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# Cytoplasmic Permeation Pathway of Neurotransmitter Transporters

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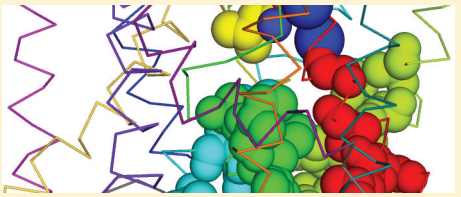
**ABSTRACT:** Ion-coupled solute transporters are responsible for transporting nutrients, ions, and signaling molecules across a variety of biological membranes. Recent high-resolution crystal structures of several transporters from protein families that were previously thought to be unrelated show common structural features indicating a large structural family representing transporters from all kingdoms of life. This review describes studies that led to an understanding of the conformational changes required for solute transport in this family. The first structure in this family showed the bacterial amino acid transporter LeuT, which is homologous to neurotransmitter transporters, in an extracellularly oriented conformation with a molecule of leucine occluded at the substrate site. Studies with the mammalian serotonin transporter identified positions, buried in the LeuT structure, that defined a potential pathway leading from the cytoplasm to the substrate binding site. Modeling studies utilized an inverted structural repeat within the LeuT crystal structure to predict the conformation of LeuT in which the cytoplasmic permeation pathway, consisting of positions identified in SERT, was open for diffusion of the substrate to the cytoplasm. From the difference between the model and the crystal structures, a simple "rocking bundle" mechanism was proposed, in which a four-helix bundle changed its orientation with respect to the rest of the protein to close the extracellular pathway and open the cytoplasmic one. Subsequent crystal structures from structurally related proteins provide evidence supporting this model for transport.



## ROLE OF NEUROTRANSMITTER TRANSPORT

Most neurotransmitter transporters belong to a family of $Na^+$-dependent amine and amino acid transporters (SLC6, NSS)[1,2] that has become a structural prototype for many other transporter families. The neurotransmitters serotonin (5-hydroxytryptamine, 5-HT), norepinephrine (NE), dopamine (DA), glycine, and γ-aminobutyric acid (GABA) all accumulate within neurons by the action of transporters in the NSS family.[3] As studied in model systems, neurotransmitter transporters are capable of using the energy of transmembrane ion gradients to power the accumulation of neurotransmitters to concentrations hundreds of times those in the external medium.[4,5] This accumulation limits the action of released transmitters, which act by binding to sites on the extracellular surface of pre- and postsynaptic receptors to cause a variety of cellular responses. In this way, the actions of transmitters released by exocytosis from nerve terminals are terminated by transport (or reuptake) back into the cells from which they were released or into neighboring cells.

The importance of these transport systems is highlighted by the effects of inhibiting their function either pharmacologically or genetically.[6] Inhibiting reuptake of the inhibitory neurotransmitter GABA by Tiagabine (Gabatril) is the basis of its anticonvulsive action. Inhibitors of SERT, the 5-HT transporter, are the most effective therapeutic agents used to treat clinical depression.[6] These drugs include fluoxetine (Prozac), citalopram (Celexa), Paroxetine (Paxil), and sertraline (Zoloft). Treatment of attention deficit hyperactivity disorder (ADHD) relies on methylphenidate (Ritalin), which inhibits transporters for DA and NE (DAT and NET, respectively).[7] Amphetamine, which is contained in Adderall (also used in ADHD therapy), stimulates efflux of the transmitter through DAT, NET, and SERT. Psychostimulant drugs of abuse that act through these transporters include cocaine, which inhibits DAT, NET, and SERT,[8] and amphetamine derivatives such as methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA, ecstasy).[9] Even when specific inhibitors are not available, genetic inactivation of neurotransmitter transporters demonstrate their importance. For example, mice in which glycine transporters have been genetically inactivated die shortly after birth from consequences caused by the failure of normal glycinergic neurotransmission.[10]

Neurotransmitter transporters, like all known members of the NSS family, utilize a transmembrane $Na^+$ concentration difference to drive the accumulation of their substrate neurotransmitter within the cell.[11] The energetically favorable influx of one or more $Na^+$ ions in response to transmembrane differences in concentration and electrical potential is coupled to the transport of a substrate molecule across the plasma membrane. This process (cotransport or symport) leads to substrate concentration ratios across the plasma membrane that are equal to, or even greater than, that of $Na^+$, depending on the coupling stoichiometry. In addition to requiring $Na^+$, many of these transporters also require $Cl^-$ for function and couple the influx of substrate to symport with $Cl^-$.[12,13] At least one transporter in the NSS family, SERT, couples influx of the substrate also to the efflux of $K^+$.[14] This exchange (antiport)

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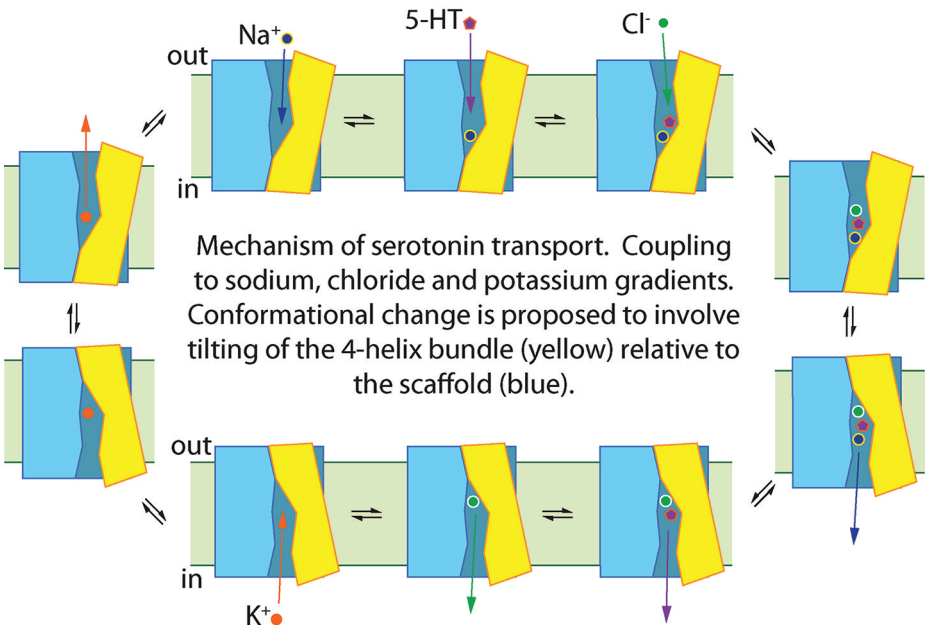


**Figure 1.** Proposed mechanism for the transport of serotonin by SERT. Binding of serotonin ($5\text{-HT}^+$), $Na^+$, and $Cl^-$ to the transporter from the cell exterior allows a conformational change, shown here as the tilting of a four-helix bundle (yellow), that closes the extracellular substrate permeation pathway and opens a cytoplasmic pathway. After dissociation of $Na^+$, $Cl^-$, and $5\text{-HT}^+$ to the cytoplasm, the transporter binds a $K^+$ ion [or a proton (see ref 96)] to allow the reverse conformational change, leading to extracellular $K^+$ dissociation and another cycle of transport.

provides additional driving force for 5-HT accumulation in addition to the $Na^+$ and $Cl^-$ gradients (Figure 1).

