The Substrate-Driven Transition to an Inward-Facing Conformation in the Functional Mechanism of the Dopamine Transporter

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Abstract

Background: The dopamine transporter (DAT), a member of the neurotransmitter:Na+ symporter (NSS) family, terminates dopaminergic neurotransmission and is a major molecular target for psychostimulants such as cocaine and amphetamine, and for the treatment of attention deficit disorder and depression. The crystal structures of the prokaryotic NSS homolog of DAT, the leucine transporter LeuT, have provided critical structural insights about the occluded and outward-facing conformations visited during the substrate transport, but only limited clues regarding mechanism. To understand the transport mechanism in DAT we have used a homology model based on the LeuT structure in a computational protocol validated previously for LeuT, in which steered molecular dynamics (SMD) simulations guide the substrate along a pathway leading from the extracellular end to the intracellular (cytoplasmic) end.

Methodology/Principal Findings: Key findings are (1) a second substrate binding site in the extracellular vestibule, and (2) models of the conformational states identified as occluded, doubly occupied, and inward-facing. The transition between these states involve a spatially ordered sequence of interactions between the two substrate-binding sites, followed by rearrangements in structural elements located between the primary binding site and the cytoplasmic end. These rearrangements are facilitated by identified conserved hinge regions and a reorganization of interaction networks that had been identified as gates.

Conclusions/Significance: Computational simulations supported by information available from experiments in DAT and other NSS transporters have produced a detailed mechanistic proposal for the dynamic changes associated with substrate transport in DAT. This allosteric mechanism is triggered by the binding of substrate in the S2 site in the presence of the substrate in the S1 site. Specific structural elements involved in this mechanism, and their roles in the conformational transitions illuminated here describe, a specific substrate-driven allosteric mechanism that is directly amenable to experiment as shown previously for LeuT.

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Introduction

The dopamine transporter (DAT) is a member of the neurotransmitter:Na+ symporter (NSS) family that includes the transporters for other biogenic amines (serotonin and norepinephrine), amino acids (GABA, glycine, proline, taurine) and osmolytes (betaine, creatine) [1]. DAT terminates dopaminergic neurotransmission by transporting dopamine (DA) against its concentration gradient from the synaptic cleft into the pre-synaptic neuron in a Na+- and Cl⁻ dependent process. DAT is recognized as the primary target of psychostimulants such as cocaine and amphetamine, and has been implicated in multiple disorders, including Attention-Deficit Hyperactivity Disorder and depression.

Structure-function relations have been studied extensively for DAT using both site-directed and deletion mutagenesis, as well as cross-linking, engineering of metal binding sites, and substituted-cysteine accessibility approaches (see [2–9] and references therein). However, the molecular details of the dynamic transport mechanism remain elusive. The high resolution structures of LeuT [10–15], a prokaryotic homolog of DAT, have provided essential structural insights that can serve to interpret the results of experimental investigations of DAT in a structural context [16], but offer only limited clues about the molecular mechanism of transport. In the first LeuT structure, the substrate is located in the center of protein, occluded from both the periplasmic and the...
cytoplasmic milieu. Although the breakthrough structural information about LeuT [10–15], and about some other related transporters [17–19] is recent, a number of models have been proposed for the functional mechanism of LeuT and cognate NSS transporters. For example, the model proposed by Gouaux et al. involves two additional conformations, outward-facing and inward-facing [15], which is in line with the alternating access model for transporters proposed earlier [20]. Structural modeling supported by experimental probing has offered ideas based on the symmetry features of the molecules in this family [21,22], and the powerful approach of computational simulation using high resolution structural information was applied to the exploration of the functional mechanisms of proteins with a LeuT-like structure fold (e.g., see [10,16,23–33]). The current mechanistic understanding emerging from the combined experimental and computational studies, while still incomplete, suggests that the functional mechanisms of the human neurotransmitter transporters in the NSS family are much more complex than would be suspected from the canonical “alternating-access model” of the transition between an outward-facing and an inward-facing form [34].

A central motif of these complex mechanisms is the allosteric effect of ion- and substrate-binding on the translocation process. Both computation and experiment suggest that in LeuT these binding events trigger a series of local perturbations that are propagated from one end of the transporter to the other, generating significant changes in the preferred state [29,34]. The large-scale structural changes are interpretable as the formation of outward- and inward-open conformations supporting the transport process. One element of the allosteric mechanism that produces the conformational changes through propagation of local perturbations, rather than large rigid body motions, is the effect of ligand binding in the extracellular vestibule of LeuT, termed the S2 binding site [29]. Using binding and flux experiments we had shown that the primary binding site (S1 site) and the S2 site could be occupied by substrate simultaneously, and that substrate in the extracellular vestibule S2 site could allosterically trigger intracellular release of Na⁺ and substrate from the S1 site, thereby functioning as a “symport effector” [29]. The S2 site also binds tricyclic antidepressants (TCAs) [11], which interact differently from the substrate and do not promote substrate release from the S1 site, thereby acting as symport uncouplers that inhibit transport [11]. In addition, we identified from computational analyses of the LeuT structures the nature of rearrangements in the extracellular region that differentiate the actions of substrates from inhibitors bound in the S2 site [10]. The likely structural commonalities among the transmembrane (TM) domains of LeuT and eukaryotic NSS [35,36] suggest that many of the details elucidated thus far for LeuT will be shared within this protein family. Other details will differ, however, leading to important functional distinctions in selectivity, sensitivity and responses to substrates, ions and various ligands, such as those evidenced among the human neurotransmitter transporters.

For DAT, previous molecular dynamics (MD) simulations have identified structural elements important for substrate binding and the formation of an occluded state [37,38]. However, the involvement of a LeuT-like S2 binding site and any mechanistic role that an S2-bound substrate might have in modulating DAT function in the manner described for LeuT, remain open questions that are examined here in the context of the allosteric mechanism responsible for conformational transitions in DAT. To study the mechanism of substrate translocation to the intracellular side we used a DAT model described previously [39] to explore the pathway with steered molecular dynamics (SMD) simulations as had been done previously for LeuT [29,40] and other transporters [33,41]. Here, this protocol was augmented with the addition of long MD equilibrations of the various DAT states, to determine properties and function-related dynamics of the S1 and S2 sites, as well as the permeation pathway and function-related states of the transporter molecule. We addressed for the first time the (i)-the molecular mechanism of communication between the S1 and S2 sites, and (ii)-the structural and dynamic elements that enable the DAT molecule to open towards the cytoplasm, which allowed us to identify structural elements responsible for the propagation of the conformational changes. These are shown here to consist, respectively, of specific residues positioned between the S1 and S2 sites, and a cluster of aromatic residues positioned below the S1 site toward the intracellular end. The conformational rearrangements are shown to involve specific “hinge residues” in the transition between the occluded and the inward-facing states. We report on remarkable agreement between the identities of the key components in the translocation mechanism we are able to identify from the simulations and experimental data in the literature. Together, these results achieve a comprehensive molecular identification of key elements of the substrate translocation pathway and the underlying allosteric mechanism in DAT, at a level of detail that is directly amenable to further experimental validation.

Results and Discussion

I. The S1 and S2 binding sites and the substrate translocation pathway of DAT

To investigate the translocation mechanism of DAT we performed SMD simulations (see Methods for details) on a homology model that we had constructed previously and simulated in explicit water and lipid environment [39]. This model of DAT in the occluded state (termed here S1-DAT) is based on the LeuT template [15] and the characterization of the Cl⁻ binding site [42], and includes in the S1 site one dopamine (DA) substrate molecule, two Na⁺ and one Cl⁻ ions. DA was docked in the S1 site by aligning its amine and hydrophobic portion with those of the leucine in the crystal structure of LeuT (see Methods for details). The permeation pathway from the extracellular side was explored in this model with SMD simulations pulling DA from the S1 site towards the extracellular side. Much like in LeuT [29], this procedure identified here a second binding site in a region above (extracellular to) the S1 site by the behavior of the steering force experienced when pulling DA that was the same as described previously for the equivalent simulation in LeuT. The force profile (see especially Figure S1 in File S1, in [29]) suggests the presence of an extracellular pocket similar to the S2 site detected computationally and validated experimentally in LeuT at a similar position [29]. After equilibrating a substrate in this S2 site, a second DA molecule was added and positioned in the S1 site, and the dual substrate configuration (S1,S2-DAT) was equilibrated for 25 ns (Figure 1A,C).

