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Review

The use of LeuT as a model in elucidating binding sites for substrates and inhibitors in neurotransmitter transporters ☆

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ABSTRACT

Background: The mammalian neurotransmitter transporters are complex proteins playing a central role in synaptic transmission between neurons by rapid reuptake of neurotransmitters. The proteins which transport dopamine, noradrenaline and serotonin belong to the Neurotransmitter:Sodium Symporters (NSS). Due to their important role, dysfunctions are associated with several psychiatric and neurological diseases and they also serve as targets for a wide range of therapeutic and illicit drugs. Despite the central physiological and pharmacological importance, direct evidence on structure–function relationships on mammalian NSS proteins has so far been unsuccessful. The crystal structure of the bacterial NSS protein, LeuT, has been a turning point in structural investigations.
Scope of review: To provide an update on what is known about the binding sites for substrates and inhibitors in the LeuT. The different binding modes and binding sites will be discussed with special emphasis on the possible existence of a second substrate binding site. It is the goal to give an insight into how investigations on ligand binding in LeuT have provided basic knowledge about transporter conformations and translocation mechanism which can pave the road for a deeper understanding of drug binding and function of the mammalian transporters.
Major conclusions: The LeuT is a suitable model for the structural investigation of NSS proteins including the possible location of drug binding sites. It is still debated whether the LeuT is a suitable model for the molecular mechanisms behind substrate translocation.
General significance: Structure and functional aspects of NSS proteins are central for understanding synaptic transmission. With the purification and crystallization of LeuT as well as the dopamine transporter from *Drosophila melanogaster*, the application of biophysical methods such as fluorescence spectroscopy, neutron- or x-ray scattering and NMR for understanding its function becomes increasingly available. This article is part of a Special Issue entitled Structural biochemistry and biophysics of membrane proteins.

1. Introduction

The mammalian neurotransmitter transporters are complex proteins that play a central role in controlling the synaptic transmission between neurons by rapid reuptake of neurotransmitter into the presynaptic neuron or neighboring glial cells. The process of transporting its solute against its concentration gradient is energetically driven by the sodium gradient through the cell membrane which is sustained by the Na^+/K^+ ATPase. This way the transport proteins ensure several important processes: (i) without the neurotransmitter transporters, the termination of the signal would be slowed by more than 100-fold [24]; (ii) they minimize lateral diffusion of transmitter to ensure that no uncontrolled chemical cross-talk on adjacent neurons takes place [21,31], and (iii) the subsequent re-packing of neurotransmitter into vesicles allows substrate recycling and thereby decrease in the cost of de novo synthesis [20]. The majority of known neurotransmitter transporters belong to the Neurotransmitter:Sodium Symporter (NSS) family which includes transporters for the monoamines, dopamine, norepinephrine and serotonin as well as for GABA and glycine [69]. They are characterized by their 12 transmembrane (TM) segment topology [25,37,54] and the utilization of the sodium gradient across the plasma membrane as driving force for the transport of solute against its concentration gradient [67]. The transport process is believed to follow the alternating access mechanism in which the accessibility to substrate binding site is alternatively exposed to either the intracellular or extracellular environment. The transport stoichiometry between ions and its solute varies within the family, but they all seem to transport one substrate molecule pr. transport cycle along with one or more Na^+ ions, a Cl^- ion and, for the serotonin transporter, the counter transport of K^+ [67]. The importance of this transporter class is cemented by their extensive use as drug targets: Drugs against depression, anxiety, epilepsy

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as well as psychoactive drugs have been shown to target NSS proteins [43]. However, in spite of their physiological and pharmacological importance, relatively little is known about structure–function relationships in these proteins. The direct assessment of binding site(s) for substrate, ions as well as blockers of transport, has been hampered by the challenge in purifying mammalian transporters in large enough quantities, sufficient purity and stability, to obtain direct structural information about this class of proteins. The lack of structural information about mammalian NSS proteins has been greatly helped by the solvation of the LeuT structure, an amino acid transporter from the thermophilic archaebacteria, *Aquifex aeolicus* [94].

1.1. Structure of the amino acid transporter, LeuT

A breakthrough in the structure–function analysis of NSS proteins was the crystallization of the LeuT in a stunning 1.65 Å resolution [94]. The crystal structure showed a protein with 12 TMs organized, at that time, in a unique fold where the first TMs 1–5 share a similar structural repeat with the following TMs 6–10 but inverted in the plane of the membrane. The last TMs 11–12 participated in a dimerization motif in the crystal lattice (Fig. 1). The core of the protein was formed mainly by TMs 1, 3, 6, and 8 with participation from TM 10 on the extracellular side and TM 5 on the intracellular end of the protein. TM 3 and TM 8 are long helices that are related by the antiparallel symmetry axis and are strongly tilted (~50°). TM 1 and TM 6 possess unwound breaks in the helical structure (dividing both in an a- and a b-section) in the middle of the lipid bilayer exposing main carbonyl oxygen and nitrogen atoms that participate in the binding site for the substrate, here found to be a leucine molecule. The substrate binding site is completed by residues in TMs 3 and 8 as well as the coordination of a sodium ion (Na1). A second sodium, Na2, has no direct interaction with the leucine, but rather plays an indirect role in the formation of the substrate binding site. The symmetry seems to be lost in the loop structures where the extracellular loops (ECL) 2 and 4 are considerably longer and ordered helical structures whereas the intracellular loops (ICL) seem much shorter and only ICL 5 bears a short helical stretch. The central binding site was occluded from the solution on both the extracellular and cytoplasmic sides; that is, both the extracellular and intracellular gates were closed. The access to the substrate binding site from the extracellular side is primarily obstructed by a thin extracellular gate formed by a charged pair composed of Arg30 and Asp404. Further down towards the substrate binding site two additional aromatic residues interact, Tyr108 and Phe253, further obstructing the access [94]. All four residues are highly conserved in the NSS family [7]. Access from the intracellular side is closed by a thick gate of ~20 Å of ordered protein structure mostly consisting of the scaffolds of TMs 1, 6 and 8. Near the cytoplasmic face of the protein, another conserved charged pair, Arg5 and Asp369, forms a partially buried salt bridge. Arg5 also forms a hydrogen bond with Ser267 and a cation–π interaction with Tyr268. Adjacent to these residues lies Trp8, stabilizing the conformation of TM 1a–TM 6b and anchors the amino terminus, including Arg5 [94]. Also on the intracellular side, the network of interacting residues forming the gate is strictly conserved among other NSS family members and extensive studies on the corresponding residues to Arg5, Tyr268 and Asp369 in the DAT and SERT have also shown that the disruption of these interactions causes a conformational bias towards the inward facing structure [15,39,47,48,84].

