Cholesterol binding to a conserved site modulates conformation, pharmacology and transport kinetics of the human serotonin transporter

Louise Laursen‡#, Kasper Severinsen‡#, Kristina Birch Kristensen‡, Xavier Periole§, Malene Overby‡, Heidi Kaastrup Müller‡, Birgit Schiøtt§, Steffen Sinning‡, &, 1

From the ‡Translational Neuropsychiatry Unit, Department of Clinical Medicine, Aarhus University, Aarhus University Hospital, Skovagervej 2, DK-8240 Risskov, Denmark and §Department of Chemistry, Aarhus University, Langelandsgade 140, 8000 Aarhus C, Denmark.
§Current address: Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510, USA.
¹To whom correspondence may be addressed: Telephone (+45) 78471115, E-mail steffen.sinning@clin.au.dk

#These authors contributed equally

Keywords: Serotonin transporter, depression, cholesterol, LeuT, antidepressants, serotonin

ABSTRACT

The serotonin transporter (SERT) is important for reuptake of the neurotransmitter serotonin from the synaptic cleft and is also the target of most antidepressants. It has previously been shown that cholesterol in the membrane bilayer affects the conformation of the serotonin transporter. Although recent crystal structures have identified several potential cholesterol binding sites it is unclear whether any of these potential cholesterol sites are occupieed by cholesterol and functionally relevant.

In the present study we focus on the conserved Cholesterol Site 1 (CHOL1) located in a hydrophobic groove between TM1a, TM5 and TM7. By molecular dynamics simulations we demonstrate a strong binding of cholesterol to CHOL1 in a membrane bilayer environment. In biochemical experiments we find that cholesterol depletion induces a more inward-facing conformation favoring substrate analog binding. Consistent with this, we find that mutations in CHOL1 with a negative impact on cholesterol binding induce a more inward-facing conformation and vice versa mutations with a positive impact on cholesterol binding induce a more outward-facing conformation. This shift in transporter conformation dictated by the ability to bind cholesterol in CHOL1 affects the apparent substrate affinity, maximum transport velocity and turnover rates. Taken together we show that occupation of CHOL1 by cholesterol is of major importance in the transporter conformational equilibrium which in turn dictates ligand potency and serotonin transport activity. Based on our findings, we propose a mechanistic model that incorporates the role of cholesterol binding to CHOL1 in the function of SERT.

INTRODUCTION

Depression is a severe psychiatric disorder with enormous socioeconomic impact. The underlying etiology of depression remains elusive but the serotonergic neurotransmitter system is believed to play a prominent role. Most antidepressants target the human serotonin transporter (hSERT) where they act as competitive inhibitors, which reduces serotonin reuptake from the synaptic cleft. The activity of hSERT can also be modulated by other mechanisms. As a transmembrane protein that undergoes considerable conformational changes in order to translocate Na⁺, Cl⁻ and serotonin across the membrane, the transporter is most likely sensitive to the physiochemical characteristics of the surrounding membrane as well as specific interactions with particular lipids. Despite only accounting for 2% of the total body mass the human brain contains approximately 23% of the total cholesterol in the body (1) making cholesterol the most abundant cell membrane component in the brain. Cholesterol is very important to brain development as well as neuronal function and synaptogenesis (2). Much of brain cholesterol is found in myelin sheaths where it is crucial for function (3) but cholesterol is also
enriched in the plasma membrane in general compared to other cellular compartments (4) and especially in synapses (5), where endo- and exocytosis (6) events are dependent on cholesterol and occur along with cholesterol-dependent receptor-mediated signaling (7,8) and transmembrane transport of neurotransmitters. Although cholesterol is enriched in specific sphingomyelin-rich microdomains with unique biophysical properties, so-called lipid rafts, cholesterol is also distributed heterogeneously in the plasma membrane (9) and therefore interaction between membrane protein and cholesterol is also possible independent of lipid raft association.

A role for cholesterol in modulating ligand binding to SERT has earlier been described (10) but it remains an open question whether that is caused by an indirect biophysical effect due to partitioning of the transporter into cholesterol-rich membrane microdomains (11) or via a direct interaction between transporter and sterol, potentially utilizing a specific cholesterol binding site.

A conformational effect of cholesterol on dopamine transporter (DAT) conformation and mobility has also been demonstrated (12-14) and for hSERT we have shown a conformational effect of depleting membrane cholesterol on transporter conformation in an intricate interplay with substrate-induced conformational changes (15). Specifically, we have earlier shown that cholesterol depletion shifts the conformational equilibrium of hSERT towards a more inward-facing conformation (15).

In crystal structures of the Drosophila dopamine transporter (dDAT) Gouaux and coworkers found first a cholesterol site (CHOL1) (16) and later a cholesterol hemisuccinate site (CHOL2) (17) and lastly a cholesterol hemisuccinate site in hSERT (CHOL3) (18), which may well also be a bona fide cholesterol site.

The location of CHOL1 at the interface between the scaffold (TM5) and the conformationally flexible bundle (TM1a and TM7) (19) (see Figure 1) makes it an interesting candidate as a specific cholesterol binding site that modulates the conformational dynamics of the transporters.

TM1a is of particular interest in the conformational role of cholesterol because this region has been shown to undergo large conformational changes in LeuT and MhsT during the transition from outward-facing to inward-facing conformation (20-24). Similarly, TM5 has been shown to line the intracellular permeation pathway in SERT and be more accessible in the inward-facing conformation (25,26) and may also respond to cholesterol binding at CHOL1 with a conformational change. TM7 is part of the conformationally mobile bundle consisting of TM1-2 and TM6-7 and may move in concert with TM1a relative to TM5.

CHOL1 is a shallow hydrophobic cavity facing the inner leaflet of the membrane (Figure 1A). The main hypotheses in the current study is therefore, 1) that a cholesterol molecule bound to CHOL1 is responsible for the conformational effect of cholesterol on hSERT and 2) that this conformational effect of cholesterol is crucial for transporter function.

We decided to test the hypothesis that cholesterol bound to the site lined by TM1a, TM5 and TM7 is responsible for a conformational effect of cholesterol by introducing mutations in this cholesterol site and determining the effect of these mutations on the conformation and function of hSERT and by performing molecular dynamics simulations to study the occupancy of specific cholesterol sites by cholesterol.

Molecular dynamics simulations indicate that site 1 is a highly occupied binding site for cholesterol in a lipid environment. Furthermore, we find that mutations in the cholesterol site shifts the conformational equilibrium in a predictable pattern. We also find that this shift in conformation by mutation of the cholesterol site is accompanied by a simultaneous shift in apparent substrate affinity and velocity that closely mirrors the extent and direction of the conformational change induced by mutating CHOL1. Overall, we find that cholesterol binding to CHOL1 lined by TM1a, TM5 and TM7 stabilizes the outward-facing conformation, which accelerates serotonin uptake, and that cholesterol dissociation from this site results in a more inward-facing conformation, which impedes serotonin uptake. These findings point to cholesterol binding to CHOL1 as an important modulator of hSERT transport activity and ligand potency.

RESULTS

Cholesterol depletion with MβCD shifts the conformation of hSERT

We first established the Amplex Red method for quantifying membrane cholesterol (Figure 2B). The use of methyl-β-cyclodextrin (MβCD) has been the preferred method for manipulating the plasma membrane cholesterol content to study the
effect on monoamine transporter function, usually removing cholesterol from both raft and non-raft domains (27).

MβCD depleted cholesterol from HEK293 membranes in a dose-dependent manner (Figure 2C).

To measure conformational changes in hSERT we rely on the hSERT_C5X_S277C construct earlier described (15,19) expressed in HEK293MSR cells. Crude membranes with the construct were subjected to a conformation-dependent inactivation with MTSEA, where [125I]-RTI-55 binding was subsequently used to quantify remaining active transporter. We find that increased cholesterol depletion increases MTSEA sensitivity (Figure 2D), reflecting an increased accessibility of S277C and thus a more inward-facing conformation of hSERT.

The conformation of hSERT (represented by MTSEA IC50) is linearly dependent on membrane cholesterol (Figure 2E).

