	12 ± 8	2 ± 4	486 ± 112	20 ± 5	18 ± 5	46 ± 7
ND10		NET (F474)	110	4 ± 3	6 ± 3	1 ± 1	402 ± 84	19 ± 2	20 ± 2	51 ± 4
ND11		NET (F550)	130	7 ± 3	7 ± 3	1 ± 1	346 ± 67	20 ± 2	19 ± 2	48 ± 4

[a] Chimera junctions are identified by the amino acid residue (using standard one-letter code) immediately preceding the junction and its position in DAT or NET.
[b] V_{max} for DA uptake, expressed relative to DAT (as percent of DAT V_{max} for DA).

marked sensitivity to many antidepressants, including desipramine (K_i = 4 ± 2 nM) and nisoxetine (K_i = 6 ± 2 nM), whereas these therapeutic agents are relatively impotent inhibitors of DAT (K_i = 9365 ± 1260 nM desipramine and K_i = 1945 ± 144 nM nisoxetine). Mazindol is also a more potent inhibitor of NET (K_i = 2 ± 1 nM) than of DAT (K_i = 60 ± 5 nM). In contrast, DAT is ~20-fold more sensitive to inhibition of [^{3}H]dopamine uptake by GBR12909, GBR12935, and LR1111 (K_i values of 1–3 nM) compared with NET (K_i values of 20–51 nM). DAT had only slightly higher affinity than NET for cocaine, a relatively nonselective inhibitor of monoamine uptake (K_i values of 391 ± 39 nM for DAT and 612 ± 53 nM for NET). The pharmacological selectivity of wild-type and chimeric transporters are summarized in Table 1.

Delineation of structural domains involved in blockade of dopamine uptake by psychomotor stimulants. To determine which structural domains are involved in blockade of DAT, we examined inhibition of [^{3}H]dopamine uptake by a variety of psychomotor stimulants (e.g., GBR12909, GBR12935, LR1111, mazindol, and cocaine). The diphenyl-substituted piperazine derivatives such as GBR12909, GBR12935, and LR1111 have been shown to be potent and highly selective inhibitors of synaptosomal dopamine uptake *in vivo* and *in vitro* (13, 19–22). Previous studies have suggested that these agents can be as much as 400-fold more selective for the inhibition of dopamine over norepinephrine uptake in synaptosomal preparations (22), but the experiments outlined here indicate a more limited 20-fold selectivity in their inhibition of the two cloned catecholamine carriers (Table 1). These compounds also produce a spectrum of cocaine-like behavioral actions. For example, GBR12909 maintains intravenous self-administration in monkeys trained to self-administer cocaine (23) and supports increasing fixed-interval responding (23, 24). In contrast, selective inhibitors of norepinephrine uptake (e.g., nisoxetine) or serotonin transport (e.g., imipramine) do not maintain self-administration (25, 26). Similarly, there are virtually no reports of abuse for mazindol, a more potent inhibitor of NET than for DAT, which produces dysphoria in humans (27).

Functional analyses of the chimeric transporters indicate that chimeras that possess DAT sequence elements within the region, including TMDs 5–7, all demonstrate the same inhibition profile as DAT, i.e., GBR12909 ~ GBR12935 ≈ LR1111 < mazindol < cocaine < nisoxetine < desipramine (e.g., ND8 and DN3; see Fig. 1, D and F). In contrast, chimeras with sequence within TMDs 5–7 from NET generally show an inhibition profile resembling NET, i.e., mazindol < desipramine ~ nisoxetine < GBR12909 ~ GBR12935 ≈ LR1111 < cocaine (e.g., DN5 and ND7; see Fig. 1, C and E). The region spanning TMDs 1–3 of the dopamine transporter appears to have a modest influence on the binding of piperazine derivatives such as GBR12909 and LR1111 (e.g., DN2, DN3, and DN5; see Table 1), whereas the analogous region of the norepinephrine transporter can influence the affinity for mazindol, as seen in the chimera ND3. Most chimeras demonstrate K_i values for cocaine in the range of DAT (391 ± 39 nM) and NET (612 ± 53 nM), suggesting no gross disruption in the structural determinants of cocaine recognition. However, two chimeras that junction in or near the first TMD (e.g., DN1 and ND1 with K_i values of 1119 ± 93 nM and 1101 ± 107 nM, respectively) demonstrate lower affinity for cocaine than wild-type or other chimeric transporters, suggesting that determinants within this structural domain may influence interaction with cocaine.

