The Substrate Import Mechanism of the Human Serotonin Transporter

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Abstract

The serotonin transporter, SERT, initiates the reuptake of extracellular serotonin

in the synapse to terminate neurotransmission. Recently, the cryo-EM structures of SERT bound to ibogaine resolved in different states provided a glimpse of functional conformations at atomistic resolution. However, the conformational dynamics and structural transitions to various intermediate states are not fully understood. Furthermore, while experimental SERT structures were complexed with drug molecules and inhibitors, the molecular basis of how the physiological substrate, serotonin, is recognized, bound, and transported remains unclear. In this study, we performed microsecond long simulations of the human SERT to investigate the structural dynamics to various intermediate states and elucidated the complete substrate import pathway. Using Markov state models, we characterized a sequential order of conformational driven ion-coupled substrate binding and transport events and calculated the free energy barriers of conformation transitions associated with the import mechanism. We identified a set of residues that recognize the substrate at the extracellular surface of SERT and our simulations also revealed a third sodium ion binding site coordinated by Glu136 and Glu508 in a buried cavity which helps maintain the conserved fold adjacent to the orthosteric site for transport function. The mutation of these residues results in a complete loss of transport activity. Our study provides novel insights on the molecular basis of dynamics driven ion-substrate recognition and transport of SERT that can serve as a model for other closely related neurotransmitter transporters.

Introduction

The serotonin transporter (SERT) terminates synaptic transmission by catalyzing the reuptake of extracellular serotonin from the synapse. Reuptake is critical for normal serotonergic signaling in the brain with implications on mood, cognition, behavior, and appetite.¹ Consequently, improper SERT function is associated with numerous neurological disorders including depression, autism, and bipolar disorder.² Additionally, SERT is expressed in platelet membranes and regulates blood coagulation throughout the circulatory system.³ Given its

30 medical importance, SERT is a major molecular target for therapeutic drugs and drugs of
31 abuse.^{4,5} Similar to other members of the solute carrier 6 (SLC6) neurotransmitter trans-
32 porter family, SERT mediated serotonin (5-hydroxytryptamine; 5HT) translocation from the
33 synapse and surrounding area is coupled to favorable ion co-transport of one Na^+ with a Cl^-
34 ion dependence, and export of one K^+ to complete an overall electroneutral cycle.⁶⁻¹⁰ Other
35 conduction states and stoichiometries with unclear physiological significance may occur un-
36 der different conditions.¹¹⁻¹⁵

37 SERT, and the closely related dopamine transporter (DAT) and norepinephrine trans-
38 porter (NET), belongs to a class of monoamine transporters in the neurotransmitter:sodium
39 symporter (NSS) family. These members share a distinct 12 transmembrane (TM) helix
40 architecture known as the LeuT fold, which consist of 12 transmembrane (TM) helices, with
41 TM1-5 and TM6-10 forming inverted pentahelical repeats around a pseudo two-fold axis
42 of symmetry.^{16,17} Cysteine labeling studies on SERT revealed that the 5HT binding site is
43 accessible from both extracellular and intracellular sides of the membrane, providing the
44 first glimpse of evidence of an alternating access model.¹⁸ Quick and Javitch developed a
45 proteomic approach to characterize the sodium-dependent substrate transport mechanism in
46 the tyrosine transporter Tyt1.¹⁹ These biochemical studies elucidated that the NSS family
47 of transporters function based on the principle of an alternating access mechanism.²⁰ Crys-
48 tal structures of the bacterial NSS homolog leucine transporter (LeuT) obtained in three
49 functional states, outward-facing (OF), occluded (OC), and inward-facing (IF) states, have
50 validated the NSS transport process is by an alternating access mechanism, in which the sub-
51 strate and ions first access their central binding sites via an open extracellular vestibule, and
52 then are released within the cell through the sequential closure of an extracellular gate and
53 opening of an intracellular exit pathway.^{17,20-23} Historically, LeuT has served as a structural
54 template to study monoamine transporters²⁴⁻²⁸ and based on in-depth studies of bacterial
55 transporters, including electron paramagnetic resonance (EPR) spectroscopy,²⁹⁻³¹ molec-
56 ular modeling,^{32,33} and single-molecule fluorescence resonance energy transfer (smFRET)

experiments,^{28,34} substrate permeation through the NSS family transporters is facilitated by reorientation of helices around the central axis, in particular the movement of TM1a away from the helical bundle to open an intracellular vestibule for substrate release.^{21,35,36} Despite low sequence similarity with human NSS transporters, these efforts paved the way for rational drug design for treating various psychiatric disorders.^{37–39}

Structural investigations into human NSS transporters have further benefited from the more recent resolution of outward-facing conformations of eukaryotic monoamine transporters *Drosophila* DAT (*dDAT*) and human SERT (*hSERT*).^{40–43} The screening and docking studies using these crystal structures provide the structural basis of antidepressant recognition and inhibition.^{44–49} Most recently, cryogenic electron microscopy (cryo-EM) structures of *hSERT* complexed with the psychedelic non-competitive inhibitor ibogaine reveal the occluded and inward-facing states with similar structural arrangements as seen in LeuT.^{17,23,50,51} However, given the structural discrepancies between SERT and other NSS structural models, the molecular basis of transitions between the intermediate states remains unknown. Closure of the extracellular vestibule is coordinated by helix motions of TM1b and TM6a where Arg79 and Glu493 are proposed to serve as extracellular gating residues to stabilize the OC and IF states. The helix orientation of TM1b in the SERT OC conformation is closely aligned to that of OF LeuT. Moreover, among the current SERT OF structures, the distance between the guanidinium group of Arg79 and carboxyl of Glu493 varies from 4.4 Å to 7.4 Å, while in the OC and IF states, this distance is 7.2 Å and 4.6 Å, respectively. As a result, the role of these gating residues and their interactions during conformational transitions is unclear. The N-terminal loop preceding TM1a and its interactions with TM6 and TM8 regulates the helix motion of TM1a during substrate release and acts as an intracellular gate.^{36,52,53} Hydrogen-deuterium exchange (HDX) experiments have provided an alternative approach to understand the conformational dynamics within the NSS family and have shown that ion-substrate binding facilitates changes in dynamics in TM1a, TM6, and TM7.^{24,54–56} Intricate loop dynamics, specifically motions of extracellular loops (EL) 3 and 4

fluctuates significantly during substrate transport.^{24,54–56} The combined structural and biochemical studies have provided invaluable insights in the functional mechanism of the NSS family. However, the realistic motions of structural transitions at atomistic resolution are not fully known to understand the conformational driven substrate transport cycle.

In this study, we performed unbiased all-atom molecular dynamics (MD) simulations to obtain a comprehensive understanding of the import mechanism for the physiological substrate serotonin in *hSERT*. Our study shows the key binding and transport events, including substrate interactions at an extracellular allosteric site, neurotransmitter binding within subsite B, coordination of three metal ions, and a single symported sodium ion being displaced into the cytosol by the movement of serotonin into the exit pathway. Using a Markov state model (MSM)-based adaptive sampling approach to explore the conformational landscape, we report a sequential order of the ion-substrate binding and transport processes for any NSS family transporter. The free energy conformational landscape plots reveal that structural isomerization from OC to IF is a rate-limiting step for import that is facilitated by the presence of 5HT in the orthosteric site. We identified a third sodium ion binding site in a buried cavity close to the orthosteric site which helps maintain the fold for substrate transport. We determined the key residues that are involved in 5HT recognition, binding, and transport, and these residues show to have some role in transport using site-directed mutagenesis. Our results provide an in-depth perspective into the molecular recognition and transport of 5HT in SERT and may aid for the development of conformational selective inhibitors for the treatment of psychiatric disorders.

Results

Substrate binding decreases the free energy barrier for SERT conformational transitions to the IF state.

To understand the effects of substrate-induced protein dynamics, the entire import process of 5HT was studied using molecular dynamics (MD) simulations. A Markov state model (MSM)-based adaptive sampling approach was used to explore the entire accessible conformational space of the SERT.⁵⁷⁻⁶¹ Simulations were initiated from the OF crystal structure of *h*SERT (PDB: 5I73) and a total of 130 μ s of 5HT-free SERT (referred to as Na⁺-SERT) was obtained. Na⁺-bound SERT in an OF conformation obtained from Na⁺-SERT simulations, with the Na1 and Na2 sites occupied, was used to seed simulations of the 5HT import process (referred to as 5HT-SERT). 100 mM 5HT was added to the simulation box (equivalent to 12 5HT molecules) and a total of 210 μ s data were collected. All simulation data were used to construct an MSM, which parses the simulation data into kinetically relevant states and calculates the transition probabilities between the states (See Methods for additional details). MSM-weighted simulation data were projected onto a coordinate system defined by distances between extracellular and intracellular gating residues (Figure 1 and S1).

The conformational landscape plots reveal that despite the absence of 5HT binding, Na⁺-SERT may undergo transitions from the OF state to the IF state (Figure 1A). Extracellular gating residues Arg104 (TM1b) and Glu493 (TM10) can separate to 10 Å, enlarging the extracellular permeation pathway. The equivalent charged residues in the bacterial transporter LeuT (Arg20 and Asp404) have been previously implicated in the gating mechanism.^{17,62} The OF states are stable, with a relative free energy of ~ 1.5 kcal/mol. The distance between gating residues Arg104-Glu493 decreases to 3 Å and is associated with electrostatic interactions (Figure 1 and S2), forming OC conformations that are more stable than the OF state. Previous studies have shown that binding of Na⁺ stabilizes the extracellular open conformation in SERT and related transporters, agreeing with the free energy landscapes

Figure 1: Conformational free energy landscapes of SERT obtained from MD simulations. Relative free energies from MSM-weighted simulation data plotted against the distances between extracellular and intracellular gates for (A) Na⁺-SERT and (B) 5HT-SERT. An outward-facing (OF) SERT crystal structure (PDB 5I73, pink star) was used as the starting structure for MD simulations and transitioned to occluded (OC) and inward-facing (IF) states. An hourglass-like (HG) state, in which both gates are open, was also observed. The SERT structure is represented as cartoon with TM 1, 5, 6, 8, and 10 colored in teal, green, magenta, yellow, and orange respectively. (C) Cross-section through SERT conformational states viewed from the membrane plane, shown as surface representations. The channel pore volume across the transporter is depicted as dark blue spheres and extracellular gates Arg104 and Glu493 are shown as teal and orange sticks, respectively.

131 calculated from MD simulation.^{28,29,63–65} Distance distribution plots reveal that the extra-
 132 cellular surface remains open while the intracellular vestibule is closed in OF and OC states,
 133 and vice versa in the IF state (Figure S3). Closure of the extracellular pathway as SERT
 134 isomerizes from the OF to OC state weakens contacts on the intracellular side of the trans-
 135 porter, creating an energetically accessible pathway towards a partial IF state, in which the
 136 intracellular pathway measured by the opening of TM1a and TM5 extends to ~ 6 Å. The
 137 free energy barrier for transition from the OC-IF state in Na⁺-SERT is estimated as ~ 2
 138 kcal/mol, which is higher compared to the OF-OC transition (~ 1 kcal/mol). Biophysical
 139 investigations of LeuT also reveal that IF states are less populated and that transitions to
 140 IF states are facilitated by the presence of the substrate.^{28,29,31,34} Formation of the IF state
 141 is associated with the partial unwinding of the cytoplasmic base of TM5, the breakage of
 142 electrostatic interactions between Arg79 (N-Term) and Asp452 (TM8) and Glu80 (N-Term)
 143 and Lys275 (TM5) at the intracellular gate, and increased dynamics of the flanking loops
 144 (Figure S2). Simulated helix rearrangements involved in opening and closing of the trans-
 145 porter agree with the recent cryo-EM structures⁵⁰ and other NSS crystal structures^{23,44,46}
 146 (Figure S4).

147 The substrate-present conformational landscape plot exhibits deviations in the relative
 148 free energies of conformational states and reduced free energy barriers between states (Figure
 149 1B). Binding of 5HT in the entrance pathway stabilizes the OF states to a greater extent
 150 compared to Na⁺-SERT simulations (Figure 1, S3C).²⁸ The gating residues form alternative
 151 interactions with Gln332 (TM6) and Lys490 (TM10), thereby widening the extracellular
 152 vestibule (Figure S2). Similar to observations seen in human DAT simulations,⁴⁰ the diffusion
 153 of 5HT to the orthosteric (S1) site via the allosteric (S2) site promotes the inward closure
 154 of extracellular gating helices TM1b, TM6a and TM10 to facilitate formation of the OC
 155 state (Figure S5). The OF-OC transition has a free energy barrier of ~ 1.5 kcal/mol, similar
 156 to Na⁺-SERT. The 'downward' movement of 5HT facilitates opening of the intracellular
 157 gate and isomerization to the IF state. The free energy barrier for the OC-IF structural

158 transition is estimated as ~ 2 kcal/mol. The presence of 5HT in the intracellular pathway
 159 stabilizes SERT in a greater IF state, with a relative free energy of ~ 1 kcal/mol as compared
 160 to ~ 2 kcal/mol in Na^+ -SERT. Compared to Na^+ -SERT, the intracellular vestibule in the IF
 161 state now extends to 7-10 Å, thus allowing for the substrate to be released and is associated
 162 with the breakage of hydrogen bonding networks between intracellular gating helices (Figure
 163 S2, S6, S7, S8, S9). Terry *et al.* has demonstrated that the selective substrate-driven
 164 conformational transition to the IF state in LeuT is highly favorable compared to non-
 165 selective substrates and ion binding alone.²⁸ Using Transition path theory, we estimated
 166 the mean first passage time (MFPT) for different transitions observed in the simulations.
 167 We found that the rate of transition between OC-IF states is rate-limiting as compared to
 168 transitions between OF-OC states both in the presence and absence of 5HT (Figure S10). We
 169 also observed partial OF-IF like conformations, which we have termed as an hourglass-like
 170 (HG) state in which both gates are open but constricted at the center (Figure S11C). Terry
 171 *et al.* identified intermediate conformations in LeuT which are open at both extracellular
 172 and cytoplasmic halves and may enable the transport of Na^+ and substrate molecules. The
 173 conformational landscape plots reveal that these states are less stable (> 3 kcal/mol) as
 174 compared to canonical intermediate states OF, OC and IF. Therefore, the release of Na^+
 175 ions and substrate molecules through HG states is a more energetically demanding process
 176 than the traditional substrate transport via the IF state. In Na^+ -SERT simulations, we
 177 observed uncoupled ion leaks, in contrast, Na^+ binds tightly to its respective site and no
 178 ion leaks were observed in 5HT-SERT (Figure S12). This state has been observed in other
 179 membrane transporters⁶⁶⁻⁶⁸ including a disease-associated mutant of DAT,⁶⁹ but transitions
 180 from HG to other intermediate states are restricted as the free energy barriers are high and
 181 the physiological relevance of this state in SERT remains elusive.

182 The calculated root mean squared fluctuation (RMSF) plots illustrate that in both Na^+
 183 and 5HT-SERT simulations, EL2 and EL4 are highly flexible during the OF to OC transi-
 184 tions. Alternatively, the loop regions are more stable during OC to IF in 5HT-SERT simula-

Figure 2: Molecular dynamics analysis of global fluctuations during conformational transitions. Normalized RMSF of Na⁺ (A,B) and 5HT transport (C,D) for OF to OC transition and OC to IF transition mapped to simulated SERT structure. Tube thickness corresponds to the RMSF values of each residue.

tions compared to Na^+ simulations (Figure 2). Hydrogen deuterium exchange (HDX) mass spectroscopy studies hint that EL2 and EL4 regions are destabilized and show increased deuterium exchange upon ion and substrate binding.^{24,54,56,68} Furthermore, EL2 exhibits higher deuterium uptake kinetics in the OF state compared to the IF state in LeuT.²⁴ In 5HT-SERT simulations, EL4 shows less pronounced fluctuations during OC to IF transitions. The experimental results from HDX show EL4 regions are more stabilized during K^+ uptake which is hypothesized to stabilize the IF state of SERT based on LeuT studies.^{56,70} The increased deuterium uptake of TM1a in the presence of K^+ agrees with the large fluctuations we observed for transitions to the IF state. The comparison of calculated deuterium exchange fraction of Na^+ and 5HT-SERT simulation data agree with previous HDX studies (Figure S13).²⁴

Identification of a new sodium ion binding site in a buried cavity stabilizes the fold for substrate transport.

Monoamine transporters utilize an electrochemical gradient to transport substrates across the cellular membrane. 5HT-mediated transport involves the symport of 1 Na^+ ion and antiport of 1 K^+ ion.^{7,71} Upon the transition of 5HT from the allosteric to the orthosteric binding site, the Na^+ ion in the Na1 site shifts to a third metal coordination center, which we call the Na3 site to be consistent with prior nomenclature.^{72,73} The vacant Na1 site is then filled by two to three water molecules that interact with the charged site until another Na^+ ion binds to the Na1 site (Figure S14). The calculated electrostatic potential map of SERT OF state cryo-EM structure reveals that the permeation pathway is negatively charged, which allows Na^+ ions to rapidly diffuse and bind to the Na1 site (Figure S15). At the Na3 site, the Na^+ ion is coordinated by the carboxylates of Glu136 and Glu508, and the sulfur of Met135 through water molecules (Figure 3). A third metal ion site has not previously been described in SERT, but computational modeling, biochemical analysis, and electrophysiology recordings indicate that equivalent residues of the neuronal GlyT2

211 transporter also form a third Na^+ site.⁷³ The simulation reveals that the presence of Na^+
212 in the Na3 site stabilizes TM6 unwinding and the proper orientations of residues in the
213 orthosteric site. There are two pieces of experimental evidence for this *in silico* discovery.
214 First, a structural alignment of SERT from the MD simulation with the crystal structure
215 shows weak but discernible electron density, comparable to the density of surrounding side
216 chains, near the modeled third Na^+ (Figure 3). Second, previous experiments have shown
217 a reduction of serotonin transport when Glu136 is mutated, underscoring the role of this
218 residue for appropriate conformational dynamics.⁷⁴

219 We note that the presence of buried glutamates within hydrophobic transmembrane re-
220 gions is highly unusual; in this case, Glu136 hydrogen bonds to exposed backbone N-H
221 groups to support the unwinding of TM6 near the central substrate binding site. We calcu-
222 lated the pKa of these residues with and without Na3 bound and found that they are not
223 protonated under physiological conditions (Table S1). The calculated pKa values for SERT
224 resolved structures and other NSS structures were also predicted to be in the range of 3-4,
225 given the intracellular neuron pH is 7.17⁷⁵ (Table S1). Our simulation shows that the Na3
226 cavity is well solvated before and after Na3 binding, with an average of 4-5 and 7-8 water
227 molecules with 5 Å of the Na3 site, respectively (Figure S16A, S16B). We also calculated the
228 protein tunnels and channel-like pores that could facilitate the transport of small molecules
229 and ions for the OF, OC and IF states of SERT. The tunnel plot reveals that the Na3
230 site is completely buried inside the transporter and is not exposed to the cytoplasmic half
231 (Figure S16C). Therefore, from our simulations, we observed that Na3 is not released to the
232 intracellular side (Figure S17E).

233 **Simulations reveal a sequential order of substrate binding and trans-** 234 **port in SERT.**

235 An aspect of the current NSS transport model that remains unaddressed is the sequential
236 order of substrate binding and transport events. Using transition path theory, the highest

Figure 3: A third Na^+ ion binding site buried beneath the orthosteric pocket. MD snapshot (TM2 colored pale blue and TM10 orange) superimposed with the SERT crystal structure (PDB 5I73, grey), with electron density shown at 0.5σ .

flux pathway for conformational change and 5HT import can be determined from the Markov state model and used to predict an ordered sequence of binding events and structural changes. SERT undergoes complete transitions to the IF state in the simulations, with permeation towards the intracellular side upon binding of substrates in the order following Na^+ , 5HT, and Cl^- ions (Figure 4). We describe each step of the import process in detail.