**Cholesterol depletion does not alter equilibrium binding of RTI-55 in hSERT wt and hSERT_C5X_S277C**

Confident that we had a reliable system for cholesterol depletion and quantification of SERT conformation we proceeded to test whether the hSERT_C5X_S277C construct behaved pharmacologically similar to hSERT wt when subjected to cholesterol depletion. In order to do so, we subjected the hSERT_C5X_S277C construct along with hSERT wt to cholesterol depletion with MβCD followed by a radioligand equilibrium binding experiment (Figure 3).

We find that cholesterol depletion does not induce any statistically significant changes in the parameters for RTI-55 equilibrium binding to either hSERT wt or hSERT_C5X_S277C (Figure 3). We therefore conclude that hSERT_C5X_S277C is a valid model of hSERT wt in cholesterol depletion and RTI-55 binding.

**Cholesterol depletion primarily increases the potency of ligands that induce an inward-facing conformation**

Earlier studies on SERT and DAT have reported moderate changes in ligand affinity or inhibitor potency after cholesterol depletion as a result of conformational changes. We hypothesized that if cholesterol depletion favored a more inward-facing conformation then the potency of ligands that bind to or induce an inward-facing conformation, i.e. serotonin, ibogaine and noribogaine, would likely increase in potency. Indeed, in an equilibrium binding competition experiment with [125I]-RTI-55 as the radioligand we observe a dose-dependent increase in potency of serotonin, ibogaine and noribogaine at increasing concentrations of MβCD (Figure 4A-C).

For ligands that are believed to bind to an outward-facing conformation, e.g. imipramine, S-citalopram and cocaine (28), we observe no significant changes in potency except for cocaine inhibition of RTI-55 binding to hSERT wt after treatment with 20 mg/mL MβCD (Figure 4C) similar to what has been described by others (13). We also observe very similar responses in hSERT wt and hSERT_C5X_S277C (Figure 4C), again supporting that hSERT_C5X_S277C is a valid model of hSERT wt in studying ligand binding as a result of conformational changes induced by cholesterol depletion.

**Cholesterol binding to site 1 of hSERT in a biological membrane environment**

We hypothesized that cholesterol binding to CHOL1 may be responsible for the conformational effect of cholesterol on hSERT. To qualify whether CHOL1 is indeed a bona fide cholesterol site we performed molecular dynamics (MD) simulation of hSERT in a POPC:CHOL lipid bilayer with a 4:1 molecular ratio. We used the Martini coarse grain (CG) force field to model the system (29). The simplification of the interactions while keeping close to atomistic resolution allows us to sample time scales relevant to the study of lipid-protein interactions (30,31). The volume map of cholesterol density extracted from the CGMD simulation indicates that cholesterol preferentially visits a few “hot spots” on the surface of hSERT (Figure 5A). Most notably, we find high cholesterol densities in the region around TM1. Two of these densities correspond to CHOL1 (TM1/TM5) and CHOL2 (TM2/TM7) previously describe in the dDAT crystal structure (Figure 5B) (17). These results demonstrate that the binding of cholesterol at the TM7/TM1/TM5 interface is relevant for hSERT in biological membranes. The details of the other cholesterol binding sites go beyond the scope of the present paper and they will be described in a subsequent publication.

**Mutations in the cholesterol site 1 affects hSERT conformation**

If cholesterol binding to the site lined by TM1a, TM5 and TM7 stabilizes the outward-facing
conformation and *vice versa* if cholesterol dissociation from this site stabilizes a more inward-facing conformation, then mutations that weaken cholesterol binding would result in a more inward-facing conformation and conversely mutations improving cholesterol affinity would result in a more outward-facing conformation. In order to study conformational changes, we introduced all CHOL1 mutants in the hSERT_C5X_S277C background and isolated crude membranes from cells expressing these mutants. Using the SCAM-assay both prior to and after cholesterol depletion we were able to determine the conformational effect of the CHOL1 mutants and the conformational effect of cholesterol depletion on these mutants (Figure 6). We chose the mutants based on the structure of dDAT (16) with cholesterol (PDB 4m48) and hSERT (18) (PDB 5i73), see Figure 1C.

V86 is located in TM1a at the bottom of the cholesterol site but at 5.4 Å, most likely too far from cholesterol to interact directly (Figure 1C). Nevertheless, we wanted to see if introduction of a potential hydrogen bonding partner for the cholesterol hydroxyl group by the V86S mutation would increase cholesterol affinity and result in a shift towards a more outward-facing conformation. Although we see a minor shift of the conformation in this direction as a consequence of the mutation it is not significantly different from that observed for the wt or V86A and suggests that V86 is too distant from cholesterol for mutations to affect cholesterol affinity.

L89 interacts with the A-ring of the cyclic sterane substructure in cholesterol with 3.7 Å between the dDAT L37 (corresponding to hSERT L89) side chain and C4 of cholesterol (Figure 1C). The almost isosteric L89N mutation primarily introduces polarity in this region which we predict would repel the hydrophobic sterane substructure of cholesterol and weaken cholesterol binding. Indeed, this mutation results in the largest shift of the conformational equilibrium among the mutants and the shift is, as predicted, towards a more inward-facing conformation, similar to the conformational effect of depleting cholesterol. On the other hand, the L89V mutation was intended as a relatively neutral mutation that shortens the sidechain by a single methylene thus possibly decreasing the ability to interact favorably with cholesterol slightly but not introducing any repulsion as such. As expected, we observe a blunted conformational effect of the mutation relative to L89N and that L89V does not introduce any conformational change that is significantly different from the wt.

L90 lines the hydrophobic groove utilized by cholesterol and is located on the hydrophobic face of the sterane substructure only 4.2 Å from the C19 methyl group of cholesterol (Figure 1C). Similar to the two mutations in L89, we expect that introducing polarity in the form of the L90N mutation or weakening the hydrophobic interaction by retracting the sidechain of L90 in the form of the L90V mutation would result in a more inward-facing conformation if cholesterol binding to CHOL1 is responsible for the conformational effect of cholesterol. As expected, we observe that both mutations result in a significantly more inward-facing conformation consistent with our hypothesis that cholesterol induces a more inward-facing conformation when bound to CHOL1.

The aromatic part of Y289 is facing cholesterol at a distance of 4.6 Å but at the same time the hydroxyl group of the tyrosine appears to form a hydrogen bond to T371 (Figure 1C). No mutations can address the role of Y289 in interacting with cholesterol while not at the same time affecting the hydrogen bond to T371. However, we were also interested in studying the potential role of this evolutionarily conserved hydrogen bond between Y289 and T371 in the conformational equilibrium of hSERT because it may be important when the bundle and T371 move relative to the scaffold and Y289. This hydrogen bond is accessible via the Y289F and T371A mutations and the prediction would be that disrupting the hydrogen bond by either mutation could possibly facilitate the transition to an inward-facing conformation. We do not observe any conformational effect of the Y289F mutation but we do observe a more inward-facing conformation for T371A (Figure 6). A conformational effect from only one of the mutations but not both points to a negligible role of breaking the hydrogen bond in the conformational equilibrium but the conservation of the two residues may instead suggest a role in the folding of the transporter. T371A would appear to be too distant from cholesterol to coordinate it directly (Figure 1C) but aside from its hydrogen bond to Y289 it also forms a hydrogen bond to the backbone carbonyl of V367 one helix turn away and may to some extent line up V367 to interact favorably with the B and D cycles in cholesterol. Indeed, as expected we do observe that introducing polarity via the mutation V367N does result in a more inward-facing
conformation (Figure 6) consistent with decreased cholesterol binding and an ensuing shift in conformation relative to wt or the control V367A. Alternatively, T371A could also destabilize Y289 and thereby indirectly destabilize cholesterol binding.

In the dDAT structure the isooctyl tail of cholesterol is nestled in between L276 (hSERT L292), L277 (hSERT S293), L280 (hSERT L296) and I358 (hSERT V374), see Figure 1C. It is obvious that the two diverging residues in hSERT will result in a larger and less hydrophobic cavity than in dDAT. However, the inherent flexibility of the cholesterol isooctyl tail makes it difficult to disrupt the binding of cholesterol by mutations in this region. The sidechain of dDAT L277 at 4.3 Å and dDAT I358 at 3.8 Å from the cholesterol tail is the nearest possible points of interaction. When mutating the corresponding hSERT S293 and V374 to alanine we observe no effect of the V374A mutation on conformation but a significant effect of the S293A mutation on conformation (Figure 6). The hydrophilic sidechain of S293 is expected to be less favorable than the alanine for interacting with the cholesterol alkane tail. Thus, the significantly more outward-facing conformation that we observe for S293A is fully consistent with creating a better cholesterol site and the bound cholesterol to stabilize an outward-facing conformation.

