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Commentary

Emerging structure–function relationships defining monoamine NSS transporter substrate and ligand affinity

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ABSTRACT

Monoamine transporters are a group of transmembrane neurotransmitter sodium symporter (NSS) transporters that play a crucial role in regulating biogenic monoamine concentrations at peripheral and central synapses. Given the key role played by serotonin, dopamine and noradrenaline in addictive and disease states, structure–function studies have been conducted to help guide the development of improved central nervous system therapeutics. Extensive pharmacological, immunological and biochemical studies, in conjunction with three-dimensional homology modeling, have been performed to structurally and functionally characterise the monoamine transporter substrate permeation pathway, substrate selectivity, and binding sites for ions, substrates and inhibitors at the molecular level. However, only recently has it been possible to start to construct an accurate molecular interaction network for the monoamine transporters and their corresponding substrates and inhibitors. Crystal structures of *Aquifex aeolicus* leucine transporter ($LeuT_{Aa}$), a homologous protein to monoamine transporters that has been experimentally demonstrated to share similar structural folds with monoamine transporters, have been determined in complex with amino acids and inhibitors. The molecular interactions of leucine and tricyclic antidepressants (TCA) has supported many of the predictions based on the mutational studies. Models constructed from $LeuT_{Aa}$ are now allowing a rational approach to further clarify the molecular determinants of NSS transporter–ligand complexes, and potentially the ability to better manipulate drug specificity and affinity. In this review, we compare the structure–function relationships of other SLC6 NSS family transporters with monoamine transporters, and discuss possible mechanisms involved in substrate binding and transport, and modes of inhibition by TCAs.

1. Introduction

Intracellular communication in the central nervous system is driven by the controlled release and reuptake of neurotransmitters at the synapse. Excess accumulation or shortage of monoamine and amino acid neurotransmitters contributes to many psychiatric and neurological disorders, including Parkinson's disease, attention deficit hyperactivity disorder (ADHD) and depression. The extent and duration of action of these released neurotransmitters are tightly regulated by a group of specialised transmembrane neurotransmitter sodium symporter (NSS) transporters localised to neurons and glial cells. The NSS transporters use the Na^+/Cl^- electrochemical gradient to remove neurotransmitters from the synapse against their concentration gradient [1,2]. Being crucial regulators of synaptic transmission in neuronal systems, these NSS transporters have become important targets for therapeutic intervention in a broad range of central nervous system (CNS) disorders.

The NSS transporters can be categorised into two major subclasses, the SLC1 and SLC6 transporters. The SLC1 subclass transports glutamate [3], whereas the larger SLC6 [2] NSS transporter family is involved in the transport of the biogenic monoamines dopamine (DA), noradrenaline (NE) and serotonin (5-HT), and amino acids including γ-aminobutyric acid (GABA) and glycine (Gly) [2]. SLC6 transporters share a number of common structural features, including 12 predicted transmembrane helices

Abbreviations: NSS, neurotransmitter sodium symporter; DAT, dopamine transporter; NET, noradrenaline transporter; SERT, serotonin transporter; GAT, γ-aminobutyric acid transporter; GlyT, glycine transporter; TCA, tricyclic antidepressant; SSRI, selective serotonin reuptake inhibitor; SNRI, serotonin and noradrenaline reuptake inhibitor; NDRI, noradrenaline and dopamine reuptake inhibitor; MAOI, monoamine oxidase inhibitor; $LeuT_{Aa}$, *Aquifex aeolicus* leucine transporter; TMH, transmembrane helix; EL, extracellular loop.

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(TMHs) [4,5], potential for oligomerisation [6–14], multiple N-linked glycosylation sites in the loop between TMH3 and TMH4 [10,15–17], intracellular N- and C-termini [4,5], and several phosphorylation sites [18–20]. These structural features have been shown to influence the transporter function and surface expression and may represent targets for future therapeutic intervention.

SLC6 NSS transporters are the primary targets for addictive substances such as cocaine [21] and amphetamine [22], and their malfunction can cause multiple CNS disorders [23–27]. The clinical relevance of monoamine transporters in psychiatric and neurological disorders has been supported by animal knockout and brain imaging studies. For instance, DAT (dopamine transporter; SLC6A3) [28], NET (noradrenaline transporter; SLC62) [29] and SERT (serotonin transporter; SLC6A4) [30] knockout mice models showed significantly increased concentrations of DA, NE or 5-HT at the synaptic cleft, and a noticeable reduction in the contents of DAT, NET or SERT in several brain regions, reminiscent of brain imaging studies of patients with central nervous system disorders. Alterations have also been reported for the levels of DAT in Parkinson's disease [31], Wilson disease [32], Lesch–Nyhan disease [33] and ADHD [34], NET in orthostatic hypotension [23] and major depression [35], and SERT in impulsive aggressive behaviour [36], Asperger's syndrome [26] and autism [27], highlighting the importance of monoamine transporters in regulating the level of monoamines at the synaptic cleft. In this review, we examine the implications of recent advances in understanding of the structure–function of monoamine transporters derived from the crystal structure of the bacterial leucine transporter [37], and co-crystal structures with amino acids, tricyclic antidepressants [38] and selective serotonin reuptake inhibitors [39] for substrate binding and transport mechanisms, the development of new and improved therapies for NSS transporter related diseases.

