A Conserved Asparagine Residue in Transmembrane Segment 1 (TM1) of Serotonin Transporter Dictates Chloride-coupled Neurotransmitter Transport

Received for publication, April 13, 2011, and in revised form, June 30, 2011 Published, JBC Papers in Press, July 7, 2011, DOI 10.1074/jbc.M111.250308

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Na+- and Cl−-dependent uptake of neurotransmitters via transporters of the SLC6 family, including the human serotonin transporter (SLC6A4), is critical for efficient synaptic transmission. Although residues in the human serotonin transporter involved in direct Cl− coordination of human serotonin transport have been identified, the role of Cl− in the transport mechanism remains unclear. Through a combination of mutagenesis, chemical modification, substrate and charge flux measurements, and molecular modeling studies, we reveal an unexpected role for the highly conserved transmembrane segment 1 residue Asn-101 in coupling Cl− binding to concentrative neurotransmitter uptake.

Chemical signaling by neurotransmitters in the nervous system depends upon the opposing mechanisms of vesicular release and transporter-mediated neurotransmitter clearance (1). In humans, two gene families, designated SLC1 and SLC6, compose the majority of transporters responsible for neurotransmitter inactivation (2–4). Whereas the SLC1 family encodes multiple transporters that inactivate the brain’s predominant excitatory neurotransmitter, 1-glutamate, the SLC6 gene family includes transporters that clear the brain’s major inhibitory neurotransmitters, γ-aminobutyric acid and glycine, as well as transporters for the neurotransmitters dopamine, norepinephrine (NE),6 and serotonin (5-hydroxytryptamine, 5-HT). The human 5-HT transporter (SLC6A4, hSERT) is of particular clinical significance, being a target for psychostimulants, including cocaine and 3,4-methylenedioxymethamphetamine (“ecstasy”), as well as the site of action for widely prescribed 5-HT selective reuptake inhibitors (5) used in the treatment of mood disorders.

hSERT, like other neurotransmitter transporters of the SLC1 and SLC6 families, displays secondary-active substrate transport (6), coupling the concentrative movement of neurotransmitter to the transmembrane gradients of Na+ and other ions (7). A distinguishing feature of neurotransmitter transport by the SLC6 family relative to the SLC1 family is a strong dependence on extracellular Cl− for transport (8, 9). During a single cycle of substrate transport in hSERT, a stoichiometry of 1–5-HTin:1–Na+in:1–Cl−in:1–K+ out has been advanced on the basis of ion dependence studies in cells and resealed membrane vesicles that predicts an overall electroneutral coupling mechanism. Such studies have led to the belief that the energy stored in the Cl− concentration gradient contributes directly to transmembrane 5-HT flux (7, 10). However, electrophysiological experiments reveal that SERT can exhibit nonstoichiometric flux states where additional 5-HT-induced charge movements occur (10–13). For the hSERT homolog dopamine transporter, Cl− has been implicated as a charge carrier in nonstoichiometric flux states, (14, 15) suggesting that the role of Cl− in neurotransmitter transport is more complex than originally sus-

6 The abbreviations used are: NE, norepinephrine; 5-HT, 5-hydroxytryptamine; hSERT, human serotonin transporter; rSERT, rat serotonin transporter; MTS, methanethiosulfonate; MTS2, 2-(trimethylammonium) ethyl methanethiosulfonate hydrobromide; TM, transmembrane segment; EL, extracellular loop; MD, molecular dynamics simulations; FEP, free energy perturbation; ANOVA, analysis of variance; TEVC, two-electrode voltage clamp; NMDG-Cl, N-methyl-d-glucamine-HCl.
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Expected. Interacting proteins, including syntaxin 1A, can modulate the stoichiometry of charge movements across hSERT and other monoamine transporters (10, 15–17), changes that can alter neuronal firing rates (15, 17). In addition, SERTs are expressed early in the embryo (18, 19), and in many distinct membrane environments (e.g. neurons, placenta, lymphoblasts, platelets, and epithelial cells), where Cl− gradients can change over time (20, 21). Together these findings indicate that the contribution of Cl− to neurotransmitter transport deserves further investigation.

Recently, high resolution structures of a Cl−-independent SLC6 family member, the leucine transporter from *Aquifex aeolicus* (LeuTαα) (22), has afforded opportunities to elucidate details of neurotransmitter transporter ionic coupling. Although overall sequence identity between LeuTαα and neurotransmitter transporters is low, amino acid identity approaches 50% for residues surrounding the binding sites for leucine and Na+, propelling homology-guided structural studies. In addition, crystal structures of the transporters ApcT (23), BetP (24), VSGLT (25), and Mhp1 (26) that have no significant sequence homology to SLC6 family members exhibit the same helical packing pattern as LeuT (27). Therefore, a model-guided study of SLC6 family members may identify important mechanisms that are likely difficult to derive from patterns of sequence conservation.

In a prior study (28), we demonstrated that an asparagine (Asn-101) in hSERT transmembrane segment (TM) 1 tolerates substitution by cysteine and that cells transfected with N101C are sensitive to transport inactivation by positively charged cysteine-directed MTS reagents. Because this inactivation was largely eliminated by the presence of 5-HT, we proposed that Asn-101 might lie at or near the substrate-binding site. Asn-101-sensitive conformational movements, rSERT Asn-101 replacements assays, NMDG-cl−-free assay buffer (same as chloride-containing buffer except 120 mM NaCl is replaced with 120 mM NaX) and hSERT C109A backgrounds was performed using the chloride-binding assay buffer (5.4 mM potassium gluconate, 1.2 mM calcium gluconate, 7.5 mM HEPES, and either NaCl or NMDG-cl− at 120 mM) cl−-free assay buffer (same as chloride-containing buffer except 120 mM NaCl is replaced with 120 mM NaX (X = Br, I, NO2,NO3, thiocyanate, acetate, methanesulfonate, or gluconate) and assayed for [3H]5-HT (5-hydroxy[3H]tryptamine trifuoroacetate, (121 Ci/mmol) Amersham Biosciences) transport as described previously (28). For Na+ replacements assays, NMDG-cl− was used in place of NaCl. Transport was linear with time under these conditions for up to 15 min. Saturation kinetic profiles for derivation of 5-HT Km and Vmax values were established in 24-well plates as described above except 2-fold serial dilutions were used maintaining 5-HT-specific activity, starting at 5 μM of a mixture of labeled and unlabeled 5-HT. Transport assays were terminated by three washes with ice-cold assay buffer, and cells were then dissolved in MicroScint 20 (Packard) scintillation fluid. Uptake from mock-transfected cells was subtracted from transporter-transfected cells to determine specific uptake. Nontransfected cells exhibited comparable uptake to assays performed in the presence of 1 μM paroxetine, 1 μM RTI-55, or 100 nM cocaine. Km and Vmax values were derived using a nonlinear curve fit as a function of 5-HT or NE concentration (40 nM to 5 μM) (Prism 4 for Mac, Graphpad software). All experiments were performed in triplicate and repeated in three or more separate assays.

**Spontaneous 5-HT Efflux**—Cells were loaded as described above for transport studies with 40 nM [3H]5-HT. Loading was allowed to proceed for 30 or 90 min at 37 °C and was terminated by aspiration of assay buffer and a single wash with 0.5 ml of ice-cold MKRHG buffer. MKRHG buffer (0.5 ml) was added to one-half of the wells and returned to 37 °C for 30 min. The wells that did not receive buffer represent the total 5-HT taken up at T = 0. Buffer from the efflux wells (representing 5-HT efflux) was collected, transferred to scintillation vials with 5 ml of EcoScint H, and counted. 5-HT remaining in the cells at T = 30 or 90 min was assessed by scintillation spectrometry. Percent efflux was calculated as the ratio (×100) of 5-HT efflux divided by 5-HT accumulated in parallel plates, not subjected to efflux, at T = 0. No difference was observed in % efflux for plates pre-loaded for 30 or 90 min (data not shown).

**Total and Cell Surface Expression Protein Analysis**—To determine total and surface expression of hSERT with wild type and/or mutant constructs, HeLa cells were plated in 24 or 12 well dishes at 100,000 or 500,000 cells per well, respectively, and transfected 18–24 h later as detailed above. Seventy two hours after transfection, cell surface proteins were biotinylated and...
analyzed via Western blotting as described previously (28). Oocyte biotinylation experiments to quantitate surface expression of hSERT and hSERT mutants were performed as described previously (12) using 1.5 ng of cRNA and substituting EZ-Link Sulfo-NHS-biotin (Pierce) with EZ-Link Sulfo-NHS-SS-Biotin. Blots of total and surface protein were probed with ST-01 from Mab Technologies, Inc. (Stone Mountain, GA), and developed using Western Lightening Chemiluminescent Plus reagent (PerkinElmer Life Sciences).

