Review

LeuT

A prokaryotic stepping stone on the way to a eukaryotic neurotransmitter transporter structure

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Key words: transporter, antidepressant, alternating access, channel activity, occluded, ion-coupled, neurotransmitter, competitive, noncompetitive inhibition

Ion-coupled secondary transport is utilized by a broad range of integral membrane proteins to catalyze the energetically unfavorable movement of solute molecules across a lipid bilayer. Members of the solute carrier 6 (SLC6) family, present in both prokaryotes and eukaryotes, are sodium-coupled symporters that play crucial roles in processes as diverse as nutrient uptake and neurotransmitter clearance. The crystal structure of LeuT, a bacterial member of this family, provided the first atomic-level glimpse into overall architecture, pinpointed the substrate and sodium binding sites and implicated candidate helices and residues in the “gating” conformational changes that accompany ion binding and release. The structure is consistent with a wealth of elegant biochemical data on the eukaryotic counterparts and has for the first time permitted the construction of accurate homology models that can be directly tested experimentally. Sequence identity is especially high near the substrate and sodium binding sites and, thus, molecular insights within these regions have been substantial. However, there are several topics relevant to transport mechanism, inhibition and regulation that structure/function studies of LeuT cannot adequately address, suggesting the need for a eukaryotic transporter crystal structure.

Introduction

Sodium-coupled transporters are ubiquitous integral membrane proteins that rely on pre-existing ion gradients to transport their substrates across the lipid bilayer. They play diverse physiological roles, ranging from nutrient uptake in hyperthermophilic eubacteria to neurotransmitter transport in the mammalian brain.1 Within the central nervous system, neurotransmitter symporters are localized to the plasma membrane of presynaptic neurons and glia, where they actively clear the synapse of small molecule neurotransmitters subsequent to postsynaptic receptor activation. Functionally and structurally, they can be partitioned into two distinct families,4 the first (SLC1) dependent on Na+/K+ to transport dicarboxylate amino acids such as glutamate and aspartate and the second (SLC6) dependent on Na+/Cl- to transport a more diverse array of small molecules such as amino acids (γ-aminobutyric acid [GABA], glycine, proline, taurine); osmolytes (betaine, creatine); and the biogenic amines (serotonin [5-HT], norepinephrine [NE] and dopamine [DA]).

SLC6 members, also known as neurotransmitter sodium symporters (NSS), have garnered significant attention because their dysfunction has been implicated in multiple debilitating central and peripheral nervous system diseases, including depression, anxiety, autism, epilepsy, attention deficit hyperactivity disorder (ADHD), obsessive-compulsive disorder (OCD), orthostatic intolerance, X-linked creatine deficiency syndrome and, to a lesser extent, bipolar disorder, schizophrenia, Parkinson’s disease and Tourette’s syndrome. Notably, these molecular machines are the target of therapeutic agents such as antidepressants, anticonvulsants and some psychostimulants as well as deleterious substances such as cocaine and amphetamines.

The first clues to the biological roles of SLC6 members were observed over 40 years ago with seminal discoveries of norepinephrine transport in the heart and nerve endings. The requirement for a sodium gradient was discovered a few years later. The notion that an integral membrane protein was responsible for the observed uptake was reinforced by evidence that the process was stereospecific, saturable and susceptible to inhibition by cocaine and tricyclic antidepressants. The use of preparations enriched in transporter, including membrane vesicles derived from brain synaptosomes and blood platelets, as well as subsequent purification and reconstitution of partially-purified transporter, enabled progressively more detailed studies defining substrate/inhibitor specificity, ion selectivity and substrate-to-ion stoichiometry. Cloning of the rat GABA transporter type 1 (GAT1), followed by the NE transporter (NET) and others heralded a new era in structure/function analysis. The effects of mutating specific residues on transporter activity, posttranslational modifications, trafficking and regulation could now be examined. Particularly important was application of the substituted cysteine accessibility method to indirectly probe structure and conformational dynamics in neurotransmitter transporters.