Transporters from distinct structural families are responsible for other aspects of neurotransmitter transport. The major excitatory neurotransmitter, glutamate, is transported across the plasma membrane by a family of transporters (SLC1, DAACS) that utilize $Na^+$ and $K^+$ gradients.[15] Accumulation of intracellular neurotransmitters within synaptic vesicles is accomplished by transporters from several families that use the low intravesicular pH and interior positive potential as a driving force to accumulate intravesicular transmitter by $H^+$ antiport.[16]

The energetics of each neurotransmitter transport process is defined by its symport and antiport stoichiometry.[11] For a transporter like NET, which catalyzes NE symport with $Na^+$ and $Cl^-$, the maximum NE gradient achieved should be determined by the $Na^+$ gradient, the $Cl^-$ gradient, and the transmembrane electrical potential (because NE is transported in its cationic form). For GABA transport, two $Na^+$ ions and one $Cl^-$ are symported with each GABA molecule and the maximum GABA gradient is also determined by the $Na^+$ and $Cl^-$ gradients and the electrical potential. For SERT, the $K^+$ gradient (in > out) also contributes to accumulation, but the potential does not, because the overall reaction moves no net charge across the membrane.

How transporters utilize transmembrane ion gradients and electrical potentials to drive neurotransmitter accumulation has been the topic of research and speculation for more than half a century.[17,18] However, only recently have convincing mechanisms been proposed. These recent advances spring from the availability of high-resolution structures of the transport proteins themselves or homologous bacterial proteins. For another type of transport protein, the $Ca^{2+}$-ATPase, multiple structures of the same protein in different steps of the catalytic cycle allowed an unprecedented understanding of molecular details.[19,20] Among ion-coupled transporters, the lac permease is the best understood, with copious biochemical and biophysical evidence directing interpretation of the crystal structure in the context of a conformational mechanism.[21] For many other transporters, much less information is available, although the expectation is that most, if not all, of these proteins work by what has been called an alternating access mechanism.

Alternating access essentially means that the binding sites for substrate and ions are exposed alternately to one side of the membrane or the other[17,18](Figure 1). Some early mechanistic descriptions of this model suggested that the transporter (carrier) bound its substrate on one side of the membrane and then diffused to the other side, where substrate could be released.[22] This was termed the moving carrier model, and it is probably an accurate description of the way that ionophores such as valinomycin, nigericin, and 2,4-dinitrophenol work. However, it is energetically prohibitive for the hydrophilic binding site on a membrane protein to move through the hydrophobic interior of the lipid bilayer. Peter Mitchell suggested that rather than the carrier itself moving, a barrier separating the binding site from one side of the membrane could move or be replaced by another barrier, so that the site would be exposed to the other side and separated from the side from which it bound, thus effectively transporting the substrate across the membrane.[23] Understanding how this movement of barriers is accomplished represents one of the most important milestones in transport research and is intimately tied to the understanding of transporter structure.

## ■ ASSESSMENT OF TOPOLOGY

When sequences of transport proteins became available in the 1980s and 1990s, predictions about their transmembrane topology were made on the basis of hydropathy plots, estimating the likelihood that each part of the primary sequence was located in a membrane-spanning region. The NSS family of neurotransmitter


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transporters was predicted to have 12 transmembrane (TM) helices,[24,25] similar to lac permease[26] and the facilitative glucose transporter.[27] In contrast, glutamate transporters provided some obvious hydrophobic regions that could span the membrane, but other parts that were difficult to assign.[28] Ultimately, it was the crystal structure of bacterial homologues that resolved the transmembrane topology of these proteins.[29,30] However, before these structures were available, site-directed chemical modification provided a way to assess which regions of these proteins were accessible from the medium.

Initially, several methods were used to reveal the topology of NSS neurotransmitter transporters. Fusing a marker protein to the C-terminus of truncated transporters was used for the glycine transporter GlyT1, and utilization of potential glycosylation sites was measured for both GlyT1 and the GABA transporter GAT-1.[31,32] Remarkably, both methods indicated that the first predicted intracellular loop (IL-1) was exposed on the cell surface (see Figure 2 for a current view of the topology of NSS transporters). From epitope-tagged transporters, the N- and C-terminal regions were known to be intracellular. The authors proposed that TM1, which was shorter than a typical transmembrane helix, did not span the membrane, that TM2 was actually the first TM helix (but with a topology opposite to what had been predicted), and that TM3, which was longer than a typical TM, spanned the membrane twice. However, the mutant transporters on which these conclusions depended were devoid of activity, leaving some of the conclusions in doubt.

For the homologous transporter SERT, a different approach was used, leading to a different topology. Chen et al.[33] found that Cys-109, one of the three endogenous cysteine residues predicted to be extracellular in SERT, was responsible for the transporter's sensitivity to modification by membrane-impermeant cysteine reagents, the other two extracellular cysteines being in a disulfide bond (Figure 2). Replacing Cys-109 with alanine allowed the construction of mutants with novel cysteine residues in various locations predicted to face the extracellular medium. Mutants containing a cysteine in each of the predicted extracellular loops (EL1−6) were modified by MTSEA-biotin, although the C109A mutant was not labeled, despite the presence of at least four intracellular cysteines.[34] Importantly, EL1 (connecting TM1 and 2) was accessible on the cell exterior, contrary to the prediction that TM1 did not cross the membrane (Figure 2).

A similar approach examined the reactivity of cysteine residues predicted to be on the cytoplasmic face of SERT. In unsealed membrane preparations of HeLa cells expressing SERT, both sides of the plasma membrane are accessible. SERT function in these membranes was measured by binding of the cocaine analogue $\beta$-CIT. Inactivation of $\beta$-CIT binding by MTS reagents required only one of the seven endogenous cytoplasmic cysteine residues, Cys-357 in IL3 between TM6 and 7.[35] Replacement of endogenous cysteines and adding novel ones demonstrated the cytoplasmic accessibility of IL1, IL2, IL5, and the N- and C-termini in addition to IL3[36] (Figure 2). A cysteine replacing Ala-441, predicted to lie within IL4, was also reactive, although it was later realized that this position was close to the center of the membrane in TM8.[37] None of these positions were accessible to extracellular MTS reagents in intact cells, indicating that they were exposed to the cytoplasm. Importantly, the sensitivity of mutants with cysteines at position 137 or 157 in IL1 indicated that this loop was cytoplasmic as originally proposed and not extracellular as suggested by nonfunctional glycosylation site mutants in GAT1 and GlyT1.[31,32]

## EVIDENCE OF CONFORMATIONAL CHANGES

In the context of the rough topological model of SERT, chemical modification studies provided an early indication of conformational changes upon ligand and ion binding. Several residues in TM1 and 3 were shown to be close to the substrate binding site as judged by substrate protection of cysteine residues placed in those positions. In TM1, replacement of Asp-98, Gly-100, or Asn-101 with cysteine led to MTS sensitive mutants that were protected from inactivation by 5-HT,[38] and in TM3, Ile-172 and Tyr-176 had similar characteristics.[39] These positions (yellow in Figure 2) were later identified as binding site residues using structural homology models.[37] However, many residues clearly outside of the binding site (blue in Figure 2) were also sensitive to modification in a way that was modulated by substrate. One clue about the nature of these peripheral sites was that many were protected by 5-HT but only in the presence of $Na^+$ or both $Na^+$ and $Cl^-$. $Na^+$ and $Cl^-$ are both required for 5-HT transport, and it was expected that these ions must bind together with 5-HT for transport to occur. However, 5-HT binding does not require $Na^+$,[40] and protection of central sites like Ile-172 also occurs in the presence or absence of this ion.[39] At other positions, such as Ile-179, 5-HT protected only when $Na^+$ was present,[41] indicating that some $Na^+$-dependent step subsequent to 5-HT

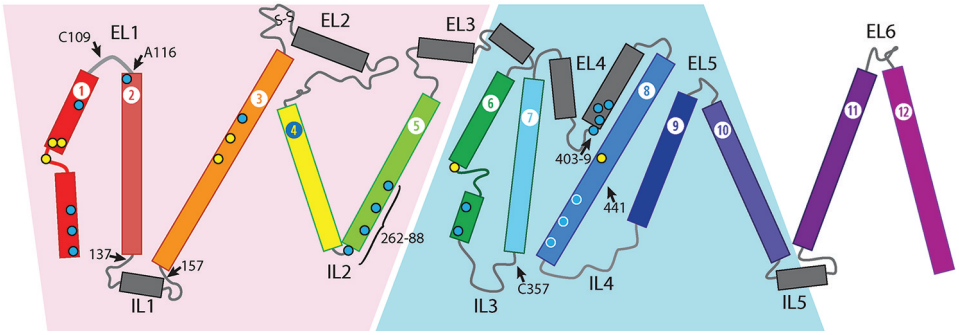


**Figure 2.** SERT topology. The topological organization of SERT was deduced from the accessibility of lysine and cysteine residues placed in proposed extracellular and cytoplasmic loops. From the structure of LeuT, the structure of transmembrane helices 1−5 (pink) is repeated, with an inverse topological orientation, by helices 6−10 (blue). Binding site residues in TM1, 3, 6, and 8 are colored yellow, and residues outside the binding site affected by conformational changes are colored blue.