2. SLC6 transporters in diseases and current treatments

The most important drugs for the treatment of central nervous system (CNS) disorders include tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), serotonin and noradrenaline reuptake inhibitors (SNRIs), noradrenaline and dopamine reuptake inhibitor (NDRI), and monoamine oxidase inhibitors (MAOIs). These drugs modulate the level of monoamines by either inhibiting their reuptake or degradation [40], supporting the hypothesis that many CNS disorders such as depression result from monoamine deficiency [40–42]. However, the molecular pathways underlying the benefits produced by these compounds remain to be established. For example, it is unclear why the therapeutic effects of these drugs take several weeks or even months to become fully effective, whereas the synaptic effects take place within hours or days [43], although altered gene expression levels of transporters and/or post-synaptic receptors may be involved [44]. Despite the ambiguity in their precise mode of action, monoamine reuptake inhibitors have proven to be clinically effective for the treatment of depression, psychiatric illnesses and neuropathic pain. SSRIs in particular are first-line treatments of anxiety disorders, depressions, premenstrual dysphoric disorders and bulimia nervosa [45], possessing several advantages over the less selective TCAs or the SNRI. In addition to greater safety in overdose [46], SSRIs produce similar levels of efficacy with less side effects compared to that of TCAs and SNRIs by avoiding other pre-synaptic transporters and several post-synaptic receptors including acetylcholine, histamine and adrenergic receptors [47,48]. However, high doses of SSRIs still cause diarrhoea, headache, tremor, insomnia and sexual dysfunction [49]. Thus, there remains a need to develop monoamine transporter inhibitors that have improved specificity for desired transporter (or specific conformational states of a transporter) over other targets (or transporter states).

3. Structure–function relationships of SLC6 transporters

The major role of SLC6 transporters is to transport biogenic amines and amino acids across the cellular membrane using the Na^+/Cl^- electrochemical gradient [1]. Importantly, disruptions in their structure and post-translation modifications impair transporter trafficking and surface expression [4–20]. For decades, researchers have attempted to understand substrate translocation mechanisms and identify ligand–receptor interactions of SLC6 transporters at the molecular level by analysing amino acid sequence differences. However, this is problematic in the absence of detailed knowledge of their three-dimensional (3D) structures since direct and indirect effects cannot easily be differentiated. Early attempts to construct models of the 3D structures of DAT, NET and SERT were based on sequence analysis, an electron density projection map, the crystal structure of *E. coli* Na^+/H^+ antiporter (NhAa) [50], site-direct mutagenesis studies [51–61] and energy calculations [62–65]. These structural models were further refined using the crystal structure of the protein lactose permease symporter (PDB ID 1PV6 [66]) from major facilitator superfamily (MFS), despite limited homology (11–13% protein sequence identity) with SLC6 transporters [65], on the assumption that they have similar structural folds [4,5]. However, docking simulations at these proposed 3D structure models with *S*-citalopram, cocaine, dopamine and *S*-amphetamine revealed a relatively similar putative substrate binding site in DAT, NET and SERT, and well-conserved substrate interacting residues in the permeation pathway formed by transmembrane helices (TMH) 1, 2, 4, 5, 7, 10 and 11 [62–65]. In addition, and a number of likely functionally important residues identified by previous site-direct mutagenesis studies [51–61] could be rationalised by these models. Together, these early studies provided the first insights into the possible substrate binding modes and transport mechanisms of the SLC6 transporters. However, these modeling approaches predicted SLC6 protein structure based solely on the protein sequence, or low resolution (~7 Å) or low homology templates, without proper 3D folding constraints [67].

A remarkable breakthrough in the understanding of structure–function of SLC6 NSS transporters came from the Gouaux laboratory with the determination of the high resolution crystal structure of *Aquifex aeolicus* leucine transporter ($LeuT_{Aa}$), a prokaryotic member of the SLC6 NSS family [37]. Given that the structural fold of proteins from the same protein family are generally highly conserved [68], it is not surprising that $LeuT_{Aa}$ has become the established template for studying both prokaryotic and eukaryotic SLC6 transporters, despite sharing only 20–25% protein sequence identity [69] [Fig. 1]. This novel structure contained a bundle of 12 TMHs formed by a pseudo 2-fold symmetry, where TMH1, TMH3, TMH6 and TMH8 come together to form the leucine binding pocket [37] [Fig. 2A]. The substrate leucine and two Na^+ and one Cl^- ions were also observed in the crystal structure [37] [Fig. 2A]. Out of 22 residues forming the substrate binding site, 18 were direct H bond, ionic or hydrophobic interactions with leucine, and the remaining four coordinated one of the Na^+ ions (Na1) [37]. Importantly, a high degree of sequence conservation was observed between the binding site of $LeuT_{Aa}$ and that of many other SLC6 transporters, particularly DAT, NET and SERT, implying that $LeuT_{Aa}$ is not only a suitable template for SLC6 transporters but may also have a similar substrate binding pocket. The carboxyl group of leucine was found to coordinate Na1, which is located between TMH1 and TMH6 [37]. Interestingly, this coordination was postulated to be contributed by a conserved aspartate in DAT (D79), NET (D75) and SERT (D98) at a position