Despite these momentous strides, the need for a structural paradigm for the interpretation of this wealth of functional data remained. Valiant attempts have been made to express, solubilize and purify...
representative SLC6 proteins to near homogeneity,40-42 but these proteins have proven labile and, thus, have yet to unveil their atomic secrets. Proteins from prokaryotic organisms are generally more stable than their eukaryotic counterparts and can be expressed at much higher levels. More than 200 bacterial SLC6 orthologs exist,43 and three of them have been functionally characterized. These include a tryptophan transporter (TnaT) from *Symbiobacterium thermophilum*,44 a tyrosine transporter (Tyt1) from *Fusobacterium nucleatum*,45 and a leucine/alanine transporter (LeuT) from *Aquifex aeolicus*.46,47 LeuT has been successfully crystallized and its structure solved.46 Although the overall sequence identity between LeuT and eukaryotic members is low (~20–25%), there are several regions within transmembrane segments 1, 3, 6 and 8 where conservation approaches -50%.43,46 Indeed, examination of the LeuT structure, construction of homology models, and structure/function analyses based on these models have begun to yield unprecedented insights into eukaryotic SLC6 transporter function.48 Nevertheless, there are limits to the revelations that a distantly related bacterial homologue can provide about its eukaryotic cousins. These include, but are not limited to, the fact that LeuT is resistant to inhibition by many SLC6 antagonists; that it is a monomer in solution, which may affect specific drug interactions; that it does not appear amenable to the electrophysiological techniques required to gauge putative channel activity; and that it is missing large portions of the amino and carboxy termini, which are known to interact with modulatory presynaptic proteins absent in bacteria. This review will address topics relevant to transporter mechanism and inhibition in SLC6 family members, what the LeuT crystal structure has specifically revealed about these topics, and where this prokaryotic model remains inadequate, thus suggesting the need for a eukaryotic transporter structure.

**Architecture**

Most SLC6 family members, with the exception of some bacterial homologues,45 consist of twelve transmembrane domains (TMs) and have their amino (N) and carboxy (C) termini located intracellularly.49 Connecting these helices are numerous intra- and extracellular loops. The most prominent include extracellular loop 2 (EL2), which, in eukaryotic members, contains glycosylation sites40 and a probable disulfide bond,49,51,52 and extracellular loop 4 (EL4), which is proximal to EL2.53 Many of the intracellular loops contain consensus phosphorylation sites, the state of which is known to regulate both endogenous transporter activity54 as well as endocytotic processes.55 Other critical transporter elements include extensive N- and C-termini, which interact with modulatory proteins such as syntaxin 1A,56-58 nitric oxide synthase,59 the PDZ-domain-containing protein PICK1,60 and the LIM homeodomain-containing protein Hic-5.61

The LeuT architecture reveals some similarities and surprises when examined in the context of functional data from eukaryotic SLC6 counterparts. As predicted, LeuT contains 12 TM segments with EL2 and EL4 juxtaposed (Fig. 1). Unexpectedly, but akin to other transporters such as the human red cell aquaporin (AQP1),62 the *Escherichia coli* sodium-proton exchanger (NhaA),63 and the *Vibrio parahaemolyticus* sodium-dependent galactose symporter (sSGLT),64 LeuT possesses an internal structural repeat relating TMs 1–5 to 6–10 by a pseudo two-fold in the membrane plane. The pseudo repeats are oriented antiparallel to one another with the two central TMs, 1 and 6, unwound near the substrate and sodium binding sites, approximately halfway across the lipid bilayer. This unwinding is critical because it exposes helix dipoles as well as mainchain carbonyl oxygens and amide nitrogens for substrate binding and ion coordination (see “Ion Dependence” section).46 Eukaryotic SLC6 members most likely possess this internal structural repeat as well as the unwound helical elements.

Despite these probable similarities, there are notable architectural disparities that might impact endogenous transporter activity: EL2 as well as the N- and C-termini are considerably shorter and none of the intracellular loops harbor consensus phosphorylation sites. The possibility that differences within EL2 could be consequential is suggested by two observations. First, transport activity is disrupted when the disulfide bond in SERT is removed65 or when EL2 of SERT is replaced with that from NET.66,67 Second, a number of SLC6 members, including DAT,53,68 GlyT1b,69 and GAT4,70 are modulated by Zn2+, and the coordinating residues, at least for DAT and GlyT1b, are located within EL2 and EL4. The notion that disparities within intracellular loops and the N- and C-termini might also affect transport activity is suggested by the interactions these regions form with modulatory presynaptic proteins. Indeed, the SNARE protein syntaxin 1A complexes with the N-terminus to regulate ion conductance (refer to “Ion Dependence” section below) in the glycine transporter (GlyT1/GlyT2),71 GAT1,72 DAT,58 NET72 and SERT,56 presumably by recognizing negatively-charged aspartate residues,66 perhaps via a Ca2+-dependent process.73 From a clinical perspective, the missing sections of EL2 as well as the N- and C-termini are significant because several disease-associated polymorphisms that affect transporter trafficking and regulation reside within these areas.74

**Putative Oligomerization**

Eukaryotic SLC6 family members are thought to exist as oligomers, possibly in a tetrameric arrangement of a dimer of dimers or as an array of dimers.75-77 Evidence for oligomerization from the
clinical realm comes from the detrimental dominant-negative effect of a human NET (hNET) A457P missense mutation, correlated with orthostatic intolerance, on surface expression of wild-type hNET.

Amino acid sequences implicated in multimerization include a leucine heptad repeat in TM2 and a glycosphingolipid motif in TM6, both of which have been verified in GAT1 and DAT by cysteine cross-linking, co-immunoprecipitation and FRET microscopy. These motifs, however, are not present in all members, and there is some heterogeneity within the family. For example, although the leucine heptad repeat is present and critical for oligomerization in GAT1 and DAT, it does not exist nor is it essential in SERT, which appears to have another contact site within TMs 11–12. In addition, a unique contact site in TM4 has been identified in DAT.

Multimerization appears to be a requirement for proper trafficking of the transporter from the endoplasmic reticulum to the plasma membrane rather than a prerequisite for substrate translocation. However, in the biogenic amine transporters, it may also be pertinent to the reported allosteric effect exhibited by some antidepressants as well as to the mechanism of amphetamine-induced substrate efflux.

What is the oligomeric state of LeuT? LeuT behaves as a monomer in solution unlike eukaryotic SLC6 members. However, the fact that TM6 is almost completely buried in the structure and TM2 is only partially accessible raise the possibility that these two domains do not mediate extensive contacts between protomers in the eukaryotic counterparts, although some involvement cannot be excluded. As far as what an actual oligomeric complex might look like, some clues might be obtained from analysis of the LeuT crystal. Despite being monomeric in the crystallographic asymmetric unit, LeuT does form a dimer along the crystallographic two-fold axis mediated by TM9, TM12 and EL2 together with their symmetry mates. But the sequence identity between these three regions and those in eukaryotic SLC6 members is virtually nonexistent, and the arrangement could just be a result of crystal lattice packing phenomena. Although there are data on SERT that support the existence of an interface involving TMs 11 and 12, the physiological relevance of the interface observed in the LeuT crystal remains uncertain.

Substrate Specificity and Ion Dependence

SLC6 family members collectively transport a wide array of substrates, ranging from glycerol, the smallest, to serotonin, one of the largest. This, in turn, reflects variability among amino acids lining the active site, both in terms of size and chemical properties. Despite this diversity, there are several universally conserved residues, especially in TM 1–3. Examples include a tyrosine in TM3 whose indispensability has been demonstrated in GAT1, SERT and GlyT2a, and an aspartate/glycine in TM1 that is at least partially responsible for distinguishing between monoamine and amino acid substrates.

Residues implicated in ion binding have proven more difficult to identify but for sodium include those in TM1, TM6 and TM7 in GAT1, SERT and DAT. Among the biogenic amine transporters, the same aspartate in TM1 hypothesized to interact with the positively-charged amine in biogenic amine substrates is also thought to be critical for sodium binding and permeation. Until recently (see below), less was known about chloride, although chimera studies had roughly defined domains in TMs 1–2 and TMs 9–12. What was indisputable is that the chloride requirement for transport activity is not as strict as that for sodium, and it had been hypothesized that chloride probably does not provide a driving force but rather enhances sodium binding.

In addition to substrate variability, another intriguing variation among SLC6 members is their ion-to-substrate stoichiometry. Whereas GAT1, GLYT1b and possibly DAT all transport Na+, Cl- and their respective substrate in a ratio of 2:1:1, GlyT2a transports them in a ratio of 3:1:1, while NET and SERT transport them in a ratio of 1:1:1. SERT is even more complex because it also counter transports potassium or protons. Although these stoichiometries frequently result in transporters being designated as electrogenic or electroneutral, characterization is not always that straightforward. This is because eukaryotic SLC6 members often exhibit substrate-dependent currents that exceed those predicted by the above stoichiometries and or display substrate-independent leak currents occasionally mimicking ion channels.

Some data from the eukaryotic transporters on substrate and sodium binding sites have been validated by the LeuT crystal structure. The structure further reveals that many critical interactions are formed by backbone carbonyl oxygens and amide nitrogens from the unwound sections of TMs 1 and 6 (Figs. 1B and 2). The universally conserved tyrosine (Tyr108) in TM3 serves a dual role in both interacting with the substrate carboxylate and in stabilizing TM1 near the unwound region (Fig. 2). In the biogenic amine transporters, whose substrates lack a carboxylate, the hydroxyl of this tyrosine may form a hydrogen bond with the amine. The glycine at position 24 does not unwind region (Fig. 2). In the biogenic amine transporters, whose substrates lack a carboxylate, the hydroxyl of this tyrosine may form a hydrogen bond with the amine. The glycine at position 24 does not unwind sections of TMs 1 and 6 (Figs. 1B and 2). The universally conserved tyrosine (Tyr108) in TM3 serves a dual role in both interacting with the substrate carboxylate and in stabilizing TM1 near the unwound region (Fig. 2). In the biogenic amine transporters, whose substrates lack a carboxylate, the hydroxyl of this tyrosine may form a hydrogen bond with the amine. The glycine at position 24 does not unwind sections of TMs 1 and 6 (Figs. 1B and 2). The universally conserved tyrosine (Tyr108) in TM3 serves a dual role in both interacting with the substrate carboxylate and in stabilizing TM1 near the unwound region (Fig. 2). In the biogenic amine transporters, whose substrates lack a carboxylate, the hydroxyl of this tyrosine may form a hydrogen bond with the amine. The glycine at position 24 does not unwind sections of TMs 1 and 6 (Figs. 1B and 2). The universally conserved tyrosine (Tyr108) in TM3 serves a dual role in both interacting with the substrate carboxylate and in stabilizing TM1 near the unwound region (Fig. 2). In the biogenic amine transporters, whose substrates lack a carboxylate, the hydroxyl of this tyrosine may form a hydrogen bond with the amine. The glycine at position 24
Na2, coupled with lower sequence conservation, lends ambiguity to the presence of this site in eukaryotic SLC6 members. Transporters such as SERT and NET that exhibit a 1:1 sodium-to-substrate stoichiometry probably possess at least the Na1 site and those such as GAT1 and GlyT1b that exhibit a 2:1 stoichiometry may have the Na2 site as well. Perhaps one of the most significant insights from the LeuT structure is that the proximity of substrate and sodium binding sites indicates that the coupling of substrate and sodium transport in LeuT and probably eukaryotic NSS members is a result of direct interactions.

Possibly one of the better testaments to the utility of LeuT as a three-dimensional template for its eukaryotic cousins is the successful transfer of substrate specificities or ion dependencies between transporter subtypes and/or orthologs. For example, Vandenberg et al., conferred the ability to transport sarcosine, a GlyT1b-selective porter subtypes and/or orthologs. For example, Vandenberg et al., conferred the ability to transport sarcosine, a GlyT1b-selective substrate, onto GlyT2a.116 Similarly, Dodd and Christie conferred the ability to transport GABA onto a creatine transporter, albeit with lower-than-expected catalytic efficiency.117 While substrate uptake by LeuT is not chloride-dependent, sequence comparisons between LeuT and eukaryotic members permitted two groups, working independently and with divergent techniques, to not only pinpoint the elusive chloride binding site in SERT,118 GAT1,119 GAT4,119 and DAT119 but to also demonstrate that it is the negative charge provided by the chloride (or Glu290 in LeuT) that promotes sodium binding and substrate translocation. As proof of principle, both groups were subsequently able to render the chloride-independent bacterial SLC6 transporters, TnaT,118 Tyt1,119 and LeuT,119 chloride-dependent.

Despite such impressive detail, the LeuT structure does not explain every observation related to substrate/ion permeation. First, it does not suggest where the third sodium in GlyT2a or the potassium in SERT may bind. Second, it does not indicate whether it is possible for one sodium to remain bound during the translocation cycle in those transporters exhibiting a 1:1 transport stoichiometry. Third, although LeuT has yielded sufficient insight to alter specificities between amino-acid-like substrates (see above), it has not yet allowed anyone to switch substrate specificities between an amino acid and its cognate biogenic amine. Fourth, the LeuT structure does not indicate how the binding of some tricyclic antidepressants, which is enhanced by sodium,120 is structurally coupled to sodium (refer to “Mechanism of Inhibition” section). Finally, LeuT has so far not offered a definitive structural basis for the channel mode of activity (see below).

Transport Mechanism and Gating

The most prevalent model to describe transport mechanism in SLC6 members is one in which substrates and ions bind to their unique sites on the transporter and an ensuing conformational change permits alternating access to either the extracellular or cytoplasmic side.121-124 A corollary of this model is that the protein assumes only two states during the translocation cycle: “open-to-out/closed-to-in” or “closed-to-out/open-to-in”, where only one of the gates (extracellular or cytoplasmic) is open at any one time. Experimental data for this model are indirect and include extensive kinetics via electrophysiology125,126 and fluorescence127 on GAT1, in conjunction with computational modeling.128 Many experiments have also employed site-directed mutagenesis, sulfhydryl modifications, engineered Zn2+ sites, and cross-species chimeras coupled with radioligand binding, transport, and exchange experiments to identify specific segments of TMs or loops as either forming part of a presumed gate or engaging in conformational rearrangements during the transport cycle. Notably, all of the identified amino acids are either strictly conserved or conservatively substituted within the SLC6 family. Critical residues residing within the extracellular-facing half of the protein include an arginine in TM1 of GAT1,129 and an aspartate or glutamate in TM10 of SERT.130 Also essential are sections of EL2 in SERT/NET66 and DAT53 and parts of EL4 in GAT1,131 DAT,68,132 GlyT1,69 and SERT.133 Critical residues residing within the intracellular-facing half of the protein include an arginine and tryptophan in the N-terminal domain/TM1 of GAT1,134 an aspartate in TM8 of DAT,135 and a tyrosine in the intracellular loop between TMs 6 and 7 of DAT.136

The location and interactions of all of these residues in the LeuT structure reaffirm their postulated roles in the conformational changes that accompany substrate/ion binding and release. Near the extracellular “gate”, at the bottom of a vestibule, is a water-mediated salt bridge between Asp404 (TM10) and Arg30 (TM1), the latter of which is structurally coupled to the substrate-binding site via a hydrogen bond network (Fig. 3). Movement of EL4 during the translocation cycle is implied by the lid-like structure it forms over the extracellular-facing vestibule (Fig. 1B) and movement of EL2 is suggested by its proximal position on top of EL4. The fact that access to the substrate binding site from the extracellular side is blocked by only a few residues (Fig. 3) indicates that only modest rotations in key helices such as TMs 1b, 6a, 3 and 8, along with more dramatic shifts in EL2 and EL4, would be required for the transporter to open to the outside.
At the opposite end of the bilayer, comprising part of the cytoplasmic “gate,” is another salt bridge, an ionic latch between Asp369 (TM8) and Arg5 (N-terminal domain/TM1), the latter of which is partly stabilized by a hydrogen bond to Tyr268 (TM6–7). Trp8 (TM8) fits snugly into a hydrophobic pocket formed by TMs 1 and 6, further anchoring the N-terminus and Arg5 in their current positions. The 25 Å of ordered protein structure between this “gate” and the substrate/ion binding sites in the middle of the membrane bilayer hints that the entire region must rearrange considerably in order for the transporter to open to the inside (Fig. 4).

Although the LeuT structure corroborates the crucial role many residues play during transport, its completely occluded state, with no solvent-accessible channels traversing the protein in the membrane plane, was unpredicted. Its existence implies that, in addition to the “open-to-out” and “open-to-in” conformations of the paradigmatic alternating access model, there is a third occluded state where both gates are closed (Fig. 5). There is no experimental evidence to suggest that this third state exists in eukaryotic SLC6 members, and it is possible that this conformation is an artifact of lattice packing interactions. However, structure/function studies with LeuT and the tricyclic antidepressant (TCA) clomipramine (CMI) indicates that the occluded state can be stabilized in solution to trap substrate on the transporter to open to the inside (Fig. 4).

A slightly different model proposes that LeuT and by analogy, other SLC6 members, undergo a direct conformational change from open-to-out to open-to-in states by exploiting the two-fold internal symmetry and “rocking” a 4-helix bundle composed of TMs 1, 2, 6 and 7. This general idea seems to be supported by the recent crystal structure determination of an unrelated prokaryotic SLC5 family member, the sodium-dependent galactose transporter from Vibrio parahaemolyticus (vSGLT) whose overall three-dimensional fold resembles that of LeuT and whose substrate galactose is occluded but clearly more accessible from the cytoplasmic side. As mentioned earlier, eukaryotic SLC6 members probably also possess this internal structural repeat and may, in fact, undergo this type of direct conformational change, a conjecture that seems to be supported by accessibility data on SERT.

A third study with LeuT proposes that the occluded state is an essential intermediate but that binding of a second substrate molecule in the extracellular vestibule acts as an allosteric trigger for substrate and sodium release to the intracellular milieu. Although the functional data presented within this paper are self-consistent, they cannot be reconciled with the inability to crystallographically isolate an occluded-state LeuT structure with a second substrate bound in the extracellular vestibule. At the present time, however, the factors accounting for this disparity are unclear and, thus, the two divergent views have emerged. Whether or not the occluded state or a second, conformationally coupled allosteric substrate binding site exists in the eukaryotic counterparts is also uncertain. Probably the best way to address these issues would be with a combination of dissociation kinetics, steady-state flux and direct structural methods on a eukaryotic SLC6 member.

An important topic relevant to transport mechanism that LeuT so far has failed to address is the reported channel-like activity exhibited by some SLC6 members. There are a number of instances in which the current measured by patch-clamp or two-electrode voltage-clamp experiments exceeds that predicted by transport stoichiometries, implying that the prevalent alternating access model may be too simplistic. In fact, it has been theorized that this excess current is actually conducted by a single-file pore through the transporter. Understanding this phenomenon may be as elementary as envisioning both extracellular and cytoplasmic “gates” open simultaneously, but actually capturing such a conformation crystallographically is an entirely separate matter. Because LeuT has so far not yielded to the standard electrophysiological techniques that would allow one to evaluate the conditions under which it might display channel-like activity, attempting to isolate a channel-like state remains a blind search. Moreover, the lack of an N-terminus means that one certainly cannot possibly hope to comprehend, at the atomic level, how presynaptic proteins like syntaxin 1A regulate ion conductances and flux stoichiometry. All of these limitations highlight the need for a eukaryotic SLC6 transporter structure.

**Mechanism of Inhibition**

Much SLC6 research is concentrated on the mechanism by which small molecules modulate transport activity because many of these agents are clinically relevant psychotropics. While some compounds, such as amphetamines, stimulate neurotransmitter efflux from the presynaptic neuron, most, including antidepressants (TCAs, SSRIs [selective serotonergic reuptake inhibitors], SNRIs [dual serotonin-norepinephrine reuptake inhibitors]), cocaine and...
Advances and limitations of a bacterial neurotransmitter sodium symporter

models and biochemical analysis on the eukaryotic SLC6 member can be a particularly powerful combination in pinpointing antagonist binding sites. Ideally, however, one would like to apply structural and functional methods to the same protein.

The crystallizability of LeuT and subsequent design of binding/transport assays afforded this unique opportunity but also illustrates the limitations of employing a distantly related ortholog in such experiments. Two groups reported that the TCAs clomipramine (CMI), imipramine (IMI), or desipramine (DMI), noncompetitively inhibits substrate uptake by LeuT and do so by stabilizing the occluded state. A single TCA molecule binds to LeuT in the extracellular-facing vestibule, about 11 Å above the substrate and directly above the Arg30-Asp404 ion pair. Intriguingly, its presence triggers a flip in the guanidium group of Arg30 so that the latter forms a direct salt bridge with Asp404, a presumably stronger interaction than one mediated by water molecules. The functional relevance of the “stabilized” structure is anticonvulsants, block reuptake of the respective neurotransmitter from the synapse. For inhibitors, kinetic data have revealed the existence of both competitive and noncompetitive modes of inhibition. For some nontransportable, competitive drugs like the SSRI citalopram, convincing biochemical evidence for a primary binding site that overlaps with that of the substrate 5-HT was acquired before the LeuT structure was published but is now also supported by LeuT-based homology models of SERT. For other drugs, like cocaine, a combination of LeuT-based homology models and mutagenesis on DAT suggests that the binding sites of dopamine and cocaine also overlap. Although these studies are reassuring and probably identify the correct antagonist binding sites, they still do not detail the conformational changes predicted to be associated with competitive inhibition and an extracellular inhibitor like cocaine or the GAT1 inhibitor SKF100330, i.e., stabilization of an open-to-out state. In lieu of direct structural studies on a eukaryotic SLC6 member, a combination of LeuT-based homology models and biochemical analysis on the eukaryotic transporter can be a particularly powerful combination in pinpointing antagonist binding sites. Ideally, however, one would like to apply structural and functional methods to the same protein.

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Advances and limitations of a bacterial neurotransmitter sodium symporter whose Oδ1 atom forms a salt bridge with the protonated N2 atom of CMI, IMI or DMI in LeuT, is actually a lysine in hSERT and a threonine in hNET. Furthermore, alanine-scanning mutagenesis in hSERT of residues within 5 Å of a putative CMI-binding site yields a decrease in inhibitory potential (increase in IC50) of only 2-5-fold (Singh SK and Gouaux E, unpublished). The greatest effect (~5-fold increase in IC50) occurs at residue Y175 (Y107 in LeuT), which is actually closer to the substrate-binding pocket than to the extracellular vestibule site. Despite the fact that similar values are reported by Zhou et al., the authors still contend that such modest effects are indicative of TCA binding in a putative extracellular vestibule of the biogenic amine transporters and conservation of the inhibition mechanism. However, as mentioned above, the stark disparity in inhibition kinetics (noncompetitive in LeuT vs. competitive in SERT/NET) implies otherwise. Finally, when I172 in hSERT (V104 in LeuT), a residue that points directly into the substrate binding pocket, is mutated to methionine, a much more substantial increase in IC50 (~178-fold) for CMI inhibition of serotonin transport results. Although the extracellular vestibule site cannot be excluded as the low-affinity allosteric site reported in SERT, the primary, high-affinity binding site for the TCAs (at least for CMI, IMI and DMI) probably resides a bit deeper in the protein core, close to or partly overlapping with the substrate binding site. Removing all ambiguities and conclusively pinpointing the actual TCA binding site will require crystal structure determination of a eukaryotic SERT/NET and/or an invertebrate DAT in complex with a TCA.

Nevertheless, these facts do not mitigate the significance of the TCA findings with LeuT. First, the data advance a tangible, testable hypothesis for the general phenomenon of noncompetitive inhibition (Fig. 7) that may be applicable to eukaryotic SLC6 members, at least within the protomer. Second, they identify a potential antagonist-binding site that most likely exists in the eukaryotic counterparts and that might be exploited in rational drug design efforts. However, determining if this mechanism of noncompetitive inhibition is relevant would still demand structural work on a eukaryotic SLC6 member.

Conclusions

There is little doubt that the LeuT structure has had a significant impact on the neurotransmitter transporter field. It has served as a structural paradigm for interpretation of the wealth of functional data available on the eukaryotic counterparts. Despite the fact that LeuT shares only 20–22% sequence identity with its eukaryotic cousins, several mechanistically critical residues, including those implicated in substrate/sodium binding as well as gating, are conserved. This evolutionary kinship has kindled a flurry of additional experiments on the eukaryotic transporters, including the building of homology models, to test structure-based predictions, and these avenues of investigation

![Figure 6](image-url)

Figure 6. (A) Clomipramine (CMI) binds to LeuT in the extracellular vestibule about 11 Å above the substrate. (B) Magnification of the CMI binding site from the LeuT-Leu-CMI complex (pink) (PDB ID 2Q6H) overlaid onto that of the original LeuT structure (green) (PDB ID 2A65), depicting the flip of the guanidium group of R30 to form a direct salt bridge with D404 and the displacement of two water molecules. (C) Space-filling model of CMI (yellow), R30 (blue) and F253 (gray), illustrating how CMI seems to stabilize R30 in its flipped position via a “layered” cation-π interaction with F253. (D) Dissociation of [3H]Leu form LeuT in the absence (filled circles) and presence (open circles) of 3 mM CMI. Reproduced from Singh et al. Supported by kinetic data demonstrating that CMI greatly reduces the rate at which [3H]leucine dissociates from LeuT (Fig. 6D). While it is tantalizing to surmise that the TCA site observed in LeuT is also present in NET and SERT, the actual targets of the TCAs in humans, there are several discrepancies that cast doubt on this conclusion and, instead, suggest that the TCAs (at least CMI, IMI and DMI) bind closer to the substrate binding pocket. First, binding of IMI to SERT is sodium-dependent. Second, IMI and DMI are competitive inhibitors of 5HT and NE transport by SERT and NET, respectively, while CMI is a noncompetitive inhibitor of alanine transport by LeuT. Third, there is at least a five-order of magnitude increase in the TCA IC50 for substrate transport by LeuT relative to the IC50 for substrate transport by human SERT/NET.

Additional sobering evidence is provided by closer inspection of hNET/hSERT homology models. For example, the glutamine (Gln34) whose Ne2 atom forms a seemingly indispensable interaction with the chloride of CMI in LeuT, is actually an isoleucine in hSERT and a valine in hNET. Similarly, the aspartate (Asp401) supported by kinetic data demonstrating that CMI greatly reduces the rate at which [3H]leucine dissociates from LeuT (Fig. 6D).
have generated undeniable dividends. However, work with LeuT and TCAs, while informative from a purely mechanistic perspective, cautions us against employing LeuT as a faithful replica of all aspects of eukaryotic SLC6 transporter function. Moreover, the fact that LeuT lacks large portions of EL2 as well as the N- and C-termini, the latter two of which are regions known to interact with modulatory presynaptic proteins such as syntaxin 1A and protein kinase G and be the location of several disease-associated polymorphisms, limits the insight we can glean from studying prokaryotic orthologs alone.

Acknowledgements

The author is supported by a K99/R00 NIH/NIMH “Pathway to Independence” Award.

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