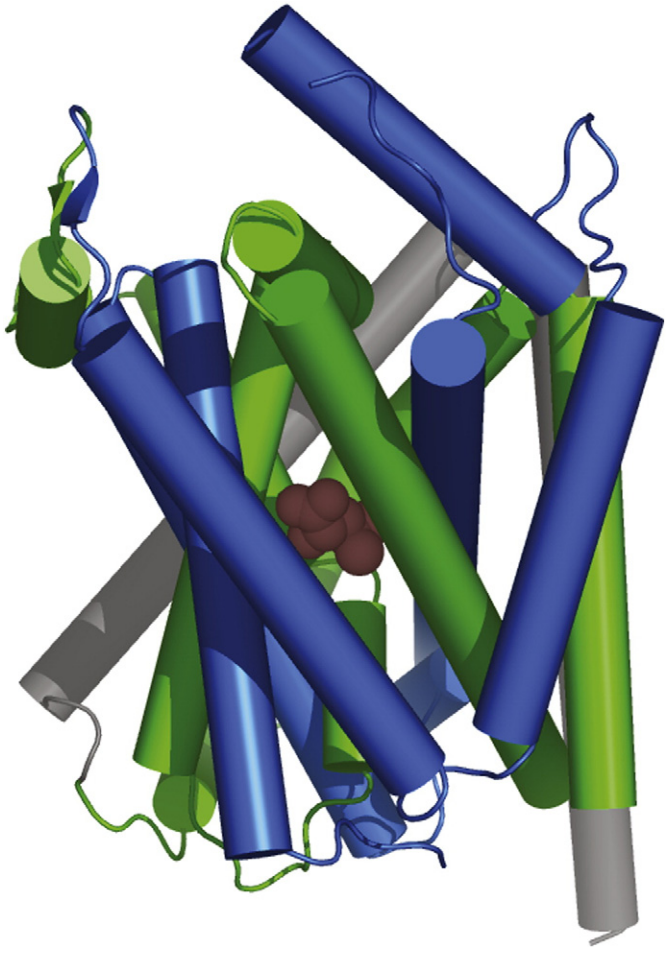

Fig. 1. Structural representation of the LeuT in the outward occluded conformation. The 5 + 5 structural repeat is emphasized by TMs 1 to 5 shown in blue and TMs 6 to 10 in green. TMs 11 and 12 are gray. The bound leucine is located in the middle (brown spheres).

The LeuT bears only 20–25% amino acid homology with the mammalian NSS, however, comparison of biochemical evidence from the mammalian NSS proteins to the LeuT structure substantiates that – at least in terms of the transmembrane domains – the LeuT shows high homology and is a suitable candidate as a model protein [13,38]. In particular the residues which seem to control the accessibility to the central binding site from either side of the protein as well as those forming the actual binding site for substrate and sodium ions, show a high degree of conservation between LeuT and its mammalian counterparts [43]. This notion is further substantiated by the subsequent elucidation of crystal structures from other classes of secondary active transporters. Even though these transporters have no sequence homology to the LeuT, the crystal structures reveal a similar 5 + 5 inverted repeat motif as the one first observed for the LeuT. Interestingly, the repeat can be located to different segments of the protein: Transporters, such as the ApcT [75] and AdiC [22], are also 12 TMs with the first 10 TMs being part of a 5 + 5 repeat, but ApcT is proton coupled and AdiC is an exchanger. The sodium-glucose transporter vSGLT [18], has in total 14 TMs, one N-terminal and three C-terminal to the 5 + 5 repeat. The multisubstrate transporter MhsT, is thought to have only 11 TMs [64] whereas others again have inserted 2 TMs before the repeat instead of after, as is the case for the betaine transporter BetP [65] and carnitine transporter CaiT [85] (Fig. 2). The reuse of a common structural fold underlines the evolutionary advantage in simplicity, and it suggests that its overall structure and function could be preserved from archaebacterial amino acid transporters to mammalian neurotransmitter transporters. In support of this, the recent publication of the first eukaryotic NSS structure, the drosophila DAT shows an almost identical structure to LeuT [55]. The LeuT has also been crystalized in both an inward facing and an outward facing conformation [42], providing valuable insight into the translocation mechanism by this class of transport proteins.

The purpose of this review is to provide an update on the current knowledge about the binding sites for substrates and inhibitors in the LeuT. The different binding modes and binding sites will be discussed with special emphasis on the possible existence of a second substrate binding site. It is the goal to give an insight into how investigations on

