

X-ray structures of LeuT in substrate-free outward-open and apo inward-open states

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Neurotransmitter sodium symporters are integral membrane proteins that remove chemical transmitters from the synapse and terminate neurotransmission mediated by serotonin, dopamine, noradrenaline, glycine and GABA (γ -aminobutyric acid). Crystal structures of the bacterial homologue, LeuT, in substrate-bound outward-occluded and competitive inhibitor-bound outward-facing states have advanced our mechanistic understanding of neurotransmitter sodium symporters but have left fundamental questions unanswered. Here we report crystal structures of LeuT mutants in complexes with conformation-specific antibody fragments in the outward-open and inward-open states. In the absence of substrate but in the presence of sodium the transporter is outward-open, illustrating how the binding of substrate closes the extracellular gate through local conformational changes: hinge-bending movements of the extracellular halves of transmembrane domains 1, 2 and 6, together with translation of extracellular loop 4. The inward-open conformation, by contrast, involves large-scale conformational changes, including a reorientation of transmembrane domains 1, 2, 5, 6 and 7, a marked hinge bending of transmembrane domain 1a and occlusion of the extracellular vestibule by extracellular loop 4. These changes close the extracellular gate, open an intracellular vestibule, and largely disrupt the two sodium sites, thus providing a mechanism by which ions and substrate are released to the cytoplasm. The new structures establish a structural framework for the mechanism of neurotransmitter sodium symporters and their modulation by therapeutic and illicit substances.

Chemical neurotransmission in the central nervous system is terminated through re-uptake of neurotransmitters from the synapse into surrounding neuronal and glial cells, a process first characterized in 1961 (ref. 1). Transmitter uptake is mediated by neurotransmitter sodium symporters (NSSs)^{2,3}, integral membrane proteins that exploit energetically favourable Na^+ electrochemical gradients for the thermodynamically uphill transport of neurotransmitters. Members of the NSS family include transporters for GABA, glycine, noradrenaline, serotonin and dopamine—chemical transmitters that have fundamental roles in the function of the nervous system. Accordingly, dysfunction of these transporters is implicated in diseases such as depression^{4,5}, schizophrenia⁴, epilepsy⁶ and Parkinson's disease⁴, and they are targets for therapeutic drugs including tricyclic antidepressants (TCAs) and selective serotonin re-uptake inhibitors (SSRIs) as well as addictive substances such as cocaine and amphetamines⁷. Understanding the substrate translocation mechanism of NSSs is central to the development of accurate models of substrate and drug complexes and to the discovery of new therapeutic agents.

The mechanism of NSS transport is generally described by the thermodynamically coupled binding of substrate and ion(s) to a central binding site that is alternately accessible to either side of the membrane^{8,9} (Supplementary Fig. 1). Crystal structures of LeuT, a bacterial NSS homologue, elucidated the architecture of NSS proteins, demonstrated the existence of a substrate- and ion-bound occluded conformation, and illustrated how competitive and non-competitive inhibitors stabilize an outward-facing conformation^{10–14}. LeuT is, at present, the best template for modelling the structure of NSSs and their complexes with therapeutic and illicit drugs. However, our understanding of

mechanism and structure/function relationships in NSSs is incomplete due to the absence of LeuT structures in outward-open and inward-open states.

In the absence of structural knowledge of transporter intermediates, general mechanisms of transport have been proposed based on structures of other secondary transporters bearing the LeuT fold and crystallized in distinct conformational states^{15–19}. However, low amino acid sequence identity compromises the extent to which this approach can generate a detailed and accurate mechanism for NSSs. Concomitantly, spectroscopic and computational methods have focused on characterization of conformational changes accompanying substrate and ion association/dissociation events in LeuT^{20–23}. Although these approaches have yielded new insights into localized movements, they have not revealed the three-dimensional, atomic-level details of conformational changes associated with substrate binding, isomerization of the transporter to the inward-open conformation, and release of substrate and sodium ion(s). Here, we present crystal structures of the outward-open and inward-open states of LeuT and thereby establish the structural basis for transport in the NSS family and provide accurate templates for modelling eukaryotic NSSs and their complexes with substrates, ions and drugs.

Substrate-free and inward-open conformations

To stabilize the substrate-free and inward-open states of LeuT, we mutated residues in transmembrane domains (TMs) 3 and 8, helices comprising part of the 'scaffold' domain (TMs 3, 4, 8 and 9) and in TM6, one of the 'core' domain helices (TMs 1, 2, 6 and 7)^{10,22,24} in the background of the wild-type-like K288A variant (LeuT^K)²⁵ (Supplementary Table 1 and Supplementary Fig. 2). To enhance

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crystallization behaviour, we raised conformation-specific antibodies, exploiting fluorescence-detection size-exclusion chromatography (FSEC)²⁶ to select the antibodies and to demonstrate state-dependent binding. Well-diffracting crystals of substrate-free LeuT were obtained using the Tyr 108 to Phe mutant in TM3 (LeuT^K(Y108F))²⁵ and the 2B12 antibody fragment (Fab). Stabilization of LeuT in an inward-open conformation required weakening of the sodium 2 (Na2) site¹⁰ by mutation of Thr 354 to Val and Ser 355 to Ala (TM8), as well as the cytoplasmic gate by changing Tyr 268 to Ala (TM6; LeuT^K(TSY))^{23,27,28}, together with the 6A10 Fab.

