Substrate and drug binding sites in LeuT
Ajeeta Nyola¹, Nathan K Karpowich¹, Juan Zhen², Jennifer Marden¹, Maarten E Reith² and Da-Neng Wang¹

LeuT is a member of the neurotransmitter/sodium symporter family, which includes the neuronal transporters for serotonin, norepinephrine, and dopamine. The original crystal structure of LeuT shows a primary leucine-binding site at the center of the protein. LeuT is inhibited by different classes of antidepressants that act as potent inhibitors of the serotonin transporter. The newly determined crystal structures of LeuT–antidepressant complexes provide opportunities to probe drug binding in the serotonin transporter, of which the exact position remains controversial. Structure of a LeuT–tryptophan complex shows an overlapping binding site with the primary substrate site. A secondary substrate binding site was recently identified, where the binding of a leucine triggers the cytoplasmic release of the primary substrate. This two binding site model presents opportunities for a better understanding of drug binding and the mechanism of inhibition for mammalian transporters.

Introduction
The sodium-dependent neurotransmitter symporter (NSS) family of membrane proteins includes the neuronal transporters for the monoamines serotonin, norepinephrine, and dopamine (SERT, NET, and DAT, respectively) [1–3]. The subset of such transporters from the human genome also forms the solute carrier 6 (SLC6) family of transporters. Progress in genomic sequencing at the turn of the century has revealed that the NSS proteins (Figure 1). The structure consists of a twelve-helix bundle, with the helices TMs 1–5 and helices TMs 6–10 being related by inverted symmetry relative to the membrane (Figure 2a). This arrangement reveals that the substrate leucine, along with two sodium ions, binds at the center of the transporter between the two inverted domains (the S1 site). The substrate is shielded from the extramembrane space by both a cytoplasmic and an extracellular gate. The alternating opening and closing of these gates is believed to allow the translocation of the substrate across the membrane. Much of the mutagenesis, biochemical, and transport activity data on SERT, NET, and DAT can be understood within the structural framework of LeuT. Indeed, the substrate binding site is conserved enough to allow the engineering of a chloride binding site in LeuT based on the SERT and DAT data [6,7].

Secondary substrate binding site in NSS proteins
In the crystal structure of LeuT in its occluded state there is just one leucine bound [5]. Therefore, it was surprising when Shi and colleagues discovered a secondary leucine-binding (S2) site in the periplasmic vestibule [8**]. This is the same site where tricyclic antidepressant (TCA) molecules had previously been shown to bind (Figure 2a and b) [9,10], and molecular dynamics (MD) simulations had suggested passage of leucine through such a site [11]. LeuT purified in detergent solution was found to bind leucine at a ratio of 1:2 [8**]. Importantly, binding experiments showed that the binding of a TCA molecule is competitive to substrate binding not at the S1 site but at the S2 site. Also surprisingly, strong evidence was provided by these investigators that the binding of substrate to S2 triggers the cytoplasmic release of the leucine molecule from the primary S1 binding site.

Intriguingly, this two-site model for transport recently (Figure 1) [8**] received support from the crystallization studies of a related membrane transporter. Despite a lack of amino acid sequence identity, several families of membrane transporters have been shown to share the same LeuT-like fold [5], and presumably they operate using a similar transport mechanism. One such protein is the carnitine transporter (CatT) from E. coli, a member of...
**Figure 1**

Transport cycle of the leucine transporter LeuT. \(C_i\): inward-facing conformation; \(C_o\): outward-facing conformation; \(O_{oc}\): occluded conformation.

**Figure 2**

Interaction of LeuT with antidepressants. (a) Overall LeuT structure in its \(O_{oc}\) state, with leucine and two sodium ions bound at the S1 site and a sertraline bound at the S2 site (PDB ID: 3GWU). (b) Desipramine binding site in LeuT (PDB ID: 2QJU) [9]. (c) R-fluoxetine binding site in LeuT (PDB ID: 3GWV) [17**]. (d) Superposition of the three LeuT–SSRI structures at the drug binding site. The figures were prepared using PyMol [51]. Sertraline molecule is colored yellow, R-fluoxetine orange, and S-fluoxetine green.
the betaine/choline/carnitine transporter (BCCT) family [12**]. In addition to the similarities in the protein fold as well as the position of the primary substrate binding site, the crystal structure of CatT revealed a secondary substrate binding site on the cytoplasmic side of the protein – an exporter – as opposed to the periplasmic secondary site found in the importer LeuT. Such symmetry, both in structure and in functionality, probably reflects a high degree of similarity for the common ancestor of both protein families [13].