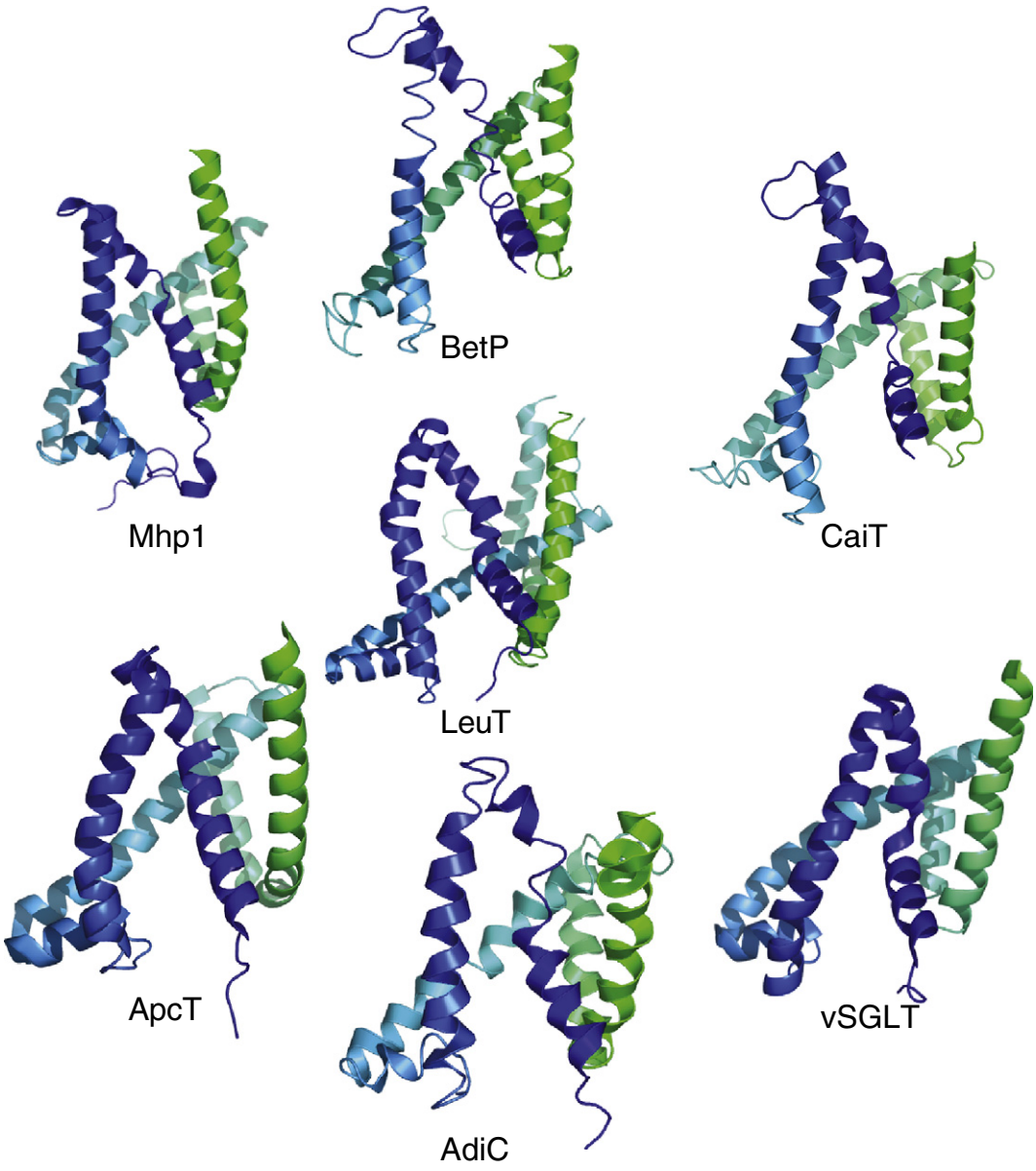

Fig. 2. Comparison of LeuT to structures of other secondary active transporters not belonging to the NSS family but bearing the LeuT 5 + 5 fold. Several secondary active transporters without sequence homology to the NSS family proteins have shown to possess a similar 5 + 5 structural repeat. Both the ion coupling and the number of pro- or preceding TMs can vary. Thus, the Hydantoin transporter [78], Mhp1 (PDB ID: 2X79) and the amino acid transporter [75], ApcT (PDB ID: 3GIA) have two TMs preceding the 5 + 5, but the ApcT is H^+-coupled. The betaine transporter [65], BetP (PDB ID: 2WIT), and the carnitine transporter [85], CaiT (PDB ID: 3HFX), have two TMs preceding the 5 + 5. BetP is Na^+ coupled, but CaiT possesses ion-independent transport but functions as an exchanger with γ-butyrobetaine. The L-arginine/agmatine antiporter [22], AdiC (PDB ID: 3LRC), has the same topology as LeuT, but is H^+-dependent. Finally, the glucose transporter [18], vSGLT (PDB ID: 3DH4), is Na^+-dependent but has one TM preceding and three TMs proceeding the 5 + 5 fold. Only the first 5 TMs in the 5 + 5 fold are shown for clarity going from blue to green from N- to the C-terminal.

ligand binding in LeuT have provided basic knowledge about transporter conformations and translocation mechanism which can pave the road for a deeper understanding of drug binding and function of the mammalian transporters.

2. The alternating access mechanism

The mechanism by which the NSS family proteins couple movement of substrate with ion movement has been the subject of much speculation. The first insight into the mechanism of secondary active transporters was in 1957 when Peter Mitchell proposed that they occupy two alternating structural states: one in which the substrate binding pocket is accessible to the extracellular solution and another in which the pocket is accessible to the intracellular solution [50]. Later, Oleg Jardetzky refined the model, calling it the 'alternating access' model, which most scientists have embraced [33]. In this model, the transporter has the capacity to change conformation upon substrate binding rendering the binding site, at a given time, accessible only from the intracellular- or the extracellular side of the membrane. At all times an impermeable barrier exists between the binding site and one side of the membrane, but the barrier can change from one side of the binding site to the other, giving the site alternate access to the two aqueous compartments that the membrane separates [33]. The structural organization of LeuT in the 5 + 5 structural repeats with the pseudo two-fold symmetry axis in the plane of the membrane did suggest a relationship between the molecular architecture of NSS family proteins and the mechanism of alternating access. One requirement for the achievement of this mechanism is the presence of gating residues; i.e. residues which undergo specific movements during the translocation mechanism, controlling access to the central binding site. These residues must be

allosterically connected to the binding of the substrate and ions and thus, only when all requirements are achieved, the conformational changes which are required for substrate transport are initiated.

The majority of inferences on the mechanism for substrate translocation have come from either molecular modeling or X-ray crystallography of LeuT structures solved in different conformational states.

2.1. Inferences about alternating access mechanism from molecular modeling

The LeuT structure in the outward occluded form made it possible to build reliable molecular models of other LeuT conformations. Forrest, Rudnick and colleagues emerged with the intriguing idea that the intrinsic symmetry in the 5 + 5 repeat could be used to deduce the inward facing conformation from the outward occluded structure [19]. They made the observation in spite of the structural similarity between TMs 1–5 and 6–10 that the overall structure of LeuT in the outward occluded conformation is not symmetrical: access to the substrate binding site is only restricted by the thin gate from the extracellular side but the thick gate from the intracellular part of the protein [94]. The asymmetry could result from differences in conformation between the two repeats and provide a key to the understanding of conformational changes in LeuT. They hypothesized that, based on the structural similarity between the 5 + 5 inverted repeats; it should be possible to generate a model of the inverted structure to the outward occluded form, by swapping the conformations of the two repeats. The 'swapped' model was consistent with an alternating access model. A comparison of the first two TMs of each repeat revealed that they have very similar arrangements. Also, the last three TMs of each repeat were similar in structure as compared to the whole repeat as such. The two domains, TMs 1–2 and 6–7 relative to TMs 3–5 and 8–10 were called 'the bundle' and 'the scaffold' domains, respectively. In the proposed model, the bundle rocks relative to the relatively stable scaffold domain. The movement is alternating between conformations, as a toggle switch, in which the substrate binding site is accessible to either the extracellular or intracellular face. Specifically, the most pronounced change from the outward occluded form is orientation of the bundle which makes a tilt by 25° around an axis constituted by the binding site region. This causes a separation of the scaffold TMs 5 and 8 from TMs 1 and 6, opening the LeuT to the cytoplasmic side. On the extracellular side, TMs 1 and 6 pack up against the scaffold TMs 3 and 10 closing accessibility to the binding side. The two Na^+ binding sites have distinct roles in this model: The Na1 site is completing the substrate binding site making it available for the substrate whereas occupation of the Na2 site could foster an interaction between the scaffold and the cytoplasmic half of the bundle, thereby closing the cytoplasmic pathway by holding the two domains together. The proposal that the conformational switch is triggered by the concerted action of substrate and Na^+ binding to the Na2 site is supported by molecular dynamics (MD) simulations [96,97]. The simulations have shown that an unbinding of the ion from the Na2 site leads to a tightening of the extracellular thin gate and a destabilization of the intracellular thick gate and thus may promote an unbinding of the co-transported substrate. This lends support to the hypothesis that one of the main drivers in the transport cycle of Na^+-coupled secondary transporters is the binding of the Na2 ion by controlling the dynamical equilibrium from an outward facing conformation to an inward facing conformation [96,97]. Conversely, Na^+ binding to the Na1 binding site seems to drive the apo-form of LeuT from the occluded form towards the outward facing conformation [98,100].

The rocking bundle model was in agreement with experimental data on SERT showing changed accessibility to the extracellular side of TM 10 and the cytoplasmic sides of TMs 1, 5, 6 and 8 upon binding of the substrates and inhibitors [19,32,95]. Also, the structure of the inward facing conformation of vSGLT [18] as well as crystal structures of several conformational states of BetP supports the rocking bundle hypothesis [56,57,65,86].

Another compelling molecular model of the LeuT inward facing structure resulted in a slightly different structure [77]. The approach and perspective for generation of the model were entirely different. Here the model was constructed by steered molecular dynamics simulations (SMD) of leucine-bound LeuT immersed in a solvated lipid bilayer. The leucine was then 'pulled' towards the cytoplasm forcing the LeuT to open to the inside. The opening pathway involves some of the same positions as observed in the rocking bundle model involving TMs 1, 5, 6 and 8, but the conformational changes in the LeuT conformation relative to the outward occluded form were overall smaller. The most interesting observation in that model was the proposal that the translocation mechanism required the binding of two substrate molecules instead of only one, because it suggested an entirely novel mechanism behind substrate translocation. This will be discussed later.