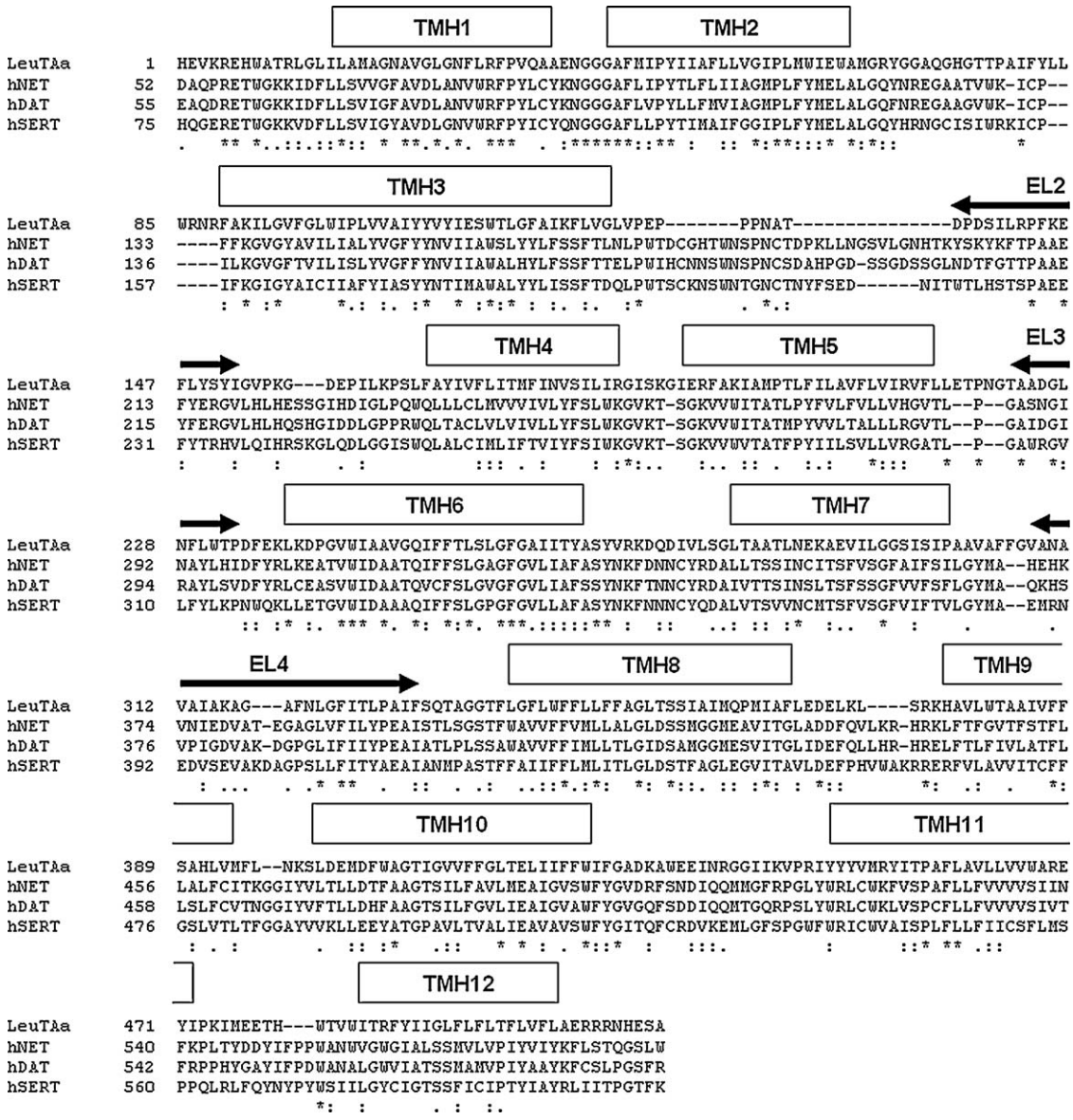

Fig. 1. Sequence alignment of $LeuT_{Aa}$ and human monoamine transporters based on the Beuming et al. [70] alignment, with secondary structure elements derived from $LeuT_{Aa}$ crystal structure [37]. The TMHs are depicted in rectangular boxes and ELs are indicated between the two arrow heads above the corresponding sequences. The conservation of the amino residues are indicated below the sequences as: (*) identical; (:) high conservation; or (.) low conservation. This figure was prepared using program ClustalX2 [118].

equivalent to G24 in $LeuT_{Aa}$, and not from the bound monoamine substrate [37,54,70–72]. In contrast to monoamine transporters, amino acid transporters posses this conserved glycine, suggesting that although SLC6 transporters are likely to share a similar binding pocket, the mode of substrate binding is expected to differ between the amino acid and monoamine transporters. In addition, the residues that have contacts with Na1 (A22, N27, T254 and N286) are found to be highly conserved in both prokaryotic and eukaryotic SLC6 transporters, thus Na1 is proposed to be functionally and structurally important for substrate binding and perhaps may be co-transported with leucine substrate [37,70].

The superimposition of the $LeuT_{Aa}$ Na1 binding sites with that of the crystal structure of the Na^+/galactose symporter from a distant NSS gene family reveals a surprisingly high degree of similarity [73]. This conservation implies that even distant members of NSS family are likely to possess a Na^+ binding site at a similar location. In addition, G20, V23, A351 and S355 in TMH1 and TMH8 have contacts with a second Na^+ ion (Na2) are also well-conserved in eukaryotic SLC6 transporters, revealing the structural importance of the Na2 binding site [37,70]. This hypothesis has been supported by mutational studies in GAT-1 (SLC6A1), where mutating D395 in GAT-1 (equivalent to T354 in $LeuT_{Aa}$) to threonine or serine abolished Na^+-dependent GABA uptake [74]. In addition, the electron density of the leucine and both Na^+ binding sites are the most well-defined regions in the entire $LeuT_{Aa}$ crystal structure [37], indicating central roles for leucine and Na^+ ions in structural stabilisation. This rigid conformation induced upon substrate and Na^+ binding has also been observed in GAT-1 [75,76], Tyt1 [77] and SERT [78]. Despite the fact that $LeuT_{Aa}$ is a Cl^--independent transporter, a bound Cl^- ion was found in between TMH3, EL2 (extracellular loop 2) and TMH4, ~25 Å distant from the leucine binding site [37] [Fig. 2A]. Although the sequence of this Cl^- binding site is not conserved across NSS family members, the construction of NET-SERT chimeras revealed the involvement of EL2 [79] and EL4 [80] in conformational changes during substrate transport, suggesting that part of these local