Presently, one can only speculate as to whether a secondary substrate-binding site exists for mammalian members of the NSS family. The lack of high quality purified protein preparations makes the sophisticated experiments for selective binding and competition, as has been done for LeuT [8**], next to impossible. Indirect support comes from our recent study on the interaction between bivalent ligands and DAT [14,15]. Bivalent ligands – compounds incorporating two receptor-interacting moieties linked by a flexible chain – often exhibit profoundly enhanced binding affinity as compared with their monovalent components, implying concurrent binding to multiple sites on the target protein. Molecules possessing two substrate-like phenylalkylamine moieties linked by a progressively longer aliphatic spacer were found to be more potent DAT inhibitors [14]. One compound bearing two dopamine-like pharmacophoric ‘heads’ separated by an 8-carbon linker achieved an 82-fold gain in inhibition of cocaine-analog binding compared with dopamine itself; bivalent 6-carbon linker combinations of dopamine-like, amphetamine-like and β-phenethylamine-like heads all resulted in considerable gains in affinity. Docking into a DAT homology model suggested simultaneous occupancy of two discrete substrate-binding domains. Similar results were obtained by Nielsen and colleagues, who used bivalent phenyl tropanes – part of the cocaine molecule – to probe binding to DAT, SERT, and NET [15]. When tethered together with an ester linker of 10 atoms, bivalent phenyl tropanes showed gains of affinity of up to 45-fold. These observations point to a secondary binding site in DAT that has affinity for both dopamine-like substrates and phenyl tropane-like inhibitors; the distance of approximately 13 Å between the two heads of the bivalent ligands in both the Schmitt et al. [14] and Nielsen et al. [15] studies fit the distance of 11–13 Å between the S1 and S2 sites in the LeuT structure model [5**].

**LeuT–TCA/SSRI structures**

Serotonin selective reuptake inhibitors (SSRIs, e.g. fluoxetine and sertraline) and tricyclic antidepressants (TCAs) (Figure 3a and e) are the most widely prescribed medications for clinical depression. Compounds from both classes bind to SERT and inhibit serotonin reuptake, in most studies, in a competitive manner (e.g. see [16]). Surprisingly, these compounds also inhibit the transport activity of LeuT although with lower potency than the human NSS proteins. Crystal structures of LeuT in complex individually with TCAs and SSRIs have been published recently (Figure 2) [9,10,17**]. In LeuT, all of the co-crystallized antidepressant compounds were found to bind at the S2 substrate site, separated from the S1 leucine-binding site by the transporter’s extracellular gate. In contrast to the binding of a second substrate at this S2 site, bound TCA and SSRI molecules prevent conformational changes and block subsequent substrate transport by directly locking the gate.

LeuT co-crystal structures with desipramine [9,10], imipramine, and chlorimipramine [10] show that TCAs bind in the extracellular vestibule S2 site, about 11 Å above the substrate and the two sodium ions located at the S1 site (Figure 2d and Table 1). In all three of the complex structures, the tricyclic rings are superimposable and the dipole moments of the drug molecules align in the same direction. The drug molecule is held in place by the EL4 hairpin loop on the extracellular side and by a salt bridge. The third ring of the drug forms cation–π interactions with gate residues Arg30 and Phe253, stabilizing the occluded conformation. This in turn prevents gate opening on the cytoplasmic side, supported by the measured reduction of the leucine dissociation off-rate [10].