Evaluation of Cysteine Accessibility—To probe for ion-dependent hSERT conformational movements, HeLa cells were plated on poly-L-lysine-coated 24-well TopCount plates at a density of 50,000 cells/well and transfected as described above. Twenty four hours post-transfection, cells were washed once with 2 ml, one time with 1 ml, and one time with 500 μl of MKRH (MKRHG without glucose) ± 120 mM Cl⁻ and ± 50 μM 5-HT. Following a 5-min incubation, solutions were aspirated and replaced with 500 μl of 2 mM MTSET in MKRH. MKRH alone was added to one set of wells as a control. MTSET-treated wells were washed twice with 750 μl of MKRH followed by aspiration and addition of 225 μl of MKRHG. Cells were allowed to equilibrate to 37 °C for 10 min followed by addition of 25 μl of 200 mM [3H]5-HT containing 100 mM ascorbic acid/HCl and iproniazid phosphate. After 10 min at 37 °C, wells were washed three times with 500 μl of ice-cold MKRH. MicroScint 20 (0.5 ml) was added to each well, and the accumulated 5-HT was quantified.

hSERT N101A/S404C accessibility was examined using MTSEA. HeLa cells were plated in 96-well plates at a density of 50,000 cells/well and transfected and infected with vTF7-3 as described above. Twenty four hours post-transfection, cells were washed three times with 500 μl of PBSCM. After 10 min, the cells were washed five times with 100 μl of PBSCM followed by incubation with 20 mM [3H]5-HT for 10 min in 50 μl of PBSCM. After 10 min, wells were washed three times with 100 μl of PBSCM. OptiFluor (PerkinElmer Life Sciences) (150 μl) was added to each well, and accumulated 5-HT was measured in a Wallac MicroBeta plate counter. Cells transfected with S404C was used as the control.

hSERT Expression in Xenopus laevis Oocytes—Oocytes were isolated, and cRNA was prepared as described previously (12, 30). cRNA was injected on the day of oocyte harvest. hSERT, N101A, N101C, S336C, N101A/S336C, and N101C/S336C cRNA were transcribed from NotI-linearized constructs in pOTV vector (a gift of Dr. Mark Sonders, Columbia University) using Ambion mMessage Machine T7 kit (Ambion, Austin, TX). Each oocyte was injected with 1.5 ng of cRNA and incubated at 18 °C for 4–6 days in Ringer’s buffer (100 mM NaCl, 2 mM KCl, 5 mM MgCl₂, 5 mM HEPES, pH 7.4) supplemented with 550 μM/mL sodium pyruvate, 100 μg/mL streptomycin, 50 μg/mL tetracycline, and 5% dialyzed horse serum. Whole-cell currents were measured by two-electrode voltage clamp techniques using a GeneClamp 500 (Molecular Devices, Palo Alto, CA). Micro-electrodes were pulled using a programmable puller (Model P-87, Sutter Instrument, Novato, CA) and filled with 3 M KCl (0.5–3-megohm resistance). A 16-bit A/D converter (Digidata 1322A, Molecular Devices) interfaced to a PC computer running Clampex 9 software (Molecular Devices) was used to control membrane voltage and to acquire data. To induce hSERT-associated current, oocytes were perfused with 5-HT (typically 5 μM) in buffer (120 mM NaCl, 5.4 mM potassium gluconate, 1.2 mM calcium gluconate, 7.2 mM HEPES, 0.1 mM iproniazid, pH 7.4) using a gravity flow system (4–5 ml/min). Buffer pH was adjusted with KOH or KHPO₄. The 5-HT-induced current was defined as current in the presence of 5-HT minus current in the absence of 5-HT. Substitution of Cl⁻ was performed as with the tissue culture studies above and are indicated in the text and figures. To minimize liquid junction potentials, Cl⁻ substitution experiments were performed using a 1 mM KCl, 2% agar salt bridge to isolate the Ag-AgCl electrode from the bath. For constant voltage recordings, data were low pass filtered at 10 Hz and digitized at 20 Hz. For current–voltage (I–V) recordings, the voltage was changed stepwise every 500 ms. Currents were low pass filtered at 100 Hz and digitized at 200 Hz. All analyses were performed using Origin 7 (OriginLab, Northampton, MA) and GraphPad Prism (GraphPad software, San Diego).

Simultaneous Measurement of 5-HT Uptake and 5-HT-induced Currents—Simultaneous measurement of 5-HT uptake and 5-HT-induced current was performed under voltage clamp conditions, using techniques described previously (31). Oocytes were perfused for 150 s under voltage clamp with 1 μM [3H]5-HT (specific activity = 3.12 Ci/mmol). To minimize loss of 5-HT, oocytes were perfused with ice-cold buffer for 250 s prior to removal from the chamber. The total charge movement was calculated by time integration of 5-HT-induced inward currents and related to the amount of 5-HT taken up in the same oocyte. Nonspecific 5-HT uptake was determined using water-injected control oocytes analyzed under the same conditions. Oocytes were solubilized with 200 μl of 0.1% SDS and 10 ml of EcoScint H (National Diagnostics, Atlanta, GA), and 5-HT accumulation was quantified by liquid scintillation spectrometry (Packard Instrument Co.).

hSERT Molecular Modeling—Molecular models for hSERT were generated using the template structure of LeuT_A (PDB ID 2A65) as described elsewhere (32). The binding mode for 5-HT identified as the one most consistent with available experimental data by Kaufmann et al. (32) was taken as the starting point for model refinement using the AMBER force-field. Briefly, ions were added to the energy-minimized model of 5-HT in complex with hSERT to generate refined models that either contained NaCl or omitted Cl⁻ (Na⁺ only). Two Na⁺ ions were added to both the Na⁺ (without Cl⁻) and NaCl models by superimposing the hSERT model reported by Kaufmann et al. (32) with the x-ray structure of LeuT_A and utilizing the coordinates of atom NA 752 (Na₁-binding site) and NA 751 (Na₂-binding site). For the NaCl model, a single Cl⁻ ion was centered on the region of the hSERT model occupied by the side-chain carboxyl group of residue Glu-290 in LeuT_A, as recently validated in studies of SERT and GAT (33, 34). Models
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RESULTS

hSERT Asn-101 Mutation Eliminates Cl⁻ Dependence of 5-HT Uptake—To examine a role of hSERT Asn-101 in the Cl⁻ dependence of 5-HT transport, we transfected HeLa cells with hSERT or hSERT N101A and N101C mutants and measured the effect of extracellular Cl⁻ on 5-HT transport saturation kinetics (Fig. 1). As described previously (44), removal of external Cl⁻ from the medium of hSERT-transfected cells resulted in a significant (5-fold) decrease in 5-HT transport V_max (NaCl, 0.068 ± 0.006 fmol/cell/min; sodium gluconate, 0.014 ± 0.002) and a significant (3.6-fold) increase in 5-HT K_m (NaCl, 0.9 ± 0.2 μM; sodium gluconate, 3.2 ± 0.36 μM, one-way ANOVA, Dunnnett’s post hoc, p < 0.002). Remarkably, the hSERT N101A and N101C mutants were largely insensitive to Cl⁻ substitution with gluconate (Fig. 1, b and c). Moreover, the 5-HT K_m values of N101A and N101C were not influenced by the presence of Cl⁻, unlike hSERT, and were comparable with the 5-HT K_m values obtained for hSERT in the presence of Cl⁻ (N101A + Cl⁻ 1.45 ± 0.4 μM, N101A-Cl 1.66 ± 0.2 μM; N101C + Cl⁻ 1.20 ± 0.3 μM, N101C-Cl 1.4 ± 0.2 μM; hSERT + Cl⁻ 0.87 ± 0.2 μM, hSERT-Cl 3.2 ± 0.4 μM). The rate of 5-HT transport by hSERT was a monotonic function of extracellular Cl⁻ concentration (Hill = 0.8 ± 0.1) with an EC50 of 7.5 mM (Fig. 1d), in line with previous studies of rSERT (45). Across the same Cl⁻ concentration range, the N101C and N101A mutants exhibited virtually no further stimulation of 5-HT transport.

