Discrete Structural Domains and Cell-Specific Expression Determine Functional Selectivity of the Dopamine and Norepinephrine Transporters

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INTRODUCTION

The dopamine (DA) transporter (DAT) and norepinephrine (NE) transporter (NET) terminate catecholaminergic neurotransmission at synapses by high-affinity reuptake into presynaptic terminals and are the initial sites of action for a variety of drugs of abuse and therapeutic antidepressants. Recent cDNA cloning studies have demonstrated that NET and DAT are members of a family of sodium and chloride ion-dependent carriers, and that single cDNAs for NET and DAT effectively reconstitute many properties of the native transporters, including appro-priate pharmacology and ion dependence. Although catecholamine transporters have a high degree of sequence similarity, they are distin-guished by their monoamine substrate selectivities and their differential sensitivities to a wide spectrum of transport antagonists. DAT mediates uptake of DA, but it is an inefficient carrier of NE and other biogenic amines (Giros et al. 1991, 1992; Kilty et al. 1991; Shimada et al. 1991; Usdin et al. 1991), whereas NET transports both DA and NE (Pacholczyk et al. 1991). 1-methyl-4-phenylpyridinium (MPP+), the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), is also a substrate of DAT. This compound causes a selective and irrever-sible loss of nigrostriatal DA neurons and provides the basis for an experimental model of Parkinson's disease (Boyson 1991; Snyder and D'Amato 1986).

Reuptake systems for monoamines are also sensitive to a wide range of uptake inhibitors (e.g., therapeutic drugs including antidepressants and drugs of abuse such as cocaine). NET exhibits marked sensitivity to many therapeutic antidepressants, including desipramine (Pacholczyk et al. 1991). Clinical and behavioral studies of tricyclic antidepressant drugs suggest that inhibition of NE reuptake correlates with antidepressant activity (Baldessarini 1985). In contrast, drugs

such as cocaine, amphetamine, and methylphenidate, which nonselectively inhibit DAT and NET, are poor antidepressants, despite the fact that they have stimulant and euphoric effects in some individuals (Baldessarini 1985).

The structural determinants responsible for the functional properties of NET and DAT, such as selectivity for substrates and antidepressants, shared recognition of cocaine and amphetamine, and conserved mechanistic features (e.g., sodium and chloride ion coupling and substrate translocation) remain to be elucidated. NET and DAT cDNAs predict protein sequences of 617 and 618 amino acids, and hydropathy analyses of the sequences indicate 12 hydrophobic regions proposed to represent membrane-spanning domains (Amara and Kuhar 1993). The two transporters are most similar in the putative transmembrane domains (TM), and least conserved in the NH2 and COOH termini thought to be oriented on the cytoplasmic face of the plasma membrane. A large extracellular domain between TM3 and TM4 shows a similar lack of conservation. In order to assign the kinetic and pharmacologic properties of NET and DAT to general structural domains, a series of recombinant chimeric transporter genes was generated and expressed in mammalian cells (Buck and Amara 1994). This approach has proven successful in the structural analysis of guanosine triphosphate binding protein (G-protein)-coupled receptors (Frielle et al. 1988; Kobilka et al. 1988), G-proteins (Masters et al. 1988), and ligand-gated ion channels (Elsele et al. 1993; Li et al. 1992). Unlike other conventional methods of mapping functional domains, such as analyses of site-directed or deletion mutants in which the function of interest is frequently destroyed, chimeras can provide an assayable phenotype that allows positive inferences to be drawn from functions associated with specific protein domains. Analyses of the functional properties of NET/DAT chimeric transporters have delineated structural domains that determine apparent transport affinity and translocation selectivity of catecholamines and MPP+ (Buck and Amara 1994), and sensitivity to desipramine and GBR12935 (1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)-piperazine).

Pharmacologic and Kinetic Selectivity of Wild-Type Catecholamine Transporters

NET and DAT have strikingly similar sequences, with 64 percent amino acid identity and nearly 85 percent sequence similarity considering conservative amino acid differences. Despite their structural similarities, these two transporters demonstrate

physiologically and clinically important differences in selectivity for substrates and for a variety of uptake inhibitors. The functional properties of NET and DAT were examined by expression in HeLa cells using a vaccinia virus-based transient expression system. These studies show that NET has high apparent affinity for both NE and DA transport kinetic constant (KT)=0.4±.1 micromolars (µM) and 0.2±0.1 µM, respectively), whereas DAT expresses lower apparent affinity for NE and DA uptake (KT=5.7±2.7 µM and 3.2±0.4 µM, respectively) (figure 1, see also table1). In these kinetic analyses, differences in substrate affinity for the kinetically relevant site(s) are reflected in the Michaelis constant (KT). However, this constant is also influenced by rate constants for a number of events that occur subsequent to substrate recognition (e.g., trans-location and dissociation). In addition to the apparent affinity constant for substrate transport (KT), a second kinetic parameter, the velocity of transport at steadystate (Vmax), can be used to assess the capacity of a carrier to catalyze translocation. Vmax reflects the turnover number of the carrier, which varies with different substrates. However, Vmax is also influenced by expression levels, and therefore cannot be compared between cells with different numbers of carriers at the surface. Thus, for each transporter, translocation efficacy is reported as a rank order Vmax in cells transfected and assayed in parallel for dopamine and norepinephrine transport (table 1). Differences in the rank order Vmax between trans-porters reflect the relative efficiency with which different substrates are translocated. For example, cells transfected with DAT efficiently translocate DA as compared to NE (rank Vmax 1:9 for NE and DA). In contrast, NET accumulates dopamine nearly as efficiently as NE (rank Vmax 2:1 for NE and DA). These data clearly show that KT and rank Vmax can vary independently (figure 1).