To explore the intracellular permeation pathway, two independent SMD simulations were initiated from S1,S2-DAT. After DA from the S1 site reached the intracellular side, the system was further equilibrated with 15 ns MD simulation in each of them. The two separate runs converged to the same final inward-facing conformation. In the following sections, we delineate our characterization of the S1 and S2 sites and the substrate translocation pathway based on the analysis of three equilibrated conformational states, namely, S1-DAT, S1,S2-DAT and the inward-facing conformation.

The S1 and S2 binding sites. The residues forming the S1 site were identified from the equilibrium trajectories of both S1-DAT and S1,S2-DAT as those close to the substrate during the simulation
trajectories (Figure 1A,B). The resulting binding pose of the DA substrate in the S1 site is consistent with previous studies [37,38,43]. Most residues in contact with the S1 substrate were from TMs 1, 3, 6 and 8 and remained the same in both trajectories (Figure 1B, Table 1). However, the identities and the orientations of several S1 residues were different when assigned in the presence or absence of substrate in the S2 site (Figure 2B, Table 1), suggesting that S2 binding has an allosteric effect on the S1 site (see below).

The S2 site residues identified in the equilibration trajectory of S1,S2-DAT were from TMs 1, 3, and 10, and the extracellular loops EL2 and EL4 (Figure 1C, Table 2). The composition of the S2 site in DAT is similar to that in LeuT [29], and includes the corresponding (aligned) hydrophobic residues F1553.49, I1593.53, W1623.56, and F47210.44 and a pair of corresponding charged residues, D47610.48 and R851.51. Note, however, that EL2 is much longer in eukaryotic NSS than in LeuT, and is involved in the S2 site of the DAT model, but not in the S2 site of LeuT.

The permeation pathway. Residues contacted as the substrate moved from the S1 site outward toward the S2 site during the SMD/MD simulation were classified as belonging to the extracellular transport pathway that is lined by two layers of hydrophobic residues along TMs 1, 3, 8 and 10 (Table 3). Following the same criterion, residues in contact with the substrate as it moved from the S1 site toward the cytoplasmic side in the two independent SMD simulations, were similarly classified as belonging to the intracellular translocation pathway lined mainly by residues from TMs 1, 3, 6 and 8 (Table 4). The endpoint for SMD pulling in the intracellular pathway was determined by monitoring the interaction energy between DA and solvating water molecules. In the S1 site the water-DA interaction energy was $\sim -19$ kcal/mol, reflecting minimal direct contact. The water-DA interaction became stronger as the substrate moved toward the cytoplasm, indicating increasing solvation until DA established an equilibrated interaction with the conserved E4288.66, when the interaction energy stabilized at $\sim -60$ kcal/mol suggesting full solvation by surrounding waters; this was supported by visual inspection. SMD pulling was terminated at this position. Notably, the residue corresponding to E4288.66 in various transporters has been shown to be important for substrate transport [44,45] and shown to become solvent exposed in the inward-facing conformation of GAT-1 [45]. The observed interaction between DA and E4288.66 suggests that this functionally important glutamate may be an anchoring point along the transport pathway where the substrate makes stable interactions before it moves to the cytoplasm, or reversely in the initial step of efflux.

II. Substrate binding in the S2 site prepares DAT for the transport of dopamine

The changes observed in the S1 site when substrate is present in the S2 site suggest a mode of allosteric interaction between the binding sites. The elements of this allosteric mechanism are the coordinated rearrangements of key residues and structural elements (TM segments and loops) discussed below.
An allosteric interaction network between the S1 and S2 sites. The presence of substrate in the S2 site is associated with a downward repositioning of the center of mass of the S1-bound substrate by 1 Å (Figure 2A,B). This is accompanied by the dissociation of several DA-protein contacts, and the formation of new ones (Figure 2B). Comparison of the equilibrated S1-DAT and S1,S2-DAT models showed that the downward repositioning is enabled by the rearrangement of (i) several conserved hydrophobic residues within the S1 site, or in between the S1 and S2 sites (e.g., the significant changes of F761.42, Y1563.50 and F3206.53 shown in Figure 2B), and (ii) the interposed water molecules. The rearrangement involved in the S1/S2 allosteric interaction is accomplished by a set of highly conserved residues that are not sequential in the primary structure, but form a network in space as indicated in Figure 2.

Compared to S1-DAT, which corresponds to the LeuT crystal structure, in the S1,S2-DAT model the EL2 and EL4 loops have moved towards the S2 substrate and pushed the extracellular segment of TM3 inward to the S2 site (see Figure S1 in File S1). This appears to cause conformational changes in the S1 site, propagated through a conserved interaction network (Table S1 in File S1). The dynamic sequence of events has I390EL4 and F391EL4 pushing the sidechain of W841.50, and then of L801.46 down toward the S1 site (Figure 2C). As a result, the sidechains of Y1563.50 and F3206.53 that are located between the S1 and S2 sites and have their phenyl rings above the catechol moiety of DA, rotated to push downward on the ligand in the S1 site. F1553.49 facilitated the rotation of Y1563.50 in a rearrangement that is likely due to its interaction with the substrate in the S2 site. Thus, the communication between the S1 and S2 sites appears to depend on this sequence of rearrangements of the highly conserved set of hydrophobic and aromatic residues (L801.46, W841.50, F1553.49, Y1563.50, F3206.53, I390EL4 and F391EL4).

Table 1. Composition of the S1 site.

| Index | Residue | Co-z* | S1-DAT %b | S1,S2-DAT %c |
|-------|---------|-------|-----------|--------------|
| 1.42  | F76     | -1.24 | 100       | 100          |
| 1.43  | A77     | 0.78  | 99        | 99           |
| 1.45  | D79     | 3.32  | 100       | 100          |
| 3.43  | S149    | 0.39  | 8         | 20           |
| 3.46  | V152    | 5.11  | 99        | 18           |
| 3.47  | G153    | 3.19  | 23        | 0            |
| 3.50  | Y156    | 6.30  | 95        | 95           |
| 6.53  | F320    | 5.58  | 100       | 100          |
| 6.54  | S321    | 5.64  | 100       | 100          |
| 6.55  | L322    | 2.00  | 99        | 100          |
| 6.56  | G323    | 1.16  | 100       | 100          |
| 6.59  | F326    | -1.28 | 99        | 98           |
| 6.61  | V328    | -4.64 | 73        | 100          |
| 8.59  | D421    | -0.98 | 0         | 20           |
| 8.60  | S422    | 1.13  | 100       | 100          |
| 8.61  | A423    | 1.73  | 24        | 0            |
| 8.63  | G425    | -3.18 | 19        | 100          |
| 8.64  | G426    | -1.17 | 98        | 99           |

*The z-coordinates for residues at the S1 site of DAT. S1-DAT equilibrated at 14 ns was used for the analysis since it is the starting structure for pulling DA towards the extracellular side.

The percentage of time spent by the residue within 3.5 Å of DA (see Methods) during the 6-16 ns segment of equilibration of the S1-DAT model.

The percentage of time spent by the residue within 3.5 Å of DA during the 6-25 ns segment of equilibration of the S1,S2-DAT model.