Asn-101 Dictates Cl⁻-dependent Conformational Changes in TM1 and EL4—Asn-101 could dictate the Cl⁻ sensitivity of hSERT simply by stabilizing binding of the anion. Alternatively, Asn-101 may be required to translate anion binding into critical conformational changes linked to the Na⁺-coupled, 5-HT transport process. To examine these issues, we first examined the ability of Cl⁻ to alter aqueous exposure of three residues proposed to report steps in the transport cycle, Cys-109, Ser-404, and Ser-277 (Fig. 2). Each of these positions is distant from the proposed ion-binding sites, so changes in their accessibility are likely to reflect conformational changes because of ligand binding rather than direct binding itself. Cys-109 lies at the extracellular end of TM1 and is the major determinant of wild type SERT sensitivity to MTS reagents (Fig. 2) (46, 47). MTS-mediated inactivation of Cys-109 is sensitive to Na⁺ replacement with Li⁺ and is also modulated by 5-HT (46, 48), possibly a sign that this residue sits within a conformationally active domain that is mobilized by ion and neurotransmitter binding. We found that the presence of Cl⁻ significantly protected Cys-109 against inactivation by the positively charged MTS (Met) (Fig. 3a), independent of the presence of 5-HT. In contrast, hSERT N101A was not protected against MTS inactivation by Cl⁻, consistent with the lack of Cl⁻ dependence measured for N101A in 5-HT transport assays (Fig. 1). That Cys-109 was the target for inactivation by MTS in hSERT N101A, as opposed to another endogenous cysteine, we found that the C109A/N101A double mutant was insensitive to MTS (data not shown). Strikingly, whereas 5-HT had little or no ability to protect wild type hSERT against MTS inactivation in Cl⁻-free conditions, 5-HT provided significant protection to hSERT N101A (Fig. 3a) in the absence or presence of...
the anion. These data suggest that Asn-101 is required to transduce Cl\(^{-}\) binding to allow 5-HT-dependent conformational changes that involve TM1 and associated elements, as reported by Cys-109 modification. In the Asn-101 mutants, 5-HT induced a similar conformational change, but this effect did not require the presence of Cl\(^{-}\). Ser-404, located in the middle of EL4 (Fig. 2), has been proposed to report reorientation of another external component of the 5-HT permeation pathway (49). Unlike Cys-109 in hSERT, however, Cl\(^{-}\) substitution in the absence of 5-HT does not afford protection of S404C (in a C109A background) to the membrane-permeant MTSEA (Fig. 3), although Cl\(^{-}\) and 5-HT together do induce protection. We found that, just as with Cys-109, the aqueous accessibility of S404C in an N101A background is sensitive to 5-HT in either Cl\(^{-}\)-containing or Cl\(^{-}\)-free medium. These studies confirm previous data (49) that transport-linked conformational changes in EL4 are associated with the loading of all three substrates and demonstrate that mutation of Asn-101 allows just 5-HT and Na\(^{+}\)/H\(^{+}\) to trigger conformational changes in EL4. Cys-109 and Ser-404 are external reporters of conformational changes arising with substrate binding. In contrast, Ser-277 in TM5 (Fig. 2) is positioned to contribute to the cytoplasmic permeation pathway for 5-HT. Indeed, increased Ser-277 accessibility is believed to report an “open-to-in” conformation of the transporter (50, 51). Na\(^{+}\) and Cl\(^{-}\) are required for the 5-HT-dependent exposure of residues in the cytoplasmic permeation pathway of SERT (50, 51). Fig. 3c demonstrates that 5-HT increases the extent of MTSEA inactivation in rSERT S277C.
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FIGURE 3. Assessment of MTSET accessibility of targeted cysteines as a prediction of conformation change. Tissue culture cells expressing hSERT or hSERT N101A (a), rSERT S404C or rSERT N101A/S404C (b), and rSERT S277C or rSERT N101A/S277C (c) were pretreated with MTSET in the absence (open bars) or presence (black bars) of 5-HT (50 μM) and in the absence or presence of Cl− as indicated. Following treatment, 5-HT uptake assays were performed in normal uptake buffer to quantitate activity. Percent remaining activity is plotted and is defined as the amount of 5-HT uptake of MTSET- (a) or MTSEA (b and c)-treated cells as a percent of untreated cells. A two-tailed t test was performed on sample sets as indicated with brackets, *, p < 0.05; **, p < 0.01; ***, p < 0.001. n.s., not significant.

only when Cl− is present. In the N101A/S277C double mutant, however, 5-HT alone increased the extent of MTSEA inactivation demonstrating Cl− independence. As the sensitization of S277C afforded by 5-HT in the N101A background still required the presence of Na+ (data not shown), the cytoplasmic pathway appears to still be coupled to Na+ with Asn-101 substitution, reinforcing a specificity for disruption of Cl− coupling in Asn-101 mutants.

hSERT Asn-101 Dictates Ion Selectivity of 5-HT-independent Charge Flux—hSERT expressed in X. laevis oocytes (52) and mammalian cells (53) conducts both 5-HT-independent and -dependent currents, in addition to transporting 5-HT. Both 5-HT-independent (“leak currents”) and -dependent currents require extracellular Na+ and Cl− (54, 55), although it is unknown whether the 5-HT-dependent and -independent currents share a common pathway and/or molecular contacts as they permeate SERT. To assess whether mutation of Asn-101 removes the Cl− dependence of SERT currents or affects stoichiometric charge movements, or both, we monitored hSERT-mediated currents in oocytes recorded under two-electrode voltage clamp. Prior to measuring currents, we determined the abundance of wild type and Asn-101 mutant hSERT expressed on the oocyte surface by biotinylation. Consistent with prior measurements of hSERT N101C surface expression in HeLa cells (28), whole-oocyte biotinylation studies revealed reduced cell surface expression of Asn-101 mutants (N101A and N101C, 61 ± 5.7 and 32 ± 8.7% of hSERT, respectively; Fig. 4d), although SERT-dependent transport and currents were readily detectable. In the oocyte biotinylation studies, a band was observed in the total (uninjected) control lane similar in size to mature hSERT. However, the band appears to be nonspecific as the same control lane lacks the immature and oligomeric hSERT bands observed in the hSERT-expressing oocytes, and this band is not observed in the surface control lane. As shown in Fig. 4a, 5-HT induced larger currents in Asn-101 mutants compared with wild type hSERT despite reduced surface expression. These currents were absent from mock-injected oocytes and were blocked by co-application of SERT antagonists (data not shown). Additionally, as first described by Mager et al. (56), antagonist (RTI-55) treatment of hSERT-expressing oocytes revealed a 5-HT-independent current that appears as an outward current at −60 mV (Fig. 4a) and that reverses at approximately +30 mV (Fig. 4b). The apparent outward current is interpreted as RTI-55 block of inward leak current. In contrast to 5-HT transport (56), the hSERT leak current was insensitive to extracellular Cl−, except at high positive potentials where a slight reduction in maximal outward current was evident (Fig. 4b). These data indicate that Cl− flux does not constitute a significant fraction of the 5-HT-independent current, consistent with the reversal potential +30 mV. Like hSERT, the N101A mutant was insensitive to external Cl−. However, in this mutant, the reversal potential for these currents (using RTI-55 to define the leak) shifted from approximately +30 to approximately +70 mV (Fig. 4c). The movement of ions during these experiments is insufficient to alter the internal ionic concentration of the oocyte, even less so in the presence of endogenous ion pumps. Thus, the shift in reversal potential we observe toward $E_{Na}$ is most likely an inherent effect of the mutant on the ion selectivity of 5-HT independent currents. In support of this hypothesis, replacing all Na+ with NMDG in normal Cl− buffer shifts the hSERT reversal negative by 40.1 ± 3.8 mV, p < 0.05, n = 7, whereas N101A shifts by 52.6 ± 3.3 mV (S.E.), p < 0.05, n = 6. These changes are consistent with a shift toward a greater contribution of Na+ to 5-HT independent currents in the N101A mutant than in hSERT.

Asn-101 Dictates Cl− Dependence of 5-HT-induced Currents— Similar to leak currents, currents elicited by 5-HT were significantly larger in the Asn-101 mutants than in wild type hSERT when normalized for surface expression (Fig. 5a). Even more striking was the loss of Cl− dependence for these currents in Asn-101 mutants (Fig. 5, a–d). In the hSERT N101C mutant, 5-HT actually induced slightly larger current ($I_{max}$) at negative potentials in the absence of Cl− and current decreased in response to increasing Cl− concentrations (Fig. 5d). These data can be explained if Cl− is still transported through the N101C mutant, offsetting the Na+ current. As described previously, 5-HT-induced currents in hSERT and rSERT did not reverse at positive potentials (52) because of outward leak currents that begin to dominate at positive potentials (Fig. 5b) (57). In contrast, a reversal of 5-HT-induced currents is evident at approximately +75 mV for both hSERT N101A and N101C. This finding is consistent with the loss of outward leak currents in the Asn-101 mutants at positive potentials (Fig. 5, c and d). Analysis of the Cl− concentration dependence of 5-HT-induced currents (−60 mV, 5 μM 5-HT) confirms that although 5-HT-
activated current in hSERT required Cl\(^-\) (EC_{50} = 0.5 \text{ mM}) At 101 mutants did not require Cl\(^-\) over the same concentration range (Fig. 5e). Unlike the shifted K_m value for 5-HT transport in the absence of Cl\(^-\) (Fig. 1a), the EC_{50} values for 5-HT-elicited currents in hSERT were unaffected by external Cl\(^-\) (1.6 versus 1.8 \text{ mM}, respectively) (Fig. 5f). The 5-HT EC_{50} values for N101A and N101C were also Cl\(^-\)-insensitive but were significantly decreased relative to hSERT values (N101A\_Cl 0.78 \pm 0.14 \text{ mM}, N101A_{+Cl} 0.64 \pm 0.11 \text{ mM}; N101C\_Cl 0.72 \pm 0.13 \text{ mM}, N101C_{+Cl} 0.39 \pm 0.05 \text{ mM}, p < 0.05 one-way ANOVA, Dunnett’s post hoc test) consistent with the lower K_m value for 5-HT measured in the mutants (Fig. 1).