We were also curious if cholesterol depletion would affect the CHOL1 mutants to a different extent than wt. In general, depletion of cholesterol with 20 mg/mL MβCD produces the same conformational shift toward a more inward-facing conformation as seen for wt except for L90N (Figure 6B), which appears to be significantly less sensitive to cholesterol depletion than the wt-like hSERT_C5X_S277C reference construct (Figure 6B). This may reflect the fact that cholesterol binding is already compromised, consistent with the findings above.

The conformational changes induced by mutations of CHOL1 result in altered transport kinetics

hSERT relies on large conformational changes to translocate serotonin and complete the transport cycle. Thus the conformational equilibrium and the transport function are intimately linked for hSERT. We hypothesize that the conformational changes in the CHOL1 mutants should translate directly to altered transport velocity, $V_{\text{Max}}$, by combining three observations: First, the rate-limiting step in the transport cycle is the return step from inward-facing to outward-facing (32,33). Secondly, cholesterol binding pushes the conformational equilibrium of SERT towards a more outward-facing conformation. Thirdly, cholesterol depletion pushes the conformation to a more inward-facing conformation as we have shown above. Therefore, we hypothesized that if CHOL1 is the cholesterol site important for the functional modulation by cholesterol, then the conformational changes for the CHOL1 mutants should mimic cholesterol binding and unbinding which would translate directly to altered transport velocity, $V_{\text{Max}}$. Specifically, if cholesterol binding to CHOL1 is the main determinant of how cholesterol affects hSERT conformation we expect that the mutants interfering negatively with cholesterol binding should also have decreased $V_{\text{Max}}$ and that the mutants interfering positively with cholesterol binding should have increased $V_{\text{Max}}$.

To test this hypothesis we subjected a subset of our CHOL1 mutants spanning the spectrum of conformational changes observed to $K_{\text{M}}/V_{\text{Max}}$ determinations (Figure 7) in serotonin uptake experiments in transiently transfected HEK 293MSR cells. As predicted, we find that $V_{\text{Max}}$ of the mutants mirror the conformation of the CHOL1 mutants; inward-facing mutants are slower and outward-facing mutants are faster (Figure 7C). Perhaps surprisingly, the apparent substrate affinity is also changed in the same way as the transport rates; inward-facing mutants have lower $K_{\text{M}}$ and outward-facing mutants have higher $K_{\text{M}}$ (Figure 7B), suggesting that also apparent substrate affinity is to a large extent a product of conformation.

When plotting the transport rate or apparent substrate affinity as a function of conformation the points are almost identical (Figure 7E). Importantly, in a linear regression analysis the regression lines of $K_{\text{M}}$ and $V_{\text{Max}}$ are not significantly different (Figure 7E) from each other and both have slopes that are significantly non-zero (P=0.0052 and 0.016, respectively). These clear correlations suggest that both kinetic parameters, $K_{\text{M}}$ and $V_{\text{Max}}$, are dictated by the conformation which again is dictated by cholesterol binding to CHOL1. The decrease in $V_{\text{Max}}$ could be a result of either decreased turn-over or simply by compromised surface expression of the CHOL1 mutants. We performed biotinylation of surface proteins to control for surface expression levels (Figure 7D).
When using the surface expression levels to convert $V_{\text{Max}}$ to relative turn-over rates we find that the strong correlation between conformation and the kinetic parameters, $V_{\text{Max}}$ and $K_{M}$, is also mirrored in the turn-over rate (Figure 7E). In conclusion, we observe that all three kinetic parameters, $V_{\text{Max}}$, $K_{M}$ and turn-over rate, are dictated by the conformational equilibrium which again is dictated by cholesterol binding to CHOL1.

**DISCUSSION**

Cholesterol distribution in the neuronal membrane is heterogeneous and enriched in the axon of the mature neuron (34) with a particular high concentration in synapses (35). Antidepressants also appear to concentrate in the cholesterol-rich microdomains (36). Therefore, neuronal proteins modulated by cholesterol are likely to display different functional and pharmacological characteristics depending on their location on the neuronal surface which again enables fine-tuning of protein activity by controlled trafficking to certain membrane regions.

It has been known for long that the GABA transporter, GAT-1, requires cholesterol to remain active in the synapse (37) or after reconstitution (38). The dopamine transporter and hSERT have been shown to distribute between two different membrane environments in living cells; one with free lateral diffusion and one with restricted diffusion that is also enriched in cholesterol and gangliosides (14,39), where the latter domain is most likely identical to or reminiscent of the so-called “lipid rafts” that have been shown to both contain hDAT (12) and hSERT (11).

Different ligands induce different conformations in hSERT and therefore their potency may be modulated differently by the conformational changes induced by cholesterol. We here show that cholesterol depletion increases the affinity of substrate analogs, which induce the inward-facing conformation, but we are unable to show a statistically change in potency for the antidepressants imipramine and S-citalopram, which are known to induce an outward-facing conformation (18,28). This partially conflicts with Scanlon et al (10) who observed first a decrease in S-citalopram affinity when depleting cholesterol with moderate concentrations of MβCD whereas at very high concentrations of MβCD they also observed a decrease in maximum binding. These differences may arise from the use of different species of SERT, where we employ the human SERT and Scanlon et al (10) employ the rat SERT. Similarly, the affinity of RTI-55 appears to be unaffected by cholesterol depletion similar to what has been shown for CFT affinity for DAT (12). However, the inhibitor, cocaine, has been shown to induce a more outward-facing conformation than imipramine (28) and for cocaine we do observe a significant increase in potency against hSERT that appears to rely on cholesterol content of the membrane in a dose-dependent manner similar to what has been observed for hDAT (13).

The notion, that cholesterol binding can directly affect inhibitor potency has been supported in preclinical studies, where systemic depletion of cholesterol by treatment with lovastatin, a statin that crosses the blood-brain barrier (40), augmented fluoxetine efficacy (41). Also, roles for both cholesterol and sphingolipid homeostasis in psychiatric disorders have been noted (42,43).

Recent crystal structures of SERT (18) and DAT (16) identified cholesterol or cholesterol analogs binding to the membrane-embedded periphery of the transporters but while these studies provide excellent structural information about potential cholesterol binding sites it is not possible to determine whether these cholesterol analogs are merely crystallization by-products or whether their binding sites are of any functional relevance. For example, in hSERT a cholesteryl hemisuccinate molecule was identified next to the extracellular part of TM12 (CHOL3), which has demonstrated little functional importance. Drosophila DAT was found to bind a cholesteryl hemisuccinate molecule in a shallow crevice lined by TM2, TM7 and TM11 (CHOL2) and a cholesterol molecule partially burrowed in a deeper crevice (CHOL1) formed by the intracellular parts of TM1, TM5 and TM7 (16). Because these latter three helices have also been proposed to be part of the most flexible regions of hSERT (19,20) we hypothesized that the conformational modulation exerted by cholesterol on monoamine transporters could be via direct binding to CHOL1.

Initial bioinformatics analysis showed that the residues lining CHOL1 appear to exhibit a higher degree of evolutionary conservation than what is generally observed for the protein face within the lipid bilayer (Figure 1B). This evolutionary selection pressure for maintaining the properties of this protein interface indicates a functional role of this region: namely cholesterol binding to this specific site.
First, we studied whether these cholesterol sites were likely to be occupied by cholesterol in a native membrane environment. In coarse-grained molecular dynamics simulations we found that cholesterol can occupy both CHOL1 and CHOL2 but found CHOL1 to be more occupied.