Similarly, in co-crystal structures of LeuT with three SSRIs (sertraline, R-fluoxetine, and S-fluoxetine), the

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**Table 1**

| LeuT residues that interact with the drug molecule and their equivalents from human NSS proteins |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Type of contact | LeuT* | SERT | NET | DAT |
| Desipramine | | | | |
| van der Waals and cation-π | | | | |
| van der Waals | | | | |
| van der Waals | | | | |
| van der Waals | | | | |
| van der Waals | | | | |
| van der Waals | | | | |
| Salt-bridge | | | | |
| Sertraline | | | | |
| van der Waals | | | | |
| van der Waals | | | | |
| van der Waals | | | | |
| van der Waals | | | | |
| Salt-bridge and van der Waals | | | | |

Notes: Atomic coordinates are taken from crystal structures of LeuT in complex with desipramine (PDB ID: 2QJU) [9] and with sertraline (PDB ID: 3GWU) [17**], respectively.
drug molecules all bind to LeuT at the S2 site and in a very similar manner (Figure 2 and Table 1). The SSRI binding site shares a number of residues with the S1 substrate binding site. For example, Leu25 is simultaneously within a 4 Å distance to both the bound sertraline and the S1 leucine (Figure 2a). The electronic dipole moment in each drug points in approximately the same direction, from TM10 to TM1. Not only are the phenyl and phenoxy groups of the three SSRIs roughly superimposable, but in each compound, the halogen moieties were found to insert into the same halogen-binding pocket (HBP) of LeuT (Figure 2d). It is of note that all of the shared key features of SSRI molecules are important for binding to LeuT.

**Antidepressant binding in SERT**

Whether the antidepressant binding site found in LeuT is conserved in the human NSS protein is intriguing and controversial. TCAs and SSRIs bind and inhibit NSS proteins from *Drosophila* and *C. elegans* as tightly as they do to the mammalian proteins, prompting Roubert and colleagues to propose in 2001 that “… in the evolution process the first amine transporters already had the ability to strongly bind TCA in a binding pocket formed by disseminated residues.” [18]. Can one extend the hypotheses further back in evolution to include the bacterial amino acid transporters in the NSS family? Indeed, we have interpreted initial results from our work on LeuT and its corresponding human monoamine transporters as pointing to the S2 site being a tricyclic antidepressant binding site in both the bacterial LeuT and human SERT [9]. Subsequently, more extensive site-directed mutagenesis was carried out related to SSRI binding [17]. At the HBP, mutations of Ile179 or Ile108 in human SERT caused dramatic reductions in the protein’s affinity to bind sertraline, with increases in IC50 ranging from 180 to 1080-fold. Mutating Ile179 reduced the affinity of the other two SSRIs that were studied in the LeuT crystal structures, R-fluoxetine and...
S-fluoxetine, by 7–20-fold. Losses of SSRI affinity were also found upon mutations in two other areas, on the extracellular side and on the cytoplasmic side of the S2 site [17**]. Compared with the changes observed in this study for fluoxetine interacting with the S2 site, SERT mutations related to S1 have generated effects of similar [19] or greater [20–22] magnitude. Mutations at the HBP in the S2 site of SERT in the form of Gly100Ala and Thr178Ala produced a modest reduction in affinity for citalopram [23]. Similarly, Lys448 in the GABA transporter rGAT1, another member of the NSS family of transporters, corresponds to Asp401 in the S2 site of LeuT; the Lys448Asp or Lys448Glu mutants were found to be more sensitive to desipramine [24].

Some other recent studies, however, support the notion that antidepressant molecules instead bind at the S1 site in SERT. Mutation of Ser438 in the S1 site of SERT into threonine caused a loss of affinity of 20–300-fold for three TCAs (imipramine, clomipramine, and amitriptyline) [25]. The loss of affinity to S-citalopram by the same mutation was as high as 2000-fold. Part of this effect can be compensated for by reciprocal removal of a methyl group from the inhibitor [26]. In computer docking into the SERT homology model, TCA molecules could be docked into the S1 site by induced fit, while allowing for protein flexibility in the docking calculations; docking into the S2 site pointed to a diffuse site with no particular drug orientation [22]. In addition, extensive mutagenesis data supported a role for S1 residues Asp98, Ala173, Phe335, and Thr439 in drug binding. These results, together with the previously identified Tyr95 and Ile172 residues believed to be involved in antidepressant binding [20], favor the S1 site as the substrate binding site. However, the cause of the Ile172Met mutation to the observed loss of antidepressant binding affinity has recently been attributed to be indirect via conformational changes [21,26].