2.2. The crystal structure of the inward facing conformation proposes a bending helix mechanism

Today, 50 crystal structures of LeuT have been posted in the NCBI database, but only in three distinct conformational rearrangements. These are, however, believed to be representing the most fundamental conformational transitions necessary for translocation to occur: the outward facing, the outward occluded and the inward facing conformations. The predicted movements required for the transition between the three crystallized states are somewhat different from those predicted by rigid helical movements as in the rocking bundle model.

To obtain the outward open and inward open structures, it was necessary to further stabilize the LeuT by specific mutations: The outward open was stabilized by the mutation of a central residue located in the substrate binding pocket (Y108F) [42]. The Y108F ablates a hydrogen bond between the OH-group of tyrosine and the carboxylate of leucine causing an almost completely abolishment of substrate binding at any concentration [59]. The inward open structure was stabilized by the mutation of residues in the Na2 binding site (T354V and S355A) as well as the cytoplasmic gating residue Tyr268. The protein conformation was also stabilized with a Fab fragment. Naturally, the specific information about the mutated residues (e.g. the Tyr268 which is believed to be a central gating residue [39,94,99]) is restricted in the crystals, but otherwise the obtained information provides detailed insight into the helical movements and role of specific residues in the transition between conformational states. The outward facing structure has been solved both as a sodium-bound substrate-free form and with bound tryptophan [42,79]. Because the outward facing structure has both sodium sites, it was proposed that the transition from outward to occluded form would be triggered by substrate binding, and the binding of Na^+ keeps the intracellular thick gate closed by bridging interactions between the intracellular halves of the core and scaffold domains [42]. The transition from the sodium-bound outward open structure to the substrate and sodium-bound outward occluded structure is basically the formation of the thin gate, and hence substrate occlusion from the extracellular side. This transition is governed by the helical hinge-like movements of the extracellular halves of TMs 1, 2 and 6. The movements pivot at Val23 (TM 1), Gly55 (TM 2) and Leu257 (TM 6) suggesting that substrate binding forges the interaction between the core and scaffold domains and poses constraints on TMs 1 and 6. TM 11 and ECL 3 move towards the center of the LeuT as a consequence of TMs 1 and 6 closing in. The movement brings central gating residues in position: Phe253 comprises a 90° lateral rotation upon substrate binding functioning as a lid on top of the binding site 'locking' the substrate into the site. This movement is followed further up the protein by the formation of the Arg30–Asp404 interaction through two water molecules. These interactions demarcate the central binding pocket and gating residues from the extracellular vestibule forming a crevice into the extracellular face of the LeuT in the outward occluded conformation. Based on the structure of the inward facing conformation, further transition away from the outward occluded form involves a tilting of

the N-terminal part of TM 1 by ~45° protruding into the predicted location of the membrane. The intracellular part of TM 6 does also rotate away from the central binding site, albeit less drastic than TM 1. In concordance with the intracellular part of TM 1 and 6 opening, the extracellular part moves towards the scaffold domain blocking the extracellular pathway in what comprises a thick gate. In contrast, on the intracellular side, the thick gate opens allowing access to the substrate binding side from the inside. TMs 2, 5 and 7 also move, but they undergo a bending rather than a tilt as observed for TMs 1 and 6. The movement of TM 7 causes ECL 4 to plug into the extracellular vestibule creating an extracellular thick gate.

Even though there are significant differences, inferences from the rocking bundle model and the solved crystal structures do not necessarily have to be mutually exclusive. The overall domains which are proposed to constitute the substrate pathway are the same. The directions of the helical movements are also similar. Where they disagree are basically, how they move – hinge bending or rocking bundle – and how much (Fig. 3). Both results come up with an inward facing structure, but they are static models and do not provide direct information about the dynamic transitions which take place between the states. One or both of the inward facing structures could be intermediates residing structurally very close to the actual inward facing structure. In principle, the rocking bundle model could pass an intermediate which involves a bending of the hinge regions in TMs 1 and/or 6, either right after substrate release or further on towards the return to the outward facing conformation. Several mutations and other structural constraints had to be imposed on LeuT to form the crystal structure which could affect the structure. The biggest difference between the two models is the 45° movement of TM 1a and the less pronounced outer movement of TM 6b in the crystal structure. Both movements are very unsymmetrical relative to the outward facing structure and will therefore not be revealed in a model based on swapping conformations in symmetrical repeats as in the rocking bundle model. The question is whether the marked TM 1a movement is only possible in a detergent solution devoid of steric or charged restrictions on the movement of the terminal polar residues or if it would also take place when LeuT is embedded into a lipid bilayer. The TM 6b interacts with the Fab fragment in the crystal structure which could bias the structure. The swapped model is based on the outward occluded form available at the time and not the more outward facing conformation which was only available later. The outward occluded form is an intermediate which opens the possibility that the swapped model also could be an intermediate state going to or from the actual inward facing conformation. Also only conformational endpoints are used for the model and changes due to substrate binding could not be taken into account.

There are good reasons to believe that the overall mechanism behind translocation is preserved from the LeuT to the mammalian NSS proteins. The apparent gating residues controlling access from both the extra- and intracellular sides are, to a large extent, identical and mutations of e.g. the intracellular gating residues produce comparable effects from LeuT to DAT and SERT. Also the observation that the LeuT fold is preserved throughout different families of secondary active transporters suggests a common translocation mechanism which could also be preserved through evolution within the same family. The almost identical structure between LeuT and the dDAT points towards this direction. The domains where information from LeuT is limited are in the loops and the termini. Here sequence conservation is very limited and, if at all revealed in the structures, much shorter. Possibly, the LeuT is not apt for interacting with intracellular proteins to regulate activity and localization as is the case for the mammalian NSS proteins [43].

3. The central substrate binding site in LeuT

The central binding site for the substrate in the LeuT is located in the center of the protein approximately at equal distance from the extra- and intracellular faces (Fig. 4). The binding site is formed by the middle regions of TMs 3 and 8 as well as from the unwound regions of TMs 1 and 6. The Na^+ ions do also play a crucial role in forming the substrate binding site. In particular Na^+ bound to the Na1 site is in direct contact with the substrates making it a crucial component in forming the substrate binding pocket. As discussed, the Na2 site is not directly coordinating the substrate and has most probably an indirect stabilizing role than a direct influence on substrate binding. In the original crystal structure, the bound substrate was a leucine molecule. The leuT was co-crystallized without addition of a substrate and, thus, leucine was simply 'caught' there as a result of the presence of leucine in the bacterial growth medium and remained bound throughout the subsequent purification and crystallization process [94].