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binding, such as a conformational change, was required. Cysteine residues inserted at several positions that are now known to be distant from the substrate site rendered SERT sensitive to MTS reagents.[35,36,42−46] 5-HT modified the rate of reaction at these positions, usually by slowing the process but in some cases by enhancing it. For many of these cysteine mutants, the effect of 5-HT also required $Na^+$ and $Cl^-$. For example, a cysteine replacing Ala-116 at the extracellular end of TM2 reacted with MTSEA faster in the presence of 5-HT but the 5-HT effect required NaCl.[44] In EL4, between TM7 and 8, cysteines at positions 403, 404, 407, and 409 were all protected by 5-HT but only in the presence of NaCl.[47] These results suggested that conformational changes linked to substrate and ion binding could be detected by their effect on the accessibility of inserted cysteine residues.

## ■ ACCESSIBILITY IN IL2

In an attempt to obtain structural and conformational information about the region surrounding a phosphorylation site at Thr-276,[48,49] the region from Ile-270 to Ser-293 was scanned by replacing each residue, one at a time, with cysteine.[50] Replacement with cysteine at any position from Lys-262 to Pro-288 was found to make SERT sensitive to modification by MTS reagents (either MTSEA or MTSES). Consistent with a region predicted to be an internal loop, none of the cysteine mutants were sensitive to reagents added extracellularly to intact cells, but all were sensitive in broken cell membrane preparations. Transporter modification was measured by a decrease in the level of binding for the high-affinity ligand $\beta$-CIT because no transport could be measured in these unsealed membranes.

The extent of the MTS-accessible sequence was larger than the region predicted from hydropathy profiles (Trp-271−Lys-279). Moreover, rates of reaction with MTSEA in this region varied in a characteristic pattern, with some positions more than 100-fold more reactive than others (Figure 3). The pattern was consistent with an $\alpha$-helical conformation with one face of the helix highly accessible to the cytoplasm and the rest of the residues protected, presumably by packing against other parts of the protein or the membrane[50] (Figure 4).

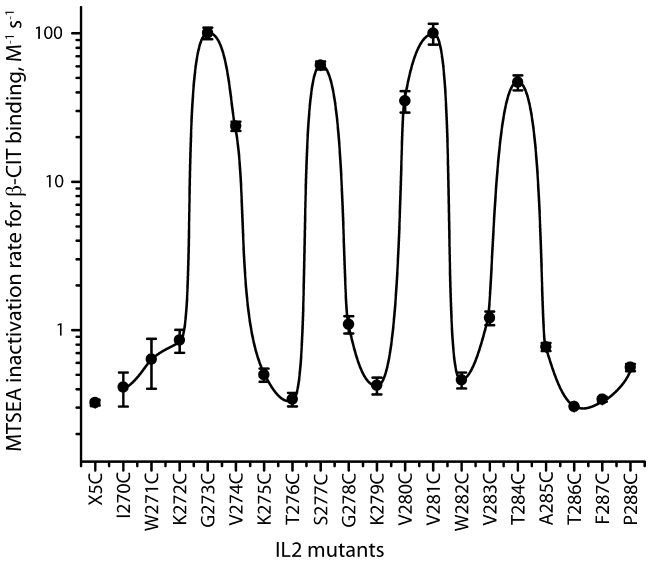


**Figure 3.** Reactivity of TM5 cysteine mutants. Rates of reaction were measured by inhibition of $\beta$-CIT (a cocaine analogue) binding to SERT. Modifying residues in the cytoplasmic permeation pathway prevents that pathway from closing, which is required for opening the extracellular pathway and binding of $\beta$-CIT.[45,50]

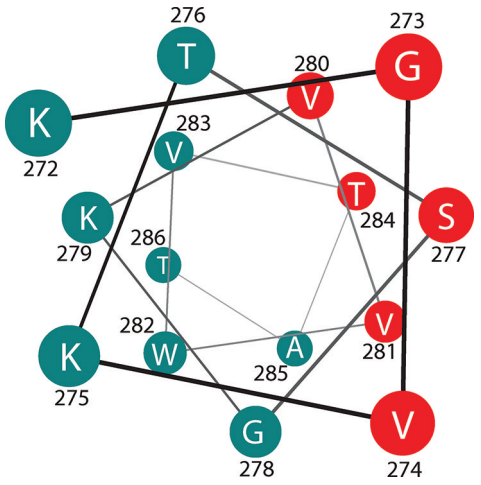


**Figure 4.** Helical wheel representation of TM5, showing reactive positions. TM5 represented as an $\alpha$-helical wheel. Mutants with positions colored red replaced by cysteine were highly reactive towards MTS reagents, and those colored teal were much less reactive (Figure 3).

## ■ ENTER LEUT

It was becoming increasingly obvious that without a structural model of an NSS transporter, it would be extremely difficult to interpret the changes in reactivity observed in SERT and other proteins in the family. Unfortunately, the known mammalian members of this family were difficult or impossible to purify, and it seemed unlikely that any of them would be crystallized. However, sequence information was becoming available for prokaryotic genomes, and many of these contained genes similar to those encoding the NSS family. One of these, *TnaT*, was found in an operon together with tryptophan metabolizing enzymes in *Symbiobacterium thermophilum* and was proposed to be a tryptophan transporter.[51] Expressing the TnaT gene product in an *Escherichia coli* strain lacking endogenous tryptophan transport systems led to $Na^+$-dependent tryptophan transport.[52] The observation that TnaT was an authentic transporter made the hundreds of prokaryotic NSS homologues attractive targets for purification and crystallization attempts.

In 2005, Gouaux and colleagues published a high-resolution crystal structure of LeuT, an amino acid transporter from the bacterium *Aquifex aeolicus*.[30] LeuT was named for the molecule of leucine found at the substrate site in the crystallized transporter, although leucine is a poor substrate for LeuT, which prefers alanine.[53] More importantly, LeuT is a homologue with a sequence 20−25% identical to that of neurotransmitter transporters in the NSS family, and that identity is clustered in regions close to the binding sites for leucine and two $Na^+$ ions. This structure made sense out of data that had been collected for NSS transporters for the 15 years that had elapsed since their primary sequences had been revealed.

