Coordination of Na^+ by Monoamine Ligands in Dopamine, Norepinephrine, and Serotonin Transporters

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Received July 17, 2007

The reuptake of neurotransmitters by dopamine, norepinephrine, and serotonin transporters during neuronal transmission requires a sodium gradient. An "ionic mode" of binding proposes that aspartate anchors the ligand's positive charge but ignores the direct role of sodium in ligand binding seen in the only representative structure, the prokaryotic leucine transporter LeuT. Here, we built structural models of human transporters of dopamine, norepinephrine, and serotonin using the LeuT structure. The ligand and sodium-binding sites are highly conserved. We examined the possibilities for ligand binding given the available experimental evidence, including examples of catechol−cation chelates in X-ray structures of protein and other complexes. We conclude that a "chelation mode" of binding with direct interaction between the catechol hydroxyls and sodium is a valid alternative, with consequences for pharmaceutical design. In the modeled serotonin transporter complexes, Y95 is placed where it could select for serotonin through hydrogen bonding to the indole nitrogen.

INTRODUCTION

Transporters of dopamine (DAT), norepinephrine (NET), and serotonin (SERT) belong to a family of proteins called neurotransmitter:Na^+/Cl^- symporters (NSS, also called SLC6, and TC#2.A.22 according to the five-component Transport Classification, TC, codes, http://www.tcdb.org/tcdb/; see the Abbreviations Used section for abbreviations used in this work). These transporters are involved in the reuptake of neurotransmitters against their concentration gradients during synaptic transmission. DAT, NET, and SERT are targeted by major classes of antidepressants, psychostimulants, and antihypertensive drugs, making them prominent pharmaceutical targets.

Since transport is against the concentration gradient of the neurotransmitters, the required energy is provided by a gradient of Na^+ ions. The Na^+-to-neurotransmitter stoichiometry is 1:1 for SERT (see, e.g., ref 1) and NET (see, e.g., refs 2 and 3) and 2:1 for DAT, although 1:1 has also been proposed for DAT since one Na^+ may not be transported across the membrane.[4,5] Although under physiological conditions there is no significant gradient associated with Cl^-, many members of the family including DAT, NET, and SERT have been shown to cotransport Cl^-, where the location of the chloride binding site has been identified using a combination of molecular modeling and site-directed mutagenesis.[6,7] In SERT, the exchange for one cytoplasmic K^+ has been suggested, too.[8]

Recently, the first three-dimensional structure of a NSS family member was solved with a resolution of 1.65 Å, that of the leucine transporter (LeuT; PDB code 2A65) from the bacteria *Aquifex aeolicus*.[9] A major feature of the LeuT structure is the bundle of 12 transmembrane helices (TMs) formed by two antiparallel 6-TM domains, a topology likely to have been generated by a single gene duplication and fusion event. LeuT was cocrystallized with the natural ligand leucine, which in the X-ray structure is tightly bound by amino acids from TMs 1, 3, 6, and 8 in a pocket located centrally within LeuT and within the plane of the membrane. In LeuT, sodium is cotransported with leucine, and two sodium ions are found in the LeuT structure, one directly coordinated by the carboxylate group of bound leucine and the second located nearby, ~6 Å from the nearest atom from leucine. The structure of LeuT is consistent with earlier predictions, based on sequence analyses, whereby most members of the NSS family would have 12 TM helices where the amino and carboxyl termini are located on the cytoplasmic side of the cell membrane.[10–12] The dicarboxylate/amino acid:cation (Na^+ or H^+) symporters (TC#2.A.23) are sometimes grouped with the NSS transporters on the basis of their cellular location and mode of transport, but the fold of the representative structure of this family—from the X-ray structure of the 8-TM glutamate transporter homologue from *Pyrococcus horikoshii* (PDB code 1XFH[13])—is clearly different from that of LeuT.

The earliest attempts to model these eukaryotic transporters[14] (see also ref 15) were based erroneously on the X-ray structures of three transporters from the major facilitator symporter family (MFS; TC#2.A.1) that are now known *not* to be related to the NSS family. Although the NSS and MFS families generally share 12 TM helices (some MFS transporters are duplicated, having 2 × 12 TMs), the similarity ends there since the folds are different: compare the LeuT structure representing the NSS family with any of the three representative structures of the MFS family, the glycerol-3-phosphate antiporter (PDB code 1PW4,[16] TC#2.A.1.4.3),

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10.1021/ci700255d CCC: $40.75
Published on Web 06/11/2008

the lactose permease (PDB code 1PV6, 1PV7;[17] TC#2.A.1.5.1), and the 6.5 Å cryoelectron microscopy structure of the oxalate transporter.[18,19]

Given the LeuT structure, it is now possible to model the three-dimensional structures of DAT, NET, and SERT with reasonable accuracy. On the basis of the LeuT structure, Yamashita et al.,[9] and more recently several others,[20–23] proposed models in which an aspartate side chain in TM1 would function to anchor the positive charge of dopamine, norepinephrine, and serotonin in the neurotransmitter transporters. This had already been suggested by Kitayama and co-workers[24] on the basis of several observations: there is an analogy with the binding modes proposed for G protein-coupled receptors (GPCRs) that bind neurotransmitters and the substantial decrease in the uptake of dopamine that occurs when D79 in DAT is mutated to alanine, glutamic acid, or glycine. Furthermore, it had been noted that aspartic acid is conserved among DAT, NET, and SERT but not among other transporters of the NSS family.[25]

Despite us being aware of the proposed binding modes for neurotransmitters by their transporters, involving an ion pair as they do in their GPCRs, we were struck by the lack of a role for sodium in these published models given the important role sodium plays in leucine binding in the LeuT crystal structure. Consequently, we have examined known molecular structures to see whether catecholamines can function to coordinate cations and, if they do indeed coordinate sodium within DAT, NET, and SERT, what would be the implications for neurotransmitter binding? The sodium-binding sites present in LeuT appear to be highly conserved in DAT, NET, and SERT. As we shall show, catechol hydroxyl groups are well-suited for bidentate interactions with cations, and a binding mode that invokes the direct coordination by the catechol ligands of one of the two sodium ions seen in the LeuT structure provides an attractive explanation for binding and cotransport by DAT, NET, and SERT.

MATERIALS AND METHODS

Amino Acid Sequence Comparisons and Molecular Modeling. The 1.65 Å resolution structure of the LeuT transporter from *Aquifex aeolicus* (PDB code 2A65[9]) was retrieved from the PDB database and its amino acid sequence extracted. Amino acid sequences of the 10 closest homologues of LeuT, DAT, NET, and SERT, were retrieved from the Swissprot database using BLAST searches. A multiple-sequence alignment containing these 14 amino acid sequences was then computed using Malign[26] with default parameters (Birkbeck structure-based matrix,[26] gap penalty of 40), and the sequence alignment was manually corrected to avoid placing gaps in the alignment that would be matched to secondary structure elements found in LeuT. At all positions that form the binding site of LeuT, our alignment of LeuT with DAT, NET, and SERT is identical to the alignments proposed by others,[9,20,22,23] suggesting that structural models based on this alignment will be consistent with the common view. (Note that there is an error starting from position 359 in Figure 1 but not in Figures 2 and 3 of Indarte et al. (ref 20), leading to a one-residue shift in the LeuT sequence relative to DAT for the remainder of the alignment.)

This multiple sequence alignment was used to identify unambiguously aligned segments common among the LeuT, DAT, NET, and SERT transporters that are likely to be structurally conserved regions and form a similar local fold. Pairwise sequence alignments of LeuT with DAT, NET, and SERT were extracted from the multiple sequence alignment and used to construct structural models of individual monoamine transporters using Modeler version 8.1.[27] In the process of building the models, Modeler considers a large set of spatial restraints derived from the template structure and from protein structures in general, followed by limited molecular dynamics.

Manual Docking of Dopamine, Norepinephrine, and Serotonin to DAT, NET, and SERT. Examples of small-molecule crystal structures were retrieved from Cambridge Structure Database (CSD).[28] Structures of proteins crystallized in complex with catecholic ligands were retrieved from the PDB using the freely accessible interface of RELIBASE at www.relibase.ebi.ac.uk.

Structural models of the transporter−ligand complexes were constructed manually using Sybyl followed by energy minimization (500 steps, MMF94 force-field). During the docking procedure, the side-chain conformation of the aspartate equivalent to G24 in LeuT, initially selected by Modeler, was modified in order to optimize its position for ligand binding in the ionic mode and in the chelation mode. Prior to docking, low-energy conformations of norepinephrine (entry code: ADRTAR) and serotonin (SERHOX) were first extracted from the CSD. Note that the catecholic and indole rings discussed in this article are rigid and only the ethylamine chain can adopt varying conformations. Dopamine was prepared by taking the norepinephrine structure, ADRTAR, and removing the β-hydroxyl group.

The ionic binding modes were constructed by positioning the structures of dopamine, norepinephrine, and serotonin within the modeled structures of DAT, NET, and SERT. As we shall show below (see Results and Discussion), all evidence suggests that the location of the binding site in DAT, NET, and SERT for their neurotransmitter ligands is equivalent to the location where the leucine ligand is bound in the LeuT structure. The protonated amine of each ligand was positioned at a distance of 2.5 Å from the closest atom of the carboxylate side chain of the aspartate (equivalent to G24 in LeuT) in DAT, NET, and SERT. The chelation binding modes were constructed as follows. The iron atom in phenylananine hydroxylase was superposed on Na_1 in the transporter model structures, and then the catechol ring system of the bound ligand plus the coordinating polar atoms, all from the 4PAH structure file, were rotated within the putative binding cavity. A superposition was identified where the four polar atoms coordinating Fe^{3+} in the phenylananine hydroxylase structure (4PAH[29]) matched four of the five possible coordinating groups involved in Na_1 coordination in the LeuT structure. This procedure not only identified the likely positions of the polar groups involved in coordinating Na_1 but also specified the placement of the catechol ring system with respect to Na_1 in the binding pocket. An oxygen−metal distance of 2.5 Å instead of 2.0 Å observed in the Fe^{3+} complex was enforced during the minimization step, since 2.5 Å was observed in LeuT and in a sodium−catechol complex (FAHROV) from the CSD. This procedure

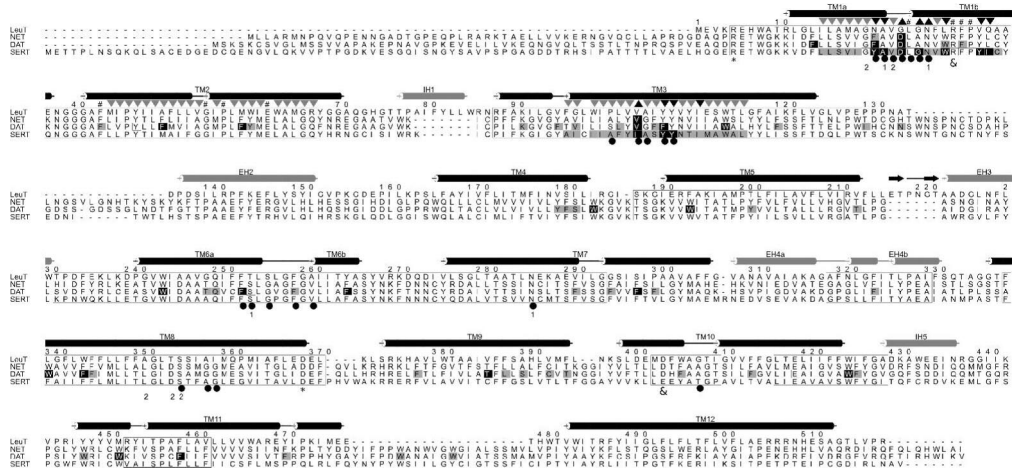

Figure 1. Alignment of the sequences of human NET, DAT, and SERT with that of LeuT. The secondary structure for the LeuT crystal structure (PDB code: 2A65[9]) is indicated above the alignment (TM, transmembrane helix; IH, intracellular helix; EH, extracellular helix). The structurally conserved regions are enclosed with boxes. Solid circles (●) indicate the residues (and equivalent positions in NET, DAT, and SERT) that bind the leucine ligand in LeuT, (*) the extracellular "gate", and (&) the intracellular "gate". The residues involved in binding Na_1 and Na_2 in the LeuT structure are respectively enumerated with 1 and 2. Residues are highlighted according to site-directed mutagenesis results[24,52,53,55–58] from human and other species (see Table 2 for predicted ligand-binding residues): black background, 3-fold reduction in ligand binding or transport; gray shading, no significant effect; boxed residue, not assayable. Results from substituted cysteine accessibility method (SCAM) studies on human and rat SERT[52–54] are indicated above the alignment: (▲), transport sensitive to (trimethylammonium)ethyl methanethiosulfonate (MTSET) and inactivation protected by ligand; (▼), transport sensitive to MTSET; (inverted gray triangle), transport insensitive to MTSET; (#), transport activity too low to be assayed.

also positioned the ring system of the respective ligand in the modeled structures of DAT, NET, and SERT.

Automated docking was performed using the program GOLD 3.2 with standard parameters. The modeled structures of DAT, NET, and SERT were prepared by removing the manually docked ligand after energy minimization. One of the side-chain oxygen atoms from the aspartate equivalent to G24 in the LeuT structure was given as the centroid for the docking search space. Dopamine, norepinephrine, and serotonin were separately docked where the amino group of the ligand was positively charged or was neutral, but the results were quite similar to each other.

Figure 1 was prepared using Alscript.[30] Figures 4–7 were prepared using Bodil[31] (www.abo.fi/bodil) and Molscript[32] and rendered using Raster3D.[33]

RESULT AND DISCUSSION

The LeuT Structure—A Stable Conformational State in the Alternating Access Model. Our first concern in this study was to evaluate the appropriateness of the LeuT static structure as a basis to model the binding modes for ligands of other transporters given the inherent dynamic nature of transporter function. For transporters, a canonical model, the alternating access model, was proposed long ago,[34] see also refs 35 and 36. In that model, a single central binding cavity in the transporter is positioned within the plane of the cellular membrane, but access to and from the cavity is under the strict conformational control of gating substructures at either end of the cavity, and the gating residues are not simultaneously open. The alternating access model suggests that, for maximum efficiency, transport would occur only when all cotransported components are bound, and not when the binding site is partly empty. This simple model was proposed before reports of the first X-ray structure of a transporter, and it agrees quite well with the known structures from different families of transporters, although it should not be surprising that crystallography has revealed the presence of several intermediate states, including an occluded state where the ligand is bound and both gates are closed. For each monomer of the trimeric glutamate transporters, ligands are found bound centrally within the plane of the membrane, and two conformational intermediates, one open to the cytoplasm[37] and an occluded state,[13] have been observed. For the MFS transporters, for example, lactose permease, glucose binds similarly in the middle of the plane of the membrane, and transport is best explained by the alternating access model.[38–40] ATP-binding cassette transporters (also called ABC transporters) form a large family of transporters that use ATP as an energy source for transport instead of an ionic gradient. Structures are known for two intermediates, one where the binding cavity is open at the cytoplasmic side of the transporter (PDB code 2NQ2[41]) and others where the cavity would be open to the extracellular space (1LV7[42] and 2HYD[43]), suggesting that the ABC transporters follow the alternating access model, too.[41]

Leucine is bound to LeuT centrally within the plane of the membrane, but as with the occluded state of the glutamate transporter,[13] both gates are apparently closed.[9] Moreover, Yamashita and co-workers[9] identified two sets of oppositely charged residues, R30 and D404, and R5 and D369, which would respectively contribute to forming the extracellular and intracellular "gates" together with other parts of the structure, and water molecules in the case of the extracellular gate. For DAT, NET, and SERT, oppositely charged amino acids are found at equivalent locations in the sequence (Figure 1), suggesting that a similar gating mechanism may be operating in these transporters, too. The structure of LeuT thus appears to be consistent with a stable state of the alternating access model; however, electro-physiological studies on the NSS family suggest the existence of an open-channel mode where diffusion occurs according to the electrochemical gradient (see, e.g., ref 44).

Table 1. Amino Acids Lining the Binding Site of LeuT, Their Equivalents in NET, DAT, and SERT[a]

TM	1	1	1	1	1	1	1	3	3	3	3
LeuT	N21	A22	V23	G24	L25	G26	N27	P101	V104	A105	Y107
DAT	F76	A77	V78	D79	L80	A81	N82	S149	V152	G153	F155
NET	F72	A73	V74	D75	L76	A77	N78	A145	V148	G149	Y151
SERT	Y95	A96	V97	D98	L99	G100	N101	A169	I172	A173	Y175
Na_1		sc					sc				

TM	3	6	6	6	6	6	7	8	8	8	10
LeuT	Y108	F253	T254	S256	F259	A261	N286	S355	A358	I359	G408
DAT	Y156	F320	S321	G323	F326	V328	N353	S422	G425	G426	A480
NET	Y152	F317	S318	G320	F323	V325	N350	S419	G422	G423	A477
SERT	Y176	F335	S336	G338	F341	V343	N368	S438	A441	G442	T497
Na_1			sc, mc				sc				

[a] The transmembrane segment (TM) to which the residue belongs and the residues functioning to coordinate Na_1 in the LeuT structure (sc, side-chain; mc, main-chain) are indicated.

Table 2. Tabulation of Published Data from Site-Directed Mutagenesis Studies (NET, DAT, and SERT from Human, Rat, Mouse, and Drosophila) and Substituted Cysteine Accessibility Studies (SCAM; for Human and Rat SERT) Conducted at the Putative Binding Sites of the Transporters[a]

DAT		NET		SERT		SCAM
F76	rat: A, ↓[j]	F72	human: Y, †[c]	Y95	human: C, ⇓[f] human: F, †[b,c,g]	human: ⇓[f]
A77		A73		A96	human: C, ⇓[f]	human: ⇓[f]
V78		V74		V97	human: C, †[f]	human: †[f]
D79	rat: A, ↓[h] rat: A, ↓!; E, ↓; N, #[l] rat: A/G/E, ↓[i]	D75	human: A/E/G/N, ↓[d]	D98	human: C, ⇓[f] rat: A/G/N/T, ↓!; E, ↓[d]	human: ⇓⇓[f]
L80		L76		L99	human: C, #[f]	human: #[f]
A81		A77		G100	human: C, ⇓[f]	human: ⇓⇓[f]
N82		N78		N101	human: C, †[f]	human: ⇓⇓[f]
S149		A145		A169	rat: C, †[e]	rat: †[e]
V152	mouse: M, ↓![g] human: I, ↓[k]	V148	human: M, ↓![g]	I172	human: M/A/C/T/V, †[g] human: D/Q/F, ↓; R/K, ↓![g] mouse: M, †[g] drosophila: I, †[g] rat: C, †[e]	rat: ⇓⇓[e]
G153		G149		A173	rat: C, †[e]	rat: †[e]
F155	rat: A, ⇓[j]	Y151		Y175	rat: C, ↓[e]	rat: ↓[e]
Y156		Y152		Y176	rat: C, ↓[e]	rat: ↓[e]
F320	rat: A, ↓![j]	F317		F335		
S321	rat: A, #[h]	S318		S336		
F326	rat: A, †[j]	F323		F341		

[a] For site-directed mutagenesis studies, effects on the natural ligands are noted as follows: (↓!), mutants where transport is totally abolished; (↓), mutants leading to more than 3-fold decrease in binding affinity (K_i or K_M) and/or transport (V_{max}); (†), no significant effect; (#), unassayable mutant. For SCAM studies: (⇓⇓), transport is sensitive to MTSET (2-(trimethylammonium) ethylmethanethiosulfonate bromide), but the introduction of the ligand protects from this inactivation by MTSET; that is, these positions are the most likely to be exposed to the binding site; (⇓), transport is sensitive to MTSET, and this inactivation is not protected by the ligand; (†), transport is insensitive to MTSET; (#), transport activity of the cysteine mutant was too low to be assayed. [b] ref 79. [c] ref 80. [d] ref 81. [e] ref 53. [f] ref 52. [g] ref 59. [h] ref 56. [i] ref 24. [j] ref 55. [k] ref 57. [l] ref 25.

Equivalent Positions in DAT, NET, and SERT Align without Ambiguities to the LeuT Binding Site. In order to model the structures, a multiple-sequence alignment was first constructed that included amino acid sequences from the LeuT, DAT, NET, and SERT transporters, together with 10 additional homologous Na^+/Cl^- transporters (Supporting Information S1). The secondary structure assigned to the LeuT structure is mapped to the sequence alignment in Figure 1. About 44% (267/601) of the amino acids of LeuT could be aligned without any ambiguities with the corresponding sequences of the DAT, NET, and SERT transporters. These regions are likely to be structurally conserved in comparison to the LeuT structure and are denoted as SCRs. These SCRs accounted for 9 out of the 12 TMs but did not include TM4, TM9, and TM12. Within the SCRs, DAT and NET are 80% identical, and both DAT and NET share with SERT about 50% sequence identity. The regions external to the TMs, intracellular loops 1 and 5 and extracellular loops 2, 3, 4, and 5, form large domains roughly two-thirds the size of the transmembrane bundle domain and contain α-helical regions. In these domains, in contrast to the TMs, the conservation between LeuT and DAT, NET, and SERT is low. The SCRs do include the TM6−TM7 loop and an ∼10-residue fragment of the loop connecting TM7 and TM8.

Although the SCRs do not correspond to contiguous segments of amino acid sequences, when mapped onto the X-ray structure of LeuT (Figure 2), these SCRs cluster together in space, forming a large core region, corresponding to that portion of the LeuT structure that would be positioned within the plane of the membrane. The binding site is located

Figure 2. Three-dimensional structure of LeuT indicating the structurally conserved regions (SCR) shared with the monoamine transporters and clustered about the ligand-binding site (derived from the multiple sequence alignment of LeuT with other transporters; boxed regions of Figure 1). SCRs: TM1−TM2 (R1 to Q63 in LeuT), in red; TM3 (F79 to W118), in green; TM5 (F163 to G191), in blue; TM6−TM7 (H206 to G268), in yellow/green; EL4 and TM8 (G293 to A304 and F313 to F345), in magenta; TM10 (L382 to T389 and L396 to V408), in cyan; and TM11 (K432 to F441), in orange. The leucine ligand (yellow) and the sodium cations Na_1 and Na_2 (purple) are shown. Viewed from the extracellular surface at an angle of 30° relative to the plane where the membrane would be located.

centrally within this core of SCRs, and all of the amino acids forming the binding site where leucine is bound belong to the SCRs. Consequently, the structural data from LeuT should provide a good basis to build models that reasonably describe the binding site of the DAT, NET, and SERT transporters even if we expect the alignment outside the SCRs—away from the binding site—to be unreliable for structural modeling.

Modeling the Binding Site of DAT, NET, and SERT on the Basis of the Structure of LeuT. We have derived structural models for the DAT, NET, and SERT transporters from the 1.65 Å resolution X-ray structure of the LeuT transporter from *Aquifex aeolicus* (PDB code 2A65[9]) using Modeler 8.1[27] and the individual pairwise sequence alignments of LeuT with DAT, NET, or SERT, as extracted from the multiple sequence alignment in Figure 1. Within the SCRs, LeuT shares with the human DAT, NET, and SERT transporters about 35% sequence identity, which is commonly considered enough to derive reasonably accurate modeled structures. Among the few examples of membrane proteins where structures for several family members have

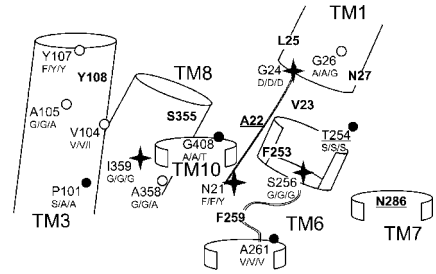

Figure 3. Schematic representation of the binding site indicating the level of conservation between LeuT and the monoamine transporters DAT, NET, and SERT. Equivalent positions are numbered according to LeuT and annotated as follows: entirely conserved, bold type; residue from LeuT is identical to the equivalent residue in at least one of the monoamine transporters, open small circle; residue conservatively varies among the four sequences, solid small circle; and divergent positions in comparison to LeuT, four-branch star. Positions (side chains or main chain) involved in binding Na_1 are underlined.

been solved, a high level of structural conservation takes place at an even lower level of sequence identity than for globular proteins.[45–47] Our experience seems to support this notion. For example, for GPCRs, the level of shared sequence identity is about 20% between adrenoceptor sequences and the sequence from the only known atomic-resolution structure of a GPCR, that is, the 2.8 Å resolution structure of bovine rhodopsin. Nonetheless, these studies have proved to be quite useful in interpreting experimental data, predicting interactions, and guiding new experimentation (see, e.g., refs 47–50).

In the X-ray structure of LeuT, the binding site is formed by about 22 amino acids located on TM1, TM3, TM6, TM8, and TM10 (Figure 3). In all, 18 residues are in direct contact with the bound leucine ligand, and four amino acids coordinate a sodium ion referred to as Na_1.[9] The binding cavity is rather elongated, with a largely hydrophobic and aromatic region located close to TM3. A polar region forming a network of hydrogen bonds that involves the ligand as well as side-chain and main-chain atoms is located at the opposite end of the cavity, near TM1 and TM6 where Na_1 is bound.

The degree of sequence conservation between the binding site of LeuT and the putative sites in DAT, NET, and SERT is quite high (Figure 3, Table 1), which further supports the use of LeuT as a structural template. Like for LeuT, the putative binding cavities in the modeled structures of DAT, NET, and SERT can generally be divided into polar and hydrophobic halves. Out of the 18 amino acids forming the binding pocket for leucine in LeuT, amino acids at six positions are identical: V23, L25, Y108, F253, F259, and S355. Seven positions vary conservatively, G26A/A/G (G26, numbered relative to LeuT and correspondingly A/A/G in DAT/NET/SERT; for the numbering of the corresponding sequence positions of DAT, NET, and SERT, see Table 1), P101S/A/A, V104V/V/I, A105G/G/A, A261V/V/V, A358G/G/G, and G408A/A/T. Four positions, N21F/F/Y, G24D/D/D, S256G/G/G, and I359G/G/G, differ substantially in LeuT versus the other transporters.

Sodium generally requires hexadentate coordination. Three out of the four side chains involved in coordinating Na_1 are entirely conserved, N27, A22, and N286. The fourth position varies conservatively as T254S/S/S and interacts using both the side-chain hydroxyl group and the main-chain carbonyl.

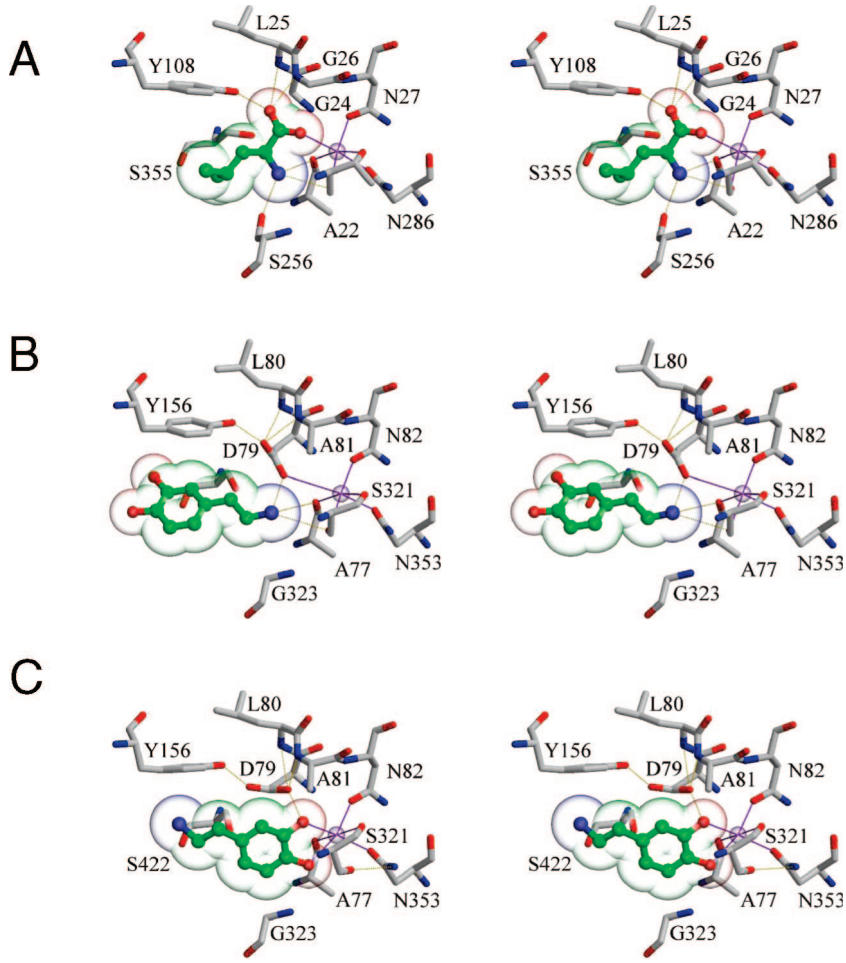

Figure 4. Polar region of the binding site in (A) the X-ray structure of leucine bound to LeuT and in the molecular models of DAT with bound dopamine: (B) the ionic mode and (C) the chelation mode. Carbons atoms of ligands, green; Na_1, purple sphere. Groups, metal and polar groups, close enough to form interactions are joined by lines. The view corresponds to that seen within the plane of the membrane for the embedded protein and is similar to that in Figure 3. In stereo.

Conservation of these side chains lends great support to an equivalently positioned sodium cation in DAT, NET, and SERT, too. In the X-ray structure of LeuT, the sixth coordinating group is supplied by a carboxylate oxygen of the bound leucine ligand.

In LeuT, a second sodium cation, Na_2, is located 6.9 Å from Na_1 and 5.9 Å from bound leucine (closest approach). The side chain of S355 is located between leucine and Na_2, effectively blocking the accessibility of Na_2 to the ligand binding cavity (the hydroxyl group of S355 is 2.3 Å away from Na_2 and, at its closest approach, 3.8 Å from bound leucine). Three residues, G20, V23, and S355, coordinating Na_2, are fully conserved in the monoamine transporters, while two positions, A351L/L/L and T354D/D/D, vary. In LeuT, T354 and S355 coordinate Na_2 via their side-chain hydroxyl groups, whereas coordination from the remaining residues is in each case via the main-chain carbonyl oxygen. In LeuT, five out of the six possible positions are thus coordinating Na_2, whereas at the expected sixth position, the peptide bond between T354 and S355 is nearby, but the polar groups do not face Na_2.

Experimental Studies Support a Common Location for Ligands in DAT, NET, SERT, and LeuT. At the moment, the experimental evidence (summarized on the sequence alignment in Figure 1, and in Table 2) largely supports the presence of a single binding site for the transported ligand in the NSS family. In particular, the location of the ligand binding site in SERT was mapped to the same site as in LeuT using the substituted cysteine accessibility method (SCAM).[51] With SCAM, a set of cysteine mutants are constructed, covering the region of interest; local reactive cysteines are mutated to alanine, and the reaction with sulfhydryl-reactive compounds, as well as protection by ligands against labeling, is tested. With mammalian SERT (studies from rat and human), SCAM identified five positions where amino acid side chains are both exposed and protected by the ligand from modification—D98 (G24 in LeuT), G100 (G26), and N101 (N27) from TM1 of the human SERT (20 mutants were tested[52]) and I172 (V104) and Y176 (Y108) from TM3 of a mouse SERT (20 mutants[54])—and ruled out the participation of residues from TM2 (25 mutants[54]). The results are consistent with the periodicity expected for an α-helix, supporting the proposed local structure of the tested region.[52–54]

Extensive site-directed mutagenesis, over 100 equivalent positions tested among the three monoamine transporters, has also been used to identify possible key residues involved in ligand binding and transport (see, e.g., refs 24, 52, 53, 55–59). Among these, 18 mutants aligned with residues forming the binding site in the LeuT structure, of which 13 had a significant effect on the binding or transport of neurotransmitters: one mutant of NET, five out of six

expressible mutants from DAT, and 7 out of 11 for SERT (Figure 1, Table 2). Still, 18 out of 76 positions mutated outside the binding site had an effect. Regarding these latter mutants, it is well-known that residues remote from any binding site can exert indirect effects (e.g., improper folding) or be involved in another functional process (e.g., transporter dynamics), rather than reflect actual perturbations at the binding site, and the effects of mutagenesis are often dependent on the type of replaced side chains, too.

Predicted Modes of Ligand Binding to DAT, NET, and SERT Invoke an Aspartate−Ligand Ion Pair. All three of the transported natural ligands, dopamine, norepinephrine, and serotonin, share a protonated amine, a benzene, or an indole aromatic ring as a spacer, and at the opposite end from the amine, one or two hydroxyl groups attached to the ring system. Kitayama et al.[24] initially proposed that an ion pair is key to ligand binding, on the basis of the substantial decrease of dopamine binding to DAT that occurred when D79 was mutated to alanine, glutamic acid, or glycine and on the basis of an analogy with the accepted view of catecholamine binding to GPCRs.[24,56] With their report of the crystal structure of LeuT, Yamashita and co-workers[9] made preliminary models for the monoamine transporters in which D79/D75/D98 in DAT/NET/SERT is exposed to the putative binding site. Moreover, Yamashita and co-workers[9] suggested that the carboxylate side chain of D79/D75/D98 would substitute functionally for the carboxylate group of leucine that in the LeuT structure participates in coordinating Na_1. Consequently, several groups have now proposed that an ion pair between aspartate of the monoamine transporters and the amine of the ligand is a key feature of the binding mode.[20–23] Indeed, all known transporters of monoamine ligands have aspartate at a position equivalent to G24 in LeuT, while all of the other known transporters have glycine,[20,25] suggesting that the functional importance of D79/D75/D98 has led to its conservation among the monoamine transporters. On the other hand, related non-monoamine transporters for many other ligands, for example, γ-aminobutyric acid, glycine, proline, leucine, creatine, betaine, and taurine, do not have an equivalent aspartate despite having a positively charged nitrogen as part of the ligand.

We will refer to this ion pair binding mode as the "ionic mode".

Ligand Interactions with Na_1 Are Neglected by the Ionic Mode of Binding. In the LeuT structure itself, Na_1 plays an important role in binding leucine to the transporter (Figures 4A and 5A); however, in the previously proposed models for the monoamine transporters, Na_1 does not appear to have any role in ligand binding. Indeed, with the amino group of the monoamine ligands oriented similarly to the amine of leucine in LeuT, there is no nearby functional group on the ligands that could coordinate Na_1 (as we shall show below, catechol hydroxyls can function as good bidentate ligands for cations). In addition to a lack of ligand interactions with Na_1 in ligand binding (Figure 4B), the ionic mode would also place the polar hydroxyl group(s) of serotonin and dopamine/norepinephrine within an aromatic/hydrophobic pocket, which is clearly unfavorable (Figure 5B). These are the two major weaknesses of the ionic mode of binding given the conserved nature of the Na_1 binding site, its location within the ligand binding cavity, and the observed role in binding leucine within the LeuT X-ray structure. Additionally, the strong electrostatic interaction created by the aspartate binding the amine in the ionic mode of binding is perhaps too strong for the purposes of a transporter.

Leucine, like the catecholamines, contains a positively charged nitrogen, but in LeuT, there is no formal charge anchoring leucine to the transporter.[9] Instead, the protonated amine of leucine is stabilized through hydrogen bonding to the hydroxyl group of S256 (TM6) as well as to the main-chain carbonyl groups of A22 (TM1) and T254 (TM6). These three interactions are enhanced by the presence of the partial negative charge at the carboxyl-terminal end of the α-helical dipoles on TM1 and TM6, helices that are centrally unwound at this location.[9] Serine 256 is *not* conserved in the monoamine transporters where glycine is found instead, an amino acid change that deprives the binding site of an analogous contact but would provide more space for a bulkier group. Leucine contains a second charged group, the carboxylate group. In LeuT, one oxygen atom from the carboxylate group of leucine interacts with Na_1, while the other oxygen is within hydrogen-bonding distance of the side-chain hydroxyl group of Y108 as well as the backbone amide groups of L25 and G26 of TM1. Interaction between the main-chain amide groups and the negatively charged oxygen from the carboxylate group of leucine would be enhanced by the partial positive charge at the amino terminus of the centrally unwound TM1 (TM1a−TM1b junction).[9]

Experimental evidence also questions the presence of an ionic complex. Wang et al.[25] did not find support for a direct ionic interaction between aspartate D79 on DAT and a positively charged group on a ligand, having instead proposed that aspartate would face the catecholic ring, not the charged amine constituent. In their studies, they compared the binding of dopamine with dihydroxybenzylamine, in which the position of the positive charge is shifted relative to the hydroxyl groups since dihydroxybenzylamine is shorter than dopamine by one methylene group. The idea being that a D79E mutant, where glutamate is one methylene longer than aspartate, would be able to reach the positive charge on the "shorter" ligand and counteract/rescue the lower binding affinity observed in comparison with dopamine. In fact, both dopamine and dihydroxybenzylamine bound to the mutant transporter at the same level as with the wild-type transporter, in contrast to variants of dopamine lacking the *meta*- or *para*-catechol hydroxyl groups and amphetamine lacking both hydroxyl groups, all of which were adversely affected by the D79E mutation.[25]

Taken together, there are sufficient reasons to question the charge-pair hypothesis of the ionic mode of binding and to examine whether alternative binding modes are possible that are compatible with the natural ligands, our available knowledge, and the participation of sodium.

Chelation of Na_1 by the Ligand Hydroxyl Group(s) Provides an Alternative to the Ionic Mode. When we began modeling the monoamine transporters DAT, NET, and SERT in complex with their natural ligands, we first examined how conserved the binding sites are likely to be with respect to LeuT and then evaluated the key features of the LeuT−leucine complex likely to be important for binding. We were initially struck by the high conservation of the Na_1 binding site and the intimate interaction Na_1 has with bound

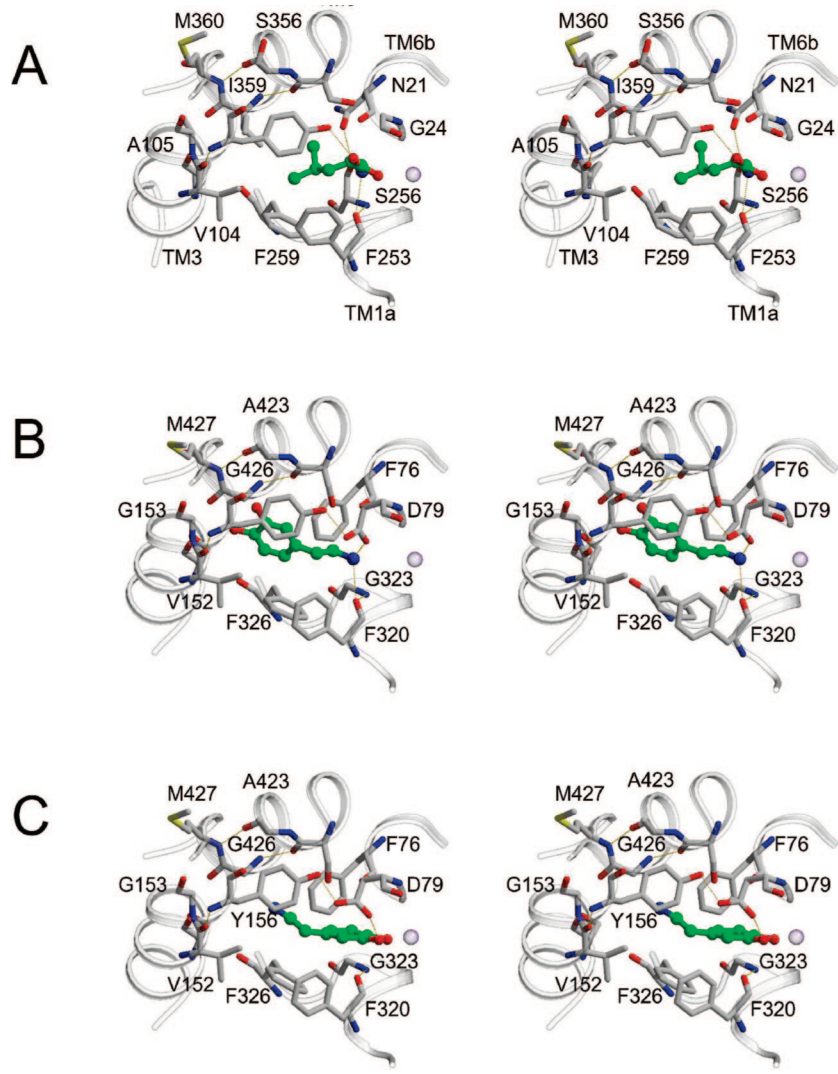

Figure 5. Hydrophobic region of the binding site in (A) LeuT and in the molecular models of DAT with bound dopamine: (B) the ionic mode and (C) the chelation mode. Carbons atoms of ligands, green; Na_1, purple sphere; potential hydrogen bonds, lines. Portions of the TM helices are indicated by transparent ribbons. Possible interactions with the metal are shown in Figure 4. The view would correspond to the extracellular surface of the membrane-embedded protein. In stereo.

leucine. Second, we knew from experience that ionized catecholamines do chelate metals by means of bidentate interactions via their catechol hydroxyl groups, and we found multiple examples of such interactions. Third, we noted the high concentration of aromatic residues predicted to be within the proposed catecholamine binding sites, and we were well aware that aromatic rings could effectively stabilize positive charges. This led us to propose a "chelation mode" of binding for DAT, NET, and SERT to their natural ligands, which differs from that reported by others. In the chelation mode, the ligands are flipped 180° along the short axis of the ligand with respect to the ionic mode; hence, the catechol hydroxyl groups would face Na_1 (Figure 5C).

There is precedence for such a binding mode since cation-bound catechol groups have been observed in three-dimensional structures of proteins (Figure 6; see also Supporting Information S2). Iron (Fe^{3+}) in phenylananine hydroxylase is consistently coordinated by two catechol hydroxyls present as part of different inhibitors cocrystallized with the enzyme (PDB codes 3PAH, 4PAH, 5PAH, and 6PAH[29]). Another enzyme, catechol *O*-methyl transferase, is crystallized with Mg^{2+} where two out of the six interactions with the metal are made by interactions with the dinitrocatechol ligand (1VID[60]). Both of these catecholamine−metal complexes are biologically relevant: phenylalanine hydroxylase is subject to feedback inhibition by Fe-bound catecholamines, and catechol *O*-methyltransferase uses Mg^{2+} as a cofactor for methylation of the catechol hydroxyl groups.

When we took a catechol ring and made a search against the CSD,[28] we identified 378 catechol-containing ligands among which 38 of the structures contained metal ions (see Supporting Information S3). Bidentate interactions were mainly observed for the catecholic hydroxyls: for example, as part of the six-coordination of Na^+ (CSD code FAHROV), CrOH (WULHIU), and Zn^{2+} (FIFKEK) and the eight-coordination of K^+ (DIJWAU and FOKHIW) and Ca^{2+} (OMARAV).

Consequently, we used details from these known structures and the LeuT structure in order to help position the catechol group of the monoamine ligands with respect to Na_1 in the modeled structures. Below, we detail the chelation mode of binding, considering the main functional groups of the ligands and the proposed interactions they would make with the transporters.

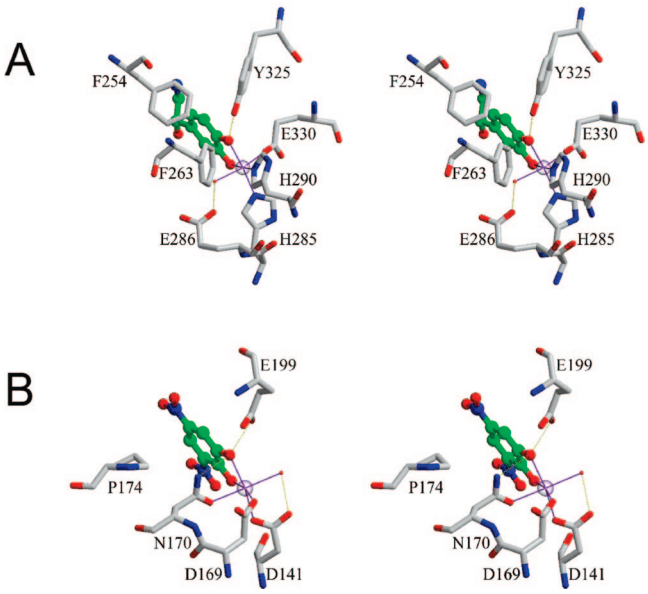

Figure 6. Examples of catecholic ligands bound to metal ions via bidentate interactions in known X-ray structures. The chelation mode is seen in A, norepinephrine bound to Fe^{3+} in phenylalanine hydroxylase (PDB code 4PAH[29]) and in B, dinitrocatechol bound to Mg^{2+} in catechol *O*-methyltransferase (PDB code 1VID[60]). Note the presence of the nearby side-chain carboxylates. In stereo. Additional examples of bidentate chelation from protein complexes and from small-molecule crystal structures are given in the Supporting Information, S2 and S3.

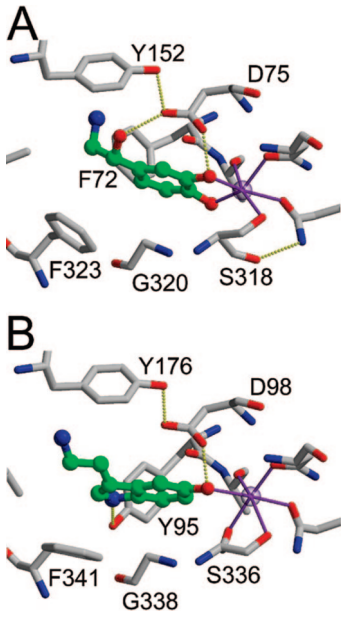

Figure 7. Close-up view of (A) norepinephrine docked to the model structure of NET and serotonin docked to the model structure of SERT. Chelation mode of binding. Selectivity in SERT may be partly governed by Y95 that would be in position to hydrogen bond with the indole ring nitrogen. In DAT and NET, phenylalanine is present at that sequence position.

The Hydroxyl Groups. Noradrenaline and dopamine both have two hydroxyl groups that could function to coordinate Na_1. Serotonin only has a single hydroxyl group attached to the indole ring, but bound to SERT, it could easily mimic the case of leucine bound to LeuT in the sense that a single carboxylate oxygen of leucine coordinates the Na_1 cation, and each of the five polar groups equivalent to those in LeuT bind Na_1. In steric terms, the fit of the ligand within the binding pocket would be good. In contrast, a monodentate interaction of dopamine/noradrenaline with Na_1 in DAT and NET, where both catecholic oxygens would effectively substitute for the carboxylate group of leucine in LeuT, does not appear feasible for steric reasons, since the catecholic ring occupies a larger volume than the carboxylate group of leucine, while there is less room where it would be needed because of the G24D/D/D changes (see Figure 4C). A bidentate interaction with Na_1 in the case of dopamine/DAT and noradrenaline/NET would require only four oxygen atoms from the protein in comparison to the five atoms coordinating Na_1 in LeuT (the main-chain carbonyl oxygens of A22/A/A/A and T254S/S/S, and the side-chain oxygen atoms of T254S/S/S, N27N/N/N, and N286N/N/N).

In order to identify the likely ligand positions in DAT and NET in which two catechol hydroxyl groups would coordinate Na_1, we consulted the example of the catechol ring system coordinating Fe^{3+} in phenylalanine hydroxylase (PDB code 4PAH;[29] using the catechol−Mg^{2+} complex from the catechol *O*-methyltransferase structure would lead to the same result). The iron atom in phenylalanine hydroxylase was superposed on Na_1 in the transporter model structures, and then the catechol ring system of the bound ligand plus the coordinating polar atoms, all from the 4PAH structure file, were rotated within the putative binding cavity. The placement of the catechol ring system with respect to Na_1 in the binding pocket was specified by matching the available space within the modeled binding cavities. A superposition was identified where the four polar atoms coordinating Fe^{3+} in 4PAH closely matched four of the five possible sodium-coordinating groups in the transporter models that are equivalent to the groups that coordinate Na_1 in the LeuT structure: the carbonyl oxygen atom of A/A/A (A22 in LeuT) and the side-chain oxygen atoms of S/S/S (T254), N/N/N (N27), and N/N/N (N286), whereas the carbonyl oxygen atom of S/S/S (T254) would not be needed. The serine in the monoamine transporters equivalent to T254 in LeuT would be located at the carboxyl-terminal end of TM6a preceding the nonhelical hinge region before TM6b; a nearby glycine present in DAT, NET, and SERT but not in LeuT may impart added flexibility allowing the main-chain carbonyl of serine to reorient itself to hydrogen-bond with the side-chain amide nitrogen of the asparagine equivalent to N286 in LeuT.

In the ionic mode, where the amine of the ligand would ion-pair with aspartate (G24 in LeuT), the catechol hydroxyl groups of dopamine and norepinephrine and the indolic hydroxyl of serotonin are then placed within the hydrophobic part of the binding pocket. In this location, the hydroxyl groups would need either to be hydrogen-bonded to polar groups or to be solvated by water molecules, and the hydrophobic location would reduce the chances of either of these two possibilities. In contrast, in the chelation mode of binding, these hydroxyl groups of the monoamines would directly participate in the coordination of Na_1 (Figure 4C), and in the chelation mode, there are other polar groups in the vicinity of the hydroxyl groups that could form additional interactions: the side chain of the conserved aspartate (G24 in LeuT) is near the 5-hydroxyl group of serotonin and the *meta*-hydroxyl group of the catecholamines, as is the main-chain amide nitrogen from A/A/G (G26 in LeuT).

Table 3. Main Characteristics of Ligand Binding in LeuT and in the Ionic and Chelation Modes of Binding for the Monoamine Transporters

LeuT - leucine X-ray structure	ionic mode of binding	chelation mode of binding
coordination of Na_1 by ligand (monodentate)	no interaction with Na_1	coordination of Na_1 by ligand (monodentate (serotonin) or bidentate)
ligand has two formal charges ($-NH_3^+$, $-COO^-$)	ligand has one formal charge ($-NH_3^+$) and one or two $-OH$ groups	ligand: one charged $-NH_3^+$ group and one or two partially/fully ionized hydroxyl groups
no full charge−charge interactions: • $-NH_3^+$ is neutralized by S256 (side-chain $-OH$) and A22 and T254 (main chain $-C{=}O$ and negative dipole, carboxyl termini of TM1a and TM6a) • $-COO^-$ is neutralized by Na_1 and G24 and G26 (main-chain $-NH$ group and positive dipole at amino terminus of TM1b)	full charge−charge interaction: • an ion pair with aspartate neutralizes the ligand's positive charge	no full charge−charge interactions: • $-NH_3^+$ group is neutralized by cation−π interactions • ionized $-OH$ groups are neutralized by interactions with Na_1
all hydrogen-bonding groups of leucine are satisfied	hydroxyl group(s) do not have hydrogen-bonding partners	all hydrogen-bonding groups are satisfied
in the "polar" region of the binding site: • seven hydrogen bonds received/donated by the two polar groups	in the "polar" region of the binding site: • three hydrogen bonds donated by the polar group, $-NH_3^+$	in the "polar" region of the binding site: • two (serotonin), four (dopamine), or five (norepinephrine) hydrogen bonds received/donated by the polar hydroxyl groups
in the "hydrophobic/aromatic" region of the binding site: • no hydrogen-bonding groups	in the "hydrophobic/aromatic" region of the binding site: • ring hydroxyl groups do not have distinct interacting partners • ring located between TM3 and TM8 • nearby polar groups from the α-helical backbone may be able to interact with hydroxyl groups if the helix is locally unwound • hydrogen bond to indole nitrogen (SERT)	in the "hydrophobic/aromatic" region of the binding site: • $-NH_3^+$ group is neutralized by cation−π interactions • hydrogen bond to indole nitrogen (SERT)
the bulk of the ligand is located in the polar region of binding pocket	the bulk of the ligand is located in the hydrophobic region	the bulk of the ligand is located in the polar region

A comparative summary of characteristic features of the chelation and ionic modes of binding is presented in Table 3. More information on the interacting groups and their distances from each other in LeuT and in the DAT model complexes are reported as Supporting Information: S4, interactions of Na_1 with ligands and binding sites; S5, interactions in the polar region of the binding pocket; S6, interactions in the hydrophobic/aromatic region of the binding pocket in the ionic mode of binding.

The Charged Amine. As a consequence of placing the hydroxyl group(s) in contact with Na_1, the protonated amine at the other end of the ligands will be oriented toward the far end of the putative binding pocket, near TM3 and TM6 (Figure 5C). Consequently, in DAT, NET, and SERT, the protonated nitrogen of each ligand would be located in a highly aromatic region of the binding site surrounded by N21F/F/Y (TM1) and Y108Y/Y/Y (TM3) and near Y107F/Y/Y (TM3), F253F/F/F (TM6), and F259F/F/F (TM6); the ring systems of the ligands also interact with these aromatic residues, which is favorable for binding. Although hydrophobic, aromatic rings are highly favorable for interactions with positive charges, accommodating ligands whereby the π electrons of the aromatic rings would neutralize the positive charge (for a review, see ref 61). Several recent X-ray structures have demonstrated the importance of cation−π interactions for ligand binding, for example, in the structure of acetylcholinesterase with bound donepezil (PDB code 1EVE[62]), in the ligand binding domain of the nicotinic acetylcholine receptor bound to HEPES from the buffer solution (PDB code 2BR7[63]), and in the adrenaline-synthesizing enzyme phenylethanolamine *N*-methyltransferase bound with the ligand *para*-octopamine. In the latter structure, the amine of *para*-octopamine is within 3.5 Å of the ring of Y222 (PDB code 2AN4[64]). This structure also suggests that water-mediated interactions occur between the amine and oxygen atoms from the side chains of E185 and E219.[64] On the basis of modeling studies, cation−π interactions have also been proposed for the binding of antagonists to α_2-adrenoceptors.[65] More generally, transporters probably need to form interactions that provide the necessary level of specificity to transport but which are easily disrupted; cation−π interactions would perhaps form a more malleable interaction than a strongly anchoring ion pair. In line with this idea, in the LeuT structure, despite having both positively and negatively charged groups, leucine does not interact with residues having a formal charge.[9]

In the chelation mode, the protonated amine of dopamine, norepinephrine, and serotonin would be positioned such that the amine could also form stabilizing hydrogen-bonding interactions with a conserved serine. In LeuT, the corresponding S355 is located centrally with respect to the binding site, sandwiched in between the leucine ligand and Na_2; in DAT, NET, and SERT, a valid conformer of the serine side chain would be sufficient to reach the amine, but this would remove the interaction with Na_2. This central location between both sodium ions and the ligand may indicate the importance of this position for dynamic transporter function.

The Indole and Catechol Aromatic Rings. The bidentate catechol rings of norepinephrine and dopamine can have two orientations, where the *meta*-hydroxyl and *para*-hydroxyl

swap their interactions with respect to Na_1 (rotation about the long axis of the ligands). Both orientations were observed in the X-ray structures of phenylalanine hydroxylase.[29] Serotonin, on the other hand, is monodentate, and in the ionic mode, we have oriented the single hydroxyl group so that it would interact with Na_1 in a way analogous to the single carboxylate oxygen of leucine bound to LeuT. For norepinephrine and dopamine, the catechol ring of the neurotransmitters was "similarly" oriented (Figure 7) with the *meta*-hydroxyl group occupying the position equivalent to the oxygen interacting with Na_1 seen for leucine in LeuT. This consequently positions the ethylamine chains of the monoamines in the same general location. We realize, however, that for dopamine and norepinephrine this may only be part of the answer since the other or both orientations of the catechol ring might be recognized.

In the chelation mode of binding, where the hydroxyl groups are oriented to coordinate Na_1, the location of the ligands' ring(s) appears ideally positioned with respect to aromatic groups from the protein, with three "sides" occupied by F/F/F (F253 in LeuT), F/F/F (F259), and F/F/Y (N21); the fourth "side" is occupied by S/S/S (S355), which is located between the ligand and Na_2 and functions to coordinate the latter in the LeuT structure. Closer to the amine of the ligand, Y/Y/Y (Y108) would also make close interactions with the ring. One of the main differences at the binding pocket, comparing LeuT with the aligned residues from NET, DAT, and SERT, is the replacement of N21 in LeuT with phenylalanine (DAT and NET) or tyrosine (SERT). This residue would form the "bottom" of the binding cavity lying beneath the ring(s) in the monoamine transporters (Figure 7); the tyrosine hydroxyl would be ideally positioned to bind the indole ring nitrogen of serotonin. This difference in sequence, alone, may explain the specificity of SERT for serotonin, but there are seven other side-chain differences between the predicted binding sites of SERT and DAT and five differences between SERT and NET: on TM1 A/A/G (G26 in LeuT); on TM3 S/A/A (P101), V/V/I (V104), G/G/A (A105), and F/Y/Y (Y197); on TM8 G/G/A (A358); and on TM10 A/A/T (G408).

With either the chelation mode or the ionic mode of binding, the ring(s) of the ligands would be positioned centrally within the binding cavity despite being flipped by 180° (about the short axis of the ligands) with respect to each other (Figure 5B,C). Thus, the ring(s) in either binding mode would participate in some similar interactions. The presence of a tyrosine in SERT and phenylalanine in DAT and NET (at the position equivalent to N21 in LeuT) could therefore be used to explain the experimental data regarding the selectivity for dopamine and norepinephrine versus serotonin in the ionic mode, too.

The β-Hydroxyl Group of Norepinephrine. Both DAT and NET bind dopamine with higher affinity than norepinephrine, and only the dopamine/norepinephrine affinity ratio is higher in DAT than in NET.[66,67] The names DAT and NET refer to their presence on dopaminergic versus noradrenergic neurons, rather than to any pharmacological preference;[68] however, there are some reuptake inhibitors that can make clear distinctions between DAT and NET. Norepinephrine differs from dopamine by having a hydroxyl group at the β position of ethylamine. In both DAT (Figures 4C and 5C) and NET (Figure 7A), three polar side-chain groups are positioned where they could hydrogen-bond with the β-hydroxyl of norepinephrine: the carboxylate group of D/D/D (G24), the hydroxyl group of tyrosine Y/Y/Y (Y108), and the hydroxyl group of serine S/S/S (S355), which could also bind simultaneously to the amino group. In other respects, DAT and NET are highly similar proteins (sharing 80% sequence identity), and the predicted binding sites differ only by two minor changes: alanine and tyrosine in NET correspond to serine and phenylalanine in DAT (equivalent to P101 and Y107 in LeuT). A unique "ancestral" orthologue of DAT and NET is found in the teleost fish medaka (*Oryzias latipes*),[69] providing evidence of a recent gene duplication leading to present-day DAT and NET.

Potential Biological Roles for the Conserved Aspartate in DAT, NET, and SERT. Although the aspartate equivalent to G24 in LeuT has been proposed to function as an anchoring point for the positively charged amine of the monoamines in the ionic mode of binding,[9,20,22–24] in the chelation mode, aspartate does not ion-pair with the ligands. The carboxylate group of aspartate is, however, well-positioned in DAT, NET, and SERT to fulfill one or more important functions.

First, interaction between the carboxylate and the ring hydroxyl groups or the ring could function to withdraw electrons, increasing the deprotonation of the hydroxyl group, therefore reinforcing the chelation of Na_1. Catecholamines are at equilibrium between different charged species, and studies from Raman spectroscopy,[70] NMR,[71,72] EPR,[73,74] and X-ray crystallography[29,60] have shown that the chelation of metals by catecholamines takes place with at least one hydroxyl in the anionic form. It has long been known that electron-withdrawing groups, for example, nitro groups, on the catechol ring will stabilize the doubly negatively charged catecholate, a concept that has been used to develop catechol *O*-methyltransferase inhibitors.[75] The pK_a values of the catecholic hydroxyls, ranging from ~9 to 11, are substantially higher than the physiological pH of the cell and would not normally be ionized in solution: indeed, the positively charged species where only the amine is charged predominates, ~95% at pH 7.4 and 68−71% at pH 8.4 and 25 °C.[76] Nonetheless, at a higher pH, a significant concentration of zwitterionic (positively charged amine plus negatively charged catecholate) and negatively charged species (e.g., neutral amine) is seen, comprising ~3% at pH 7.4 and 22−24% at pH 8.4.[76] In reality, within a protein where there is no water (in the LeuT structure there are no water molecules within the binding site; the closest water molecules are over 9 Å from the Cα carbon of bound leucine, and this is possibly true for the monamine transporters, too), the ease of ionizing a group may be very different, especially if negatively charged groups or a general base are nearby.

In the modeled structures of the transporters, aspartate and sodium provide the local surroundings necessary for ionization of one of the hydroxyl groups, whereas the second hydroxyl present in dopamine and norepinephrine is positioned near the partly negatively charged helix dipole of TM6a. There is some precedence for such a mechanism in catecholate−metal complexes found in the PDB: in the structure of catechol *O*-methyltransferase,[60] the catecholic hydroxyl groups of the ligand are ionized as a consequence of direct interactions with the Mg^{2+} ion together with the side chains of E199 and K144.[75] In this structure, the

carboxylate of E199 is positioned directly "above" one catechol hydroxyl—2.5 Å away, at a distance and location similar to that of the carboxylate of the aspartate in the modeled structures of DAT, NET, and SERT from this study—and functions to abstract the proton upon binding. Lysine 144 is located "below" the other catecholic hydroxyl and would act as a general base. In catechol *O*-methyltransferase, modifications at the catechol hydroxyl groups are essential to the enzymatic mechanism. In the structure of phenylalanine hydroxylase,[29] an unrelated enzyme for which dopamine and norepinephrine are endogenous competitive feedback inhibitors, catecholamines chelate Fe^{3+} to form a bidentate ligand-to-metal charge-transfer complex. Nearby amino acids, Y325 and E330, directly interact with one of the ligand's catechol hydroxyls. Glutamate 286 reaches the other catechol hydroxyl *via* an intervening water molecule. A similar negatively charged environment is seen in our models of DAT, NET, and SERT and includes the conserved aspartic acid residue. In addition to the aspartate equivalent to G24 in LeuT, the carboxyl-terminal helix dipole of TM6 would provide another partial negative charge in the vicinity of the ligand's second hydroxyl group in DAT and NET. In the LeuT structure, this partial negative charge helps to anchor the amino group of leucine.

An additional role of the aspartate could be to form hydrogen bonds with neighboring polar groups and, more particularly, to neutralize the helix dipole at the amino-terminal end of helix TM1b near the amide of both D/D/D (G24 in LeuT) and A/A/G (G26). In the leucine−LeuT complex, glycine (no side chain) is present instead of aspartate, making room for one carboxylate oxygen of leucine to neutralize the amino-terminal end of helix TM1b, at G24 and G26.[9] Additionally in LeuT, the carboxylate of the leucine ligand participates in a network of hydrogen bonds together with the hydroxyl groups of Y108Y/Y/Y and S335S/S/S. In DAT, NET, and SERT, the side-chain carboxylate of D/D/D (G24) could participate in a similar hydrogen-bonding network together with these conserved side chains.

Third, the side-chain conformation of aspartate could change such that it would interact with Na_2, and there are other sequence changes near Na_2, for example, D/D/D (T354 in LeuT), that indicate some alteration in the site in comparison to LeuT might take place (Supporting Information S7). Such changes of conformation, if they occur, may be related to the transport mechanism.

Thus, in the chelation mode of binding, there are several roles that could be suggested to explain the biological conservation of aspartic acid among DAT, NET, and SERT, but the most notable function in the chelation mode would be in helping to deprotonate a hydroxyl group on the ligand, thus enhancing its interaction with bound sodium.

Both Ionic and Chelation Modes of Binding Can Be Reproduced by Automated Docking Methods. In a preliminary study, only the ionic mode of binding was found through automated methods of docking made by us using GOLD (while Na^+ was recognized by the program, the program could not deduce the coordination parameters), and made by Indarte and co-workers[22] (using MOE-DOCK 2004.03 GA, MOE-Dock 2005.06, and ASEDock; only MOE-Dock 2005.06 had parameters for metal ligation in its energy function). As a consequence, we decided to make the following test study where we asked whether the program we were using could even reconstruct an X-ray complex where catechol coordination of a metal ion is actually observed.

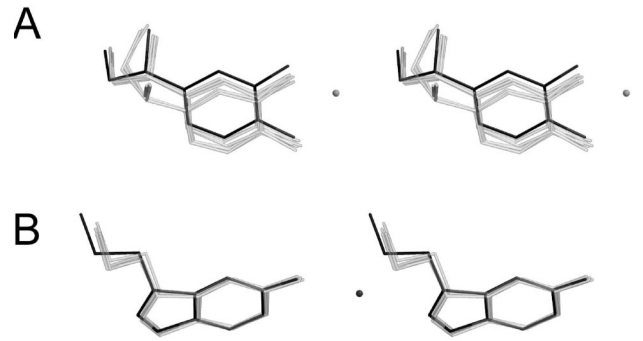

Figure 8. Chelation mode of binding (in stereo) obtained by automated docking of (A) norepinephrine to the model structure of NET and (B) serotonin to the model structure of SERT. The different solutions produced by the automated docking (transparent) closely match the pose previously obtained by manual docking (black, reported in Figures 3–7). For clarity, the protein and the hydrogen atoms are not shown. The Na_1 ion is shown as a small sphere. In a similar exercise, leucine docked to the LeuT crystal structure produced a conformation nearly identical to that in the known structure (data not shown). The program GOLD 3.2 was used for ligand docking, and the sodium ions were relabeled as magnesium; without relabeling, the metal ion would not be taken into account by the docking method and would produce instead the ionic mode of binding (data not shown). Of the six solutions shown for NET and SET, in three poses, the nitrogen atom of the ligand was neutral and, in three poses, the nitrogen atom was positively charged (the algorithm produces three solutions for each docking run, and the program reports the final result when the three best solutions are within a rmsd of 1.5 A of each other).

We docked the catechol-containing ligands norepinephrine and dinitrocatechol respectively to the structures of phenylalanine hydroxylase (PDB code 4PAH[29]) and catechol *O*-methyltransferase (PDB code 1VID[60]), where the catechol groups directly coordinate Fe^{3+} (4PAH) or Mg^{2+} (1VID) in the crystal structures. For catechol *O*-methyltransferase, the observed binding mode was recreated very closely to the X-ray structure of the complex. With phenylalanine hydroxylase, one of the catechol groups does coordinate the metal, while the position of the second group fluctuated among several positions. With LeuT, when Na^+ is present, the coordination number is not deduced by the program; the ion is ignored, but leucine is docked close (amino, carboxy, and side-chain functional groups correctly positioned) to the pose seen in the X-ray complex. We then relabeled the sodium ion as magnesium in the PDB file; the ion was recognized; its parameters were deduced by GOLD and a complex produced that was almost perfectly superimposed to the X-ray structure of leucine bound to LeuT. As a result of these findings, we reconsidered the cases of DAT, NET, and SERT. In completely automated dockings, with sodium relabeled as magnesium in the coordinate files of the modeled structures used as input for docking, the chelation mode but not the ionic mode was recreated in the docked complexes (Figure 8). Despite these results, we urge a cautious interpretation; computationally, the problem of evaluating the relative energy of binding in both the ionic and chelation modes with contributions of the ligand, binding site, sodium, and water molecules is challenging and faces major theoretical issues such as the lack of adequate force fields, the poor estimation of solvation and desolvation energies, and an

inability to deduce parameters for the coordination of some metal ions, in addition to uncertainties and errors in the modeled complexes.

CONCLUDING REMARKS

There is extensive experimental support for a similar location of binding for leucine in LeuT and for monoamines to DAT, NET, and SERT transporters (Figure 1 and Table 1). There are four prominent differences between the 22 amino acids that define the binding site of the prokaryotic transporter LeuT and the corresponding sites in the eukaryotic transporters DAT, NET, and SERT: serine (S256) in LeuT (which stabilizes the amine of leucine in LeuT) is replaced by glycine; isoleucine (I359) in LeuT is replaced by glycine (which would allow the binding site to accommodate larger ligands in DAT, NET, and SERT); asparagine (N21) in LeuT is replaced by phenylalanine in DAT and NET (forming the "bottom" of the binding cavity and contributing to aromatic interactions with the ligand ring systems) or tyrosine in SERT (in SERT, Y95 is well-positioned to hydrogen-bond with the indole group of serotonin, and as we suggest here, the residue at this position may be partly responsible for ligand selectivity in SERT, DAT, and NET). The fourth major difference, glycine (G24) in LeuT is replaced by an aspartic acid in DAT, NET, and SERT, could function to bind ligands in either of two ways: (1) in the ionic mode by contributing to the ion pair with the transported ligand or (2) within the chelation mode by contributing to a network of hydrogen-bonding with the ligand (ring systems, hydroxyl groups), thus increasing the ionization of the catecholic hydroxyl(s), leading to a catecholamine−sodium complex. A comparison of the key features of both binding modes is summarized in Table 3.

Although the ionic binding mode has been proposed previously for the endogenous catecholamines dopamine and norepinephrine to DAT and NET and serotonin to SERT, the binding mode does not take advantage of bound Na_1 that is very likely to be present in these transporters since the sites are so well conserved. The evidence from Wang and co-workers[25] that binding to a D79E mutation in DAT (versus wild-type DAT) was affected most by alterations of ligand structure at the ring (loss of hydroxyl groups) suggested that the aromatic ring would be in the vicinity of aspartic acid in DAT, in contradiction to the ionic mode of binding. Nonetheless, these experiments are insufficient to rule out the ionic mode of binding.[25]

We have proposed an alternative mode of binding, the chelation modes, whereby Na_1 plays an important role through the coordination of the two catechol hydroxyls of dopamine and norepinephrine in DAT and NET or the single hydroxyl of serotonin in SERT. In the chelation mode, the amine at the other end of the ligands is neutralized by cation−π interactions. For transporters such as DAT, NET, and SERT, nonaminergic ligands such as cocaine analogues, where the nitrogen has been substituted by carbon, retain potent activity as transport inhibitors despite the absence of a positive charge.[77]

Coordination of a metal by the ligand in the chelation binding mode would represent an attractive mechanism probably leading to lower costs, energetically, for solvating and desolvating both the catecholic hydroxyls and sodium. Direct interaction between the metal and ligand does not, however, appear to be required for the transport mechanism, since transport can occur even for ligands lacking the catecholic hydroxyls, for example, amphetamines.[66] The requirements of sodium for ligand binding are difficult to investigate experimentally, as trace amounts of metal can and do remain constitutively in proteins, or the sodium sites could be occupied by other metals with similar properties. Studies have nonetheless suggested that the presence or absence of sodium does not alter the affinity of catecholamines for their transporters, but for some other ligands, sodium is required.[78]

We contend that, in the absence of definitive data, that is, a crystal structure of a relevant complex, that the chelation mode for monoamine binding to their transporters provides an attractive alternative to the ionic mode, is supported by available data, and could lead to new insights for the design of selective molecules targeted to monoamine transporters.

Abbreviations Used. DAT, dopamine transporter; NET, norepinephrine transporter; SERT, serotonin transporter; LeuT, leucine transporter; SCRs, structurally conserved regions; GPCR, G protein-coupled receptor; NSS, neurotransmitter:sodium symporter; rmsd, root mean-squared deviation; TM, α-helical transmembrane segment.

ACKNOWLEDGMENT

We thank Gianluca Rossato for preparing Figure 2. This study was supported by grants to M.S.J. from the Technology Development Center of Finland (NeoBiology Program), the Academy of Finland, and from the Sigrid Jusélius Foundation; to V.B. from ISB, the National Graduate School of Informational and Structural Biology; to K.D. a postdoctoral fellowship from the Academy of Finland; and to H.X. from the Foundation of Åbo Akademi University (Center of Excellence in Cell Stress), the Tor, Joe and Pentti Borg Memorial Fund, the Emil Aaltonen Foundation, and ISB. CSC—Finnish IT Center for Science is acknowledged for organizing software and data bank consortia to which we belong.

Supporting Information Available: (S1) Multiple sequence alignment of 14 transporter sequences including LeuT and human DAT, NET, and SERT; (S2) additional examples of bidentate metal coordination by catechol hydroxyl groups, from ligands found in the Protein Data Bank; (S3) additional examples of bidentate metal coordination by catechol hydroxyl groups, from ligands found in the Cambridge Structure Database; (S4) groups coordinating Na_1 via hexadentate coordination in the X-ray structure of LeuT and in the modeled ionic and chelation modes for dopamine bound to DAT; (S5) polar interactions, leucine bound to LeuT and dopamine bound to DAT (modeled in both the ionic and chelation modes of binding); (S6) dopamine bound to the DAT model structure, ionic mode of binding: chemical groups in the vicinity of the catechol hydroxyls; and (S7) groups coordinating Na_2 in the X-ray structure of LeuT and equivalent groups in DAT, NET, and SERT. This material is available free of charge via the Internet at http://pubs.acs.org. The coordinates of the complete models for DAT, NET, and SERT are available at http://www.abo.fi/fak/mnf/bkf/research/johnson/supplementary.php.

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CI700255D