The substrate binding pocket can be divided into two segments: a polar and a hydrophobic segment. In the polar segment, the α-carboxy and α-amino groups of the leucine substrate form hydrogen bonds with exposed backbone amide groups from the unwound regions of TM 1 and TM 6. In addition to providing hydrogen-bonding partners for the substrate within the backbone, the helical breaks have been suggested to allow the substrate to interact directly with the ends of the helical segments; thus maximizing α-helical dipole moments [94]. The α-amino group of Leucine interacts with backbone carbonyls from Ala22 (TM 1), Phe253 and Thr254 (TM 6), and with the side-chain hydroxyl from Ser256 (TM 6). The α-carboxy group coordinates Na^+ from the Na1 site and backbone amide from Leu25 and Gly26 (TM 1), and the side chain of Tyr108 (TM 3). The aliphatic side chain of leucine is accommodated by the hydrophobic segment formed by the side chains of Val104 and Tyr108 (TM 3), Phe253, Ser256 and Phe259 (TM

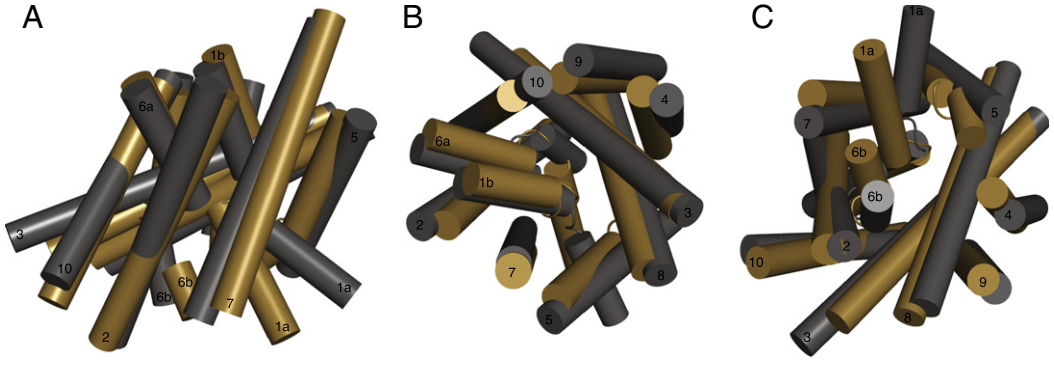

Fig. 3. Comparison of the proposed inward facing conformation from the swapped model to the crystal structure. (A) Side view. (B) Top view. (C) Bottom view. The crystal structure of the inward facing conformation (PDB ID: 3TT3) is shown in gray and the swapped model in sand color. Only TMs 1–10 are shown. The loop structures are omitted for clarity. The major differences are seen in the TM 1a and TM 6b helices. The swapped model was kindly provided by Lucy Forrest.

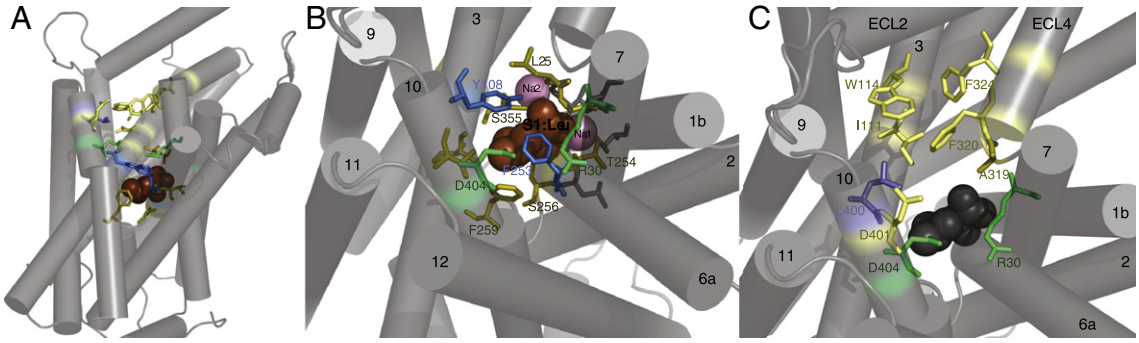

Fig. 4. Leucine binding sites in LeuT. (A) Side view of the LeuT in the outward occluded state (PDB ID: 2A63) with leucine bound in the S1 site. The residues shown to interact with leucine bound to either the S1 or the S2 bound leucine are shown in sticks. The residues constituting the extracellular thin gate are depicted in green. Residues shown to knock out leucine binding to either the S1 or S2 site are shown in blue. Other interacting residues are in yellow. Residues participating in S1 binding are in darker shades than the ones presumed to constitute the S2 binding site. TMs 11 and 12 are removed for clarity. (B) The S1 binding site found in the crystal structure viewed from the outside into the vestibule. The bound leucine (brown spheres) is shown in the middle. The two Na^+ ions are shown as pink spheres. Also here the Arg30–D404 thin gate is shown in green. The Tyr108 and the Phe253 shown to completely and partly eliminate leucine binding, respectively, are depicted in marine blue. Other interacting residues are shown in olive. (C) The proposed S2 site in the extracellular vestibule viewed from the same angle as in (B). Residues proposed to interact with S2-bound leucine are shown as sticks. The Leu400 shown to – when mutated to either a cysteine or serine residue – eliminate S2 binding is shown in blue. The Arg30–Asp404 thin gate is in green. The S1 bound leucine is shown in gray.

6), and Ser355 and Ile359 (TM 8). The binding site is completely dehydrated with the closest water >9 Å away from the leucine α-carbon atom [94] (Fig. 4).

The substrate specificity is not restricted to leucine, rather, it is capable of binding a variety of aliphatic and aromatic amino acids such as alanine, glycine, valine, isoleucine, methionine, phenylalanine and tyrosine [79]. They all bind to LeuT with lower affinity than Leucine, which is probably why this molecule initially was identified in the binding site. However, alanine, methionine and glycine are transported with a higher velocity than leucine making the LeuT rather an alanine transporter than a transporter for leucine. It is believed that the lower affinity for alanine facilitates substrate release on the intracellular side thereby increasing the transport rate. Indeed, the binding of alanine facilitates the isomerization of LeuT from outward to inward form [100]. Crystal structures of all LeuT:substrate complexes reveal an occluded state analogous to the first leucine-bound structure. All substrates (glycine, alanine, leucine, methionine, 4-F-phenylalanine (4-F-Phe) — a more soluble form of phenylalanine) bind to the same binding site and induce overall the same conformation of the protein [79]. As for the first structure with bound leucine, access to the central substrate binding site is occluded from the extracellular side with just a few residues whereas access from the intracellular side is blocked by approx. 25 Å of compact protein. Albeit the overall structure is the same there are small but important differences in the structure compared to the initial leucine-bound structure. Primarily the difference is within the location of Phe259 and Ile359. In the case of glycine and alanine binding, the phenyl ring of Phe259 makes a turn into the binding pocket to accommodate the smaller ligand. Also the *sec*-butyl of Ile359 moves into the pocket in the alanine and glycine bound states. In contrast, when the larger 4-F-Phe ligand is bound, the I359 *sec*-butyl rotates 180°. Also the backbone of the unwound region of TM 6 is moved 0.5 Å outward. It is hypothesized that this minor distortion of the binding site results in a strained occluded state which makes it less likely to isomerize to the open-to-in state and thereby could account for the lower transport rate of the aromatic substrates [79]. Even though the structural rearrangement might seem subtle, the Phe259 and Ile359 might be important for the transition towards the inward facing conformation and subsequent substrate release. Indeed the phenyl ring of Phe259 shows a 3 Å movement from the outward occluded to the inward facing structure, collapsing into the empty binding site when the substrate and inhibitors have been released.

The molecular mechanisms behind the substrate specificity of LeuT have been investigated with a combination of molecular dynamics (MD) simulations and free energy perturbations (FEP) [53]. The study suggests that the ability of LeuT to discriminate between substrates relies in particular on the dynamics of residues Phe253 and Gln250 that directly participate in forming its binding pocket by closing access to the extracellular environment. Also the charged side chains of the Arg30–Asp404 interaction are different depending on the bound substrate. The two charged amino acids Glu287 and Glu290 show large changes in interaction energies in the FEP-MD simulation, depending on the substrate present in the binding pocket. This could participate in the determination of substrate specificity also for the mammalian NSS proteins [9,53].

Tryptophan is the single amino acid which binds LeuT but is not transported. It binds to the same binding pocket as the transported amino acids, but 'locks' the LeuT in its open-to-out conformation [79] very similar to the substrate-free Na^+-bound structure. Relative to the substrate bound outward occluded conformation, this means that, indole rings of the bound tryptophan brace the binding pocket open. The Tyr106 does not form hydrogen bond with the carboxylate of tryptophan as it does for the substrates. Also Phe253 which seems to initiate closure of the extracellular thin gate in the substrate bound complexes is here about 3 Å father away from Tyr108. The increased distance is sufficient to allow the solvent to access the binding pocket because the widening is extended into the extracellular vestibule. This is achieved by a concerted rearrangement of the upper part of TMs 1, 2 and 6 together with an outward rotation of the helix structure in ECL 4. The lack of transport ability is probably due to its larger size. Indeed, substitution of just a single residue (I359Q) within the substrate binding site from the tryptophan transporter TnaT, revealed a LeuT with changed substrate specificity, now capable of transporting tryptophan [58]. A crystal structure of the Trp-bound LeuT I359Q mutant does also reveal a protein which has now adopted the occluded-state conformation [58].

There is little doubt that the substrate binding site in the mammalian NSS protein is identical to the site found in LeuT. Seven of the 11 residues in LeuT that have direct interactions with leucine are conserved in the mammalian homologues, but a few differences exist which likely determine substrate specificity: in the monoamine transporters residues are replaced with smaller amino acids (e.g. Ser256 to glycine, Ile359 to glycine), to accommodate the larger substrate molecule whereas in the glycine transporters, residues surrounding the isopropyl moiety of leucine are replaced with amino acids of a larger size (Val104 to isoleucine, and Phe259 to tryptophan) to reduce the volume of the binding pocket, thereby rendering it more complementary to glycine [43,94]. It is also consistent with experimental evidence identifying S1 residues controlling substrate affinity [7,27,35,68]. In addition the

docking of 5-HT, dopamine and GABA into their respective transporters has also revealed a similar pose [6,9,29,30,92].

4. The putative second substrate binding site

Apart from the primary substrate binding site, experimental evidence suggests a second binding site for substrates, located in the extracellular vestibule [36,62,63,76,77,100]. The proposal for an extra substrate binding site was based on steered molecular dynamics (SMD) simulations where a leucine molecule is "pulled" from its binding site and towards the extracellular environment in a LeuT model immersed in a solvent lipid bilayer [77]. The pulling revealed a new favorable position, approximately 10 Å above the primary leucine binding site (from here on termed the S1 site) towards the extracellular environment. As for the primary binding site, this secondary site, S2, consists of two components: the hydrophobic pocket composed of Leu29, Tyr107, Ile111, Trp114, Ala319, Phe320, Phe324 and Leu400 and an ionic cleft composed of Arg30 and Asp404 (Fig. 4). The SMD analysis was further supported by experimental data showing that the [^{3}H] Leucine binding stoichiometry is 2 leucine per LeuT molecule. When mutations were introduced in either the S1 or the S2 site as done with the F253A and L400S mutations, respectively, the binding capacity was reduced by 50% [62]. A similar result was obtained for [^{3}H]alanine [62]. The data suggested a paradigm shift for the alternating access mechanism in which the binding of a second substrate to S2 is required for the intracellular release of the S1-bound substrate. The proposal was in stark contrast to the solved crystal structures where only one substrate molecule could be seen. It was suggested that this was because the crystal structures were produced in the detergent octyl-β-D-glycopyranoside (β-OG) which occupies the S2 site and thus inhibits binding of the second substrate [63]. In contrast, all functional binding data have been performed in n-dodecyl-β-D-maltoside (DDM) which – at least in low concentrations [36,62] – apparently does not seem to interfere with the S2 site. However subsequent crystal structures using DMPC-CHAPSO bicells, a lipid:detergent mixture that mimics the native membrane bilayer, also failed to reveal a second substrate in the S2 site [89] but a density was observed in the S2 site, although too small to be a substrate molecule.

It has been criticized that binding data has been performed with the scintillation proximity assay (SPA) which involves the binding of His-tagged LeuT molecules to NTA-coated beads containing scintillation liquid [64,77]. Thus, only radioligands bound to LeuT will elicit a signal due to the proximity principle. In such a setup, the affinity or accessibility for the His-tag to the beads does influence the signal, e.g. by imposing mutations in the LeuT the affinity to the beads could change. Also the specific activity is important for the calculation of stoichiometry. This can only be measured with the addition of scintillation liquid and it is not clear whether the counting efficiency in that setup is the same as in the SPA mode. These issues are solved when using the equilibrium dialysis assay. In concordance with the SPA data, equilibrium dialysis binding of [^{3}H]leucine to LeuT wild type also showed a binding stoichiometry of two leucine molecules bound pr. LeuT. With the F253A or the L400S mutations introduced the stoichiometry was reduced to one pr. LeuT [62]. In support of the binding experiments it was shown that, in reconstituted proteoliposomes, either the S1 or the S2 site mutations impair transport function of LeuT as assessed by its ability to transport [^{3}H]alanine. Single-molecule imaging also demonstrated that the L400S mutation prevents not only transport but also the enhancement of intracellular gate dynamics induced by alanine binding [100]. A question raised is that the two mutations – F253A and L400S – both seem to have an all-or-nothing effect on substrate binding. In most cases when dealing with pharmacological issues, a mutation will impair binding to a certain extent but not cause a complete disruption. This might not be the case here either: A crystal structure of LeuT F253A did show leucine binding to the S1 site when applying 1 mM leucine in the crystallization media [91]. Interestingly, even under these conditions, with a destabilization of the S1 site and very high concentrations of leucine, it was not possible to detect the binding of a leucine molecule to the S2 site. However, also in this structure, an electron density was detected immediately adjacent to the leucine slightly protruding into the extracellular vestibule [91]. The density could not be fitted to a leucine molecule, but its presence could potentially obstruct the binding of the second substrate. The most intriguing aspect of the discrepancy between the two binding site models from Javitch and colleagues and the data from the laboratory of Gouaux is that it has not been possible to reproduce the two-leucine stoichiometry pr. LeuT in Gouaux' laboratory even when they attempted to reproduce the preparation of protein according to the methods of Javitch and colleagues or in the recently developed lauryl maltose neopentyl glycol (MNG-3) detergent [91] which has been shown to increase stability and functionality of purified LeuT [10,11,12]. Neither has it been possible for them to reduce [^{3}H]leucine binding by 50% by introducing the L400S mutant or by the addition of clomipramine which should bind exclusively to the S2 site [91]. The role of clomipramine will be discussed in more detail in the next section. Also Sinning and colleagues were unable to reproduce the inhibition of leucine binding to S2 by clomipramine [17]. Furthermore, isothermal titration calorimetry was not able to detect the binding of a second substrate [59]. The lack of reproducibility in pharmacological assays as well as in the crystallographic trials could suggests that very specific conditions have to be fulfilled before S2 binding is obtained. Of note, in our laboratory we have observed a 50% reduction of binding sites in both the S1 and S2 mutants relative to LeuT wild type (Billesbølle & Loland, unpublished results).

