Steric hindrance mutagenesis in the conserved extracellular vestibule impedes allosteric binding of antidepressants to the serotonin transporter*

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**Background:** The serotonin transporter contains an allosteric binding site with unknown location.

**Results:** We use molecular modeling, mutagenesis and zinc site engineering to locate and inhibit the allosteric binding site.

**Conclusion:** Data are consistent with the allosteric binding site being located in the extracellular vestibule.

**Significance:** This opens the search for a new generation of antidepressant drugs directed towards the proposed allosteric binding site.

**SUMMARY**

The serotonin transporter (SERT) controls synaptic serotonin levels and is the primary target for antidepressants including selective serotonin reuptake inhibitors (e.g. S-citalopram) and tricyclic antidepressants (e.g. clomipramine). In addition to a high-affinity binding site, SERT possesses a low-affinity allosteric site for antidepressants. Binding to the allosteric site impedes dissociation of antidepressants from the high affinity site, which may enhance antidepressant efficacy. Here we employ an induced-fit docking/molecular dynamics protocol to identify the residues that may be involved in the allosteric binding in the extracellular vestibule located above the central substrate binding site (S1). Indeed, mutagenesis of selected residues in the vestibule reduces the allosteric potency of S-CIT and CMI. The identified site is further supported by the inhibitory effects of Zn2+ binding in an engineered site and the covalent attachment of benzocaine-methanethiosulfonate to a cysteine introduced in the extracellular vestibule. The data provide a mechanistic explanation for the allosteric action of antidepressants at SERT and suggest that the role of the vestibule is evolutionary conserved among NSS proteins as a binding pocket for small-molecule ligands.
The neurotransmitter serotonin (5-HT) plays a critical role in the central nervous system where it modulates physiological and psychological functions such as mood, sleep, appetite and sexual drive (1). The serotonin transporter (SERT) mediates rapid reuptake of 5-HT following its release from presynaptic nerve terminals and is thereby essential for maintaining 5-HT homeostasis in brain. SERT belongs to the Neurotransmitter:Sodium Symporter (NSS) family that also includes transporters for the neurotransmitters dopamine, norepinephrine, glycine and γ-amino butyric acid (2). SERT is the main pharmacological target in the treatments of major depression and anxiety disorders. Both classical tricyclic antidepressants (TCAs), including e.g. clomipramine (CMI) and imipramine, and the selective serotonin reuptake inhibitors (SSRIs), including e.g. S-citalopram (S-CIT, Lexapro), sertraline (Zoloft) and fluoxetine (Prozac), exert their actions as potent inhibitors of SERT (3).

It has been a sustained goal in the mechanistic study of these compounds to gain insight into the structural basis underlying their action at SERT. Crystallization of the amino acid transporter LeuT, a bacterial homologue to the mammalian NSS proteins, provided the first insight into the tertiary structure of this transporter family (4). Interestingly, LeuT has been crystallized not only in its substrate bound form but also together with TCAs (5,6) or with the SSRIs fluoxetine and sertraline (7), for which LeuT was found to possess low affinity. These structures revealed a binding site for TCAs and SSRIs in the LeuT located to an extracellular vestibule (termed the S2 site) that is ~13 Å above the central substrate binding site (S1 site). Based on these observations and further mutagenesis of the corresponding site in SERT, it was proposed that the high-affinity binding site of TCAs and SSRIs is located in the aligned cavity, a putative S2 site in SERT (6-8).

However, this view was challenged by other studies strongly suggesting that both SSRIs (S-CIT, fluoxetine and sertraline) and TCAs (CMI, imipramine, and amitriptyline) are classical competitive inhibitors with their primary high affinity binding site located in the S1 site (9-14). Correspondingly, we have shown in the dopamine transporter (DAT) that the high-affinity binding site for both cocaine and the benzztropine class inhibitors is the S1 site (15,16).

In addition to the high-affinity binding site, it has been known for almost three decades that SERT also possesses a low-affinity allosteric site (17). Its existence was demonstrated by showing that several SERT ligands, of both the TCA and SSRI class, as well as 5-HT itself, can modulate the dissociation rates of other SERT ligands (18-21). For example, S-CIT has a marked allosteric effect resulting in dramatic inhibition of the dissociation of a high-affinity bound inhibitor such as S-CIT itself or other SSRIs (19,21). Notably, it has been suggested that this putative dual action of S-CIT at two binding sites in the SERT is responsible for the higher efficacy and faster onset observed in clinical trials for S-CIT as compared to racemic citalopram (CIT) (22-26). However, despite previous attempts to locate the binding site (27-30) the molecular mechanism underlying the allosteric effect of SERT inhibitors has remained essentially unknown. The site is considered distinct from the high affinity binding site since mutations that disrupt high-affinity binding of S-CIT to SERT did not affect the general allosteric effect of S-CIT (27). Conversely, a series of mutations in the transmembrane segment (TM) 10 and 12, which impaired the allosteric effect of S-CIT, did not affect its high affinity binding (28-30).

Here we provide evidence, by combining computational approaches with mutagenesis, Zn$^{2+}$ site engineering, and cysteine reactivity assays, that the allosteric binding site in SERT is in the vestibule located extracellular to the S1 site. Accordingly, our data support a model in which the dual action of S-CIT at SERT might involve the high-affinity S1 site as well as binding to the S2 site. This model also identifies the S2 site as an evolutionary conserved binding site for small molecule ligands in the NSS proteins, in line with the recent suggestion that S2 represents a secondary substrate binding site at least in LeuT (31,32).

**EXPERIMENTAL PROCEDURES**

**Molecular Modeling of SERT with ligand complexes and Zn$^{2+}$ binding sites** – The homology model of SERT was built based on the LeuT structure in an outward-occluded state (PDB:2A65). Using the protocols as previously described for our DAT models (33), the molecular...
dynamics simulations system with the SERT model immersed in the explicit water-lipid-water environment was established and then relaxed with molecular dynamics simulations. The available crystal structures of outward-occlude and outward-open LeuT have very limited differences, for example, the RMSD of all-Cα atoms between 2A65 and 3F3A is only 1.2 Å. Importantly, the conformational rearrangements may not be relevant in addressing the divergence between LeuT and SERT in the extracellular vestibule (see Supplementary Figure 1).

Thus, to characterize the simultaneous binding of two compounds in two binding sites we developed an integrated and iterative protocol that combines induced-fit docking (IFD) and molecular dynamics (MD). The IFD protocol (34) induces conformational changes in the binding site to accommodate the ligand and exhaustively identify possible binding modes and associated conformational changes by side-chain sampling and backbone minimization. To explore the conformational changes associated with the binding, and enable the sampling of structural rearrangements related to any allosteric coupling between the S1 and S2 sites, we performed MD runs using Desmond (35) on the resulting top-ranked IFD poses in order to equilibrate the complex and optimize the ligand-transporter interactions. If significant rotation and translation freedom was observed for the ligand in the MD simulation stage, we facilitated convergence by re-docking the molecule into the MD-altered binding site, using IFD and re-equilibration of the complex with MD. This iterative approach, described in the illustration below, was applied until the ligand binding mode was stable and converged for at least 30 ns. Its practical advantage is the increased sampling of binding modes so as to overcome the difficulties presented by the large molecular sizes of the inhibitors of SERT and their relatively low affinities for the S2 site. Moreover, the iterative relaxation and sampling through MD reduce the time required for convergence and the uncertainty associated with the use of a homology model (36) for SERT.