Second, we performed a mutational analysis of CHOL1. We aimed to produce either isosteric mutations that would change the hydrophobicity of the cholesterol site or mutations that would disrupt potential hydrogen bonds. We find that the conformational consequences of cholesterol binding and unbinding to CHOL1 are profound. With a series of mutations that were predicted to compromise cholesterol binding we obtained a conformational phenotype, a more inward-facing conformation, identical to the one obtained when depleting cholesterol, consistent with the notion that the inward-facing conformation of hSERT is more likely when CHOL1 is empty. Conversely, the S293A mutation predicted to improve cholesterol binding to CHOL1 produced the opposite conformational phenotype, a more outward-facing conformation, consistent with the notion that cholesterol binding to CHOL1 stabilizes an outward-facing conformation. From these observations we conclude that cholesterol binding to CHOL1 induces a more outward-facing conformation and cholesterol unbinding from CHOL1 induces a more inward-facing conformation in hSERT.

Furthermore, the observation that the S293A mutation favoring cholesterol binding can obtain a more outward-facing conformation than wt hSERT suggests that under native conditions the CHOL1 in wt hSERT is not fully saturated with cholesterol, thus a subpopulation of transporters do not have cholesterol bound to CHOL1. This is an important realization because it indicates a delicate equilibrium for cholesterol binding and unbinding to CHOL1 with a resulting dynamic probability for conformational transitions depending on cholesterol occupation of CHOL1. This hypothesis points to an interesting dynamic role for cholesterol in the fundamental function of hSERT, which we decided to probe by establishing a relationship between cholesterol binding, conformational transitions and 5-HT uptake kinetic parameters.

It has been shown earlier that cholesterol depletion affects cellular uptake of serotonin or dopamine negatively (10,14). However, a number of reasons for this observation are possible and it is unclear to which extent it is attributable to indirect effects of cholesterol by changing the biophysical properties of the cell membrane or through a direct effect of cholesterol on monoamine transporter function because uptake assays on cholesterol-depleted live cells may be inherently compromised by permeabilization of the membrane and/or viability and adherence issues for the cells. For example, selectively removing cholesterol from the membrane with saponin leaves large holes behind that would obviously affect radiotracer flux assays negatively (44).

In our uptake assays, we have left the membrane cholesterol concentration and membrane biophysical properties untouched but emulated the effect of lower cholesterol concentrations by mutating the CHOL1.

We observe that the mutations in CHOL1 that shifts the conformational equilibrium also affect uptake velocity and apparent substrate affinity in a predictable and systematic manner; mutations that compromise cholesterol binding to CHOL1 produce more inward-facing transporters that are slow but with increased apparent substrate affinity, whereas mutations that favor cholesterol binding produce outward-facing transporters that are faster but with decreased apparent substrate affinity (Figure 7E).

It is noteworthy that there is such a clear correlation between turn-over rates, \( V_{\text{Max}} \) and \( K_M \) (Figure 7E) and it strongly suggests that these parameters are mirrored readouts caused by the same phenomenon - in transporters where the mutations are located far from the substrate site and there is a strong correlation with conformation (Figure 7E), the conformation appears as the most likely cause for these changes in transport kinetic parameters.

These observations are all fully consistent with 1) cholesterol binding to CHOL1 stabilizing an outward-facing conformation with lower substrate affinity and 2) cholesterol unbinding from CHOL1 stabilizing an inward-facing conformation with higher substrate affinity (see Figure 8).

The interplay between cholesterol binding and unbinding in relation to the molecular mechanism of the transport cycle prompted us to combine these observations into a model that accounts for our observations (see Figure 8).

We know that the rate-limiting step in the transport cycle for hSERT is the transition from an inward-facing conformation, where K+ is bound and the empty substrate site is filled by L99 (32,33), towards the outward-facing conformation;
cholesterol binding to CHOL1 appears to accelerate this transition (Inapo, chol $\rightarrow$ Outapo, chol in Figure 8). Conversely, the unsaturated state of the CHOL1 suggests a dynamic equilibrium between cholesterol binding and unbinding that allows a second pathway in the transport cycle where hSERT without bound cholesterol in CHOL1 but with substrate, Na$^+$ and Cl$^-$ bound, can transition faster from the outward-facing to the inward-facing conformation (Out5HT $\rightarrow$ In5HT in Figure 8). In this way, cholesterol binding and unbinding to CHOL1 may assist in lowering the energetic hilltops associated with the most important conformational changes in the transport cycle. The model accounting for the two routes are illustrated in Figure 8; the route with the black arrows represents the conventional pathway that assumes constant cholesterol binding to a cholesterol site, whereas the route with the green arrows represents the additional and kinetically more favorable route supported by our data where cholesterol is in a dynamic equilibrium with CHOL1.

The model proposed here (Figure 8) is also fully consistent with the findings in our earlier work (15), where the inward-facing conformation was found to possess higher affinity for 5-HT and where low concentrations of the substrate together with Na$^+$ and Cl$^-$ ions, and the substrate are not expected to affect our modeling experiments and were not included in the CG model. All molecular dynamics (MD) simulations were performed using the GROMACS simulation package version 5.1 (48) and the Martini-2.0 CG force field for biomolecules (49,50) and its extension 2.1 to protein (50,51) together with the EINeDyn approach (51,52). This CGMD approach (30) is well suited to study lipid binding (31,49,52-55). Conventional simulation setups associated with the use of the Martini force field were used (30,56). The system was simulated for 100 µs. For analysis the density map of cholesterol at the surface of the protein were determined using the VolMap tool of VMD (56) using a 0.26 nm radius for the CG beads. The map is shown at level about four times higher than found in the buck.

Mutagenesis and DNA purification
Site-directed mutagenesis of hSERT in the pCDNA3.1 vector was performed and validated by sequencing as described earlier (15). DNA for transfection of mammalian cells was produced from a clonal colony of E. coli XL10 Gold (Stratagene) cultured in 60 mL LB medium with ampicillin selection and purified using the PureYield Plasmid Midiprep System (Promega).

Cell culture
Human embryonic kidney cells, HEK-293-MSR (Invitrogen), were grown in monolayer culture essentially as described earlier (15). For transfection a complex of 0.2 µg of plasmid and 0.5 µl of Lipofectamine 2000 transfection reagent (Invitrogen) per cm² of plating area was formed in Dulbecco's modified Eagle's medium and added to already adherent HEK-293-MSR cells in cell culture dishes 48 h before harvest.
Membrane preparations
48 h after transfection with hSERT adherent HEK-293-MSR cells were harvested by scraping. In brief, cells were harvested in 50 mM Tris-base buffer (150 mM NaCl, 20 mM EDTA, pH 7.4), centrifugated at 4,700 × g, washed, and homogenized in Tris-base buffer using an Ultra-turrax T25 (IKA Works, Wilmington, NC) before centrifugation at minimum 12,500 × g to sediment the membrane fraction. Homogenization, centrifugation at minimum 12,500 × g and aspiration were performed twice. All steps were performed at 4 °C. The membrane preparations were stored in 10 mM HEPES with 150 mM NaCl (adjusted to pH 8.0 with N-methyl-D-glucamine (NMDG⁺)) at −20 °C.

Cholesterol depletion
Membrane cholesterol was removed by the addition of methyl-β-cyclodextrin (Sigma Aldrich) to a final concentration of 2, 5, or 20 mg/ml in 10 mM HEPES with 150 mM NaCl (adjusted to pH 8.0 with NMDG⁺) and allowed to incubate at 37 °C for 30 min with gentle shaking followed by centrifugation at 16,500 × g at 4 °C for 15 min. The supernatant with the MβCD-cholesterol complex was removed by aspiration and the pellet containing hSERT in cholesterol-depleted membranes was resuspended in 10 mM HEPES (150 mM NaCl, pH 8.0, with NMDG⁺) with a Ultra-turrax T25 (IKA Works, Wilmington, NC) for 20 seconds and used immediately in the substituted cysteine accessibility method.

Determination of membrane cholesterol concentration using Amplex Red
Cholesterol content of the membranes was performed using the Amplex Red cholesterol assay kit (Molecular Probes) according to the manufacturer’s instructions. In brief, a dilution series of a cholesterol standard in binding buffer is used to produce a standard curve and along with the membranes is incubated with the Amplex Red working solution containing cholesterol oxidase, cholesterol esterase and Horseradish peroxidase. The production of hydrogen peroxide is proportional to the cholesterol content and the hydrogen peroxide converts Amplex Red to resorufin. After incubation for 30 minutes at 37 °C the fluorescence is detected at 590 nm (20 nm bandwidth) after excitation at 485 nm (20 nm bandwidth) in a BioTek Flx800 fluorescence plate reader. The total cholesterol content is calculated from the linear standard curve.