The existence of a second substrate binding site is still an open question. The experimental setup and conductance have for all groups involved been performed with state-of-the art techniques and of high quality addressing the problem with several methods and assays. Still only Javitch and colleagues have reported its existence. Even though one tends to perceive a crystal structure as the true structure, it might be difficult to trap the LeuT in a transient or unstable high energy state which could be revealed in a functional assay. Indeed as for the outward occluded structures, an unspecified electron density was also observed adjacent to the proposed S2 site of the LeuT crystal structure of the inward facing conformation [42]. As will also be discussed later, it is indeed possible to bind molecules to the S2 site in the LeuT as well as in its mammalian cousins. The challenge is to coordinate the substrates. But if the translocation mechanism is conserved within, not only in the NSS family, but in all the solved structures of proteins with the 5 + 5 LeuT fold, then why should not at least one of them reveal a second substrate site? The answer might be that the LeuT is the only transporter that requires a second substrate for transport: An important residue in the S2 site is Leu29 which is supposed to be coordinating the leucine directly. This residue is a tryptophan in all other studied NSS transporters both in bacteria (TnaT, Mhp1, Tyt1) and mammals (DAT, NET, SERT etc.). In the crystal structure of the inward facing conformation of Mhp1 [78] this tryptophan seems to collapse into the S2 site and thereby stabilizes the extracellular vestibule. Indeed, mutation of the tryptophan (Trp84) in the DAT, renders the transporter in an outward facing structure [14,73].

One transporter with a 5 + 5 LeuT-like fold has been crystallized with several substrate bound: the L-carnitine/γ-butyrobetaine exchanger, CaiT, from *Escherichia coli* [85]. The CaiT belongs to the betaine/choline/carnitine transporter (BCCT) family. Unlike the other members of this family, the CaiT independently of an ion gradient [34]. Of the solved structures with a LeuT-like fold, the CaiT is most closely related to the BetP. Both of them are 12 TM proteins with two TMs preceding the 5 + 5 fold. The conformation of CaiT is different from the solved LeuT structures [85]. The binding site is accessible from the cytoplasmic side, but it is not as inward facing as the one solved for LeuT. Interestingly, in this structure, four L-carnitine molecules are associated: one at the central binding site (LC-I) corresponding to the S1 site in LeuT, one at the base of the intracellular vestibule about

6 Å from LC-1, one at the entrance of the extracellular vestibule (LC-III) and one in a shallow cavity on the extracellular surface (LC-IV). Thus, the CaiT does not have a substrate bound to a location corresponding to the S2 site in LeuT, but the fact that several substrate binding sites are present in a crystal structure opens the possibility for a similar mechanism in LeuT. According to MD simulations the substrates are not stably bound and substrates quickly dislodge from the reported binding sites [103]. This is in contrast to LeuT where substrates should bind with equal affinity to both sites [62]. Another explanation is that because the CaiT is an exchanger, not a symporter, it is possible that the protein has evolved a different transport mechanism as compared to the NSS family proteins.

The use of accelerated MD to facilitate conformational transitions on a set of 13 trajectories to explore stable intermediate states, provided a full atomic map of the sequence of events from alanine binding to the release by LeuT [16]. In this model a highly stable intermediate conformation between the outward occluded and inward occluded was observed. The conformation is considerably different from the solved crystal structures of LeuT. Here, two alanines are stably bound to the S1 and S2 sites, respectively. A further MD run showed an opening of the intracellular gate and release of S1 bound alanine. In this transition, the S2 site was practically partitioned into two loci, the inner and the outer, by the Arg30–Asp404 salt bridge. If the alanine resides in the outer locus, it was prone to escape to the extracellular environment, but dislocation to the inner locus caused a rotation of Phe320 which again dislodged the S1:alanine by 4 Å towards the intracellular environment. However, the completion of translocation and release of S1:alanine is not solely dependent on Phe320, but requires further cooperative arrangements which could not be revealed by the MD simulation.

A study on human DAT provides evidence which favors a second substrate binding site [72]. Here, bivalent ligands bearing two dopamine-like pharmacophoric heads separated by a carbon linker were developed. Relative to dopamine the bivalent compounds had higher affinity for the DAT. In particular, a ligand with an 8 carbon linker between the two heads possessed an 82-fold higher affinity for the DAT than dopamine. Eight carbons gives a distance between the two heads of approximately 13 Å, which fit the distance of 11–13 Å between the S1 and S2 sites in the LeuT structure model [77]. Mutation of the aforementioned S2 site residue, Trp84 further increased the affinity. Also in the SERT it has been shown that 5-HT can inhibit the dissociation of bound imipramine. If imipramine binds to S1 in SERT this suggests that 5-HT can bind to an additional allosteric site, at least in the presence of a pre-bound imipramine [93].

5. Binding site for inhibitors

The LeuT has so far proven to be an excellent model for the mammalian NSS proteins. As described above the mammalian neurotransmitter transporters – DAT, NET and SERT – are important drug targets for the treatment of e.g. depression and anxiety. The discovery of the first antidepressant, imipramine, was done in the 1950s by serendipity [44]. Later, it was found that imipramine inhibited noradrenaline reuptake in tissues [4]. The molecular mechanism behind the inhibitory action as well as its target protein, was unknown. Only much later the cloning of the monoamine transporters were performed [8,23,54] and with that, the molecular pharmacology and the search for the actual binding sites for the drugs began on the transporters. Before the crystal structure of LeuT and the possibility to perform reliable homology models emerged, the identification of inhibitor binding sites on mammalian monoamine transporters were, to a large extent, based on site-directed mutagenesis in which it is practically impossible to discriminate between direct- and indirect effects of the introduced mutations (for extensive reviews, see [87,88]). Retrospectively, many of the observed mutations which possessed a significant change in inhibitor affinity were likely due to allosteric rearrangements of protein conformations rather than a direct interaction in the binding site [45,46,48,52]. Mutagenesis studies are still an ongoing experimental procedure, but with the availability of the current molecular models of mammalian NSS family proteins; much progress has been made in the generation of detailed hypotheses and more directed experiments can be performed [1,2,6,41,61,71,81,82]. Until the crystal structures of mammalian NSS proteins are solved, direct evidence for a detailed insight into the binding mode of inhibitors will still remain to be elucidated.

5.1. Inhibitors binding to the S2 site of LeuT

In 2007 two papers reported crystal structures of the binding of antidepressants to the LeuT. It was shown that a number of TCAs including clomipramine and desipramine could cause a non-competitive inhibition substrate uptake by the LeuT [80,101]. The TCA bound LeuT structures showed that the TCAs were nested in the S2 site. Specifically, the inhibitors were cradled by the carboxy-terminal regions of TMs 6 and 10, the midpoint of TM 3, and the sharp turn of ECL 4, about 11 Å above the S1-bound leucine and Na^+ ions. Most of the residues lining the S2 site are nonpolar which complements the hydrophobic nature of the TCAs. This probably also explains the promiscuity of the S2 site enabling it to mediate low-affinity binding for many ligands of different nature. The structures showed a LeuT in a fold almost identical to the originally solved outward occluded conformation. Neither the leucine nor the two Na^+ ions had moved. Only two accommodations were observed as a result of the bound inhibitor: The first is the guadinium group of Arg30, the amino acid which together with the Asp404, forms the extracellular thin gate. Here the two water molecules have been expelled and a direct salt bridge is formed. A similar action occurs when LeuT transitions into the inward facing structure [42]. The new position of Arg30 is further buttressed by a cation–π interaction with Phe253. The second change is localized to the ECL 4 where Ala319 participates in a sharp turn of the loop. Here, the inhibitors push the pin of the loop including Ala317–Ala319, towards the extracellular environment by as much as 1.4 Å. Later it was shown that the selective serotonin reuptake inhibitors (SSRIs) sertraline and fluoxetine were also able to bind the S2 site of LeuT [102]. The findings were surprising. Even though the binding of antidepressants had quite low affinity, it was unexpected that a transporter from an archeal bacterium possessed a binding site for the drugs at all. Put into perspective: the binding of desipramine to the human DAT is in the high micromolar range even though it possesses 75% amino acid identity with the NET which binds the same drug with low nanomolar affinity. The LeuT has only ~20% homology with the NET which would thus be expected to cause a complete disruption of desipramine binding. On the other hand, both TCAs and SSRIs bind and inhibit NSS proteins from *Drosophila melanogaster* and *Caenorhabditis elegans* as tightly as they do the mammalian proteins [66]. However, TCAs are small, hydrophobic, planar molecules, which are a suitable pharmacophore for low-affinity interaction with proteins in general. The mutagenesis of the residues in DAT, NET and SERT correlating with the interacting residues in the LeuT crystal showed a modest, but significant effect on both TCA and SSRI binding supporting the location of the high affinity binding site to the S2 [101,102]. In contrast to these, molecular docking models and experimental evidence on mammalian DAT, NET and SERT have suggested the binding of TCAs and SSRIs as well as for cocaine to be located at the S1 binding site [1–3,5,6,9,26,28,40,41,74,81,82]. The explanation for the controversy is probably that the mammalian monoamine transporters have two binding sites for antidepressants: The high affinity binding site located at S1 and a low-affinity allosteric site most likely corresponding to the S2 site in LeuT. It has been known for some time that several SERT ligands, particularly citalopram, clomipramine, paroxetine and 5-HT itself have allosteric effects on the high affinity binding of antidepressants [12,60,61,93]. The allosteric effect has been assessed by the ability of ligands to impair the dissociation of a pre-bound radioligand. The most pronounced effect has been the allosteric effect

of S-citalopram on the dissociation of [^{3}H]S-citalopram [12]. It has been suggested that the dual action of S-citalopram at two binding sites in the SERT is responsible for the higher efficacy and faster onset of antidepressant action for S-citalopram as compared with the racemic compound [49,51,70,83]. Generation of a molecular docking model of SERT with two S-citalopram molecules bound showed that the allosteric site corresponds to the S2 site in SERT [61]. The model was supported by experimental evidence. Also the DAT was shown to contain a similar site [61].

5.2. Inhibitors located at the S1 binding site of LeuT

The discrepancy still persisted between structural data from LeuT showing the binding site for antidepressants located at the S2 site as opposed to the experimental evidence from the monoamine transporters, suggesting that the binding site is directed towards the S1 site. Two comprehensive papers from Gouaux and colleagues probably solved the problem [55,90]. In one study, chimeras of LeuT and the monoamine transporters provided new insight into the binding of all classes of inhibitors. The LeuT was engineered to harbor the pharmacology of the monoamine transporters by substituting key residues around the primary binding site with the corresponding residues from the SERT [90]. All residues within a 10 Å radius surrounding the primary binding site of LeuT with bound tryptophan was analyzed to identify about 20 residues which point towards the primary binding pocket and are divergent from SERT. The residues from SERT were inserted singly and in combinations into the LeuT with the goal of increasing the binding affinity for the SSRI [^{3}H]paroxetine to the LeuT mutant. The screen resulted in three LeuT:biogenic amine transporter chimeras (LeuBATs), bearing significantly increased affinities for the SSRIs sertraline, paroxetine, fluoxetine and fluvoxamine; the selective noradrenaline reuptake inhibitors (S)-duloxetine and desvenlafaxine; the TCA clomipramine and the stimulant mazindol. Crystal structures of the LeuBAT:inhibitor complexes showed that, they all bind to the central S1 binding site in an outward open LeuT conformation similar to the one solved for the tryptophan bound state [79,90]. The pharmacophores of all inhibitors include inserted residues in TM 1 (Tyr21, Asp24 — corresponding to Asp98 in SERT and Asp79 in DAT), TM 3 (Pro101, Val104, Ala105, Tyr108), TM 6 (Phe253, Gly256, Phe259), TM 8 (Ser355, Ser356, Gly359), and TM 10 (Asp404, Thr408). Collectively, these observations invalidate the notion that SSRIs and TCAs elicit their effects on SERT by binding to the extracellular vestibule [71,101,102]. Even though the data leaves little doubt about the binding site, the fact that LeuBAT possesses the same conformation in all structures is not a coincidence: The crystal structures were grown in the presence of saturating concentrations of mazindol or serotonin. All other LeuBAT:drug complexes were formed by soaking LeuBAT crystals in solutions containing 3–20 mM of each drug. This does not leave any doubt that the drugs bind to the S1 in the outward facing conformation of the LeuBAT. The question is whether this is the preferred conformation.

In the other study, Gouaux and colleagues solved the structure of the DAT from *D. melanogaster* [55]. The transporter is locked in an outward facing conformation with nortriptyline coordinated between TMs 1, 3, 6 and 8. The binding site is identical to the one identified for clomipramine in LeuBAT [90] and very similar to experimentally validated MD simulations of imipramine binding to S1 in SERT [81]. Even though the dDAT is much different from LeuT and bears more than 50% sequence identity with its mammalian counterparts, the most stunning about the solved structure is the high similarity it has to the LeuT in spite of their evolutionary distance. It does also possess differences: The TM 12 has a kink about halfway through the membrane and the C-terminal contains a helix which lies perpendicular to the membrane and probably functions as a lid for the accessibility to the central binding site. Also a cholesterol molecule is wedged in between TMs 1, 5 and 7 probably maintaining an outward open state of the transporter. With the structure of the dDAT, the biophysical investigations on Na^+-coupled transporters have taken a giant leap towards the biochemical and molecular investigations in the mammalian NSS proteins. Someday all applications will probably be performed on mammalian NSS.

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