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X-ray structure of the dopamine transporter in complex with tricyclic antidepressant

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Abstract

Antidepressants targeting Na⁺/Cl[−]-coupled neurotransmitter uptake define a major therapeutic strategy to treat clinical depression and neuropathic pain. However, identifying the molecular interactions that underlie the pharmacological activity of these transport inhibitors and thus the mechanism by which the inhibitors lead to increased synaptic neurotransmitter levels has proven elusive. Here we present the crystal structure of the *Drosophila melanogaster* dopamine transporter (dDAT) at 3.0 Å resolution bound to the tricyclic antidepressant nortriptyline. The transporter is locked in an outward-open conformation with nortriptyline wedged between TMs1/6 and 3/8, blocking the transporter from binding substrate and from isomerizing to an inward facing conformation. While the overall structure of dDAT is similar to that of its prokaryotic relative LeuT, there are multiple distinctions that include a kink in TM12 halfway across the membrane bilayer, a latch-like C-terminal helix that caps the cytoplasmic gate, and a cholesterol molecule wedged within a groove formed by TMs 1a, 5 and 7. Taken together, the dDAT structure reveals the molecular basis for antidepressant action on sodium-coupled neurotransmitter symporters and illuminates critical elements of eukaryotic transporter structure and modulation by lipids, thus expanding our understanding of mechanism and regulation of neurotransmitter uptake at chemical synapses.

Introduction

Chemical neurotransmission involves release of neurotransmitters upon Ca²⁺-induced depolarization of presynaptic nerve cells¹. Upon release into the synaptic cleft, neurotransmitters such as glutamate, dopamine, norepinephrine, serotonin, glycine, and GABA (γ-aminobutyric acid) activate GPCRs and ligand-gated ion channels resulting in excitatory or inhibitory postsynaptic signaling cascades and currents^{1–3}. The widespread and critical roles played by neurotransmitters in both central and peripheral nervous systems necessitate a requirement for strict spatio-temporal control of their levels at neural synapses. The primary mode of neurotransmitter clearance from the synaptic cleft is through

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Author Contributions

A.P., K.W., and E.G. designed the project. A.P. and K.H.W. performed protein purification, crystallography, and biochemical assays. A.P., K.H.W., and E.G. wrote the manuscript.

The coordinates for the structure have been deposited in the Protein Data Bank under the accession code xxxx. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests.

secondary active transporters localized in presynaptic and glial cells that harness ionic gradients, across the cell membrane, to drive the uphill transport of neurotransmitters⁴. This symport process requires both Na⁺ and Cl⁻ ions⁵, which therefore, has led to the solute carrier 6 (SLC6) family of secondary transporters³, to be referred to as neurotransmitter sodium symporters (NSSs)².

Dysregulation of NSS function is associated with several debilitating disorders that include depression², attention deficit hyperactivity disorder (ADHD)⁶, orthostatic intolerance⁷, epilepsy⁸, Parkinson's disease² and infantile parkinsonism dystonia⁹. NSSs are also the primary targets of antidepressants, drugs to treat neuropathic pain, ADHD, anxiety and habit-forming substances of abuse such as cocaine and amphetamines³. Development of antidepressants had a serendipitous beginning in the 1950s¹⁰, followed by the discovery that the tricyclic antidepressant (TCA) imipramine, inhibits norepinephrine reuptake in tissues¹¹. Numerous variants of imipramine, and the subsequent discovery of selective serotonin reuptake inhibitors have revolutionized antidepressant treatment^{12, 13}. To date, inhibition of neurotransmitter uptake remains the most widely used strategy for antidepressant therapy¹², despite numerous side-effects¹⁴.

Gains in our understanding of the molecular mechanisms underlying sodium-coupled transport have benefited from the structures of multiple conformations of LeuT^{15, 16, 17}, a bacterial sodium-coupled amino acid transporter with ~20% sequence identity to the eukaryotic NSSs. Models of eukaryotic NSSs based on LeuT have provided valuable insights into substrate and ion specificities, pharmacology, and transport mechanisms in NSS members^{18, 19}. However, bacterial NSS models fall short of answering questions concerning the elements of NSS structure and function that include the local structure of NSSs in regions that are unrelated to LeuT in amino acid sequence, the determinants of substrate selectivity and the atomic level details of transport inhibition by antidepressants and addictive compounds. Moreover, there is no understanding, at the level of three-dimensional structure, on the role of lipids and post-translational modifications in NSS structure and mechanism.

Here we present a 3.0 Å x-ray crystal structure of the *Drosophila melanogaster* dopamine transporter (dDAT)²⁰ in complex with the TCA nortriptyline. The dDAT has greater than 50% sequence identity with its mammalian counterparts and harbors a pharmacological profile that is a hybrid of the mammalian dopamine (DAT), norepinephrine (NET) and serotonin transporters (SERT), thus rendering dDAT a powerful vehicle to study NSS pharmacology and substrate specificity²⁰. The dDAT structure reveals atomic details of TCA recognition, novel structural elements of NSS protein architecture and suggests a role for cholesterol in the allosteric control of transport in eukaryotic NSS members.

Thermostabilization and crystallization

Wild-type dDAT is labile, loses ligand binding activity upon detergent extraction from the cellular membranes and is refractory to crystallization. To stabilize dDAT for functional characterization, antibody generation and crystallization, we screened single point mutants for ligand binding activity at elevated temperatures²¹, ultimately combining 5 mutations into the construct used for crystallization and structure determination (dDAT_{cryst}; Supplementary Fig. 1). Purified dDAT_{cryst} binds to the high-affinity inhibitor nisoxetine with a K_D of 29 nM (Supplementary Fig. 2a), and the TCA nortriptyline exhibits a K_i of 156 nM (Supplementary Fig. 2b). Unfortunately we were unable to measure the binding of nortriptyline to wild-type dDAT because of its instability. Nortriptyline has a K_i of 18 nM at hSERT and 4.4 nM at hNET²², values that are ~9-fold and ~35-fold lower than that for dDAT_{cryst}. In dopamine uptake measurements with wild-type dDAT and nortriptyline, a

precursor of nortriptyline, inhibits wild-type dDAT transport with a K_i of 30 nM²⁰, whereas the dDAT_{cryst} construct is inactive in transport (Supplementary Fig. 2c, d). Crystallization was further enhanced by the use of a complex with a Fab, resulting in crystals of a dDAT_{cryst}-Fab complex that diffract X-rays to 3.0 Å resolution.

Architecture of dDAT

The structure of dDAT_{cryst} bound to nortriptyline exhibits an outward-open conformation whereby the antidepressant is bound in a cavity halfway across the membrane bilayer and accessible to solvent from only the extracellular side of the membrane (Fig. 1). The transporter displays an overall LeuT-like fold with 12 transmembrane (TM) helices where helices 1–5 and 6–10 are related by inherent pseudosymmetry, akin to LeuT¹⁵ (Supplementary Fig. 3). Residues in TM1 and TM6 make numerous interactions with the ligand and ions via non helical, hinge-like regions at the approximate mid-points of these TMs, connecting the bonding networks of all three ions with the inhibitor. Residues at the bend in TM3 contribute to the hydrophobic pocket that cradles the tricyclic moiety of the ligand, which lays approximately perpendicular to the TMs, mimicking a wedge separating the jaws of a vise. One cholesterol molecule is located in a groove between TM5 and TM7 and poised to modulate the movement of TM1a that occurs during the transport cycle (Fig. 1a)¹⁷.

The primary binding site accommodates nortriptyline but cannot adopt the subsequent helical movements of TMs 1b and 6a required to form the occluded state. Using LeuT for comparison, the occluded state of LeuT is formed in the presence of sodium and leucine substrate, but not in the presence of tryptophan, which binds to the primary site, comparable to the TCA in the context of dDAT_{cryst}. We propose that both tryptophan and TCA stabilize the outward-open conformations of LeuT and dDAT_{cryst}, respectively, by targeting the primary binding site and sterically blocking the extracellular domains of the transporter, preventing the extracellular gate from closing and thus acting by way of a foot-in-the-door mechanism (Supplementary Fig. 4a, b; Supplementary Table 2).

Whereas the core of dDAT_{cryst} closely resembles that of LeuT, the periphery of dDAT_{cryst} exhibits several features distinct from LeuT and important for neurotransmitter transport and cellular localization. In TM12, a kink in the center at Pro 572 causes the second half of the helix to turn away from the transporter, indicating that the dimerization interface of LeuT is not the same as potential oligomerization interfaces of eukaryotic NSSs (Fig. 1a and b, Supplementary Fig. 5). While previous studies indicate that NSSs oligomerize^{23,24}, dDAT_{cryst} is monomeric in detergent micelles and in the crystal lattice (Supplementary Fig. 6), thus suggesting that a membrane bilayer or additional molecules may be required for NSS assembly. The variable EL2 region has numerous predicted *N*-linked glycosylation sites²⁵ and one disulfide bond²⁶, modifications that play critical roles in proper trafficking of NSSs to the plasma membrane³. The strictly conserved disulfide linkage²⁶ was observed in the structure between two conserved cysteines, Cys 148 and Cys 157 (Supplementary Fig. 7). In the crystal, EL2 plays a central role in lattice contacts, packing against a neighboring Fab with an 870 Å² interface (Supplementary Fig. 6). Because 43 residues were deleted from EL2 in the dDAT_{cryst} construct, further studies are required to determine the role of the full-length EL2 in transporter structure and function (Supplementary Fig. 8b). Together with EL2, EL4 harbors a Zn²⁺-binding site in mammalian DATs that modulates transport²⁷. The equivalent residues in dDAT_{cryst} are within a Ca-Ca distance of 10 Å, yet because their identities are Glu 161, Leu 374, and Ala 395, they do not form a high affinity zinc binding site in dDAT_{cryst}.

Tricyclic antidepressant binding site

Unambiguous density for nortriptyline in dDAT_{cryst} was observed in the primary site, approximately halfway across the membrane bilayer (Fig. 2a). In accordance with previous chimeric studies, swapping TM regions between NET and DAT²⁸, the drug-binding site is surrounded primarily by helices 1, 3, 6 and 8 in a region equivalent to the substrate binding pocket of LeuT¹⁵, and in close proximity to the densities for sodium and chloride ions (Fig. 2a). The dibenzocycloheptene ring of nortriptyline is oriented as a saddle, curving around the central region of TM3 and engaging in hydrophobic interactions with Val 120, Tyr 124 and Ala 117 (Fig. 2b). Val 120 is extensively conserved and faces the cycloheptene ring (Supplementary Fig. 4a), and replacement of the corresponding Ile 172 in human SERT with larger substitutions such as methionine markedly reduce affinity towards most NSS inhibitors¹⁹. This location was previously found in human SERT to be protected from crosslinking agents in the presence of inhibitor or substrate²⁹. Phe 325 in TM6b forms a π -stack with one of the benzyl groups of nortriptyline. Residues Gly 425 (TM8) and Ala 479 (TM10) also interact with the tricyclic group of the drug. The *N*-methylpropylamine group of the drug extends across the width of the drug binding site and prevents TMs 1b and 6a from closing the extracellular gate, 'above' the drug. The amine group forms a hydrogen bond with the main chain carbonyl of Phe 43 and a cation- π interaction with the side chain of Phe 43 (Fig. 2b). Interestingly, residues equivalent to Val 120 and Phe 43 (Ile 172 and Tyr 95) in SERT are necessary for interactions with antidepressants³⁰.

The biogenic amine transporters harbor a crucial aspartate residue in TM1 and in the dDAT_{cryst} structure we see how Asp 46 substitutes for the absence of the carboxylate group in biogenic amines as compared to amino acid substrates transported by LeuT, GATs and GlyT (Supplementary Fig. 8a)¹⁵. The side chain of Asp 46 forms a hydrogen bond with the hydroxyl of Tyr 124, which is equivalent to Tyr 108 in LeuT, which plays a role in substrate recognition³¹ (Fig. 2c). Mutations at this aspartate result in substantial losses in transport activity and reduced binding affinities for cocaine³². Ser 421 (TM8), which coordinates a sodium ion at site 2, is within 3.5 Å to the propylamine group of the TCA and also forms a hydrogen bond with the carbonyl of Phe 43. Ser 421 therefore participates in a network of hydrogen bonds that interconnects nortriptyline with the Na2 site and was also found to be crucial for high affinity recognition of antidepressants by human SERT³³.

The *N*-methyl group of nortriptyline is 3.2 Å away from the main chain carbonyl of Phe 319 and sterically prevents Phe 319 and TM6a from closing the extracellular gate above the drug, thereby stabilizing the outward-open state of the transporter. Phe 319 is the equivalent of Phe 253 in LeuT, which gates the substrate binding pocket (Fig. 2c)^{15–17}. The relative position of Phe 319 is markedly different from Phe 253 in the substrate-bound, occluded structure of LeuT, and instead resembles the positions of Phe 253 in the substrate-free and inhibitor-bound structures. To address the question of whether nortriptyline could bind to dDAT_{cryst} in a LeuT-like, occluded conformation, we superimposed dDAT_{cryst} onto the occluded state of LeuT and found that Phe 319 and Phe 325 would clash with the dibenzocycloheptene ring of the TCA (Fig. 2c, Supplementary Fig. 4c). Identification of nortriptyline bound in the substrate binding pocket of dDAT_{cryst} provides the first structural evidence that TCA antidepressants inhibit neurotransmitter transporters by preventing substrate binding and stabilizing the outward open conformation^{18,19,34}. The dDAT_{cryst}-NTT complex, together with the LeuBAT-antidepressant complexes³⁵, conclusively demonstrate that antidepressants inhibit NSSs by acting at the primary or S1 site, in stark contrast to how TCAs inhibit LeuT via a non competitive mechanism³⁶ by binding within the extracellular vestibule^{36–38}.

Ion-binding sites

Locations of ions essential for transport could be identified in dDAT_{cryst} with electron densities ($>4.0 \sigma$) at three locations near the non helical hinge-like regions of TMs 1 and 6, and close to the TCA. Densities at the two sites coincided exactly with Na1 and Na2 sites identified in LeuT (Fig. 3a, b)¹⁵. A chloride ion was positioned at the third position of high omit density nestled in between TMs 2, 6 and 7 and close to Na1 (Fig. 3a). Placing ions in the omit densities during model building led to a concomitant loss of *Fo-Fc* density during refinement. The atomic displacement factors of the ions matched the *B*-values of surrounding atoms. The sodium at site 1 is located $\sim 5.2 \text{ \AA}$ away from the amino group of nortriptyline and is coordinated with an octahedral geometry by side chain oxygens of Asn 49, Ser 320, Asn 352 and main chain carbonyls of Ala 44 and Ser 320 (Fig. 3a, Supplementary Fig. 8a). Interestingly, the sodium at Na1 is also coordinated by one water molecule which in turn is within hydrogen bonding distance to Asp 46, thus showing that the Asp in TM1 indirectly participates in the sodium ion coordination. The mean ion coordinating distances ($2.7 \text{ \AA}$) at this site are longer than the distances ($2.42 \text{ \AA}$) reported for Na^+ ions in solution but shorter than the distances reported for K^+ ions ($2.84 \text{ \AA}$; Supplementary Table 3)³⁹.

The chloride ion is located $5.0 \text{ \AA}$ away from the Na1 site at a position previously identified by computational and mutational studies based on LeuT⁴⁰ and GATs⁴¹. A recent structural study of a chloride-dependent E290S mutant of LeuT also identified a chloride ion at this location⁴². Chloride is coordinated in a tetrahedral fashion through residues in TM6 (Ser 320, Gln 316), TM7 (Ser 356) and TM2 (Tyr 69) (Fig. 3a). Interestingly, the hydroxyl group of Ser 320 bridges the Na1 and Cl^- sites and is positioned to interact with both ions. The mean ion-ligand distances at the Cl^- site are $3.0 \text{ \AA}$ (Supplementary Table 3) and the *B*-factors of surrounding atoms are similar to that of chloride, supporting the placement of chloride at this site.

The sodium at the Na2 site is located 'below' the plane of the drug toward the cytoplasmic face, in between TMs 1 and 8, and is coordinated in a trigonal bipyramidal fashion by main chain carbonyls from Gly 42 (TM1a), Val 45 (TM1-hinge), Leu 417 (TM8) and the side chain oxygens from Ser 421 and Asp 420 (TM8) (Fig. 3b). The mean ion-oxygen distances are $2.4 \text{ \AA}$, in line with reported values for sodium coordination in solution (Supplementary Table 3). While the interconnected network of interactions between TMs 1, 6, nortriptyline, sodium, and chloride provides a structure-based mechanism for the coupling of ion and inhibitor binding⁴³, we do not have a comprehensive understanding of the ion dependence of inhibitor binding in NSSs.

Cholesterol binding site

A cholesterol molecule is lodged in a trough-shaped cavity bordered by TM5, TM7, and TM1a at a depth equivalent to the inner leaflet of the membrane (Fig. 4a). Branched aliphatic residues are primarily involved in forming the protein-cholesterol interface ($357 \text{ \AA}^2$), thus allowing cholesterol to bury $\sim 57\%$ of its solvent accessible surface area. *Fo-Fc* density for this site clearly demarcated the orientation of the isooctyl group of cholesterol anchored at the junction of TMs 5 and 7 by residues Leu 276, Leu 277, and Ile 358. The β -face of the sterol ring primarily faces residues Tyr 273, Leu 270 and Trp 266 in TM5 and also interacts with residues Val 34, Leu 37, Leu 38, and Ile 41 on TM1a. The α -face of cholesterol interfaces with residues Leu 347 and Ile 351 in TM7.

Cholesterol plays an important role in modulating the function of NSS members^{44,45}, stabilizing an outward-open state of DAT with a concomitant increase in B_{max} for cocaine⁴⁶.

In LeuT, TM1a undergoes a large conformational change upon transition from the outward-facing open and occluded states to the inward-open state¹⁷. If a similar conformational change were to occur in dDAT, it would entirely disrupt the cholesterol site (Fig. 4b). We hypothesize that one mechanism for the action of cholesterol on dDAT is that by occupying its binding site in the outward-open, inhibitor-bound state, cholesterol stabilizes the outward-open conformation of the transporter⁴⁶.

Extracellular and cytoplasmic gates

The ion and ligand binding sites in dDAT_{cryst} are accessible to solvent from the extracellular face due to the open gate above the primary binding pocket. The distance between Tyr 124 of TM3 and Phe 319 in TM6a is 10 Å, whereas in the substrate-bound occluded state of LeuT the corresponding distance is half as long (Fig. 5a, b). Similarly, the 10 Å separation between Arg 52 on TM1b and Asp 475 on TM10 renders the primary binding site accessible to extracellular solution. The steric bulk of the tricyclic moiety combined with the extended *N*-methylpropylamine chain of nortriptyline prevents both TM1b and TM6a from approaching TM3 and TM8 to cap the putative substrate pocket and close the gate.

In contrast to the extracellular gate, extensive polar interactions at the intracellular face of the transporter form a thick barrier of ~24 Å between the ligand and ion pockets and solvent to keep the cytoplasmic gate shut. At the cytoplasmic face of the transporter, the indole nitrogen of Trp 30 caps the carbonyl oxygen of Tyr 331 in TM6b, and Arg 27 forms a salt bridge to Asp 435 of TM8 (Fig. 5c). Arg 27, Trp 30, and Asp 435 are strictly conserved in NSS orthologs and LeuT, suggesting that these intracellular gate interactions are general and important facets of the transport mechanism for this family of sodium symporters^{47,48}. Tyr 334, the residue corresponding to Tyr 335 in human DAT, was previously shown to be responsible for shifting the conformational equilibrium of the dopamine transporter towards an inward-open state⁴⁹.

C-terminal latch

Two novel attributes at the C-terminus of dDAT_{cryst} were immediately evident from the structure. Helix 12b is shifted by 22° in comparison to its position in LeuT, resulting in the exposure of TM3 to solvent and lipid (Supplementary Fig. 5). Pro 572, conserved in most eukaryotic NSS members, is likely at the root of this kink, and thus plays an important role orienting the second half of the helix away from the rest of the transporter. The hairpin between TM12b and the intracellular C-terminus of dDAT_{cryst} is stabilized by hydrogen-bonding between the ε-nitrogen of Arg 589 and carbonyl oxygen of Thr 582 (Fig. 5d). The second feature is the C-terminal helix, which contains 2½ turns from residues 586 to 595, where several hydrogen bonds and a p-cation interaction between Trp 597 and Arg 101 restrain this C-terminal helix near IL1 at the cytoplasmic face of dDAT_{cryst}. Although sequence conservation within TM12 and the C-terminus is rather low across NSS orthologs, Gly 584 is strictly conserved and only Lys or Arg is present at the position equivalent to Arg 589 of dDAT_{cryst}, suggesting that the redirection of the C-terminus in the structure is a conserved feature with functional relevance. Studies of human DAT have identified the region following TM12 to contain sites for protein kinase C-mediated endocytic trafficking^{3,50}, and it is plausible that phosphorylation may alter the conformation or accessibility of the C-terminus, allowing it to interact with cellular machinery for internalization. We also note that latch participates in interactions with IL1, which in turn interacts with TM1a, thus suggesting that the C-terminal latch may modulate transporter activity.

Conclusion

The structure of dDAT_{cryst} captures the transporter in an inhibitor-bound, outward-open conformation. The TCA nortriptyline targets the primary substrate site and stabilizes the open conformation by sterically preventing closure of the extracellular gate (Fig. 6a). One chloride and two sodium ions are located adjacent to the ligand, suggesting that the binding of ions and inhibitor are directly coupled. A cholesterol molecule bound to a crevice flanking TM1a likely stabilizes the outward-open, inhibitor-bound conformation (Fig. 6b). The structure reveals a C-terminal latch that makes extensive interactions with the cytoplasmic face of the transporter, proximal to the cytoplasmic gate, and thus in a position to modulate transport activity. Taken together, the structure of a eukaryotic dopamine transporter reveals novel insights into antidepressant recognition and structural elements implicated in the regulation of neurotransmitter transport, providing a foundation for drug design strategies.

Methods summary

The dDAT_{cryst} construct (Supplementary Fig. 1) was expressed in virus-infected mammalian cells and purified by affinity and size-exclusion chromatography. Fab 9D5 was added prior to crystallization along with nortriptyline (1mM) at a DAT:Fab molar ratio of 1:1.1 and concentrated down to 3 mg/ml. Crystals of the complex were obtained in the presence of 100 mM glycine pH 9 and 38% PEG 350 MME. The structure was solved by molecular replacement using a poly-alanine model of LeuT (PDB id. 3F3A) and an ensemble of Fab variable and constant domains. Data processing, model building, and refinement were performed using standard crystallographic software (Supplementary Table 1).

Methods

Screening, construct optimization and protein expression

The dDAT was selected as a promising candidate for structural studies after screening multiple orthologues of DATs and NETs by fluorescence-detection size-exclusion chromatography (FSEC)⁵¹. In addition, FSEC was employed to screen other parameters such as detergent efficacy, thermostability, lipid effects, tertiary epitope-specific monoclonal antibodies and sample homogeneity following purification. The DNA encoding the *Drosophila melanogaster* DAT was provided by Susan Amara and was modified by removal of the first 20 amino acids (D1–20), by a deletion in EL2 (D164–206) and by point mutations to enhance thermostability (V74A V275A V311A L415A G538L) by PCR-based methods. This modified dDAT sequence, deemed dDAT_{cryst}, was fused to a C-terminal GFP-His₈ tag with a thrombin cleavage site (LVPRGS) in place of residues 602–607. dDAT_{cryst}-GFP-His₈ was produced by virus-mediated expression in mammalian cells^{52–54}.

Antibody production

Monoclonal antibodies against dDAT_{cryst} were raised by Dan Cawley (Vector and Gene Therapy Institute, OHSU) using standard methods. Antibodies were screened by fluorescence-based size-exclusion chromatography and western blot to select clones that recognized natively folded dDAT_{cryst} protein. Sequencing of Fab regions was performed on mouse hybridoma cells (Fusion Antibodies Ltd.) and on the intact antibody protein by Edman degradation (Mary Ann Gawinowicz, Columbia University). Antibody was purified from hybridoma supernatant using 4-mercapto-ethyl-pyridine resin. Fab protein was generated by papain cleavage of full-length antibody, followed by F_c capture on Protein A resin and cation exchange. Fab was stored in 20 mM sodium acetate pH 5, 250 mM NaCl, and 10% glycerol.

Purification of dDAT_{cryst}

Membranes were solubilized in TBS (20 mM Tris pH 8, 150 mM NaCl) containing n-dodecyl- β -D-maltoside (DDM) at a w/w ratio of 0.1g detergent per 1g membrane. The detergent-soluble fraction was incubated with cobalt-charged metal ion affinity resin, and dDAT_{cryst}-GFP-His₈ was eluted with 100 mM imidazole in 20 mM Tris pH 8, 300 mM NaCl, 5% glycerol, 14 μ M lipids (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC): 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE): 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (POPG) at a weight ratio of 3:1:1), 1 mM DDM, and 0.1 mM CHS. After thrombin digestion to remove the GFP-his₈ tag, dDAT_{cryst} was isolated by size-exclusion chromatography in 20 mM Tris pH 8, 100 mM NaCl, 5% glycerol, 14 μ M POPE, 4 mM decyl-maltoside, and 0.1 mM cholesterol hemisuccinate. The purified dDAT_{cryst} protein was mixed with Fab 9D5 at a molar ratio of 1:1.1 and used for crystallization trials at 3 mg/ml in the presence of 1 mM nortriptyline.

Crystallization

Crystals grew in 100 mM glycine pH 9 and 38% PEG 350 MME using a drop ratio of 1 μ l protein and 0.5 μ l reservoir solution by hanging drop vapor diffusion. Initial crystals appeared at 4°C after 2 days, reaching full size after 7 days. Crystals were flash frozen in liquid N₂ directly and used for X-ray diffraction data collection.

Structure determination

X-ray data were collected at the Advanced Photon Source (Argonne National Laboratory, beamline 24-ID-C). Data were indexed, integrated, and scaled using HKL2000⁵⁵ (Supplementary Table 1). The structure was solved using molecular replacement, with ensembles of constant (constant domains of heavy and light chains as one set) and variable domains (variable domains of heavy and light chains as a second set) of Fab coordinates in the PDB along with a poly alanine model of LeuT (PDB id. 3F3A). A multi-model search was done using PHASER⁵⁶. Initial phases were improved by iterative steps of manual model building, refinement and maximum likelihood density modification using COOT⁵⁷, PHENIX Refine⁵⁸ and PHENIX Phase and Build⁵⁹, respectively. Multiple rounds of refinement led to the placement of a majority of main chain and side chain atoms for both the Fab and dDAT_{cryst}. The structure was refined to acceptable R-factors (Supplementary Table 1) with residues 20–24 and 600–605 in dDAT_{cryst}, and 135–138 in the heavy chain unmodeled due to poor density. Nortriptyline, ions and cholesterol molecules were placed into $F_o - F_c$ density contoured at 2s or greater in the putative substrate pocket, ion sites, and at the periphery of the transporter. Stereochemistry was evaluated using MolProbity⁶⁰.

Ligand binding and uptake measurements

Scintillation-proximity assays using transporter solubilized in detergent⁶¹ were carried out using copper yttrium silicate (Cu-YSi) beads (Perkin Elmer) at 0.5 mg/ml, 30 nM ³H-labeled nisoxetine (1:9 ³H: ¹H), and 10 nM dDAT_{cryst}-GFP-His₈ protein in the same buffer as that used for size-exclusion chromatography. Unlabeled nortriptyline was used as the competitor ligand. Assay plates were read using a MicroBeta TriLux 1450 LSC & Luminescence counter. Data were fitted using a standard single site competition equation, and K_i values were calculated from the IC₅₀ values using the Cheng-Prusoff equation.

Uptake assays were performed using human embryonic kidney (HEK 293) cells expressing respective mutant constructs. Cells were resuspended in 10 μ M ³H-dopamine (1:49 ³H: ¹H) containing uptake buffer made with 25 mM Hepes-Tris, pH 7.1, 130 mM NaCl, 1 mM MgSO₄, 5 mM KCl, 1 mM CaCl₂, 5 mM D-glucose and 1 mM L-ascorbic acid⁶². Control samples were preincubated with 10 μ M cold desipramine prior to addition of label. Assays

were quenched with cold uptake buffer containing 1 μ M desipramine after 10 min, cells were washed twice with cold uptake buffer and activity was measured from solubilized cells by scintillation counting. Data were plotted using Origin 7.0.

Thermostability screening of dDAT_{cryst}

Sites for mutagenesis were selected based on model of dDAT built on the template of LeuT and residues were altered to Ala, Leu, or Phe²¹. Individual mutants along with the wild-type construct were transfected into HEK cells and kept in culture for 48 hours, then tested for binding activity after detergent solubilization. Samples were split and one part was kept at 4°C, and the other portion of lysate heated at 40°C for 10 minutes. ³H-nisoxetine was added prior to heating to select for mutants that stabilize an inhibitor-bound state of the transporter. Scintillation proximity assay was used to monitor activity in a high-throughput format. Mutants that consistently had an increased melting temperature (*T_m*) compared to wild-type (*T_m* = 35° C) were chosen and pooled into one construct, which yielded a 5 mutant construct with a *T_m* of ~60° C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Architecture of dDAT_{cryst}

a, Structure of dDAT_{cryst} viewed parallel to membrane. Nortriptyline, sodium ions, a chloride ion, and a cholesterol molecule are shown in sphere representation in magenta, purple, green, and yellow, respectively. **b**, View of dDAT_{cryst} from the extracellular face. **c**, Surface representation showing that ligand and ion binding sites are accessible from the extracellular vestibule. Nortriptyline and TMs 1, 3, 6, and 8 are colored as in panel a.

Figure 2. Antidepressant binding site

a, Overall view of the nortriptyline-bound dDAT_{cryst} *Fo-Fc* densities (blue mesh) for drug and ions are contoured at σ levels of 2.5 and 3.0, respectively. **b**, Close-up view of the drug binding pocket. Na⁺ and Cl⁻ ions are shown as spheres. Nortriptyline is represented as sticks (magenta). The amino group of nortriptyline is 2.8 Å from the carbonyl oxygen of F43 (TM1a) and the *N*-methyl group of nortriptyline is 3.2 Å from the carbonyl oxygen of F319. Residues lining the drug binding pocket with interfacial areas greater than 10 Å² are represented as sticks. **c**, Comparison of the drug or substrate-binding pocket of dDAT_{cryst} with that of LeuT (PDB id. 2A65), respectively. The distance between the carboxylate group of leucine and Tyr 108 (spheres) is 2.7 Å in the occluded state (2A65) and 5.1 Å in the inhibitor-bound state (3F3A) of LeuT, whereas the equivalent interaction in dDAT_{cryst} between Asp 46 and Tyr 124 is 3.2 Å.

Figure 3. Ion binding sites

a, Na1 and chloride ion-binding sites. Na⁺ is purple and Cl is green and both are modeled as spheres. **b**, Coordination at the Na2 site. Coordination at the site is trigonal bipyramidal with the water molecule (red sphere) 3.3 Å from the sodium ion. Distances are in ångströms for residues that are in the coordination sphere and interactions are shown by dashed lines. Residues are colored according to their respective TMs.

Figure 4. Cholesterol site

a, Cholesterol (yellow sticks) shown with F_o-F_c density (light magenta) contoured at 2.0σ . Residues that interface with the cholesterol group are represented as sticks. **b**, Potential role of cholesterol in maintaining an outward-open state of transporter. Cholesterol (sticks with transparent surface) sterically clashes with the position of TM1a in the inward-open conformation of LeuT (PDB id. 3TT3)¹⁷.

Figure 5. Extracellular and cytoplasmic gates and the C-terminal latch

a, Relative locations of the open extracellular gate (red box), closed cytoplasmic gate (blue box), and C-terminal latch (green box) in dDAT_{cryst}. **b**, The width of the extracellular gate is depicted by the distances between Y124 and F319 (10 Å), and R52 and D475 (10 Å). Nortriptyline, ions, and helices are colored as in figure 1. **c**, The cytoplasmic gate is closed by polar and electrostatic interactions between TM1a, TM2, IL1, TM6b, and TM8. **d**, The C-terminal helix following TM12 is bound to the cytoplasmic face of the transporter via polar interactions with IL1. Polar and electrostatic bonds are represented as gray dashed lines.

Figure 6. Mechanisms of antidepressants and cholesterol

a. The TCA nortriptyline (magenta) wedges between scaffold helices 3 and 8 and the core helices 1 and 6, preventing the movement of TMs 1b and 6a from closing the extracellular vestibule. **b.** Cholesterol (yellow) is bound in an intracellular pocket and prevents the movement of TM1a from opening, thereby stabilizing the outward-open conformation of DAT. The C-terminal latch interacts with IL1 as part of the cytoplasmic gate.