Sodium-bound state is outward-open

The substrate-free LeuT^K(Y108F) structure (Supplementary Table 2 and Supplementary Fig. 3) adopts an outward-open conformation as a consequence of hinge-like movements in TMs 1b, 2a (residues 41–55), and 6a, relative to the outward-occluded state (Fig. 1a–c, e). Extracellular loop 3 (EL3) and TM11 are displaced by as much as 2.8 Å and 2.2 Å, respectively, due to the movements of TM1b and TM6a (Fig. 1a). Notably, TMs 1b, 2a and 6a pivot at Val 23, Gly 55 and Leu 257, respectively, suggesting that when substrate no longer forges interactions between the core and scaffold domains, constraints on TM1b and TM6a are released, allowing them to move outward and the transporter to adopt an outward-open conformation. The LeuT^K(Y108F) structure is similar to the previously reported Trp-bound LeuT structure¹² with a root mean squared deviation (r.m.s.d.) value of 0.4 Å for all C α atoms (Supplementary Fig. 4), supporting the

general principle that inhibitors bind to conformational states populated by the wild-type transporter.

The ‘thin’ extracellular gate¹⁹ of the outward-occluded state is ruptured in the LeuT^K(Y108F) structure. Residues that bridge TM1b/TM6a to TM3/TM10 in the outward-occluded state have separated, opening a pathway to the extracellular solution (Fig. 1d, e). Arg 30 no longer forms a water-mediated salt bridge with Asp 404 (TM10) and the side chain of Phe 253 has rotated away from the binding site by a $\sim 90^\circ$ rotation about the χ_1 dihedral angle, in agreement with molecular dynamics simulations^{21,29}. The coordinated movement of Arg 30 and Phe 253 enables the retention of an important cation- π interaction between their respective guanidinium and phenyl groups. The phenyl ring of residue 253 now occupies the same position as the indole ring of Trp 602 bound to the extracellular vestibule in the Trp-bound LeuT complex¹².

Prominent electron density peaks (4.0σ) observed in the Na1 and Na2 sites together with ion-oxygen distances of ~ 2.4 Å (ref. 30) are consistent with sodium occupancy of these sites, although higher resolution diffraction data will be required to confirm their identity (Fig. 2). Most importantly, the outward-open structure suggests that the presence of sodium ions keeps the intracellular thick gate¹⁹ closed by bridging interactions between the intracellular halves of the core and scaffold domains. Even though the Na1 site is located close to the pivot points for TM1b and TM6a (Fig. 2a), the concerted movement of the helices allows for retention of ion coordination geometry, excepting the loss of the carboxylate oxygen from the absent substrate.

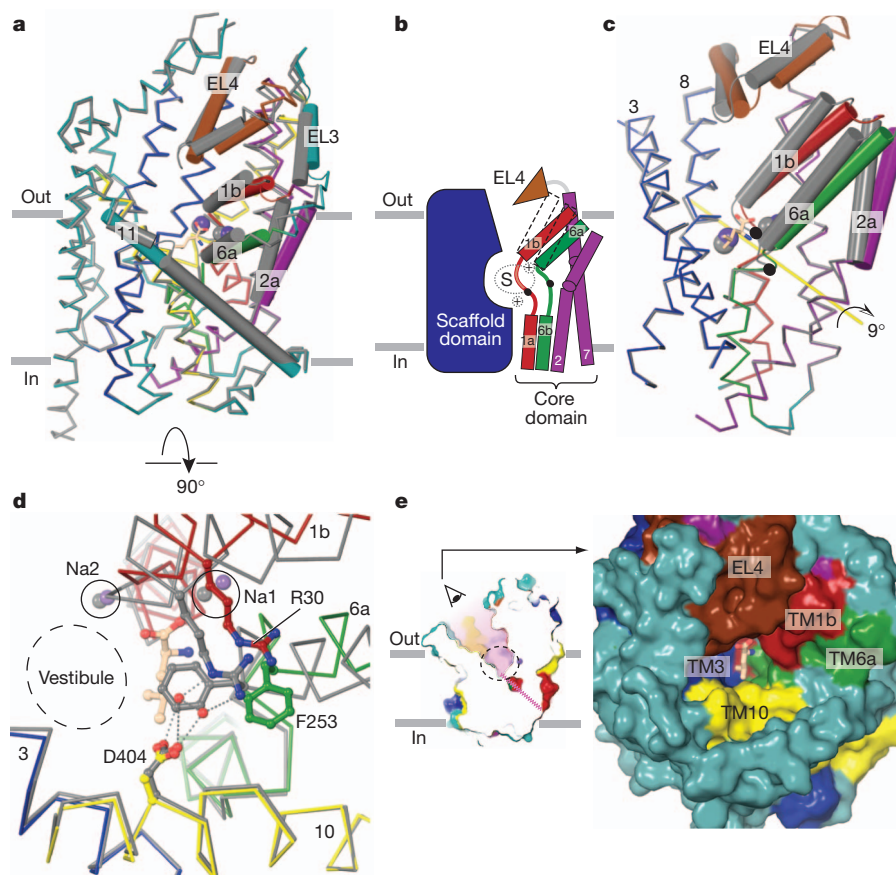


Figure 1 | The substrate-free, Na⁺-bound state is outward-open.

a, Superposition of the outward-open and leucine-bound outward-occluded conformations. Outward-open structure is coloured with Na⁺ ions as purple spheres. The outward-occluded structure with Na⁺ ions (spheres) is grey and leucine is shown in stick representation. **b**, Schematic of scaffold and core domains, EL4, the pivot points of hinge movements in TMs 1 and 6 (solid black circles) and the substrate (S) and sodium sites (+). **c**, Superposition, as in panel **a**, illustrating that a $\sim 9^\circ$ rotation about an axis passing through the middle of

the core domain (yellow arrow) describes the conformational change associated with opening to the outside. Pivot points are shown as in panel **b**. **d**, Rupture of extracellular gate interactions (grey dashed lines) in the outward-open structure. Two water molecules that bridge Arg 30 and Asp 404 in the outward-occluded state are shown as red spheres. **e**, Surface representation of the outward-open structure with the zig-zag pink line indicating a closed intracellular pathway. Leucine, where shown, is from the outward-occluded Leu-bound structure.

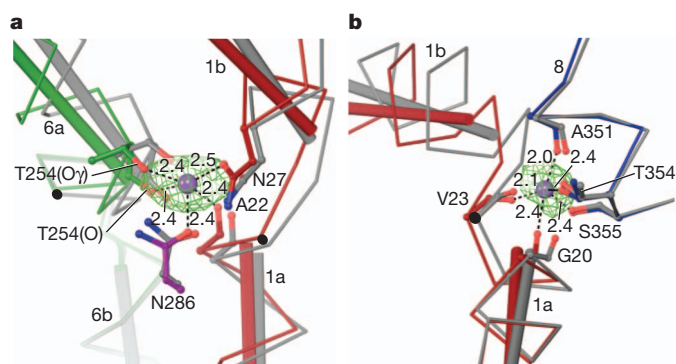


Figure 2 | Sodium sites in the outward-open state. **a**, The Na1 site showing positions of the coordinating residues within the framework of global changes in the outward-open structure. **b**, The Na2 site. $F_o - F_c$ omit density is contoured at 4σ and represented as green mesh. Colour scheme and representations are the same as in Fig. 1. Dashed lines indicate interactions between sodium ions and coordinating atoms with distances in Å, solid black circles are approximate pivot points for hinge movement of helices and red and green cylinders define the TM1 and six helix axes, respectively.

Binding of Na^+ at this site may precede substrate binding, as also suggested by simulation studies²⁹, thereby stabilizing local conformations of TMs 1 and 6 and engaging the main-chain carbonyl oxygen of Ala 22 (TM1a) with side-chain oxygens of Asn 27 (TM1b) and Thr 254 (TM6a). The Na2 site is located towards the intracellular region of TM1, stitching TM1a to TM8 through the main-chain carbonyls of Gly 20 and Val 23 (Fig. 2b). Thus, the Na^+ ions, through their interaction with TM1a, stabilize an intracellular-closed conformation, a finding supported by single-molecule Förster resonance energy transfer (FRET) studies²³.

Structure of the inward-open conformation

We hypothesized that weakening of the Na2 binding site by mutation of Thr 354 and Ser 355 to valine and alanine, respectively, and perturbation of the intracellular gate by mutation of Tyr 268 to Ala²⁷,

together yielding the LeuT^K(TSY) variant, would shift the conformational equilibrium of LeuT towards the inward-open state (Supplementary Discussion and Supplementary Figs 2, 5, 8 and 9). We proceeded to crystallize the LeuT^K(TSY)-6A10 Fab complex in the presence of lipids and solved the structure by molecular replacement at 3.2 Å resolution (Supplementary Table 2 and Supplementary Figs 10 and 11). Electron density for LeuT residues 1–10 was not observed and that for amino acids 11–24 was weak (mean B factor of 158.0 Å² versus 121.2 Å² for the rest of LeuT). The Fab fragment binds on the intracellular side of LeuT^K(TSY) (Supplementary Fig. 11).

LeuT^K(TSY) adopts an inward-open conformation as a consequence of large hinge-like movements within the core domain relative to the scaffold domain, and shifts in extracellular loops (Figs 3a–c). Perhaps the most notable change involves TM1a. Tilted by $\sim 45^\circ$ from its position in the closed state, it protrudes into the predicted location of the membrane. TM6b, in comparison, is rotated away from the central binding site by only 17° . In contrast to the uncoupled movements of TMs 1a and 6b, TMs 1b and 6a tilt by a similar extent (24° and 21° , respectively) towards the scaffold domain, blocking the extracellular pathway. Because TM1 and TM6 do not move as a unit, we superimposed the inward-open and outward-occluded structures using only the scaffold domain, resulting in an r.m.s.d. in $\text{C}\alpha$ positions of 3.0 Å. Helices buttressing TM1 and TM6, namely TM2, TM7 and TM5, also undergo substantial rearrangements but their extracellular and intracellular halves move to significantly different extents (Fig. 3c). Thus, these helices bend, rather than tilt as rigid bodies, facilitated by either a glycine or proline residue located in their midsections. Movement of TM7 causes EL4 to dip down further into the extracellular vestibule, thus closing off the extracellular solvent pathway. To make room for TM6a, TM11 moves away from the centre of the transporter, a direction opposite to that seen in the outward-open state (Fig. 3b).

Access to the substrate-binding site

In the inward-open LeuT structure, formation of a thick extracellular gate cuts off solvent access from the extracellular side while the thick

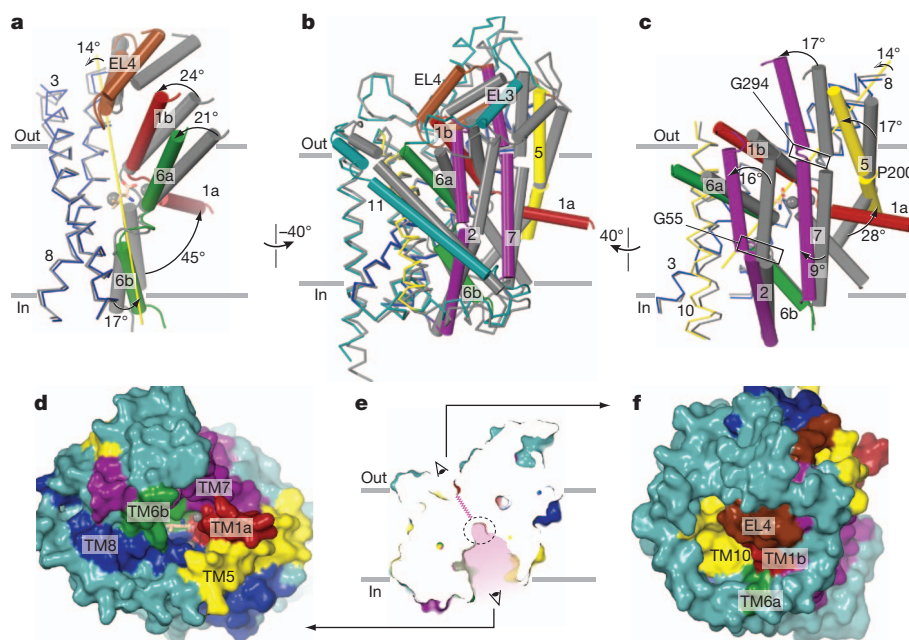


Figure 3 | Inward-open conformation. **a–c**, Superposition of inward-open and outward-occluded state structures using the scaffold domain. The overall changes shown in **b** are divided in two parts, **a** and **c**, for clarity. The extent of rotation for the key TMs between the outward-occluded and inward-open conformations are indicated in **a** and **c**. The axis of rotation of core domain with the exclusion of TM1a is depicted in yellow. **d**, Surface representation of the

inward-open structure looking 'up' into the binding site from the intracellular side, as depicted in **e**. **f**, Surface representation of the inward-open structure showing the elements forming the extracellular thick gate. Colour coding and representations are as in Fig. 1. Leucine and sodium, where shown, are from the outward-occluded Leu-bound structure.

intracellular gate opens, allowing access to the substrate binding site from the inside (Figs 3d–f). Consistent with cysteine accessibility studies on the serotonin (SERT)^{22,31,32} and GABA (GAT-1)³³ transporters, TM1a, TM5 and TM8 line the intracellular cavity. In particular, residues Leu 14 and Ala 19 of TM1a are solvent accessible, in line with the marked increase in reactivity observed for the corresponding residues in SERT in the inward-open conformation²². Accessibility and cross-linking studies also indicate that TM1b and TM6a move closer to TM3 (ref. 34) and, together with EL4, become inaccessible in the absence of sodium^{35–38}. Consistent with its essential role in substrate transport³⁹, EL4 occludes the extracellular pathway by packing tightly against TM1b and TM7 on one side and TM3, TM8 and EL2 on the other side, making extensive contacts that include hydrophobic interactions and a hydrogen bond between Asp 401 (TM10) and Ala 319 (EL4).

The collapse of the extracellular vestibule in the inward-open state is central to our understanding of how therapeutic agents, including TCAs and SSRIs, inhibit NSSs. Large, bulky molecules such as tryptophan¹², the cocaine analogue 2 β -carbomethoxy-3 β -(4-iodophenyl)tropane³¹ and SKF-8997A⁴⁰ arrest LeuT, SERT and GAT-1, respectively, in the outward-facing conformation by blocking collapse of the extracellular vestibule, which in turn precludes opening of the intracellular gate. The inward-open LeuT structure now provides a new template for designing novel therapeutic agents that arrest NSS transporters in the inward-facing conformation. Ibogaine, shown to bind in the intracellular vestibule of SERT⁴¹, is the only known inhibitor of this type.

The closure of the extracellular gate radically changes the nature of the proposed S2 substrate-binding site, which is hypothesized to be occupied by substrate in occluded and inward-facing conformational states^{42,43}. Residues Ile 111 and Leu 400, stated to line the S2 site, are now deeply buried (Supplementary Fig. 12). Although we do see a

small non-protein electron density feature near EL4, it is probably not a substrate molecule, not only because no substrates were included in the protein preparation, but also because the density cannot be well fit by leucine or a related amino acid. We suggest that the density is due to buffer, detergent or several water molecules.

Rupture and formation of gating interactions

The extracellular gate of the inward-open state is closed through interactions between residues of TM1b (Arg 30) and TM10 (Asp 404, Gly 408, Thr 409), EL4 (Ala 319) and TM10 (Asp 401) (Fig. 4a) and TM6a (Asp 240) and TM11 (Tyr 471). The guanidinium group of Arg 30, riding 'on top' of the aromatic ring of Phe 253 (TM6a), makes multiple interactions with TM10 that include a direct salt bridge with Asp 404 and hydrogen bonds to Thr 409 and the carbonyl oxygen of Gly 408. In GAT-1, conservative mutation to Lys of the residue equivalent to Arg 30 severely compromises uptake activity⁴⁴, an outcome in accord with the manner in which interactions of Arg 30 are modulated in the outward-open, outward-occluded and inward-open states of LeuT (Fig. 4a, b).

In contrast to the extracellular gate, a network of interactions within the thick intracellular gate of outward-facing and outward-occluded states is disrupted (Fig. 4b, c). Ionic interactions linking the N terminus and TM1a (Arg 5, Trp 8) to TM6b (Ser 267, Tyr 268) and TM8 (Gln 361, Asp 369) on the intracellular side no longer exist. The splaying apart of TM5, TM7 and TM1a also abrogates interactions between Lys 196 and Thr 10 and between Ser 278 and Arg 11. Consistent with the disruption of these interactions, mutation of residues homologous to Arg 5 or Trp 8 (ref. 45), Tyr 268 (refs 27, 28), Asp 369, Lys 196 and Ser 278 (refs 46, 47) in eukaryotic transporters alters substrate uptake and shifts the conformational equilibrium of the transporter towards an inward-open state.

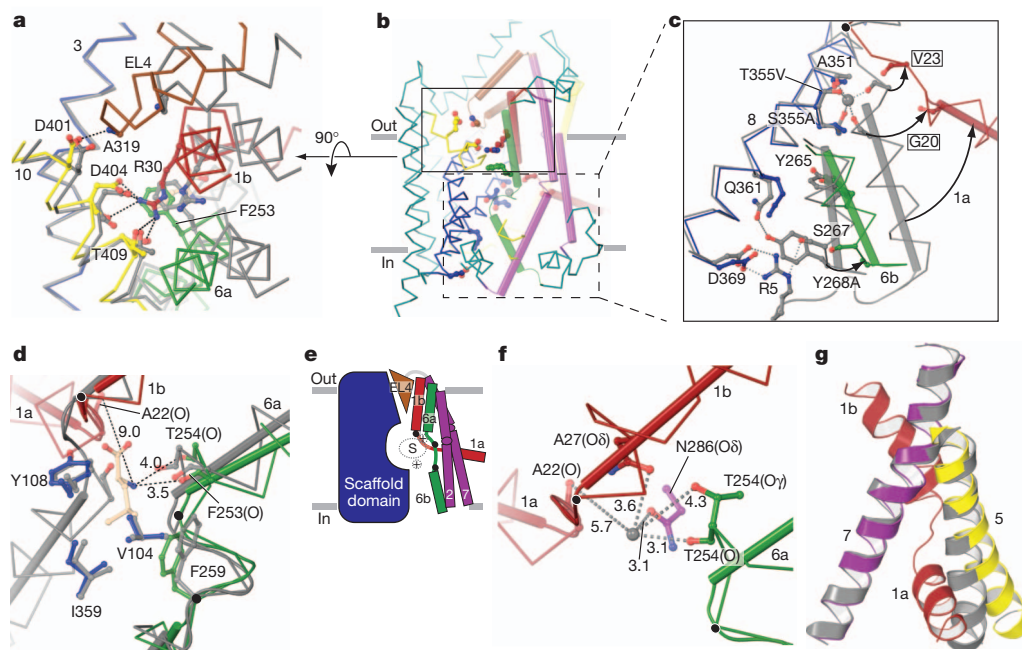


Figure 4 | Changes in gate, substrate and ion site interactions and coupling to helix movements. **a**, Comparison of the extracellular gating interactions in the inward-open and outward-occluded structures. View is from extracellular side. Polar contacts in the inward-open structure are shown as black dashed lines. **b**, Overall view of inward-open structure showing closed extracellular gate (box with solid lines) and open intracellular gate (box with dashed lines). **c**, Comparison of the intracellular gating interactions in the inward-open and outward-occluded structures. Interactions forming the intracellular gate in the outward-occluded state are shown as grey dashed lines. **d**, Changes in the central substrate-binding site. In comparing the outward-occluded and inward-

open structures, A22, T254 and F253 move away from the binding site. Distances were measured relative to leucine from the outward-occluded structure. **e**, A cartoon representing changes in the core domain relative to the scaffold domain and the location of hinges relative to the position of substrate- and ion-binding sites is shown. **f**, Changes in the Na1 site. Distances of the coordinating residues from the sodium ion of the outward-occluded structure are shown. **g**, Superposition of the inward-open and outward-occluded structures using TM7. TM1a of only the inward-open structure is shown for clarity. Colour scheme is as in Fig. 1. Location of TM1 and TM6 hinges are shown as black spheres in c–f.

Perturbation of ion and substrate sites

The location of hinges for the conformational changes in TM1 and TM6 of the inward-open state has profound consequences for the substrate and sodium ion sites. The hinge for TM1a is located at Leu 25, considerably 'above' the Na2 site, leading to separation of residues on TM1a and TM8 that define this site, thus demonstrating how the release of sodium from the Na2 site, the movement of TM1a and the opening of the transporter to the cytoplasm are coupled (Fig. 4c), a mechanism that has also been suggested for the *Vibrio parahaemolyticus* sodium/galactose transporter (vSGLT)⁴⁸. There are two hinges in TM6, Ser 256 for TM6a and Phe 259 for TM6b, while TM1b also pivots at Leu 25. Because these residues are all close to the substrate-binding site but further away from the Na1 site (Fig. 4e), there is minimal perturbation of the former (Fig. 4d) but considerable weakening of the latter (Fig. 4e, f). Thus, although changes in TM1 and TM6 affect residues coordinating the α -amino group of leucine and cause small shifts in the side chains of Phe 253 and Phe 259, residues from TM3 and TM8 that engage the aliphatic moiety of the substrate maintain their positions. Consequently, an important portion of the substrate-binding pocket is retained in the inward-open state, preserving the ability of the transporter to bind substrate under conditions of reversed substrate flux.

Structural principles of transporter mechanism

The pseudo two-fold symmetric relationship of TMs 1–5 and 6–10 together with the organization of TMs 1, 2, 6 and 7 as a four-helix bundle led to the rocking bundle mechanism of transport, in which the core moves as a unit about a rotation axis oriented approximately parallel to the membrane and intersecting the substrate-binding site²². Detailed analysis of the inward-open state, however, indicates that only a portion of the core moves as a unit and that there is not strict adherence to the pseudo two-fold symmetry. The conformational transition from the outward-occluded to the inward-open state is brought about by multiple adjustments in individual TM helices, including the bending of TM2 and TM7 at conserved glycine residues and the independent movements of TMs 1a and 6b. Indeed, the core domains in the inward-open and outward-occluded states align with a r.m.s.d. of 3.5 Å (C α atoms), whereas the scaffold domains align with a r.m.s.d. of 0.7 Å. If TM1a is excluded from consideration, however, the core domain helices superpose with a r.m.s.d. of 0.83 Å (C α atoms), thus demonstrating that a portion of the core helices do reorient as a unit, undergoing a rotation of $\sim 14^\circ$ about an axis passing through Leu 25 and Phe 259 in the plane of the membrane (Fig. 3a, c). The intracellular loop 1 (IL1) and EL4 are also related by the pseudo two-fold symmetry axis, yet whereas EL4 undergoes large relative movements, the position of IL1 is constant.

The structural rearrangement of TM1a in the inward-open conformation of LeuT is different from that seen in Mhp1 (refs 15, 18), in which TM1 moves as a rigid body (Supplementary Fig. 13)²². At present it is unclear whether the extent to which TM1a is seen to tilt away from its position in the occluded state reflects its true position in a native membrane environment. The fact that TM1a is neither involved in crystal contacts nor interacts with the Fab (Supplementary Fig. 11) diminishes the possibility that its position is a crystallization artefact. The weak density of TM1a suggests that it is highly flexible in the detergent/lipid micelles in which LeuT^K(TSY) was crystallized. Indeed, the apparent mobility and large extent of movement of TM1a are in general agreement with findings of single-molecule FRET studies and molecular dynamics simulations^{23,49,50}.

Coupling of binding sites and helix movements

The inward-open structure of LeuT suggests the manner by which changes in the sodium- and substrate-binding pockets are coupled to the larger structural changes that simultaneously open the intracellular side and close the extracellular side. The location of the TM1 hinge at Leu 25 in the inward-open state, at a position extracellular to the

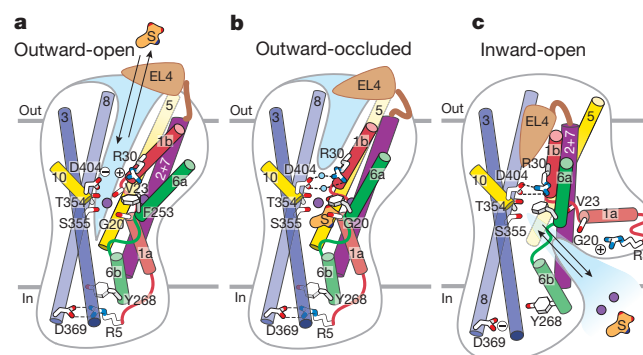


Figure 5 | Schematic of transport in LeuT. a–c, Shown are structural elements and gating residues instrumental to conformational changes associated with the transition from the outward-open (a) to the outward-occluded state (b) and the inward-open state (c). At present there is no crystal structure for an inward-occluded state and thus no schematic is provided.

substrate and sodium sites, is consistent with the observation that in the absence of Na⁺ ions TM1a moves away from the scaffold domain and TM6b, not only initiating conformational changes that open an intracellular pathway, but also resulting in the disruption of the sodium ion sites. We propose that it is energetically unfavourable for TM1a to remain in an intracellular gate 'closed' conformation without the compensating contributions from bound Na⁺. We further suggest that the movement of TM1a initiates a cascade of structural rearrangements that result in closure of the extracellular gate as follows. A change in the position of TM1a requires movement of the intracellular region of TM5 (Fig. 4g). Yet because TM5 lacks the hinge-like regions of TM1 and TM6, the entire helix tilts, 'pushing' TM7 and TM1b and closing the extracellular gate (Fig. 3c). Similar structural adjustments involving TM2 and TM6a also occur, ensuring that the intracellular gate cannot open without simultaneous closure of the extracellular gate.

Mechanism

Crystal structures of LeuT captured in three distinct conformational states show how both local conformational changes and rigid body movements of groups of helices are associated with the transport mechanism (Fig. 5). Opening and closure of the extracellular and intracellular gates exploit hinge-like bending of helices at pivot points within short non-helical regions halfway across the bilayer, together with the rigid body rotation of helical bundles, movements of extracellular loops and flexing of numerous transmembrane helices (Supplementary Movie). General principles emerging from these studies are that local hinge-like movements of transmembrane helices are coupled to the formation and disruption of substrate- and sodium-binding sites, which are translated through nearly rigid body movements of groups of helices and loops into opening and closing of the 'thick' extracellular and intracellular gates. Most importantly, the relative locations of substrate/ion-binding sites and hinge pivots define the extent to which hinge motions perturb the sites. More broadly, these structures demonstrate how the overall conformation and shape of LeuT changes during the transport cycle, thereby providing a general mechanistic framework to understand how substrates, ions, mutations and drugs modulate the conformational equilibria and transport activity of LeuT and related NSS proteins.

METHODS SUMMARY

LeuT mutants were expressed as previously described¹⁰, monoclonal antibodies and Fab fragments were generated by standard methods, and X-ray crystal structures of the Fab complexes were solved by molecular replacement. Final models were obtained by an iterative process of manual model building and refinement against X-ray diffraction data. The functional properties of LeuT mutants were examined using scintillation proximity binding assays and uptake or exchange assays with LeuT reconstituted into proteoliposomes.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information Atomic coordinates and structure factors have been deposited with the Protein Data Bank under accession codes 3TT1, 3TT3 and 3TU0 for the LeuT^K(Y108F)–2B12 complex, the LeuT^K(TSY)–6A10 complex and the LeuT^K(TS) complex with alanine, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to E.G. (gouauxe@ohsu.edu).

METHODS

Purification of LeuT mutants. The site-directed mutants LeuT^K(Y108F), LeuT^K(TS) and LeuT^K(TSY) were produced by polymerase chain reaction (PCR) and subcloned into a pET16b plasmid containing a thrombin-cleavage site and a C-terminal octa-histidine tag. All mutants were verified by DNA sequencing. The resulting proteins were expressed and purified as previously described^{10,25} with the following modifications. To obtain leucine-free transporter, buffers for purification did not contain sodium salts and membranes were washed three times in sodium-free buffer to ensure that no endogenously bound leucine was carried along. LeuT mutants were solubilized with *n*-dodecyl- β -D-maltopyranoside (C₁₂M) and purified by Ni-affinity chromatography. Protein for functional and biochemical assays was further purified by gel filtration in buffer containing C₁₂M.

Monoclonal antibodies and Fab fragments. Mouse monoclonal antibodies against LeuT^K(Y108F) and LeuT^K(TSY) were raised by standard methods using corresponding purified protein in detergent as antigen. Antibodies recognizing a conformational epitope in LeuT^K(Y108F) were selected using FSEC²⁶ and western blot analysis, resulting in the identification of the 2B12 monoclonal antibody. Antibodies specific to the inward-facing mutant LeuT^K(TSY) were selected based on their relative affinities for LeuT^K(TSY) and wild-type LeuT as determined by FSEC. From these screens we isolated the 6A10 monoclonal antibody. All FSEC analysis relied on C-terminal GFP fusions of the LeuT variants. Antibodies were purified from hybridoma supernatants using a SP sepharose cation-exchange column. Fab fragments were generated by papain digestion at 37 °C in 50 mM sodium phosphate pH 7.2, 1 mM EDTA and a papain to monoclonal antibody ratio of 1:20 w/w (2B12) or 1:100 w/w (6A10). Cysteine at 10 mM final concentration was added to the digestion reaction for 6A10. Digestion of 2B12 was stopped with 30 mM iodoacetamide after 4 h, while digestion of 6A10 was quenched by transferring the reaction to 4 °C after 2 h. Fab fragments were purified on a Protein A column, followed by ion-exchange chromatography. DNA encoding the light and heavy chains of 2B12 (IgG2a, κ) and 6A10 (IgG1, κ) Fab fragments were cloned and sequenced from hybridoma cells using rapid amplification of 5' complementary DNA ends (5'-RACE).

Purification of Fab complexes for crystallization. The His-tag of LeuT destined for crystallization was cleaved by thrombin. Transporter was then mixed with excess Fab and the complex was subjected to gel filtration in *n*-octyl- β -D-thiogluco-pyranoside (C₈SG)-containing buffer. The buffer consisted of 20 mM Tris, pH 8.0, 50 mM (LeuT^K(TSY)-Fab) or 100 mM (LeuT^K(Y108F)-Fab) KCl and 12 mM (LeuT^K(TSY)-Fab) or 15 mM (LeuT^K(Y108F)-Fab) C₈SG. For crystallization of LeuT^K(TSY)-Fab complex, the protein was then supplemented with a fivefold molar excess of 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) and incubated at 4 °C for 1 h before removing insoluble lipids by centrifugation. A 55 mM DMPE stock was prepared in 20% DMSO and 80% gel filtration buffer. Protein was concentrated to 3.0–3.2 mg ml⁻¹ (LeuT^K(Y108F)-Fab) or 4.5–5 mg ml⁻¹ (LeuT^K(TSY)-Fab) for crystallization.

Crystallization. Crystallization was carried out by vapour diffusion in hanging drops at 20 °C. LeuT^K(Y108F)-Fab crystals were grown in 40 mM Tris pH 7.5, 23–26% PEG 550 monomethyl ether and 50–100 mM NaCl, and cryo-protected in 35% PEG 550 monomethyl ether. Crystals were allowed to sit in the final cryo-solution for 4–5 h before flash freezing in liquid nitrogen.

Different crystal forms were obtained for the LeuT^K(TSY)-Fab complex in similar crystallization conditions with the best crystals belonging to space group C222₁. These crystals were grown in 100 mM HEPES pH 7.6, 0.1 M Mg(NO₃)₂, 12–14% PEG 1500 and 1.5% w/v trimethylamine *N*-oxide dehydrate in a protein to precipitant ratio of 1:2. LeuT^K(TSY)-Fab crystals were cryo-protected with 15% PEG 1500 and 20% glycerol before flash-freezing.

Crystals of alanine-bound LeuT^K(TS) were obtained from protein purified in 20 mM Tris pH 8.0, 100 mM L-Ala, 200 mM NaCl and 40 mM *n*-octyl- β -D-glucoside (β -OG). Purification and crystallization were carried out as described for wild-type LeuT¹⁰.