Two domains are required for high affinity interaction with antidepressants. NETs and serotonin transporters are important initial targets for a number of tricyclic and other antidepressants used in the treatment of human depression. Desipramine is one of the most potent tricyclic antidepressants in blocking NET but is >5000-fold less potent as an inhibitor of DAT. Nisoxetine, a benzenepropanamide antidepressant, is >300-fold selective as an inhibitor of NET than DAT (Table 1). An important goal of the present study was to delineate the structural domains responsible for high affinity interaction of NET with tricyclic and nontricyclic antidepressants. Analyses of the chimeric transporters identify two domains that are required for effective blockade of transport by desipramine and nisoxetine. Our data indicate that the most influential determinant(s) of NET selectivity for antidepressants lie within a region spanning TMDs 5–7. The affinities of chimeras DN5 and DN8, which differ from each another in the region between the fifth and eighth TMDs, differ 50-fold for desipramine (K_i = 189 ± 44 nM and 9426 ± 2078 nM, respectively). Similarly, ND3 and ND7 differ within a region spanning TMDs 4–7 and differ 180-fold in sensitivity to desipramine (K_i = 2724 ± 558 nM and 15 ± 8 nM, respectively). Moreover, our data show that all chimeras with high affinity for desipramine include NET sequence elements within TMDs 5–7 (e.g., ND7, ND8, ND10, ND11, DN1, and DN2, with K_i values in the range of 4–35 nM), whereas chimeras with low affinity for desipramine possess DAT sequence elements across this region (e.g., DN8, DN9, DN10, and ND1 with K_i values of 9133–9995 nM).

Chimeric transporters with intermediate sensitivity for desipramine and nisoxetine delineate a secondary domain spanning TMDs 1–3 that also influences interaction with antidepressants. Thus, chimeras with DAT sequence in TMDs 5–7 and NET sequence in TMDs 1–3 display intermediate sensitivity to desipramine (e.g., K_i = 2724 ± 558 nM for ND3) but more closely resemble DAT. Chimeras with NET sequence in TMDs 5–7 and DAT sequence in TMDs 1–3 also show intermediate sensitivity for desipramine (e.g., K_i = 178 ± 35 nM for DN3) but more closely resemble NET. Chimeric transporters with NET sequence elements in both TMDs 5–7 and TMDs 1–3 demonstrate high affinity for desipramine (e.g., ND7 with K_i = 15 ± 8 nM), similar to NET. Thus, structural determinants within TMDs 5–7 and TMDs 1–3 are involved in high affinity blockade of catecholamine transporters by tricyclic and nontricyclic antidepressants.

Several regions appear to have no influence on the selective interactions of various inhibitors. The chimeras DN3 and DN5 do not differ in their affinities for desipramine and nisoxetine, suggesting that TMD 4 and the large extracellular loop between TMD 3 and TMD 4 do not differentiate NET and DAT interaction with antidepressants. NET and DN1 both show high affinity for desipramine and nisoxetine, indicating that the NH_2 terminus of NET is not required for selective interaction with antidepressants. Chimeras ND7, ND8, ND10, and ND11 all demonstrate high affinity for desipramine (K_i = 6–12 nM) and nisoxetine, indicating that the COOH-terminal region of NET (including TMDs 8–12) does not contribute to differences between NET and DAT in interaction with antidepressants.