The LeuT crystal structure with bound leucine shows an aqueous pathway from the extracellular medium leading to, but not including, the binding site for amino acid and $Na^+$ ions


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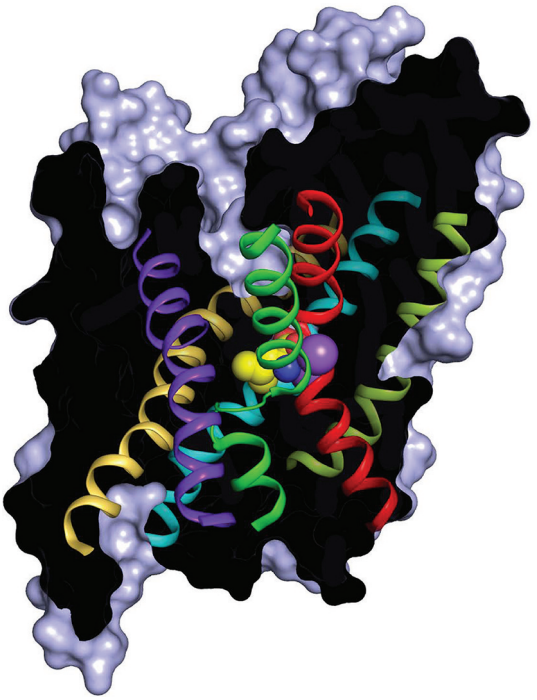

**Figure 5.** Cut-away view of LeuT. An occluded molecule of bound leucine is shown, together with $Na^+$ ions as spheres. Part of the extracellular permeation pathway is visible leading to the binding site. Transmembrane helices contributing to the binding site and the pathways are shown in cartoon form: red for TM1, yellow for TM3, yellow-green for TM5, green for TM6, cyan for TM8, and violet for TM10.

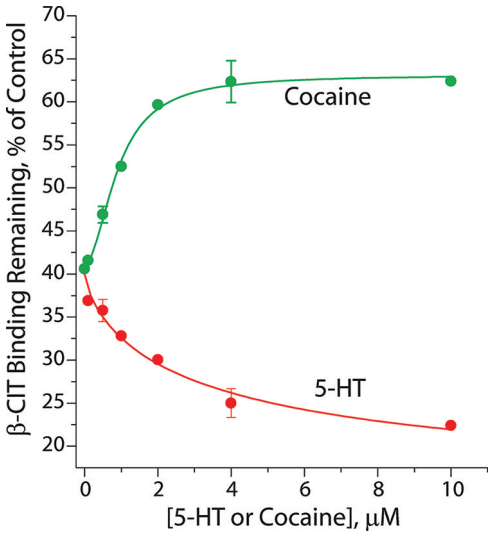


**Figure 6.** Opposite effect of 5-HT and cocaine on cytoplasmic pathway accessibility. Using an MTSEA concentration and incubation time chosen to inactivate ~50% of $\beta$-CIT binding to the S277C mutant of SERT, cocaine decreased the level of inactivation and 5-HT increased it. These results are consistent with cocaine stabilizing the extracellular conformation of SERT and 5-HT allowing conversion to the cytoplasmic form.[45]

(Figure 5). This pathway opens to include the binding sites in a subsequent structure with tryptophan bound.[53] On the cytoplasmic side of the membrane, however, there is no pathway visible for substrate diffusion. Indeed, 20 Å of packed protein structure lies between the amino acid binding site and the cytoplasm. Access of bound leucine to the extracellular side is also obstructed in the LeuT-leucine structure, although only by a few amino acid side chains. Leucine was described as being occluded within its binding site by barriers to both sides of the membrane,[30] as predicted by some alternating access models of transport.

The LeuT structure also shed light on the apparent helical pattern of reactivity in the IL2 region of SERT. When aligned with the LeuT sequence, this region corresponded not only to the loop between TM4 and TM5 but also to approximately half of the TM5 helix, including residues buried in the core of LeuT and inaccessible to solvent.[45] Therefore, the LeuT structure provided an explanation for the helical structure of the reactive region, but the predicted accessibility was clearly different from that of the corresponding residues in SERT. For these positions to react with MTSEA, SERT would need to be in a conformation different from that of the LeuT crystal. Either the LeuT structure was not a good model for SERT, or it represented a conformation different from the one responsible for the MTSEA reactivity of TM5.

The possibility that conformational differences were responsible for the accessibility of TM5 in SERT was supported by changes in the rates of reaction in the presence of 5-HT and cocaine.[45] Cocaine protected the most reactive positions in TM5, while 5-HT, conversely, increased their reactivity (Figure 6). As a substrate, 5-HT is expected to induce the conformational change that leads to its translocation across the membrane. Part of this change involves opening a pathway through which 5-HT, $Na^+$, and $Cl^-$ diffuse to the cytoplasm. Moreover, the 5-HT effect required $Na^+$ and $Cl^-$, as expected if the effect of 5-HT was to allow the transporter to initiate a cycle of transport.[45] For cocaine, the most likely mechanism of inhibition is by binding to the substrate site of SERT (as proposed for DAT[54]) and preventing substrate access and conformational change. Thus, cocaine would stabilize a conformation resembling that of the LeuT crystal, in which the cytoplasmic pathway was closed and TM5 reactivity would be minimal. The opposite effects of cocaine and 5-HT suggested that cocaine stabilized an "extracellular-facing" conformation of SERT and 5-HT allowed a change to a "cytoplasm-facing" conformation that increased the accessibility of the reactive positions in TM5. Although the nature of the conformational change was not initially apparent, we tentatively concluded that accessibility of TM5 represented opening of a permeability pathway from the cytoplasm to the substrate binding site.[45] We proposed that TM5 contributed one face to the pathway and predicted that other TM helices constituted the remainder of the pathway.

## AN INVERTED STRUCTURAL REPEAT IN LEUT PROVIDES CLUES ABOUT THE MECHANISM

An important feature that LeuT shares with several other membrane transport families is an inverted structural repeat.[30] The structure of TM1−5 is strikingly similar to that of TM6−10, despite the lack of significant sequence homology between these two regions (Figure 2). The repeated structure contains an odd number of transmembrane spans, and as a consequence, the topology of the second repeat is inverted with respect to the first. The first repeat begins with TM1 on the cytoplasmic side

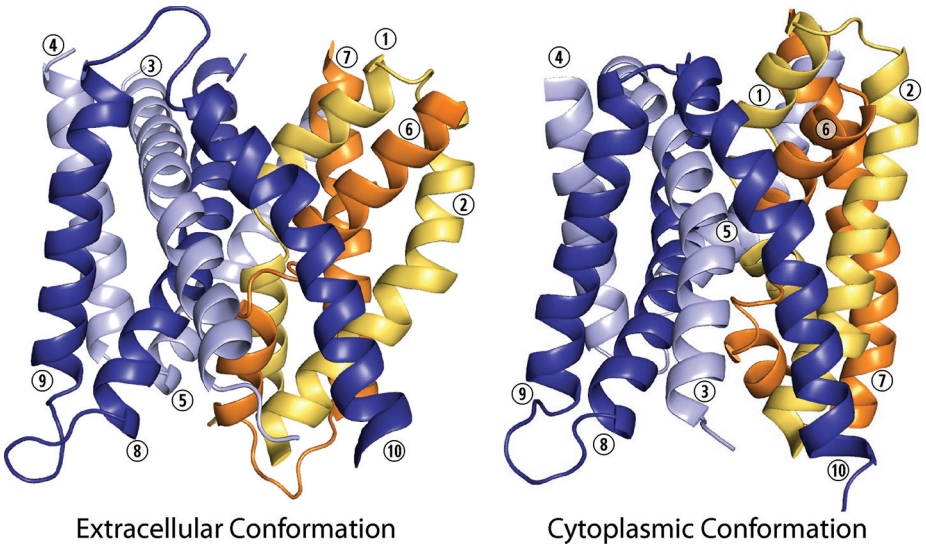


**Figure 7.** Extracellular and cytoplasmic conformations of LeuT. The extracellular conformation represents the X-ray structure of LeuT with occluded leucine.[30] The cytoplasmic conformation represents the model created by exchanging the conformations of the first and second structural repeats of LeuT.[46] The first repeat is shown in pale colors and the second in more intense colors. Gray and purple indicate the scaffold helices and yellow and orange the bundle helices. TM11 and 12 and several loops have been omitted for the sake of clarity. In the X-ray structure (left), the cytoplasmic half of the bundle packs closely against the scaffold, whereas in the model (right), the cytoplasmic half of the bundle moves away from the scaffold to create the cytoplasmic pathway and the extracellular half of the bundle packs closely against the scaffold, closing the extracellular pathway.

of the membrane and ends with the extracellular end of TM5, and the second repeat begins with TM6 on the extracellular side and ends with the cytoplasmic end of TM10. For each segment in a repeat, there is a complementary segment in the other repeat that echoes its structural characteristics. For example, the long tilted helix of TM3 in the first repeat lies next to TM8, another long and tilted helix in the corresponding position of the second repeat (Figure 2). Two V-shaped helix pairs, TM4 and 5 from the first repeat and TM9 and 10 from the second, trap the two tilted helices at each end. In TM1, there is an unwound region that contributes to the substrate and $Na^+$ binding site that is complemented by an unwound region of TM6 that also contributes to these sites. Finally, TM1 and 6 are found within a four-helix bundle containing TM2 and 7 (Figure 7). The pseudo-2-fold symmetry inherent in this structure supplied an important clue toward revealing the pathway through which substrate diffuses from the binding site to the cytoplasm.

## COMPOSITION OF THE CYTOPLASMIC PATHWAY IN SERT

Cysteine scanning of EL5, connecting TM9 with TM10, demonstrated that this region was exposed to the extracellular medium.[43] The accessible region associated with EL5 corresponds to LeuT positions that include EL5 but also encompasses approximately half of TM10. EL5–TM10 accessibility is remarkably similar to IL2–TM5 accessibility on the cytoplasmic face of SERT (Figure 8). TM10 is one of the helices lining the aqueous pathway leading from the extracellular surface to the binding site and is related to TM5 as they are each the last helix in one of the five-helix repeats. This similarity raised the possibility that the cytoplasmic pathway was composed of elements corresponding to those that comprised the LeuT extracellular pathway [TM1, 3, 6, and 10 (Figure 5)].[30] For each of these helical elements, the corresponding element in the opposite repeat of SERT proved to be part of the cytoplasmic pathway, namely, the cytoplasmic

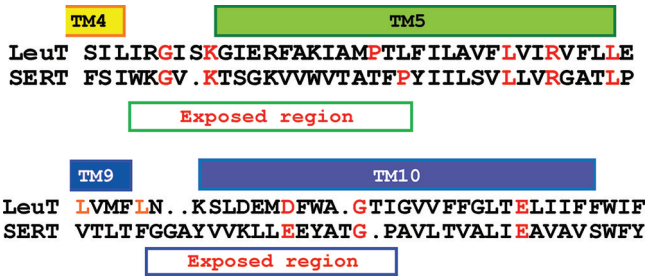


**Figure 8.** Exposed regions of IL2–TM5 and EL5–TM10 structures. The cytoplasmically accessible region between TM4 and 5 (IL2) and the cytoplasmic half of TM5 are shown to correspond to the extracellularly accessible region between TM9 and 10 (EL5) and the extracellular half of TM10. Accessibility in IL2 and TM5 was determined by inactivation of $\beta$-CIT binding in membrane preparations,[50] and in EL5 and TM10, accessibility was measured by inactivation of 5-HT transport in intact HeLa cells.[43]

halves of TM6, 8, 1, and 5, respectively.[46] Cysteine scanning of these helices indicated that they each contained positions readily accessible to cytoplasmic cysteine reagents, along one side of each helix stretching from the binding site to the cytoplasm[46](Figure 9). Thus, the cytoplasmic permeation pathway is made of elements similar to those that constitute the extracellular pathway. Importantly, both permeation pathways are composed of helices from each of the two repeats. These findings highlight an important relationship between the inverted structural repeat of LeuT and the functional elements responsible for the mechanism of transport.

Initial experiments that aimed to examine the effect of ligands on accessibility in TM1, 6, and 8 were puzzling. Cocaine had an inhibitory effect on the reactivity of cysteine residues in these helices, similar to TM5, but 5-HT, rather than stimulating reactivity as it did in TM5, decreased reactivity. Apparently, the effect of 5-HT varied in different cysteine mutants. In most but not all of the TM5 cysteine mutants, 5-HT binding favored the


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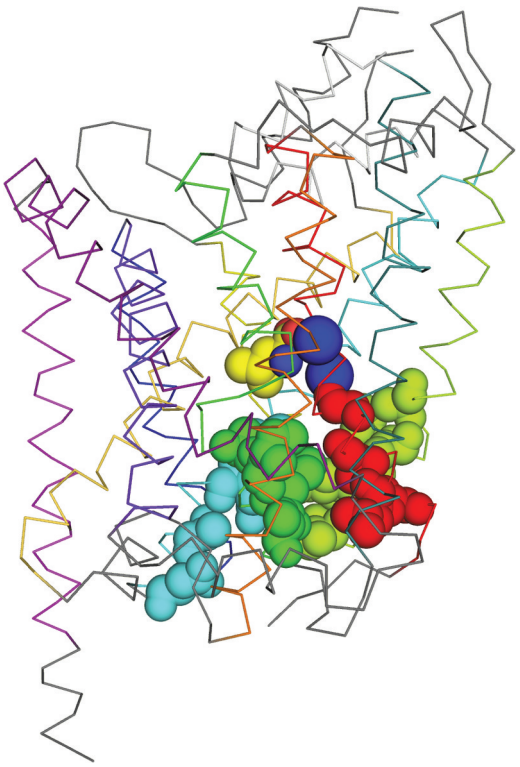

**Figure 9.** Residues lining the cytoplasmic pathway of SERT. The $\alpha$-carbon chain of LeuT is shown with bound leucine (yellow) and $Na^+$ (blue) as spheres. Residues corresponding to the reactive positions in the SERT cytoplasmic pathway are colored red (TM1), lime (TM5), green (TM6), and cyan (TM8).

cytoplasmic conformation of SERT, in which the cytoplasmic pathway is open and reactivity of inserted cysteine residues is increased. However, in other mutants, particularly those in TM1, 6, and 8, 5-HT binding favored the extracellular conformation, which resembles the LeuT crystal structure. In retrospect, this is not surprising, because 5-HT must bind to both conformations at different points during the transport cycle. To reliably favor the cytoplasmic conformation though, we needed another ligand with affinity for the cytoplasmic form of SERT. This ligand proved to be an alkaloid called ibogaine.[55]

## IBOGAINE INFLUENCES THE CONFORMATION OF SERT

Ibogaine is a hallucinogenic alkaloid used for ritual purposes in West Africa and proposed as a treatment for opiate withdrawal.[56] It had been reported to competitively inhibit activity of SERT and DAT[57] and, when examined more carefully, demonstrated itself to be a noncompetitive inhibitor of SERT.[58] All previously known SERT inhibitors were competitive, and it was not immediately clear how ibogaine acted. However, there were precedents for noncompetitive inhibitors of two other transporters. In the red cell glucose transporter, inhibition by cytochalasin B was noncompetitive,[59] as was inhibition of the mitochondrial adenine nucleotide exchanger by bongkrekic acid.[60] Both of these inhibitors were known to bind to the conformation of their target transporter in which the substrate binding site was exposed on the internal but not external side of the membrane. This also proved to be the case for ibogaine binding to SERT.[58] Ibogaine increased the reactivity of cysteines at the highly accessible TM5 positions that were proposed to line the cytoplasmic permeation pathway, in contrast to cocaine, which decreased reactivity at the same positions (Figure 10). However, TM5 mutants with cysteine on other faces of the helix did not react faster in the presence of ibogaine, suggesting that the effect represented opening of the pathway and not a generalized increase in accessibility. On the extracellular side of the binding site, the opposite effect was observed. Y107C and S404C are two mutants with reactive cysteine residues in the extracellular permeation pathway. 5-HT protected these two mutants from inactivation by the MTS reagents, and the protection required $Na^+$ and $Cl^-$, indicating that it resulted from conformational changes.[38,47] In these mutants, ibogaine also protected against inactivation by MTSEA, the opposite effect that it had on positions in the cytoplasmic pathway (Figure 10). The results suggest that ibogaine induces a concerted conformational change in SERT that opens the cytoplasmic pathway and closes the extracellular one.

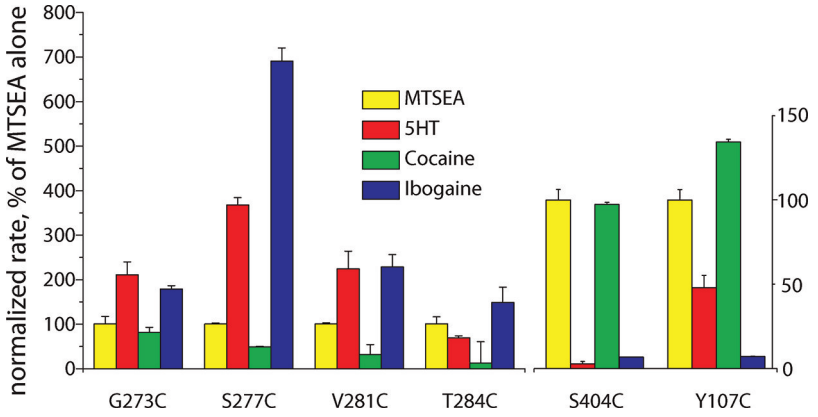


**Figure 10.** Effect of 5-HT, cocaine, and ibogaine on reactivity of TM5 and extracellular pathway positions. 5-HT and ibogaine enhanced the reactivity of TM5 cysteines (273, 277, 281, and 284) in the permeation pathway, and cocaine decreased reactivity. Cysteines in the extracellular pathway (107 and 404), in contrast, reacted much slower in the presence of 5-HT and ibogaine, and cocaine, if anything, enhanced the rate. These results suggest a concerted conformational change in both pathways due to 5-HT and ibogaine increasing the fraction of SERT in the cytoplasmic conformation and cocaine stabilizing the extracellular conformation.


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When we compared the effects of ibogaine and cocaine on reactivity in TM1, 5, 6, and 8, we found that almost all the accessible positions were more reactive in the presence of ibogaine and less reactive in the presence of cocaine.[46] The exceptions were two positions at the cytoplasmic ends of TM5 and 8, where the already high reactivity was not enhanced by ibogaine, presumably because these positions were already quite accessible even in the conformation with the pathway closed. The only other exception was V343C in TM6 near the substrate binding site. Ibogaine decreased the reactivity of this position for unknown reasons, although it is possible that ibogaine binding sterically occludes that position. The complementary effects of cocaine and ibogaine on accessibility support the idea that TM1, 5, 6, and 8 all experience increased accessibility when the cytoplasmic pathway opens during transport. However, the accessibility data alone were insufficient to deduce the mechanism by which this conformational change occurred.

## MODELING THE CYTOPLASMIC CONFORMATION OF LEUT

Computational modeling provided the key to understanding the conformational change responsible for opening the cytoplasmic permeation pathway. The two repeats in LeuT, although similar in structure, are in different conformations. Indeed, if they were identical, the structure of the TM1−10 core would be symmetrical with respect to the membrane. Instead, the asymmetry of the structure (with an open pathway on the extracellular side of the binding site and a packed structure on the cytoplasmic side) indicated that the two repeats were in different conformations, in addition to having opposite topologies.[30] Lucy Forrest, at the time a postdoctoral associate with Barry Honig at Columbia University (New York, NY), examined the two five-TM repeats and found that each one was composed of two parts. The first two TMs of each repeat superimposed nicely on each other, as did the last three. However, the angle between the first and second parts differed by ~25°.[46] In the crystal structure, the first two TMs in each repeat form a four-helix bundle that is tilted with respect to the rest of the protein (Figure 7). The six remaining helices in the core of the protein (TM3−5 and TM8−10) form a scaffold that wraps around three sides of the bundle. The tilt in this bundle relative to the scaffold is responsible for most of the conformational difference between the two repeats, and it also is responsible for the asymmetry in the structure, because the cytoplasmic half of the bundle packed closely against the scaffold while the extracellular half was separated from it, thus creating the extracellular permeation pathway (Figure 7).

These observations suggested a possible mechanism for transport. If the bundle could tilt one way, it might also tilt in the opposite way to close the extracellular pathway and open the cytoplasmic one. For this to happen, the loops connecting the bundle with the rest of the protein should be long enough to allow the motion. TM1, 2, 6, and 7 are connected to the rest of the protein by three loops, IL1, EL3, and EL4. Each of these loops is large enough and has helical elements that would allow the required motion. In contrast, the loops within the bundle (EL1 and IL3) and most of the loops between the remaining helices (EL5, EL6, IL2, and IL4) are short and should limit relative motion between the helices they connect (Figure 2).

A strategy for visualizing a cytoplasm-facing conformation was developed on the basis of the idea that the two conformations of the transporter, with the binding site exposed either to the extracellular medium or to the cytoplasm, were related by the tilt of the four-helix bundle. The tilt was reflected in the conformational difference between the two repeats. Therefore, exchanging the conformations of the two LeuT repeats should generate a cytoplasm-facing conformation opposite to that in the crystal structure. Lucy Forrest aligned the first repeat (TM1−5) with the second repeat (TM6−10) guided by similarities in structure, sequence conservation, and accessibility. She generated a structure in which the first repeat was constrained to the conformation that the second repeat assumed in the LeuT crystal structure but retained its N-terminal cytoplasmic topology. Likewise, the second repeat was modeled to assume the conformation that the first repeat had in the crystal. By careful adjustment of the alignment, she was able to generate a model (Figure 7) of a cytoplasm-facing conformation that optimized the interaction of conserved positions with protein and maintained contacts within the binding sites for $Na^+$ and substrate, at the same time allowing cytoplasmic access to residues corresponding to the accessible positions in SERT and closing the extracellular permeation pathway.[46]

## ROCKING BUNDLE MECHANISM OF TRANSPORT

The principal difference between this model and the crystal structure is the orientation of the four-helix bundle relative to the remainder of the protein. Specifically, in the model, the bundle is tilted approximately 25° from its position in the LeuT crystal structure, so that the extracellular half is closer to the scaffold and the cytoplasmic half is removed from it to create a permeation pathway (Figure 7). This pathway is lined by the four helices that correspond to those identified in SERT as part of the cytoplasmic permeation pathway, TM1, 5, 6, and 8. Conversion between the position of the four-helix bundle in the LeuT crystal structure and the cytoplasm-facing model could constitute the principal conformational change that allows the transporter to bind substrates on one side of the membrane and release them to the other side.[46]

Although the process by which the conformational change occurs is not known, the simplest mechanism would be for the four-helix bundle to tilt or rock back and forth as a structural unit. This "rocking bundle" mechanism of transport has many appealing characteristics. It provides a simple way to couple the opening of one permeation pathway with the closing of another. Moreover, if the bundle rocked back and forth on an axis close to the bound substrate and ions, it would be expected to affect those binding sites minimally relative to other points along the bundle−scaffold interface. Also, it provides a way to couple ligand binding to conformational change, which is crucial for coupled transporters such as SERT and related proteins. By binding at the interface between the two parts of the protein that move relative to one another, the substrates are in an ideal position to influence those movements. In this way, binding of $Na^+$, $Cl^-$, and 5-HT together allows movement of the bundle relative to the scaffold that would not occur if one of the substrates was missing.

## LEUT-LIKE STRUCTURES FROM OTHER TRANSPORTER FAMILIES

There are several indications that the model of LeuT in the cytoplasmic conformation represents a reasonable approximation

of the state of this transporter that releases substrates to the cytoplasm. Some of this information came from unlikely sources. In 2008, Faham et al.[61] published the structure of a bacterial sugar transporter in the solute:sodium symporter (SSS) family thought to be unrelated to the neurotransmitter transporters. Despite the lack of sequence homology with LeuT, this protein, vSGLT, had a very similar structure. vSGLT, unlike LeuT, has 14 transmembrane helices, but TM2−11 form a core transporter structure very similar to that of TM1−10 in LeuT. The conformation of these TMs, however, is more similar to that in the cytoplasm-facing model of LeuT rather than in the crystal structure.[62] Consequently, the vSGLT structure shows a permeation pathway between the sugar binding site and the cytoplasm, lined by TM2, 6, 7, and 9, which corresponds to the pathway identified in SERT and visualized in the cytoplasm-facing LeuT model. It was surprising to find that an apparently unrelated transporter family adopted the same structure. Evidently, the SSS family, containing vSGLT, diverged from the NSS family so long ago that no sequence identity remained, although the structure was maintained, presumably because it was essential for transport function.

Subsequently, members of three additional transporter families, all unrelated by sequence to LeuT, were crystallized and also found to have structures resembling that of LeuT. These include a sodium:benzyl-hydantoin symporter, Mhp1 from *Microbacterium liquefaciens* and belonging to the nucleobase:cation symporter (NCS1) family,[63] two members of the betaine/choline/carnitine transporter family (BCCT), a sodium:betaine-glycine transporter, BetP from *Corynebacterium glutamicum*,[64] and the $Na^+$-independent carnitine/butyrobetaine antiporter CaiT from *Proteus mirabilis*,[65,66] and two members of the amino acid-polyamine-organocation (APC) family, the arginine-agmatine antiport AdiC from *Salmonella enterica*[67−69] and the proton-coupled amino acid antiporter ApcT from *Methanocaldococcus jannaschii*.[70] Thus, it appears as if LeuT represents a large transporter family related by a common structure but divided by sequence homology into subfamilies (Figure 11). This similarity in structure, in the face of a wide variety of coupling mechanisms in these transporters, suggests that the conformational mechanism of transport may be similar in all of the transporters within this large structural family. Also evident is a diversity of substrates and coupling mechanisms indicating that this structure is sufficiently versatile to transport a variety of substrates using $Na^+$ and $Cl^-$ symport, $H^+$ or $K^+$ symport or antiport, and amino acid antiport.

Most relevant, however, to the discussion of transport mechanism is the case of Mhp1. There are now three structures of this protein, two in extracellular-facing conformations, with and without substrate bound,[63] and a more recent structure in a cytoplasm-facing conformation.[71] The substrate-free extracellular conformation of Mhp1 resembles the LeuT crystal structure with leucine bound,[30] and the cytoplasmic conformation resembles the model of LeuT in that conformation.[46]

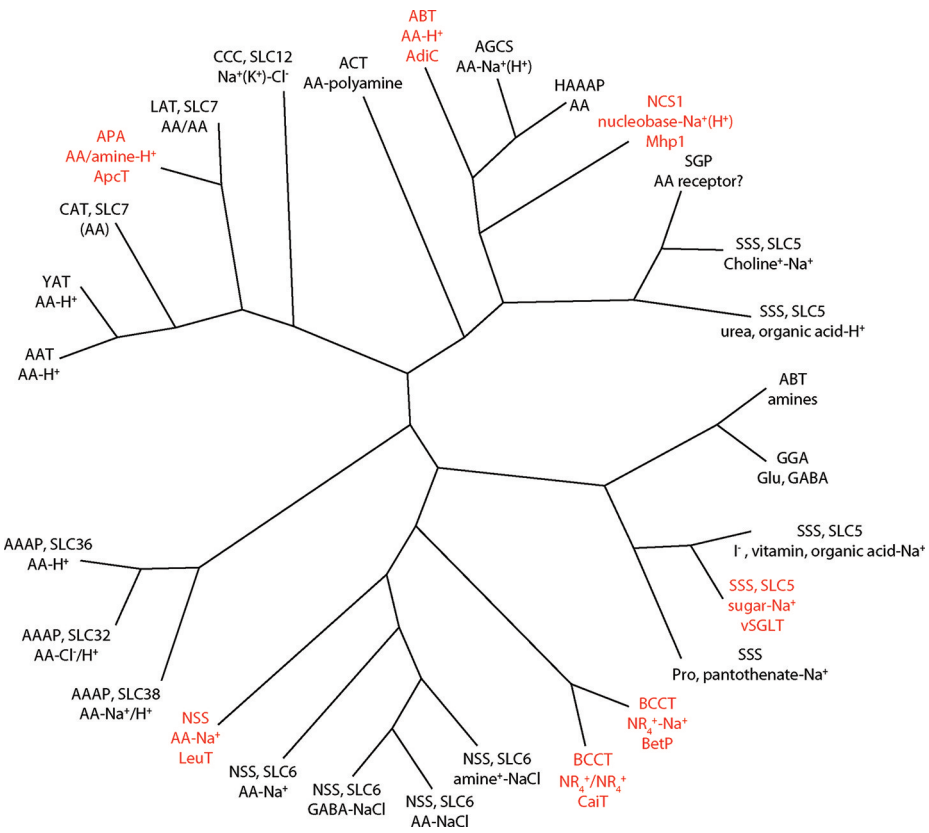


**Figure 11.** Relationships between transporters in the LeuT structural family. Transporter families with sequences homologous to those of proteins structurally similar to LeuT were identified with the Transporter Classification Database (TCDB).[97] Consensus sequences from each of the related families were aligned using Clustal W, and the resulting relationships between the families are presented here. Where the families include human members, the relevant SLC family is given along with the TCDB name. Following each family name is a brief description of the transported substrate and any other substrates coupled by symport or antiport. Symported substrates are separated by a hyphen and antiported substrates by a slash. Commas separate alternative substrates within each family. Highlighted in red are families in which there is high-resolution structural information and the name of the protein for which structures have been obtained.

Furthermore, when the computational method used to construct the cytoplasmic conformation of LeuT was applied to Mhp1, it predicted a conformation very similar to the crystal structure of the cytoplasmic conformation (L. R. Forrest, personal communication). These observations validate the method of exchanging conformations between repeats as a way to predict the alternate conformation of a transporter. They also provide further support for the mechanism in which the four-helix bundle tilts relative to the scaffold [or "hash" domain (see below)] in the transition between extracellular- and cytoplasm-facing conformations. The apparent similarity in mechanism between the families containing Mhp1 and LeuT also supports the proposal that members of the larger structural family of LeuT-like transporters all function by the same conformational mechanism.

## OCCLUDED STATES AND GATING

An occluded state, where substrates are bound and unable to dissociate to either side of the membrane, is usually invoked to allow a coupled transporter to translocate its substrates without creating a transmembrane pore that would allow uncoupled solute flux. This occluded state would be an intermediate or transition state through which the transporter passes in switching between the conformations that face the two sides of the membrane. In the original extracellular-facing LeuT structure with leucine bound and in the cytoplasm-facing vSGLT structure with galactose bound, several amino acid side chains separate the bound substrate from the open pathway leading to the cell exterior in LeuT[30] and to the cytoplasm in vSGLT.[61] These structures were consequently described as occluded conformations from which the substrate could not dissociate, although the extent to which these side chains could impede substrate dissociation is uncertain. In LeuT, another structure with tryptophan bound shows the pathway even more open, allowing aqueous access from the cell exterior to the binding site.[53] Singh et al., therefore, proposed that substrates bind to a conformation similar to that of the LeuT-trp structure, followed by substrate occlusion as the protein assumes the LeuT-leu conformation.

Alternative modes of occlusion have been proposed. The observation that the central portions of TM1 and 6 are unwound where they contribute to the binding site prompted the suggestion that TM1 and 6 might bend at these "hinges" so that the bundle could close both pathways simultaneously in the transition from extracellular- to cytoplasm-facing conformations.[30,72] The problem with such a mechanism is that TM2 and 7, which are also part of the bundle, do not contain unwound regions to allow the entire bundle to bend. If TM1 and 6 closed both pathways simultaneously, the integrity of the bundle might be disrupted and binding sites that depend on interactions within the bundle, such as the sugar binding site in vSGLT[61] or the proposed $Cl^-$ binding site in neurotransmitter transporters,[73,74] would be destroyed before the ligand could be delivered to the other side of the membrane. Morover, the internal structures of the four-helix bundle in extracellular- and cytoplasm-facing conformations of Mhp1 are identical, supporting the proposal that the bundle remains intact during the transition.[71]

The extracellular-facing structures of Mhp1 with and without bound substrate provide another potential mode of occluding the binding site. The apoprotein shows an open extracellular permeation pathway, but in the substrate-bound structure, this pathway is blocked by TM10, which bends at a proline residue (conserved as proline or glycine in many but not all LeuT structural homologues) so that the extracellular part of this helix tilts into the open pathway, preventing substrate release.[63] In the cytoplasm-facing Mhp1 structure, a break in TM5 (the helix in the first repeat that corresponds to TM10) suggests that a similar conformational change could block the cytoplasmic pathway to create another occluded state in the cytoplasm-facing conformation.[71] These observations led Shimamura et al. to divide the six-helix scaffold identified in LeuT into a four-helix hash motif containing TM3, 4, 8, and 9 and two flexible helices, TM5 and 10, that contribute to occlusion.[71] The break in TM5 was not observed in structures of LeuT, although that does not rule out the possibility that the flexibility of TM5 and 10 is important in occlusion throughout the greater family of LeuT-like transporters. Indeed, in SERT, the helical pattern of cytoplasmic TM5 accessibility shown in Figures 3 and 4 was disrupted in low-$Na^+$, high-$K^+$ solution[45] and was absent in the extracellular half of TM10,[43] suggesting conformational flexibility in those regions. It is also possible that the overall mechanism of alternating access is similar among transporters in this family, but the specifics of substrate occlusion might vary between subfamilies.

## ALTERNATIVE MECHANISM

A different mechanism for conformational change in LeuT was proposed on the basis of steered molecular dynamics simulations. In these simulations, a force on bound leucine sufficient to dislodge it from the binding site was applied in the direction of the cytoplasm.[75] Equilibration of the protein structure at intermediate positions along the pathway allowed an estimate of conformational changes that accompanied substrate movement. These conformational changes, which mainly involve movement of TM1,[76] were facilitated by a second leucine molecule inserted for the simulations at a previously identified antidepressant binding site in the extracellular permeation pathway. Evidence of binding of a second leucine consistent with this mechanism was obtained from a slowly formed complex containing a labeled leucine molecule that was released if $Na^+$ was removed and unlabeled leucine was added.[75]

Although the existence of a second leucine binding site is somewhat controversial,[77] several biophysical approaches provide support for movements within the LeuT structure. Electron paramagnetic resonance studies suggest a more condensed structure of the extracellular pathway in LeuT upon leucine binding, consistent with formation of the occluded state.[78] Förster resonance energy transfer measurements of conformational changes at the N-terminus of LeuT were invoked as support for TM1 movements predicted by the steered molecular dynamics simulations.[76,79] Such movement of TM1, however, is also consistent with several other proposals for conformational change in LeuT and its structural homologues.[46,71,72]

## ROLE OF STRUCTURAL SYMMETRY IN TRANSPORT

The inverted structural repeat in LeuT and related transporters is a common, although not universal, feature of transport proteins. It has been found also in the dicarboxylate/amino acid:cation symporter (DAACS) family that includes neuronal and glial glutamate transporters.[80] Chloride channels and transporters that belong to the ClC family also contain an inverted structural repeat.[81] Other transporters with this feature

include those in the major facilitator superfamily (MFS)[82−85] and the sodium:proton antiporter (NhaA) family.[86] Moreover, this feature is not restricted to solute transporters because it has also been found in the major intrinsic protein (MIP) and ammonia transport (Amt) families, which contain many solute channels.[87,88] Even the protein translocation channel SecY contains an inverted structural repeat.[89] Remarkably, there is little structural similarity between LeuT and any of these families, suggesting that each structural family evolved independently. The DAACS family serves to reinforce the utility of computational methods in identifying an alternate conformation. A model of the archaeal aspartate transporter $Glt_{Ph}$ in the cytoplasm-facing conformation was generated from the crystal structure of the extracellular-facing form by Crisman et al.[80] This model accurately predicted the conformation of the cytoplasm-facing $Glt_{Ph}$ crystal structure,[90] both of which suggested a conformational mechanism for the transition between the two conformations. Similarly, the extracellular-facing conformation of the MFS transporter LacY was modeled on the basis of a cytoplasm-facing crystal structure and the presence of inverted structural repeats.[85] The unexpected frequency of such a remarkable structural feature in a wide variety of transporters suggests that it may play an important role in the function of these proteins.

The pseudo-2-fold symmetry of the inverted structural repeat may reflect an intrinsic symmetry in the transport process. The principal energetic barrier to moving a small polar molecule across a lipid bilayer is the energetic cost of losing favorable interactions with water that are not replaced by interactions with the lipid side chains in the center of that bilayer. Transporters solve this problem by creating a binding site within the membrane that provides polar and ionic interactions to stabilize the substrate. Getting into and out of this binding site are essentially similar processes whether it is from one side of the membrane or the other. Therefore, the pathways from the binding site to the two sides of the membrane perform the same function and could be formed from similar elements. The inverted structural repeat provides a way to create both pathways using similar building blocks. As an example, each element forming the extracellular pathway apparent in the LeuT crystal structure has a corresponding element in the other repeat that contributes to the cytoplasmic pathway, as modeled in LeuT and shown to be accessible in SERT.[46] Likewise, in $Glt_{Ph}$, the gates to the binding site from either side of the membrane are complementary elements from the two repeats. The inverted structural repeat may be nature's solution to the problem of creating symmetrical pathways from each side of the membrane leading into and out of a central binding site.

Although the evolution of inverted structural repeats is generally believed to involve gene duplication and fusion, no examples of a precursor five-TM half-transporter with the LeuT fold have been identified, although candidates have been proposed.[91] However, another transporter family may provide an example of the process leading to the evolution of transporters with inverted repeats. The small multidrug resistance (SMR) family of transporters contains EmrE, which functions as a homodimer,[92] and others that are heterodimeric,[93,94] with two subunits of opposite topology. The topology of EmrE has been controversial, but there is evidence to indicate that it functions as both a parallel (both subunits have the same topology) and an antiparallel dimer.[95] The SMR family, then, could represent the early evolution of inverted repeats in transporters, with a transporter functioning as a parallel homodimer evolving to work as an antiparallel heterodimer. This would be followed by gene duplication and specialization in an antiparallel configuration. Finally, gene fusion would lead to a single polypeptide containing the inverted structural repeat.

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## ABBREVIATIONS

SLC, solute carrier family; NSS, neurotransmitter:sodium symporter; 5-HT, 5-hydroxytryptamine (serotonin); NE, norepinephrine; DA, dopamine; GABA, γ-aminobutyric acid; SERT, serotonin transporter; NET, norepinephrine transporter; DAT, dopamine transporter; MDMA, 3,4-methylenedioxymethamphetamine (ecstasy); DAACS, dicarboxylate/amino acid:cation ($Na^+$ or $H^+$) symporter; TM, transmembrane helix; IL, intracellular loop; EL, extracellular loop; MTSEA, 2-aminoethyl methanethiosulfonate; MTSES, 2-sulfonatoethyl methanethiosulfonate; SSS, solute:sodium symporter; NCS1, nucleobase:cation symporter; BCCT, betaine/choline/carnitine transporter; APC, amino acid-polyamine-organocation transporter; ClC, chloride carrier/channel; MFS, major facilitator superfamily; NhaA, $Na^+$:$H^+$ antiporter; MIP, major intrinsic protein; Amt, ammonia transporter channel; SMR, small multidrug resistance transporter.

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