Determinatation of Residues in the Norepinephrine Transporter That Are Critical for Tricyclic Antidepressant Affinity*

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The norepinephrine (NET) and dopamine (DAT) transporters are highly homologous proteins, displaying many pharmacological similarities. Both transport dopamine with higher affinity than norepinephrine and are targets for the psychostimulants cocaine and amphetamine. However, they strikingly contrast in their affinities for tricyclic antidepressants (TCA). Previous studies, based on chimeric proteins between DAT and NET suggest that domains ranging from putative transmembrane domain (TMD) 5 to 8 are involved in the high affinity binding of TCA to NET. We substituted 24 amino acids within this region in the human NET with their counterparts in the human DAT, resulting in 22 different mutants. Mutations of residues located in extra- or intracytoplasmic loops have no effect on binding affinity of neither TCA nor cocaine. Three point mutations in TMD6 (F316C), -7 (V356S), and -8 (G400L) induced a loss of TCA binding affinity of 8-, 5-, and 4-fold, respectively, without affecting the affinity of cocaine. The triple mutation F316C/V356S/G400L produced a 40-fold shift in desipramine affinity. These three residues are strongly conserved in all TCA-sensitive transporters cloned in mammalian and nonmammalian species. A strong shift in TCA affinity (IC50) was also observed for double mutants F316C/D336T (35-fold) and S399P/G400L (80-fold for nortriptyline and 1000-fold for desipramine). Reverse mutations P401S/L402G in hDAT did not elicit any gain in TCA affinities, whereas C318F and S358V reverse mutations P401S/L402G in hDAT did not elicit any gain in TCA affinities, whereas C318F and S358V resulted in a 3- and 10-fold increase in affinity, respectively. Our results clearly indicate that two residues located in TMD6 and -7 of hNET may play an important role in TCA interaction and that a critical region in TMD8 is likely to be involved in the tertiary structure allowing the high affinity binding of TCA.

The dopamine (DAT)1 and norepinephrine (NET) transporters mediate reuptake of catecholamines into presynaptic terminals, thus limiting the extracellular concentration of norepinephrine and dopamine and their availability for receptor activation (1–3). NET (4–7) and DAT (8–13) have been cloned from different species, establishing their membership in the protein superfamily defined as Na+/Cl−-dependent transporters (SERT) (17–20) form the subfamily of monoamine transporters. DAT and NET are the most closely related members of this subfamily with about 80% similarity (65% identity) in their amino acid sequences. These two transporters also share several pharmacological properties, e.g. both transport DA with a higher affinity than NE (16, 21, 22), and both are targets for psychostimulants such as cocaine and amphetamine. On the other hand, tricyclic antidepressants (TCA), which elicit their antidepressant effects through blockade of NET (23, 24) and/or SERT (25), are highly discriminative drugs between NET and DAT. For example, the TCA desipramine and nortriptyline inhibit NET in the nanomolar range whereas they inhibit DAT in the micromolar range (21, 26).

To investigate the structure/activity relationships of the family of Na+/Cl−-dependent transporters, several groups have used site-directed mutagenesis and/or generation of chimeric proteins. Experiments using mutagenesis have been designed to investigate the role of amino acids endowed with a functional moiety possibly involved in mechanisms such as charge transfer (e.g. Lys, Arg, Glu, and Asp) (27, 28), amine fixation (Ser) (27, 29), N-glycosylation (Asn) (30, 31), or tertiary structure stabilization (Pro and Cys) (32–34).

Functional chimeras have been successfully constructed between the two closely related monoamine transporters rat DAT and human NET (21, 35), between human DAT and NET (26), between rat and human SERT (36), and between the SERT and NET second extracellular loop (37). Because DAT and NET are similar, yet with distinct pharmacological differences, chimeras between DAT and NET were extremely informative: the loss of TCA binding in NET is observed either for proteins that comprise TMD6 to TMD8 of DAT (chimera M (26)), or for proteins that fuse the NH2-terminal region of DAT to the COOH-terminal region of NET between TMD5 and TMD9 (chimera DN5 (35)) or between TMD8 and TMD9 (chimera DN8)

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1 The abbreviations used are: DAT, dopamine; NET, norepinephrine; SERT, serotonin transporter; TCA, tricyclic antidepressants; PBS, phosphate-buffered saline; TMD, transmembrane domain; EL, extracellular loop; IL, intracytoplasmic loop.
(35). Thus, determinants within a region spanning TMD5–8 appear to be important for conferring TCA sensitivity. In addition, cocain had absolutely no inhibitory effect on human DAT (comprising Val to Ala changes). We suspected that one of the 24 nonconserved residues could be implicated in the high affinity for TCA. Therefore, we systematically mutated these 24 amino acids in TMD6 to TMD8 of human NET and substituted them with their counterparts in human DAT. The aim of this study was to generate NET mutants that lose their affinity for TCA and eventually to identify particular amino acids responsible for the high affinity binding of TCA to NET.

Materials and Methods

Construction of Human NET Mutants—All the mutants were constructed using polymerase chain reaction with primers containing a de- scribed mutation. For hNET mutants H296S, K303C, A330S, D336T, I384F, and H370Q/E371K/K373S/N375P, E377G, E382D, A384P, and S402A the po-

ed in extra- (EL) or intracytoplasmic (IL) loops, were

mid integration with 800 µg/ml Geneticin (G418, Life Technologies, Inc.) for 4 weeks. Stable clones were then grown in Dulbecco’s modified Eagle’s medium/Ham’s F-12 without G418. Immunofluorescence staining was performed as already described (38). Brieﬂy, stably transfected and nontransfected HEK293 cells were grown on polyornithine-coated coverslips inserted in the wells of six-well culture dishes. After washing with phosphate-buffered saline (PBS), the cells were ﬁxed with freshly prepared PBS containing 4% paraformaldehyde for 20 min at room temperature, followed by three washes with ice-cold PBS for 2 min each. Permeabilization of cell membranes was performed with PBS containing 0.25% Triton X-100 and 0.12% gelatin for 20 min at 4 °C. Labeling with primary antibodies directed against a COOH-terminal peptide sequence of the human NET (C590–607 (39), 1:250 dilution) was achieved in the same solution for 2 h at room temperature, followed by three washes with ice-cold PBS. Immunofluorescence labeling was performed with an ﬂuorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma; 1:200 dilution) in PBS for 1 h. After three washes with ice-cold PBS, the coverslips were mounted in Vectashield (Vector, Burlingame, CA). Immunostaining was visualized by confocal laser micros- copy (Leica TCS-NT, New York) at an excitation wavelength of 488 nm (emission at 514 nm) at 600-fold magnification.

Uptake Experiments—We transfected either LLC-PK1 cells by elec- troporation (Equibio, St. Louis, MO) using 1 to 4 µg of plasmid or COS-7 cells by the calcium-phosphate method using 5 to 10 µg of plasmid. Cells were harvested 2 days later. For hNET mutants P401S, L402G, S420H, and mutant S420A (IL4) clearly exhibited less than 10% of native transporters) that were not signiﬁcantly different from those of the wild-type transporters. However, hNET mutants S288I, S8255N8292F, located in the third extracellular loop (EL3) exhibited a T-fold increased capacity for DA. Three mutants, modiﬁed in extra- (EL) or intracytoplasmic (IL) loops, were devoid of normal uptake activity. Mutant H296S (EL3) and H370Q/E371K/K373S/N375P (EL4) were completely inactive and mutant S420A (ILA) clearly exhibited less than 10% of wild-type NET transport activity. As this uptake was measured on whole intact cells, we wondered whether these mutants were correctly targeted and expressed at the plasma membrane in transfected cells. To address this issue all mutants were stably transfected in eukaryotic cells (HEK293). Transporter protein expression was examined in permeabilized cells by indirect immunofluorescence using primary antibodies directed against a COOH-terminal peptide sequence of the COOH-terminal end of NET (39). All mutants exhibiting an uptake activity (>10% of native transporters) were correctly expressed at the plasma membrane (not shown). Fig. 2 shows that mutant hNET-H296S was not targeted to the plasma membrane, and that plasma membrane localization of

Functional Analysis of Transporter Mutants—We have generated 22 mutants of the human NET, in which amino acids in TMD4 to TMD8 were replaced with their counterparts in DAT (Fig. 1) as well as five reverse mutations in the strategic residues of hDAT. After transient transfection in eukaryotic cells, most of the hNET mutants were able to transport tritiated DA (Table I) and (NE (data not shown) with kinetic parameters (Km and Vmax) that were not signiﬁcantly different from those of the wild-type transporters. However, hNET mutants S288I/ N8292F/N8292F, located in the third extracellular loop (EL3) exhibited a T-fold increased capacity for DA. Three mutants, modified in extra- (EL) or intracytoplasmic (IL) loops, were devoid of normal uptake activity. Mutant H296S (EL3) and H370Q/E371K/K373S/N375P (EL4) were completely inactive and mutant S420A (ILA) clearly exhibited less than 10% of wild-type NET transport activity. As this uptake was measured on whole intact cells, we wondered whether these mutants were correctly targeted and expressed at the plasma membrane in transfected cells. To address this issue all mutants were stably transfected in eukaryotic cells (HEK293). Transporter protein expression was examined in permeabilized cells by indirect immunofluorescence using primary antibodies directed against a COOH-terminal peptide sequence of the COOH-terminal end of NET (39). All mutants exhibiting an uptake activity (>10% of native transporters) were correctly expressed at the plasma membrane (not shown). Fig. 2 shows that mutant hNET-H296S was not targeted to the plasma membrane, and that plasma membrane localization of

Inhibitor-binding Sites in the Norepinephrine Transporter

Chemicals—[3H]Dopamine (42 Ci/mmol) and [3H]norepinephrine (36 Ci/mmol) were purchased from NEN (Boston, MA). All other chemicals were obtained from Sigma (St. Louis, MO) or RBI (Natick, MA). Cocaine was kindly provided by Dr. M.-H. Thiebot (INSERM U288, Paris, France).

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H370Q/E371K/E377G/N375P, and S420A was very low and similar to background (measured in the presence of 10 μM nomifensine).

Inhibitor-binding Sites in the Norepinephrine Transporter

Kinetic parameters ($K_m$ and $V_{max}$) of $[^{3}H]$dopamine uptake in COS-7 cells expressing wild-type hNET, hDAT, hNET mutants or hDAT mutants

$K_m$ values are mean ± S.E. of three to six experiments, each performed in triplicate. $V_{max}$ value for the uptake of $[^{3}H]$DA by the wild-type NET was 36 ± 15 fmol/min/10^6 cells and this value was considered 100% uptake. $[^{3}H]$DA uptake by mutants hNET-H296S, hNET-H370Q/E371K/K373S/N375P, and S420A was very low and similar to background (measured in the presence of 10 μM nomifensine).

| $K_m$ (nM) | $V_{max}$ (% of wild-type) |
|-----------|-----------------------------|
| hNET      | 730 ± 155                   |
| hNET-H296S| 580 ± 300                   |
| hNET-S396A| 700 ± 447                   |
| hNET-H296S| No uptake                   |
| hNET-K303C| 600 ± 238                   |
| hNET-F316C| 800 ± 116                   |
| hNET-A330S| 600 ± 42                    |
| hNET-D336T| 300 ± 102                   |
| hNET-F316C-D336T| 350 ± 364 |
| hNET-V356S| 980 ± 136                   |
| hNET-1364F| 410 ± 122                   |
| hNET-H370Q/E371K/K373S/N375P| No uptake |
| hNET-H370Q/E371K/K373S/E377G| 190 ± 86           |
| hNET-H370Q| 400 ± 104                   |
| hNET-N375P| 500 ± 240                   |
| hNET-S396A| 920 ± 230                   |
| hNET-S399P| 620 ± 300                   |
| hNET-S399P/G400L| 690 ± 404 |
| hNET-F403A| 460 ± 80                    |
| hNET-A413T| 230 ± 15                    |
| hNET-S420A| No uptake                   |
| hNET-F316C/V356S/G400L| 957 ± 170          |
| hDAT      | 2,140 ± 920                 |
| hDAT-C318F| 1,940 ± 300                 |
| hDAT-S356V| 2,650 ± 960                 |
| hDAT-P401S| 1,680 ± 510                 |
| hDAT-L402S| 3,000 ± 300                 |
| hDAT-F401G/L402S| 870 ± 230      |

Our data clearly showed (Table II) that exchanges of amino acids located in the intracytoplasmic or extracytoplasmic loops had no significant effect on TCA affinities. This applies to mutants S288I/N289D/N292R and K303C within EL3, mutants A330S and D336T within IL3, and mutants H370Q/E371K/K373S/E377G/N375P, and S420A was very low and similar to background (measured in the presence of 10 μM nomifensine).
S399P/G400L. The desipramine competition curve fitted a two-site model (IC\textsubscript{50}(1) = 1 nM and IC\textsubscript{50}(2) = 2690 nM), whereas the nortriptyline competition curve did not fit adequately neither a one- nor a two-site model (global IC\textsubscript{50} = 1670 nM) (Table II, Fig. 3). Therefore, as these mutations modify the classical one-site inhibition curve, it can be inferred that the different affinity states are reflecting the presence of allosteric conformational changes in this mutant transporter. Finally, we constructed a triple mutant combining the three individual changes that induce an affinity shift, F316C, V356S, and G400L. This mutant displayed a very strong shift for both desipramine (36-fold) and nortriptyline (9-fold), thus confirming the involvement of these three residues in the binding affinity of TCAs (Table II).

Inhibition of [\textsuperscript{3}H]DA Uptake by Desipramine and Nortriptyline in hDAT Mutants—To determine whether these changes in TMD6, TMD7, and TMD8 were important for TCA inhibition of hDAT, we also mutated the corresponding amino acids in hDAT to their counterpart in hNET. In TMD6, a change from Cys to Phe in position 318 of the hDAT triggered a significant 2–3-fold gain in affinity for TCAs (Table III). In TMD8, single mutant P401S showed a tendency toward a better affinity for desipramine and a significant 1.5-fold better affinity for nortriptyline. However, double mutant hDAT P401S/L402G did not exhibit any affinity increase for TCA but on the contrary displayed a loss of affinity for both desipramine and nortriptyline (without exhibiting a multiple site competition curve to these compounds). Mutant hDAT-S358V was the most impressive, with an IC\textsubscript{50} value of 940 nM for desipramine (Table III), 10-fold better than what was observed on the wild-type hDAT.

**DISCUSSION**

Chimeric transporters from different members of the monoamine subfamily have already provided information about the participation of restricted regions in drug or substrate binding (21, 26, 35–37). However, single amino acids that may be directly involved in the high-affinity binding of tricyclic antidepressants in NET are not yet known. The present study was designed to determine precisely the role of individual residues of DAT and NET in determining their pharmacological characteristics. It seems now quite obvious that all amine transporters cloned in nonmammalian species are sensitive to TCA. This is the case for species as far from mammalians as *Caenorhabditis elegans* (13) for ceDAT, *Drosophila melanogaster* (17, 18) for dSERT, *Rana catesbiana* (7) for fET, and the teleost fish

**TABLE II**

| IC\textsubscript{50} values for inhibition of [\textsuperscript{3}H]DA uptake by desipramine, nortriptyline, and cocaine in COS-7 or LLC-PK1 cells expressing wild-type NET, DAT, or NET mutants |
|---|
| **IC\textsubscript{50} ± SE (nM)** | Desipramine | Nortriptyline | Cocaine |
| hNET | 1.2 ± 0.2 | 21 ± 2 | 91 ± 6.0 |
| hNET-H280R | 2.9 ± 0.9 | 24 ± 9 | 172 ± 110 |
| hNET-S288I/N289D/N292R | 2.2 ± 0.5 | 28 ± 5 | 135 ± 80 |
| hNET-K303S | 2.2 ± 1.2 | 15 ± 1 | 189 ± 70 |
| hNET-F316C | 7.8 ± 0.7\* | 160 ± 20\* | 41 ± 6\* |
| hNET-N375P | 1.0 ± 0.2 | 24 ± 12 | 133 ± 5 |
| hNET-D336T | 1.4 ± 0.2 | 28 ± 5 | 85 ± 33 |
| hNET-F316C/D336T | 34 ± 3.6\* | 970 ± 220\* | 94 ± 46 |
| hNET-V356S | 4.6 ± 0.2\* | 210 ± 91\* | 61 ± 4 |
| hNET-I364F | 1.3 ± 0.2 | 22 ± 7 | 130 ± 30 |
| hNET-H370Q/E371K/K373S/E377G | 1.7 ± 0.3 | 22 ± 1 | 48 ± 9 |
| hNET-N375P | 0.5 ± 0.1 | 36 ± 8 | 120 ± 19 |
| hNET-E377G/E382D/A384P | 0.7 ± 0.2 | 28 ± 7 | 125 ± 65 |
| hNET-S396A | 1.8 ± 0.3 | 96 ± 52 | 66 ± 20 |
| hNET-S399P | 1.7 ± 0.2 | 40 ± 26 | 72 ± 34 |
| hNET-G400L | 3.8 ± 0.5\* | 133 ± 38\* | 60 ± 20 |
| hNET-S399P/G400L | 1.4 ± 0.4 | 1,670 ± 280\* | 71 ± 28 |
| hNET-F403A | 2,890 ± 450\* | 123 ± 60 | 115 ± 49 |
| hNET-A413T | 0.8 ± 0.1 | 123 ± 60 | 115 ± 49 |
| hNET-A413T | 1.2 ± 0.3 | 33 ± 7 | 61 ± 16 |
| hNET-F316C/V356S/G400L | 43 ± 14\* | 182 ± 51\* | ND\* |
| hDAT | 9,250 ± 580 | 28,200 ± 1,550 | 260 ± 22 |

\* p < 0.05 as compared to NET.

\* ND, not determined.
our work on mutation from NET to DAT was to search for a loss of function, possibly revealed by a single point mutation. We generated 22 mutants of the hNET, in which amino acids in TMD5 to TMD8 were replaced by their counterparts in hDAT. We chose to target this domain because two independent studies had clearly demonstrated the involvement of the central region of the NET in high affinity binding of TCA (21, 26). To cover as many mutations as possible, we occasionally changed neighboring bases to include up to 4 amino acid changes (Fig. 1). Furthermore, some reversed mutations of special interest were also performed in hDAT, and these amino acids were replaced by their counterparts in hNET (Fig. 1). However, it should be reminded that we have not engineered any changes in the 17 residues displaying conservative changes (Fig. 1) between DAT and NET, which may deserve further analysis.

**Efficacy of the Uptake Activity**—We directly studied the uptake properties of our mutant collection but did not investigate their binding properties, as this would not be informative regarding uptake mechanisms. All mutants in TMDs displayed a measurable uptake after transient transfection in eukaryotic cells (Table I) and were correctly expressed at the plasma membrane (not shown). Among NET mutational within the loops, three displayed a severe loss of uptake activity. These mutants were in EL3 (H296S), EL4 (H370Q/E371K/K373S/N375P), and IL4 (S420A) (Fig. 2). It is interesting to note that hNET mutants H370Q/E371K/K373S/E377G and E375P, which partially overlap mutations of the nonexpressed H370Q/E371K/K373S/N375P, were able to transport DA and NE with the same efficacy as the wild-type NET. These findings imply that the changed amino acids in the loop of these mutants may play a role in correct membrane processing or targeting of NET, although presently the mechanism involved is unknown. This finding is in agreement with the importance of this central domain of catecholamine transporters in their functional expression at the plasma membrane. Furthermore, it was previously shown that some chimeric proteins engineered in this region were devoid of uptake activities (26, 35).

**Interactions with Cocaine**—It was previously described that chimeras having predominantly the NET sequence but with TMD5 to TMD8 replaced by the DAT cassette, displayed a significant loss of affinity for cocaine (26). None of our 19 functional hNET mutants (Table II) displayed any loss of cocaine inhibition, which indicates that this property previously observed in DAT/NET chimeras was more the consequence of a perturbation of inter-domain interactions, rather than the elimination of a cocaine-binding residue. No differences relative to cocaine inhibition were observed in hNET S399P/G400L or in the corresponding hDAT (not shown) or hNET single mutants in TMD8 (Table II). It can be inferred that these mutations in TMD8 of hDAT are somehow affecting the ability of cocaine to block the transporter by inducing conformational changes and perhaps preventing access of cocaine to its site of action. Cocaine-binding sites are thought to involve common residues in all cocaine-sensitive transporters, that are probably located in disparate regions of the transporters and take into account the tertiary (37) or oligomeric structure of these proteins (41, 42).

**Interactions with TCA**—In contrast to the affinity for cocaine, which is conserved among all monoamine transporters, the affinities for TCA such as desipramine or nortriptyline are 3 orders of magnitude higher for SERT and NET than for DAT (16, 22). In hNET-F316C there was a significant loss of affinity for TCA. Structure/function analyses are often supported by studies of phylogenetic relations in a homologous family. Involvement of Phe316 in TCA affinity is supported by the fact that this residue is conserved among all tricyclic-sensitive

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**TABLE III**

| Inhibitor-binding Sites in the Norepinephrine Transporter |
|-----------------------------------------------------------|
| **IC<sub>50</sub> values for inhibition of [3H]DA uptake by desipramine and nortriptyline in COS-7 or LLC-PK1 cells expressing wild-type hDAT or hDAT mutants** |
| ----------------------------------------------------------|
| Similar values were found whether wild-type DAT or DAT-mutants were expressed in COS-7 or LLC-PK1 cells, and calculations were performed on pooled values from experiments with both cell lines for wild-type and mutant DAT. Values are mean ± S.E. of four to six experiments, each performed in triplicate. Statistics were performed using Student's t test. |

| Inhibitor          | hDATasso<sup>a</sup> | hDAT-C318F <sup>b</sup> | hDAT-S358V <sup>c</sup> | hDAT-P401S <sup>d</sup> | hDAT-L402G <sup>e</sup> | hDAT-P401G/L402S <sup>c</sup> |
|--------------------|-----------------------|--------------------------|-------------------------|--------------------------|--------------------------|-------------------------------|
| Desipramine        | 8.1 ± 0.5             | 8.8 ± 0.6                | 8.3 ± 0.6               | 8.5 ± 0.6                | 8.4 ± 0.6                | 8.5 ± 0.6                     |
| Nortryptiline      | 10 ± 1                | 10 ± 1                   | 10 ± 1                  | 10 ± 1                   | 10 ± 1                   | 10 ± 1                        |

<sup>a</sup> p < 0.05 as compared to hDAT.<br>
<sup>b</sup> p < 0.001 as compared to hDAT.<br>
<sup>c</sup> p < 0.01 as compared to hDAT.

**Oryzias latipes** (medaka) for meNET. Actually, the only amine transporters not sensitive to TCA are DAT cloned from rat, bovine, or human. This means that in the evolution process the first amine transporters already had the ability to strongly bind TCA in a binding pocket formed by disseminated residues, and that this ability was lost in DAT. Thus, it was unlikely we could find a single residue that would increase TCA affinities in DAT to what is observed in NET and SERT. The rationale in...
Inhibitor-binding Sites in the Norepinephrine Transporter

Table IV
Sequence alignment within the amine transporter family

Alignment was performed by using amino acid sequences of the cloned medaka NET (meNET, Roubert et al., submitted), frog ET (fET (7)), human NET (hNET (6)), rat NET (rNET (4)), bovine NET (bNET (5)), D. melanogaster SERT (dSERT (18)), human SERT (hSERT (20)), rat SERT (rSERT (19)), bovine DAT (bDAT (12)), rat DAT (rDAT (8)), human DAT (hDAT (9)), and C. elegans DAT (ceDAT (13)). Only a subset of sequences in TMD6, TMD7, TMD8, and IL3 are shown. Residues that have been mutated in this study are bold. IC_{50} values for desipramine are from the studies cited above, except for hDAT and hNET, which are from the present work.

| TMD6 | IL3 | TMD7 | TMD8 | IC_{50} desipramine nM |
|------|-----|------|------|------------------------|
| hNET | DAAIQFFSLG...YKFDNN...TSTFGSPA | YPEAITLPSGTFW | 1.2 |
| bNET | DAAIQFFSLG...YKFDNN...TSTFGSPA | YPEAITLPSGTFW | 1.2 |
| rNET | DAAIQFFSLG...YKFDNN...TSTFGSPA | YPEAITLPSGTFW | 4.0 |
| meNET | DAAIQFFSLG...YKFDNN...TSTFGSPA | YPEAITLPSGTFW | 0.9 |
| fET | DAAIQFFSLG...YKFDNN...TSTFGSPA | YPEAITLPSGTFW | 0.8 |
| hSERT | DAAIQFFSLG...YKFDNN...TSTFGSPA | YAEAITMPSATTFF | 54.0 |
| rSERT | DAAIQFFSLG...YKFDNN...TSTFGSPA | YAEAITMPSATTFF | 437.0 |
| dSERT | DAAIQFFSLG...YKFDNN...TSTFGSPA | YAEAITMPSATTFF | 580.0 |
| ceDAT | DAAIQFFSLG...YKFDNN...TSTFGSPA | YAEAITLPS5VWF | 3.0 |
| hDAT | DAAIQFFSLG...YKFDNN...TSTFGSPA | YAEAITLPS5AVW | 4800.0 |
| rDAT | DAAIQFFSLG...YKFDNN...TSTFGSPA | YAEAITLPS5AVW | 9250.0 |

cloned transporters (Table IV), including mammalian SERT (19, 20, 43) and NET (4–6) and also in homologous transporters cloned in nonmammalian species like in the bullfrog R. catesbiana, fET (7), in the nematode C. elegans, ceDAT (13), in the fruit fly D. melanogaster, dSERT (17, 18), and in the fish O. latipes, meNET (2). In the SERT, it was also reported that a Phe residue in TMD12, Phe^{356}, is responsible for the 5–10-fold difference in TCA affinity between the rat and the human protein (25). However, this Phe residue is not conserved in the Drosophila SERT (17, 18) or in all hitherto cloned NETs, and thus is probably not involved in a TCA binding pocket that would be conserved in all TCA-sensitive transporters. Both in this SERK mutant and in hNET-F316C, this affinity shift is the consequence of a phenylalanine substitution, suggesting that the aromatic ring may be involved in stacking (or hydrophobic) interactions with the rings forming the core of the TCA.

The double mutant hNET-F316C/D336T displayed a 30-fold shift in the IC_{50} value for TCA. The reason for this affinity shift, when comparing hNET-F316C to hNET-F316C-D336T, is presently not fully understood. It can be speculated that the short IL3 (12 amino acids) might act as a stabilizer of TCA binding in NET, possibly through a charge-stabilizing effect of the acidic residue Asp on conformational changes. This Asp residue is present in all mammalian and nonmammalian NET, with the exception of ceDAT where it is substituted by an His residue.

Mutation of residue Val^{356} to Ser also had a significant effect on TCA inhibition of DA uptake (Table II). This Val residue Val^{356} is strictly conserved in mammalian SERTs and meNET, but it is changed to a conservative Ile or Leu in the other TCA-sensitive transporters including rNET, bNET, fET, hSERT, and dSERT (Table IV). The Ser residue is found in the other TCA-sensitive transporters including rNET, bNET, fET, hNET, and is changed to a conservative Ile or Leu in the other members of the NET/SERT family; Table IV could establish hydrophobic interactions with the TCA or the Ser residue could directly interfere with TCA binding. In conclusion, our site-directed mutagenesis

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Inhibitor-binding Sites in the Norepinephrine Transporter

experiments underline the complex structural equilibrium that is achieved in the Na\(^{+}/\)Cl\(^{-}\)-dependent transporters. These proteins are known to exhibit a functional channel together with a ligand-binding domain, inside the same structure (44, 45). In this respect, amino acids leading to conformational changes or involved in the tertiary organization of these transporters are particularly important for these complex proteins. This is supported by the high degree of conservation observed in all Na\(^{+}/\)Cl\(^{-}\)-dependent transporters, particularly among the monoamine transporters.

The most important outcome of the present study is the first evidence that binding site(s) for TCA are presumably formed or induced by residues that are contained in TMDs (Fig. 1), whereas no interaction could be ascribed to residues in the putative intra- or inter-cytoplasmic loops (Fig. 1). We clearly identified a Phe residue in TMD6 and a Val residue in TMD7 that may directly interact with TCA, because their loss in hNET decreases the affinity, whereas their introduction in hDAT increases the affinity for these inhibitors. It cannot yet be definitively inferred from our results whether TCA are acting by inducing conformational changes in the transporter or by a direct interaction at the precise sites where the substrate will bind and/or translocate. Further exploration of whether these residues are accessible to aqueous modifying reagents (e.g. MTSET or MTS-biotin) could help to resolve this issue. However, the NET and DAT mutants that lose their affinity for TCA still retain their property to transport NE and DA (with unchanged apparent affinity and efficacy), meaning that the TCA-binding residues are not involved in the substrate translocation mechanisms.

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