In contemplating the location of drug binding sites in human SERT, the first consideration should be that the bacterial and human transporter could be sufficiently divergent as to impart different drug binding properties as a function of the drug studied. Another consideration is that there could be more than one binding pocket for a given drug, for example, a particular antidepressant. Siring and colleagues [22] suggest that the S2 site is the allosteric site previously proposed for citalopram [27]. However, a detailed mutagenesis study of residues impacting allosteric interactions with citalopram did not point to a cluster of S2 residues [26,28]. In interpreting mutagenesis studies, one always needs to consider indirect conformational changes distal from the site of interest. Furthermore, in contemplating drug binding sites, it is important to recall that different drugs – cocaine-like phenyltropanes, TCAs, and structurally heterologous SSRI antidepressants – often show dramatically different effects to the same mutation [17**,20–22,25]. This calls for the separation of results for individual drugs, even among SSRIs, when interpreting mutagenesis and binding experiments. Another consideration is that the close spatial proximity between the S1 and S2 sites makes the total separation of the roles between the two sites difficult to ascertain. The two sites even share a number of common residues (Figure 2a). Therefore, even if the inhibitor and the substrate do not compete for space, they can still compete for those common residues. This issue is further complicated by the flexible nature of substrate binding sites in transporter proteins, which operate by an induced-fit mechanism [29]. It should thus be considered that some drugs could bind to both S1 and S2 sites, with differing impact on substrate translocation. Ultimately crystal structures of human SERT, or related mammalian transporters, with different compounds will provide the decisive information.

SERT specificity for SSRIs

SSRIs bind SERT with high specificity relative to NET and DAT (Figure 3) [30,31]. Both the position and type of substitution found on an aromatic moiety of the SSRI molecule are important determinants for SERT specificity [32,33]. All SSRIs have a halogen substitution at the 3rd (meta) or 4th (para) position of the phenyl or phenoxy ring [32,34–38], which, together with the amine group at the other end of the molecule, yields a dipole moment (Figure 3d). It is this halogen substitution and this characteristic dipole moment that appears to be largely responsible for the drugs’ specificity to SERT over NET [39,40]. Similar halogen substitutions also exist in serotonin-norepinephrine reuptake inhibitors [41,42] as well as in serotonin–norepinephrine–dopamine triple reuptake inhibitors [43–46] but not in norepinephrine selective reuptake inhibitors (NRIs) [32,37]. Strikingly, in fluoxetine-related antidepressants, [35,36] substitutions with CH3– or OCH3– at the 2nd (ortho) position in the phenoxy ring yield NRIs like toloxetine and nisoxetine (Figures 2b and c).

Amino acids around the S2 site in SERT are largely homologous with those in LeuT, and within the HBP six of the seven residues are conserved between SERT, NET, and DAT (Table 1). The only difference in primary sequence of the HBP between the three human proteins is found at Gly100 (SERT). When the equivalent residue in the HBP was mutated to a glycine, both NET and DAT showed modestly increased affinities to all three SSRIs tested, sertraline, R-fluoxetine, and S-fluoxetine [17**]. Indeed, both NET and DAT are the two very neurotransmitter transporters that SSRIs were developed to select against, and a single mutation can partially reverse the selectivity. The findings are consistent with the possibility that the specificity of the human SERT to this class of antidepressants is defined largely by interaction of the
drug’s halogens with the protein’s halogen-binding pocket. Obviously, given the size of the SSRI molecules, additional structural determinants in SERT must be implicated in its specificity for SSRIs.

**LeuT-Trp structure**

Tryptophan has recently been shown to be a competitive inhibitor of LeuT [47**]. It binds at the primary leucine S1 site, trapping the protein in an outward-facing (Coc) conformation (Figure 4a). In the LeuT–tryptophan crystal structure [47**], tryptophan bound at the S1 site interacts with the same residues as leucine, except it fails to form a hydrogen-bond with the hydroxyl group of the extracellular gate residue Tyr108. The structure shows that the gating residues Tyr108 and Phe253 are 3 Å farther apart in comparison to the leucine bound occluded (Ococ) state, leaving the S1 site accessible from the extracellular side. In addition, there is a second tryptophan molecule bound 4 Å on the extracellular side of the S1 site sandwiched between the Arg30 and Asp404 gating residues (Figure 4b). This secondary tryptophan molecule has been hypothesized to be the substrate-in-waiting, to be recruited as substrate after a desolvating passage through the Arg30 and Asp404 gate [47**].