The iterative approach is briefly illustrated for the SERT configuration with S-CIT in the S1 site and CMI in the S2 site: i) an S-CIT molecule was first docked into the S1 site of the SERT model (36); ii) the top ranked ligand-transporter complex was re-immersed in an all-atom representation of a bilayer-water environment, and was subjected to 48 ns MD simulations; iii) a CMI molecule was subsequently docked in the S2 site, based on an equilibrated MD snapshot from stage (ii); iv) an MD simulation in explicit-solvent was carried out following the protocol described in (ii). For this particular case we observed a significant rearrangement of the interactions between the transporter and the CMI ligand that moved from its initial docking pose (~2 Å translation). To efficiently explore the CMI in its altered environment, based on the snapshot at the end of 6 ns, we reiterated step (iii) and then re-equilibrated the new complex for 48 ns in step (iv), which resulted in a more stable complex. For another construct, with S-CIT bound in both the S1 and S2 sites, there was no major rotation or translation of the S-CIT in the S2 site during the 60 ns run in the initial step (iv), precluding the need for an iteration back to step (iii).

The procedures for modeling of the Zn\(^{2+}\) binding sites and the covalent BZ-MTS complex are described in detail in the Supplementary Methods.

Site-directed mutagenesis - The hSERT was cloned into the pUbi1z expression vector using NotI and XbaI. Mutations herein were generated either using the QuickChange method (adapted from Stratagene, La Jolla, CA) or ordered through GeneArt (Regensburg, Germany). All mutations were confirmed by DNA sequencing.

Membrane preparation - Membranes were prepared from COS7 cells 2 days after transient transfection with WT hSERT or mutant plasmid using the Lipo2000 transfection protocol (Invitrogen) as described previously (15). After detachment, cells were lysed with two ultra-sound bursts (Branson Sonifier with microtip) in membrane buffer (120 mM NaCl, 5 mM KCl, 1.2 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 25 mM HEPES, pH 7.5) and pelleted (4900xG, 10' (Sigma SK15 swing-out rotor)) and resuspended in membrane buffer containing 0.3 M sucrose.

\[^{3}H\]S-CIT dissociation rate assay - Dissociation rates were measured as previously described (27). In brief, \[^{3}H\]S-CIT (15-20 nM, 70 Ci/mmol) was added to the membranes in final volume of 400 µL...
and incubated at 0°C for 30 minutes in binding buffer (25 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl2, 1.2 mM MgSO4, 1 mM L-ascorbic acid, 5 mM D-glucose, pH 7.4). For the cysteine reactivity experiments, BZ-MTS (0.5 mM) was added and membranes were further incubated for 10 min. Dissociation was initiated by diluting the samples 12x in binding buffer containing 0.1 μM paroxetine (note that 0.1μM paroxetine has no allosteric effect on [3H]S-CIT (27)) and S-CIT or CMI in the indicated concentrations. For the DAT constructs, 6 nM [3H]CFT (WIN 35,428, PerkinElmer) and 50 nM nomifensine were used. The dissociation was assessed over 5 to 120 minutes and stopped by rapid filtration of the samples through GF/B filters using a Tomtec cell harvester and washed for 20s with ice cold 0.2 M NaCl. The amount of bound [3H]S-CIT were determined using a Wallac microplate scintillation counter. Non-specific binding was determined with 1µM paroxetine/nomifensine at 37°C for 90 min. The temperature for the dissociation rate was set for each mutant so that the precise T½ could be determined. Dissociation rates were determined in triplicate in at least three independent experiments for all constructs.

[3H]S-CIT binding experiments - The affinity of S-CIT to the high-affinity binding site in SERT WT and mutants was determined by the addition of 3-5 nM [3H]S-CIT in binding buffer together with increasing concentrations of S-CIT in the concentration range from 0.01 to 2500 nM using a consecutive factor 3 dilution (10 determinations in triplicate) in 96-well plates. Subsequently, membranes expressing SERT WT or mutants were added to a total volume of 400 μL. The binding mixture was incubated for 1 hour at room temperature and subsequently filtered, washed and counted as described for the dissociation rate assay. Non-specific binding was determined by adding 5 μM paroxetine.

[3H]5-HT uptake experiments - Uptake assays were performed using 5-[1,2-3H(N)]- hydroxytryptamine ([3H]5-HT) (28 Ci/mmol) (PerkinElmer). Transfected COS-7 cells were plated in 24-well dishes (10^5 cells/well) coated with poly-ornithine to achieve an uptake level of no more than 10% of total added [3H]5-HT. The uptake assays were carried out 2 days after transfection. Prior to the experiment, the cells were washed once in 500 μl of binding buffer at room temperature (RT). 5-HT was added to the cells in 10 concentrations from 1 nM to 1 mM equally distributes around the expected IC50 value, and uptake was initiated by addition of ~10 nM [3H]5-HT in a final volume of 500 μl. After 3 (for the WT) or 5 (for the mutants) min of incubation, the cells were washed twice with 500 μl of ice cold uptake buffer, lysed in 250 μl 1% SDS and left for 30 min at 37 °C. All samples were transferred to 24-well counting plates and 500 μl of Opti-phase Hi Safe 3 scintillation fluid (Perkin Elmer) was added followed by counting of the plates in a Wallac Tri-Lux β-scintillation counter (Perkin Elmer). Non-specific uptake was determined in the presence of 5 μM paroxetine. All determinations were performed in triplicate.

Data calculations - The allosteric potency was calculated as described previously (29). The calculated dissociation rate constants (k_{drug}) at different S-CIT or CMI concentrations are expressed relative to the dissociation rate constant without the presence of unlabeled ligand (k_{buffer}). The allosteric potency was determined as the drug concentration that impairs the dissociation rate by 50% compared with dissociation in buffer. IC50 values were calculated from concentration effect curves of normalized dissociation ratio (k_{drug}/k_{buffer}) versus log[drug] and are shown as mean values calculated from means of plC50 and the SE interval from the pIC50 ±S.E. All data were subjected to linear or nonlinear regression analysis using Prism 5.0 (GraphPad Software Inc., San Diego, CA).