Substituted Cysteine Accessibility Method (SCAM)
Multiscreen HTS 96-well filtration plates (Millipore) pretreated with 0.1% PEI were used to capture homogenized membrane preparations of hSERT-transfected HEK-293-MSR cells. After membranes were bound to the filters in the filtration plates they were subjected to at least three washing steps with 10 mM HEPES buffer (supplemented with 150 mM NaCl, pH 8.0, adjusted with NMDG⁺). Incubation with the ligand proceeded for at least 25 min at room temperature before 2-aminoethyl MTS hydrobromide (MTSEA) (Apollo Scientific) was added in given concentrations (0.1 µM – 10 mM) and incubated with the hSERT-containing crude membranes for 15 min simultaneously with ligand. At least three washes with 10 mM HEPES buffer (150 mM NaCl, NMDG⁺-adjusted pH 8.0) terminated the MTS reaction with hSERT. To quantify residual unreacted hSERT, the membranes were then incubated with 0.1 nM [¹²⁵I]-RTI-55 (PerkinElmer) for at least 60 min. At least 5 washes of the filters with ice-cold 10 mM HEPES (150 mM NaCl) were performed to remove unbound radioligand. The dry filters were then dissolved in Microscint20 (Packard), and bound [¹²⁵I]-RTI-55 was quantified on a Packard Topcounter NXT scintillation counter.

Equilibrium binding
To determine the inhibitory potency of ligands and the affinity of [¹²⁵I]-RTI-55 equilibrium binding experiments were performed on native and cholesterol-depleted membranes.
For saturation binding, membranes were incubated with increasing concentrations of [¹²⁵I]-RTI-55 at RT for 60 minutes in binding buffer (10 mM HEPES buffer, 150 mM NaCl, pH 8.0, adjusted with NMDG⁺). Non-specific binding was assessed using 200 µM imipramine. For IC50 experiments, membranes were co-incubated with increasing concentrations of unlabeled ligand and 0.1 nM [¹²⁵I]-RTI-55 at RT for 60 minutes in binding buffer (10 mM HEPES buffer, 150 mM NaCl, pH 8.0, adjusted with NMDG⁺). The membranes with bound [¹²⁵I]-RTI-55 were then harvested on white Unifilter GF/B filterplates (PerkinElmer) pretreated with 0.1% PEI. The filters were washed three times with binding buffer. The dry filter were solubilized in
Microscint20 (Packard), and bound $[^{125}I]$-RTI-55 was quantified on a Packard Topcounter NXT scintillation counter.

**Cell-based serotonin uptake assay**

The uptake measurements were performed as previously described (57). HEK-293 MSR cells (Invitrogen) were cultured as monolayer cultures in DMEM (BioWhitaker) supplemented with 10% FCS (Gibco Life Technologies), 100 U ml$^{-1}$ penicillin, 100 $\mu$g ml$^{-1}$ streptomycin (BioWhitaker) and 6 $\mu$g ml$^{-1}$ of Geneticin (Invitrogen) at 95% humidity and 5% CO$_2$ at 37°C. Two days before the uptake experiment, cells were detached from the culture flask with trypsin/EDTA (BioWhitaker), transfected with midiprep DNA-Lipofectamine 2000 (Life Technologies) complex and seeded into white tissue culture treated 96-well microtiter plates (Nunc). Immediately before the uptake experiment was initiated, medium was aspirated and cells were washed once with PBSCM (137 mM NaCl, 27 mM KCl, 4.7 mM Na$_2$HPO$_4$, 1.2 mM KH$_2$PO$_4$, 0.1 mM CaCl$_2$, 1 mM MgCl$_2$, pH 7.4). Cells for determination of non-specific uptake were preincubated with 200 $\mu$M imipramine for 30 min, while cells for determination of total uptake were incubated with PBSCM. Uptake was initiated by the addition of 40 $\mu$l of a dilution of the $[^3]$H-5-HT mixed with unlabelled 5-HT in a 1:10 ratio. Uptake was terminated after 10 min by aspiration and washing with PBSCM. All wash steps were done on a BioTek Instruments ELx50 automatic strip washer. 50 $\mu$l of Microscint 20 (Packard) was dispensed into each well resulting in cell lysis and release of accumulated radiolabelled substrate from the adherent cells allowing direct quantitation on a Packard Topcounter. Uptake data were fitted to Michaelis–Menten kinetics by nonlinear regression analysis using the built-in tools in Prism5 (Graphpad).

**Quantification of cell surface hSERT**

HEK-293-MSR cells (Invitrogen) were cultured as monolayer cultures in DMEM (Sigma) supplemented with 10% FCS (Gibco Life Technologies), 100 U/ml penicillin, 100 $\mu$g/ml streptomycin (Sigma) and 6 $\mu$g/ml Geneticin (Apollo Scientific) at 95% humidity and 5% CO$_2$ at 37°C. Cells were detached from the culture flask with trypsin/EDTA (Sigma) seeded into clear tissue culture treated 6-well microplates (Cell+, F, Sarstedt) and allowed to adhere. After 24 hours the cells were transfected with midiprep DNA in complex with Lipofectamine 2000 (Life Technologies) and incubated for 48 h. Prior to biotinylation the transfected HEK-293-MSR cells were washed three times with ice-cold PBSCM (137 mM NaCl, 27 mM KCl, 4.7 mM Na$_2$HPO$_4$, 1.2 mM KH$_2$PO$_4$, 0.1 mM CaCl$_2$, 1 mM MgCl$_2$, pH 7.4) and the incubated with 1.0 mg/mL EZ-Link Sulfo-NHS-SS-biotin (ThermoScientific) in PBSCM for 45 minutes on ice with gentle agitation. The biotinylation reaction was quenched by washing the cells three times with ice-cold quench buffer (100 mM glycine in PBSCM) and incubated for additional 30 minutes in quench buffer on ice. Cells were washed three times with PBSCM before being lysed in lysis buffer (100mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1X Complete EDTA-free proteinase inhibitors (Roche)) for 45 minutes. The lysate was centrifuged and incubated with NeutrAvidin UltraLink Resin (ThermoScientific) for 1 hour at RT. Beads were washed three times in lysis buffer, and the bound (biotinylated) proteins were eluted in SDS sample buffer with DTT (125 nM Tris-HCl, pH 6.8, 20 % glycerol, 4 % SDS, 0.02 % bromphenol blue, and 125 mM dithiothreitol) at 50 °C for 15 min. The samples were separated on a NuPAGE® 10% Bis-Tris Gel (Life Technologies) with the NuPAGE® MOPS SDS Running Buffer (Life Technologies), transferred to a 0.2 µm nitrocellulose membrane (Bio-Rad) using the Trans-Blot® Turbo™ Transfer Pack (Bio-Rad). The membrane was probed with primary antibodies (goat anti-SERT (C-20), Santa Cruz (sc-1458), 1:1000 dilution and rabbit anti-β-actin, LI-COR Biosciences 926-42212, 1:3000 dilution) overnight at 4 °C followed by incubation with the appropriate IRDye conjugated secondary antibody (IRDye 680RD donkey anti-Rabbit and IRDye 800CW donkey anti-goat IgG, LI-COR Biosciences 925-68073/925-32212, 1:10,000 dilution) for 1 hour at RT. Infrared signals were detected using Odyssey CLx infrared imaging system (LI-COR), and bands were quantified using Image Studio software (LI-COR Bioscience).

**Data Calculations**

IC$_{50}$ data for inhibitor binding and SCAM were fitted by nonlinear regression to a sigmoidal dose-response curve with variable slope with the in-built tools in GraphPad Prism 5.0. Non-specific binding was subtracted from the total saturation binding data and the resulting specific
saturation binding data were fitted to a hyperbolic one-site binding curve with the in-built tools in GraphPad Prism 5.0.
Uptake data were fitted to Michaelis–Menten kinetics by nonlinear regression analysis using the built-in tools in GraphPad Prism 5.0.

ACKNOWLEDGEMENTS
We are grateful for skilled technical assistance by Bente Ladegaard.
The project was kindly supported by the Lundbeck Foundation.