**FIGURE 4. Current-voltage relationship analysis of the hSERT leak conductance of N101A mutant.** a, current traces of 5-HT-induced (5 \text{ mM} 5-HT) and leak current (revealed by application of the SERT antagonist RTI-55 (5 \text{ mM} RTI-55)) from oocytes expressing hSERT mutants. Oocytes were injected with equimolar amounts of cRNA. Steady-state I/V analysis of leak currents for hSERT (b) and N101A (c) recorded in Ringer’s buffer containing either 120 mM NaCl (E) or 120 mM sodium methanesulfonate (f). Values plotted represent the difference between conductance in buffer alone versus addition of 5 \text{ mM} RTI-55. Because of the low levels of leak current observed in the hSERT-expressing oocytes compared with the Asn-101 mutants, only hSERT-expressing oocytes showing relatively higher leak currents were used to have sufficient signal for the I/V analysis. The traces in a reflect the average observed leak for hSERT and Asn-101 mutants. d, Western blot analysis of total and surface expression of hSERT detected with hSERT-specific monoclonal antibody ST-01. Equal amounts of protein were loaded in each lane. A band of similar size to mature hSERT was noted in the total (uninjected) control lane. However, the band is nonspecific as the total control lane lacks the immature and oligomeric hSERT bands observed in the hSERT-expressing oocytes, and no bands are observed in the surface control (CTL) lane.

Asn-101 Dictates hSERT Coupling and Stoichiometry—To investigate the larger 5-HT-induced currents exhibited by the Asn-101 mutants, we determined 5-HT flux and total charge movements in hSERT and hSERT Asn-101 mutants in single voltage-clamped (−600-mV) oocytes (12). As shown in Fig. 6a, in normal extracellular Cl\(^-\), hSERT supported the net inward movement of ∼7 positive charges per 5-HT molecule, consistent with previous studies (10, 56). Removal of Cl\(^-\) caused a modest but significant increase in the flux ratio (∼10 charges/5-HT), arising from a relative retention of 5-HT-gated currents despite a reduction in 5-HT transport. Charge/5-HT flux ratios in the Asn-101 mutants were significantly greater than for
hSERT, in the presence or absence of Cl\(^{-}\), with \(-40\) charges moved per 5-HT. When normalized for transporter surface expression, this dramatic increase in the charge/5-HT flux ratio resulted from increased charge movement (rather than a reduction in 5-HT transport; data not shown), suggesting a disruption of coupling between transmembrane flux of Na\(^{+}\) (or other ions) and 5-HT. To test this idea, we returned to mammalian cells where the smaller internal volume facilitates an assessment of equilibrium accumulation of 5-HT. After 2 h of incubation in 50 mM 5-HT, hSERT-transfected HeLa cells established a 281-fold (\(14\)) gradient of 5-HT in/5-HT out (Fig. 6b). In contrast, the Asn-101 mutants concentrated 5-HT to a significantly lesser extent (N101A, 18-fold (\(1\)); N101C, 31-fold (\(1\)); \(p < 0.01\), one-way ANOVA and Dunnett’s post hoc analysis). Removal of the Cl\(^{-}\) gradient as a driving force in platelet plasma membrane vesicles led to a much smaller (\(\sim 50\%\)) decrease in 5-HT accumulation (58), consistent with an important role for Asn-101 in optimal coupling of 5-HT transport to the Na\(^{+}\) gradient. Moreover, when transfected cells with accumulated 5-HT were washed and then incubated in 5-HT-free medium, hSERT Asn-101 mutant cells exhibited significantly more efflux of 5-HT (hSERT 31 \(1.3\%\) in 30 min; N101A 76 \(1.7\%\) (\(p < 0.001\); N101C 71 \(1.1\%\) (\(p < 0.001\), one-way ANOVA and Dunnett’s post hoc analysis) (Fig. 6c and supplemental Fig. S9). Together, these data reveal that mutation of Asn-101 disrupts the ability of hSERT to utilize energy stored in transmem-

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**FIGURE 5. TEVC analysis of Asn-101 mutants.** Dependence of \(i_{\text{Na}}\) on Cl\(^{-}\) concentration. a, raw traces of Cl\(^{-}\) dose-dependent 5-HT-induced currents (5-HT, 5 \(\mu\)M). Cl\(^{-}\) addition is designated by bars along with concentration used. Anion concentration was adjusted with 120 mM with MS. Current-voltage relationship for the 5-HT-induced current reveals a reversal equilibrium for Asn-101 mutants. Steady-state currents evoked upon application of 5 \(\mu\)M 5-HT in the presence (**) or absence (**) of Cl\(^{-}\) and normalized to \% current obtained with 120 mM Cl\(^{-}\) at \(-60\) mV are plotted in relation to membrane potential for hSERT (b), N101A (c), and N101C (d). e, plot of induced current amplitudes from hSERT (**), N101A (\(\bullet\)), and N101C (\(\square\)) from a. Currents were normalized to current obtained at 120 mM Cl\(^{-}\). Cl\(^{-}\) was replaced with 120 mM methane sulfonate. f, plot of induced current as a function of 5-HT concentration in the presence (**) or absence (**) of Cl\(^{-}\) and normalized to percent current at saturation conditions in the presence of Cl\(^{-}\). Cl\(^{-}\) was replaced with 120 mM MS.
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brane ion gradients to support the intracellular accumulation of 5-HT.

Molecular Modeling Suggests a Mechanism for Asn-101 Participation in Ion-coupled 5-HT Transport—The lack of evidence for direct interaction between Asn-101 and Cl− (33, 34, 59, 60) suggests that Asn-101 effects derive not from Cl− binding. We investigated the possibility that Asn-101 served an essential role in propagating Cl− participation in Ion-coupled 5-HT Transport.

FIGURE 6. Ion-substrate coupling analysis of hSERT Asn-101 mutants. a, TEVC analysis of charge to substrate flux ratio. TEVC oocytes expressing hSERT or the Asn-101 mutants were exposed to [3H]5-HT in the presence or absence of Cl−. Induced current was monitored during the incubation period followed by quantitation of total 5-HT incorporated. Total current was converted to allowed to efflux for 30 min in MKRH buffer. % [3H]5-HT efflux is plotted and normalized by calculating the concentration ratio of [3H]5-HT inside the cell compared with the concentration in the buffer. The data were fit to a Michaelis-Menten nonlinear regression equation. b, steady-state uptake kinetics. [3H]5-HT (20 nM) uptake in NaCl-containing assay buffer by HeLa cells transiently transfected with hSERT (○), N101A (▲), N101C (■), or nontransfected (●) is monitored over 120 min. Data are normalized by calculating the concentration ratio of [3H]5-HT inside the cell with the concentration in the buffer. The data were fit to a Michaelis-Menten nonlinear regression equation. c, substrate efflux from cells preloaded with [3H]5-HT. HeLa cells transiently transfected with hSERT, N101A, or N101C are incubated with 20 nM [3H]5-HT for 30 min washed and allowed to efflux for 30 min in MKRH buffer. % [3H]5-HT efflux is plotted and determined by comparing [3H]5-HT remaining in the cells from efflux assay to duplicate samples halted prior to the efflux step. (***, p < 0.001).

The placement of 5-HT in our model is consistent with biochemical data that indicate coordination of the 5-HT amine by Asp-98 as well as sensitivity of 5-HT to substitution at various positions around the indole ring (62, 63). In hSERT, with Na+, Cl−, and 5-HT bound, our depiction illustrates these three substrates co-localized around the Na1 binding pocket through their coordination by residues of TM1, -2, -6, and -7 (supplemental Table S3). These four helices have been proposed to form a bundle whose movement within the protein closes the extracellular permeation pathway and opens a pathway to the cytoplasm (50). Our minimized structures also predict that Cl− and Na+ coordination is linked via dual interacting residues Ser-336 (TM6) and Asn-368 (TM7). 5-HT engages both the Na+ and Cl− coordination networks via a salt bridge provided by its ethylamine nitrogen. Asn-101 participates in Cl− interactions in these models through stabilization of Asn-368 in TM7 and Na1. To test predictions from the gas-phase computations, we constructed a solvated lipid membrane system with our SERT models and performed all-atom MD simulations with the CHARMM-27 force field (supplemental Fig. S7).