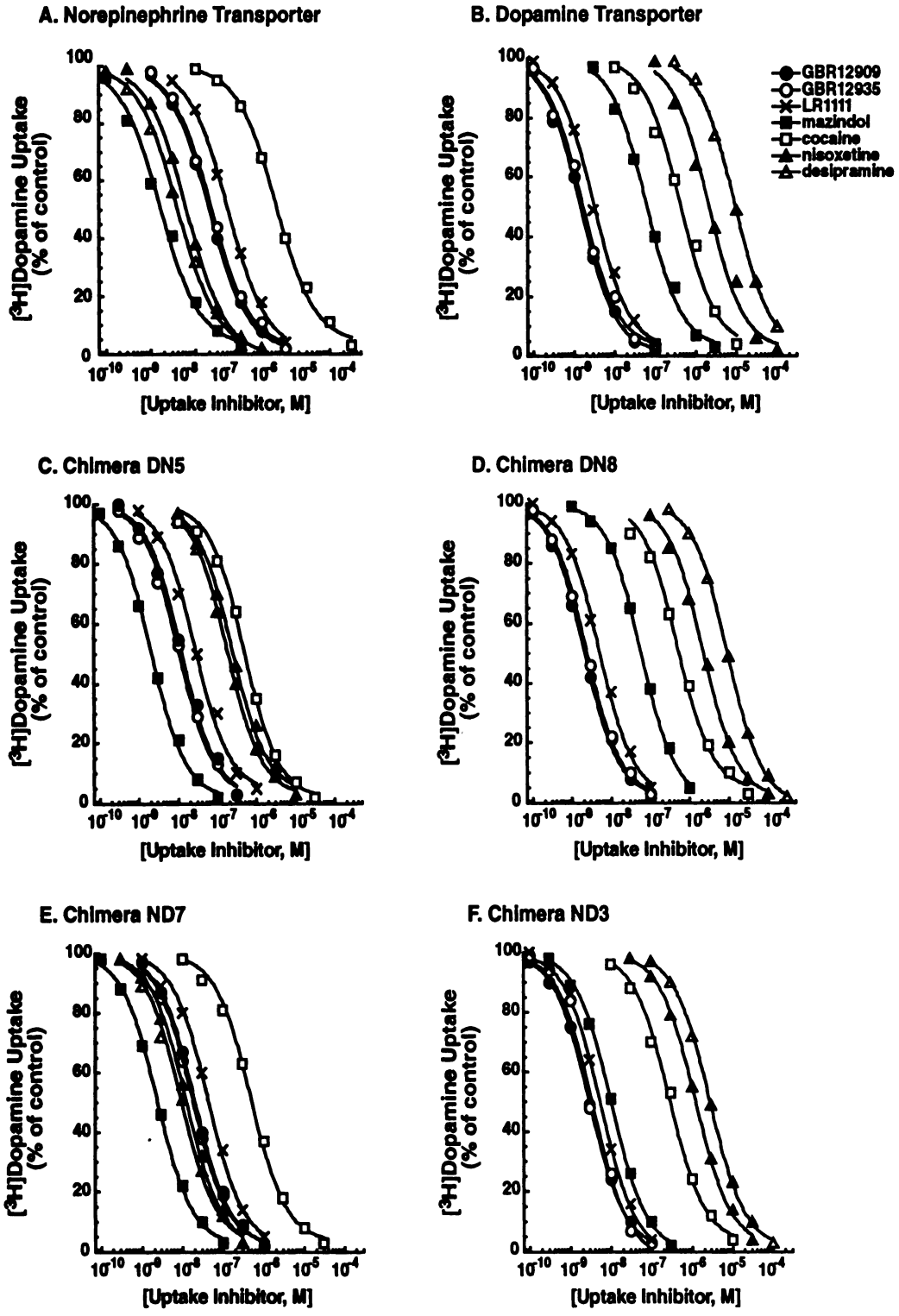

Fig. 1. Inhibition profiles for catecholamine transporter chimeras. Accumulation of [^{3}H]dopamine into transfected cells was assessed in the absence or presence of increasing concentrations of uptake inhibitors. The velocity (V) of dopamine uptake was normalized for transport in the absence of antagonist (V_o). Results represent the mean $V/V_o \pm$ standard error of four to six independent experiments performed in triplicate. All chimeras that possess DAT sequence elements within TMDs 5–7, including DN8 and ND3 (D and F), DN9, DN10, and ND1, demonstrate the same inhibition profile as DAT (B): GBR12909 ~ GBR12935 ~ LR1111 < mazindol < cocaine < nisoxetine < desipramine. Most chimeras with sequence elements within TMDs 5–7 from NET, including ND7 (E), ND8, ND10, ND11, and DN1, demonstrate the same pharmacological profile as NET (A): mazindol < desipramine ~ nisoxetine < GBR12909 ~ GBR12935 ~ LR1111 < cocaine. However, chimeras that include DAT sequence elements within TMDs 1–2, i.e., DN2, DN3, and DN5 (C), show higher affinity for GBR12909, GBR12935, and LR1111 compared with chimeras with NET sequence elements within TMDs 1–2.

Discussion

Little information has been available on the protein domains involved in the interaction of catecholamine carriers with transport inhibitors or on the key structural features of the carriers (e.g., the orientation and proximity of transmembrane domains) that might contribute to the formation of an antagonist binding site. To define the regions of NET and DAT that contribute to the selective binding of inhibitors and blockade of substrate translocation, we constructed and expressed a series of recombinant chimeras in which analogous sequence domains of the two transporters are exchanged. These structure-function analyses have identified specific domains of NET and DAT that influence their selectivity for clinical antidepressants and anorectics and drugs with reinforcing properties. Recognition of both nonselective inhibitors, such as cocaine, and recognition of inhibitors selective for each carrier are likely to involve some shared determinants of NET and DAT. However, the present study focuses on structural domains that distinguish NET and DAT and account for the differences in their affinities and functional selectivity for a variety of reuptake inhibitors.

Overlapping domains influence substrate translocation and selective interaction with uptake blockers. Structure-function analysis of the chimeric transporters indicates that the most influential determinants of DAT and NET selectivity for reuptake inhibitors lie within a domain spanning TMDs 5–7. DAT and all chimeras that possess DAT sequence elements within TMDs 5–7 demonstrate the profile GBR12909 < mazindol < cocaine < nisoxetine < desipramine. In contrast, NET and chimeras with sequence within TMDs 5–7 from NET generally demonstrate the profile mazindol < desipramine ≈ nisoxetine < GBR12909 < cocaine. Thus, determinants within a region spanning TMDs 5–7 appear to be important for conferring sensitivity to a variety of transport inhibitors, including mazindol, the tricyclic and nontricyclic antidepressants, and the diphenyl-piperazine derivatives such as GBR12935, GBR12909, and LR1111. This structural domain may also play a critical role in the mechanism of substrate translocation (Fig. 2) (5). Thirteen chimeras demonstrating substantially less translocation of catecholamine substrates (i.e., <10% the capacity for dopamine uptake compared with the wild-type DAT) each junction in a region spanning TMDs 5–8 (5), suggesting that

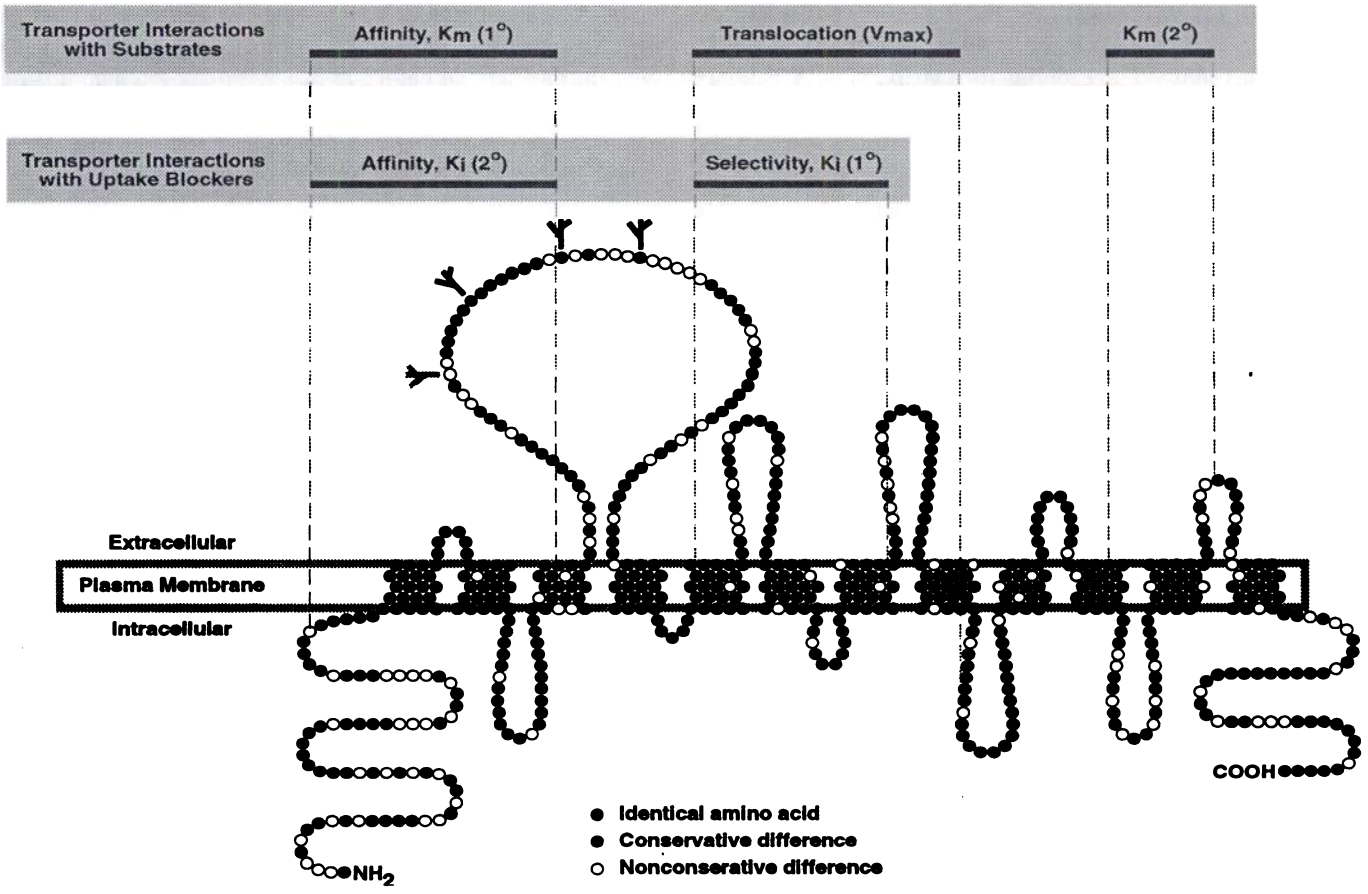

Fig. 2. Current model of the structural domains influencing the substrate and inhibitor selectivity of catecholamine transporters. Structural domains that contribute to the transport properties and selective blockade of DAT and NET are illustrated in this schematic representation of a catecholamine transporter. Domains that influence interaction of DAT and/or NET with uptake inhibitors are identified. Conservative and nonconservative amino acid differences between NET and DAT are indicated. The positions of domains that influence recognition or functional sensitivity to transport antagonists and the nature of interactions between these domains is largely unknown. Delineation of the structural domains involved in high affinity recognition of antidepressants suggest that determinants within TMDs 1–3 and TMDs 5–7 interact with antidepressants. Functional analyses of the chimeras have previously suggested that TMDs 1–3 influence apparent substrate affinity and that TMDs 5–8 may play a role in the mechanism of substrate translocation (5). These structural domains may be in proximity to one another during substrate recognition or translocation or interaction with uptake inhibitors.

this domain may be important for substrate translocation or for appropriate processing and insertion of transporters into the plasma membrane. Chimeras that junction in or near TMD 4 and TMD 9, flanking TMDs 5–8, do not differ in apparent substrate affinity (5). Thus, it appears that TMDs 5–8 are primarily responsible for events occurring subsequent to substrate recognition. It has been suggested that substrate dissociation or recycling processes after substrate recognition may be rate limiting in transport kinetics (28–30).

Giros *et al.* reported data on human catecholamine transporter chimeras suggesting the involvement of the last four TMDs in determining the apparent affinity for substrates (6). Our previous kinetic analyses of DAT/NET and NET/DAT using chimeras have shown a more pronounced effect of TMDs 1–3 in determining apparent K_m values for transport of catecholamines and a more subtle influence of TMD 10 and TMD 11 on the K_m for 1-methyl-4-phenylpyridinium (5). Whether this discrepancy results from the use of chimeras with different junctions, from different species, or from the use of different expression systems will require further investigation. For the serotonin transporter, studies of functional rat-human SERT chimeras have implicated residues in or near TM 12 in determining species-specific differences in inhibitor sensitivities (7).

Structural determinants of antidepressant selectivity. The present studies delineate two regions, a primary domain within TMDs 5–7 and a secondary domain within TMDs 1–3, that influence sensitivity to two norepinephrine-selective reuptake inhibitors, desipramine and nisoxetine. Although another report suggested a role for TMDs 6–8 in the inhibition of NET by desipramine, the contribution by TMDs 1–3 was not noted (6). We have previously demonstrated that TMDs 1–3 have a profound effect on the apparent affinity of NET and DAT for substrate catecholamines (5). Our analyses indicate that this region is also important in determining high affinity of NET for antidepressants (Fig. 2). Thus, the present study suggests an overlap between regions involved in the recognition of monoamine substrates and highly selective antidepressant inhibitors. Analyses of structure-activity relationships using monoamines and a number of therapeutic antidepressants have indicated that a protonated amine group is critical for transporter interaction of these compounds, suggesting that this chemical moiety may associate with a negatively charged residue of the transporters (31–35). An aspartate residue in TMD 1 is conserved among the monoamine transporters but not in other members of the gene family. The speculation that this residue interacts with the protonated amino groups of both substrates and selected inhibitors remains to be demonstrated.

TMDs 1–3 influence interaction with cocaine and selective blockers of DAT. Although the present study is more informative regarding the binding sites for selective inhibitors, it suggests the importance of a region encompassing TMD 1 for cocaine recognition. Chimeras that junction within or near TMD 1 (e.g., DN1 and ND1) demonstrate lower affinity for cocaine compared with other chimeras or wild-type transporters (Table 1). Similarly, RTI-55 [3β-(4-iodophenyl)-tropane-2β-carboxylic acid methyl ester], a structural analogue of cocaine, is less potent in blocking chimera DN1 than DAT or NET (data not shown). Kitayama *et al.* (36) have shown that mutation of the aspartate residue at position 79 in TMD 1 of the human DAT to glycine or alanine significantly reduces binding of a cocaine analogue, [^{3}H]-CFT [3β-(4-fluorophenyl)tropane-2β-carboxylic acid methyl ester], and dopamine transport. Others have shown that substitution of sequences from TMD 5 to TMD 8 of DAT into NET results in a dramatic (>30 fold) loss of affinity for cocaine (6). This suggests that cocaine's interactions are likely to involve several domains, including those shown to be important for substrate translocation (5, 6).

Data presented in Table 1 also suggests a possible influence of TMDs 1–3 on the affinity for GBR12909 and GBR12935. These compounds are more potent relative to other antagonists in blocking chimeras with DAT sequence elements within TMDs 1–3 (e.g., DN3 and DN5) than otherwise observed for chimeras possessing NET sequence elements within TMDs 5–7. In a recent report (37), 1-[2-(diphenylmethoxy)ethyl]-4-[2-(4-azido-3-[^{125}I]iodophenyl)-ethyl]piperazine, a photoactive GBR derivative, appears to react with residues near TMDs 1 and 2 of the DAT protein in the nucleus accumbens.

To date, no information is available on the positioning of transmembrane domains relative to each other or on whether the transporter is multimeric. Functional analyses of chimeric transporters demonstrate that two regions spanning TMDs 1–3 and TMDs 5–7 are important in determining high affinity interaction with desipramine and nisoxetine and suggest that structural determinants within these domains are important for interaction of monoamine transporters with antidepressants (Fig. 2). Similarly, TMDs 1–3 and TMDs 10–11 have recently been shown to influence apparent substrate affinity (4) and may be in proximity to one another during substrate recognition or translocation (Fig. 2). Thus, structure-function studies of chimeric transporters can provide evidence that some domains are more important for certain functions than for others, although they may not distinguish between direct effects on residues directly contacting the substrate and indirect effects in modifying transporter conformation. As more information becomes available on the higher-order structure of members of the family of sodium dependent transporters, precise interactions between residues and domains identified in these studies and their role in transport will become apparent.

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