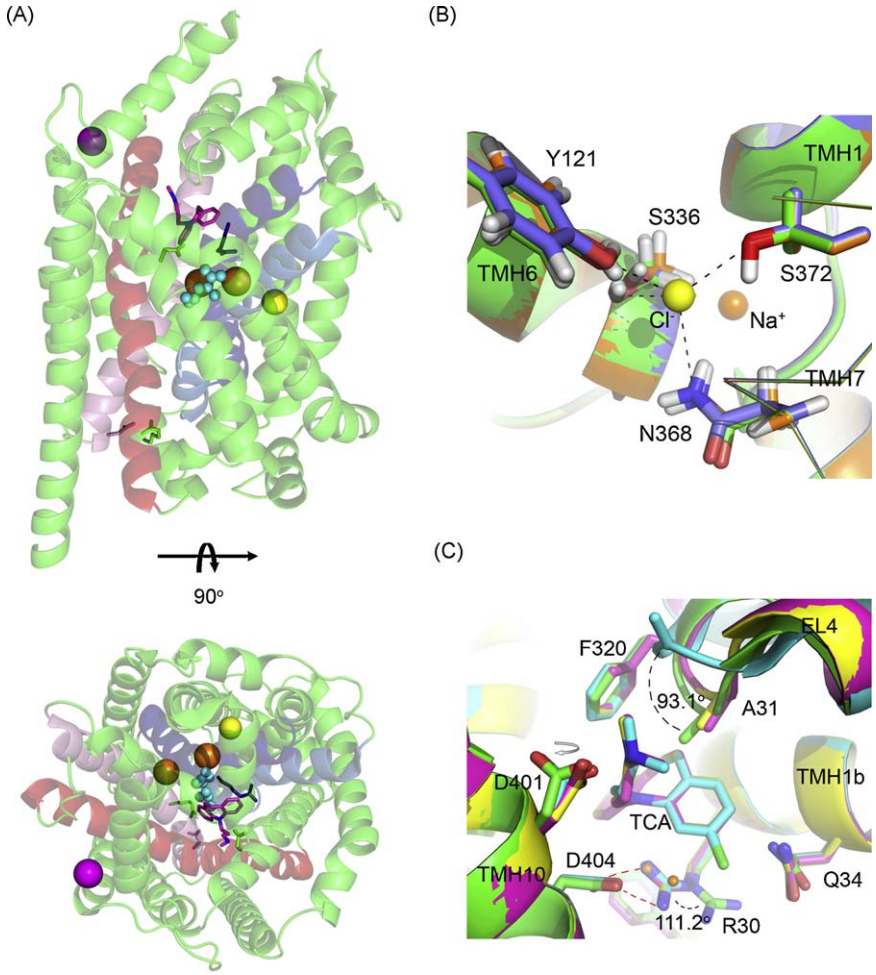

Fig. 2. $LeuT_{Aa}$ ligand and ion binding sites. (A) The positions of TCA [38], leucine [37], Na^+ [37] and Cl^- [37,81,82] in the crystal structure of $LeuT_{Aa}$. The 3D structure is presented with 50% transparency for clarity. The top panel shows the side view of $LeuT_{Aa,}$ and the bottom panel the top view after 90° rotatation. The binding site forming TMHs are coloured: TMH1 deep blue; TMH3 red; TMH6 light blue; TMH8 pink. The Na^+ and Cl^- ions found initially in the $LeuT_{Aa}$ crystal structure are shown as orange and magenta spheres, respectively. The Cl^- ion in the proposed binding site in SERT is superimposed and shown as yellow sphere [81]. The leucine substrate is depicted as small cyan spheres and desipramine in magenta stick view. The residues involved in salt bridge formation are shown in stick view: R30 (dark blue; top right), D404 (green, top left), R5 (green, bottom right) and D369 (pink, bottom left). (B) Models of the predicted Cl^- binding site in SERT (blue), DAT (orange) and NET (green) are shown [81]. The numbering of the interacting amino residues follows that of the SERT sequence and TMH7 is partially removed and presented in thin ribbon for clarity. Side chains of the residues that form interactions (black dashed lines) with Cl^- ion (yellow sphere) are presented in stick view. The Na1 binding site, modeled as equivalent to it location in $LeuT_{Aa}$, is indicated by an orange sphere. The homology models were built using *Modeller* 9v2 [67] followed the published methods by Forrest et al. for consistency [81]. (C) Superimposition of the TCA binding sites of the original $LeuT_{Aa}$ (green, PDB ID 2A65 [37]), $LeuT_{Aa}$-Clomipramine (cyan, PDB ID 2Q6H [38]), $LeuT_{Aa}$-imipramine (magenta, PDB ID 2Q72 [38]) and $LeuT_{Aa}$-desipramine (yellow, PDB ID 2QB4 [89]). All TCAs and interacting residues are presented in stick view. Local structural movements (°) with respect to the original $LeuT_{Aa}$ structure are shown along the black dashed lines and indicated by the curved left arrow. The direct salt bridge between D404 and R30 is indicated by the red dashed lines. The two water molecules displaced upon TCA binding are presented as small orange spheres.