Using different methods (comparative modeling, docking, minimization, and then all-atom MD simulations), we consistently observed distinct backbone conformational shifts at residue Ser-336 in response to Cl− removal (Fig. 7, a and b and d) and e that result in a shift of hydrogen-bonding interactions of the Ser-336 side-chain OH away from interaction with the Asn-368 side-chain amide (Fig. 7, a and d) and e), where many residue contacts in TM1, -2, -6, and -7 are affected, including Tyr-121 and Ser-372. The multiple interactions of Asn-101 observed in hSERT are likely critical to coupling as they lead to an extensive hydrogen bond and coordination network around the bound ion substrates. Conversely, Cl− coordination of Asn-368 permits an interaction of the Ser-336 side-chain OH with Asn-368 via an improved geometry for coordination of the Na1 site Na+ ion. In turn, these interactions link TM2 and TM7 to TM1, where critical aspects of 5-HT coordination are located (residue Asp-98 and the backbone carbonyl of Tyr-95) (Fig. 7, a and d). Notably, our recent substituted cysteine accessibility method analysis of TM6 (64) revealed S336C exhibits the same phenotype as the N101C mutant in that it is sensitive to +charged MTS reagents and insensitive to −charged MTS reagents.

Importantly, analysis of MD trajectories allow us to predict the residue interaction changes likely to arise in Asn-101 mutants that allow for Cl−-independent 5-HT transport. Analysis of the inter-residue contacts from MD simulations predicted that the N101A mutation can considerably disrupt the H-bond interaction network found in the hSERT substrate-Na1 ion-binding pocket (supplemental Table S3 and supplemental Fig. S8). Coordination of Na+ by the Asn-101 side-chain amide oxygen is predicted to be lost as is the H-bond between 5-HT and Ser-336. A number of long-lived (stable) bonds connecting TM2 and −7 are also lost in the hSERT WT sodium only models (Fig. 7, b and e, and supplemental materials). However, the Ala-101 side chain, being considerably smaller than Asn-101, permits a local repacking of the residues forming the Na1-binding
site and displacement of the Na⁺ ion by ~2.0 Å (Fig. 7f, gray and magenta spheres). This change allows formation of a novel Na⁺ coordination site in which the backbone carbonyl of Ser-336 supplements the Ser-336 side chain, restoring WT-like ion coordination number (n = 6) with the backbone carbonyl of Leu-337 (TM6) forming an H-bond with the 5-HT ethylamine moiety. Interestingly, a conservative but bulkier N101Q mutation results in almost complete loss of function (data not shown) suggesting side chains larger than Asn may significantly impact interactions in the binding site. The Na1 site is further stabilized by Ser-336 forming an H-bond to the side chain of the Asn-368 amide. There is also a long living hydrogen bond (80% of all analyzed MD frames) between Tyr-121 and Ser-372 (TM6). These interactions effectively mimic the relative positioning of the same residues from TM7/TM2/TM6/TM1 found in the WT hSERT Cl⁻ ion coordination models (Fig. 7, a and d and e and f).

Effect of Cl⁻ and Different Mutations on Ion and Solute Binding to the Transporter—To better understand the role of Cl⁻ in binding of Na⁺ and 5-HT, we computed binding enthalpies using MM/PBSA approximation (65). The evaluation of binding enthalpies for mutants (101A and 336C) may help highlight the role of the anion in the transport cycle and its modulation by Asn-101 and Ser-336. The results from binding computations are summarized in supplemental Table S4. The ΔΔH for the Cl⁻-free WT protein shows significant inhibition of both Na⁺ and 5-HT binding using MM/PBSA approximation (65). The evaluation of binding enthalpies for mutants (101A and 336C) may help highlight the role of the anion in the transport cycle and its modulation by Asn-101 and Ser-336. The results from binding computations are summarized in supplemental Table S4. The ΔΔH for the Cl⁻-free WT protein shows significant inhibition of both Na⁺ and 5-HT binding using MM/PBSA approximation (65). The evaluation of binding enthalpies for mutants (101A and 336C) may help highlight the role of the anion in the transport cycle and its modulation by Asn-101 and Ser-336. The results from binding computations are summarized in supplemental Table S4. The ΔΔH for the Cl⁻-free WT protein shows significant inhibition of both Na⁺ and 5-HT binding using MM/PBSA approximation (65). The evaluation of binding enthalpies for mutants (101A and 336C) may help highlight the role of the anion in the transport cycle and its modulation by Asn-101 and Ser-336. The results from binding computations are summarized in supplemental Table S4. The ΔΔH for the Cl⁻-free WT protein shows significant inhibition of both Na⁺ and 5-HT binding using MM/PBSA approximation (65). The evaluation of binding enthalpies for mutants (101A and 336C) may help highlight the role of the anion in the transport cycle and its modulation by Asn-101 and Ser-336. The results from binding computations are summarized in supplemental Table S4. The ΔΔH for the Cl⁻-free WT protein shows significant inhibition of both Na⁺ and 5-HT binding using MM/PBSA approximation (65). The evaluation of binding enthalpies for mutants (101A and 336C) may help highlight the role of the anion in the transport cycle and its modulation by Asn-101 and Ser-336. The results from binding computations are summarized in supplemental Table S4. The ΔΔH for the Cl⁻-free WT protein shows significant inhibition of both Na⁺ and 5-HT binding using MM/PBSA approximation (65). The evaluation of binding enthalpies for mutants (101A and 336C) may help highlight the role of the anion in the transport cycle and its modulation by Asn-101 and Ser-336. The results from binding computations are summarized in supplemental Table S4. The ΔΔH for the Cl⁻-free WT protein shows significant inhibition of both Na⁺ and 5-HT binding using MM/PBSA approximation (65). The evaluation of binding enthalpies for mutants (101A and 336C) may help highlight the role of the anion in the transport cycle and its modulation by Asn-101 and Ser-336. The results from binding computations are summarized in supplemental Table S4. The ΔΔH for the Cl⁻-free WT protein shows significant inhibition of both Na⁺ and 5-HT binding using MM/PBSA approximation (65).
on ion binding affinity to Na1. In contrast, removal of Cl− from the N101A mutant led to relatively modest changes in the binding enthalpies for solute and ions to the Na1/Na2 sites as compared with WT. In the N101A system, both side-chain carboxylate oxygens of Asp-98 and the side-chain OH and backbone carbonyl oxygen of Ser-336 are now participating in the coordination shell for the Na+ ion bound to the Na1 site thereby compensating for the substitution at Asn-101 (Fig. 7f). Ser-336 contributes both main chain and side-chain oxygens to ion coordination in the N101A mutant (with and without Cl− bound). However, binding of Cl− to the N101A mutant destabilizes ion coordination at the Na2 site such that the affinity of Cl− for the transporter is reduced relative to that of a wild type transporter. Thermodynamic analysis of binding enthalpies suggest that Asn-101 plays an important role in modulation of binding affinity at the Na1 and Na2 sites as well as a contribution to the modulation of Cl− binding.

Validation of a Critical Partnership between Asn-101 and Ser-336 in Cl−-dependent 5-HT Transport—As noted above, our models suggest a critical relationship between Asn-101 in TM1 and Ser-336 in TM6 on the coupling of Na+ and Cl− binding to 5-HT transport. To test this hypothesis, we generated the Cys substitution S336C, reducing the length of the side-chain hydrogen bond donor because our model proposes that this side chain directly coordinates Cl− as well as Asn-368 (Fig. 7a) (22, 34). Indeed, although surface expression of hSERT S336C was 70% of hSERT (supplemental Fig. S6d), transport activity was only 11 ± 2.7% (n = 3) that of wild type. Importantly, the residual 5-HT transport activity observed with S336C was largely Cl−-independent and was actually enhanced by full anion replacement with acetate (supplemental Fig. S6, a–c).

The findings with S336C are similar to results obtained by Forrest et al. (34) who proposed Ser-372 was a Cl−-coordinating residue in SERT (Fig. 7a) and found that mutation of Ser-372 to the negatively charged Asp or Glu yielded Cl− independence. However, unlike N101C, S372C did not result in Cl−-independent uptake but rather yielded an increase in the apparent $K_m$ value for Cl−. This difference in Cys substitution at these two sites in terms of Cl− independence could be explained by the p$K_a$ at Ser-336 and Ser-372 in the absence of Cl−. Cys-372 is proposed to be charged in the reverse SH form (34). We constructed equilibrated and minimized hSERT models in a solvated membrane system and used free energy perturbation FEP analysis using dual topology methods (66) to evaluate p$K_a$ shift upon deprotonation for cysteines at the positions 336 and 372 relative to model solution (150 mM aqueous solution of NaCl). FEP simulations of the p$K_a$ shift for buried cysteines. These analyses reveal that in the presence of Na+ in the Na1 site, Cys-336 can be deprotonated at pH 7 with a large Δp$K_a$ shift of approximately −5.0. The experimental p$K_a$ for the Cys side chain is −8.0, and therefore the p$K_a$ for Cys-336 is −3.0, suggesting that there is a high probability that the side chain of Cys-336 is deprotonated with Na+ present at the Na1 site. However, analysis of Cys-372 shows the resulting Δp$K_a$ shift is approximately −0.9 and the net p$K_a$ for Cys-372 is −7.1. The reason for the apparent difference in protonation state at these two positions lies in the proximity to bound Na+.

![Figure 8. Kinetic analysis of 5-HT uptake in hSERT S336C and N101A/S336C mutants.](https://www.jbc.org/)

**DISCUSSION**

In our previous biochemical analysis of hSERT TM1, we suggested that the conserved residue Asn-101 was proximal to the substrate- and cocaine-binding sites (28). A position proximal to the substrate-binding site was later substantiated by the LeuT crystal structure (22) and in relation to the competitive antagonist cocaine by subsequent LeuT model-guided studies.
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of dopamine transporter (68). Interestingly, during the substituted cysteine accessibility method analysis, we found N101C could be modified by the positively charged reagent MTSET but not by the negatively charged MTSES (28). This distinction does not reflect a direct antagonism of MTSES by Cl\(^-\) as Asn-101 is not likely to be directly involved in Cl\(^-\) binding (34, 59, 60). In fact, Cl\(^-\)-free conditions fail to promote MTSES inactivation of the N101C transporter.\(^7\) The inability of MTSES to inactivate the N101C mutant may arise from repulsion by the transmembrane dipoles that are predicted to exist at the central unwound regions in TM1 and -6 (69). Moreover, four recent reports that identify Cl\(^-\)-binding sites and the coordinating residues in SERT and GAT1 do not implicate Asn-101 in this role (33, 34, 59, 60).

In this study, we provide several lines of evidence regarding a critical role that Asn-101 plays in coupling Cl\(^-\) binding to the conformational changes that are essential for Na\(^+\)-coupled 5-HT transport. In particular, our substituted cysteine accessibility method analysis reveals that the Asn-101 mutants no longer require Cl\(^-\) for 5-HT-induced conformational changes, providing more specific evidence for the involvement of Asn-101 in Cl\(^-\) dependence. The Asn-101 mutations increase non-stoichiometric charge movements carried by hSERT, both in the presence and the absence of 5-HT, indicating uncoupled ion movements. Conversely, although Asn-101 mutants transported 5-HT at initial rates comparable with that of hSERT, steady-state 5-HT accumulation was reduced ~90%, further indicating a loss of thermodynamic coupling between transmembrane gradients of ions and 5-HT. This loss of coupling was even more severe than would be expected simply from the contribution of a transmembrane Cl\(^-\) gradient to 5-HT accumulation, which was less than 2-fold in measurements with resealed vesicles expressing native SERT (58), suggesting a more profound defect in ion coupling in the Asn-101 mutants.

Slight differences in the extent of Cl\(^-\) substitution by the anions acetate (105%), methanesulfonate (78%), and gluconate (70%) correlate with their Stokes diameters of 4.5, 5, and 6.2 Å, respectively (supplemental Figs. S5 and S6), and suggest that Asn-101 substitution may impart changes in anion selectivity at the Cl\(^-\)-binding site in SERT. These results indicate that anions may still interact with hSERT in the Asn-101 mutants and are consistent with the presence of all previously proposed Cl\(^-\)-binding site residues (33, 34) in these mutants. Further evidence comes from Cl\(^-\) substitution with multivalent anions such as phosphate, resulting in poor functional replacement compared with monovalent species (~40% compared with Cl\(^-\)-containing conditions, data not shown). These data also suggest that the anion-binding site is still available, and its functional role would limit adoption of a Cl\(^-\)-like geometry (supplemental Fig. S4, a, c, d, and f). In N101A, the Asn-368 amide side chain is no longer constrained by the H-bond network imposed by Asn-101 and is thus free to interact with acetate in a manner that reproduces the ion coordination geometry of the WT NaCl model (supplemental Fig. S4, b and e).

More generally, our findings illustrate that both the direct interaction of Asn-101 with Asn-368 and the more indirect interaction with Ser-336 via coordination of the Na\(^+\) sodium ion suggest that establishment of physical interactions between TM1 and -6 is a critical facet of ion-coupled substrate movement. This idea is further supported and expanded by our inter-helical interaction energy analysis conducted on MD trajectories for WT and mutants (see supplemental material), which suggests that Cl\(^-\) removal has an adverse impact on transport by disruption of interactions between TM1 and -3, TM1 and -6, and TM5 and -8. In contrast, the removal of Cl\(^-\) is energetically much less disruptive to inter-helical interactions in the Asn-101 mutants. Recent crystal structure and MD studies suggest that the primary transport mechanism for the LeuT structural superfamily involves helical bundles that move relative to one another to allow entry to and exit from the substrate- and ion-binding sites (24, 50, 71, 72) via gating residues originally described by Yamashita et al. (22). However, there do exist disagreements between the models, and these differences may reflect mechanistic subtleties between transporters influenced by the substrates and ions involved.

Within the SLC6 family, some bacterial, insect, and mammalian family members differ in the identity of the residue homol-

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7 L. K. Henry and R. D. Blakely, unpublished observations.
ogous to hSERT Asn-101 and contain instead a His, Ala, Cys, Gly, Thr, Ser, or Asp (supplemental Fig. S2 and supplemental Table S2) (73), whereas all known Cl\(^{-}\)-dependent SLC6 neurotransmitter transporters contain Asn at this site. This observation suggests that this residue bears a critical role in promoting optimal coordination of sodium, Cl\(^{-}\), and substrate while limiting movement of additional charges, a role that appears relaxed in the Asn-101 mutants. As we and others (10, 14, 15, 17) have published that serotonin and dopamine transporter charge flux is an important contributor to neuronal excitability, we believe that the charge flux-limiting property of this residue is particularly important at synapses.

Neurotransmitter transporters are now known to exist in regulated protein complexes that can modulate multiple aspects of transport, including substrate affinity, membrane trafficking, and ion conductance states. Our observation of a >7-fold increase in Na\(^{+}\) flux in the Asn-101 mutants and the finding that syntaxin 1a interaction with the N terminus of SERT can modulate Na\(^{+}\) stoichiometry during the transport cycle (10) raise the possibility that syntaxin 1a binding may modulate transporter conductance states by orienting residues in TM1, likely including Asn-101, to restrict nonstoichiometric ion flow during the transport cycle. Although Ala (or Cys) can functionally replace Asn-101, the loss of optimal coupling is accompanied by dramatic increases in both leak and 5-HT-gated currents, properties that may be captured by syntaxin 1A-linked regulatory mechanisms. The remarkable >2-fold increase in 5-HT efflux from cells expressing the Asn-101 mutants also supports the idea that precise orientation of the amide side chain of this residue can control coupling between regulated protein complexes that can modulate multiple

REFERENCES

41. Brooks, B. R., Brooks, C. L., 3rd, Mackerrell, A. D., Jr., Nilsson, L., Petrella,
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Supplemental Figure-S1  Molecular Dynamic Analysis of N101A/S336C Double Mutant. These models were obtained starting from the NaCl model relaxed using 1ns of molecular with a second Na\textsuperscript{+} ion bound in the Na2 site (Yamashita et al. 2005) and the Dunbrack rotamer libraries in UCSF Chimera. Comparison with predicted rotamers and clashes at the S336C/N101A double mutant are indicated below (Table S1). Fewer clashes are predicted in S336C/N101A consistent with significant steric effects in the ion binding site that possibly contribute to the observed biological phenotypes in single and double mutants.

Supplemental Table S1. Rotamers of S336C in UCSF Chimera

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Supplemental Table-S2 Legend for figure S2 alignment. Table lists the organism, protein length, identity of relation to N101 and documented function of the sequences listed in figure S2. While only one sequence is displayed here with Ala at the position homologous to N101, Ala can be found in 27% of the prokaryotic members of the family identified to date.

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Supplemental Figure-S3 Na⁺ dependence of hSERT N101A and N101C mutants. Uptake of [³H]5-HT (20 nM) after incubation for 15 minutes in medium with and without Na⁺ (replaced with NMDG-Cl⁻) is plotted as (a) percent uptake of Na⁺ containing buffer and (b) raw cpm. CPM counts for N101A and N101C in panel b are ~30% those of the WT and are expected based upon the mutants surface expression relative to wild-type.
Supplemental Figure-S4 Molecular models of putative hSERT ion binding sites with Acetate. Using the same energy minimization and MD model relaxation protocol described in Methods section, the N101A and WT hSERT models were refined with manually docked acetate in the putative Cl ion binding site. The charged group of acetate was positioned to coordinate with the bound Na+ ion in the NA1 site. Comparative analysis of the resulting ion bindsite geometries with that of the WT NaCl model (Panel a) reveals similarity in the ion bindsite geometries of residues N368,S336,S372 and Y121 for the N101A/acetate model (Panel b). The WT/acetate model (Panel c) featured a different conformation for N368 due to interactions with N101 side chain and S336 backbone carbonyl moieties that apparently prevented hydrogen bond formation with acetate (ACT) to stabilize the ion coordination pattern observed in WT NaCl models (Panel a). Coordinates from the final minimized structures are shown in Panels d, e and f for three-dimensional reference. Note the N368 side chain conformer similarities (Panel d and e) and differences (Panel e and f) that apparently facilitate changes in ion coordination geometry in N101A mutant versus WT models.
Supplemental Figure-S5. Dose response of N101 and S336 mutants to anion replacement. Minimal buffers were used to analyze ability of acetate (AC-buffer), methanesulfonate (MS-buffer) and gluconate (GL-buffer) to replace Cl in a dose dependent manner. Buffers were prepared using plasma grade water (Aristar Ultra ®, BDH) to AC buffer (120 mM Na acetate, 4.7 mM K acetate, 2.2 mM Caacetate, and 10 mM HEPES, pH 7.4, glucose 1.8 g/L); MS-buffer (120 mM Na methanesulfonate, 4.7 mM K methanesulfonate, 2.2 mM Ca methanesulfonate, and 10 mM HEPES, pH 7.4, glucose 1.8 g/L); or MS-buffer (120 mM Na gluconate, 4.7 mM K gluconate, 2.2 mM Ca gluconate, and 10 mM HEPES, pH 7.4, glucose 1.8 g/L). Cl containing buffers where 120 mM NaCl was substituted for either NaAcetate, Na methanesulfonate or Na gluconate were mixed in appropriate ratios with the AC, MS or GL-buffer to obtain the Cl concentrations given above.
Supplemental Figure-S6 Anion replacement impact on 5-HT transport and substrate induced current in N101 and S336 mutants. \([^{3}H]5\text{-HT}\) uptake in HeLa cells was determined under Cl\(^{-}\)-replacement with the anions (a) acetate (AC), (b) methanesulfonate (MS) and (c) D-gluconate (GL) in hSERT and mutants as marked. \([^{3}H]5\text{-HT}\) was held at 30 nM. Data were normalized to uptake of Cl\(^{-}\)-containing buffer for each mutant. (d) Western blot analysis of cells used in panel a, b and c to verify similar levels of surface expression of hSERT mutants. Surface proteins were labeled with NHS-S-S-Biotin, purified were normalized to uptake of Cl\(^{-}\) (b) methanesulfonate (MS) and (c) D-gluconate (GL) in hSERT and mutants as marked. (e) TEVC analysis in oocytes of S336C, N101A/S336C and N101C/S336C double mutants plotting steady-state currents evoked upon application of 5 \(\mu\text{M}\) 5HT in response to Cl\(^{-}\) concentration. Currents were normalized to % current obtained with 120 mM Cl\(^{-}\) at -60 mV.
Figure S7. Root-mean-square deviations (RMSD) for simulations of hSERT protein systems. RMSD was performed on the backbone atoms at 2fs intervals for a duration of 15ns. The wild type system is shown in blue, with the chloride site occupied (dark blue) and vacant (light blue). The N101A mutants are shown in green with chloride site occupied (dark) and vacant (light). And lastly the S336C mutants are shown in orange with chloride site occupied (dark) and vacant (light). RMSD is shown in angstroms (Å) based on the deviation from the average structure and the time is shown in nanoseconds (ns) as a convention used in the MD simulations.

Root-mean-square deviation (RMSD) is one of primary and important screens used in validating the homology model and overall stability of the MD simulations. The RMSD is used to judge the stability of the model over the course of the molecular dynamics (MD) simulation relative to the starting structure. The length of the full MD for all six systems studied was 15 ns. As expected, the RMSD converges to a reasonable range of 2.5-3.0 Å in a very short time period for all the systems. The RMSD was calculated on the backbone atoms (C, CA, N, and O) on every second frame. That is a total of 1500 frames, equivalent to 15ns of simulation. Most of the systems during this time frame converged even below 2.5 Å regardless of their mutation or ion presence/absence. The only exception was S336C mutant with the chloride ion bound, which was on track till the last 3 ns when it jumped to just under 3.0 Å. In conclusion it is safe to say that the homology model, based on its RMSD convergence, indicate it is a viable system for present analysis and future studies.
Figure S8. Analysis of changes in inter-helical interactions ($\Delta E_{\text{int}}$) for (A) a Cl-free wild type SERT, (B) a Cl-bound N101A SERT mutant and (C) a Cl-free N101A SERT mutant transporter. Shown on the x-axis are residues of interest, comprising the inner/core region (TM1 to TM8). On the y-axis are the respective interacting residue. Each circle on the graph represents an interacting amino acid pair (x,y). Blue filled circles are favorable (positive $\Delta \Delta E$) interactions, while the empty circles are negative $\Delta \Delta E$. The size of the circles corresponds to the magnitude of the energy.
Supplemental Table S3. Ion coordination in hSERT modeled transporter systems.

WT:

Na\(_1\): A\(_{96}\) O, D\(_{98}\) O\(\delta\), N\(_{101}\) O\(\delta\), S\(_{336}\) O, S\(_{336}\) O\(\gamma\); n=6
Na\(_2\): G\(_{94}\) O, V\(_{77}\) O, L\(_{434}\) O, D\(_{437}\) O\(\delta\), S\(_{438}\) O\(\gamma\); n=5
Cl\(^-\): Y\(_{121}\) HH, Q\(_{332}\) He, S\(_{336}\) H\(\delta\), N\(_{368}\) H\(\delta\), S\(_{372}\) H\(\gamma\); n=5

WT: No Cl

Na\(_1\): A\(_{96}\) O (0.3), D\(_{98}\) O\(\delta\), N\(_{101}\) O\(\delta\), F335 O, S\(_{336}\) O, n=5.1
(due to coordination) S336 hydroxyl oxygen is not coordinating Na\(^+\) and it has no-
table effect on ion stability in Na1.
Na\(_2\): G\(_{94}\) O, V\(_{77}\) O, L\(_{434}\) O, D\(_{437}\) O\(\delta\); n=5 (bi-dentate coordination by D437)

N101A: No Cl

Na\(_1\): A\(_{96}\) O, D\(_{98}\) O\(\delta\), O\(\delta\), S\(_{336}\) O, O\(\gamma\), N368(O\(\delta\)), n=6 (S336 replaces 101)
Na\(_2\): G\(_{94}\) O, V\(_{77}\) O, L\(_{434}\) O, D\(_{437}\) O\(\delta\); n=5 (bi-dentate coordination by D437)

N101A: Cl

Na\(_1\): A\(_{96}\) O, D\(_{98}\) O\(\delta\), O\(\delta\), S\(_{336}\) O, O\(\gamma\), N368(O\(\delta\)), n=5.8 (S336 replaces 101)
Cl\(^-\): Y\(_{121}\) HH, Q\(_{332}\) He, S\(_{336}\) H\(\gamma\), N\(_{368}\) H\(\delta\), S\(_{372}\) H\(\gamma\); n=5

C336: Cl

Na\(_1\): A\(_{96}\) O, D\(_{98}\) O\(\delta\), O\(\delta\), N101(O\(\delta\)), C\(_{336}\) O, N368 O\(\delta\) (0.1), n=5.7

C336: Cl

Na\(_1\): A\(_{96}\) O, D\(_{98}\) O\(\delta\), N101(O\(\delta\)), C\(_{336}\) O, N368(O\(\delta\)), n=5.3

Supplemental Table-S4: Relative (to WT) enthalpies of binding (kcal/mol) for the solute
(5HT), co-transported ions to the transport

<table>
<thead>
<tr>
<th>Site</th>
<th>WT (Cl)</th>
<th>WT (no Cl)</th>
<th>N101A (no Cl)</th>
<th>N101A (no Cl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\Delta H)</td>
<td>(\Delta H)</td>
<td>(\Delta H)</td>
<td>(\Delta H)</td>
</tr>
<tr>
<td>Na(_1)</td>
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<td>+6.0</td>
<td>0.0</td>
<td>+0.9</td>
</tr>
<tr>
<td>Na(_2)</td>
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<td>+5.0</td>
<td>+5.5</td>
<td>+1.7</td>
</tr>
<tr>
<td>Cl</td>
<td>-7.4</td>
<td>N/A</td>
<td>+3.1</td>
<td>N/A</td>
</tr>
<tr>
<td>5HT</td>
<td>-37.0</td>
<td>+15</td>
<td>-1.8</td>
<td>+1.0</td>
</tr>
</tbody>
</table>
Figure S9. Pre-loading of Hela cells expressing hSERT and mutants with [3H]5-HT for efflux assay.
Hela cells transiently expressing hSERT, N101A or N101C mutant.
Figure S10. 5-HT uptake equilibrium experiment upon transfection with different amounts of transfecting DNA. Plots represent time course of [3H]5-HT uptake (5 to 120 minutes) with cells transfected with (a) 100 ng or (b) 300 ng of hSERT or mutant containing plasmid DNA. The results indicate that surface expression of SERT (or the mutants) does not significantly impact the extent to which 5-HT can be concentrated in the cell.
Supplemental Methods and Results:

Free Energy Perturbation Simulations: pKa computations:

pKa computations (see below) suggest that thiol group coordinating sodium ion may be deprotonated. The target relative free energy and its relation to pKa can be expressed as:

\[ \Delta pK_a = \frac{\Delta \Delta G}{2.3 k_B T}, \]

where

\[
\Delta \Delta G = \Delta G_{\text{protein}} - \Delta G_{\text{solution}} = (\Delta G_{\text{protein}}^{(u)} - \Delta G_{\text{solution}}^{(u)}) - (\Delta G_{\text{protein}}^{(p)} - \Delta G_{\text{solution}}^{(p)})
\]

The \( \Delta pK_a \) is estimated as a difference between protonated (p) and unprotonated (u) states isolated in solution and protein environment. To ensure accurate treatment of environmental effects we opted to perform free energy simulations as previously described by Simonson et al. and Li et al. for studies of membrane proteins. This protocol utilizes free energy perturbation to transform charges on the thiol group of cysteine into deprotonated thiolate. The starting structures for FEP perturbation have been extracted from MD simulations with neutral form of 336C and 372C systems. The setup of simulation has been identical to that used for equilibrium MD simulations. The reference environment was modeled as a cysteine molecule at the center of origin, cubic box 512 water molecules (TIP3P) and \( \text{Na}^+/\text{Cl}^- \) ions to mimic 150mM aqueous solution of table salt. Each FEP perturbation was run in 44 \( \lambda \)-windows (22 for forward and 22 for backward perturbations) with the thermodynamic coupling parameter \( \lambda \) varying between 0.0 and 1.0 by increments of 0.1. The integration time-step used was set to 1 fs. The FEP simulations time per window was set to 4 ns for better sampling. The free energy of protonation/deprotonation has been assessed using Weighted Histogram Method (WHAM).

Free Energy of Binding Computations: MM/PBSA simulations

Free energy of binding for solute and co-transported ions to the transporter were evaluated with a traditional MM/PBSA approach used previously for studies of LeuT. Briefly, a dielectric constant of 4 was assigned to protein, the membrane environment was modeled as a low dielectric continuum (\( \epsilon=2 \)) with thickness of 30 Å. The Poisson-Boltzmann equation was solved on a fine grid covering the whole protein with spacing set at 0.5 Å. The focusing method was used with a coarse grid spacing of 1.0 Å. The set of C27 charges were used together with atomic radii optimized for continuum electrostatics computations. The non-electrostatic interaction between protein and its ligands was evaluated with CHARMM27 force-field parameters. The binding free energy for WT and
mutants was expressed as the difference in self-energies (sum of electrostatic and van-der-Waals terms) between complex, protein and bound substrate. If the ligand of the interest is evaluated (for example 5-HT) then the complex would be comprised of the protein contain remaining ions/substrate bound e.g. occupied Na1/Na2 sites and Cl-, if present in the complex, is introduced in its binding site.

**Analysis of Inter-Helical Interaction Energies**

The effect of the mutation on the inter-helical ($\Delta \Delta E_{\text{int}}$) interaction energies was evaluated from 10,000 measurements extracted from production trajectory frames. The infinite cut-offs were used to compute electrostatic and van der Waals components of $\Delta \Delta E_{\text{int}}$. The interactions were computed over the last 10ns of the MD simulation averaging every 20 ps. This resulted in a total of 500 frames that were analyzed for all possible pair-wise amino acid interactions for each of the transmembrane helices forming transporter’s core (e.g. from TM1 to TM8). TM1 to 8 were used as the show highest sequence identity to the LeuT X-ray structure, also they comprise the core region which of greatest interest to us. The total energies where then averaged over all the frames for each interaction pair and a cut-off was set at -5 kcal/mol in accordance with previous studies. The data for each of the systems was organized into pair-wise interactions with a corresponding total energy.

**Stability of the protein in MD simulations.**

Root-mean-square deviation (RMSD) is a primary and important screen for validating homology models and overall stability of the MD simulations. The RMSD is used to judge the stability of the model over the course of the molecular dynamics (MD) simulation relative to the starting structure. The length of the full MD for all six systems studied was 15 ns. As expected, the RMSD converges to a reasonable range of 2.5-3.0 Å in a very short time period for all the systems (Figure S1). The RMSD was calculated on the backbone atoms (C, CA, N, and O) on every second frame. That is a total of 1500 frames, equivalent to 15ns of simulation. During this time frame, most of the systems converged even below 2.5 Å regardless of their mutation or ion presence/absence. The only exception was S336C mutant with the chloride ion bound, which was on track till the last 3 ns when it jumped to just under 3.0 Å. In conclusion, based on its RMSD convergence, the homology model used appeared to be a viable system for present analysis and future studies.

**Effect of Cl$^-$ and different mutations on ion and solute binding to the transporter: Binding Enthalpies**

To further our understanding of the role of Cl$^-$ in binding of sodium and serotonin, we computed binding enthalpies using MM/PBSA approximation. The evaluation of binding enthalpies for mutants (101A and 336C) helps to elucidate
the exact role of the anion in the transport cycle. The results from binding computations are summarized in Table S1. ΔΔH for the Cl⁻ free WT protein shows significant inhibition in both Na⁺ and serotonin binding to the transporter indicating an important role of Cl⁻ in the stabilization of entire binding pocket. The analysis of the ion coordination within the Na1 site shows that the anion-depleted transporter displays different ion coordination for Na1 than the anion bound complex. In particular, the side-chain of S336 no longer coordinates an ion and its coordination number is reduced from 6 to 5 ligands. Removal of Cl⁻ from the N101A mutant led to relatively modest changes in the binding enthalpies for solute and ions to the Na1/Na2 sites as compared to WT. In the N101A system the side-chain of N368 is now participating in the coordination shell for the Na⁺ ion bound to the Na1 site thereby compensating replacing N101. S336 contributes both main-chain and side-chain oxygens to ion coordination in the N101A mutant (with and without Cl⁻ bound). On the other hand, binding of Cl⁻ to the N101A mutant destabilizes ion coordination at the Na2 site such that the affinity of Cl⁻ for the transporter is reduced relative to that of a wild-type transporter. Thermodynamic analysis of binding enthalpies suggest that N101 may play an important role in modulation of binding affinity to sites Na1 and Na2 as well as partake regulation of Cl⁻ binding.

**Inter-helical interaction energies.**

Data from our study suggests that removal of Cl⁻ results in the disruption of several critical helix-helix contacts. To shed additional light on the anion stabilization of the binding pocket, we performed per-residue decomposition of the inter-helical interaction energies to elucidate structural elements affected the most by removal of Cl⁻ and/or by the N101A mutation. Figures S8 A,B and C shows change in the inter-helical interaction energies for different systems relative to WT. Each of the systems was then compared to its most closely related native counterpart. Thus, the wild-type chloride-free system was compared to the wild-type chloride bound system (Figure S8A), and the N101A mutants were compared to the wild-type with the same chloride present/free system (Figure S8B & S8C). When the two energies are subtracted the resulting number is the ΔΔE. A positive value corresponds to a more favorable interaction for the wild-type system, meaning that the given mutation or chloride removal resulted in that particular interaction becoming unfavorable (filled circle) or disrupted. On the contrary, an increase in the strength of the interaction due to a mutation or chloride removal results in a negative energy (empty circles).

Aside from the obvious effect on the coordination of Na⁺ at the Na1 site, removal of Cl⁻ from WT led to disruption of the entire interaction network between TM1 and TM3, TM1 and TM6, as well as having a major impact on interactions between TM5 and TM8. Obviously MD simulations with a homology models are
not expected to be exact, but they qualitatively show that removal of Cl\(^-\) has a strong effect on the overall stability of the transporter. The overall effect of Cl\(^-\) removal can be measured by \(\Delta\Delta E_{(\text{helix-helix})}\), which is \(~+90\) kcal/mol. Interestingly, Cl\(^-\)-free N101A mutant show less disruption in the inter-helical interactions compared to that of Cl\(^-\)-free WT and Cl\(^-\)-bound N101A system with \(\Delta\Delta E = +12.1\) kcal/mol vs \(~90\) kcal/mol and 29 kcal/mol, respectively. These numbers are indicative of the direction for the change and do not represent the “true” relative free energies.

**Ion coordination in WT and mutant proteins**

Ion coordination was determined by counting all contacts within a 3.5 Å sphere that roughly corresponds to the position of the first minima in ion-oxygen radial distribution function for Na\(^+\) and Cl\(^-\) ions in water. The numbers in parenthesis correspond to instances of non-unitary (<1) occupancy in the coordination shell.

REFERENCES: