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Putative drug binding conformations of monoamine transporters

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Abstract—Structural information about monoamine transporters and their interactions with psychotropic drugs is important for understanding their molecular mechanisms of action and for drug development. The crystal structure of a Major Facilitator Superfamily (MFS) transporter, the lactose permease symporter (lac permease), has provided insight into the three-dimensional structure and mechanisms of secondary transporters. Based on the hypothesis that the 12 transmembrane α-helix (TMH) secondary transporters belong to a common folding class, the lac permease structure was used for molecular modeling of the serotonin transporter (SERT), the dopamine transporter (DAT), and the noradrenaline transporter (NET). The molecular modeling methods used included amino acid sequence alignment, homology modeling, and molecular mechanical energy calculations. The lac permease crystal structure has an inward-facing conformation, and construction of outward-facing SERT, DAT, and NET conformations allowing ligand binding was the most challenging step of the modeling procedure. The psychomotor stimulants cocaine and *S*-amphetamine, and the selective serotonin reuptake inhibitor (SSRI) *S*-citalopram, were docked into putative binding sites on the transporters to examine their molecular binding mechanisms. In the inward-facing conformation of SERT the translocation pore was closed towards the extracellular side by hydrophobic interactions between the conserved amino acids Phe105, Pro106, Phe117, and Ala372. An unconserved amino acid, Asp499 in TMH10 in NET, may contribute to the low affinity of *S*-citalopram to NET.

1. Introduction

Secondary transporters are ion-coupled membrane transport proteins that include the sodium:neurotransmitter symporter family (SNF), the Na^+/H^+ antiporter (NhaA), and the major facilitator superfamily (MFS) transporter, lactose permease symporter (lac permease). Secondary transporter proteins carry small molecules across cell membranes against a concentration gradient using ion gradients as energy source, thus facilitating solute accumulation and toxin removal. Most secondary transporters are predicted to have 12 transmembrane α-helices (TMHs) and intracellular localization of the amino- and carboxy-terminals.[1–4]

The serotonin transporter (SERT), the dopamine transporter (DAT), and the noradrenalin transporter (NET) belong to the SNF transporters. The sequence identity within the SNF family is 40–90%.[3] Experimental studies have indicated that SERT[5,6] and DAT[7,8] may exist as oligomers, but the functional role of oligomerization is unclear. The SNF proteins act as co-transporters of sodium and chloride ions, and of transmitter molecules.[9] The energy required for the inward movement of the transmitter molecules is provided by the sodium gradient across the cell membrane, which regulates the action of the neurotransmitter.

The three-dimensional molecular structure has not been reported for any SNF transporter. However, the crystal structures of the *Escherichia coli* MFS transporters lac permease[10] and glycerol-3-phosphate transporter[11] have provided insight into the three-dimensional structure and molecular mechanisms of secondary transporters, and an experimental basis for molecular modeling of the secondary transporters, SERT, NET, and DAT. Such transporter models are based on the hypothesis that the 12 TMH secondary transporters belong to a common folding class. The crystal structures revealed that the MFS transporters lac permease[10] and the glycerol-3-phosphate transporter[11] do share a common folding motif.

We have previously used a three-dimensional NhaA model,[12] which was constructed from a low-resolution three-dimensional projection map of the *E. coli* NhaA,[1] to make molecular models of SERT, DAT, and NET.[13,14] Despite the lack of a high resolution structural

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template, the NhaA model had general structural features that corresponded quite well with those of the crystal structures of the MFS transporters lac permease[10] and the glycerol-3-phosphate transporter.[11] The main common features were the relative positions of TMHs 1–6, and a binding site formed by TMHs 1, 2, 3, 4, 5, 7, 8, and 11. The main differences between the NhaA model[12] and the MFS transporter crystal structures[10,11] were in the positions of TMH10 and TMH12.[15]

In the present study, the crystal structure of lac permease[10] was chosen as the template for modeling of SERT, DAT, and NET. Lac permease is an intensively studied member of the MFS superfamily,[4,10] and the sequence identity is 11–13% within the transmembrane regions between lac permease and the SNF proteins. The lac permease crystal structure has an N-terminal and a C-terminal domain, each with six transmembrane helices. These domains are symmetrically positioned within the transporter, and a hydrophilic cavity is open to the cytoplasmic side in the inward-facing conformation. A possible model for the outward-facing conformation, based on chemical modification and cross-linking experiments, was proposed by Abramson et al. by applying a relative rigid-body rotation of ~60° to the N- and C-terminal domains.[10] Lac permease uses the electrochemical proton gradient to provide an inward movement of galactosides against a concentration gradient. Hydropathy plots indicate that secondary transporters have similar secondary structures, and it is possible that their tertiary structures and mechanisms may have been preserved throughout evolution.[10]

The ligand binding sites of SERT, DAT, and NET may have a similar core area for cocaine binding, since cocaine has similar affinities for these three transporters. Binding studies of SSRIs showed that they are 300–3500 times selective for SERT over NET, and generally have lower affinities for DAT,[16] indicating differences in their SSRI binding areas. Both cocaine and *S*-citalopram block transmitter reuptake competitively. While cocaine is non-selective, *S*-citalopram is a selective SERT inhibitor.[9] Amphetamine and related compounds, such as methamphetamine, methylphenidate, fenfluramine, and 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy'), act as 'false substrates' for monoamine transporters and inhibit monoamine reuptake. Mapping of the ligand binding areas may be used for rational design of more specific antidepressants with less side effects, and of drugs that might be used to treat cocaine addiction.

The models of DAT, SERT, and NET presented in this study were used to dock the psychomotor stimulants cocaine and *S*-amphetamine, and the SSRI *S*-citalopram, into their putative binding sites and examine the molecular mechanisms of their different binding affinities.

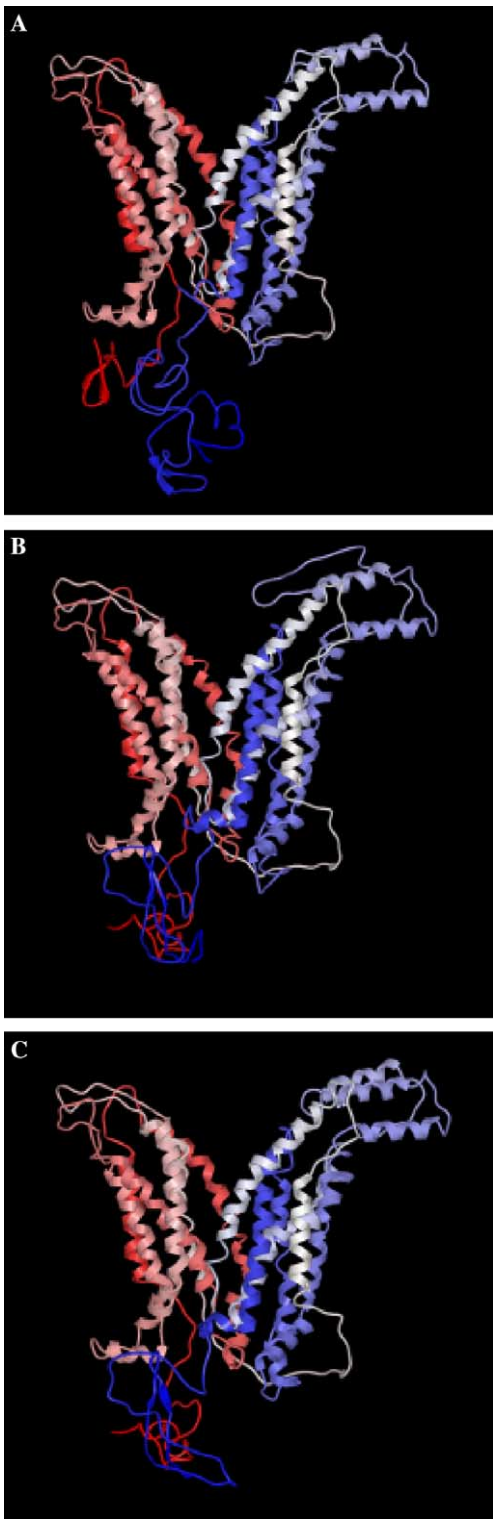

Figure 1. (A) Energy minimized SERT model viewed in the membrane plane. (B) Energy minimized DAT model viewed in the membrane plane. (C) Energy minimized NET model viewed in the membrane plane. The C_α-traces of each transporter model are colored according to residue order, with the N-terminal being dark blue and the C-terminal being dark red.

2. Results

The energy minimized models of SERT, DAT, and NET are shown in Figures 1A–C. The transporter models are structurally organized as their template, lac permease, with symmetrically positioned N- and C-terminal domains, each with six TMHs. In contrast to the template,

their hydrophilic putative ligand binding cavities are open to the extracellular side, representing an outward-facing conformation. The Procheck option of the Savs Metaserver for analyzing and validating protein structures (http://nihserver.mbi.ucla.edu/SAVS/) was used to check the stereochemical quality of the models. According to the Ramachandran plot, residues in the most favored regions were 80.0% (SERT), 78.3% (DAT), and 75.9% (NET), residues in additional allowed regions were 17.6% (SERT), 19.8% (DAT), and 21.5% (NET), residues in generously allowed regions were 2.2% (SERT), 1.1% (DAT), and 2.0% (NET), and residues in disallowed regions were 0.2% (SERT), 0.7% (DAT), and 0.6% (NET). These results indicate that the models were geometrically acceptable.

Conserved amino acids in lac permease, and SERT, DAT, and NET are Leu89, Phe105, and Pro106 (TMH1), Phe117 (TMH2), Thr301 (TMH5), Trp326 (TMH6), Ala387 (TMH7), Ala424 and Phe428 (TMH8), Val512 (TMH10), Phe551 (TMH11), and Gly578 (TMH12). These amino acid numbers refer to SERT. In the inward-facing conformation of SERT, the substrate translocation pore was closed toward the extracellular side by hydrophobic interactions between the conserved amino acids Phe105, Pro106, Phe117, and Ala387. In TMH2 facing the cytoplasm there is a conserved negative charge; Glu135 in SERT and Asp68 in lac permease.

A putative ligand binding area and a substrate translocation area are located between TMHs 1, 2, 4, 5, 7, 8, 10, and 11 in the SERT, DAT, and NET models, in accordance with the lac permease structure and site-directed mutagenesis studies (Table 2) on DAT and SERT. The ligands had overlapping, but not identical binding sites in the three transporter models. The most important TMHs forming the ligand binding site are TMHs 1, 4, 5, 7, and 11, as also observed for the sugar binding site in the lac permease structure.[10]

2.1. SERT–ligand interactions

The ligands docked into SERT are shown in Figures 2A–C, displaying the amino acids suggested as most important for ligand interaction from the present docking results. All the ligands interacted with Asp98 and Val102 (TMH1), Phe117, Tyr121, and Met124 (TMH2), Met260, Phe263, and Tyr267 (TMH4), Tyr289 (TMH5), Tyr516 and Thr519 (TMH10), and Leu547, Phe548, Phe551, and Ile552 (TMH11) (Table 1). In addition, *S*-citalopram and *S*-amphetamine inter-

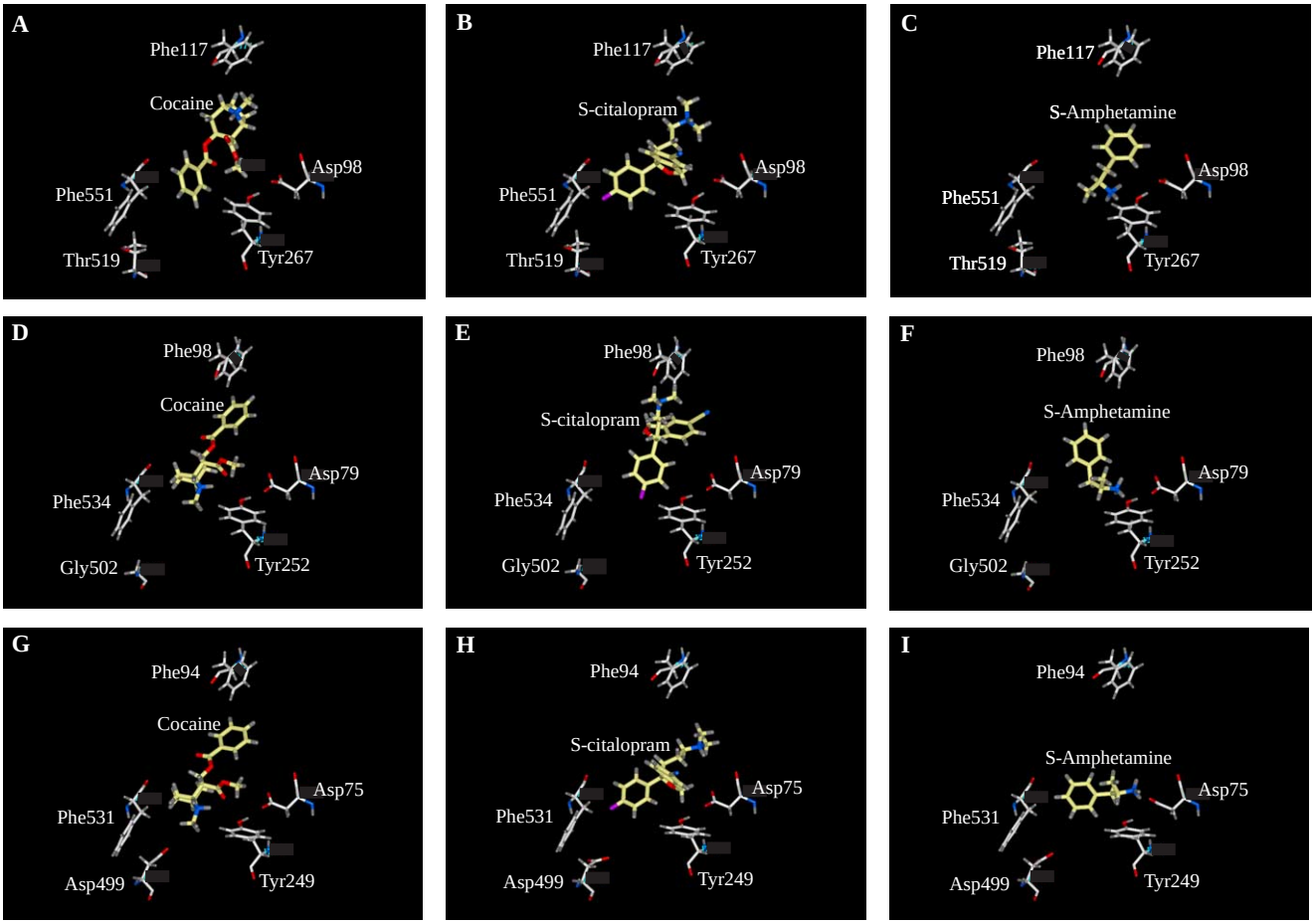

Figure 2. Cocaine (A), *S*-citalopram (B), and *S*-amphetamine (C) docked into SERT model. Displayed amino acids interacting with the ligand are Asp98, Phe117, Tyr267, Thr519, and Phe551. Cocaine (D), *S*-citalopram (E), and *S*-amphetamine (F) docked into DAT model. Displayed amino acids interacting with the ligand are Asp79, Phe98, Tyr252, Gly502, and Phe534. Cocaine (G), *S*-citalopram (H), and *S*-amphetamine (I) docked into NET model. Displayed amino acids interacting with the ligand are Asp75, Phe94, Tyr249, Asp499, and Phe531.

Table 1. Amino acids in the ligand binding area identified by ligand docking

TMH	SERT	DAT	NET
1	Tyr95	Phe76	Phe72
	Asp98	**Asp79**	**Asp75**
	Val102	**Val83**	**Val79**
	Pro106	**Pro87**	**Pro83**
2	**Phe117**	**Phe98**	**Phe94**
	Tyr121	**Tyr102**	**Tyr98**
	Met124	Phe105	Phe101
4	Met260	Val245	Met242
	Phe263	Ile248	Val245
	Thr264	Val249	Ile246
	Tyr267	**Tyr252**	**Tyr249**
5	**Tyr289**	**Tyr274**	**Tyr271**
7	**Ser372**	**Ser357**	**Ser354**
	Gly376	**Gly361**	**Gly358**
	Phe380	**Phe365**	**Phe362**
10	**Tyr516**	**Tyr499**	**Tyr496**
	Thr519	Gly502	Asp499
11	Leu547	Cys530	Ala527
	Phe548	**Phe531**	**Phe528**
	Phe551	**Phe534**	**Phe531**
	Ile552	Val534	Val532

Each line represents amino acids in corresponding positions in each transporter. Positions with identical amino acids within SERT, DAT, and NET are shown in boldface.

acted with Tyr95 (TMH1), and cocaine and *S*-citalopram interacted with Ser372 (TMH7).

In the docking position of cocaine predicted by the ICM program as the most energetically favorable, the benzene ring of cocaine formed stacking interactions with Phe551 (TMH11). The ester groups of cocaine formed hydrogen bonds with Tyr121 (TMH2), Tyr267 (TMH4), and Tyr289 (TMH5). The tropane ring interacted with Phe117 (TMH2) forming hydrophobic interactions (Figure 3).

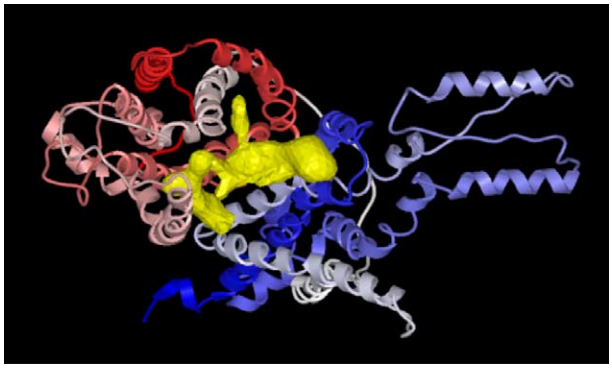

Figure 3. Possible binding pockets (yellow) at the outward-facing SERT model viewed from the extracellular side as evaluated by ICMPocketFinder. The SERT C_α-traces are colored according to residue order, with the N-terminal being dark blue and the C-terminal being dark red.

The nitril group of *S*-citalopram was oriented toward Tyr289 (TMH5), and the fluoride atom toward Thr519 (TMH10). Stacking interactions were formed between Phe551 (TMH11) and the fluorobenzyl group of *S*-citalopram.

S-Amphetamine formed stacking interactions with Phe263 (TMH4).

2.2. DAT–ligand interactions

Figures 2D–F show the ligand–DAT complexes after energy minimization, displaying only the amino acids suggested as most important for ligand interaction from the present docking results. All ligands interacted with Asp79 and Val83 (TMH1), Tyr102 and Phe105 (TMH2), Ile248, Val249, and Tyr252 (TMH4), and Phe531 and Phe534 (TMH11).

In addition, cocaine interacted with Phe98 (TMH2), Val245 (TMH4), Tyr274 (TMH5), (Ser357, Tyr499 (TMH10)), and Cys530 (TMH11). The ester groups of cocaine formed hydrogen bonds with Tyr102 (TMH2) and Tyr274 (TMH5) in DAT. Stacking interactions were formed between the benzene group of cocaine and Phe98 (TMH2), while hydrophobic interactions were formed between the tropane ring of cocaine, and Phe105 (TMH2) and Phe534 (TMH11).

S-Citalopram also interacted with Phe76 and Pro87 (TMH1), Phe98 (TMH2), Val245 (TMH4), Tyr274 (TMH5), and Tyr499 (TMH10). Hydrogen bonds were formed between the ether oxygen of *S*-citalopram and Tyr102 (TMH2) in DAT. The nitril-benzene group of *S*-citalopram formed stacking interactions with Phe98 (TMH2), while the fluorobenzyl group formed stacking interactions with Phe105 (TMH2), Phe531, and Phe534 (TMH11). Hydrophobic interactions were formed between the nitril-benzene group of *S*-citalopram and Val245 (TMH4), and between the alkyl chain of *S*-citalopram and Val83 (TMH1).

S-Amphetamine interacted with Phe76 (TMH1), Ser357, and Phe365 (TMH7). The *S*-amphetamine molecule formed stacking interactions with Phe105 (TMH2), Phe531, and Phe534 (TMH11).

2.3. NET–ligand interactions

The docking complexes of NET after energy minimizations are shown in Figures 2G–I, displaying only ligands and interacting amino acids. All three ligands interacted with Asp75 and Val79 (TMH1), Tyr98, and Phe101 (TMH2), Val245, Ile246, and Tyr249 (TMH4), Tyr271 (TMH5), Ser354 (TMH7), Tyr496 (TMH10), and Ala527, Phe531, and Val532 (TMH11).

Cocaine also interacted with Phe94 (TMH2), Met242 (TMH4), and Phe528 (TMH11). Hydrogen bonds were formed between the ester groups of cocaine, and Tyr98 (TMH2), Tyr249 (TMH4), and Tyr271 (TMH5) in NET. Stacking interactions occurred between the benzene ring of cocaine, and Tyr98 and Phe94 (TMH2) in

NET. Hydrophobic interactions were formed between the tropane ring of cocaine, and Phe101 (TMH2) and Phe531 (TMH11), and between the benzene ring and Ile246 (TMH4).

The nitril group of *S*-citalopram interacted with Tyr271 (TMH5), and the fluoride atom was oriented toward Asp499 (TMH10). Stacking interactions were formed between the fluorobenzyl group of *S*-citalopram, and Phe101 (TMH2) and Phe531 (TMH11). In addition, *S*-citalopram interacted with Phe72 (TMH1), Phe94 (TMH2), Met242 (TMH4), and with Phe528 (TMH11).

S-Amphetamine also interacted with Phe528 (TMH11) and formed stacking interactions with Phe98 and Phe101 (TMH2), Tyr249 (TMH4), and Phe531 (TMH11) in NET.

3. Discussion

The SERT, DAT, and NET models presented in this study were based on an assumed structural resemblance between the SNF transporters and lac permease, due to their common functional mechanism using an ion gradient as energy source for translocation of molecules against a concentration gradient. This common mechanism suggests that these transporters may have a common folding motif, but this has not been confirmed experimentally. The sequence identity within the transmembrane regions between lac permease and the SNF proteins SERT, DAT, and NET is 11–13%. Within the transmembrane regions the sequence identity is 6–11% between G-protein coupled receptors (GPCR) and bacteriorhodopsin, which share a common 7 TMH overall structure.[17] The crystal structure of bacteriorhodopsin[18] was previously used as a template for molecular modeling of GPCRs.[19] The crystal structure of the GPCR rhodopsin[20] confirmed the existence of 7 TMHs and that the GPCR models based on bacteriorhodopsin were valuable for the prediction of ligand binding sites. The sequence similarities between the neurotransmitter transporters and lac permease are comparable with the similarities between GPCRs and bacteriorhodopsin, which indicates that the lac permease structure may be a suitable template for modeling of neurotransmitter transporters.

We have previously used knowledge-based methods to construct molecular models of SERT, DAT, and NET, when a detailed structural template was not available.[13,14] The previous models of the 12 membrane spanning α-helices in SERT, DAT, and NET were based on an NhaA model,[12] that was constructed from the low-resolution electron density projection map of NhaA[1] and site-directed mutagenesis studies on SERT and DAT. After publication of the models, the crystal structures of the MFS transporters lac permease[10] and the glycerol-3-phosphate transporter[11] were reported. Interestingly, there was a striking resemblance between the membrane spanning domains of the MFS crystal structures and the previous SERT, DAT, and NET models.[15] For example, both in the previous NhaA based SERT model and the present lac permease based SERT model, the cocaine binding area includes residues Tyr95, Asp98 (TMH1), Tyr267 (TMH4), Tyr289 (TMH5), and Phe551 (TMH11).

When the cocaine molecule was docked automatically into the putative binding sites of each transporter model by the interactive docking option provided by the ICM program, the cocaine positions and orientations were almost identical in DAT and NET (Fig. 2). The benzene ring of cocaine formed stacking interactions with the conserved phenylalanines Phe98 (DAT)/Phe94 (NET), and the tropane ring formed hydrophobic interactions with Phe534 (DAT)/Phe531 (NET). The methyl ester group of cocaine formed hydrogen bonds with the conserved Tyr252 (DAT)/Tyr249 (NET). The position of the cocaine molecule when docked into the putative binding site in SERT was similar to that in DAT and NET. However, the docking procedure of cocaine into SERT resulted in a 180° rotation compared with the docking into DAT and NET. In SERT, there was also a hydrogen bond between the methyl ester group of cocaine and the conserved Tyr267, similar to the interactions of cocaine with DAT and NET. In addition, there were stacking interactions between the conserved Phe551 in SERT, and the benzene group of cocaine, and hydrophobic interactions between the tropane ring of cocaine and the conserved Tyr117 in SERT. A hydrogen bond interaction between the benzoate carboxyl of cocaine and a tyrosine residue was observed in a crystal structure of a bacterial cocaine esterase complex.[21] In the present docking studies, the benzoate carboxyl of cocaine interacted with tyrosines being conserved in DAT, NET, and SERT, namely Tyr102 (DAT), Tyr98 (NET), and Tyr121 (SERT).

Docking of *S*-citalopram resulted in almost identical positions for *S*-citalopram in SERT and NET. In general, the amino acids involved in *S*-citalopram binding were conserved between SERT and NET, namely Asp98 (SERT)/Asp75 (NET), Val102 (SERT)/Val79 (NET), Phe117 (SERT)/Phe94 (NET), Tyr121 (SERT)/Tyr98 (NET), Tyr267 (SERT)/Tyr249 (NET), Tyr289 (SERT)/Tyr271 (NET), Tyr516 (SERT)/Tyr496 (NET), Phe548 (SERT)/Phe528 (NET), and Phe551 (SERT)/Phe531 (NET). The involvement of the SERT residue Phe263 in ligand binding, which was seen in the docking complex, has recently been confirmed by site-directed mutagenesis studies.[22]

The nitril group of *S*-citalopram was oriented toward Tyr289 (SERT)/Tyr271 (NET), and the positively charged nitrogen atom of *S*-citalopram was oriented toward Asp98 (SERT)/Asp75 (NET). The main differences in the binding area were Tyr95 (SERT)/Phe72 (NET) and Thr519 (SERT)/Asp499 (NET). The fluorobenzyl group of *S*-citalopram interacts with Phe551 (SERT)/Phe531 (NET) and Thr519 (SERT)/Asp499 (NET). The selectivity factor of *S*-citalopram, which is calculated from the K_D (dissociation constants) ratios for *S*-citalopram binding to SERT over NET, is 3500.[16] An unfavorable anion–π repulsion[23] between the fluorobenzyl group of *S*-citalopram and Asp499 of NET, as

observed in the *S*-citalopram-NET docking, may explain the lower affinity of *S*-citalopram for NET than for SERT.

The main difference in orientation of *S*-citalopram in DAT compared to SERT and NET was the position of the nitril group. In DAT, the nitril group interacted with Val245 (TMH4), while in SERT and NET it was oriented toward Tyr289/Tyr271 (TMH5), respectively. The fluorobenzyl group of *S*-citalopram was oriented in the same direction in all transporters, pointing in the direction of the amino acid corresponding to Gly502 in DAT. However, in SERT this amino acid is a threonine (Thr519), while in NET it is an aspartate (Asp499). Gly502 was not in van der Waals contact with *S*-citalopram and might not contribute to stabilize the transporter–ligand complex.

Studies using human and *Drosophila* SERT chimeras have indicated that Tyr95 in SERT contributes to *S*-citalopram potency.[24] In the present docking studies, Tyr95 was localized in the binding area and might interact with the nitril group of *S*-citalopram.

The positions of *S*-amphetamine after the docking into SERT, DAT, and NET were similar in the three transporters. However, the benzene ring was oriented somewhat differently in each transporter. In SERT, the benzene group of *S*-amphetamine was oriented toward TMH4, stacking with Phe263, in DAT it was oriented toward TMH2, stacking with Phe98 and Phe105 (TMH2), and in NET it was oriented toward TMH11, stacking with Phe531.

ICMPocketFinder indicated that the conserved residues Asp98/79/75 (SERT/DAT/NET), Tyr267/252/249 (SERT/DAT/NET), and Tyr289/274/271 (SERT/DAT/NET) are important for ligand binding. The interactive docking option provided by the ICM program placed the ligands into this binding pocket, yielding interactions between these residues and the ligands. This finding is in accordance with site-directed mutagenesis studies.[25–27]

The amino acids in lac permease corresponding to Asp98, Tyr267, and Tyr289 in SERT are Met23, Ala122, and Trp151, respectively. In the lac permease crystal structure, these residues are involved in sugar binding.[10]

Some amino acids are conserved between lac permease and SERT, DAT, and NET: Leu89, Phe105, and Pro106 (TMH1), Phe117 (TMH2), Thr301 (TMH5), Trp326 (TMH6), Ala387 (TMH7), Ala424 and Phe428 (TMH8), Val512 (TMH10), Phe551 (TMH11), and Gly578 (TMH12), SERT numbering. This suggests that these residues are involved in transporter function or ligand binding. Interestingly, three of these amino acids, Pro106, Phe117, and Phe551 (SERT numbering), take part in the putative cocaine/SSRI binding site. Phe105, Pro106, Phe117, and Ala387, which 'close' the substrate translocation pore by hydrophobic interactions between the side chains in the inward-facing conformation, might be involved in an alternating access transporter mechanism. The conserved negative charge; Glu135 (SERT numbering), corresponding to Asp68 in lac permease, which is in contact with the cytoplasm, may also be important for the transporter mechanism, possibly involving charge transfer leading to conformational changes in the transporter.

Although there is agreement between the present docking studies and data from site directed mutagenesis studies, some of the results from the docking have to be experimentally confirmed: (1) Whether the crystal structure of lac permease is a sufficiently similar template for homology modeling of SNF proteins. (2) The proposed outward-facing conformation of SERT. (3) The interpretation of site directed mutagenesis data. Such results are not unambiguous, since they do not show whether the observed effects are due to direct disruption of side-chain-ligand interactions or caused by overall structural changes of the binding region induced by the mutations.

The involvement of TMH3 in ligand binding has been suggested by some experimental studies (Table 2).

Table 2. Transporter protein residues shown by site directed mutagenesis studies to interact with cocaine/CFT, citalopram, MPP^+ or dopamine

TMH	Transporter	Residue	Corresponding hSERT residue	Ligand	Reference
1	hSERT	Tyr95		Citalopram	24
	rDAT	Phe76	Tyr95	Cocaine/CFT	37
	rSERT	Asp98	Asp98	Citalopram	25
	rDAT	Asp79	Asp98	Cocaine/CFT	26
3	hSERT	Ile172,Tyr176		Cocaine/CFT	38
	bDAT	Val152	Ile172	Cocaine/CFT	39
7	rDAT	Ser350, Ser353	Val366, Cys369	MPP^+, dopamine	40
	rDAT	Ser356, Ser359	Ser372, Ser375	MPP^+, dopamine, CFT	26
4	rDAT	Tyr251	Tyr267	Cocaine/CFT	27
5	rDAT	Tyr273	Tyr289	Cocaine/CFT	27
11	rDAT	Tyr533	Phe551	Cocaine/CFT	41

hSERT, human SERT; rSERT, rat SERT; hDAT, human DAT; rDAT, rat DAT; bDAT, bovine DAT; MPP^+ = 1-methyl-4-phenylpyridinium; CFT = (−)-2β-carbomethoxy-3β-(4-fluorophenyl)tropane (cocaine analogue).

Recently, a study involving cross-species chimera followed by species-scanning mutagenesis suggested that the TMH3 residues Ala169 and Ile172 of hSERT are important for ligand binding.[28] Assuming that the 12 TMHs of secondary transporters may be loosely packed in an active state associated with transport of substrate molecules, TMH3 could easily slide or tilt toward the ligand binding site during ligand binding. Widespread cooperative conformational changes including sliding and tilting motions of the TMHs have been suggested to occur during ion and substrate transport in lac permease.[4] Therefore, the SERT transport mechanism may involve several conformational changes in the transporter, both in TMHs and in loop segments.

In conclusion, the present models may be used as tools for designing further experimental structural studies of SERT, DAT, NET, and their ligand interactions, which may contribute to the iterative process of structural investigation of SNF proteins. Investigation of the molecular interactions of psychotropic drugs with such transporters may be useful in drug development. These models may have pharmacological explanatory power, in spite of several uncertainties, due to the fact that they are modeled by analogy with the crystal structure of other distant relative transporters.

Coordinates of the SERT, DAT, and NET models are available from the authors upon request.

4. Experimental

4.1. Computational procedures

The ICM software version 3.0-28i[29] was used for interactive molecular graphics, homology modeling, and ligand docking, and the AMBER 8.0 program package[30] was used for empirical force field calculations of molecular structures. All models and complexes were first energy minimized with restrained backbone by 500 cycles of steepest descent minimization followed by 500 steps of conjugate gradient minimization, and then energy minimized with no restraints by 1000 cycles of steepest descent minimization followed by 1500 steps of conjugate gradient minimization. A 10 Å cutoff radius and a distance dependent dielectric function ($\varepsilon = r_{ij}$), where r_{ij} is the distance between atoms i and j, were used in the molecular mechanics calculations.

4.2. Homology modeling of SERT based on lac permease X-ray structure

The amino acid sequences of human SERT,[31,32] human DAT,[33] and human NET[34] were aligned manually to the lac permease amino acid sequence using ICM. The alignment was adjusted according to data reported from site directed mutagenesis studies on SERT and DAT (Table 2), such that residues shown to interact with ligands in experimental studies would be directed inwards toward a putative binding site in the transporter model. The alignment used for the homology modeling is shown in Figure 4.

A model of SERT was constructed using the homology modeling module of ICM, with the lac permease crystal structure as template.[10] The ICM program constructs the model from a few core sections defined by the average of C_α atom positions in the conserved regions. This procedure provides a homologue model of the TMHs and loops, but not of the N- and C-terminals. The N- and C-terminals were derived from a previous SERT model,[14] based on structures in the Protein Data Bank (PDB) database.[35] The SERT model was refined using a RefineModel macro of ICM including: (1) Monte Carlo simulation on side chains; (2) 50 steps of energy minimizations; and (3) a second Monte Carlo simulation on side chains. Restraints were used in step 2 to keep the backbone atoms at the same positions as in the template.

4.3. Modeling of an outward-facing conformation of SERT

The lac permease structure[10] that was used as a template for the SERT model represents the inward-facing conformation with bound substrate. The sugar-binding site is open toward the cytoplasmic side in this structure. To bind ligands such as cocaine and SSRIs, an alternative outward-facing conformation open to the extracellular side is required. The construction of the outward-facing model represented the most challenging step of this modeling study. Based on chemical modification and cross-linking experiments, Abramson et al. suggested a model for the outward-facing conformation of lac permease.[10] Using their description as a guideline, a relative rigid-body rotation of ~60° between the N- and C-terminal domains around the axis perpendicular to the membrane was applied to obtain an initial putative outward-facing conformation of the SERT model. The cocaine molecule was docked into the inward-facing SERT model in a position equivalent to that of the sugar molecule in lac permease.[10] The cocaine molecule was used since it binds to all three monoamine transporters, and it has been shown by site directed mutagenesis studies to interact with Asp98, Tyr267, and Tyr289 (Table 2). Cocaine was kept in the same position during the rotation, maintaining contact with Asp98, Tyr267, and Tyr289. Figure 5 shows the inward-facing and the outward-facing SERT conformations, respectively.

4.4. Homology modeling of DAT and NET

The outward-facing SERT model was used as a template for modeling of DAT and NET, using the homology modeling option of the ICM program. The initial DAT and NET models were refined using the RefineModel macro of ICM. The SERT, DAT, and NET models were then energy minimized using the AMBER 8.0 program package.

4.5. Ligand molecules

The chemical structures of citalopram, cocaine, and amphetamine are shown in Figure 6. Atomic coordinates, restrained electrostatic point (RESP) charges for the atoms, and empirical force field parameters for the

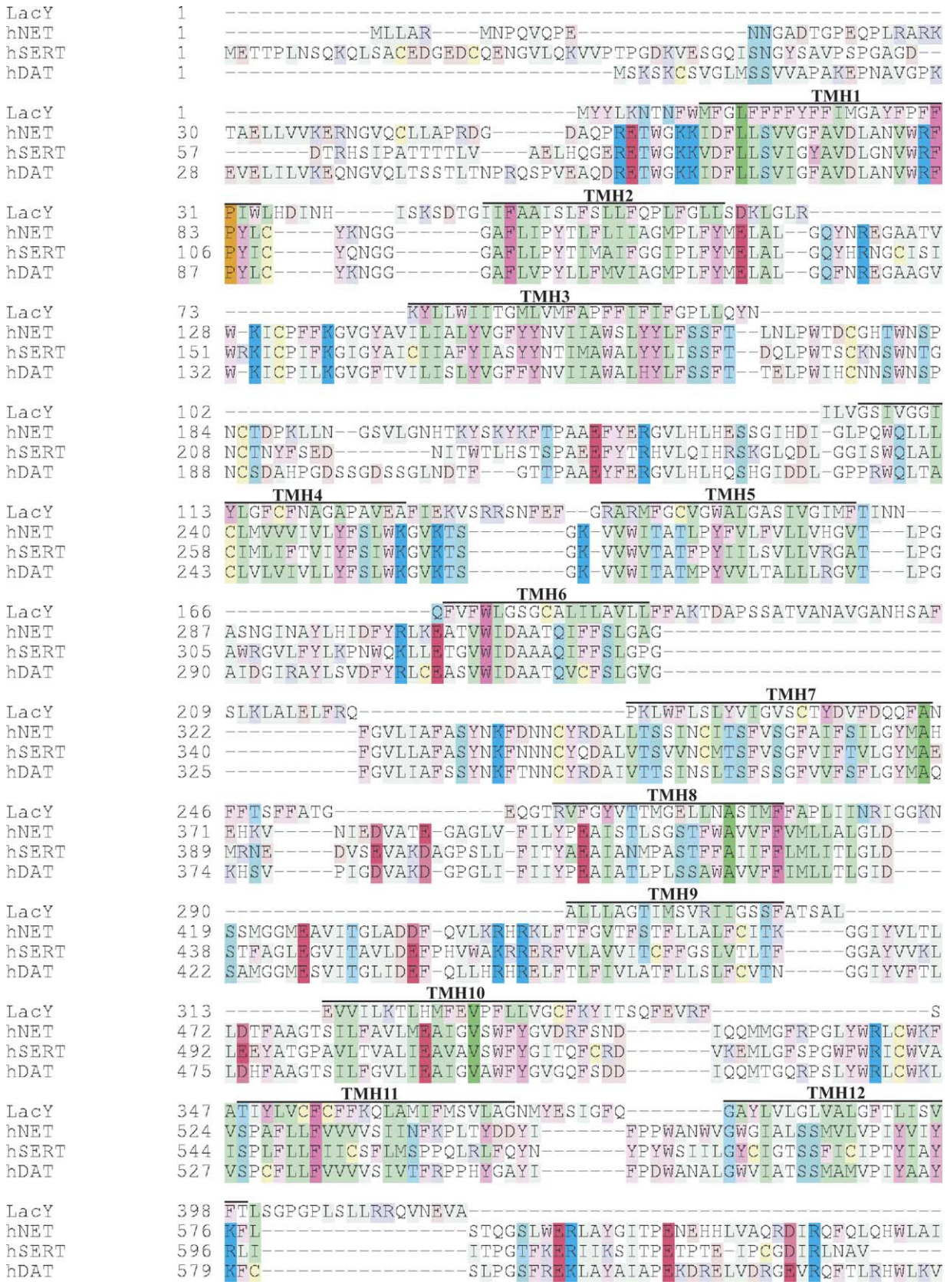

Figure 4. The amino acid sequences of hSERT, hDAT, and hNET aligned to the lac permease amino acid sequence (LacY).

molecular mechanics calculations of *S*-citalopram and cocaine were taken from previous molecular modeling studies.[13,36] Input for the AMBER molecular mechanics programs of the *S*-amphetamine molecule was prepared using the Leap and antechamber programs of the AMBER 8.0 program package. The molecule was drawn

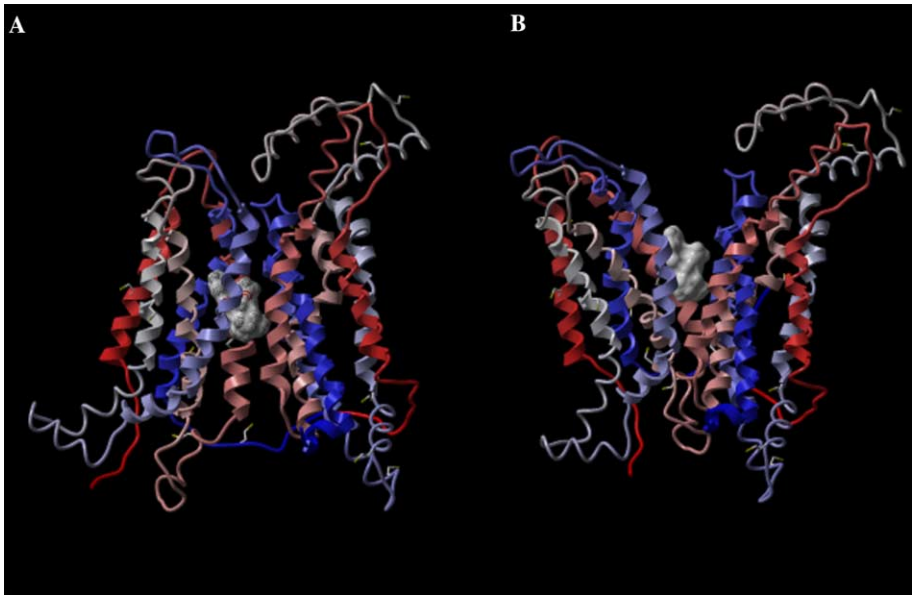

Figure 5. (A) Inward-facing SERT model with cocaine molecule in the putative binding site. (B) Outward-facing SERT model with cocaine molecule in the putative binding site.

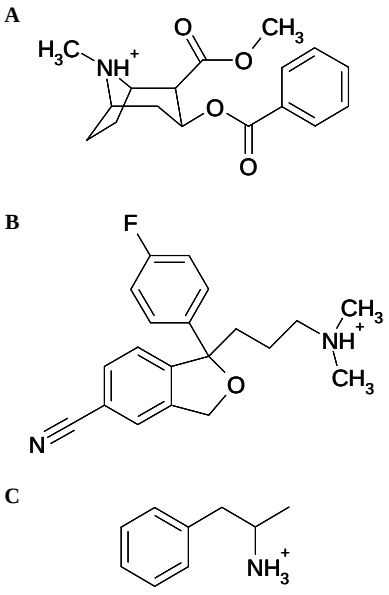

Figure 6. Molecular structures of cocaine (A), citalopram (B), and amphetamine (C).

using the X-windows graphical interface of the xleap shell scripts and the antechamber program was used to assign atomic charges and atom types. The Sander program of the AMBER 8.0 program package was used for energy minimization of the *S*-amphetamine molecule.

4.6. Ligand docking

The binding pockets at the outward-facing SERT model were characterized using ICMPocketFinder, which detects cavities of sufficient size to bind ligands. The yellow areas indicated in the figure represent a binding pocket suggested by the ICM program. The areas include Asp98, a residue shown to interact with *S*-citalopram in site directed mutagenesis studies (Table 2). Other residues in the putative binding pocket in the yellow areas were Phe117 (TMH2), Tyr267 and Trp271 (TMH4), Tyr289 (TMH5), Phe423 (TMH8), and Phe556 (TMH11).

The cocaine-, *S*-citalopram- and *S*-amphetamine molecules were first docked manually into the outward-facing SERT model into the cavity consisting of the yellow pockets shown in Figure 3. The manually generated ligand position was adjusted by the interactive docking option provided by the ICM program, using Asp98 as an anchoring point.

Asp79 was used as the anchoring point for automatically docking of the cocaine-, *S*-citalopram-, and *S*-amphetamine molecules into the DAT model, as described for SERT. Asp79, which corresponds to Asp98 in SERT, has been shown to interact with cocaine in site directed mutagenesis studies (Table 2).

The anchoring point for docking the cocaine-, *S*-citalopram-, and *S*-amphetamine molecules into the NET model was Asp75, which corresponds to Asp98 in SERT and Asp79 in DAT. The docking was performed as described for SERT.

For each ligand docking, the complex with the lowest interaction energy of 20 positions examined by ICM was energy minimized using the Sander program in the AMBER 8.0 program package.

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