structural changes might be initiated by Cl^- binding. Forrest et al. and Zomot et al. revealed the possible Cl^- binding site and role of Cl^- ion in Cl^--dependent SLC6 transporters [81,82]. This electroneutral Cl^- binding site in SERT was formed by Y121 (TMH2), S336 (TMH6), N368 and S372 (TMH7) [81] [Fig. 2B], which are conserved in GAT-1, DAT, NET and GlyT-1 (SLC6A9) and is ~5 Å distant from Na1 site in $LeuT_{Aa}$ [Fig. 2A and B]. Remarkably, replacement of S372 and N368 in SERT (E290 and N286 in $LeuT_{Aa}$, respectively) by negatively charged residues Glu or Asp, which are conserved in Cl^--independent SLC6 transporters, allow the transport of substrate 5-HT in the absence of Cl^- ion [81]. In a parallel study, Zomot et al. also revealed the significance of the Cl^- ion in substrate transport by mutating E290S in $LeuT_{Aa}$, which reduced leucine binding to 12% in the absence of Cl^- ion, compared to that in its presence, and successfully transformed GAT-1, GAT-4 (SLC6A11) and DAT to Cl^--independent transporters by mutating residues corresponding to E290 in $LeuT_{Aa}$ (S331, S340 and S357, respectively) to a negatively charged residue [82]. Furthermore, recent studies conducted by Tavoulari et al. and Erreger et al. demonstrated that Cl^- ions facilitate a conformational change in SERT [83] and DAT [84] from the open-to-in conformation back to the substrate binding state [Fig. 3]. This interpretation suggests that Cl^- ions favor movements of the TMHs that are essential for efficient substrate turnover by SLC6 transporters [Fig. 3C and D]. The identification and characterisation of these solvent exposed Na^+ and Cl^- binding sites offer prospects for designing novel agonists and potentially antagonists of SLC6 transporters that interfere with the roles of Na^+ and Cl^- ions.

The structures of $LeuT_{Aa}$ co-crystallised with the tricyclic antidepressants (TCAs) clomipramine, imipramine and desipramine reveal all three TCAs share the same binding site formed by R30, Q34, F253, A319, F320, D401 and D404, located approximately 11 Å above the leucine substrate binding site [38] [Fig. 2A and C]. All three TCAs induce conformational movements in the backbone of EL4 that was postulated to accommodate the binding of TCAs [38]. Conformational changes in EL4 were also identified to occur

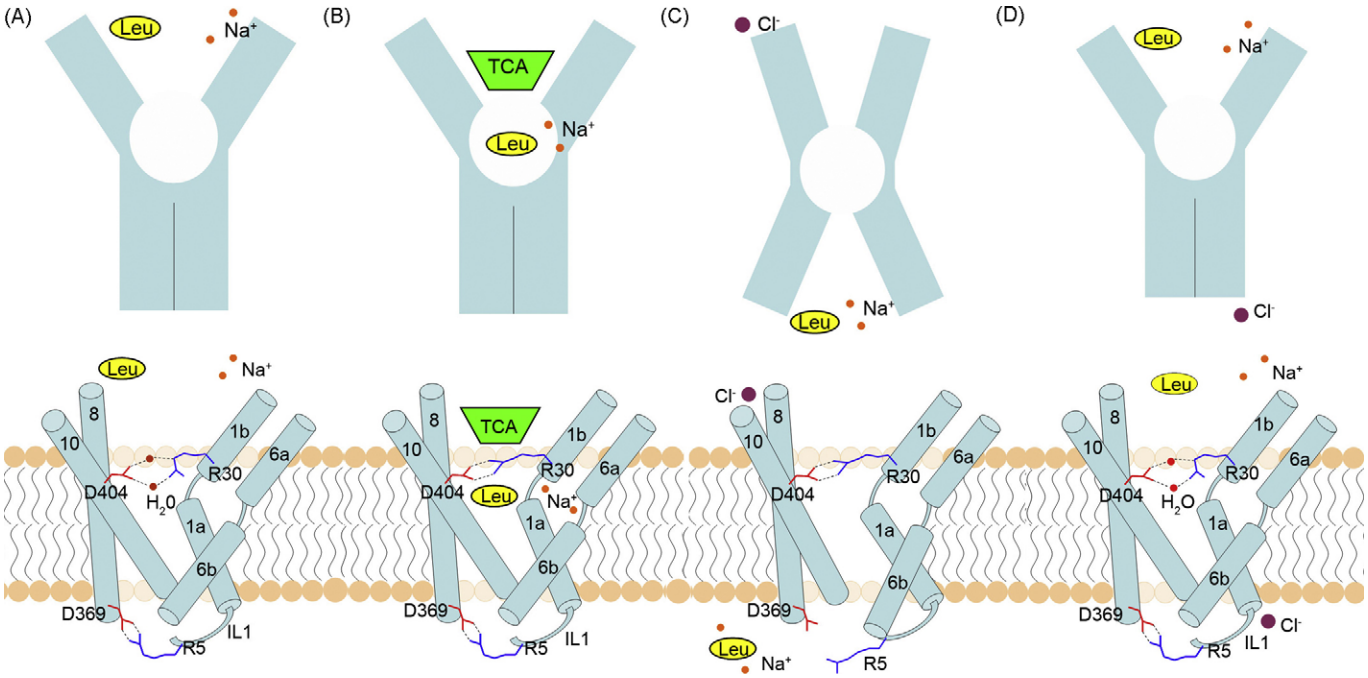

Fig. 3. The proposed substrate transport mechanisms of SLC6 transporters. The top panel shows a schematic representation of the $LeuT_{Aa}$ transporter at different stages of the proposed substrate transport mechanism, while the corresponding bottom panel illustrates the detail interaction involved. The co-crystal structure of $LeuT_{Aa}$ and substrate leucine [37] (A) is proposed to be the substrate binding conformation, while the formation of the extracellular salt bridges between D404 and R30 is proposed to be the gating mechanism that prevents the substrate from being released extracellularly (interestingly, both residues are identical in most of the SLC6 transporters). From the co-crystal structure [38], the formation of the salt bridge observed in the presence of the TCAs (B) may play a role in inhibiting substrate transport by stabilising the transporter structure and TCA binding. Two Cl^- ions, one external (C) and one internal (D) are positioned to help facilitate the transitions between the substrate release and resting conformations, explaining their affect on substrate transport rate [83,84,97–99].

in GAT-1 [85], DAT [86], GlyT1b [87] and SERT [88] during substrate transport. The backbone of the EL4 was found to be tilted upwards by ~93° with respect to the original $LeuT_{Aa}$ structure when bound with clomipramine [38] [Fig. 2C]. However, when imipramine and desipramine were bound to $LeuT_{Aa}$, the backbone of EL4 was shifted up by ~1.4 Å instead [38] [Fig. 2C]. In addition, mutational studies in EL4 implied that desipramine bound to the same site in DAT and SERT as in $LeuT_{Aa}$ [89], further emphasising the high degree of conservation in the TCA binding site across SLC6 transporters. Since TCAs are competitive inhibitors of substrate transport in SERT and NET [90–92], the location of leucine identified in the in $LeuT_{Aa}$ may not be the only monoamine binding site. This observation points to the existence of a multi-step substrate transport cycle in eukaryotic SLC6 transporters, and highlights the difference in substrate transport and recognition mechanisms between the monoamine and amino acid transporters.

Interestingly, comparison of the original $LeuT_{Aa}$ [37] and TCA–$LeuT_{Aa}$ [38] complexes reveals a hypothetical extracellular gate in a closed conformation formed by the conserved R30 in TMH1 and D404 in TMH10 that that is formed when two coordinating water molecules are displaced [Fig. 2C and 3B]. This salt bridge is proposed to have a role in preventing leucine from being released extracellularly by inhibiting local conformational movement in TMH1 [89,93]. A second salt bridge observed in the original $LeuT_{Aa}$ structure, formed intracellularly between R5 and D369 (TMH8), has been proposed to play a role in regulating intracellular gating [37][Figs. 2A and 3]. All four of these charged residues involved in extracellular and intracellular gating have been found to be identical in almost all SLC6 transporters, suggesting that members of the SLC6 family are likely to share the same substrate translocation mechanism.

Co-crystal structures of the SSRIs sertraline, *R*-fluoxetine and *S*-fluoxetine with $LeuT_{Aa}$ showed that all three SSRIs share similar binding modes with the key specificity determinant, the electronegative halogen, shown to interact with identical residues within a halogen binding pocket (HBP; L25, G26, L29, R30, Y108, I111 and F253) [39]. Importantly, the HBP of all three monoamine transporters are identical, except for G100 in hSERT (A77 and A81 in hNET and hDAT), suggesting that SSRIs may bind similarly in human hSERT. This conclusion is supported by site-directed mutagenesis studies in hSERT, where changing I179 (I111 in $LeuT_{Aa}$) to Ala, Phe or Asp increased the IC_{50} for SSRIs by 7–1080 fold [39]. In addition, mutations of A77G in hNET and A81G in hDAT improved SSRI affinity, suggesting that the HBP of $LeuT_{Aa}$ and monoamine transporters is as a key factor regulating antidepressant specificity.

In contrast to other amino acids investigated, tryptophan acts as an inhibitor and traps $LeuT_{Aa}$ in an open-to-out conformation [94]. This conformational changes result in a significantly increased binding site accessible area compared to that of the occluded (Leu bound) conformation, permitting the accommodation of substrate and has been postulated to be the initial substrate binding state [94]. This open-to-out conformation is not observed with any other $LeuT_{Aa}$-amino acid or $LeuT_{Aa}$–TCAs co-crystal structures, indicating that it may be a tryptophan-specific, though potentially drugable, state of $LeuT_{Aa}$.

Together, these observations are helping to unravel the important molecular and structural patterns in common between the $LeuT_{Aa}$ and other SLC6 transporters. The conservation of TCA and SSRI binding sites between $LeuT_{Aa}$ and the clinically important monoamine transporters, as well as the identification of local conformational movements required to accommodate binding, may allow the development of novel compounds and improvements on current existing antidepressants. Establishing the structure of the open-to-in conformation of NSS transporters is required to more fully understand the mechanisms of transport and may reveal additional drugable sites on NSS transporters.

4. Homology modeling of monoamine transporters

Regardless of the low overall protein sequence identity between $LeuT_{Aa}$ and other members of SLC6 NSS family, a high degree of conservation in protein sequence and architecture of the TMHs that form the TCA, leucine and Na^+/Cl^- binding sites are observed. These features indicate that SLC6 transporters are likely to share a similar substrate permeation pathway, implying that the core regions of therapeutically important DAT, NET and SERT can be modeled with a reasonable accuracy using the $LeuT_{Aa}$ structures as a template. Not surprisingly, predictions from previous models of SERT based on the electron density map projection and crystal structure of NhAa and lactose permease [62–65] were significantly improved by using the $LeuT_{Aa}$ structure as the template [95] to the point where accurate structure–function predictions can now be made for SLC6 transporters. For example, studies by Dodd and Christie successfully switched the substrate specificity of creatine transporter (CRT; SLC6A8), a member of SLC6 family to a transporter selective for GABA, by mutating three or four residues in the putative binding sites selected from the sequence alignment of CRT and GAT-1 based on the $LeuT_{Aa}$ structure template [96]. Forrest et al. and Zomot et al. were also able to identify key regulating residues in the potential Cl^- binding site of the Cl^--dependent transporters by generating LeuT-based homology models [81,82]. Tavoulari et al. recently confirmed the structural role of Cl^- ions in modulating the conformational changes in SERT required for different antidepressant binding using modeling, docking simulations and cystine accessibility assays [84], supporting previous studies using MTSEA (2-(Aminoethyl)methanethiosulfonate hydrobromide) and cysteine scanning mutagenesis [97–99] that identified distinct conformational states for SERT, NET and DAT when bound with different inhibitors in the presence of Cl^- ions [97–99]. For example, cocaine binds to all three monoamine transporters in a substrate bound conformation, while ibogaine appears to trap SERT in an open-to-in (cytoplasm open) conformation [98,100] and may potentially facilitate crystallisation of this key conformational state in the transport cycle.

The studies described above experimentally demonstrate that $LeuT_{Aa}$ is a good structural template for the identification of the residues involved in substrate binding activity and selectivity. However, any structural template for proteins with low sequence identity requires careful optimisation of sequence alignment to ensure the accuracy of resulting models. Several studies have attempted to produce the DAT, NET and SERT homology models with different sequence alignment algorithms. A recent study by Indarte et al. attempted to generate homology models of DAT using the sequence alignments proposed by Yamashita et al. and by two other algorithms [72]. This approach revealed conformational differences in the loop between TMH9 and TMH10, although the Cα superimposition of all three models showed extensive overlap in their backbones [72]. The observation by Celik et al. also showed that although models generated using different alignments were similar, variations between the alignments led to differences in the volume of binding sites and in distances between substrate and residues [101]. This suggests the subtle variation in alignment can influence the analysis of substrate recognition. The DAT model built by Indarte et al. applied three different automated docking approaches to identify two likely positions for dopamine and amphetamine binding [72], a deeper binding site was almost identical to that of leucine in $LeuT_{Aa}$ [37], and a shallower binding site (near the TCA binding site in $LeuT_{Aa}$ [38]) proposed to also contribute to substrate translocation [72]. Direct interactions between the substrate and two conserved residues (D79 in TMH1 and V152 in TMH3) identified in earlier mutational studies, were also identified with their model [51,58,102,103].

Since many residues that disrupt substrate and inhibitor binding [104–110] and surface expression [23,111] in SLC6 transporters are located distantly from the substrate binding sites and are poorly conserved, including TMH2, TMH4 and TMH12, these regions are more difficult to model. Another homology modeling study of SLC6 NSS transporters performed by Beuming et al. using a comprehensive alignment strategy revealed residues that are likely to regulate the substrate binding selectivity and transport mechanisms [70]. Topology, interior and lipid-exposed faces of TMHs and secondary structure predictions were utilised to guide and facilitate the alignments of the poorly conserved TMH4, TMH9 and TMH12 as well as EL2 and EL4 [70]. This structure-based alignment significantly increased the sequence identity of the dissimilar regions in contrast to the sequence alignments published earlier [37] and was supported by the experimental data, including side chain accessibility data and metal ion binding site constraints [112]. The homology models of the SLC6 NSS transporters generated using this alignment, in conjunction with the docking experiments using the corresponding substrates, revealed the roles of the non-conserved residues, which formed the bottom of the substrate binding pocket [70]. Here, variation at the position corresponding to F259 in $LeuT_{Aa}$ was found to play a role in complementing the substrates spatially. For instance, the smallest amino acid substrate Gly was found to couple in GlyT1 with the bulkiest residue Trp [70]; conversely the bulkiest amino acid substrates Trp and Tyr were found to bind to TnaT (SLC6A14) [70,113] and Tyt1[70,77] with small residues Val and Gly at this position, respectively. This complementarity may well be involved in regulating the substrate binding selectivity, as the majority of the binding site residues are highly conserved. Another SLC6 NSS homology model study performed by Andersen et al. using the sequence alignment of Beuming et al. constructed a SERT model that predicted the direct involvement of S438 in recognition of SSRI citalopram and TCAs imipramine, clomipramine and amitriptyline [114]. In the study, it was found that a conservative mutation S438T, which was predicted to cause a steric clash only with the inhibitors containing dimethyl on the aminopropyl chain, resulted up to ~175 fold decrease in inhibitory potency for citalopram, clomipramine and imipramine with no significant effect observed for amitriptyline [114]. This direct interaction between the inhibitors and S438 revealed that the TCA and SSRI binding sites overlap the 5-HT binding site, suggesting a different (or additional) substrate binding site to the deep leucine site in $LeuT_{Aa}$ [38,89]. In fact, Shi et al. [115] identified a secondary Leu binding site, which shares the similar binding site residues with TCAs and SSRIs observed in co-crystal structures [38,39], and site-directed mutagenesis, binding and flux experiments suggested a possible competitive inhibition by TCAs and SSRIs in $LeuT_{Aa}$, as found in NET and SERT [90–92]. It was also suggested that both the deep and shallow Leu binding sites are simultaneously occupied, where the secondary Leu (top) acts as a symport-effector facilitating the transport of Na^+ ions and the primary Leu [115]. The presence of multiple substrate binding sites interacting in the transport cycle complicate the interpretation of structure–function relationship of SLC6 transporters [115].

The sequence alignment of Beuming et al. [70] was also used to generate a model of NET that helped identify residues responsible for the uptake of NE and binding of the non-competitve conopeptide inhibitor MrIA, nisoxitine and the TCA desipramine [116]. Out of eight identified residues, four were located at the poorly conserved EL2 (F207, S225) and EL4 (T381, E382), two on TMH10 (D469, D473) and one of each remaining residue located on the distant EL3 (H296) and the intracellular part of TMH2 (L114) [116] [Fig. 4]. From the wide geometry distribution of these key residues, it appears that the effects of these residues on NE and nisoxitine binding are more likely to be structural than functional

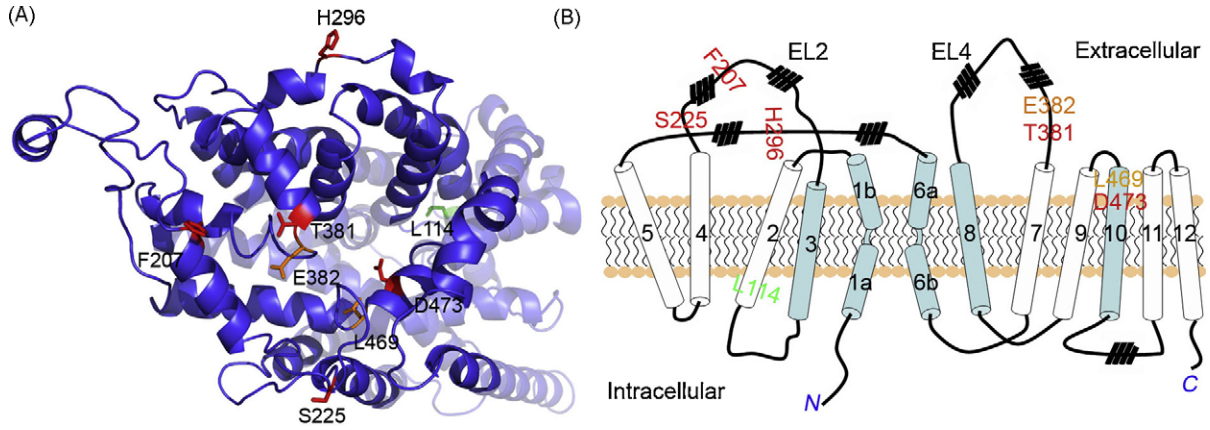

Fig. 4. NET homology model. (A) Extracellular (top) view of the NET homology model [116] and (B) the corresponding topology map. Residues that effect inhibitor affinity and substrate uptake or/and binding are presented in stick view and coloured: red for effects on substrate and/or nisoxitine binding; orange for effects on MrIA affinity; green for effects on desipramine affinity. The transmembrane helices are represented in cylinders, the intra- and extra-cellular helices shown as black bars in the topology map, and the conserved regions coloured cyan. The PDB file of this homology model is available from the website of Institute for Molecular Bioscience, University of Queensland (http://www.imb.uq.edu.au/index.html?page=15581&pid=12142).

[Fig. 4]. Thus, it is reasonable to speculate that the induction of the local structural changes can affect ligand binding and substrate uptake. Notably, L469 located on TMH10 was found to contribute to the MrIA binding activity as L469F mutation caused a reduction in inhibition potency of ~30 fold, while L469A had no effect on MrIA binding, implying that the L469F is likely to cause a steric clash with MrIA or to perturb subsequent local conformational changes required for MrIA binding [116]. This side chain-substrate complementarity is also observed in GlyT [70], TnaT [70,113] and Tyt1 [70,77]. Reconstitution of the entire NET EL2 (residue 166–210) by DAT EL2 had no effect on the desipramine binding [116], indicating that the native conformation of both transporters may be conserved across this region and perhaps contributes to their similar selectivity for DA and NA [117]. These studies highlight the significant improvements in building the homology models using advanced sequence alignment algorithms that may allow successful high throughput virtual compound screening. However, iterative sequence alignment based on the sequence analysis and experimental data, together with new transporter crystal structures, are likely to unlock further details of the structure–function relationships of the SLC6 NSS transporters.

5. Future direction

Structural biology is playing an increasingly important role in drug discovery and design. This approach can provide a clear picture of the myriad of factors contributing to interactions between ligand and receptor protein, and thus allows chemists to rationally design molecules at the molecular level to fit the specific requirements of different ligand binding sites. However, this approach is hampered by the number of available experimental 3D structures, as greater than 60% of the pharmaceutically significant drug targets are membrane proteins and crystallisation of membrane proteins has proved technically challenging. Homology modeling is a powerful computational tool that can supplement structure–function studies of membrane proteins. Despite concerns about discrepancies between homology models and real conformations, studies discussed in this review demonstrate the value of using homology models to dissect and better understand structure–function relationships between substrates and ligands acting at clinically important SLC6 NSS transporters. These homology models, together with docking simulations and mutational studies, are starting to reveal the roles of individual residues identified as being functionally significant by site-directed mutagenesis. Already identified are residues that switch substrate selectivity [70,81,82,96,116] or change transporter orientation and conformation upon ligand binding [38,75–77,113]. Determining the structures of additional SLC6 transporters, including one or more in the open-to-in conformation, will help further elucidate the molecular basis of ligand selectivity and transport, and facilitate drug discovery and development at therapeutically relevant transporters.

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