Data collection and structure determination. For the LeuT^K(Y108F)-Fab complex, X-ray diffraction data sets for the crystals were indexed, integrated and scaled using HKL2000 and the CCP4 suite of computer programs^{51,52}. The crystals diffract to ~3.1 Å resolution, belong to the space group *P*2₁2₁2₁ and harbour two LeuT^K(Y108F)-Fab complexes per asymmetric unit, giving a Matthews coefficient⁵³ of 3.9 Å Da⁻³. The structure was determined by molecular replacement using the program PHASER⁵⁴. The search probes were the tryptophan-bound LeuT structure (PDB accession 3F3A) and a Fab homology model built using SWISS-MODEL⁵⁵. The model was built using COOT⁵⁶ into 2F_o - F_c maps, cross-validated using simulated annealing composite omit maps and refined with PHENIX⁵⁷ using non-crystallographic symmetry, individual atomic displacement parameters and translation/libration/screw (TLS)⁵⁸ subdomains. Six TLS groups composed of two LeuT molecules, two Fab constant domains and two

Fab variable domains were defined. This iterative model building and refinement procedure yielded a structure with good crystallographic and stereochemical statistics. Regions of weak or no electron density were excluded from the model such that the final structure consists of LeuT residues 5–129 and 135–507, Fab light chain residues 1–215, and Fab heavy chain residues 1–135, 143–219.

For the LeuT^K(TSY)-Fab complex, the crystals diffract to ~3.2 Å resolution and belong to the space group C222₁. There is one complex in the asymmetric unit, resulting in a Matthews coefficient of 3.4 Å Da⁻³. Initial phases were derived from a molecular replacement solution using PHASER⁵⁴ in which a partial structure of leucine-bound LeuT (PDB accession 2A65) and a high-resolution IgG1 Fab structure (PDB accession 1Q9Q) were used as search probes. The partial LeuT search probe was obtained by deleting TMs 1, 6 and 12 from the 2A65 structure. Electron density maps calculated using the 'prime and switch' method⁵⁹ clearly showed the LeuT regions where there were deviations from the 2A65 structure. This initial molecular replacement solution was used as a starting point for automated building using Phenix. Further changes were made to the LeuT portion using COOT⁵⁶. At this stage, electron density for the Fab portion was poor, making it difficult for manual building. A homology model built using PDB accession 1EJO for the light chain and PDB accession 2Z4Q for the heavy chain by PHYRE⁶⁰ resulted in a substantial decrease in the crystallographic *R* factors, yielding *R*_{work} and *R*_{free} values of 0.33 and 0.36, respectively. This model and a 2F_o - F_c map calculated with exclusion of the test set of reflections (*R*_{free} set) were then submitted to a crystallographic refinement server (<http://godzilla.uchicago.edu>). The resulting model had improved stereochemistry and *R*_{work} and *R*_{free} values of 0.29 and 0.32, respectively.

Further improvements to the model were made until the *R*_{work}/*R*_{free} values converged to 0.26/0.30 and structure quality assessed using Molprobity⁶¹ was satisfactory. Throughout the model building procedure, bias-minimized 'prime and switch' maps⁵⁹ and simulated annealing composite omit maps were used. Individual isotropic *B* factors and TLS parameters were refined using 11 TLS groups identified using Phenix⁵⁷: three LeuT domains comprised of residues 11–184, 185–254 and 255–511, four Fab heavy-chain domains involving residues 1–83, 84–140, 141–180 and 181–219, together with residues 1–94, 95–131, 132–177 and 178–215 forming the 4 Fab light-chain domains. The final model consists of LeuT residues 11–511, Fab light-chain residues 1–216, Fab heavy-chain residues 1–102, 104–219, and several detergent molecules.

The changes in helix and domain orientation were analysed using the program Interhlx (K. Yap, University of Toronto) and DynDom, respectively, and molecular figures were prepared using PyMOL.

Saturation binding assays. Dissociation constants of LeuT mutants for leucine were determined by scintillation proximity assays as described previously²⁵ at a protein concentration of 1 μ M in 20 mM Tris-MES pH 7.0, 200 mM NaCl and 1 mM C₁₂M. Non-specific binding was measured in duplicate using 1 mM L-leucine as cold competitor and total binding was measured in triplicate. Data were fit with GraphPad Prism.

Transport time course. LeuT mutants were reconstituted into liposomes as previously described²⁵, at a protein to lipid ratio of 1:100, and loaded with either Buffer 1 (20 mM Tris-MES pH 6.0 and 500 mM KCl) for uptake or Buffer 2 (20 mM Tris-MES pH 6.0, 500 mM NaCl and 10 mM L-leucine) for exchange. Transport was initiated at 27 °C by diluting proteoliposomes to 10 μ g ml⁻¹ into external buffer (20 mM Tris-MES pH 6.0, 500 mM NaCl) containing either 200 nM (for LeuT^K(Y108F)) or 400 nM (for LeuT^K(TS) and LeuT^K(TSY)) or 10 nM [³H]-leucine. Uptake or exchange was followed by quenching 100 μ l samples of the reaction mixture in 1.8 ml of ice-cold Buffer 1 for uptake or Buffer 2 for exchange. Background counts were measured with control reactions performed in the absence of sodium. Reactions were measured in replicates of 3–5 and analysed using GraphPad Prism.

FSEC. FSEC experiments²⁶ were carried out using a Superose 6 10/300 column with the mobile phase consisting of 20 mM Tris, pH 8.0, 1 mM C₁₂M, and either 200–400 mM NaCl and 10 mM leucine or 200 mM KCl for analyses of LeuT in substrate-bound and apo states, respectively. Fab–LeuT complexes were prepared by mixing LeuT and Fab in a molar ratio of 1:2. Elution was followed using tryptophan fluorescence with excitation and emission wavelengths at 280 nm and 335 nm, respectively.

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