RESULTS

Computational characterizations of S-citalopram or clomipramine binding in the S2 site - To test the hypothesis that the putative S2 site in SERT may be the long-sought allosteric binding site in SERT for inhibitors such as S-citalopram (S-CIT) and clomipramine (CMI), we developed an iterative induced-fit docking (IFD)/molecular dynamics (MD) protocol (see Experimental Procedures). Applying this protocol to characterize binding poses of either S-CIT or CMI in the S2 site with S-CIT bound in the S1 site, we sought to identify experimentally testable interacting residues in the
S2 site. To establish a proper context for the S2 binding, we first used the protocol to characterize the binding pose of S-CIT bound in the S1 site (S1:S-CIT). Compared to results from previous efforts our S1:S-CIT pose was very similar to the experimentally validated results (10,13) (see Supplementary Discussion). We then docked either S-CIT or CMI in the S2 site of the SERT model equilibrated with S1:S-CIT.

During the equilibration of our SERT model with S-CIT bound in both sites, when the poses of the ligands and surrounding SERT regions were stabilized, we observed the coordination of S2:S-CIT to include residues Leu99, Trp103 and Arg104 in TM1, Ile179 in TM3, Ala486, Val489 and Lys490 in TM10, Val236 and Leu237 in extracellular loop 2 (ECL2), and Gly402 in ECL4 (Fig. 1, A and B). The center-of-mass distance between the S1:S-CIT and S2:S-CIT was ~17 Å. In our equilibrated SERT complex with S2:CMI in the presence of S1:S-CIT, the binding pose of S2:CMI overlapped significantly with that of S2:S-CIT resulting in a similar interaction pattern (Fig. 1C). However, CMI protrudes less towards TM10 and ECL2 and has no direct interaction with Ala486 (Fig. 1C). Compared to the previously reported binding modes of CMI in the extracellular vestibule (8,14), both the location and orientation of the stabilized CMI in our SERT model are different, especially with the alkylation pointing towards Val489. The differences are likely due to the presence of a bound S-CIT molecule in the S1 site, which is unique in our study. Note that although the S2 site in SERT is comparable to the binding site for CMI found in LeuT (5), and they share a few common positions of coordinated residues, the S2:CMI in SERT is positioned more extracellular to the S1 site (Supplementary Fig. 1).

The coordination of S2:S-CIT and S2:CMI as seen in the results from the computations would obstruct the release of S1:S-CIT to the extracellular side along the proposed transport pathway (Supplementary Fig. 1) (31). We note, however, that unlike the reports from molecular docking of two substrates bound in the S1 and S2 sites in DAT (32), binding modes of S1:S-CIT in SERT do not seem to be affected by the presence or absence of the S2:S-CIT/CMI. The S2-bound inhibitors had no noticeable structural impact either on the SERT or on the position of the ligand in the simulations; rather they were simply situated so as to block the entry and exit route to the high affinity S1 site.

**Site-directed mutagenesis of proposed interacting residues in S2 decreases the allosteric potency of S-CIT and CMI** - To validate the SERT models with S2:S-CIT/CMI, we employed mutations that would be expected to cause steric hindrance (37,38) and/or affect the conformations of residues predicted to have side chain interactions with the bound molecules. Thus, we mutated residues from beneath (L99H in TM1), from the sides (W103H and R104K in TM1 and I179H in TM3), or from the top (A486E, V489H and K490A in TM10, V236X and L237X - where X is H, Y or E - in ECL2, and G402H in ECL4). The mutants were analyzed in [3H]S-CIT binding experiments on membranes from transiently transfected COS7 cells. All mutants except V236X and L237X displayed high affinity [3H]S-CIT binding, with the highest change in affinity observed for W103H (10-fold decrease for [3H]S-CIT as compared to WT (Table 1)), which suggests that the mutations do not severely compromise the integrity of the protein. The minor effects on high-affinity binding can likely be attributed to indirect conformational rearrangements, although we cannot rule out completely that a mutation such as W103H interferes directly with the high-affinity site. However, in the subsequent dissociation experiments, the differences in high-affinity [3H]S-CIT binding were canceled out when we calculated and compared the allosteric potencies. This makes it possible to compare the effect on allosteric binding for mutants with different [3H]S-CIT binding affinities.

To characterize the effect of the mutations in terms of their impact on overall transporter functionality, we also conducted [3H]5-HT uptake experiments. Most of the mutants showed [3H]5-HT uptake with K<sub>m</sub> values similar to SERT WT. However, L99H, I179H and G402H rendered the SERT devoid of any measurable [3H]5-HT transport despite preserved [3H]S-CIT binding (Table 1).

Because mutations of either Val236 or Leu237 produced complete loss of [3H]S-CIT binding activity (data not shown) they were not investigated further. The other mutants were evaluated in [3H]S-CIT dissociation experiments in the absence of additional unlabeled inhibitors.
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(see below). Interestingly, although the affinity for [\(^3\)H]S-CIT was largely unaffected, histidine introduced at positions 99 (L99H) or 179 (I179H) caused a decrease in the dissociation rate of [\(^3\)H]S-CIT (Supplementary Fig. 2 and Table 2). This suggests that the changes in volume distribution and polarity produced by the inserted histidine may cause an obstruction of the exit route from the binding site, likely analogous to the effect of an allosteric inhibitor. Interestingly, such an obstruction effect was not observed for mutations more distal to the S1 binding site like G402H and A486E (Supplementary Fig. 2 and Table 2).

The potency of S-CIT and CMI in inhibiting the dissociation of high-affinity bound [\(^3\)H]S-CIT was measured according to described methods (27,29) by pre-binding of [\(^3\)H]S-CIT to the membrane preparations followed by determination of [\(^3\)H]S-CIT dissociation rates in the absence or presence of increasing concentrations of unlabeled inhibitor (Fig. 2, A and B). Consonant with previous observations (30), S-CIT had a profound effect on [\(^3\)H]S-CIT dissociation (Fig. 2A). By plotting the data as the [\(^3\)H]S-CIT dissociation rate in the presence of inhibitor, relative to that in the absence of inhibitor (\(k_{\text{drug}}/k_{\text{buffer}}\)), it was possible to determine the allosteric potency of the ligands. In SERT WT, [\(^3\)H]S-CIT dissociation was inhibited by S-CIT with an allosteric potency of ~4.6 µM, and by CMI with a potency of ~23 µM (Fig. 2, C and D and Table 3).

In agreement with our S2:S-CIT model, L99H, W103H, R104K, I179H, G402H, A486E, and V489H mutations significantly reduced the allosteric potency of S-CIT (Fig. 2, C and E and Table 3). The strongest effect was seen for G402H leading to complete elimination of measurable allosteric effect of S-CIT (Fig. 2, B and C). Notably, Gly402 is aligned to Ala319 in LeuT that accommodates CMI binding to S2 in LeuT (5). Consistently, in our model of the G402H mutation, the introduced histidine residue protrudes into the extracellular vestibule, which should sterically hinder the binding of S2:S-CIT (Supplementary Figure 3). The R104K mutation did also show a drastic effect on [\(^3\)H]S-CIT dissociation (Fig. 2C and Table 3) causing a 50-fold reduction in allosteric potency. In addition, to being in the S2 site and contacting S-CIT directly, Arg104 also likely forms the extracellular thin gate with Glu493 (4,39). According to the LeuT crystal structures (4,39), the opening and closing of this gate, in association with the configuration changes of the conserved Phe334-Phe335-Tyr175-Tyr176 aromatic cluster, is assumed to play a critical role in the conformational transition. To characterize the impact of R104 mutation, we modeled and simulated the R104K and compared it with WT in the presence of S1:S-CIT. The subtle but significant difference between the packing of Lys104-Phe335 and Arg104-Phe335, is propagated into the S2 site by disrupting the Glu493-Tyr175 H-bond interaction. Consequently, the Tyr175 side chain rotates into the S2 site and partially fills the cavity (Supplementary Fig. 4). While high-affinity [\(^3\)H]S-CIT and [\(^3\)H]5-HT binding are retained in R104K, the transport activity (Table 1) is disrupted, with significantly reduced \(V_{\text{MAX}}\), suggesting the mutation has limited impact on the S1 site but conformational transition is impaired. The mutation of R104 to other residues, eg. R104A resulted in an inactive transporter (Table 1).

The effects of the remaining mutants were significant but more modest, showing a ~3 to 12-fold decrease in allosteric potency for S-CIT. Because both the basal [\(^3\)H]S-CIT dissociation and the allosteric potency were decreased by the L99H and I179H mutants, we reasoned that the effect of the double mutant would be even larger if both histidines cause a steric hindrance of the allosteric bound S-CIT. Indeed, the double mutant produces >20-fold decrease in allosteric potency (Table 3). A similar augmentation was observed when combining the three mutants in TM 10, i.e. A486E-V489H-K490A, with the allosteric potency found to decrease more than 20-fold to 106[88;127] µM (Table 3).

To exclude the possibility that all mutations in the extracellular vestibule would somehow affect allosteric binding, we made T178V. The side chain of Thr178 is exposed to the vestibule but it is not in direct interaction with S2:S-CIT in our docking results. Consistent with this prediction, the T178V is WT-like for all tested parameters (Table 1-3).

Because both the extracellular and intracellular face of the transporter are accessible when the dissociation experiments are performed on membranes, we performed as well [\(^3\)H]S-CIT dissociation experiments on whole cells. The experiments were done on cells expressing SERT WT or R104K. We chose R104K because it
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retained WT-like \(^{[3]}\text{H}\)S-CIT binding (IC\(_{50}\) = 5.2[5.0;5.5] nM) and had transport activity while at the same time markedly impairing allosteric potency. Importantly, the results were comparable to those obtained on membrane preparations ([3H]S-CIT dissociation from WT (T/2) = 16±3 and 34±6 min and R104K (T/2) = 22±6 and 20±4 min, without and with 50 µM S-CIT, respectively), supporting that \(^{[3]}\text{H}\)S-CIT dissociates from S1 via the extracellular vestibule.

An important agreement of the measurements with our modeling results is underscored by the parallel way in which the allosteric potency of CMI was affected by mutants that affected S-CIT, albeit to a lesser extent: The most pronounced effect was observed for R104K which caused a 17-fold decrease in allosteric potency relative to SERT WT (Fig. 2D). The G402H mutation resulted in 7-fold decrease, and a 2-4 fold decrease was observed for the L99H, W103H and I179H mutants (Fig. 2, D and F and Table 3). A486E caused only a minor, non-significant effect on the CMI potency (Fig. 2F and Table 3). This is entirely consistent with the computational models where Ala486 was found to be the only residue that coordinates S2:S-CIT, but not S2:CMI. The different impact of A486E on the allosteric potencies of S2:S-CIT and S2:CMI is thus explained by the observed difference in the binding modes of the two ligands in the same pocket. Another mutation on TM10, V489H, also had no significant effect on the allosteric potency of CMI, which is consistent with the observation from the simulations that S2:CMI protrudes less towards TM10 than S2:S-CIT, as described above.

Engineering of a Zn\(^{2+}\)-binding site in the vestibule impairs the allosteric potency of antidepressants - To gain further evidence for the location of the allosteric binding site and its relation to the S2 site, we engineered a Zn\(^{2+}\) binding site in the vestibule. Zn\(^{2+}\) binding should impair the allosteric effects of S-CIT and CMI in the pocket affected by the presence of the ion. The structural requirements for Zn\(^{2+}\) binding to proteins are well-defined (40) and engineered Zn\(^{2+}\) binding sites have been used before to impose intramolecular constraints in membrane proteins including NSS proteins (15,41-43). An analysis of the MD simulation trajectory of SERT showed that Zn\(^{2+}\) could be coordinated between Ile179 and Val489 after substitution of these residues by histidines (I179H-V489H). The results of computational docking of Zn\(^{2+}\) into this mutant construct, in the presence of S2:S-CIT (see Supplementary Methods), showed that coordination of Zn\(^{2+}\) close to the S2:S-CIT produced electrostatic repulsion towards the dimethylammonium group (Fig. 3A). A similar repulsion was observed between Zn\(^{2+}\) and S2:CMI (Fig. 3B). To verify these models experimentally we made the double mutant (I179H-V489H) and the single mutants (I179H and V489H) and investigated the effect of Zn\(^{2+}\) on the allosteric effects of S-CIT and CMI on \(^{[3]}\text{H}\)S-CIT dissociation. In agreement with the predictions, the application of 200 µM Zn\(^{2+}\) to I179H-V489H caused a significant decrease in the allosteric potency of both S-CIT (~4-fold) and CMI (~5-fold) (Fig. 3, C and D, and Table 4). This effect was not seen for the single mutants showing that both histidine residues are required to obtain the effect of Zn\(^{2+}\), consonant with the need for both to produce a binding site for the ion (Fig. 3, C and D insets, and Table 4).

To exclude the possibility that the effect of Zn\(^{2+}\) at I179H-V489H resulted from altering the affinity of \(^{[3]}\text{H}\)S-CIT for the S1 site, we assessed \(^{[3]}\text{H}\)S-CIT high affinity binding with and without Zn\(^{2+}\) (200 µM). This showed that Zn\(^{2+}\) had no significant effect on \(^{[3]}\text{H}\)S-CIT affinity (IC\(_{50}\) = 6.2[4.8;7.9] and 10[4.8;13] nM without and with Zn\(^{2+}\), respectively, means(SE interval), N=3, and Supplementary Fig. 5). Of note, Zn\(^{2+}\) itself did not affect the dissociation rate of the high-affinity bound \(^{[3]}\text{H}\)S-CIT in I179H-V489H mutant (T/2 for \(^{[3]}\text{H}\)S-CIT dissociation from SERT I179H-V489H is 25.6±1.5 min and 22.5±2.8 min without and with 200 µM Zn\(^{2+}\) present, respectively. Data are means±SEM, N=4), suggesting that coordination of the relatively small Zn\(^{2+}\) to this site is not sufficient to impose an allosteric effect on bound \(^{[3]}\text{H}\)S-CIT.

A cysteine reactive bulky reagent in S2 impairs the allosteric potency of S-CIT - To further substantiate the role of the SERT S2 site in the allosteric effect of S-CIT, we introduced a steric hindrance to S2:S-CIT binding in the form of the bulky cysteine reagent benzocaine-methanethiosulfonate (BZ-MTS) (Fig. 4A, inset). A cysteine was introduced in S2 (L99C in TM1) in...
a Cys-less SERT background (SERT-C) in which the only reactive cysteine on the extracellular face of SERT had been mutated (C109A) (44). Like in WT SERT, addition of S-CIT (40 µM) to both SERT-C and SERT-C L99C decreased the dissociation rate (and thereby increased the T½ for dissociation) of [1H]S-CIT. In SERT-C, the effect was slightly impaired by BZ-MTS, and BZ-MTS was found to have no effect in itself (Fig. 4B). In contrast, BZ-MTS impaired the allosteric effect of S-CIT in SERT-C L99C as reflected by a significant decrease in T½ for [1H]S-CIT dissociation (Fig. 4B). In addition, in the SERT-C L99C, the reactivity of BZ-MTS did have an effect by itself on [1H]S-CIT dissociation (T½ = 31.3±2.2 min and 21.7±1.6 min with and without added BZ-MTS, respectively). These data are consistent with our covalent docking model of BZ-MTS in SERT L99C (Fig. 4A).

A low-affinity inhibitor binding site in the extracellular vestibule of the dopamine transporter - We then investigated whether the presence of a low-affinity binding site for inhibitors in the extracellular vestibule of SERT can be generalized to other mammalian transporters. Thus we assessed whether CMI was able to inhibit the dissociation of pre-bound [1H]CFT ((-)-2β-carbomethoxy-3β-(4-fluorophenyl)tropane or WIN 35,428), a cocaine analogue, in DAT. As shown in Fig. 5, CMI impaired [1H]CFT dissociation from DAT with an allosteric potency of 167±188 µM (mean±SE interval, N=3). Remarkably, and similar to what we observe in the SERT, this effect was decreased by introducing the aromatic histidine residue at position 80 (which is aligned to Leu99 of SERT), resulting in a small but significant decrease in allosteric potency (555±471;655 µM; mean±SE interval, N=3) (Fig. 5).

DISCUSSION

For almost three decades it has been proposed that SERT possesses an allosteric binding site for antidepressants (17). The allosteric activity has been demonstrated for several antidepressants including the SSRIs (S/R-CIT, sertraline, fluoxetine and paroxetine) and the TCAs (CMI and imipramine) (19-21) as well as for 5-HT itself (17). A corresponding allosteric site has also been proposed to exist in the homologous norepinephrine transporter (NET) (20). However, the molecular mechanisms behind the observations, as well as the actual binding site(s), have not been identified. In this study, we used S-CIT and CMI as model compounds for SSRIs and TCAs, respectively, and combined computational modeling and mutagenesis studies to locate the allosteric binding site for these compounds in SERT (Fig. 1). We found that the allosteric binding site is in the extracellular vestibule and is analogous to the S2 site in LeuT (Supplementary Figure 1), which has been found to bind inhibitors (5-7) and suggested to constitute a second substrate-binding site (45).

Mutation of the residues predicted to protrude into the allosteric binding site region from below (TM1: L99H and R104K), from the sides (TM1: W103H, TM3: I179H and TM10: A486E, V489H and K490A), and from above (ECL4: G402H) indeed decreased the allosteric potency of both S-CIT and CMI to various degrees (Fig. 2). The most pronounced effect was seen for G402H in which the allosteric effect of S-CIT was eliminated (Fig. 2), suggesting that the side chain of histidine may be especially disruptive of an energetically acceptable positioning of the 1,3-dihydroisobenzofuran-5-carbotitrile moiety of S-CIT in close vicinity to position 402 (Supplementary Figure 3). This inference agrees with previous observations showing that histidines are particular efficient in producing steric hindrance by virtue of the aromatic and polar character of the side chain (37,38). The R104K mutation also caused drastic decreases in allosteric potency, for both S-CIT and CMI (Fig. 2). In addition to directly disrupting the binding of the S2-inhibitors, it is likely that the large decrease of allosteric potency of R104K is also due to the participation of Arg104 in the extracellular thin gate in SERT (forming a salt bridge to Asp493 in TM10), thus the R104K may bias the conformational equilibrium so that it is not optimal to bind S2-inhibitors (Supplementary Figure 4).

Interestingly, we observed that substitutions with histidines at positions 99 and 179 already reduced the basal dissociation rate of the high-affinity bound [1H]S-CIT (Supplementary Fig. 2 and Table 2), consonant with the high-affinity binding site being located beneath the two residues (9,11-13), which then impose a constraint on the exit route from the S1 site.
Some of our mutations in S2 (i.e. W103H and A486E) reduced high affinity binding to S1 (Table 1), but the impact on S1 affinity does not bias the comparison of allosteric potencies across different mutants, as it is accommodated into the calculations (see Supplementary Methods). It is possible that the affinity change is due to an allosteric effect that propagates from the S2 site and causes minor distortions of the adjacent S1 pocket.

The structural context of the allosteric effects we proposed was further strengthened by the results obtained from constructs in which a Zn\textsuperscript{2+} binding site was engineered in S2 (I179H-V489H). Zn\textsuperscript{2+} binding impaired the allosteric effect of CMI and S-CIT, consistent with an obstruction of S2:S-CIT/CMI binding without blocking the dissociation pathway of S1:S-CIT (Fig. 3). The possibility that Zn\textsuperscript{2+} causes a major structural rearrangement by coordinating between I179H in TM3 and V489H in TM10 and thus occluding the S2 pocket, is considered unlikely because the coordination requirements of the small and inert Zn\textsuperscript{2+} ion are very strict and the binding is reversible and of low affinity. Additionally, Zn\textsuperscript{2+} did not exert any measurable allosteric effect in I179H-V489H by itself, which argues further against a major conformational rearrangement in the S2 pocket.

In parallel to the Zn\textsuperscript{2+} experiments, we found that the cysteine-reactive compound BZ-MTS significantly impaired the allosteric effect of S-CIT in SERT-C L99C compared to SERT-C (Fig. 4). The modest size of the effect is possibly due to the low labeling efficiency, but we were unable to increase further the BZ-MTS concentration and the labeling time due to non-specific effects in the background mutation (SERT-C). Nonetheless, our data suggest that conjugation of BZ-MTS to Cys99 in TM1 results in protrusion of the benzocaine side chain into the vestibule and thereby partial obstruction of S-CIT binding in S2 (Fig. 4). Notably, this is analogous to the observation of a decrease in basal \textsuperscript{[3H]}S-CIT dissociation upon steric hindrance mutagenesis in the same position (L99H).

A chimera study has previously identified five residues in TMs 10 and 12 as critical for allosteric binding. Because TM12 (together with TM9) constitutes a putative dimeric interface according to the LeuT crystal structure, it was speculated that the identified allosteric residues may be associated with the dimer interface and therefore capable of transmitting signals between the binding sites in two monomers (28-30). However, the five residues are not predicted to form a well-defined binding pocket to accommodate small-molecule ligands. Therefore, in light of our current observations and supported by recent simulations (46), we surmise that mutation of the five residues indirectly affects the conformation of the extracellular vestibule and thereby the allosteric potency of S-CIT.

It is however unexpected that we do not observe larger effects in several of the described mutations. If the inserted residues caused ‘efficient’ steric hindrance, all mutations might be expected to have effects similar to G402H and R104K. As the vestibule is relatively large and the binding is of low affinity, it is possible that the binding modes of S2:inhibitors have tolerance for changes at non-essential positions, which can partially reduce the impact of the introduced mutation, e.g a bulky histidine could simply push the ligand slightly towards the other side of the vestibule causing only minor impact on allosteric potency.

The effects of some mutations on the allosteric potencies might be indirect – by binding to another site on the transporter, the ligand could potentially alter the relative abundance of different transporter conformations that in turn change accessibility to the primary binding site through the extracellular vestibule. In such a scenario, the mutations may affect the ability of the ligand to alter the conformational state of the transporter to different extents. However, our modeling, mutational analysis, Zn\textsuperscript{2+} binding data and MTS experiments argue against this possibility. It is also important to note that all critical mutations retained \textsuperscript{[3H]}S-CIT binding, supporting the notion that the mutations did not cause major changes in conformational equilibria.

Taken together with the previously accumulated data, the present observations suggest that the extracellular vestibule in NSS proteins constitutes a pocket capable of binding small molecule ligands. Crystal structures of LeuT demonstrated how TCAs and some SSRIs could be accommodated in the vestibule of this transporter and the present data support the binding of TCAs and SSRIs, such as CMI and S-CIT, in the
The allosteric binding site in SERT

vestibule of SERT, and of CMI in DAT, though in different binding modes. The vestibule has also been suggested to be important for the translocation process by constituting a possible second substrate binding site needed for transport (45-48). However, multiple crystal structures of LeuT have failed to support the biochemical findings (49-52). Our observation that some of the S2 mutations impair 5-HT transport is consistent with the suggestion that the vestibule is involved in substrate translocation.

A major question is whether the allosteric inhibition of dissociation, identified here for antidepressants like S-CIT, plays a role in their antidepressant action. Compared to the nanomolar affinity of S-CIT for the high-affinity binding site, the apparent affinity for the allosteric site is low (~4.5 µM) and the therapeutic concentration of CIT in the cerebrospinal fluid (CSF) has been measured to be in the hundred nanomolar/submicromolar range (53). However, it is not possible to use a direct measurement of radioligand binding to the S2 site, and for that reason the actual affinity of the compounds for the allosteric site could potentially exceed the EC50 values obtained in our dissociation assay. Moreover, due to the hydrophobic character of CIT, the compound is likely to partition into the membrane to produce higher local concentrations than measured in the CSF. The allosteric site is thus likely to be at least partly occupied during S-CIT therapy.

It has been suggested that the higher efficacy and faster onset of antidepressant action observed for S-CIT, as compared to racemic CIT, depends on the interaction of S-CIT with the allosteric binding site. It was proposed that R-CIT (inactive at the high-affinity binding site in therapeutic concentrations) might interfere with the action of S-CIT at the allosteric site and thereby prevent stabilization of S-CIT binding to the high-affinity binding site (23). Although the model is speculative, there is also evidence from nonclinical studies that R-CIT can indeed antagonize the effects of S-CIT (22-24,26,54). To assess more precisely the significance of the allosteric binding site in S-CIT action, it will be instrumental to generate a knock-in mouse expressing a SERT mutant in which this site is selectively perturbed. Importantly, the mutational data presented here should prove critically helpful for the design of such a mutant.

By showing how compounds like S-CIT and CMI can exert their action by blocking the exit pathway for [3H]S-CIT bound to its high-affinity binding pocket (S1), our findings might furthermore challenge the notion of a classical allosteric mechanism for the antidepressant action at SERT. Thus, according to the present docking models, the binding of S-CIT and CMI to S2, do not promote a conformational change at S1 that might switch the transporter into a state with higher affinity of S-CIT for S1. Rather, S-CIT and CMI binding to S2 have a corresponding effect on the entry pathway and thereby on the association rate, i.e. resulting in no change in overall affinity. However, it is important to note that the decrease in [3H]S-CIT dissociation rate caused by S-CIT or CMI binding to S2 could just as well reflect an increase in [3H]S-CIT affinity for S1, consistent with a classical allosteric mechanism. Unfortunately this question must remain unanswered because antidepressants such as CMI and S-CIT bind the primary site with high affinity and the ‘allosteric’ site with low affinity making it technically impossible to assess the effect of CMI/S-CIT on [3H]CIT association.

Because the present data provide support for the extracellular vestibule of SERT being the site from which S-CIT and CMI exert the allosteric effect on dissociation, it is intriguing to consider the use of this information in drug discovery, by evaluating the clinical potential of the compounds with enhanced S2 affinity and lowered S1 affinity. The effect may then be exploited either directly, or as an adjuvant therapy to improve the clinical efficacy of existing drugs targeting S1.

REFERENCES
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**FOOTNOTES**

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Author Contributions: PP and CJL performed experiments. LS performed the majority of molecular modeling and contributed to writing of the manuscript and planning of computational work with HW. TB contributed to molecular modeling. AHN participated in planning of experiments and writing of the manuscript draft. HW planned computational experiments with LS and participated in writing of the manuscript. UG participated in the planning of the experiments and wrote the final manuscript together with CJL. CJL planned and performed experiments with PP, wrote the manuscript draft together with LS and the final manuscript together with UG.

**FIGURE LEGENDS**

**FIGURE 1.** Binding modes of S-CIT and CMI in the S2 site of SERT. (A) The relative locations of the S1 and S2 sites in our SERT model viewed from an angle parallel to the membrane with S-CIT (yellow spheres) docked into both sites. The TMs and the ECLs 2 and 4 are indicated (B+C) Top views of the S-CIT (yellow spheres) and CMI (blue spheres) in the S2 site, respectively, which is composed of residues from TMs 1, 3, and 10, and ECLs 2 and 4. S1 bound S-CIT (yellow) as well as the putative location of the two Na⁺ (orange) and the Cl⁻ (green) are shown.
FIGURE 2. Allosteric potency of S-CIT and CMI on [3H]S-CIT dissociation form SERT WT and S2 mutants. (A+B) Representative experiments showing the concentration-dependent effect of S-CIT on [3H]S-CIT dissociation from SERT WT (A) and G402H (B). This experiment is the basis for the curves shown in the subsequent panels in which the T½ ratios (k_{diss, S-CIT}/k_{buf}) are plotted against inhibitor concentration. (C+D) The allosteric potency of S-CIT and CMI for [3H]S-CIT dissociation from SERT WT (●), G402H (▲) and R104K (▲). (E+F) Bar graphs showing the effect of selected mutants on allosteric potency of S-CIT and CMI on [3H]S-CIT dissociation. The mutated residues were shown in the docking protocol to interact with S2-bound S-CIT and CMI. All experiments were performed on COS7 cells membranes transiently transfected with SERT WT or mutants. Data are plotted in C and D as the observed effect of the indicated concentrations of antidepressant on [3H]S-CIT dissociation half-life (T½, S-CIT: k_{diss, S-CIT} or CMI: k_{diss, CMI}), plotted relative to the T½ of [3H]S-CIT alone (k_{buf}). Data depicted in E and F as EC_{50} values for the allosteric potency of S-CIT or CMI (±SE of 3-7 experiments). Asterisk denote significantly different IC_{50} values compared to WT *P<0.05; **P<0.01; ***P<0.001, student’s unpaired t test.

FIGURE 3. Insertion of a Zn^{2+} binding site in the extracellular vestibule of SERT between I179H and V489H decreases the allosteric potency of antidepressants. (A+B) Model of Zn^{2+} (pink) coordinated between I179H and V489H (blue sticks). According to the model, Zn^{2+} impairs the binding mode of S2-bound S-CIT (yellow sphere) (A) and CMI (blue sphere) (B) with electrostatic repulsion towards the dimethylamonium group. S1 bound S-CIT (yellow sphere), the two Na+ (orange) and Cl- (green) are shown. (C+D) Effect of Zn^{2+} on allosteric potency of S-CIT (C) and CMI (D) in SERT I179H-V489H. The IC_{50} for S-CIT bound to S2 changed from 20[13;30] µM to 59[58;60] µM and for CMI from 19[16;23] µM to 79[70;88] µM by the application of 200 µM Zn^{2+} (mean±SE interval, N=4). Insets: Effect of Zn^{2+} on the allosteric potency for the single mutants depicted as bar graphs (mean±SEM, N=3). Experiments were performed and calculated as described in Fig. 2 and in Experimental Procedures.

FIGURE 4. Effect of the cysteine-reactive BZ-MTS (benzocaine-methanethiosulfonate) on the allosteric potency of S-CIT in SERT-C L99C and background mutant SERT-C (C109A). (A) Molecular docking model of S1 bound S-CIT in a SERT mutant with BZ-MTS conjugated to Cys99. S-CIT could not be docked into S2 due to steric inhibition by the benzocaine (depicted in transparent in the position found in SERT WT). (B) Bar graph showing the effect of S-CIT (40 µM) and BZ-MTS (0.5 mM) on dissociation rate of bound [3H]S-CIT from COS7 cell membranes transiently transfected with SERT-C or SERT-C L99C. The addition of S-CIT to SERT-C resulted in a ~8-fold decrease in dissociation rate of [3H]S-CIT. The effect was slightly, but non-significantly inhibited by BZ-MTS ([3H]S-CIT with 40 µM S-CIT was 8.5±0.5 and 7.5±0.4 without and with BZ-MTS, respectively, relative to no S-CIT added). In contrast, BZ-MTS caused a significant inhibition of the allosteric effect of S-CIT in SERT-C L99C ([3H]S-CIT with 40 µM S-CIT was 5.5±0.2 and 3.2±0.5 without and with BZ-MTS, respectively, relative to no S-CIT added). The reactivity of BZ-MTS to Cys99 did also itself have effect on [3H]S-CIT dissociation (T½ = 31.3±2.2** and 21.7±1.6 with and without added BZ-MTS, respectively) whereas no effect was seen on SERT-C alone (T½ = 14.3±1.2 and 13.0±0.4 with and without added BZ-MTS, respectively). Data are means±SE of 3 experiments performed in triplicate. Asterisk denote significantly different T½ values compared to no BZ-MTS added **P<0.01; students unpaired t test.

FIGURE 5. Allosteric effect of CMI in the DAT. The dissociation rate of the cocaine analogue, [3H]CFT in DAT can be allosterically modulated by CMI. Inhibition dissociation curve showing allosteric potency of CMI on DAT WT and L80H (IC_{50} values are 167[148;188] µM and 555[471;655] for the DAT WT and L80H respectively. mean[SE interval], N=3) The L80H correspond to L99H in SERT. Experiments are performed on membranes from COS7 cells transiently transfected with DAT WT or L80H and calculated as described in Experimental Procedures.
Table 1.

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>WT</td>
<td>2777 ± 497</td>
<td>0.38 [0.31;0.47]</td>
<td>2.8 [1.4;5.6]</td>
</tr>
<tr>
<td>L99H</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>W103H</td>
<td>145 ± 16</td>
<td>0.84 [0.71;1.01]</td>
<td>28 [24;33]</td>
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<tr>
<td>R104K</td>
<td>462 ± 88</td>
<td>0.52 [0.42;0.65]</td>
<td>5.2 [5.0;5.5]</td>
</tr>
<tr>
<td>R104A</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>T178V</td>
<td>3822 ± 373</td>
<td>1.02 [0.79;1.30]</td>
<td>5.8 [5.7;5.9]</td>
</tr>
<tr>
<td>I179H</td>
<td>ND</td>
<td>ND</td>
<td>3.4 [0.8;15]</td>
</tr>
<tr>
<td>G402H</td>
<td>ND</td>
<td>ND</td>
<td>8.9 [6.3;12]</td>
</tr>
<tr>
<td>A486E</td>
<td>285 ± 50</td>
<td>0.21 [0.14;0.30]</td>
<td>16 [13;19]</td>
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<tr>
<td>V489H</td>
<td>2238 ± 741</td>
<td>0.42 [0.30;0.58]</td>
<td>7.9 [4.9;12.9]</td>
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<tr>
<td>K490A</td>
<td>2264 ± 284</td>
<td>0.48 [0.30;0.77]</td>
<td>9.6 [7.2;12.9]</td>
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<tr>
<td>L99H-I179H</td>
<td>ND</td>
<td>ND</td>
<td>4.0 [1.7;9.6]</td>
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<tr>
<td>I179H-V489H</td>
<td>ND</td>
<td>ND</td>
<td>5.0 [2.5-10.1]</td>
</tr>
<tr>
<td>A486E-V489H-K490A</td>
<td>389 ± 148</td>
<td>0.40 [0.36;0.44]</td>
<td>7.2 [4.8;10.7]</td>
</tr>
</tbody>
</table>

The listed values were found by non-linear regression analysis of competition uptake or binding assays by either $[^3]H$5-HT on whole cell preparations or $[^3]H$S-CIT on membranes, prepared from COS7 cells transiently transfected with SERT WT or mutant. The mean and S.E. interval for $[^3]H$5-HT binding potency and $[^3]H$S-CIT affinity is indicated and was calculated from the pK$_I$ ± S.E. Data are means[SE interval] of 3 to 13 experiments performed in triplicate. ND: Not Detectable.
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Table 2.
Effect of mutations in the extracellular vestibule on basal \([{}^{3}H]\)S-CIT dissociation

<table>
<thead>
<tr>
<th>SERT construct</th>
<th>([{}^{3}H])S-CIT dissociation T(\frac{1}{2}) (min)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>12.9 ± 0.4</td>
</tr>
<tr>
<td>L99H</td>
<td>27.3 ± 7.5</td>
</tr>
<tr>
<td>W103H</td>
<td>25.2 ± 6.0</td>
</tr>
<tr>
<td>R104K</td>
<td>26.2 ± 3.5</td>
</tr>
<tr>
<td>T178V</td>
<td>13.6 ± 0.9</td>
</tr>
<tr>
<td>I179H</td>
<td>21.3 ± 7.4</td>
</tr>
<tr>
<td>G402H</td>
<td>13.3 ± 1.5</td>
</tr>
<tr>
<td>A486E</td>
<td>17.9 ± 0.6</td>
</tr>
<tr>
<td>V489H</td>
<td>21.7 ± 1.1</td>
</tr>
<tr>
<td>K490A</td>
<td>11.8 ± 0.3</td>
</tr>
<tr>
<td>L99H-I179H</td>
<td>23.0 ± 2.5</td>
</tr>
<tr>
<td>I179H-V489H</td>
<td>22.7 ± 1.4</td>
</tr>
<tr>
<td>I179H-V489H +Zn(^{2+})</td>
<td>20.2 ± 2.9</td>
</tr>
<tr>
<td>A486E-V489H-K490A</td>
<td>28.1 ± 0.8</td>
</tr>
<tr>
<td>SERT-C L99C</td>
<td>19.3 ± 0.7</td>
</tr>
<tr>
<td>SERT-C L99C +BZ-MTS</td>
<td>29.2 ± 0.9</td>
</tr>
</tbody>
</table>

The listed values were found by linear regression analysis of dissociation time at 20°C for prebound \([{}^{3}H]\)S-CIT on membranes prepared from COS7 cells transiently transfected with SERT WT or mutant. For the mutants with added Zn\(^{2+}\) or BZ-MTS the reagents is added in the concentration of 200 µM and 0.5 mM, respectively. The mean ± S.E is calculated from 3 experiments performed in triplicate.
<table>
<thead>
<tr>
<th>SERT construct</th>
<th>Allosteric Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S-citalopram (in µM)</td>
</tr>
<tr>
<td>WT</td>
<td>4.6[4.2;5.0]</td>
</tr>
<tr>
<td>G402H</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>R104K</td>
<td>250[226;275]</td>
</tr>
<tr>
<td>L99H</td>
<td>24[23;25]</td>
</tr>
<tr>
<td>W103H</td>
<td>26[21;31]</td>
</tr>
<tr>
<td>T178V</td>
<td>6.2[6.1;6.3]</td>
</tr>
<tr>
<td>I179H</td>
<td>46[41;52]</td>
</tr>
<tr>
<td>A486E</td>
<td>58[40;85]</td>
</tr>
<tr>
<td>V489H</td>
<td>13[9;19]</td>
</tr>
<tr>
<td>K490A</td>
<td>7.8[6.7;9.1]</td>
</tr>
<tr>
<td>L99H-I179H</td>
<td>102[67;155]</td>
</tr>
<tr>
<td>I179H-V489H</td>
<td>20[13;29]</td>
</tr>
</tbody>
</table>

The allosteric potencies are the IC$_{50}$ values obtained from non-linear regression analysis of data from [3H]S-CIT dissociation experiments in the presence of increasing concentrations S-CIT and CMI. The dissociation rate constants ($k_{\text{drug}}$) at different S-CIT or CMI concentrations were calculated by linear regression and expressed relative to the dissociation rate constant without the presence of unlabeled ligand ($k_{\text{buffer}}$). The allosteric potency is determined as the IC$_{50}$ value of the drug concentration ($\log[k_{\text{drug}}]$) that impairs the dissociation rate by 50% compared with dissociation in buffer ($k_{\text{drug}}/k_{\text{buffer}}$) and are shown as mean values calculated from means of pIC$_{50}$ and the SE interval from the pIC$_{50}$ ±S.E of 3-10 experiments performed in triplicate. n.d.: not determined.
Table 4. Effect of Zn$^{2+}$ on the allosteric potency of S-CIT and CMI from SERT histidine mutants

<table>
<thead>
<tr>
<th>SERT Construct</th>
<th>S-citalopram (in µM)</th>
<th>Clomipramine (in µM)</th>
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<tbody>
<tr>
<td>200 µM Zn$^{2+}$</td>
<td>-</td>
<td>+</td>
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<td>I179H-V489H</td>
<td>20[13;29]</td>
<td>59[58;60]</td>
</tr>
<tr>
<td>I179H</td>
<td>46[41;52]</td>
<td>44[40;48]</td>
</tr>
<tr>
<td>V489H</td>
<td>13[9;19]</td>
<td>13[9;19]</td>
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</tbody>
</table>

The allosteric potencies are the IC$_{50}$ values obtained from non-linear regression analysis of data from [$^3$H]S-CIT dissociation experiments in the presence of increasing concentrations S-CIT and CMI. The dissociation rate constants ($k_{\text{drug}}$) at different S-CIT or CMI concentrations were calculated by linear regression and expressed relative to the dissociation rate constant without the presence of unlabeled ligand ($k_{\text{buffer}}$). The allosteric potency is determined as the IC$_{50}$ value of the drug concentration ($\log[k_{\text{drug}}]$) that impairs the dissociation rate by 50% compared with dissociation in buffer ($k_{\text{drug}}/k_{\text{buffer}}$) and are shown as mean values calculated from means of pIC$_{50}$ and the [SE interval] from the pIC$_{50}$ ±S.E of 3-6 experiments performed in triplicate. n.d, not determined. Data obtained from COS7 cells transiently expressing the SERT histidine mutants.
Figure 1 - Docking models
Figure 2 - Allosteric potencies

A) SERT WT

B) SERT G402H

C) [S-CIT] dissociation T½ ratio (k[S-CIT]/kbuf)

D) [S-CIT] dissociation T½ ratio (k[S-CIT]/kbuf)

E) Allosteric potency of S-CIT in IC50 (µM)

F) Allosteric potency of CMI in IC50 (µM)
Figure 3 - The I179H-V489H Zn2+ binding site

A

B

C

D
Figure 4 - BZ-MTS

A

B

Dissociation rate (in T½ relative to ctrl)

BZ-MTS

SERT-C SERT-C L99C

ns

*
Figure 5 - Allosteric effect in DAT
Steric hindrance mutagenesis in the conserved extracellular vestibule impedes allosteric binding of antidepressants to the serotonin transporter
Per Plenge, Lei Shi, Thijs Beuming, Jerez Te, Amy Hauck Newman, Harel Weinstein, Ulrik Gether and Claus J. Løland

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