Chimeric Catecholamine Transporters

To determine which structural domains confer distinct functional properties of NET and DAT, a series of chimeric recombinant transporters from NET and DAT cDNAs was constructed using a novel in vivo method that generates chimeras which junction in regions of sequence similarity (Buck and Amara 1994) (figure2). Briefly, DAT/NET and NET/DAT chimeras were engineered by subcloning the coding regions of NET and DAT in tandem into a pB SKII- plasmid vector. The DAT/NET and NET/DAT constructs were linearized and used to transform bacteria. The chimeric constructs are formed from the

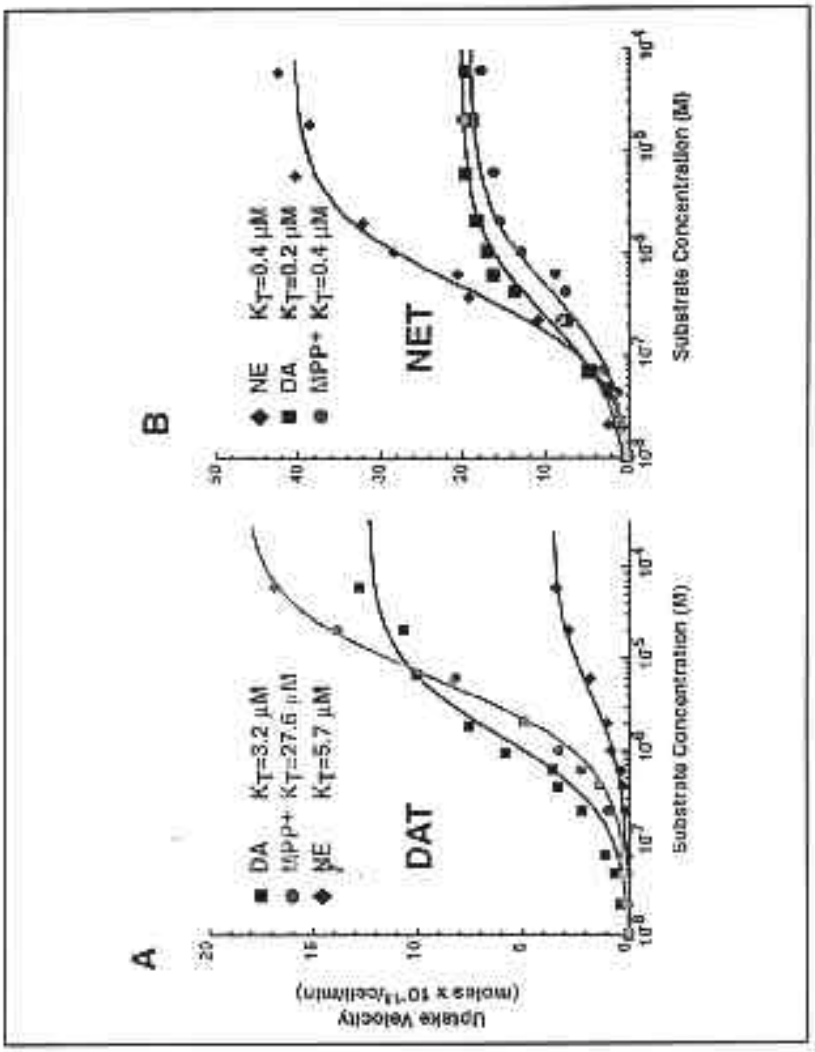

FIGURE 1. *Differences in substrate selectivity between NET and DAT. Panel A shows the uptake velocity of the DAT plotted as a function of substrate concentration for the substrates DA, NE, and MPP+. The DAT was expressed in HeLa cells as described and uptake of each of the substrates was measured in parallel assays done on the same transfected cells. The K_T for each substrate was obtained by nonlinear least-squares fits of substrate/velocity profiles using a data analysis program. Panel B shows the results of the same analysis for cells transfected with the NET.*

TABLE 1. Substrate selectivity of wild-type and chimera transporters.

KT (μM) (apparent affinity constant)			Vmax (uptake efficacy)	
Transporter	NE	DA	Relative (NE:DA)	Normalized (DA)
NET	0.4±0.1	0.2±0.1	2:1	1.6
ND 11	0.4±0.1	0.3±0.1	1:1	1.3
ND 10	0.9±0.4	0.6±0.3	1:1	1.1
ND 4	1.6±0.1	1.5±0.5	1:1	1.3
ND 3	1.7±0.5	0.8±0.2	1:1	0.6
ND 1	2.6±0.8	2.6±0.7	1:4	1.0
DAT	5.7±2.7	3.2±0.4	1:9	1.)
DN 10	2.4±0.8	2.2±0.8	1:20	1.8
DN 9	2.3±0.6	2.1±0.5	1:17	1.7
DN 3	2.0±0.6	1.1±0.4	1:5	0.8
DN2	2.1±0.3	1.8±0.2	4:5	0.9
DN1	0.4±0.1	0.2±0.1	1:2	0.7

NOTE: Kinetic analysis of substrate transport in HeLa cells expressing chimeric transporters or wild-type NET or DAT. Chimeras are identified as ND or DN, and numbered to reflect the transmembrane domain near which they junction. The kinetic constants, KT, and Vmax for each of the substrates were obtained by nonlinear least-squares fits of substrate/velocity profiles using data analysis program. The apparent affinity constant (KT) for transport of the substrates NE and DA is reported as the mean KT ± SEM as determined from three to six independent experiments performed in quadruplicate. For each transporter, translocation efficacy is reported as a rank order Vmax for DA and NE transport in cells transfected and assayed in parallel.

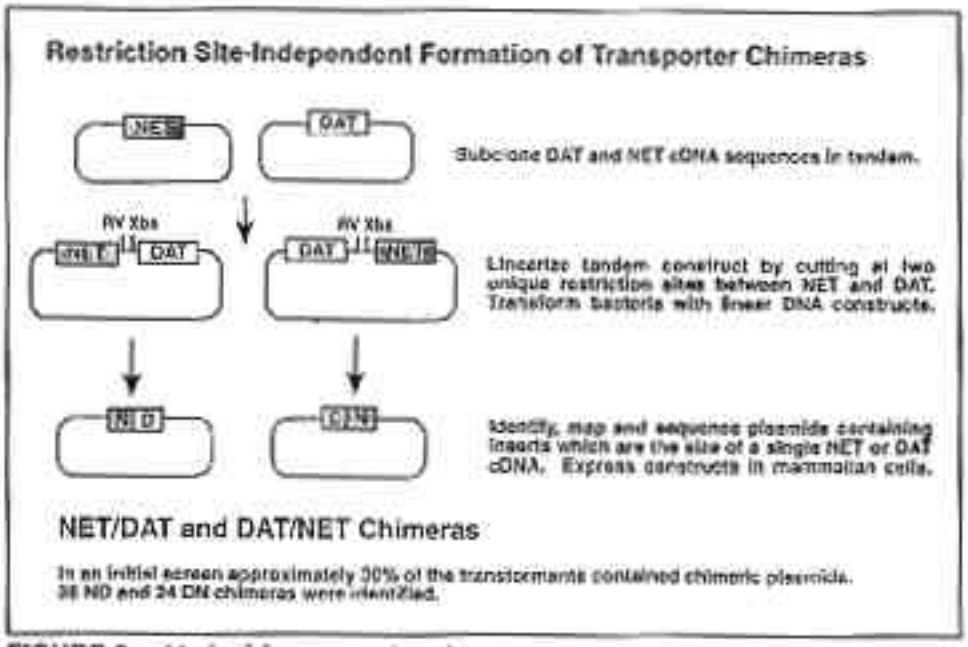

FIGURE 2. *Method for generating chimera transporters.*

linear DNA within the bacterial host in a process that probably involves endogenous exonuclease digestion of linear DNA, base pairing of homologous regions, and subsequent bacterial repair of the plasmid. The approach used favors generation of chimeras which junction at single sites in regions of conserved nucleic acid sequence, and typically resulted in functional chimeric transporters. Expression of the chimeric trans-porters in HeLa cells using a vaccinia virus/T7 polymerase-based expression system (Blakely et al. 1991) allowed an assessment of their apparent affinity constants (KT) and translocation efficacy (rank Vmax) for each catecholamine, and sensitivity to uptake antagonists such as DMI and GBR 12935. To provide an indication of the robust activity of these chimeras, the Vmax of each chimera was compared to the wild-type DAT activity observed in a parallel experiment (table1, normalized Vmax). Specific functional properties of NET and DAT were found to be correlated with particular protein sequence elements of the chimeric transporters, allowing differential properties of the two transporters to be assigned to specific domains. The structures of this series of chimeras, their apparent substrate affinity constants for uptake of NE and DA, and their selectivity for inhibition by desipramine are compared relative to wild-type NET and DAT in figure 3.

Transport is attenuated in all chimeras that junction in a region spanning TM5 through TM8 (including DN5, ND7, DN8, and ND8, figure 3c). Chimera DN5 demonstrated a dramatic reduction in transport (i.e., Vmax <5percent for NE uptake by NET) with no decrease in apparent substrate affinity relative to DN3 and DN9. However, surface [125I]RTI-55 binding to intact cells expressing these chimeric transporters was approximately 40 to 50 percent of binding to intact cells transfected with NET or 25 to 35percent of binding to cells expressing DAT. RTI-55 is a structural analog of cocaine with high affinity for DAT (Boja et al. 1991), which also recognizes NET although with lower affinity. These results suggest that this region spanning TM5 through TM8 may contain important elements of a catalytic domain involved in substrate translocation. However, in general the low transport capacity of chimeras junctioning between TM5 and TM8 limited detailed analysis of their transport kinetics, and may have restricted the ability to localize additional determinants influencing translocation efficacy and substrate KT which may also exist between TM5 and TM8. However, apparent substrate affinity and translocation efficacy were readily compared in chimeric transporters that junction in or before TM4 (e.g.,DN1, DN2, DN3, ND1, ND3, ND4) or after TM9 (e.g., DN9, DN10, ND10, and ND11) (figure 3b).

Discrete Domains Influence Catecholamine Substrate Selectivity

A goal of these studies was to identify structural domains that influence apparent affinity (KT) and catalytic efficacy (rank Vmax) of NET and DAT. The kinetics of catecholamine uptake by wild-type and chimeric trans-porters are summarized in table 1. The apparent substrate affinities of DN1 are consistent with those of NET, indicating that substitution of NET NH2-terminal sequences with that of DAT does not reduce apparent affinity relative to NET (table 1 and figure 3). DN3 has a fivefold higher KT for DA, and fivefold higher KT for NE than DN1 or NET (table1). These two chimeras define a domain that has a pronounced effect on substrate KT within a region spanning TM1 and TM3. The comple-mentary chimeras ND1 and ND3 further underscore the importance of this region in differentiating substrate KT values of NET and DAT. Furthermore, because ND1 has a modest two- to threefold lower apparent affinity than ND3 for both substrates, additional determinant(s) may reside near the amino-terminus of TM1. A region spanning TM10 and TM11 also contributes to differences in KT between NET and DAT for catecholamines.

DAT substrate selectivity for DA is largely due to the rate (Vmax) with which it transports DA relative to NE (see figure1). Chimeras DN3 and ND1 translocate DA more efficiently than NE (rank Vmax > 4:1 for DA and NE), indicating that amino-terminal determinants also influence relative Vmax. Thus, TM1 through TM3 play an important role in determining substrate KT, and TM2 through TM3 influence rank Vmax. DN9 and DN10 even more closely resemble DAT in their capacity to translocate DA relative to NE. These results suggest that some deter-minants that enhance the efficacy with which DA is transported relative to NE may fall within the central domain (TM5 through TM8) identified above as involved in catalyzing substrate translocation (see rank order Vmax in table 1).

Desipramine Specificity Involves Two Structural Domains

The NET and serotonin transporters are important initial targets for a number of tricyclic and other antidepressants used in the treatment of human depression. An important goal of the authors' studies was to identify the structural domain(s) involved in differential sensitivity of NET and DAT for antidepressants. Desipramine is one of the most potent tricyclic antidepressants in blocking NET, but is more than a

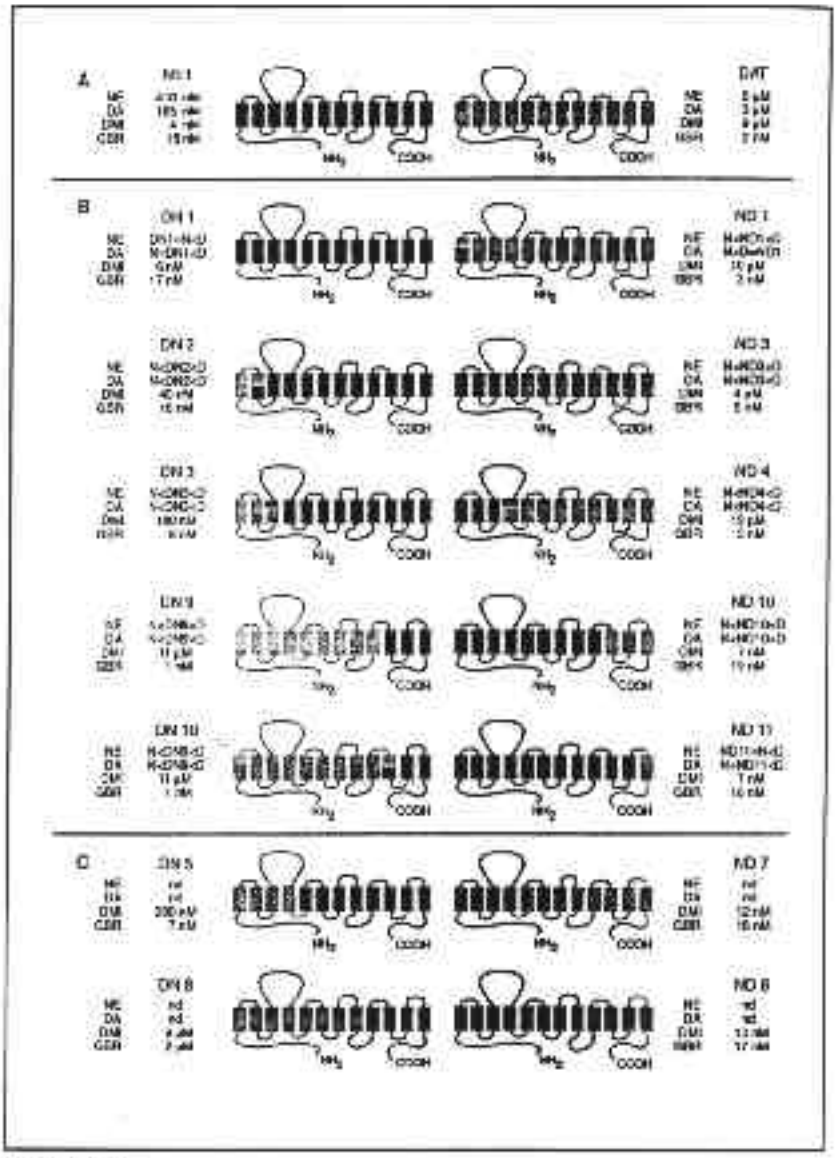

FIGURE 3.

FIGURE 3. *Wild-type and chimeric catecholamine transporters: summary of pharmacological selectivity for substrates and inhibitors. Panel A. Diagram of wild-type NET (in black) and DAT (in grey). Twelve hydrophobic domains are modeled as membrane-spanning domains. The NH_2 and COOH termini are presumed to lie in the cytoplasm (intracellular). Beside each transporter is a summary of its pharmacological characteristics. The following properties are summarized: the apparent affinity constant (K_m) for transport of the substrates NE and DA and the potency (K_i) with which desipramine (DMI) and GBR12935 inhibit [^{3}H]dopamine transport. DAT expresses lower apparent affinity for transport of the substrates DA, and NE than NET. The therapeutic antidepressant DMI demonstrates considerable selectivity for NET and GBR12935 is moderately selective for DAT. Panel B. Properties of functional chimeras. The functional characteristics of each chimera are summarized relative to the wild-type NET (N) and DAT (D). Chimeras are referred to as ND (i.e., NET/DAT) or DN (i.e., DAT/NET), and numbered to reflect the transmembrane domain nearest their junction. Uptake inhibition by desipramine and GBR 12935 was examined using [^{3}H]dopamine (10 nM). Uptake inhibition constants (K_i's) were determined using INPLOT. Data represent the mean $K_i \pm$ SEM determined from three independent experiments performed in triplicate. Panel C. Chimeras with reduced function. Apparent substrate affinities were not determined (nd) for several chimeras in which transport was significantly attenuated (e.g., DN5, ND7, DN8, and ND8), all of which function within a region spanning TM5 through TM8. For these chimeras apparent dopamine transport affinities were not readily determined. Thus, IC_{50} values determined using 200 nM substrate in the presence of increasing concentrations of unlabeled inhibitor are reported for these chimeras.*

163

thousandfold less potent as an inhibitor of DAT (figure 3a). Antagonism of DA uptake by the chimeric transporters identifies two domains of primary and secondary importance that are required for the effective blockade of catecholamine transport by desipramine (figure 3b).

The most influential determinants of desiramine selectivity for NET lie within a region spanning TM5 through TM7, delineated in part by ND7 and DN5, which differ seventeenfold in their sensitivity to desipramine. These two chimeras (as well as ND8, ND10, ND11, DN1, DN2, and DN3), each with nanomlar (nM) affinity for desipramine, share a region of sequence from NET extending from the amino-terminus of TM5 through TM7. In contrast, chimeric transporters that pharmacologically resemble DAT (e.g., despramine inhibition constant (Ki) = 9.1 to 10.0 μM for ND1, DN8, DN9, DN10) all possess sequence from the analogous region of DAT (figure 3). ND4 has lower sensitivity to desipramine than either DAT or NET, suggesting that this chimera may reflect a disruption in desiramine recognition rather than a shift toward DAT-like sensitivity. Several chimeric transporters (DN2, DN3, DN5, and ND3) displayed intermediate sensitivity for uptake antagonism by desipramine, and delineate a secondary domain spanning TM1 through TM3 that also influences desipramine recognition. Chimeras with NET sequence in the primary domain and DAT sequence in the secondary domain demonstrate intermediate selectivity for desipramine, which more closely resembles NET (e.g., Ki = 352 nM, 178 nM, and 189 nM for DN2, DN3, DN5) (figure 3). In contrast, a chimera with DAT sequence in the primary domain and NET sequence in the secondary domain displays an intermediate antidepressant sensitivity much more similar to DAT (e.g.,ND3 with Ki= 2.7 μM). Chimera DN1 displays specificity for desipramine consistent with NET, indicating the cytoplasmic NH2-terminus does not contribute to this secondary domain. Thus, secondary deter-minants for desipramine potency lie within a region spanning TM1 through TM3, and may overlap with the amino-terminal domain described above, which influences both KT and rank Vmax for the catecholamines DA and NE. In addition, the domain that has the greatest impact on desipramine Ki is within a region spanning TM5 through TM7, and may overlap the domain thought to be involved in catalyzing substrate translocation.

GBR 12935 Inhibition of Dopamine Uptake

GBR 12935, a potent uptake antagonist, is moderately selective for DAT relative to NET (Anderson 1987). Chimeric transporters (e.g.,

ND4 and ND7 with Ki values of 3±1 nM and 18±5 nM, respectively) delineate a domain spanning TM4 through TM7, which confers modest selectivity for GBR 12935 (figure 3). This domain may overlap with a region extending from TM5 through TM8, which contributes to selective translocation of DA and MPP+ by DAT. This domain may also overlap in part with a homologous region of NET, which is largely responsible for desipramine selectivity. Interestingly, antagonism of DA uptake by GBR 12935 is not impaired in ND4 relative to other chimeric trans-porters, as was inhibition by desipramine. These results suggest that ND4 may dissociate the inhibitory actions of GBR 12935 and desipramine.

Distribution of the Dopamine and Norepinephrine Transporters in the Central Nervous System

The distribution of the transporters in different neuronal populations in large part determines which substrates they are likely to encounter and which pathways they are most likely to influence. The two carriers show a distinct and nonoverlapping distribution. NE-containing cell bodies in the locus coeruleus and lateral tegmentum of the brainstem express NET messenger ribonucleic acid (mRNA), whereas DAT mRNA is expressed in dopaminergic cell groups of the mesencephalon, and the A12 and A13 dopaminergic cell groups of the diencephalon (Lorang et al. 1994). The cell type-specific expression of catecholamine transporters suggests that DAT and NET gene expression may be closely linked to cellular mechanisms that specify transmitter phenotype. Thus, the role of the transporters in DAT and NET influence catecholaminergic neurotrans-mission and appear to be determined by their distribution in the brain, as well as differences in their pharmacologic and kinetic properties.

Studies combining in situ hybridization of DAT and NET with immuno-histochemical detection of enzymes for catecholamine synthesis indicate that these two transporters are expressed only in catecholaminergic neurons. NET mRNA labeling is found in regions of the medulla oblon-gata and pons known to contain noradrenergic cell bodies (corresponding to the A4, A5, A6, and A7 cell groups) and neurons in the lateral teg-mentum and nucleus of the solitary tract that correspond to the locations of A1 and A2 catecholaminergic neurons. The locus coeruleus A6 cell group contains the greatest density of heavily labeled NET mRNA-containing neurons. No evidence for NET mRNA in tyrosine hydroxylase (TH)-positive dopaminergic neurons was observed even though NET has a higher affinity for DA than NE (Buck and Amara 1994; Pacholcyzk et al. 1991). Despite

the possibility that NET could serve as the transporter for epinephrine, no NET mRNA was detected in phenylethanolamine-N-methyl transferase (PNMT)-immunoreactive neurons.

Combined immunohistochemical/in situ hybridization analysis (see figure4) of NET mRNA in catecholaminergic neurons revealed that an overwhelming majority of dopamine ß-hydroxylase (DBH)-immuno-reactive cells in the A1-A7 cell groups also contain NET mRNA. Noradrenergic cells do not appear to express DAT mRNA, which is restricted to dopaminergic cells of the diencephalon and mesencephalon of the central nervous system (CNS). Northern blots show a single DAT

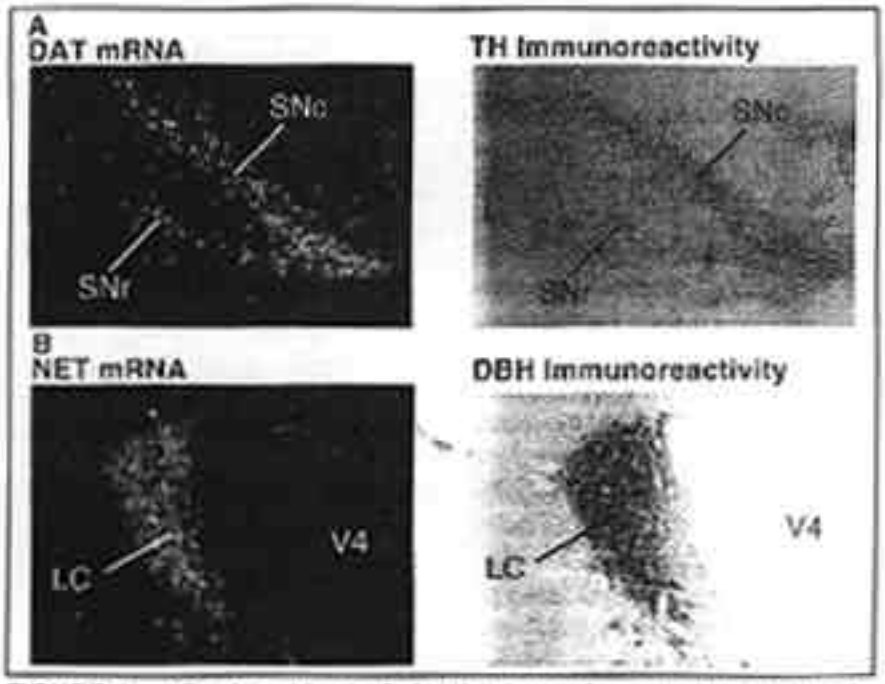

FIGURE 4. *Combined in situ hybridization and immunohistochemical analysis of the expression of the norepinephrine and dopamine transporters. Panel A. Darkfield and brightfield photomicrographs (4X) of the same field showing DAT mRNA containing neurons (left) and TH immunoreactivity (right) in the ventral tegmental area and substantia nigra (SN) of the rat. Panel B. Darkfield and brightfield photomicrographs (6.6X) of the same field showing NET mRNA containing neurons (left) and DBH immunoreactivity (right) in the locus coeruleus.*

transcript in neurons that comprise both the mesostriatal and mesocorti-colimbic dopaminergic pathways. Thus, neurons that send projections to the ventral and dorsal striatum appear to express the same DAT mRNA as do neurons that project to limbic regions, isocortex, and the spinal cord. The cells showing the most intense in situ hybridization to DAT mRNA are found in the substantia nigra and the ventral tegmental area, which corres-pond to the A8, A9, and A10 mesencephalic dopaminergic cell groups. Immunohistochemical analysis confirms that greater than 90percent of the TH-immunoreactive cells of the zona compacta and zona lateralis of the substantia nigra, and throughout the ventral tegmental area, are also intensely labeled for DAT mRNA. In the diencephalon, intense hybridi-zation with DAT mRNA is found in the zona incerta in cells presumably belonging to the A13 cell group, and moderate levels of labeling are found over neurons in the A12 cell group in the arcuate nucleus. Interestingly, TH-positive dopaminergic neurons in the preoptic area, periventricular nuclei, and the posterior hypothalamus do not appear to contain DAT mRNA, suggesting that DA release from terminals does not undergo rapid inactivation by reuptake into presynaptic cells. Thus, despite marked similarities in their primary structures and shared pharmacological properties, the nonoverlapping and differential expression patterns of DAT and NET suggest that these transporters function independently in distinct neuronal pathways. Moreover, the possibility that the expression or activity of these transporters is differentially regulated in these pathways provides a possible basis for understanding how pharmacological agents that act on both transporters exert such diverse effects on catecholaminergic neurotransmission.

DISCUSSION

In order to establish a potentially general approach to study the superfamily of sodium and chloride ion-dependent carriers, the authors constructed and expressed a series of functional recombinant chimeric transporters in which similar sequence domains and distinct functional properties of catecholamine transporters are exchanged (Buck and Amara 1994). These studies examine the structural domains responsible for differential selectivity of NET and DAT for a variety of substrates (e.g.,-DA, NE, MPP+) and uptake inhibitors (e.g.,GBR 12935 and desipramine), and are the first step in identifying the specific structural or regulatory determinants responsible for the distinct functional properties of NET and DAT. Antagonist recognition and substrate translocation are likely to involve some shared determinants of NET and DAT. However, studies

employing chimeric catecholamine transporters focus on structural features unique to NET and DAT that contribute to differences in their apparent substrate affinities, translocation efficacies, and antagonist selectivities. These data are summarized in the model shown in figure 5.

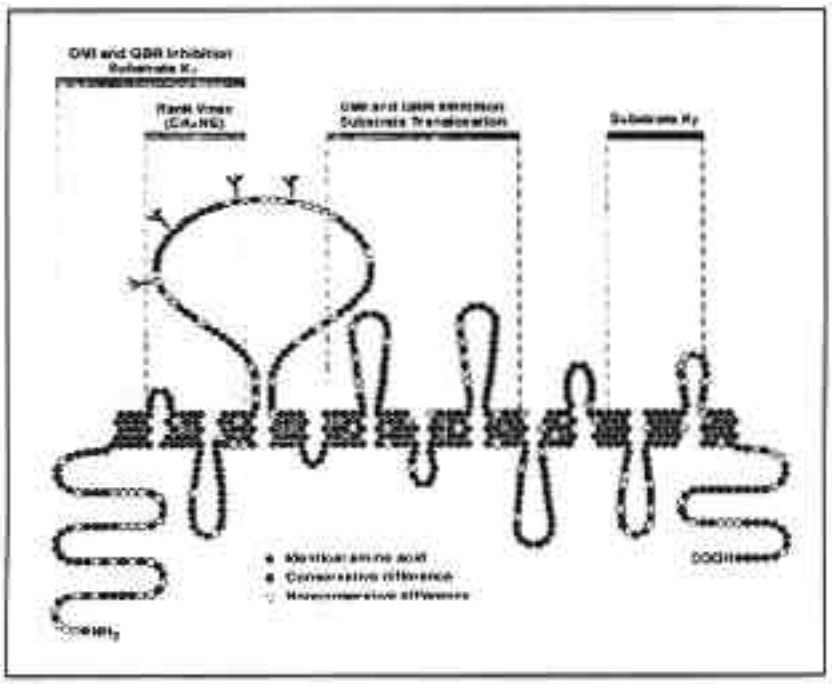

FIGURE 5. *Summary of structural domains influencing catecholamine transporter kinetics and pharmacology. Structural domains identified as having a major (1°) or secondary (2°) role in determining different kinetic properties and pharmacological selectivity of NET and DAT are illustrated in this schematic representation of a catecholamine transporter. Domains which influence apparent substrate affinity (K_m), inhibition by GBR 12935 and desipramine (K_i) and translocation capacity (rank V_{max}) are identified. Conservative and nonconservative amino acid differences between NET and DAT are indicated.*

Structural Determinants of Antidepressant and Catecholamine Selectivity

Structure activity studies indicate that a protonated amine group is a critical feature of both transported substrates and a number of therapeutic antidepressants recognized by monoamine transporters (De Paulis et al. 1978; Koe 1976; Maxwell and White 1978; Maxwell et al. 1969, 1976). The terminal ammonium of monoamines and antidepressants may associate with a negatively charged residue of the transporters, whereas planar aromatic moieties of these compounds may associate with an analogous surface by hydrophobic and/or van der Waals bonding. Current studies delineate determinants within a region spanning TM1 through TM3 which have a pronounced effect on apparent substrate affinity. Additional determinants within a region spanning TM2 and TM3 may be involved in selective translocation of DA relative to NE by DAT. A homologous region of NET may influence selective antagonism by desipramine. Within these domains, negatively charged aspartate (D) and glutamate (E) residues at positions NETD64/DATD68, NETD75/DATD79, and NETE113/DATE117 are conserved in monoamine carriers and may recognize the terminal ammonium of substrate catecholamines and tricyclic antidepressants. NETD75 and DATD79 are of particular interest because they are uniquely conserved in monoamine carriers. Mutation of aspartate residue DATD79 to glutamate or to neutral alanine or glycine residues has been reported to dramatically impair DA uptake (Vmax values were < 7 percent to 24 percent of wild-type values), and results in a threefold to sixfold decrease in the apparent affinity for DA uptake (Kitayama et al. 1992). Results obtained from studies of catecholamine transporter chimeras indicate that amino acids in close proximity to these conserved acidic residues are important in determining both the different apparent affinity and differential translocation efficacy of DAT and NET and differential sensitivity to antidepressants (figure 5). Within TM1, a single amino acid difference (valine for isoleucine) between NET and DAT is observed. In TM2, three conservative differences and a nonconservation substitution (NETT99 versus DATL103) are found, while the sequence between TM2 and TM3 is somewhat less conserved. These results underscore the possible importance of conservative amino acid substitutions, which have been shown to be critical for agonist pharmacology in other membrane proteins (Schmieden et al. 1992).

Studies of catecholamine binding to mutant adrenergic receptors indicate that serine and aspartate residues within the hydrophic

regions are also important in catecholamine recognition (Chung et al. 1988; Fraser et al. 1988; Strader et al. 1988, 1989; Wang et al. 1991). Through hydrogen bonds, serine residues may possibly interact with meta- and parahydroxyl moieties of the phenyl ring of the substrate catecholamines. In DAT, replacement of the serine residues at positions DATS356 and DATS359 in TM7 by alanine or glycine has recently been reported to cause reductions in DA and MPP+ uptake (Kitayama et al. 1992). These serine residues are conserved in NET, suggesting that these amino acids are not directly responsible for differences in the transport properties of the two carriers. However, DATS358 is not conserved in NET (which contains alanine). These serine residues fall within a region extending from TM5 to TM7, which in the current studies appear to be involved in translocation selectivity, and may also play a more basic role in the mechanism of substrate translocation. DATS358 or one of several other nonconserved residues within this domain may therefore be important in determining greater translocation efficacy for DA than NE by DAT. This domain does not appear to influence apparent substrate affinity, suggesting that substrate recognition is not altered. Thus, it appears that this central domain is primarily responsible for events occurring subsequent to substrate recognition. Interestingly, it has been suggested that a substrate dissociation or recycling processes following substrate recognition may be rate limiting in transport kinetics (Friedrich and Bonisch 1986; Schomig and Bonisch 1988; Zimanyi et al. 1989).

Overlapping Domains for Antagonist Affinity and Substrate Translocation

Structure-function analysis of the chimeric transporters indicates that overlapping domains may influence transporter affinity for desipramine (a potent NET antagonist) and GBR 12935 (a selective DAT inhibitor), and translocation efficacy (Vmax) (summarized in figure 5). Chimeras junctioning between TM5 and TM8 also demonstrate attenuated transport (i.e., the capacity of these chimeras to transport DA is < 5 percent that of NET), suggesting that they may define a structural domain involved in substrate translocation. Despite the low transport efficacy of these chimeras, the potency of various inhibitors was readily assessed. Primary determinants of desipramine affinity are found within a region spanning TM5 through TM7 delineated in part by DN5 and ND7. Similarly, determinants of substrate translocation selectivity lie within a region extending from TM3 through TM9.

An overlapping region spanning TM5 through TM8 may also define a domain critical for substrate translocation. These results imply that this domain may influence transport by positioning determinants involved in substrate translocation and antagonist recognition. To date, no infor-mation is available on the positioning of transmembrane domains relative to each other, or if the transporter is multimeric. In spite of these potential limitations, all of the chimeras reported here are functional to a significant degree, indicating that the secondary and tertiary structures of NET and DAT are largely maintained. 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 interacting with the substrates and indirect effects modifying transporter conformation. As more information becomes available on the higher order structure of members of the family of sodium-dependent transporters, precise inter-actions between residues and domains identified in these studies and their role in transport will become apparent.

SUMMARY

The successful generation and functional expression of a series of recombinant chimeric transporters, in which distinct functional properties of NET and DAT are exchanged, have allowed the assignment of a number of important functional properties of MPP+ and antidepressant-sensitive catecholamine transporters to specific domains within their primary structure. These studies are the first comprehensive structure-function analysis of members of the rapidly growing superfamily of Na+/Cl- carriers using chimeric transporters. This represents the first step in identifying the specific structural or regulatory determinants that differentiate NET and DAT. An appreciation of the potentially distinct sites for substrate recognition, translocation, and transport inhibition of NET and DAT may facilitate the development of more selective drugs for the treatment of stimulant addiction, human depression, and other affective disorders.

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Click here to go to page 176