The transport process begins with the binding of Na^+ to the Na1 site, followed by a second Na^+ binding the Na2 site (Figure S18, S19). These two sites are well-described in the SERT crystal structure and the literature.^{23,44,46,76} Na^+ bound at the Na1 site couples activity between the ion and substrate binding sites, whereas computational studies of related transporters have indicated that Na^+ coordinated at the Na2 site dissociates during the transporter cycle to become the symported metal ion.^{40,77,78} The importance of the Na2 site is underscored by its conservation in distantly related secondary transporters.⁷⁹ Na^+ ions entering the transporter interact with Asp328 and Asn112 at the extracellular surface, then rapidly diffuse into the allosteric site (Figure S18A). Here, the Na^+ ions interact with Glu493 and Glu494, and a rotameric shift in Glu493 enables the ions to descend past the extracellular gate to their central binding sites (Figure S18B, S18C). Na1 is stabilized by

Figure 4: The major flux pathway and mean first passage times for SERT conformational transitions and 5HT import determined from transition path theory. The transport process begins with the binding of 2 Na^+ ions to the Na1 and Na2 sites in the OF state (2, 3). Substrate diffusion to the orthosteric site shifts a Na^+ to the Na3 site (4). An additional Na^+ and Cl^- ion bind (5), facilitating closure of the extracellular gate to form the OC state (6). Isomerization to the IF state is associated with the release of Na^+ from the Na2 site and 5HT diffuses out (6-9). Arrow thickness represents relative flux between transitions.

Asn101, Ser336, and Asn368, while Na2 is coordinated by backbone carbonyls of Gly94, Val97, Leu434, and side chains of Asp437 and Ser438. Previous simulations of SERT have shown Asp437 as a key residue for maintaining Na2-ion interactions,⁷⁸ and mutations of Asp437 and Ser438 have confirmed their role in coordination and dissociation of Na2.^{78,80} Similar to other transporters in the NSS family,^{29,63,64} the binding of Na^+ ions to their respective sites stabilizes SERT in the OF state while neutralizing the polar cavity to allow protonated 5HT diffusion.

5HT is recognized by Tyr107, Ile108, Gln111, and Asp328 at the extracellular vestibule to initiate the binding in the OF state. Ile108 forms hydrophobic contacts with the indole ring of 5HT while other residues form polar interactions with the substrate that favors binding. 5HT then diffuses inside the translocation pore and binds to the allosteric site (Figure 5B). The substrate is stabilized by aromatic ring packing against Phe335 and Arg104, and a hydrogen bonding network with Asp328, Gln332, and Glu494 (Figure 5B). Previous mutations of residues in the allosteric site have been shown to alter inhibitor potency.⁸¹ 5HT undergoes a 90° rotation by rapidly exchanging its polar interactions and shifts towards the orthosteric site (5C). The switching of amine group interactions to Glu494 triggers the movement of 5HT from the allosteric site to the orthosteric site. 5HT rotates such that the conformation becomes perpendicular to the membrane which is further favored by polar and hydrophobic interactions by Asp328, Gln332, Leu502, and Ala331. The extracellular gating residues form a salt bridge interaction and enlarges the binding cavity such that substrate can escape to the primary binding site. This substrate binding site in the NSS family contains three well-studied subsites and has served as the basis of designing various tricyclic antidepressant molecules.⁸² In the orthosteric site, the protonated amine moiety of 5HT forms charged interactions with Asp98 of subsite A, disrupting the hydrogen bonding interactions between Asp98 and Tyr176 (Figure S20). Also within subsite A, the phenol moiety forms aromatic interactions with Tyr95 and Phe341. Site-directed mutagenesis and computational docking studies have emphasized the significance of Asp98 in 5HT recognition and transport,^{83,84} while biochemical studies show that the disruption of aromatic interactions with the substrate leads to a loss of function or decreased potency of antidepressants.^{81,85-87} The binding of 5HT from the allosteric to the orthosteric binding site promotes Na⁺ in the Na1 site to migrate to the third metal coordination center, the Na3 site.

Figure 5: MD snapshots of the simulated mechanism for SERT-catalyzed 5HT import. (A) Overlaid MD snapshots of 5HT translocation, from when 5HT enters the extracellular vestibule (blue) to its cytosolic exit (red). The positions of ions in the OC state are shown as spheres. Transmembrane helices 1, 5, 6, 8, and 10, are colored in teal, green, magenta, yellow, and orange, respectively. (B) 5HT enters the transporter by binding in the allosteric site. (C) From the allosteric site, 5HT rotates down the transport pathway to the orthosteric site. (D) Initial Cl⁻ recognition is assisted by Asp98, Arg104, Tyr175, Phe335, and indole of 5HT.

5HT mediates the binding of an electrogenic Cl⁻ ion.

Cl⁻ and Na⁺ permeate into the extracellular vestibule and bind at the Cl⁻ and vacated Na¹ coordination sites, which leads to the formation of an occluded conformation (Figure S21).

287 Na^+ is stabilized by Glu493 and Glu494 while Cl^- forms polar interactions with Arg104
288 and Tyr176. The calculated electrostatic potential map of the SERT-OF cryo-EM structure
289 reveals that the permeation pathway is negatively charged and supports our finding that the
290 binding of Cl^- ion will be accompanied by a Na^+ ion (Figure S15). The additional interaction
291 of Cl^- with the indole-NH of 5HT further stabilizes the ion in the exposed extracellular
292 recognition site (Figure 5D). The indole ring of 5HT occupies subsite C of the primary
293 binding site, which in turn favors the transition of the Cl^- ion to its binding site (Figure
294 S22).

295 As Cl^- enters, it shifts the guanidinium group of Arg104, facilitating the diffusion of
296 both the Cl^- and Na^+ ions into the central cavity (Figure S20C). The movement of Cl^-
297 through the transporter is supported by a network of interactions with Arg104, Tyr176,
298 and the indole-NH of 5HT (Figure 5D). A shift of the Arg104 side chain exposes Gln332
299 for making contacts with the Cl^- ion, facilitating its migration to the Cl^- binding site.
300 The predicted Na1, Na2, and Cl^- sites in the simulations concur with their respective sites
301 observed in crystal structures of SERT, LeuT, and DAT^{23,44,46,76} (Figure S23). Interestingly,
302 we did not observe Cl^- to bind in the absence of the substrate within the given simulation
303 timescales (Figure S24). In order to quantify the binding of Cl^- in the absence of 5HT,
304 we performed umbrella sampling simulations to compute the free energy of Cl^- binding in
305 Na^+ -SERT conditions. The free energy barrier for Cl^- binding without 5HT was estimated
306 on the order of ~ 12 kcal/mol as compared to ~ 4 kcal/mol in 5HT-SERT simulations (Figure
307 S25). The highest energy barrier observed at approximately $3\text{\AA}$ away from the Cl^- binding
308 site is associated with the dissociation of Na^+ from Cl^- to allow binding to their respective
309 sites suggesting that the substrate in the orthosteric binding site neutralizes the polar cavity
310 to allow for favorable Cl^- binding.

311 The binding of ions and the substrate to their respective sites leads to the closure of the
312 extracellular gates to obtain the OC state where the permeation pathway is closed at both
313 ends. The decrease of the pore results in shifting of the aromatic ring of 5HT from subsite C

to subsite B within the orthosteric binding pocket. The amine moiety of the neurotransmitter remains bound to Asp98, Tyr95, and the C-terminal pole of TM1a within subsite A (Figure 6A,B). The simulated configuration of 5HT in subsite B agrees with the crystal structure of dopamine-bound *d*DAT⁷⁶ (Figure S23F). The indole-NH ring of 5HT interacts with Thr473 and other hydrophobic contacts by Ala169, Ala441, Gly442, and Leu443 residues stabilizing the substrate in subsite B. Additionally, 5HT may enter into subsite B in which the indole-NH is oriented towards Phe341 adopting a binding pose also reported in previous modeling studies.^{84,88} Previous experimental studies indicate that mutations of these residues can decrease 5HT transport^{81,85} and the binding pose is further supported by several studies showing that interactions in subsite B are critical for inhibitor potency.^{82,85,86}

Mechanism of 5HT translocation down the exit pathway.

The conformational free energy landscape suggests that structural isomerization to the IF state is limited by a large free energy barrier which is decreased in the presence of the substrate. The prolonged binding of 5HT in subsite B weakens Na⁺ interactions in the Na2 site and results in its dissociation. Na⁺ loses its interaction with the backbone carbonyl of Leu434 and enters the intracellular vestibule, thereby initiating structural transitions from the OC to IF state. Koldsø *et al.* has shown that solvation of the intracellular vestibule allows Asp437 to rotate Na2 towards the intracellular pathway and facilitates transitions to the IF conformation.⁷⁸ Similarly, we also observed that the rotameric shift in Asp437 initiates the dissociation of Na⁺ from the Na2 site (Figure S26A, S26B). The Na⁺ ion then interacts with Ser91 and is further stabilized by Phe347 and Phe440 via cation- π interactions (Figure S26C). Finally, the Na⁺ ion engages with Asp87 and diffuses into the intracellular space (Figure S26D). Koldsø *et al.* also predicted that Glu444 interacts with Na2 in the exit pathway,⁷⁸ yet in our simulations, Glu444 forms an ionic interaction with Arg462 and does not interact with Na2. At this juncture, the intracellular gating residues (Arg79 and Asp452) still hold their hydrogen bond interactions, and Na⁺ can access the intracellular pathway

Figure 6: MD snapshots of 5HT in the orthosteric binding site and intracellular release. Transmembrane helices 1, 5, 6, 8, and 10, are colored in teal, green, magenta, yellow, and orange, respectively. Substrate color relates to progression of 5HT-import as shown in Figure 5A. (A,B) While 5HT is in subsite C and B, the amine moiety of 5HT at the orthosteric site interacts with Asp98, disrupting the hydrogen bonding interaction between Tyr176-Asp98. (C) The rotameric flip of the phenol ring of Tyr95 initiates the opening of the intracellular vestibule and allows for permeation of 5HT towards the intracellular exit pathway. (D) 5HT translocation through the exit pathway between TM1a and TM5.

without the breakage of ionic contacts. However, in Na⁺-SERT simulations, the Na2 ion has a tendency to rebind to the Na2 site after release (Figure S27). Such intracellular binding events were not observed in 5HT-SERT simulations. The coupling of 5HT import to the cytoplasmic release of Na⁺ from the Na2 site explains the 1:1 sodium to neurotransmitter stoichiometry of the transport cycle.

The rotameric shift of Tyr95 results in permeation of 5HT to the exit pathway (Figure 6C). The flipping of the indole ring of 5HT displaces the ionic interactions with Asp98 resulting in the aromatic ring of 5HT to be trapped between Tyr95 and Val343. The 'downward' movement of 5HT disrupts contacts between TM1a and TM5, specifically the hydrogen bonding interactions between Asp87 and Trp282 (Figure S9). The distance between Asp87 and Trp282 increases up to 16 Å, thus drives the dissociation of 5HT to the cytoplasmic half of the transporter. Additionally, the intracellular salt bridge network between Glu80-Lys279 and Arg79-Asp452 weakens as the cytoplasmic base of TM5 unwinds, further resulting in the opening of the intracellular pathway. 5HT shifts to the intracellular vestibule and occupies the Na2 site (Figure 6C). The amine group forms strong polar contacts with residues in the Na2 site, and the indole ring is lodged between Tyr95, Phe347, and Phe440. 5HT further diffuses down, however the amine group of 5HT still forms interactions with Tyr95, and the indole-NH forms additional interactions with Ser91. Finally, 5HT leaves the transporter through a widened intracellular pathway surrounded by TM1a and TM5. Our results show that the rotation of Tyr95 propagates the opening of TM1a and mediates substrate transport to the cytoplasmic half. The equivalent residue in *h*DAT, Phe76, has been shown to undergo a similar rotameric transition to allow for substrate release.⁴⁰ We also observed that the cytoplasmic base of TM5 unwinds and shifts outward by ~8-10 Å to facilitate cytoplasmic opening of the exit pathway (Figure 6D), and it is well known that these regions play a crucial role in regulating SERT activity⁶⁴.

Simulations identify key residues involved in substrate transport.

SERT has been extensively studied by targeted mutagenesis, especially within the orthosteric binding site, confirming key interacting residues with substrate and drugs.^{84,89} To provide basic evidence supporting aspects of the simulations, we instead focused mutagenesis to residues predicted to make early interactions with substrate (Figure 7A). Changes in transport activity were assessed based on cellular uptake of a fluorescent 5HT analogue, APP+, which has been shown to have similar transport properties to 5HT.⁹⁰ Furthermore, we extracted individual states from the 5HT-SERT simulation data and performed molecular docking experiments to predict the binding mode of APP+, and observed that APP+ binds in a similar fashion and shares similar residue interactions as the native substrate, 5HT (Figure S28).

We examined by immunoblot that the decreased transport activity was not a result due to poor SERT expression, with the exception of Ile108Ala (Figure 7B). Alanine substitutions of Tyr107 and Gln111, which are predicted to participate in the early recognition of 5HT as it first diffuses into the extracellular vestibule and substitution of Asp328 in the allosteric site reduce substrate transport (Figure 7B). Both mutations are deleterious for transport activity as they mediate crucial interactions with the substrate in the initial recognition as well as in the allosteric site (Figure 7B and 7C). Leu502 is packed beneath aromatic residues in the allosteric and early recognition sites, and disruption of local structure by its substitution to alanine renders SERT inactive, although we cannot exclude broader effects on the protein fold (Figure 7D). We further tested alanine substitution of Val343, which is packed against Tyr95 at the base of the orthosteric site. Once Tyr95 undergoes a rotameric shift during the OC to IF transition, a void is created between Val343 and Tyr95 that is temporarily occupied by the substrate as it moves into the intracellular vestibule (Figure 7E). SERT Val343Ala has partly reduced activity, which is notable considering the close chemical similarity between alanine and valine side chains. Additionally, we determined the K_M - V_{Max} values for a mutant in each interaction site which show a reduction in transport activity (Table S2). Finally, Glu136 and Glu508 that coordinate Na^+ in the Na3 site were

Figure 7: Alanine substitutions of residues involved in the simulated transport process cause decreased substrate uptake. (A) Overlaid MD snapshots showing the permeation of 5HT from its diffusion into the extracellular vestibule (dark blue), to binding at the orthosteric site (green), to cytosolic release (red). Mutated residues are similarly colored based on when in the import pathway they interact with 5HT. Glutamates coordinating Na^+ in the Na3 site are magenta. (B) SERT mutants were expressed in human Expi293F cells and uptake of fluorescent substrate APP+ ($0.5 \mu\text{M}$) was compared to wild type (WT) protein. Data are mean $\pm$ SD, $n = 3$. Aligned below is an anti-SERT immunoblot of total cell lysate. (C-E) MD snapshots of 5HT at the (C) extracellular vestibule, (D) allosteric site, and (E) released from the orthosteric site. Residues that were substituted with alanine colored as red sticks. Transmembrane helices 1, 6, 8, and 10, are colored in teal, magenta, yellow, and orange, respectively.

392 mutated to alanine to cause a complete loss of activity, demonstrating the importance of
 393 this region for the transport process.

Conclusions

In this study, we present an atomistic view of the substrate import process in SERT as well as characterizing the thermodynamics of key states involved in substrate transport. By implementing an MSM-based adaptive sampling protocol to sample the conformational landscape, we investigated global transitions from OF to IF for both 5HT-free and 5HT-transporting SERT. MSM-weighted conformational free energy landscapes show the OF and OC states are relatively stable, and transitions to and from OF and OC states are relatively low energy. Transitions from OC to IF are substantially higher, with energy barriers of ~ 2 kcal/mol in Na⁺-SERT; however, the presence of 5HT not only lowers the free energy barrier of OF to OC transitions, but further stabilizes SERT in the IF state as compared to Na⁺-SERT. The dependence of the IF state on the presence of substrate has experimentally been observed for the bacterial LeuT transporter, where addition of extracellular substrate promotes dynamics at the intracellular gate.⁶²

The simulated structures show similar helix orientations at the extracellular and intracellular gates with respect to experimental SERT structures.^{23,44,46} The comparison of Na⁺ and 5HT-SERT data reveal that the structural transitions from OF to OC involve minimal helix movements in TM1b, TM6a, and TM10, while OC to IF transitions show higher fluctuations of intracellular helix tips of TM1a, TM5, and TM7 to facilitate opening of the intracellular vestibule (Figure S4). Biochemical experiments in conjunction with computational modeling have shown that cholesterol binding to a conserved site comprised of TM1a, TM5, and TM7 inhibits these helix movements and may act as a regulatory mechanism for SERT function.^{46,91,92} Deviations were observed in the opening of the intracellular vestibule of the IF state between the cryo-EM and predicted MD structure, specifically the outward motion of TM1a. This might reflect the loss of lateral pressure following detergent-extraction; the membrane is anticipated to constrain the extent to which TM1a can move away from the helical bundle. Nevertheless, our results show that partial opening of the intracellular pathway is sufficient for substrate transport. Experimental studies of LeuT also indicate that a

partially-open IF conformation is suitable for substrate transport.²⁸

Conducting simulations of both serotonin-free and serotonin-present conditions allows us to examine the finer details of the substrate-induced conformational transitions and the cooperative nature between the transporter and the substrate. Motions of TM2 were highly correlated with TM1b in Na⁺-SERT, whereas the binding of 5HT decreases the cooperativity as TM1b rotates and closes the extracellular cavity to open the exit pathway (Figure S29). The conformation dynamics examined through HDX studies reveal that TM2 undergoes less fluctuations when bound to Na⁺, in contrast to when the transporter is substrate bound.⁵⁴ Similar trends were observed between TM6a and TM2, more specifically, the presence of 5HT destabilizes this region and favors the conformational change required for substrate transport (Figure S29). Gating residues Arg104-Glu493 remain open until the ions and 5HT bind to the primary binding site, and later close the extracellular cavity as the exit pathway opens to allow the release of substrate molecules (Figure S5). At the binding site, Tyr176 has the tendency to form a polar interaction with Asp98 in the OF and OC states. Interactions by the amine moiety of the substrate sever the Tyr176-Asp98 interaction, which is further disrupted when the opening of the intracellular cavity induces a rotameric shift in Tyr176 away from the Asp98 (Figure S19). Most significantly, the closure of the extracellular allosteric cavity is coupled to opening of the intracellular helix TM1a when the substrate is bound (Figure S29C). Experimental studies show that the substrate binding increases dynamics between EL4 and TM12.^{24,54-56} In simulations, EL4 inserts deep inside the extracellular cavity, which is further facilitated by the movement of TM12 (Figure S30). Furthermore, HDX studies on DAT support the coupled motions between TM7 and TM12 observed in simulations when the substrate is bound, compared to just Na⁺ binding (Figure S29).

Our simulations reveal an ordered sequence of binding and transport events that agree with the 1:1 substrate:Na⁺ stoichiometry as previously characterized for the NSS family. In 5HT-SERT simulations when Na1 and Na2 sites are occupied, both Cl⁻ ions and 5HT molecules were present in the solution, but even after extensive sampling, we only observed

448 5HT binding to the transporter. Similarly, in the Na^+ -SERT simulations, Na^+ and Cl^- ions
 449 were present in the solution, yet we observed rapid diffusion and binding of Na^+ ions to their
 450 binding sites instead of Cl^- ion binding. We further determined that the free energy barrier for
 451 Cl^- to bind without the substrate in the orthosteric cavity is significantly greater than in 5HT-
 452 SERT simulations (Figure S25). Additionally, the calculated electrostatic potential map of
 453 the OF SERT cryo-EM structure reveals that the permeation pathway is negatively charged
 454 and supports our finding that the binding of Cl^- prior to substrate binding will be associated
 455 with a large free energy barrier (Figure S15). We identified a third sodium ion binding site
 456 buried deep in the binding pocket that is conserved among various NSS transporters and
 457 critical for substrate transport. Similar to other NSS members, the release of Na^+ from the
 458 Na2 site decouples the interactions between TM1a, TM5, and TM8, increasing the solvation
 459 of the intracellular permeation pathway and allowing TM1a to open for substrate release.
 460 As unbiased molecular dynamics simulations can only sample events with low free energy
 461 barriers, we have the highest likelihood of observing the most probable binding sequence of
 462 ions and substrate. However, we do not disregard that different sequences of ion and substrate
 463 binding events with higher free energy barriers associated with them are possible and may
 464 alter the kinetics of the SERT transport cycle. Differences in stoichiometry may arise if the
 465 ion unbinding timescales for Na1, Na3, and Cl^- are much longer than the simulated timescales
 466 obtained in this study. We investigated the mechanism of 5HT recognition, binding, and
 467 translocation in SERT and through mutagenesis provided support for certain residues having
 468 potential roles in substrate binding and/or conformational transitions, though we do not
 469 exclude that they may have other roles in folding of the transport cycle.

470 In some simulations, we observed 5HT to simultaneously bind both the orthosteric and
 471 allosteric site. Substrate bound at the allosteric site hinders the fluctuations of several
 472 residues at the extracellular side, hence, allosterically communicates to the primary binding
 473 site of the transporter by limiting conformational transitions from the 5HT-bound OF state.
 474 The distance distribution plot reveals that the side chain conformation of Arg104 is restricted

when 5HT binds to both orthosteric and allosteric sites, whereas when 5HT is solely occupied the orthosteric site, Arg104 may adopt multiple conformations (Figure S31). The binding of the substrate at both sites sterically restricts opening of the exit permeation and substrate release.

This study has explored the conformational dynamics and substrate import of a monomeric unit of SERT; however, fluorescent microscopy has shown SERT to form functional oligomers in the membrane.^{93,94} The LeuT crystal structure has been resolved as a dimeric unit, and while recent modeling studies have explored possible dimeric SERT units,⁹⁵ the exact interface of SERT oligomerization and the effects of coupled dynamics remains unclear. Further investigation is required into understanding how *in vivo* regulation affects the kinetics and conformational landscape of SERT.

Methods

Molecular dynamics (MD) simulations.

The OF SERT crystal structure (PDB: 5I73) was used as the starting model for MD simulation.⁴⁴ Thermostable mutations in the crystal structure Ala110, Ala291, and Ser439 were reverted to wild type Tyr110, Ile291, and Thr439, respectively. The protein was embedded in a phosphatidylcholine (POPC) bilayer with CHARMM-GUI⁹⁶ and solvated with TIP3P water molecules.⁹⁷ 150 mM NaCl was added to neutralize the system and mimic physiological conditions. Terminal chains were capped with acetyl and methyl amide groups. Overall, the final Na⁺-SERT system consisted of ~70,000 atoms in a period box volume of 77 X 77 X 113 Å³. The MD system was built using the tleap module of AMBER14.⁹⁸ The MD system was minimized for 20,000 steps using the conjugate gradient method, heated from 0 to 300 K at NVT, and equilibrated for 40 ns under NPT conditions. A Na⁺ bound in the Na1 and Na2 site OF SERT structure, obtained from Na⁺-SERT simulations, was used as the starting model to capture the mechanism of 5HT import. 100 mM serotonin (equivalent to 12

500 5HT molecules was randomly added to the simulation box and equilibrated under the same
501 conditions described previously. We note that the simulated 5HT concentration is unphysio-
502 logical and was implemented to enhance the probability of substrate binding events. During
503 the alternate-access cycle, the transporter only interacts with at most two 5HT molecules.
504 Therefore, the free energy and kinetics of the conformational change processes are indepen-
505 dent of the additional 5HT molecules in solution. The ionic charge of 5HT is neutralized with
506 Cl^- ions. While the ionic concentration could have some effects on the protein conformation
507 such as altering the structure of the extracellular loops which directly interact with the ad-
508 ditional 5HT molecules, we do not observe any shifts in the structure of these loops between
509 the Na^+ -SERT and 5HT-SERT simulations. All simulations were implemented using Am-
510 ber14 package^{98–101} employing Amber ff14SB¹⁰² force field combined with GAFF force field
511 at constant NPT conditions (300K, 1 atm) and periodic boundary conditions. Temperature
512 and pressure were maintained with Berendsen thermostat and barostat, respectively.¹⁰³ Elec-
513 trostatic interactions were treated with the Partial Mesh Ewald method,¹⁰⁴ and hydrogen
514 bonds were constrained using SHAKE algorithm.¹⁰⁵ Nonbonded distance cutoff was set at
515 10 Å, and an integration timestep of 2 fs was used for all simulations. Snapshots were saved
516 every 100 ps during production simulations.

517 **Adaptive sampling.**

518 Obtaining sufficient sampling is a reoccurring challenge in simulating complex biological
519 processes. To overcome this issue, we adopted a Markov state model (MSM)-based adaptive
520 sampling methodology to efficiently explore the conformational landscape.^{57,106,107} In each
521 round of adaptive sampling, multiple short MD simulations are conducted in parallel. The
522 simulation data is clustered using the K-means algorithm¹⁰⁸ based on a designated metric
523 and starting structures are chosen from the least populated states to seed the subsequent
524 rounds of simulation. The sampling bias introduced from least-count selection is eliminated
525 during the construction of the MSM by estimating the reverse transition probability matrix

for transition between all conformational states. In the limit of long timescales, the sampling errors are expected to be small. We show that there is little difference between the population of states derived from the raw data and the MSM-derived equilibrium population on the conformational landscape (Figure S32). Extracellular and intracellular gating residues were used as adaptive sampling metrics for the conformational sampling of the states and z-position of substrates to capture the import process. A total of $\sim 130 \mu\text{s}$ of Na^+ - and $\sim 210 \mu\text{s}$ 5HT transport simulation data was obtained. Multiple substrate binding and transport events were captured as a direct advantage of the MSM-based adaptive sampling approach (Figure S33). The entire MD dataset was used for MSM construction and analysis.

MSM construction.

The CPPTRAJ and pytraj¹⁰⁹ modules in AmberTools and MDTraj Python library¹¹⁰ were used for post processing the trajectory data. Markov state models (MSMs) were constructed using pyEMMA 2.5.6 Python package.¹¹¹ MSMs were constructed for both Na^+ - and 5HT transport datasets. 15 residue-residue pair distances surrounding the permeation pathway for clustering (Figure S34). Additionally, the z-components of the 2 Na^+ ions were incorporated to the 15 residue-residue pair distances as featurization metrics for Na^+ -SERT simulations. The z-components of 5HT, Cl^- , and the symported Na^+ ion were added along with the 15 distances for the 5HT transport process. Time-structure independent component analysis (tICA) was performed on the feature matrix to reduce the dimension space by obtaining the slowest-relaxing degrees of freedom as a linear combination of the features.¹¹² The optimum number of clusters and time-independent components (tICs) were obtained from the set that yielded the greatest sum of the eigenvalues of the transition matrix, also known as the VAMP1 score. 700 clusters and 8 tICs were used to construct the MSM for Na^+ -SERT simulations. 700 clusters and 10 tICs were used for 5HT-SERT MSM. (Figure S35A). The lag time of 8 ns was determined for MSM construction from implied timescale plots (Figure S35B). The Chapman-Kolmogorov test, which validates the Markovian behavior of

the MSM,¹¹³ was performed on 5 macrostates implemented in pyEMMA (Figure S36, S37).

Trajectory analysis.

MSM-weighted simulation data were plotted on the coordinates of the gating distances, specifically the distances between the closest heavy atom between Arg104 and Glu493 of the extracellular gate, and the closest heavy atom between groups of residues Gly77-Ser91 of the N-terminus and TM1a and Lys297-Trp282 of TM5 for the intracellular gate (Figure S1). MSM states were further clustered into macrostates and visualized with Visual Molecular Dynamics (VMD)¹¹⁴ and PyMOL (Schrödinger, LLC). The predicted fraction deuteration was calculated according to Adhikary *et al.*²⁴ on 1000 OF and IF structures randomly extracted from the Na⁺-SERT and 5HT-SERT MSM. The 5HT-residue interactions were obtained from python scripts implemented from the GetContacts package (<https://getcontacts.github.io/>). To calculate the RMSF between transitions, 1000 structures of each OF, OC, and IF states were randomly extracted from the MSM and measured with respect to the cryo-EM structure of the prior conformational state (i.e. OF-OC: 1000 OC structures with respect to OF cryo-EM structure). In-house scripts and matplotlib Python library were used to generate plots. Channel pore radius was calculated using the HOLE program.¹¹⁵ pKa values were calculated using DelPhiPKa program as it also considers the effects of solute molecules while performing the calculation in the static structure.¹¹⁶ Cross correlation analysis of Na⁺-SERT and 5HT-SERT was performed to identify the allosteric effects of substrate on conformational changes between various states and the substrate transport. The cross-correlation value of 0 represents motions that are not correlated, while a value of 1 shows that the motions are highly coupled.

Transition path theory.

The top flux pathways for conformational changes and 5HT import were determined using transition path theory (TPT) analysis. Given a transition matrix obtained from the Markov

state model, TPT examines the transition probabilities and estimates transitions pathways connecting the source (defined as state A) and sink (defined as state B) states and the fluxes associated with the pathway.¹¹⁷ The flux between state i to j or f_{ij} is defined as

$$f_{ij} = \pi_i q_i^- T_{ij} q_j^+$$

where π_i is the stationary probability of state i as obtained from the eigenvalue decomposition of the transition matrix, T_{ij} is the probability of transitioning from state i to state j , q_i^- is the backwards committer probability of transitioning to the source state rather than the sink state from state i , and q_j^+ is the forwards committer probability of transitioning to the sink state rather than the source state from state j .¹¹⁸ The forward committer probability for a state i , or q_i^+ is computed as the sum of all the transitions from state i the sink state B and the sum of all the transitions from state i to sink state B via an intermediate state I ,¹¹⁸ or

$$q_i^+ = \sum_{k \in B} T_{ik} + \sum_{k \in I} T_{ik} q_k^+$$

The backwards probability committer probability is calculated as

$$q_i^- = 1 - q_i^+$$

TPT analysis was conducted using the pyEMMA 2.5.6 Python package.¹¹¹

Umbrella sampling of Cl⁻ binding in Na⁺-SERT

Umbrella sampling MD simulations were used to calculate the binding free energy of Cl⁻ to the Cl⁻ site without the presence of the substrate. The Cl⁻ binding reference pathway was obtained from 5HT-SERT simulations. Using the distance of Cl⁻ to the binding site as the collective variable, 73 equally spaced umbrella sampling windows from 0 to 18 Å

at 0.25 Å intervals. At each window, the Cl⁻ ion was restrained by a harmonic potential with spring constant 10 kcal/mol-Å². The umbrella sampling simulations were implemented using the Amber18 package.¹¹⁹ Each window was equilibrated for 10 ns and followed by 25 ns production. The free energy was constructed using the weighted histogram analysis (WHAM) software¹²⁰ and averaged over 5 independent runs.

Plasmid construction.

Human SERT was PCR amplified from pcDNA3-*hSERT* (Addgene 15483)¹²¹ to introduce a consensus Kozak sequence and ligated into the Kpn1-Xho1 sites of pCEP4 (Invitrogen). Alanine substitutions were introduced by overlap extension PCR, and all plasmid sequences were verified.

Transport Assay.

Expi293F cells (ThermoFisher) were cultured in Expi293F expression medium (ThermoFisher) at 37°C, 8% CO₂, 125 rpm, and transfected with 500 ng plasmid per ml at a density of 2 x 10⁶ cells/ml using Expifectamine (ThermoFisher). 24-28 hr post-transfection, cells were washed with PBS-BSA (Dulbecco's phosphate-buffered saline supplemented with 0.2% bovine serum albumin), incubated with fluorescent substrate analogue APP+ (Aobious) in PBS-BSA for 2 min at room temperature, washed twice with ice cold PBS-BSA, and analyzed on a BD LSRII cytometer. The main cell population was gated by forward and side scatter properties to exclude debris and doublets, and 10,000 gated events were collected. Cell fluorescence was measured with a 488nm laser, 505 LP dichroic mirror, and 530/30 bandpass filter. Fluorescence corresponding to non-specific uptake by vector-transfected cells was subtracted, and activity of mutants was assessed relative to the wild type construct.

Immunoblot.

Cells were collected 24 hr post-transfection, solubilized in SDS loading dye, and proteins were separated by SDS-polyacrylamide gel electrophoresis. After transferring proteins to polyvinylidene fluoride membrane (Bio-rad) at 4°C, the membrane was blocked for 1 hr at room temperature with tris-buffered saline containing 0.1% Tween 20 (TBST) and 3% skim milk powder. The membrane was incubated with 1:1,000 anti-SERT rabbit polyclonal (Abcam Cat. No. ab102048) in TBST containing 1% milk overnight at 4°C, washed 3 times with TBST for 5 min, and incubated with 1:10,000 HRP conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) in TBST containing 1% milk for 1 hr at room temperature. The membrane was washed three times with TBST for 5 min, and developed using clarity western ECL substrate (Bio-rad). The signal was visualized using Gel Doc (Bio-rad).

Molecular Docking.

Docking was performed using AutoDock 4.2.¹²² The PDBQT format files for the SERT structures and APP+ was obtained using AutoDock python utility modules. The grid center was chosen by selecting the residues within 6 Å of the ligand and grid maps were generated using AutoGrid. The ligands were docked into the active site using AutoDock. The default Lamarckian Genetic algorithm was used for performing energy evaluation of the ligand conformations.¹²³ The docked poses were visualized in PyMOL (Schrödinger, LLC).

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Notes

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Abbreviations

SERT, serotonin transporter; 5HT, 5-hydroxytryptamine; DAT, dopamine transporter; NET, norepinephrine transporter; NSS, neurotransmitter:sodium symporter; TM, transmembrane; LeuT, leucine transporter; OF, outward-facing; OC, occluded; IF, inward-facing; EPR, electron paramagnetic resonance; smFRET, single-molecule fluorescence resonance energy transfer; *d*DAT, *Drosophila* dopamine transporter; *h*SERT, human serotonin transporter; EL, extracellular loop; HDX, hydrogen-deuterium exchange; MSM, Markov state model; APP+, 4-(4-dimethylamino)phenyl-1-methylpyridinium.

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