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Figure 2. The allosteric effect of S2 on the S1 site. (A) Change in position of the DA substrate in the S1 site during equilibration of the S1,S2-DAT model. The green trace shows that the Z-coordinate of DA (center of mass) decreases by ~ 1.5 Å (i.e., DA is shifted downward toward the intracellular side), compared to its position in S1-DAT (orange trace). (B) The positional changes of DA, the rotamer changes of F761.42, Y1563.50 and F3206.53, and the changes in the composition of the S1 site in S1-DAT (residues rendered in orange) compared to S1,S2-DAT (in green). Note that in S1,S2-DAT, the substrate interacts more (i.e., a higher percentage of time) with S1493.43, V3286.61, and G4258.63 and establishes a new interaction with D4218.59 (Cα atoms shown as orange spheres) while losing interactions with V1523.46, G1533.47, and A4236.61 (Cα atom shown as green spheres). (C) The residues forming an interaction network involved in conformational transitions between S1-DAT (orange) and S1,S2-DAT (green). Residues I390EL4 and F391EL4 are in contact with DA in the S2 site. Note that changes in W841.50, L801.46, Y1563.50 and F3206.53 are coordinated with I390EL4 and F391EL4 (Table 1).

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Indeed, the difference distance matrix map (Fig. S1 in File S1) shows that residues L80\(^{1.46}\), W84\(^{1.50}\), I390\(^{EL4}\) and F391\(^{EL4}\) moved together with F155\(^{1.49}\), Y156\(^{3.50}\) or F320\(^{6.53}\). This type of allosteric communication function carried out by a conserved network in the molecular space had been proposed for allosteric networks in other molecular systems as well, e.g., in PDZ domains and other proteins [46–48].

The functional role of the rearrangements we observed extends to a change in the coordination of Na\(^{2+}\) in the S1,S2-DAT model, which may well be associated with the release of the ion in a process observed both computationally and experimentally for LeuT, as described in [29]. Compared to the S1-DAT, in the S1,S2-DAT model the backbone carbonyl of L418\(^{8.56}\) is flipped, with a 130° change in its \(\psi\) angle (Table S1 in File S1), so that it no longer coordinates Na\(^{2+}\) (Figure S1G in File S1) but interacts instead with W84\(^{1.50}\) (through a water molecule), which stabilizes it in a new position. With this set of rearrangements, the middle portion of TM8 near L418\(^{8.56}\) moves back from the Na\(^{2+}\) site and away from TM1, facilitating the observed rotamer changes in L80\(^{1.46}\) and Y156\(^{3.50}\).

The allosteric effect of S2 binding induces an open-inward conformation allowing water penetration. We found that the formation of continuous water pathways in the core of DAT (Figure 3) is associated dynamically with substrate binding in the S2 site. The pathway determined from the SMD simulations for the exit of substrate from the S1 site corresponds exactly to a water channel (Table 4), thus identifying a specific mechanism for substrate binding in the S2 site to trigger permeation. Notably, the residues in the highly conserved aromatic cluster lining this channel, which we observe in the simulations to coordinate the movement of DA, are buried in the core of the transporter in the S1-DAT state. However, when S2-bound DA triggers the formation of the channel, part of this aromatic cluster (F69\(^{1.35}\), F70\(^{1.42}\) and F332\(^{6.53}\)) becomes more solvated (Figure S2 in File S1), supporting the relation between substrate binding in the S2 site and the opening of the intracellular pathway for the penetration of water and the downward

| Table 2. Composition of the S2 site. |
|-------------------------------------|
| **Index** | **Residue** | **Co-z** | **S1,52-DAT -Average %\(^a\)** | **Inward -Average %\(^b\)** |
| 1.51 | R85 | 14.00 | 30 | 1 |
| 3.49 | F155 | 8.26 | 90 | 92 |
| 3.53 | I159 | 10.20 | 45 | 97 |
| 3.56 | W162 | 13.55 | 93 | 99 |
| EL2 | F217 | 25.71 | 51 | 94 |
| | V221 | 21.92 | 98 | 98 |
| | L222 | 23.71 | 94 | 53 |
| EL4 | D385 | 26.11 | 22 | 0 |
| | G386 | 22.68 | 31 | 2 |
| | P387 | 20.82 | 80 | 100 |
| | F391 | 23.47 | 19 | 4 |
| 8.53 | L415 | 11.26 | 3 | 98 |
| 10.41 | I469 | 20.78 | 30 | 34 |
| 10.44 | F472 | 16.09 | 71 | 0 |
| 10.45 | T473 | 18.04 | 49 | 0 |
| 10.48 | D476 | 14.19 | 99 | 100 |

\(^a\)as in Table 1.

\(^b\)The percentage of time spent by the residue within 3.5 Å of DA (see Methods) during the 6–25 ns of equilibration of the S1,S2-DAT model.

\(^c\)The percentage of time spent by the residue within 3.5 Å of DA during the 6–15 ns of equilibration of the inward-facing model.

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| Table 3. The substrate translocation pathway from the S1 site to the S2 site. |
|---------------------------------|
| **Index** | **Residue** | **Co-z** | **Max %\(^b\)** |
| 1.46 | L80 | 8.96 | 100 |
| 1.47 | A81 | 9.50 | 100 |
| 1.50 | W84 | 14.00 | 55 |
| 3.57 | A163 | 12.42 | 27 |
| EL4 | I390 | 20.48 | 10 |
| 10.49 | H477 | 14.84 | 5 |
| 10.51 | A479 | 10.00 | 76 |
| 10.52 | A480 | 12.12 | 100 |

\(^a\)as in Table 1.

\(^b\)For SMD calculations, percentages were defined as the number of frames during which substrate sees a residue (Frame No. when it first sees that residue); both the SMD and MD equilibrations were included for the calculation. The maximum percentages from individual SMD or MD simulations are reported.

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| Table 4. The substrate translocation pathway from the S1 site to the intracellular site. |
|---------------------------------|
| **Index** | **Residue** | **Co-z** | **Max (1\(^{st}\)/2\(^{nd}\) %\(^b\)** |
| 1.25 | R60 | –20.47 | 69/44 |
| 1.29 | W63 | –14.34 | 99/94 |
| 1.38 | S72 | –6.59 | 1/100 |
| 1.41 | G75 | –1.70 | 1/24 |
| 2.66 | A128 | –9.93 | 56/25 |
| 4.62 | L255 | –7.14 | 59/0 |
| 4.65 | G258 | –10.61 | 0/35 |
| 5.36 | V259 | –11.44 | 98/70 |
| 5.40 | S262 | –7.22 | 63/81 |
| 5.41 | G263 | –8.90 | 16/27 |
| 5.43 | V266 | –3.55 | 94/97 |
| 6.62 | L329 | –6.63 | 16/0 |
| 6.64 | A331 | –9.52 | 100/100 |
| 6.65 | F332 | –9.81 | 100/100 |
| 6.68 | Y335 | –15.58 | 100/100 |
| 8.62 | M424 | –1.90 | 51/47 |
| 8.66 | E428 | –6.11 | 100/100 |
| 8.67 | S429 | –5.48 | 78/84 |
| 8.70 | T432 | –8.97 | 3/66 |
| 8.71 | G433 | –9.56 | 11/58 |
| 8.74 | D436 | –14.31 | 7/44 |
| 9.38 | R445 | –13.45 | 1/50 |

\(^a\)as in Table 1.

\(^b\)as in Table 3. The results are shown as the percentage from the first simulation/percentage from the second simulation.

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movement of the substrate from the S1 site. Correspondingly, the interaction energy of the S1-bound substrate with water is \( \sim -10 \text{ kcal/mol} \) in the absence of S2-bound substrate, but becomes much stronger \((\sim -19 \text{ kcal/mol})\) when substrate occupies the S2 site due to water penetration that also facilitates the process of release into the cytoplasmic medium (Figure 3E). This is enabled by rotamer changes of the buried S2625.40 and M4248.62 residues in the core of the protein in S1,S2-DAT (Table S1 in File S1), which make room for waters to move up from the intracellular side (Figure 3F,G). Further, the change in conformation of F761.42 (see above) induced S4228.60 to move toward Y1563.56 (Figure 3F,G), resulting in the disruption of the interaction between S4228.60 and F761.42 and water penetration into the S1 site. Notably, Na2 is solvated in this process (facilitating its inward release as described in our findings for LeuT [29]), whereas Na1 remains fully sequestered from water.

Release of DA from the S1 site involves specific changes in a conserved cluster of aromatic residues. We found that ligand binding in the S2 site triggers remarkable changes in the putative permeation pathway of DAT, but the subsequent inward movement of DA from the S1 site toward the intracellular exit involves additional conformational rearrangements in a cluster of highly conserved aromatic residues in TMs1a and 6b (Table S2 in File S1) that includes F691.35, F761.42, F3326.65, as well as W631.29(NT) and Y3356.68 (Figure 4A). A set of sequential rotamer changes in these residues is propagated through a series of local conformational rearrangements in the intracellular segments of the TMs. The changes in the rotamers of F761.42, F3326.65 and Y3356.68 along the translocation pathway (Figure 4B), are correlated (Figure S3 in File S1), as indicated by the application of Spearman’s rank test [49] to the trajectory-derived data from the SMD simulations of substrate moving inward from the S1 site. Specifically, rotamer changes in F761.42 and F3326.65 allow DA to exit from the S1 site (Figure 4C,D), and rotation of F3326.65 produces the rearrangement of F691.35 that results in the significant movement of the intracellular end of TM1a away from TM6b (Figure 4D). Finally, Y3356.68 dissociates from an H-bond interaction with E4286.66, enabling further movement in TM1a and the N-terminus, which includes W631.29(NT) (Figure 4E,F). Overall, the conformational changes within the entire aromatic cluster produce an opening surrounded by TMs 1, 5, 6 and 8 that enables water penetration, as indicated by the significant increase in the values of solvent accessible surface areas (SASA) calculated for the dynamics trajectories for residues F3326.65, Y3356.68 and W631.29(NT) in a time sequence corresponding to the direction of the pathway (Figure 4B).

The dynamic mechanism emerging from our SMD and MD simulations indicated that the rearrangements of F761.42, F3326.65 and Y3356.68 in the aromatic cluster, together with E4286.66, function as “gates” along the intracellular translocation pathway. The first of these gates is composed of F761.42, F3326.65 and Y3356.68 in the aromatic cluster (discussed above) and their neighbors, underscoring the key role of this highly conserved cluster of aromatic residues (W631.29(NT), F691.35, F761.42, F3326.65 and Y3356.68) in the conformational transition between different functionally-related states of the transporter protein. This is similar to the aromatic cluster found in TMs 5–6 of Class A GPCRs that is known to contribute to switching between inactive and active states of the receptor (e.g., see [50–52]).

III. Global rearrangement from S1-DAT to the inward-facing conformation

The ordered sequence of local structure perturbations described above gives rise to a global conformational rearrangement of the transporter molecule from one state (e.g., S1-DAT) to another (e.g., inward-facing). A structural characterization of this transition to the inward-facing conformation was obtained with the RMSD TT algorithm [33] that performs an iterative alignment of structures, giving larger weight to regions that have small residue-based RMSDs. Comparing S1-DAT to the inward-facing conformation, this procedure showed that as the intracellular passage opened for the substrate DA, portions of TM segments positioned extracellular to the S1 site have small residue-based backbone RMSDs (1–2 Å), whereas the TM segment portions ranging from the S1 site downward to the intracellular side exhibited drastic conformational changes, with RMSDs of 3–6 Å (Figure S4 and Table S3 in File S1). The same structural characterization of the transition was observed when the models of the two DAT states were aligned using two other approaches, either aligning residues in the conserved TMs 1, 3, 6 and 8, or performing 3-D structural alignment of entire DAT structures (“Stamp structural alignment” in VMD [54,55]).

The global conformational rearrangement at the extracellular side caused by the S2-bound substrate is small, but noticeable. TM3 residues F1553.49, I1593.53 and W1623.56 are in direct contact with the ligand in the inward-facing conformation and alter the nearby TM packing, so that TM4 moves toward TM3, and TM6 tilts outward to TM5 and 4 (Figure 5A). Both EL2 (connecting TMs 3 and 4) and EL4 (connecting TMs 5 and 8) move downward to interact with the ligand and close the S2 site. In addition, EL3 (between TM5 and 6) also moves inward toward the bundle, consistent with the movement of EL3 observed in LeuT [10,34,56].

Conformational rearrangements at the intracellular side make room for the descending DA: TMs1, 4, 5, 8, and 9 move as one group, and TMs2, 6, 7, 10, and 11 as a second group that distances itself from the first. Notably, TM1 exhibits the largest movement, consistent with experimental data for LeuT [34], TMs3 and 12 move to fill in the space created by the rearrangements of the two groups (Figure 5B), so that TM3 moves towards the position originally occupied by the second group to maintain its associations with TMs6 and 10, and TM12 to maintain contacts with TMs3, 8 and 9.

Overall, we found the global rearrangements in DAT to be similar to those observed from the comparison of the LeuT crystal structure of the occluded form [15] to the inward-facing LeuT model obtained from SMD [57], with the difference that in LeuT some intracellular portions of TM segments exhibited either smaller-scale movements (TMs4 and 8) or did not move at all (TMs3, 9 and 10). This difference may well be due to the higher rigidity that LeuT, which is from a hyperthermophilic organism, would be expected to exhibit at the simulated room temperature; the rigidity is likely to be achieved by a combination of several factors [58].

Hinge regions enable the global conformational transitions. That specific hinge regions enable the dynamics of propagation of the observed allosteric effects was established first for the unwound regions of TMs1 and 6. In the transition from S1-DAT to the inward-facing conformation, the extracellular end segments of TM1 (TM1b) and TM6 (TM6a) (above the
unwound region) remain largely unchanged, whereas the intracellular ends, TM6a and 6b, swing outward non-symmetrically to open the substrate translocation pathway. TM1a moves substantially more, thereby distancing itself from TM6b (Figure 3C). Consistent with the mechanism proposed from the crystal structure of LeuT [15], we did not observe a big...
movement in TMs1b and 6a in the transition between S1-DAT and the inward-facing conformation. Interestingly, comparing LeuT structures in an occluded state [15] and in an outward-facing state [12], it is the intracellular segments TMs1a and 6b that were considered to remain immobile, while the extracellular TMs1b and 6a tilt outwards. The findings described here are also consistent with previously observed changes in the Tyt1 transporter upon opening of the translocation pathway, where this opening is associated with increased solvent accessibility of C181.39 and C2386.65 [28]. The increased solvent accessibility at these two positions in Tyt1 was attributed to the rearrangement in TMs1a and 6b in the inward-facing conformation [28]. In the inward-facing conformation of DAT, TMs1a and 6b also move away from each other and the corresponding residues at the two positions, V731.39 (data not shown) and F3326.65 (Dehnes et al, manuscript in prep), become more solvent accessible. These results, together with the corresponding observations in LeuT, support the view that TMs1 and 6 do not rock like a rigid bundle [21] when the transporter converts from the outward-facing to occluded conformation, and then to the inward-facing conformation (see Concluding Remarks). Indeed, the rearrangements of TMs1b-6a and TMs1a-6b appear to be driven by different local rearrangements and are thus separated during the transport cycle.

Other types of hinge regions comprise one or more conserved Gly/Pro/Thr/Ser/Cys residues (e.g., G552.40, P572.50, G2947.13, G40810.52 and P45711.50) that enable rigid-body motions of helical segments. Using the Prokink analysis tool [59] implemented in the

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**Figure 4. Conformational changes in the aromatic cluster during DA movement inward from the S1 site.**

(A) The cluster of aromatic residues from TMs 1 and 6 shown in S1-DAT is important for conformational transitions; DA in the S1 site is rendered in stick representation. Orange dashed line indicates H-bond of Y3356.68 to E4288.66. (B) Time evolution of dihedrals and SASA (bottom panel) during inward pulling in SMD and MD alternation. Time points marked by the A, B, C, D, E, F arrows on the x-axis correspond to the structures shown in the (A)–(F) panels. The lines in the SASA plot are coded in colors corresponding to the residues names in the same colors. (C) The change in the rotamer of F766.42 from the conformation in S1-DAT (orange) to the configuration at the time point indicated by the C arrow in (B) (cyan), which allows the downward movement of DA. (D) The subsequent change in the rotamer of F3326.65 (same color coding as in (C)) as DA moves to the position originally occupied by the sidechain of F3326.65. (E) When DA is slowly pulled down a bit further in the SMD protocol, its amine forms a new H-bond with the carboxyl oxygen of D4217.39 which coordinates Na2 in S1-DAT, and its hydroxyl groups forms H-bonds with the sidechain carboxyl group of E4288.66. Time is indicated by the E arrow in (B). (F) The rotamer of Y3356.68 changes last, breaking the H-bond between Y3356.68 and E4288.66. DA moves to the position originally occupied by the sidechain of Y3356.68 and E4288.66. Time is indicated by the F arrow in (B).

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Simulaid suite [60] we found that for most TMs, the bend angle and face shift values produced by TM helix breakers change significantly in the transitions (Table 5). These local conformational changes connect individual TMs to the configuration changes propagated from the S2 to the S1 site, and further from the S1 site to the intracellular side of the transporter (see Text S2 and Figures S5, S6, S7 within File S2).

Experimental support for these observations was obtained from the rich structure-function information in the literature collected through the TRAC information management platform [57]. The results show that mutations of corresponding residues in these hinge regions in various NSS transporters disrupt the binding profile and/or the translocation cycle, as indicated by decreased binding affinities of substrates and ligands, increased $K_m$ and/or decreased rates of transport, or altered DA efflux (see Table 5 and references therein). This underscores the functional importance of conformational changes associated with “hinges” in the course of the transition between functional states of the transporter.

Interaction networks are reconfigured in the transition. A series of previously identified interaction networks [16] that were viewed as “gates”, were found here to participate in the conformational rearrangements underlying the state-to-state transitions by a mechanism of reconfiguration of interaction partners. Thus, networks stabilized by specific interactions such as salt bridges and H-bonds [16] need to be replaced by newly formed interactions to compensate for the energy loss. The intricacy of this reconfiguration suggests that modeling the global configurational changes based on global 3D folding-symmetry considerations cannot offer sufficient insight into the transitions. For example, we had shown that in S1-DAT the cation-pi interaction of Y3356.68 with R601.25(NT) stabilizes a salt bridge between R601.25(NT) and D4368.74, and that this intracellular interaction network regulates conformational transitions in DAT [16]. Here we reported that Y3356.68 H-bonds to E4288.66 in S1-DAT, but not in the inward-facing conformation (Figure 6) in which Y3356.68 forms an H-bond with T621.27(NT). The loss of the Y3356.68 interaction with E4288.66 destabilizes S1-DAT and steers the transporter towards an inward-facing conformation. The remodeling of this interaction network alters the capability of the transporter to alternate freely between S1-DAT and the inward-facing conformations that is seen to require a set of local rearrangements, rather than a purely symmetric rearrangement of TM segments.

Notably, the effect of Y3356.68 mutation to Ala was shown to be rescuable by the addition of Zn$^{2+}$ [62], and the mechanism explained by the ability of Zn$^{2+}$ to replace the energetically favorable Y3356.68 interaction with R601.25(NT) thereby reinforcing S1-DAT, and restoring the equilibrium between S1-DAT and the inward-facing conformation that had been lost in the Y3356.68A mutant [16]. Zn$^{2+}$ binding to an endogenous site within the extracellular loops of the wild type (WT) DAT was shown to potentely inhibit transport, while substrate binding can still take place [3,62]. Considering the reconfiguration of the interaction network we describe, the details of S1-DAT and the inward-facing conformation we observed provide an atomistic-level mechanism for these findings related to the nature of the endogenous binding site for Zn$^{2+}$ that consists of residues H193E1.2, H375E4a and E396E6b. In S1-DAT, the average C$_a$ distance between H375E4a and E396E6b is 13 A, suitable for Zn$^{2+}$ binding [63] (Figure 7). In contrast, in the inward-facing conformation model this site is no
longer suitable for $\text{Zn}^{2+}$ binding because both EL2 and EL4b moved down toward S2, and EL4a moved away from EL4b so that the corresponding C$_a$ distance increased to 15 Å. Accordingly, $\text{Zn}^{2+}$ binding prefers the occluded conformation of WT DAT and by stabilizing it prevents the transition to the inward-open (facing) state, thus inhibiting translocation.

The detailed atomistic model of the allosteric mechanism that emerges from this study. Using SMD simulations with extended MD equilibrations we have identified detailed contributions of specific structural elements to the transition between states visited by the transporter molecule in the process of substrate translocation from the primary S1 binding site both to the extracellular and to the intracellular end of the protein. In the movement of substrate from S1 to the intracellular side, these structural elements perform an ordered sequence of local rearrangements that are triggered by the binding of substrate in the S2 site. This allosteric mechanism, identified here for DAT from the SMD simulations and extensive MD equilibrations of the resulting intermediate states, reconfigures a conserved spatial network of interactions (either direct, or through interposed substrate or water molecules) among residues in non-consecutive sequence loci, in a defined temporal sequence. Together, the local conformational changes revealed in the computational modeling of the process give rise to the global rearrangements of TM and

| Table 5. ProKink analysis. |
|---------------------------|
| Residues | bend angle | | face shift | | References |
| | inward-facing | | inward-facing | | |
| S1-DAT | inward-facing | | S1-DAT | inward-facing | |
| Avg. | std | Avg. | std | Avg. | std | Avg. | std |
| L80 | 32.8 | 2.3 | 38.5 | 2.7 | 127.3 | 6.6 | 165.0 | 5.8 | [77,78] |
| P101 | 12.0 | 3.9 | 19.4 | 3.1 | 26.8 | 13.1 | 9.9 | 11.1 | [5–9] |
| P115 | 19.3 | 3.0 | 24.1 | 2.7 | 117.7 | 40.2 | 9.1 | 144.5 | [5,6,9,79] |
| S149 | 22.0 | 2.8 | 14.5 | 2.7 | 13.3 | 6.1 | 13.7 | 6.3 | [80] |
| GI53 | 10.2 | 3.7 | 19.4 | 3.7 | 18.4 | 7.4 | 14.7 | 6.8 | [80] |
| S245 | 43.2 | 2.8 | 55.0 | 3.4 | 10.0 | 10.5 | 41.8 | 8.9 | [81–83] |
| T269 | 6.0 | 3.3 | 15.8 | 4.0 | –13.5 | 5.4 | 15.7 | 7.0 | [82,84] |
| P273 | 22.3 | 5.2 | 24.2 | 5.1 | 59.5 | 20.3 | 58.2 | 18.3 | [5,6,84,85] |
| S354 | 6.2 | 2.2 | 6.0 | 2.6 | 8.0 | 6.8 | 12.1 | 5.5 | [82,86–89] |
| S357 | 6.2 | 2.0 | 6.0 | 1.9 | 21.5 | 7.7 | 12.7 | 6.3 | [82,88,90–92] |
| S422 | 14.9 | 3.1 | 14.2 | 3.1 | 0.1 | 8.6 | 44.3 | 9.4 | [83,93] |
| G426 | 17.1 | 5.2 | 11.9 | 4.8 | 18.9 | 14.8 | 25.3 | 13.2 | n/a |
| T456 | 6.2 | 2.7 | 7.1 | 2.8 | 32.1 | 7.4 | 26.8 | 8.0 | [82,87] |
| S460 | 5.9 | 3.2 | 6.6 | 3.4 | 15.8 | 8.4 | 17.7 | 8.3 | [82,94] |
| G481 | 31.6 | 2.4 | 36.6 | 2.2 | 59.2 | 8.4 | 57.6 | 16.3 | n/a |
| S483 | 30.4 | 3.1 | 33.7 | 2.1 | 137.1 | 4.5 | 80.5 | 4.0 | [82,95,96] |

Figure 6. Changes in intracellular interaction networks. Y335$^{6,68}$ forms an H-bond with E428$^{6,66}$ in S1-DAT (A), and switches its H-bond partner to T62$^{12,70}$ in the inward-facing conformation (B).

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Transition to an Inward-Facing Conformation in DAT

Materials and Methods

Construction of function-related conformational states of DAT

DAT residue numbering. The DAT residue numbering scheme used here includes, in addition to the sequence-based numbering, a generic numbering system defined in [33,67]. According to this scheme, the most conserved residue in each TM is assigned a number 50, and then a pair of numbers (A1.A2) is used to identify each residue, where A1 refers to the TM number and A2 denotes the position of the amino acid relative to the most conserved residue in the TM (A2 numbers decrease from 50 towards the N-terminus and increase towards the C-terminus).

Homology modeling of DAT and construction of the simulation system. We had recently described results for a homology model of DAT and simulated it in explicit water and lipid environment [39]. The general protocol and the structure-based sequence alignment for homology modeling and ligand docking is as described there and earlier [35]. Briefly, the homology model uses as the template the known crystal structures for the cognate and homologous structure of LeuT [15]. DA was placed in the S1 site by aligning its amine group and hydrophobic portion with those of the structure leucine in the LeuT structure, and the long equilibration refines the interactions between DA and DAT. The two Na\(^{+}\) ions were positioned equivalently to those in LeuT and a Cl\(^{-}\) ion was placed based on the chloride binding site described in [42]. The final model was immersed in an explicit water/lipid box to construct the simulated system.

Substrate movement towards the extracellular side. Constant velocity SMD simulations were used to explore the extracellular translocation pathway and the S2 site following a protocol described previously for a similar study of LeuT [29]. The SMD simulations were performed on an equilibrated DAT model with a substrate present in the S1 site [39]. A velocity of 4 Å/ns and a harmonic constant of 4 kcal/(mol Å\(^2\)) were used in the pulling protocol of SMD for the substrate in the S1 site moving towards the extracellular side. As before, the force is applied through a connecting spring tethered at the center of mass of the ligand [29,68,69]. As described for the LeuT protocol, about 100 residues in the bottom parts of TMs 2, 4, 5, 7, 9, 10, 11 and 12 were constrained in the Z direction during the SMD. The entire simulation was performed in two phases. In Phase I, 2 ns of SMD simulation was followed by 4 ns of equilibration, while in Phase II 2 ns of SMD simulation was followed by 10 ns of equilibration. After 10 ns of equilibration a substrate was introduced again in the S1 site and the entire system with the two substrates (S1,S2-DAT) was equilibrated for 25 ns.

Substrate movement towards the cytoplasm. The SMD simulation was performed on the equilibrated S1,S2-DAT model to explore the intracellular translocation pathway and an inward-facing conformation. Step-wise decreased velocities of 10 Å/ns (for the first 200 ps), 5 Å/ns (for the subsequent 300 ps) and 2.5 Å/ns

Figure 7. The endogenous Zn\(^{2+}\) binding site. Extracellular portions of DAT containing the endogenous Zn\(^{2+}\) binding site, S1-DAT (orange) and the inward-facing DAT (cyan) are aligned with RMSD calculations using the whole structure and rendered in cartoon. The sidechains of Zn\(^{2+}\) and the inward-facing DAT (cyan) are aligned with RMSD calculations using the whole structure and rendered in cartoon. The distance increases to 15 Å between H375EL4a and E396El4b is 13 Å (orange dashed line). The distance increases to 15 Å in the inward-facing conformation (blue dashed line).
Snapshots were extracted every 10 ps. Multiple simulations were performed. For the analysis, the first 5 ns trajectories were discarded during equilibration of either S1, S2-DAT or the inward-facing conformation. Residues within 3.5 Å of the substrate at the S1 site for more than 5% of the time during equilibration of either S1-DAT or S1, S2-DAT were identified. Similarly, simulations of S1, S2-DAT that pulled the substrate towards the cytoplasm were used for identifying the residues in the transport pathway from the S1 site to the cytoplasm of DAT. Residues in either the S1 or S2 site were excluded from transport pathways.

**Structural Analysis**

Number of waters in DAT along the transport pathway. The internal water pathway in DAT was monitored from the average numbers of water molecules in the pathway along the Z coordinate (the membrane normal) every 50 ps for the last 1.5 ns of various equilibration trajectories of DAT: S1-DAT, S1, S2-DAT and the inward-facing conformation. All trajectories were aligned to a reference (S1-DAT) before counting.

Substrate-water interaction energies. The interaction energies were calculated with the CHARMM27 force field [70] using NAMD [71]. All the water molecules were treated as one group and DA as the other.

Calculation of dihedral angles. Dihedral angles \( \phi \), \( \psi \) and \( \chi_1 \), \( \chi_2 \) in the rotamers of residues F76\(^{1,42} \), F332\(^{6,65} \) and Y335\(^{6,68} \) were calculated with ptraj in AMBER9 [72] every 5 ps for the 25 ns equilibration trajectory of the S1, S2-DAT. Similarly, dihedral angles \( \chi_1 \), \( \chi_2 \) and \( \phi \), \( \psi \) angles of residues W63\(^{1,29} \), F69\(^{1,35} \), F76\(^{1,42} \), F332\(^{6,65} \), Y335\(^{6,68} \) and E428\(^{6,66} \) were calculated for the final 2 ns equilibration trajectories of S1-DAT, inward-facing and S1, S2-DAT models.

Calculation of solvent accessibility surface area (SASA). For W63\(^{1,29} \), F69\(^{1,33} \), F76\(^{1,42} \), F332\(^{6,65} \), Y335\(^{6,68} \) and E428\(^{6,66} \) values were calculated from the 25 ns S1, S2-DAT equilibration trajectory and the two intracellular pulling trajectories. SASA was recorded every 5 ps for the equilibration trajectories and every 2 ps for the SMD trajectories. Only surface area accessible to solvent was counted; surface area exposed to lipids was treated as buried. SASA percentage was obtained by dividing the SASA value for residue X by a reference value calculated for X in a Gly-X-Gly tripeptide in extended conformation [73].

Calculation of helix kink parameters. To describe local distortions in helices during the MD simulation caused by proline or consecutive glycine [74] as well as other helix-disrupting residues...
serine, threonine and cysteine [50], we calculated bend and face shift angles around the following residues: L90<sup>1.46</sup>, P101<sup>1.39</sup>, Pro12<sup>1.50</sup>, Pro17<sup>1.50</sup>, S149<sup>1.41</sup>, G153<sup>1.44</sup>, S254<sup>-1.41</sup>, T269<sup>-1.46</sup>, S354<sup>-1.39</sup>, S357<sup>-1.42</sup>, S422<sup>-1.50</sup>, G426<sup>-1.04</sup>, T569<sup>-1.49</sup>, G401<sup>-1.55</sup> and S463<sup>-1.56</sup>. Calculation was also carried out for Leu<sup>1.46</sup> to quantify the change in TM1α and TM1β. Since L90<sup>1.46</sup> is included the Pro Cα atoms of the Pro or other helix-disrupting residue in the relevant TM was positioned at the Cartesian origin. To calculate the bend angle of a helix, the coordinate system was rotated for each trajectory frame around the axis passing through the pre-proline helical segment until the long axis of the post and pre-proline parts were in the same plane. From this orientation, the bend or kink angle was measured as the angle between the axes of the two parts of the helix. To obtain the face shift angle, for each snapshot the post-proline segment, which included the Pro Cα atom was rotated so that both pre and post-proline helical parts shared a long axis [59]. The face shift angle was then calculated as the angle between projections of two vectors onto the plane perpendicular to the long axis; these vectors are the on connecting the Pro Cα atom with the Cartesian origin, and the average vector connecting the Cα atoms of the (-3) and (-4) amino acids with the origin.

**Aligning different conformational states with RMSD TT to define global movements.** Inward-facing conformations were aligned to occluded conformations for both DAT and LeuT using the plugin RMSD TT [53] of the VMD (Visual Molecular Dynamics) [75] program. The iterative fitting implemented in the RMSD TT plugin performs a by-residue weighted pairwise fitting, such that after each iteration, residues with lower average RMSD are assigned higher weights in the next iteration. A similar idea for improving the quality of the commonly used RMSD comparison had been described independently by Damm and Carlson [76]. In total, three iterations of fitting were carried out with default fitting parameters. The reported residue-based RMSD was calculated with RMSD TT when aligning the whole structure.

For additional methods, see Text S1 in File S1.

**Supporting Information**

**File S1** Supplementary Methods details, figures, and tables detailing structural rearrangements in the different conformational states of DAT.

**File S2** Hinge regions underlying the global and local conformational rearrangements in DAT and the hinge regions enabling these changes.

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**Author Contributions**

Conceived and designed the experiments: JAJ LS HW. Performed the experiments: JS. Analyzed the data: JS LS HW. Wrote the paper: JS JAJ LS HW.

**References**

1. Sonders MS, Quick M, Javitch JA (2005) How did the neurotransmitter cross the bilayer? A closer view. Curr Opin Neurobiol 15: 296–304.
2. Javitch JA (1998) Probing structure of neurotransmitter transporters by substituted-cysteine accessibility method. Methods Enzymol 296: 331–346.
3. Loland CJ, Norrugaard L, Gether U (1999) Defining proximity relationships in the tertiary structure of the dopamine transporter. Identification of a conserved glutamic acid as a third coordinate in the endogenous Zn<sup>2+</sup>-binding site. J Biol Chem 274: 36928–36934.
4. Norrugaard L, Loland CJ, Gether U (2003) Evidence for distinct sodium-, dopamine-, and cocaine-dependent conformational changes in transmembrane segments 7 and 8 of the dopamine transporter. J Biol Chem 278: 30547–30596.
5. Lin Z, Itohoka M, Uhl GR (2000) Dopamine transporter proline mutations influence dopamine uptake, cocaine analog recognition, and expression. FASEB J 14: 713–728.
6. Itohoka M, Lin Z, Uhl GR (2002) Dopamine efflux via wild-type and mutant dopamine transporters: alanine substitution for proline-572 enhances efflux and reduces dependence on extracellular dopamine, sodium and chloride concentrations. Brain Res Mol Brain Res 108: 71–80.
7. Sen N, Shi I, Beamng T, Weinstein H, Javitch JA (2005) A pincer-like configuration of TM2 in the human dopamine transporter is responsible for indirect effects on cocaine binding. Neuropharmacology 49: 780–790.
8. Lin Z, Uhl GR (2004) Proline mutations induce negative-charge effects on uptake velocity of the dopamine transporter. J Neurochem 94: 276–207.
9. Sucic S, Bryan-Lluka LJ (2005) Roles of transmembrane domain 2 and the first intracellular loop in human noradrenaline transporter function: pharmacological and SCAM analysis. J Neurochem 94: 1620–1630.
10. Quick M, Winther AM, Shi I, Nissen P, Weinstein H, et al. (2009) Binding of an octylglucoside detergent molecule in the second substrate (S2) site of LeuT establishes an inhibitor-bound conformation. Proc Natl Acad Sci U S A 106: 5563–5568.
11. Singh SK, Yamashita A, Gouaux E (2007) Antidepressant binding site in a bacterial homologue of neurotransmitter transporters. Nature 448: 952–956.
12. Singh SK, Pucetelli CL, Yamashita A, Gouaux E (2008) A Competitive Inhibitor Traps LeuT in an Open-to-Out Conformation. Science 322: 1655–1661.
13. Zhou Z, Zhen J, Jia Z, Karpovich NK, Law CJ, Reith ME, et al. (2009) Antidepressant specificity of serotonin transporter suggested by three LeuT-SSRI structures. Nat Struct Mol Biol 16: 652–657.
14. Zhou Z, Zhen J, Karpovich NK, Goeta RM, Law CJ, et al. (2007) LeuT-desipramine structure reveals how antidepressants block neurotransmitter reuptake. Science 317: 1390–1393.
15. Yamashita A, Singh SK, Kowate T, Yan Jin Y, Gouaux E (2005) Crystal structure of a bacterial homologue of Na<sup>+</sup>Cl<sup>−</sup> dependent neurotransmitter transporters. Nature 437: 213–223.
16. Kniazzeff J, Shi I, Loland CJ, Javitch JA, Weinstein H, et al. (2008) An intracellular interaction network regulates conformational transitions in the dopamine transporter. J Biol Chem 283: 17691–17701.
17. Abramson J, Wright EM (2009) Structure and function of Na<sup>+</sup>–symporters with inverted repeats. Curr Opin Struct Biol 19: 425–432.
18. Gouaux E (2009) Review. The molecular logic of sodium-coupled neurotransmitter transporters. Philos Trans R Soc Lond B Biol Sci 364: 149–154.
19. Lolkema JS, Slotboom DJ (2008) The major amino acid transporter superfamily has a similar core structure as Na<sup>+</sup>–galactose and Na<sup>+</sup>–leucine transporters. Mol Membr Biol 25: 567–570.
20. Hruby VJ, O (1966) Simple Allosteric Model for Membrane Pumps. Nature 211: 969–970.
21. Forrest LR, Tavoulari S, Zhang YW, Rudnick G, Honig B (2007) Identification of a chloride ion binding site in Na<sup>+</sup>–Cl<sup>−</sup> dependent transporters. FASEB J 21: 780–790.
22. Gouaux E, Casadio R, Cossarizza A, Pavone M, Fiser A, et al. (2007) Science 317: 1390–1393.
23. Forrest LR, Rudnick G (2009) The rocking bundle: a mechanism for ion-coupled solute flux by symmetrical transporters. Physiology (Bethesda) 24: 377–396.
24. Forrest LR, Tavoulari S, Zhang YW, Rudnick G, Honig B (2007) Identification of a chloride ion binding site in Na<sup>+</sup>–Cl<sup>−</sup> dependent transporters. Proc Natl Acad Sci U S A 104: 12761–12766.
25. Beuming T, Kniazzeff J, Bergmann ML, Shi I, Gracia L, et al. (2008) The binding sites for cocaine and dopamine in the dopamine transporter overlap. Nat Neurosci 11: 789–798.
24. Crisman TJ, Qu S, Kanner BI, Forrest LR (2009) Inward-facing conformation of glutamate transporters as revealed by their inverted-topology structural repeats. *Proc Natl Acad Sci U S A* 106: 20752–20757.

25. Enkavi G, Tajkhorshid E (2010) Simulation of spontaneous substrate binding revealing the binding pathway and mechanism and initial conformational response of GLP-T. *Biochemistry* 49: 1105–1114.

26. Khalili-Araghi F, Gumbart J, Wen PC, Sotomayor M, Tajkhorshid E, et al. (2009) Molecular dynamics simulations of membrane channels and transporters. *Compr Opin Struct Biol* 19: 129–137.

27. Li J, Tajkhorshid E (2009) Ion-releasing state of a secondary membrane transporter. *Biophys J* 97: L29–31.

28. Quick M, Yano H, Goldberg NR, Duan L, Beuming T, et al. (2006) State-dependent conformational changes of the translocation pathway in the tyrosine transporter TylT, a neutral neurotransmitter symporter from *Bacillus subtilis*. *J Biol Chem* 281: 26444–26454.

29. Shi L, Quick M, Zhao Y, Weinstein H, Javitch JA (2008) The mechanism of a neurotransmitter-sodium symporter inward release of Na+ and substrate is triggered by substrate in a second binding site. *Mol Cell* 30: 667–677.

30. Zhao Y, Quick M, Shi L, Mehler EL, Weinstein H, et al. (2010) Substrate-dependent proton antiport in neurotransmitter-sodium symporters. *Nat Chem Biol* 6: 109–116.

31. Noskov SY, Roux B (2008) Control of Ion Selectivity in LeuT: Two Na+ Binding Sites with Two Different Mechanisms. *Journal of Molecular Biology* 377: 904–918.

32. Noskov SY (2010) Molecular mechanism of substrate specificity in the bacterial neutral amino acid transporter LeuT: Proteins: Structure, Function, and Bioinformatics 75: 851–863.

33. Shi L, Weinstein H (2010) Conformational rearrangements to the intracellular occluded state in the LeuT and ApcT transporters are modulated by common mechanisms. *J Biol Chem* 285: 1105–1114.

34. Zhao Y, Terry D, Shi L, Weinstein H, Blanchard SC, et al. (2010) Single-molecule dynamics of gating in a neurotransmitter transporter homologue. *Nature* 465: 186–193.

35. Indarte M, Madura JD, Surratt CK (2008) Dopamine transporter comparative modeling Anisotropic Thermal Diffusion. *Journal of Molecular Biology* 351: 726–730.

36. Elling CE, Thirstrup K, Nielsen SM, Hjorth SA, Schwartz TW (1997) Engineering of ion-transporter distance as constraints in structural and functional analysis of TTR receptors. *Folding and Design* 2: 878–880.

37. Humphrey W, Dalke A, Schulten K (1996) VMD: visual molecular dynamics. *J Comput Chem* 14: 33–38.

38. Case DA, Darden TA, Cheatham III TE, Simmerling CL, Wang J, et al. (2006) The AMBER 8 suite: new developments in the force field, molecular dynamics, and utility programs. *J Comput Chem* 27: 2115–2131.

39. Damm KL, Carlson HA (2006) Gaussian-weighted RMSD superposition of class A G protein-coupled receptors. *Biophys J* 94: 1600–1612.

40. Henry LK, Adkins EM, Han Q, Blakely RD (2003) Serotonin and cocaine-targeted mutation of an intracellular tyrosine constitutively alters the conformational equilibrium of the transport cycle. *Proc Natl Acad Sci U S A* 99: 1683–1688.

41. Nosakov SY (2008) Molecular mechanism of substrate specificity in the bacterial neutral amino acid transporter LeuT. *Proteins: Structure, Function, and Bioinformatics* 71: 301–310.

42. Damm KL, Carlson HA (2006) Gaussian-weighted RMSD superposition of class A G protein-coupled receptors. *Biophys J* 94: 1600–1612.

43. Visiers I, Brunnheim BB, Weinstein H (2000) Prokinetics: a protocol for numerical evaluation of helix and strand motifs. *Bioinformatics* 16: 1292–1293.

44. Krishnamurthy H, Piscitelli CL, Gouaux E (2009) Unlocking the molecular mechanisms of gating in a neurotransmitter transporter homologue. *Nature* 431: 811–818.

45. Ota N, Agard DA (2005) Intramolecular Signaling Pathways Revealed by Structural probing of a microdomain in the dopamine transporter by cross-linking. *J Mol Biol* 345–354.

46. Case DA, Darden TA, Cheatham III TE, Simmerling CL, Wang J, et al. (2006) The AMBER 8 suite: new developments in the force field, molecular dynamics, and utility programs. *J Comput Chem* 27: 2115–2131.

47. Visiers I, Brunnheim BB, Weinstein H (2000) Prokinetics: a protocol for numerical evaluation of helix and strand motifs. *Bioinformatics* 16: 1292–1293.

48. Alam MM, Roux B (2007) Structural dynamics of gating in a neurotransmitter transporter homologue. *Nature* 431: 811–818.

49. Visiers I, Brunnheim BB, Weinstein H (2000) Prokinetics: a protocol for numerical evaluation of helix and strand motifs. *Bioinformatics* 16: 1292–1293.

50. Shi L, Srdanovic M, Beuming T, Skrabanek L, Javitch JA, et al. (2010) TRAC GAP dynamics and substrate-dependent conformational dynamics of a bacterial homolog of neurotransmitter-sodium symporters. *Nat Struct Mol Biol* 17: 822–828.

51. Singh R, Hurst DP, Barnett-Norris J, Lynch DL, Reggio PH, et al. (2002) Activation of the cannabinoid CB1 receptor may involve a W648/F363 rotamer toggle switch. *The Journal of Peptide Research* 60: 337–370.

52. Crisman TJ, Qu S, Kanner BI, Forrest LR (2009) Inward-facing conformation of glutamate transporters as revealed by their inverted-topology structural repeats. *Proc Natl Acad Sci U S A* 106: 20752–20757.

53. Gracia I (2005) *RMSDTT: RMSD Trajectory Tool*. 2.5 ed. Weil Medical College of Cornell University, Department of Physics and Biophysics.

54. Russell RB, Barton GJ (1992) Multiple protein sequence alignment from tertiary structure comparison: assignment of global and residue confidence levels. Proteins: 14: 309–323.

55. Eargle J, Wright D, Luther-Schulte Z (2006) Multiple alignment of protein structures and sequences for VMD. *Bioinformatics* 22: 504–506.

56. Enkavi G, Tajkhorshid E (2010) Molecular dynamics simulations of membrane channels and transporters. *Compr Opin Struct Biol* 19: 129–137.

57. Shi L, Srdanovic M, Beuming T, Skrabanek L, Javitch JA, et al. (2010) TRAC GAP dynamics and substrate-dependent conformational dynamics of a bacterial homolog of neurotransmitter-sodium symporters. *Nat Struct Mol Biol* 17: 822–828.

58. Christianson MW, Parthasarathy S, Schepartz A, Katayama A, et al. (2008) Sodium channels and substrate transport membranes domain polar mutants: AG and AAG values implicate regions important for transporter functions. *Mol Pharmacol* 73: 1103–1105.
83. Lin Z, Zhang PW, Zhu X, Melgari JM, Huff R, et al. (2003) Phosphatidylinositol 3-kinase, protein kinase C, and MEK1/2 kinase regulation of dopamine transporters (DAT) require N-terminal DAT phosphoacceptor sites. J Biol Chem 278: 20162–20170.
84. Zhang YW, Rudnick G (2005) Cysteine-scanning mutagenesis of serotonin transporter intracellular loop 2 suggests an alpha-helical conformation. J Biol Chem 280: 30807–30813.
85. Paczkowski FA, Bryan-Lluka IJ (2004) Role of proline residues in the expression and function of the human noradrenaline transporter. J Neurochem 89: 2013–211.
86. Kitayama S, Wang JB, Uhl GR (1993) Dopamine transporter site-directed mutations differentially alter substrate transport and cocaine binding. Proc Natl Acad Sci U S A 90: 7782–7785.
87. Danek Burgess KS, Justice JB, Jr. (1999) Effects of serine mutations in transmembrane domain 7 of the human norepinephrine transporter on substrate binding and transport. J Neurochem 73: 656–664.
88. Dar DE, Mayo C, Uhl GR (2005) The interaction of methylphenidate and benztropine with the dopamine transporter is different than other substrates and ligands. Biochem Pharmacol 70: 461–469.
89. Zhou Y, Zomot E, Kanner BI (2006) Identification of a lithium interaction site in the gamma-aminobutyric acid (GABA) transporter GAT-1. J Biol Chem 281: 22092–22099.
90. Kitayama S, Shimada S, Xu H, Markham L, Donovan DM, et al. (1992) Dopamine transporter site-directed mutations differentially alter substrate transport and cocaine binding. Proc Natl Acad Sci U S A 89: 7782–7785.
91. Danek Burgess KS, Justice JB, Jr. (1999) Effects of serine mutations in transmembrane domain 7 of the human norepinephrine transporter on substrate binding and transport. J Neurochem 73: 656–664.
92. Dar DE, Mayo C, Uhl GR (2005) The interaction of methylphenidate and benztropine with the dopamine transporter is different than other substrates and ligands. Biochem Pharmacol 70: 461–469.
93. Zhou Y, Zomot E, Kanner BI (2006) Identification of a lithium interaction site in the gamma-aminobutyric acid (GABA) transporter GAT-1. J Biol Chem 281: 22092–22099.
94. Paczkowski FA, Bonisch H, Bryan-Lluka IJ (2002) Pharmacological properties of the naturally occurring Ala(457)Pro variant of the human norepinephrine transporter. Pharmacogenetics 12: 165–173.
95. Keller2nd PC, Stephan M, Glomska H, Rudnick G (2004) Cysteine-scanning mutagenesis of the fifth external loop of serotonin transporter. Biochemistry 43: 8510–8516.
96. Plenge P, Wiborg O (2005) High- and low-affinity binding of S-citalopram to the human serotonin transporter mutated at 20 putatively important amino acid positions. Neurosci Lett 383: 203–208.