**Cocaine binding to DAT**

Cocaine’s behavioral action is thought to be primarily due to inhibition of the reuptake of dopamine by DAT [48], in most studies found to be of a competitive nature [49]. Recently, molecular models for binding of dopamine, cocaine, and the cocaine-analog CFT to human DAT have been proposed on the basis of the LeuT structure, verified by extensive mutagenesis and chemical cross-linking experiments [50*]. Although the binding sites for dopamine and cocaine were proposed to partially overlap, the two molecules appeared considerably different in their interaction with the gating residue Tyr156. In the DAT protein, Tyr156 appears to form a π–π stacking with the catechol ring of the dopamine and an H-bond with Asp79 via its hydroxyl group. When CFT is bound to the DAT, the interaction with Tyr156 is maintained but the hydrogen-bond with Asp79 gets disrupted leaving the non-polar pocket facing outward, open to the bulk solvent. MD simulation studies showed that disruption of this H-bond was accompanied by the permeation of water deep in the binding pocket [50*].

**Conclusions**

The bacterial leucine transporter LeuT has proven to be an informative model for understanding the structure and mechanism of the neurotransmitter/sodium symporter family. Some of these properties may extend to mammalian members of this transporter family, but it is not yet clear how much overlap actually occurs in view of substantial detailed differences in bacterial and mammalian sequences. Crystal structures of LeuT in complex with various inhibitors, TCAs, SSRIs, and amino acids, have inspired new hypotheses on how antidepressants bind and inhibit mammalian SERT. These new hypotheses, for which some mutagenesis data lend support, are still controversial, with a large body of mutagenesis data pointing to interaction of antidepressant drugs, especially tricyclics and citalopram, with the primary S1 substrate site. Purified protein samples will eventually allow stoichiometry measurements of drug binding and site specific drug inhibition, as has been done for LeuT. Ultimately, the protein structure of mammalian SERT, and other mammalian members of the NSS transporter family, needs to be solved.

The identification of a secondary substrate-binding site in LeuT represents an important breakthrough in the understanding of the protein’s transport mechanism. It also poses some key unanswered questions: How does a kinetic theory for such a two-binding-site protein differ from the original Michaelis–Menten kinetics for the single-binding-site protein, usually described by the alternating access model? How does a two-binding-site transporter display competitive inhibition? What experiments will be needed to dissect substrate/inhibitor interaction for each individual site? Could there be multiple stopover sites along the substrate permeation pathway with their primary role being to guide substrate passage? Answers to such questions will require the combination of a better theoretical frame of transport kinetics, crystal structures of mammalian NSS proteins in complex with drugs, and single molecule spectroscopic techniques that can define the complete transport cycle of the NSS protein.

**Conflict of interests**

None.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
•• of outstanding interest


10. Singh SK, Yamashita A, Gouaux E: Another substrate was found in a secondary S2 site located in the extracellular vestibule. The two binding sites can be occupied simultaneously. Binding of leucine substrate at the S2 site triggers intracellular release of substrate from the primary site. This two-site model of transport calls for new kinetics theories and provides opportunities for greater understanding of substrate-binding and drug-binding in NSS proteins.


13. Binding and flux experiments, together with molecular dynamics simulations, identified a second substrate binding (S2) site located in the extracellular vestibule. The two binding sites can be occupied simultaneously. Binding of leucine substrate at the S2 site triggers intracellular release of substrate from the primary site. This two-site model of transport calls for new kinetics theories and provides opportunities for greater understanding of substrate-binding and drug-binding in NSS proteins.


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18. Co-crystal structures of LeuT with sertraline, R-fluoxetine, or S-fluoxetine revealed that all three SSRI molecules bind at the S2 site in LeuT, with their halogens all binding to the same pocket. Mutation at this halogen-binding pocket (HBP) in human SERT markedly reduced the transporter’s affinity for SSRIs. Conversely, when the only nonconserved HBP residue in both norepinephrine and dopamine transporters was mutated into that found in SERT, their affinities for all the three SSRIs increased uniformly. These results show that the specificity of SERT for SSRIs is dependent largely on interaction of the drug halogens with the protein’s HBP.


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