CONFLICTS OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
KS and SS planned the project and the biochemical experiments while XP and BS planned the simulations. LL, KS, KBK, MO, HKM and SS performed the biochemical experiments and XP performed the computational simulations. LL, KS, KBK, XP, MO and SS analyzed the data. XP, BS and SS wrote the manuscript.
REFERENCES

1. Dietschy, J. M., and Turley, S. D. (2004) Thematic review series: brain Lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. *J Lipid Res* **45**, 1375-1397

2. Mauch, D. H., Nagler, K., Schumacher, S., Goritz, C., Muller, E. C., Otto, A., and Pfrieger, F. W. (2001) CNS synaptogenesis promoted by glia-derived cholesterol. *Science* **294**, 1354-1357

3. Saher, G., Brugger, B., Lappe-Siefke, C., Mobius, W., Tozawa, R., Wehr, M. C., Wieland, F., Ishibashi, S., and Nave, K. A. (2005) High cholesterol level is essential for myelin membrane growth. *Nat Neurosci* **8**, 468-475

4. Lange, Y., Swaisgood, M. H., Ramos, B. V., and Steck, T. L. (1989) Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts. *J Biol Chem* **264**, 3786-3793

5. Whittaker, V. P. (1966) SOME PROPERTIES OF SYNAPTIC MEMBRANES ISOLATED FROM THE CENTRAL NERVOUS SYSTEM*. *Annals of the New York Academy of Sciences* **137**, 982-998

6. Linetti, A., Fratangeli, A., Taverna, E., Valnegri, P., Francolini, M., Cappello, V., Matteoli, M., Passafaro, M., and Rosa, P. (2010) Cholesterol reduction impairs exocytosis of synaptic vesicles. *Journal of cell science* **123**, 595-605

7. Hanson, M. A., Cherezov, V., Griffith, M. T., Roth, C. B., Jaakola, V. P., Chien, E. Y. T., Velasquez, J., Kuhn, P., and Stevens, R. C. (2008) A Specific Cholesterol Binding Site Is Established by the 2.8 Å Structure of the Human β2-Adrenergic Receptor. *Structure* **16**, 897-905

8. Sooksawate, T., and Simmonds, M. A. (2001) Effects of membrane cholesterol on the sensitivity of the GABA(A) receptor to GABA in acutely dissociated rat hippocampal neurones. *Neuropharmacology* **40**, 178-184

9. Wüstner, D. (2007) Plasma membrane sterol distribution resembles the surface topography of living cells. *Molecular Biology of the Cell* **18**, 211-228

10. Scanlon, S. M., Williams, D. C., and Schloss, P. (2001) Membrane cholesterol modulates serotonin transporter activity. *Biochemistry* **40**, 10507-10513

11. Magnani, F., Tate, C. G., Wynne, S., Williams, C., and Haase, J. (2004) Partitioning of the serotonin transporter into lipid microdomains modulates transport of serotonin. *J Biol Chem* **279**, 38770-38778

12. Jones, K. T., Zhen, J., and Reith, M. E. (2012) Importance of cholesterol in dopamine transporter function. *J Neurochem* **123**, 700-715

13. Hong, W. C., and Amara, S. G. (2010) Membrane cholesterol modulates the outward facing conformation of the dopamine transporter and alters cocaine binding. *J Biol Chem* **285**, 32616-32626

14. Adkins, E. M., Samuvel, D. J., Fog, J. U., Eriksen, J., Jayanthi, L. D., Vaegter, C. B., Ramamoorthy, S., and Gether, U. (2007) Membrane mobility and microdomain association of the dopamine transporter studied with fluorescence correlation spectroscopy and fluorescence recovery after photobleaching. *Biochemistry* **46**, 10484-10497

15. Bjerregaard, H., Severinsen, K., Said, S., Wiborg, O., and Sinning, S. (2015) A dualistic conformational response to substrate binding in the human serotonin transporter reveals a high affinity state for serotonin. *J Biol Chem* **290**, 7747-7755

16. Penmatsa, A., Wang, K. H., and Gouaux, E. (2013) X-ray structure of dopamine transporter elucidates antidepressant mechanism. *Nature* **503**, 85-90

17. Wang, K. H., Penmatsa, A., and Gouaux, E. (2015) Neurotransmitter and psychostimulant recognition by the dopamine transporter. *Nature* **521**, 322-327

18. Coleman, J. A., Green, E. M., and Gouaux, E. (2016) X-ray structures and mechanism of the human serotonin transporter. *Nature*

19. Forrest, L. R., Zhang, Y. W., Jacobs, M. T., Gesmonde, J., Xie, L., Honig, B. H., and Rudnick, G. (2008) Mechanism for alternating access in neurotransmitter transporters. *Proc Natl Acad Sci U S A* **105**, 10338-10343
Cholesterol site in hSERT

20. Krishnamurthy, H., and Gouaux, E. (2012) X-ray structures of LeuT in substrate-free outward-open and apo inward-open states. *Nature* **481**, 469-474

21. Zhao, Y., Terry, D. S., Shi, L., Quick, M., Weinstein, H., Blanchard, S. C., and Javitch, J. A. (2011) Substrate-modulated gating dynamics in a Na+-coupled neurotransmitter transporter homologue. *Nature* **474**, 109-113

22. Kazmier, K., Sharma, S., Quick, M., Islam, S. M., Roux, B., and Weinstein, H. (2014) Conformational dynamics of ligand-dependent alternating access in LeuT. *Nat Struct Mol Biol* **21**, 1006-1012

23. Malinauskaite, L., Quick, M., Reinhard, L., Lyons, J. A., Yano, H., Javitch, J. A., and Nissen, P. (2014) A mechanism for intracellular release of Na+ by neurotransmitter/sodium symporters. *Nat Struct Mol Biol* **21**, 472-479

24. Stolzenberg, S., Li, Z., Quick, M., Malinauskaite, L., Nissen, P., Weinstein, H., Javitch, J. A., and Shi, L. (2017) The role of transmembrane segment 5 (TM5) in Na2 release and the conformational transition of neurotransmitter/sodium symporters toward the inward-open state. *J Biol Chem* **292**, 7372-7384

25. Zhang, Y. W., and Rudnick, G. (2006) The cytoplasmic substrate permeation pathway of serotonin transporter. *J Biol Chem.* **281**, 36213-36220

26. Zhang, Y. W., and Rudnick, G. (2005) Cysteine-scanning mutagenesis of serotonin transporter intracellular loop 2 suggests an alpha-helical conformation. *J Biol Chem.* **280**, 30807-30813

27. Zidovetzki, R., and Levitan, I. (2007) Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. *Biochim Biophys Acta* **1768**, 1311-1324

28. Tavoulari, S., Forrest, L. R., and Rudnick, G. (2009) Fluoxetine (Prozac) binding to serotonin transporter is modulated by chloride and conformational changes. *J Neurosci.* **29**, 9635-9643

29. Marrink, S. J., and Tieleman, D. P. (2013) Perspective on the Martini model. *Chemical Society reviews* **42**, 6801-6822

30. Periole, X., and Marrink, S.-J. (2013) The Martini Coarse-Grained Force Field. in *Biomolecular Simulations: Methods and Protocols* (Monticelli, L., and Salonen, E. eds.), Humana Press, Totowa, NJ. pp 533-565

31. Periole, X. (2017) Interplay of G Protein-Coupled Receptors with the Membrane: Insights from Supra-Atomic Coarse Grain Molecular Dynamics Simulations. *Chemical Reviews* **117**, 156-185

32. Schicker, K., Uzelac, Z., Gesmonde, J., Bulling, S., Stockner, T., Freissmuth, M., Boehm, S., Rudnick, G., Sitte, H. H., and Sandtner, W. (2012) Unifying concept of serotonin transporter-associated currents. *J Biol Chem.* **287**, 438-445

33. Malinauskaite, L., Said, S., Sahin, C., Grouleff, J., Shahsavar, A., Bjerregaard, H., Noer, P., Severinsen, K., Boesen, T., Schiott, B., Sinning, S., and Nissen, P. (2016) A conserved leucine occupies the empty substrate site of LeuT in the Na(+)-free return state. *Nature communications* **7**, 11673

34. Tashiro, Y., Yamazaki, T., Shimada, Y., Ohno-Iwashita, Y., and Okamoto, K. (2004) Axon-dominant localization of cell-surface cholesterol in cultured hippocampal neurons and its disappearance in Niemann-Pick type C model cells. *Eur J Neurosci.* **20**, 2015-2021

35. Kokubo, H., Helms, J. B., Ohno-Iwashita, Y., Shimada, Y., Horikoshi, Y., and Yamaguchi, H. (2003) Ultrastructural localization of flotillin-1 to cholesterol-rich membrane microdomains, rafts, in rat brain tissue. *Brain Res* **965**, 83-90

36. Eisensamer, B., Uhr, M., Meyr, S., Gimpel, G., Deiml, T., Rammes, G., Lambert, J. J., Ziegglansberger, W., Holsober, F., and Rupprecht, R. (2005) Antidepressants and antipsychotic drugs colocalize with 5-HT3 receptors in raft-like domains. *J Neurosci.* **25**, 10198-10206

37. North, P., and Fleischer, S. (1983) Alteration of synaptic membrane cholesterol/phospholipid ratio using a lipid transfer protein. Effect on gamma-aminobutyric acid uptake. *J Biol Chem.* **258**, 1242-1253

38. Shouffani, A., and Kanner, B. I. (1990) Cholesterol is required for the reconstruction of the sodium- and chloride-coupled, gamma-aminobutyric acid transporter from rat brain. *Journal of Biological Chemistry* **265**, 6002-6008

39. Chang, J. C., Tomlinson, I. D., Warnement, M. R., Ustione, A., Carneiro, A. M., Piston, D. W., Blakely, R. D., and Rosenthal, S. J. (2012) Single molecule analysis of serotonin transporter...
regulation using antagonist-conjugated quantum dots reveals restricted, p38 MAPK-dependent mobilization underlying uptake activation. *J Neurosci* **32**, 8919-8929

40. Guillot, F., Misslin, P., and Lemaire, M. (1993) Comparison of fluvastatin and lovastatin blood-brain barrier transfer using in vitro and in vivo methods. *J Cardiovasc Pharmacol* **21**, 339-346

41. Renshaw, P. F., Parsegian, A., Yang, C. K., Novero, A., Yoon, S. J., Lyoo, I. K., Cohen, B. M., and Carlezon, W. A., Jr. (2009) Lovastatin potentiates the antidepressant efficacy of fluoxetine in rats. *Pharmacol Biochem Behav* **92**, 88-92

42. Narayan, S., and Thomas, E. A. (2011) Sphingolipid abnormalities in psychiatric disorders: a missing link in pathology? *Frontiers in bioscience (Landmark edition)* **16**, 1797-1810

43. Papakostas, G. I., Ongur, D., Iosifescu, D. V., Miscoulon, D., and Fava, M. (2004) Cholesterol in mood and anxiety disorders: review of the literature and new hypotheses. *Eur Neuropsychopharmacol* **14**, 135-142

44. Seeman, P., Cheng, D., and Iles, G. H. (1973) Structure of membrane holes in osmotic and saponin hemolysis. *J Cell Biol* **56**, 519-527

45. Olsson, M. H. M., Sondergaard, C. R., Rostkowski, M., and Jensen, J. H. (2011) PROPKA3: Consistent Treatment of Internal and Surface Residues in Empirical pK(a) Predictions. *J Chem Theory Comput* **7**, 525-537

46. Schrödinger, L., New York, NY. (2016) Maestro.

47. de Jong, D. H., Singh, G., Bennett, W. F., Arnarez, C., Wassenaar, T. A., Schafer, L. V., Periole, X., Tieleman, D. P., and Marrink, S. J. (2013) Improved Parameters for the Martini Coarse-Grained Protein Force Field. *J Chem Theory Comput* **9**, 687-697

48. Abraham, M. J., Murtola, T., Schulz, R., Páll, S., Smith, J. C., Hess, B., and Lindahl, E. (2015) GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* **1–2**, 19-25

49. Ingólfsson, H. I., Arnarez, C., Periole, X., and Marrink, S. J. (2016) Computational ‘microscopy’ of cellular membranes. *Journal of cell science* **129**, 257

50. Marrink, S. J., Risselada, H. J., Yefimov, S., Tieleman, D. P., and de Vries, A. H. (2007) The MARTINI force field: coarse grained model for biomolecular simulations. *J Phys Chem B* **111**, 7812-7824

51. Monticelli, L., Kandasamy, S. K., Periole, X., Larson, R. G., Tieleman, D. P., and Marrink, S.-J. (2008) The MARTINI Coarse-Grained Force Field: Extension to Proteins. *J Chem Theory Comput* **4**, 819-834

52. Periole, X., Cavalli, M., Marrink, S.-J., and Ceruso, M. A. (2009) Combining an Elastic Network With a Coarse-Grained Molecular Force Field: Structure, Dynamics, and Intermolecular Recognition. *J Chem Theory Comput* **5**, 2531-2543

53. Hedger, G., Shorthouse, D., Koldso, H., and Sansom, M. S. P. (2016) Free Energy Landscape of Lipid Interactions with Regulatory Binding Sites on the Transmembrane Domain of the EGF Receptor. *The Journal of Physical Chemistry B* **120**, 8154-8163

54. Arnarez, C., Mazat, J.-P., Elezgaray, J., Marrink, S.-J., and Periole, X. (2013) Evidence for Cardiolipin Binding Sites on the Membrane-Exposed Surface of the Cytochrome bc1. *Journal of the American Chemical Society* **135**, 3112-3120

55. Periole, X., and Marrink, S. J. (2013) The Martini coarse-grained force field. *Methods Mol Biol* **924**, 533-565

56. Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD: Visual molecular dynamics. *J Mol Graphics* **14**, 33-&&

57. Sinning, S., Musgaard, M., Jensen, M., Severinsen, K., Celik, L., Koldso, H., Meyer, T., Bols, M., Jensen, H. H., Schiott, B., and Wiborg, O. (2010) Binding and orientation of tricyclic antidepressants within the central substrate site of the human serotonin transporter. *J Biol Chem* **285**, 8363-8374

58. Glaser, F., Pupo, T., Paz, I., Bell, R. E., Bechor-Shental, D., Martz, E., and Ben-Tal, N. (2003) ConSurf: Identification of Functional Regions in Proteins by Surface-Mapping of Phylogenetic Information. *Bioinformatics* **19**, 163-164
Figure 1:
(A) Cholesterol is bound to a hydrophobic pocket in the membrane layer on dDAT. The dDAT structure (16) (PDB 4m48) is viewed in the plane of the membrane bilayer with the protein surface within 7 Å of cholesterol (light brown) shown as either grey (hydrophobic), red (partial negative charge) or blue (partial positive charge).

(B) Higher evolutionary conservation of the cholesterol site relative to the surrounding transmembrane protein surface suggests a functional role of cholesterol binding. 38 DAT, SERT and NET sequences from 26 mammalian, four insect, three avian, two fish and two nematode were aligned using ClustalW and the resulting alignment used to color-code the surface of the dDAT structure (PDB 4m48) (16) using the ConSurf server (http://consurf.tau.ac.il/2016/) (58) with the most conserved residues in magenta, moderately conserved residues in grey and the least conserved residues in turquoise. Cholesterol is shown in light brown.

(C) The cholesterol site in the dDAT structure (PDB 4m48) (16) is lined by conserved hydrophobic residues from TM1a (purple), TM5 (blue) and TM7 (green). The corresponding residues in hSERT (PDB 5i73) are shown in turquoise. Cholesterol is shown in light brown.
Figure 2:

A

B

C

D

E

Cholesterol site in hSERT

[17]
(A) Numbering scheme for the 27 carbon atoms and four sterane rings in cholesterol.
(B) Cholesterol content is quantified using the Amplex Red method. Total content of cholesterol in the membranes are calculated from the linear standard curve.
(C) Depletion of cholesterol from membranes induces a more inward-facing conformation of hSERT as measured by the Substituted Cysteine Scanning Method (SCAM). Crude membranes from HEK-293-MSR cells transiently transfected with the wt-like construct hSERT_C5X_S277C are exposed to increasing concentrations of MβCD to deplete membrane cholesterol. A dose-dependent depletion of cholesterol in response to MβCD is observed.
(D) Depletion of membrane cholesterol shifts the conformational equilibrium towards a more inward-facing conformation. Membranes were subjected to cholesterol depletion followed by incubation with the cysteine-specific reagent 2-Aminoethyl methanethiosulfonate (MTSEA). If the conformational equilibrium is shifted towards a more inward-facing conformation where the intracellular pathway is exposed MTSEA will be more reactive to S277C. The reaction of MTSEA causes inactivation of hSERT which is measured by a reduction in $[^{125}I]$-RTI-55 binding. The IC$_{50}$ of MTSEA represents the reactivity of hSERT S277C and thus a leftward shift of the curve represents a more inward-facing conformation. These results are similar to what we have published earlier in Bjerregaard et al (15).
(E) The conformational equilibrium of hSERT as measured by MTSEA IC$_{50}$ is linearly dependent on the cholesterol content of the membrane. The remaining membrane cholesterol in the membranes used in Figure 2C is quantified using the Amplex Red method (see Figure 2B-C) and the conformation is measured using the SCAM assay (see Figure 2D). Shown in B-C are a single representative experiment out of three independent experiments. Shown in E are aggregate data from three independent experiments. All points represent mean values and error bars represent SEM.
Figure 3:

Cholesterol depletion does not affect the equilibrium binding of [125I]-RTI-55 to hSERT wt or hSERT_C5X_S277C. Crude membranes from HEK293MS cells transiently transfected with hSERT wt (A) or hSERT_C5X_S277C (B) were treated with 0, 5 or 20 mg/mL MβCD to deplete cholesterol followed by incubation with increasing concentrations of [125I]-RTI-55 in a saturation binding experiment and the data were fitted to a one site model. Error bars represent SEM. For statistical analysis each treatment with MβCD (5 or 20 mg/mL) are compared to untreated (0 mg/mL) with a two-way ANOVA with Bonferroni post-test and showed no significant difference.
Figure 4:

Cholesterol depletion increases the inhibitory potency of ligands that induce the inward-facing conformation in hSERT. Crude membranes from HEK293MS cells transiently transfected with hSERT wt (A) or hSERT_C5X_S277C (B) were treated with 0, 5 or 20 mg/mL MβCD to deplete cholesterol followed by a coincubation with 0.1 nM $[125I]$-RTI-55 and decreasing concentrations of inhibitor. Data shown in (A) and (B) are a representative data from a single experiment. (C) Normalized mean $K_i$-values of hSERT wt (unhatched bars) or hSERT_C5X_S277C (hatched bars) from three independent radioligand competition assays with six different hSERT ligands are shown as bars. Error bars represent SEM on (A)-(B) and SD on (C). For statistical analysis each treatment with MβCD (5 or 20 mg/mL) are compared to untreated (0 mg/mL) with a two-way ANOVA with Bonferroni post-test. Significance levels are $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***)
Figure 5:

Cholesterol binding site on hSERT surface. a) CGMD prediction of cholesterol (chol) high density regions (blue mesh) on the surface of hSERT (grey surface depicts the density of the backbone CG beads). POPC glycerol density is shown as a green volume map and the cholesterol molecules found in the dDAT crystal structure (17) are shown as stick. b) hSERT atomistic model (PDB entry 5I6X (18)) with the cholesterol molecules as found in dDAT crystal structure (17).
Mutation of the cholesterol site affects the overall conformation of hSERT. (A) The impact of mutating the cholesterol site on the conformational equilibrium is measured using the SCAM method. Of the mutations predicted to possibly improve cholesterol binding (V86S, S293A), only S293A induces a more outward-facing conformation. Of the mutations predicted to interfere negatively with cholesterol binding (L89N, L89V, L90N, L90V, V367N) all but L89V induce a significantly more inward-facing conformation. (B) The conformational impact of depleting cholesterol from the membranes using 20 mg/ml MβCD on the different mutants is measured using the SCAM method. The conformational
response of all mutants to cholesterol depletion is similar to wt, except for L90N, which appears to be significantly less conformationally sensitive to cholesterol depletion. All mutations are introduced in the wt-like hSERT_C5X_S277C background and expressed in HEK293MSR cells by transient transfection. Upon isolation of crude membranes the mutants are subjected to MTSEA inactivation followed by quantification of residual binding of $[^{125}\text{I}]$-RTI-55. Statistical analysis of mutant conformational change relative to the wt-like reference hSERT_C5X_S277C performed with a one-way ANOVA with a Dunnett’s test to compare to wt. Significance levels are $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***)}. Bars represent mean values from at least three independent experiments and error bars represent SEM.
Manipulation of cholesterol binding by mutation of the cholesterol binding site modulates transporter conformation which in turn changes apparent substrate affinity, transport rates and turn-over rates. (A) Representative $K_M/V_{\text{Max}}$ experiment of wt hSERT and two cholesterol site mutants that either have a more outward-facing (S293A) or more inward-facing (L89N) conformation. The apparent transport affinity for 5-HT (B) and maximum transport rate (C) is significantly modulated by mutations in the cholesterol binding site. (D) Cell surface expression levels of hSERT cholesterol site mutants are determined using biotinylation of proteins expressed on the cell surface followed by avidin purification and Western blotting with an anti-hSERT antibody to quantify relative hSERT cell surface expression levels. Depicted is a representative biotinylation experiment out of five independent experiments. (E) Correlation plot of $K_M$, $V_{\text{Max}}$ and relative turn-over rates as a function of conformation of the individual mutants shows that reductions in $K_M$ are closely mirrored by similar reduction in $V_{\text{Max}}$ and turn-over rate suggesting that all three are modulated as a result of the same mutational effect. Both $K_M$ and $V_{\text{Max}}$ of individual mutants correlate with the conformation of the mutants as measured by the SCAM method (see Figure 6A). When the relative turn-over rate is computed by correction of $V_{\text{Max}}$ with the cell surface expression levels from (D) an identical trend is observed for the turn-over rate pointing to cholesterol occupation of hSERT cholesterol site 1 as a principal modulator of hSERT functional kinetics. Linear regression analysis (the curved lines are a result of logarithmic transformation of X-axis) shows that all lines have a slope that is significantly non-zero ($P<0.016$) and cannot identify any statistically significant difference between linear fits for $K_M$, $V_{\text{Max}}$ or turn-over rate as a function of mutant conformation. Points in (A) are mean values of quadruplicate determinations from a representative experiment. Bars/points in (B-C and E) represent
mean values from at least three independent experiments. Error bars represent SEM in (A) and SD in (B), (C) and (E). Statistical analysis of mutant transport kinetic parameters relative to hSERT wt in (B-C) is performed with a one-way ANOVA with a Dunnett’s test to compare to wt. Significance levels are P<0.05 (*), P<0.01 (**) and P<0.001 (***)

Cholesterol site in hSERT

Figure 8:

Model for cholesterol modulation of the conformational equilibrium in the transport cycle for 5-HT uptake mediated by hSERT. The conventional transport cycle is described by black arrows and the new proposed modified transport cycle (where cholesterol binding to CHOL1 modulates conformational changes) is shown with dark green arrows. In our model cholesterol from the membrane pool can bind and unbind to CHOL1. When cholesterol is bound it stabilizes the outward-facing conformation (Outapo,chol and Out5HT,chol) which has low-affinity for 5-HT (see Figure 4C and 7E). Cholesterol unbinding (Out5HT,chol → Out5HT) favours the transition from an outward-facing conformation to an inward-facing conformation (In5HT), which is a high-affinity state for 5-HT (see Figure 4C and 7E). At this state (In5HT), rapid Na⁺ dissociation into the virtually Na⁺-free cytoplasm commits hSERT to release of 5-HT into the cytoplasm (In5HT → Inapo). The rate-limiting conformational transition in the transport cycle is known to be the transition from inward-facing to outward-facing (Inapo → Outapo) which is greatly accelerated by cholesterol binding to CHOL1 (Inapo,chol → Outapo,chol)(see Figure 7E).
Cholesterol binding to a conserved site modulates conformation, pharmacology and transport kinetics of the human serotonin transporter
Louise Laursen, Kasper Severinsen, Kristina Birch Kristensen, Xavier Periole, Malene Overby, Heidi Kaastrup Müller, Birgit Schiøtt and Steffen Sinning

J. Biol. Chem. published online January 19, 2018

Access the most updated version of this article at doi: 10.1074/jbc.M117.809046

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts