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A putative three-dimensional arrangement of the human serotonin transporter transmembrane helices: a tool to aid experimental studies

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Abstract

The human serotonin transporter is the molecular target for selective serotonin reuptake inhibitor drugs which are being used for treatment of depression. A three-dimensional model of the membrane spanning parts of the transporter was constructed. The transporter was assumed to consist of 12 transmembrane α-helices. The model was based on published experimental data of cocaine binding to mutant transporters, amino acid sequence analysis, and interactive molecular graphics. The model suggests that a high affinity cocaine binding site is situated in a region of the model where Asp98 acts like an anchor, while a putative low affinity site is situated in another region with Glu508 as the anchoring amino acid. A series of docking experiments with various reuptake inhibitors were conducted, using interactive molecular graphics techniques combined with energy calculations and analysis of the transporter-ligand complexes. Experiments involving molecular mapping of ligand binding areas may benefit from using the current model in experimental design. From the current model, several amino acids were proposed as prime candidates for mutagenesis and subsequent ligand binding studies. Also for evaluation of results from site directed mutagenesis experiments with SERT and similar transporters we assume the model will be helpful.

Keywords: Molecular modelling; Cocaine; SSRI; Molecular graphics; Mutant

1. Introduction

The selective serotonin (5-HT) reuptake inhibitors (SSRI) are drugs being used for the treatment of depression. SSRI act by inhibiting presynaptic reuptake of 5-HT at the 5-HT transporter (SERT) in the central nervous system and, thus, elevate the concentration of 5-HT at the synaptic cleft.

The SERT belongs to the family of SNF (sodium:neurotransmitter symporter family) proteins [1]. This family also includes the dopamine transporter (DAT), the GABA-transporter (GAT), the glycine transporter, the betaine transporter and several other transporters for neurotransmitters and hormones. All these proteins show high degrees of sequence similarity. More than 40 SNF proteins have been cloned, but the detailed three-dimensional molecular structure of any of the SNF proteins is not known. The SNF proteins act as co-transporters of sodium and chloride ions and of substrate molecules [2,3]. The transmembrane sodium gradient provides energy for the inward movement of the substrate molecules against its own concentration gradient.

The SERT has been proposed to consist of 12 transmembrane α-helices (TMH), with both the amino terminus and carboxy terminus localised intracellularly [4–6]. Hydropathy plots indicate three putative TMH followed by a large hydrophilic loop [1], which is assumed to be located extracellularly. Nine clear peaks of hydrophobicity follows after this loop, and this suggests a total of 12 TMH [1]. Alternative topologies of SNF proteins have been proposed, suggesting that either TMH1 [7,8] or TMH2 [9] does not span the membrane. Furthermore, an additional TMH between TMH3 and TMH4 of the originally proposed topology has been suggested [7–9]. However, recent results from measuring interactions of selected lysine and cysteine residues with extracellular agents support the originally proposed 12 TMH topology for SERT since none of the predicted internal cysteine or lysine residues are exposed to the external environment [5]. Furthermore, the recently determined electron density projection map of the *Escherichia coli* Na^+/H^+ antiporter reveals that this membrane transporter protein has 12 TMH [10].

The SNF amino acid sequences are more conserved in the region between TMH1 and the N-terminal than the subsequent segments of these proteins, and the highest degree of sequence similarity is observed in the relatively hydrophilic

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region where TMH1 is localised. This region contributes to an SNF signature sequence having hydrophilic character and has been suggested to form an amphipatic α-helix with conserved polar residues localised to one side of TMH1 [1]. Functional important residues for ligand interaction of both SERT (Tyr95 [11]) and DAT (Asp79 [12]) are situated in the amino acid segment predicted in the Swiss-Prot database [13] to be TMH1.

A number of psychotropic drugs interact with the SNF proteins SERT, DAT and the noradrenaline transporter, including the psychostimulant cocaine, the tricyclic antidepressant imipramine, and the SSRI (fluoxetine, fluvoxamine, paroxetine, citalopram and sertraline). Cocaine has similar affinities for DAT and SERT, imipramine has slightly higher affinity for SERT than for the noradrenaline transporter, and the SSRI are from 300 to 3500 times more selective towards SERT than to the noradrenaline transporter. SSRI and tricyclic antidepressants generally have low affinity for DAT [14]. Studies of SERT and the binding of cocaine, imipramine and the SSRI paroxetine and citalopram [15–19] indicate the existence of more than one binding site on the same transporter protein.

Other molecular models of 12 TMH transporters have been presented previously, including DAT [20], GLUT1 [21] and GLUT3 [22]. Extensive studies conducted on lac permease [23,24], have given insight into the arrangement of the TMH in this transporter. Of these modelled transporters, only DAT belongs to the same family as SERT. No detailed crystal structure of a 12 TMH transporter is available, therefore, any proposed arrangement of SERT TMH is hypothetical.

The present SERT model is based on the amino acid sequence alignment and published ligand binding studies of mutated SERT and DAT interacting with cocaine. Mutagenesis studies indicate that the drug binding area is located in the transmembrane region, therefore, the extracellular and intracellular loops and terminals of the transporter protein were not included in the model. In this study, the cocaine molecule and cocaine binding data acquired from the literature were used to guide the construction of a cocaine binding site including amino acids in TMH 1, 3, 4, 5 and 11 of the human SERT, using molecular modelling techniques. The remaining helices were positioned according to mutagenesis data on other ligands binding to SERT, DAT and GAT, and calculated electrostatic interactions between the helices. The overall architecture of the SERT model has similarities to the published electron density projection map of the *Escherichia coli* Na^+/H^+ antiporter [10]. The SSRI were docked into a putative ligand binding area in order to investigate hypothetical binding modes and differences in binding modes, and to suggest amino acids to be examined in site-directed mutagenesis and functional studies.

Additional knowledge about the three-dimensional structure and molecular interactions between SERT and SSRI is important for a better understanding of the molecular mechanisms of the central nervous action of SERT and SSRI. Molecular mapping of ligand binding areas of SERT is one approach in the development of antidepressants with improved potency and selectivity.

2. Methodology

2.1. Modelling of ligands

The AMBER molecular mechanical parameters for the SSRI citalopram, paroxetine, fluoxetine, fluvoxamine and sertraline (Fig. 1) were chosen by analogy [25], interpolated [26–28], obtained from [29–31] or derived from the Cambridge Structural Database (CSD) [32]. The ligands were energy minimised using molecular mechanical and quantum mechanical methods. The sander program in the AMBER v. 4.1 software package [28] was used for molecular mechanical calculations. The ligands were energy minimised by 10 cycles of steepest descents followed by conjugate gradient minimisation until convergence using a convergence criterion of 0.0001 for the norm of the energy gradient. A distance dependent dielectric function ($\varepsilon = 1 \times r_{ij}$) was used in order to imitate an aqueous environment. The quantum mechanical calculations were performed with Gaussian94 [33], at the Hartree–Fock level with a 6-31G* basis set, and direct self-consistent field calculations with a convergence criterion of 10^{-6}. The Berny algorithm as implemented in Gaussian94 was used for energy minimisation. Since there

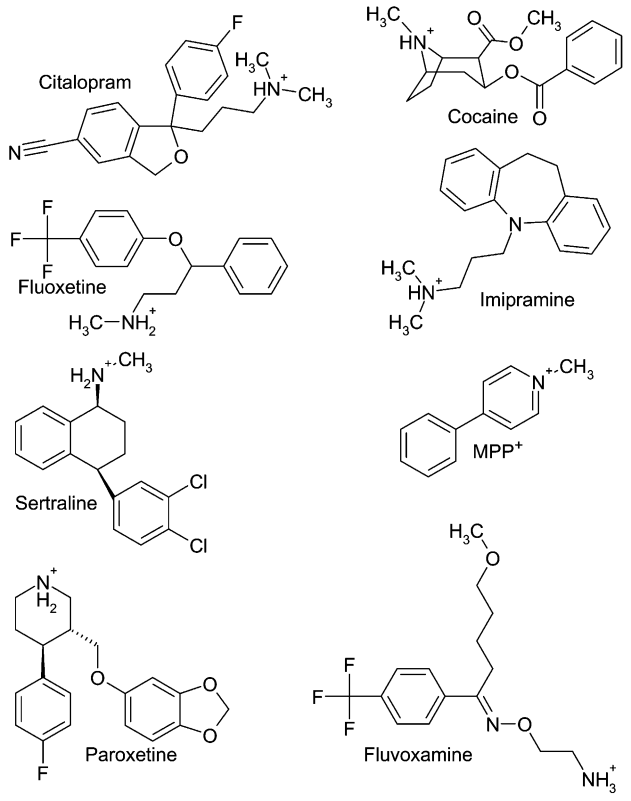

Fig. 1. Molecular structure of ligands.

are no published crystal structure available for the majority of the above compounds, the quantum mechanically energy minimised structures also served as a check on the molecular mechanical parameters which had been developed. The electrostatic potentials of the quantum mechanically energy minimised SSRI structures were calculated, and the resultant potentials were transformed into atom centred charges which were used in the molecular mechanical energy calculations. The RESP [34,35] module of the AMBER package was used for the calculation of atomic charges.

2.2. Identification of TMH

The modelling procedure for the SERT model is shown in Fig. 2. The amino acid sequence of the human SERT (Swiss-Prot accession number P31645) [36] was retrieved from the Swiss-Prot database (release 36) [13], and was submitted to the PHD Predict Protein [37] server as a query sequence. A multiple sequence alignment of the most similar proteins was obtained along with a prediction of TMH regions. The sequence alignment was used to obtain TMH predictions from other servers on the Internet (TMHMM [38], DAS [39], HMMTOP [40] TMpred and TopPred2 [41]). Thus, a series of slightly different predictions of putative TMH positions in the human SERT sequence were obtained. TMHMM predicted TMH1 with low confidence, DAS predicted TMH1 to be very short, whereas the other methods failed to predict TMH1 (Table 1). We interpreted this to indicate that TMH1 has weak characteristics of a TMH. Furthermore, this could indicate that TMH1 is surrounded by other TMH and/or water. The start and end points of each TMH as predicted by PHD for TMH 2–12 were used for modelling the SERT. The position of TMH1 in the amino acid sequence was chosen from the Swiss-Prot record, since the remainder of the TMH positions in this record corresponded well with the predictions obtained from the PHD

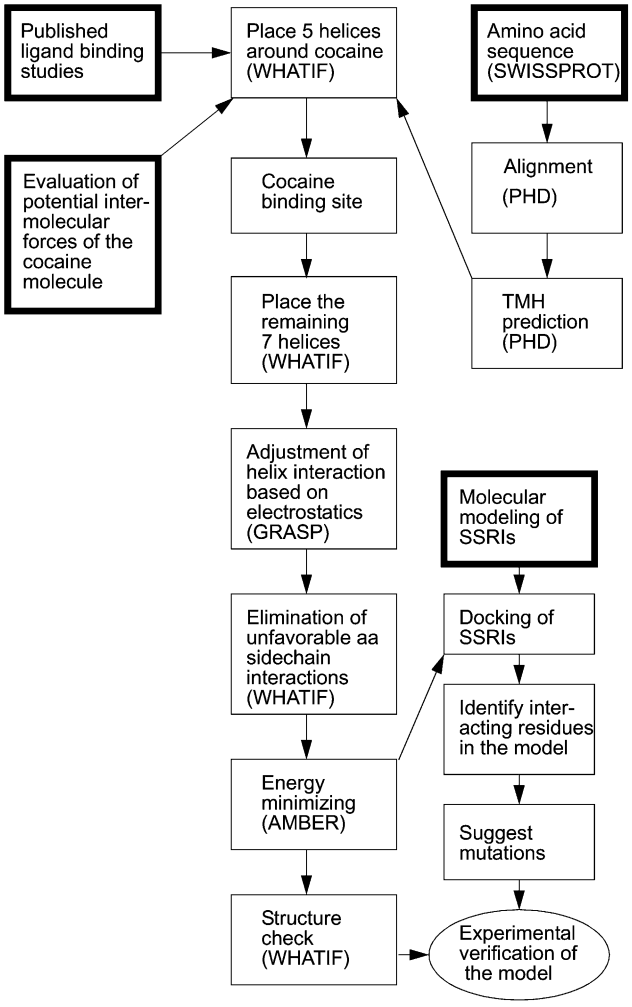

Fig. 2. Procedure for construction of the SERT model and docking studies. Software and servers that were used are indicated in parentheses.

Table 1
TMH predictions considered for the selection of membrane spanning regions for modelling the human SERT (Swiss-Prot accession number P31645)

TMH	Predicted TMH region							TMH sequence modelled[a]
	PHD	Swiss-Prot	TMHMM[b]	DAS	Tmpred	TopPred2	HMMTOP	
1		88–108	88–106	88–97				FLLsVIGyAVdLGnVWrFpyI
2	117–135	117–135	118–140	117–134	117–138	113–133	115–139	FLLpytIMAIFGGIpLFyM
3	162–185	162–185	160–182	161–175	157–176	156–176	160–184	GyAICIIAFyIAsyyntIMAWALy
4	255–272	255–272	251–273	253–270	251–271	251–271	250–271	LALCIMLIFtVIyFsIk
5	283–300	283–300	280–302	283–299	280–306	277–297	280–304	VtAtFpyIILsVLLVrGA
6	331–348	331–348	326–348	334–347	332–348	330–350	325–349	AqIFFsLGpGFGVLLAFA
7	367–384	367–384	361–383	365–385	366–387	367–387	362–386	VnCMtsFVsGFVIFtVLG
8	416–434	416–434	414–436	420–436	417–436	416–436	413–437	nMpAstFFAIIFFLMLItL
9	467–484	467–484	465–487	466–487	465–486	465–485	465–489	LAVVItCFFGsLVtLtFG
10	497–514	497–514	496–518	500–515	497–516	499–519	500–521	TGpAVLtVALIeAVAVsW
11	541–558	541–558	539–561	539–558	539–558	539–559	534–558	WVAIspLFLLFIICsFLM
12	577–594	577–594	573–595	574–598	572–595	572–592	571–595	LgyCIGtssFICIptyIA

[a] According to Swiss-Prot data (TMH1) and PHD-prediction (TMH 2–12). Hydrophobic amino acids are shown in upper case letters, hydrophilic in lower case letters.

[b] TMH1 was predicted with confidence 0.6, other TMH with confidence 1.0.

Table 2
Single amino acid substitution mutant data for the serotonin (SERT), dopamine (DAT) and γ-amino butyric acid (GAT) transporters considered when modelling the TMH bundle[a]

Transporter	Residue in transporter	Correspondig residue in SERT	TMH	Ligand binding and transport modulation	Possible mechanism, functional and structural importance
SERT	Tyr95		1	Citalopram, mazindol	TMH1 involved in formation of antagonist binding sites [11]
	Ile172		3	Cocaine, 5-HT	On the face of a TMH. Ile172 and Tyr176 near 5-HT and cocaine binding site [43,46]
	Tyr176				
	Ile179				
	Cys369		7	Complete loss of substrate transport and antidepressant binding	Important for 5HT transport and antidepressant binding [17]
	Ser545		11	Na^+, imipramine	Determinant of both the cation dependence of serotonin transport and imipramine binding [49]
	Phe586		12	Imipramine, desipramine, nortrityline	Formation of high affinity antagonist binding sites [50]
DAT	Asp79	Asp98	1	Dopamine, MPP^+, CFT	Crucial for DAT function [12]
	Tyr251	Tyr267	4	Cocaine, CFT, dopamine, MPP^+	May be involved in cocaine analogue recognition, possibly by interaction with the aromatic or tropane rings of cocaine [44]
	Tyr273[b]	Tyr289	5		
	Ser356	Ser372	7	Dopamine, MPP^+, CFT	Crucial for DAT function, residues differentially important for cocaine binding and dopamine uptake [12]
	Ser359	Ser375			
	Ser350	Val366	7	MPP^+, dopamine	Selectively involved in neurotoxin uptake [47]
	Ser353	Cys369			
	Ser527	Ser545	11		
	Ser538	Phe556			
	Tyr533	Phe551			
	Tyr533	Phe551	11	MPP^+, cocaine	May be important for function incl. species differences [45]
GAT	Arg69	Arg104	1	GABA (or possibly Cl^-)	Translocation of Na^+ through the membrane does not involve charged amino acid residues [48]

[a] MPP^+: 1-methyl-4-phenylpyridinium; CFT: (−)-2b-carbomethoxy-3b-(4-fluorophenyl)tropane (cocaine analogue).
[b] Tyr271 probably corresponds to Tyr273 [44].

server (Table 1). Only the core hydrophobic stretches were predicted, therfore, the TMH obtained from the PHD predictor are on average <20 amino acids long. The Swiss-Prot record is based on four transmembrane prediction methods.

2.3. TMH aggregation

Results from publications on ligand binding experiments with mutated SERT, DAT and GAT were used as a basis for the modelling (Table 2). Since the results from ligand binding experiments with mutant transporters where one or very few amino acids have been substituted can be interpreted to indicate amino acid participation in ligand interaction, such data were used for the construction of a cocaine binding pocket. The loops and terminals of SERT are relatively long and could not be predicted or modelled with confidence, and were not included in the model. Thus, of the 630 amino acids, the SERT model includes 227 amino acids, corresponding to the putative 12 TMH. The 12 TMH were built individually as α-helices ($\phi = \psi = -57^\circ$, $\omega = 180^\circ$) using the WHATIF package version 19970813-1517 [42], and arranged in an antiparalell way using interactive computer graphics.

Since cocaine is a relatively rigid molecule it was assumed that the three-dimensional structure of cocaine and the data from published ligand binding studies (Table 2) could be used to guide the construction of a putative cocaine binding site. Potential interaction sites with cocaine were assumed for those amino acids found to be important in ligand binding experiments with mutated transporters (Table 2). Initially, an evaluation of potential intermolecular interaction sites and binding modes between the ligand and amino acids in SERT was done, and possible interactions of cocaine with amino acids published in site directed mutagenesis studies were considered based on the initial evaluation (Fig. 3). Such intermolecular interactions of the cocaine molecule may involve ionic interactions, hydrogen bonds and hydrophobic interactions, including stacking interactions. Cocaine is a relatively rigid molecule, which means that few conformers are important. The assumption that the lowest energy conformation of cocaine interacts at the binding site may therefore be valid. Moreover, cocaine is a potent inhibitor of SERT [14,43].

Published data on cocaine interacting with mutant transporters (Table 2) [12,43–46] suggested that amino acids in TMH 1, 3, 4, 5 and 11 could form a cocaine binding site. These experimental results were used to position these five TMH around the cocaine molecule using interactive molecular graphics (WHATIF). During the interactive process it was made sure that intermolecular interactions (van der Waals surface contact) with cocaine as indicated in Fig. 3 were formed. All atoms were included at this stage of the modelling, and the α-helices were rigid. The helices were oriented perpendicular to the virtual membrane plane. Asp98 (TMH1) was oriented towards the positively charged nitrogen atom of the cocaine molecule, Tyr176 (TMH3) had van der Waals surface contact with the ester group of the methyl ester moiety of the cocaine molecule, and Ile172 (TMH3) interacted with the tropane ring of the cocaine molecule. Tyr267 (TMH4) and Tyr289 (TMH5) formed contact with the ester group of the benzoate ester moiety of the cocaine molecule, and Phe551 (TMH11) was directed towards the phenyl moiety of the cocaine molecule. Thus, by using cocaine and the ligand binding data in Table 2 as a basis, it was possible to construct a putative binding site for cocaine including TMH 1, 3, 4, 5, and 11. This solution is not unique, but is rather a step towards mapping the cocaine binding site of SERT. The cocaine molecule could be oriented differently, and the conformation of the molecule at the binding site might not be the lowest energy conformation. The intermolecular interactions between the SERT residues and the cocaine molecule are also assumptions. Experimental data indicate which residues that interact with cocaine at the binding site, but exactly which residues that interact with

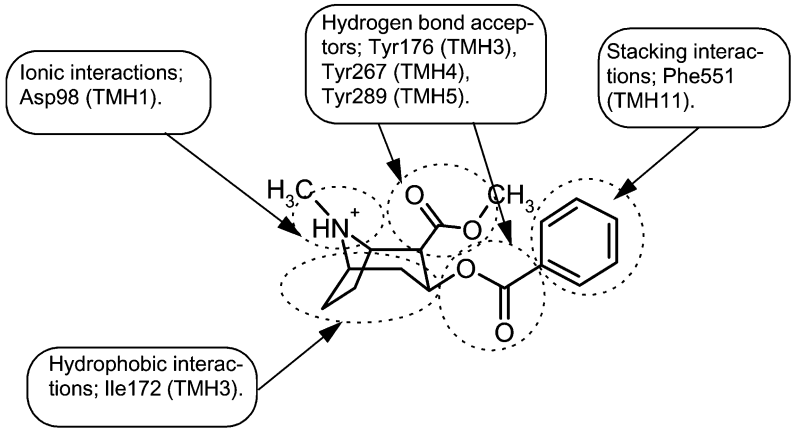

Fig. 3. Evaluation of potential intermolecular forces between cocaine and SERT, and putative interactions of cocaine with amino acids published in ligand binding studies.

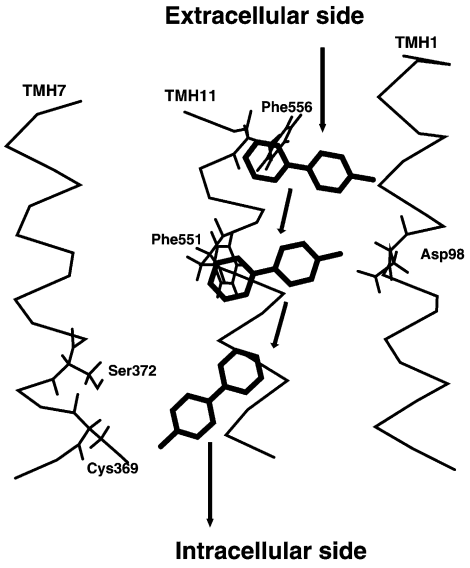

Fig. 4. A possible substrate translocation pathway with focus on MPP^+ interactions with TMHs 1, 7 and 11. The arrows indicate the MPP^+ pathway from the extracellular side through the transporter and into the intracellular side. MPP^+ (thick lines) is shown in different hypothetical positions during translocation. SERT residue side chains that correspond to residues predicted from experimental studies on TMHs 1, 7 and 11 of DAT to interact with MPP^+ are shown; Asp98, TMH1 (Asp79 of DAT [46]), Phe551, TMH11 (Tyr533 of DAT [50]), Phe556, TMH11 (Ser538 of DAT [50]), Cys369, TMH7 (Ser359 of DAT [46]) and Ser372, TMH7 (Ser356 of DAT [46]). SERT probably undergoes conformational changes during substrate translocation while this substrate docking was performed on a rigid model.

the different parts of the cocaine molecule remains to be determined. A mirror-image organisation of the TMH in the cocaine binding site can not be excluded.

Based on literature data (Table 2) on mutant DAT and their interactions with dopamine and 1-methyl-4-phenylpyridinium (MPP^+, a DAT substrate being regarded as a neurotoxin) [12,44,45,47], the localisation of TMH7 was assumed to be between TMH 5 and 11. This allowed MPP^+ to be translocated along TMH 1, 4, 5, 7 and 11, as predicted from experimental data from studies on MPP^+ binding to mutated DAT (Table 2). A possible substrate translocation pathway along TMH 1, 3, 4, 5, 7 and 11 was tested with interactive molecular graphics with focus on MPP^+ interactions with residues predicted from experimental studies on TMH 1, 7 and 11 of DAT (Fig. 4). Interactions could be seen between residues Asp98, TMH1 (Asp79 of DAT [12]), Phe551, TMH11 (Tyr533 of DAT [47]), Phe556, TMH11 (Ser538 of DAT [47]), Cys369, TMH7 (Ser359 of DAT [12]) and Ser372, TMH7 (Ser356 of DAT [12] (Fig. 4). Residues on TMH 4 and 5 were also in contact with MPP^+. One should keep in mind that SERT probably undergoes conformational changes during substrate translocation while this substrate docking was performed on a rigid model. If TMH6 and TMH7 swap positions in the helical bundle, as might seem more natural if the helices were sequentially organised, the hypothetical MPP^+ translocation pathway would not fit the experimental data.

TMH1 and TMH11 were placed in the central area of the SERT model since experimental data indicate the existence of drug interacting residues on two sides of the TMH [12,45,48,49]. This arrangement yields two possible ion translocation pathways, a feature also suggested for the Na^+/H^+ antiporter [10]. The functionally important Arg69 of the GAT [48], which corresponds to Arg104 of SERT, is situated on the opposite side of TMH1 relative to Asp98. A high affinity sodium dependent imipramine binding site seems to involve Ser545 of TMH11 [49] and Phe586 of TMH12 [50]. Ser545 is on the opposite side of TMH11 relative to Phe551, which was assumed to interact with cocaine. Thus, it was assumed that cocaine and imipramine might bind to different translocation pathways. With this information in mind TMH 1 and 11 were placed centrally in the helical bundle. TMH2 and TMH6 are relatively lipophilic, thus, these helices probably do not line any aqueous translocation pore, and consequently, TMH2 and TMH6 were surrounded by other TMH. TMH6 was positioned between TMH 7, 11, 8 and the virtual membrane. The relatively short loop between TMH6 and TMH7 suggested that these helices should be placed next to each other. Gln332 of TMH6 and Asn416 of TMH8 may interact by hydrogen bonds, according to the present model. TMH2 was placed between TMH 1, 3 and the virtual membrane. The relatively short loop between TMH1 and TMH2 suggested that these TMH should be placed close to each other. Glu508 of TMH10 was directed towards the second aqueous pore assuming that Glu508 could be an anchoring point for a second ligand interaction site [51]. TMH9 and TMH10 were placed next to each other because the extracellular loop between them is relatively short indicating that these TMH are neighbours. TMH12 was placed between TMH8 and TMH9 with Phe586 lining the second translocation pathway, thus, forming a putative imipramine binding site between Ser545 of TMH11 [49] and Phe586 of TMH12 [50]. All pore lining helices were oriented with hydrophilic residues directed towards the aqueous translocation pathways. Fig. 5 shows the relative localisation of the 12 TMH.

Electrostatic potentials of all TMH were calculated with the GRASP software [52] using the amino acid atomic charges from AMBER as a basis. The interhelical electrostatic contacts were taken into account for each helix–helix interaction when the helices were positioned in the helical bundle. The most negatively charged areas of the electrostatic potential field of one helix had helix–helix contact with the most positively charged areas of the electrostatic potential field of the neighbouring helix. Neutral electrostatic areas of the helices were matched with each other. Subsequently, removal of unrealistic side chain interactions was done with WHAT IF [42]. Finally, the TMH bundle was energy minimised as described above.

The modelling strategy described above is different from the one used in a previous DAT model [20]. However, in both models explicit membrane molecules and water and

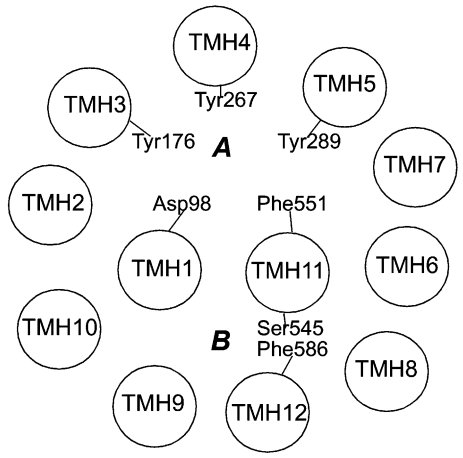

Fig. 5. Schematic view of the relative positions of the 12 TMH of the SERT model. Residues from published site directed mutagenesis studies implying cocaine binding (region A: Asp98, Tyr176, Tyr267, Tyr289 and Phe551) and imipramine binding (region B: Ser545 and Phe586) are indicated (see Table 2).

ions were omitted. It is assumed that the inclusion of such molecules in the model does not contribute to the quality of the model since the relative positions of the TMH are much more significant.

2.4. Ligand docking

The SSRI citalopram, paroxetine, fluoxetine, fluvoxamine and sertraline were manually docked into the putative cocaine binding region using the MIDASPlus software [53]. Both ligands and the transporter were kept rigid during docking. Each ligand was docked into the model in three or four different positions/orientations, and both enantiomers of citalopram and fluoxetine were docked. The positively charged nitrogen atom of each ligand was oriented towards Asp98 in each position, and the SSRI molecules were fitted into the cocaine binding pocket in different orientations. For each ligand, the two positions that appeared to be most favourable sterically and electrostatically were further

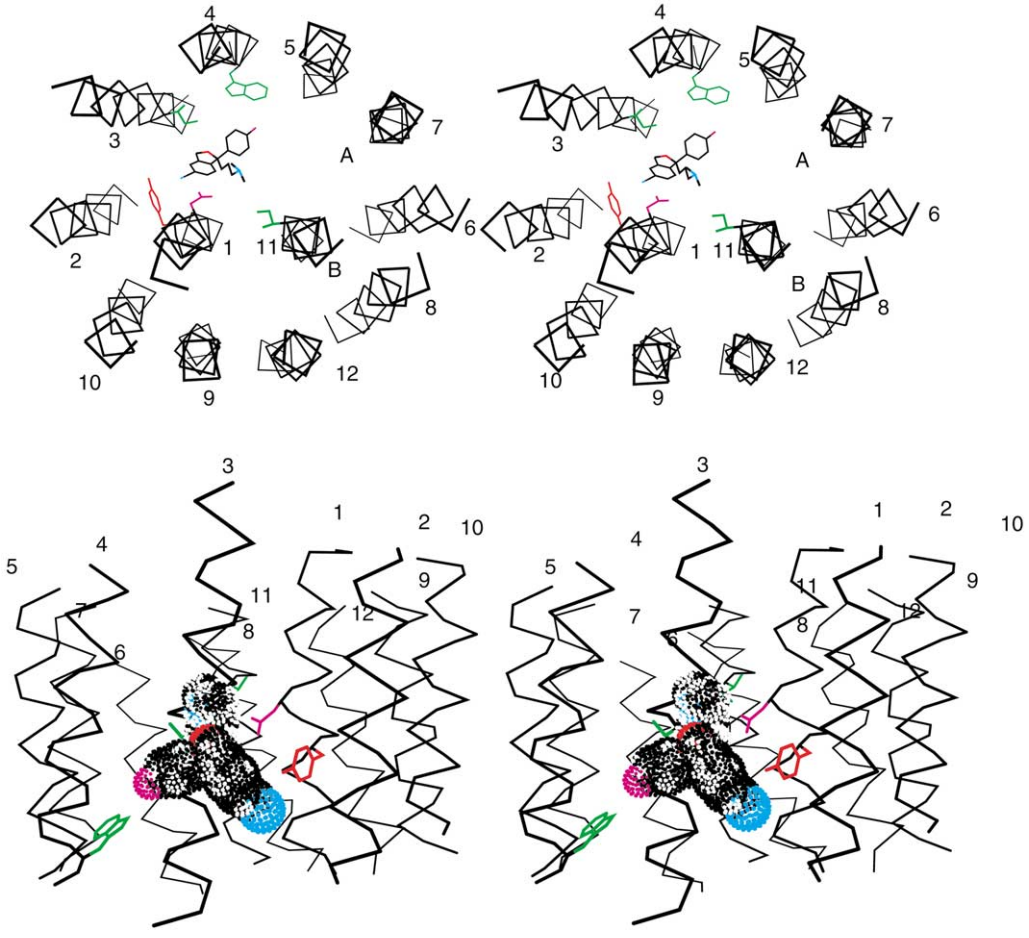

Fig. 6. Stereoscopic view of *S*-citalopram docked into the SERT model. The TMH (numbered) are shown as Cα traces and the amino acid side chains being most important for ligand–protein interactions are shown in colour. Tyr95 (TMH1, red), Asp98 (TMH1, magenta), Ile172 (TMH3, green), Trp271 (TMH4, green), Ile552 (TMH11, green). Nitrogen atoms of citalopram are shown in cyan, fluorine is shown in magenta and oxygen is shown in red. Lines closer to the viewer are drawn thicker than lines further away. Upper: view from the synaptic side. Lower: perpendicular view; synaptic side up. Citalopram is surrounded by dots indicating its van der Waals surface. The letters A and B identify two putative ligand binding regions in the transporter model (see Fig. 5).

refined by energy minimisation as described above, except that a convergence criterion of 0.002 was used. Amino acids involved in ligand binding were identified based on a distance criteria: amino acid residues having van der Waals contact with the ligand was judged to be involved in ligand binding, as well as those having van der Waals contact after increasing all van der Waals radii with 20%. Fig. 6 shows how one of the SSRI, citalopram, was docked into the putative ligand binding site.

3. Results and discussion

The SERT model shown in Fig. 6 has two regions (A and B) separated by TMH 1 and 11. These regions may be possible substrate/ion translocation pathways. TMH1 and TMH11 were positioned centrally in the model, and amino acid sequence similarity data indicated that these membrane spanning segments have special properties. TMH1 showed the highest amino acid sequence similarity (67%) and TMH11 showed the lowest similarity (14%) among SNF proteins [54]. The low similarity of TMH11 among various transporters within this superfamily could indicate that this segment is involved in ligand recognition. TMH1 fails to be predicted by several theoretical methods for secondary structure prediction. The prediction methods are partly based on amino acid lipophilicity, and TMH1 might lack direct contact with the membrane, as in the current model. TMH1 contributes to a signature sequence for the SNF proteins [1] and is assumed to be a transmembrane α-helix. TMH11 also lacks contact with the membrane and is located in an area of the putative substrate transport channel which is rich in phenylalanines (Fig. 7). Furthermore, TMH11 contains several phenylalanines and is predicted as a TMH, and the hydrophobicity of TMH11 is retained within the SNF family.

An electron density projection map of the *Escherichia coli* Na^+/H^+ antiporter was recently determined using electron cryo-microscopy [10]. Possible structural similarities between symporter and antiporter proteins remains uncertain. The Na^+/H^+ antiporter is an ion exchanger while SERT is a sodium-neurotransmitter symporter, so the mechanisms of translocation would be expected to differ between the two transporter proteins. Two ion-translocation pathway possibilities are suggested in the Na^+/H^+ antiporter structure, as for the present SERT model. The Na^+/H^+ antiporter reveals that two TMH are located centrally in the transporter with little or no membrane contact, a feature also shared with the SERT model. However, the Na^+/H^+ antiporter displays an arrangement of helices in a roughly triangular shape on one side of the membrane and a loose irregular helix packing on the other side [10]. In the SERT model, the helical bundle is arranged roughly as a cylinder (Figs. 5 and 6). The perpendicular arrangement of the SERT helices to the virtual membrane plane is an assumption that may not be true. Three-dimensional structures of membrane proteins show that the TMH often are tilted relative to the membrane plane. However, at this stage of SERT modelling we had no evidence indicating how the SERT helices may be tilted. The SERT model helix arrangement also shows similarities to the proposed helix arrangement of lac permease, the H^+/β-galactoside symporter of *Escherichia coli* [23].

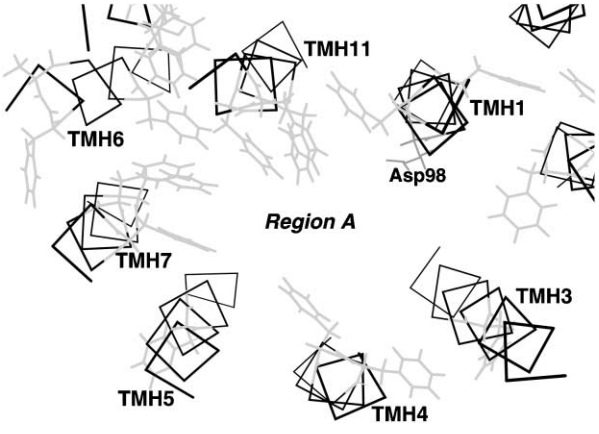

Fig. 7. The phenylalanine rich area of SERT region A. Phenylalanine side chains and Asp98 are shown explicitly the remaining amino acids are shown as Cα traces.

Some experimental data indicate one high affinity and one low affinity cocaine binding site, possibly situated on the same transporter molecule [15]. We hypothesise that the high affinity site is situated in region A with Asp98 as an anchoring point, while the low affinity site is situated in region B with Glu508 as an anchoring point (Fig. 5). Cocaine was docked into region B and it was found that fewer hydrogen bonds than in region A could be formed (Fig. 8). Thus, region B might provide a low affinity binding site for cocaine. According to the present model and published mutagenesis data, citalopram and cocaine probably have overlapping interaction areas in region A (Fig. 5). Citalopram probably binds to the 5-HT specific part [16] and inhibits 5-HT transport competitively [17]. It has been suggested that paroxetine and cocaine bind to different sites on the transporter, and that there is an allosteric modulation by paroxetine [18].

The tricyclic antidepressant imipramine is found to have one low affinity binding site in the citalopram binding region and one sodium dependent high affinity site in an another region [17]. Site directed mutagenesis studies indicate that Ser545 of TMH11 [49] and Phe586 of TMH12 [50] participate in the high affinity binding of imipramine. In the present model, these residues are situated in region B (Fig. 5). In order to check if the model could reproduce these experimental findings on imipramine binding to SERT, imipramine was docked into region B and could indeed form intermolecular contacts with the van der Waals surfaces of Ser545 (TMH11) and Phe586 (TMH12). The low affinity site is probably localised in region A in the cocaine/citalopram binding area with Asp98 as an anchor [17].

It has been suggested that there exist at least three different ligand binding areas in the SERT, and that imipramine,

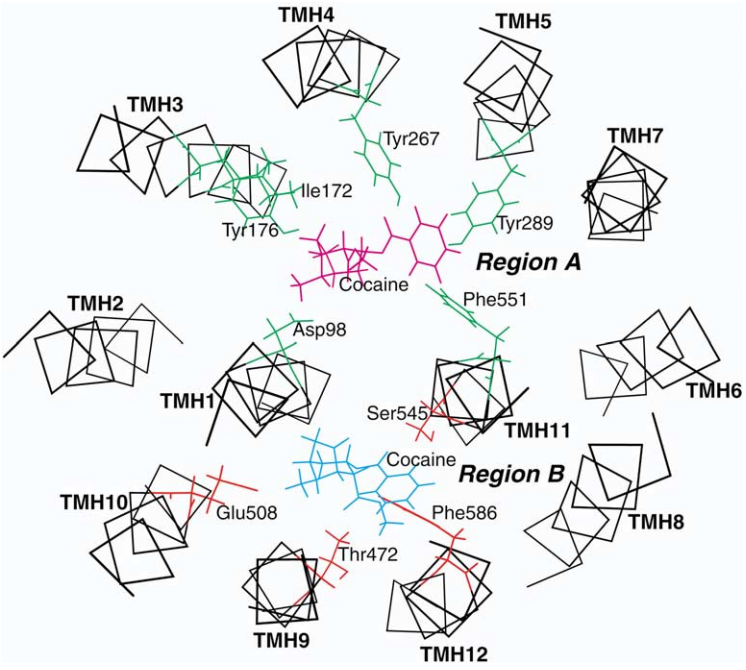

Fig. 8. Cocaine docked into both high affinity binding site (region A) and hypothetical low affinity binding site (region B) of the SERT model. The TMH (numbered) are shown as Cα traces and the amino acid side chains being most important for ligand–protein interactions are shown in colour. Region A: cocaine (magenta), Asp98 (TMH1), Ile172 (TMH3), Tyr176 (TMH3), Tyr267 (TMH4), Tyr289 (TMH5) and Phe 551 (TMH11) (green). Region B: cocaine (blue), Thr472 (TMH9), Glu408 (TMH10), Ser545 (TMH11) and Phe586 (TMH12) (red).

paroxetine and citalopram bind to different areas [16,55]. In the current model, emphasis was placed on the cocaine/citalopram interaction area in region A. A mutation of Asp98 in rat SERT, which was published after the current model had been constructed, confirms this model by the loss of antagonist potencies of cocaine, citalopram and imipramine, but not of paroxetine [19]. This mutation also diminished coupling to Na^+ and Cl^- and decreased 5-HT transport capacity, thus, suggesting that region A provides a permeation pathway for 5-HT and ions, and a binding site for cocaine and citalopram. Site directed mutagenesis studies indicated that the neurotoxin MPP^+ is translocated through the DAT molecule via interactions with serine residues [12,47]. Since DAT and SERT are relatively similar, a hypothetical translocation pathway that was tested interactively by transversing MPP^+ through region A of the SERT model involved TMH 1, 4, 5, 7 and 11 (Fig. 5). Fig. 4 shows a graphical presentation of this translocation pathway with focus on TMH 1, 7 and 11. The interactive translocation suggested that the model is in agreement with experimental data.

Several assumptions regarding ligand binding to mutated SERT may be made based on analysis of the ligand-transporter complexes, assuming that the SSRI interact in the putative cocaine interaction area. Experimental data indicate that at least paroxetine might interact in an other area of SERT than cocaine [18,19]. Residues involved in the binding of cocaine according to published ligand binding studies (Asp98, Ile172, Tyr176, Tyr267, Tyr289 and Phe551) should be tested for interactions with the SSRI. This would indicate whether the SSRI other than paroxetine and citalopram actually bind to SERT within the same area as cocaine. The importance of the putative anchoring point Asp98 in TMH1 for ligand binding should be tested for fluoxetine, sertraline and fluvoxamine. If no interaction is detected at this point with an SSRI, mutation of Glu508 of region B might be worthwhile. When SSRI were docked into the putative cocaine interaction area of region A, the following observations were made: Ile172 interacted with sertraline, citalopram, fluvoxamine, and fluoxetine. Tyr176 interacted with *R*-fluoxetine, citalopram and fluvoxamine and with sertraline. Tyr267 interacted with sertraline, *S*-fluoxetine, fluvoxamine and citalopram. Tyr289 interacted with *S*-fluoxetine in one of the docking positions, and also with both citalopram enantiomers and with fluvoxamine in one of the docking positions. Thus, a site directed mutagenesis study involving Tyr289 could give information on the orientation of the SSRI when interacting with the transporter.

In order to verify whether citalopram (and fluvoxamine) actually do interact slightly further into the transporter than cocaine, as observed in the docking complexes, residues closer to the cytosol (about one α-helix turn) than the residues interacting with cocaine should be mutated. These would include Ile168 in TMH3, Phe548 in TMH11 and possibly Ser91 in TMH1, Tyr134 in TMH2 and Trp271 in TMH4. Citalopram should also be checked for interaction with Ile552 of TMH11. Sertraline and fluoxetine should be

examined for interactions with the TMH4 residues Phe263 and Met260. In some of the modelled complexes, SSRI interact with Phe380 which has been shown to be vital to serotonin transport [3]. Other residues found by analysis of ligand-transporter complexes were: Phe551 which interacts with fluoxetine and sertraline; Tyr95 which interacts with fluvoxamine.

Experimental data indicate that at least three ligand binding sites exists on SERT [16,55]. If the binding site (region A) modelled in this study is the cocaine/citalopram binding site, the binding sites for paroxetine and imipramine remain unidentified. A mapping of a putative binding site in region B could thus reveal interesting features. This could be approached by mutating Glu508 of TMH10, assuming this amino acid is an anchoring point, and check whether paroxetine and imipramine binding are affected, as well as other ligands. Furthermore, potential extracellular anchoring points are possible candidates for mutagenesis studies. The present model of the cocaine binding site was based on the assumption that the residues identified by site directed mutagenesis studies constitute a high affinity binding site of cocaine. However, the possibility that some of these residues are localised in the low affinity binding site can not be excluded.

Mutating Gln332 of TMH6 or Asn416 of TMH8 might verify the helical packing of these two helices by interrupting the hypothetical hydrogen bond between these residues. Introduction of histidyl residues in these positions to see if a metal-ion-binding site is formed is one possibility [56]. In general, a systematic investigation of the relative positions of the helices would indeed be valuable.

In order to refine the SSRI interaction area of the SERT model, assuming the current model is reasonable, several approaches could be used. The two enantiomeres of fluoxetine have somewhat different affinity to the SERT; the (+)-isomer being slightly more potent than the (−)-isomer [57]. Investigation of transporter/enantiomer complexes could contribute to the mapping of the binding area. Examination of differences in the ligand binding areas of SERT, DAT and the noradrenaline transporter would also be interesting. Docking of the SERT-selective citalopram molecule into the noradrenaline transporter and the DAT should give fewer favourable interactions than with SERT, while cocaine theoretically should fit nicely into all three transporters. Sertraline, which is relatively potent at the DAT, should be possible to dock into the DAT in a favourable position. However, in order to investigate the binding areas further, it is necessary to verify the present model by site directed mutagenesis experiments since there are several possibilities for ligand binding positions and orientations, as well as other possibilities of TMH arrangement.

Although the present SERT model is based on a smaller number of studies with mutated transporters than desired, it is assumed that a least the putative ligand binding area in region A is reasonably well modelled. Recent biophysical studies of the cocaine binding pocket in SERT suggest that the binding site for cocaine is highly hydrophobic, and that the bound cocaine is still accessible for aqueous quenching [58]. This could indicate that the cocaine binding site is not very deeply buried in the transporter, or that the SERT conformation when cocaine is bound is not an entirely closed structure. The loops and terminals are probably important to maintain the structure of TMH [59], and the omission of these segments may influence the results. However, experimental data suggest that the external loops are not the primary determinants of substrate and inhibitor binding, but rather contribute to maintaining the stability and conformational flexibility of the transporter [60]. Hence, the loops were not included in the current model. This deficiency of the model will probably be of largest importance when performing molecular dynamics simulations, unless the loops do participate in ligand binding. The omission of solvation and membrane molecules obviously influences the structure and dynamics of the SERT, however, we believe that a crude model as this does not warrant sophisticated solvation and/or membrane models to be included. Indications that SERT might form a dimer or tetramer [61] was not considered since it is not clear whether dimerisation or tetramerisation is essential for functional activity. A recent study indicates that the favoured state of SERT in living cells is a homo-oligomeric form [62], thus, the present helical model of SERT could be modelled with an overall structure different from the cylindrical form in order to create possible inter-monomeric contacts. In the electron density projection map of the *Escherichia coli* Na^+/H^+ antiporter dimer, the distance between the two monomers suggests that the translocation pathway is not at the dimer interface [10].

In this study, conformational changes during translocation were not considered. Studies on lac permease indicate that the 12 TMH are loosely packed with water in the cavities in the active state, and that wide-spread co-operative conformational changes including sliding and tilting motions, may occur during ion transport [23]. This could also be the case for SERT. The role of TMH7 has been investigated and appears to contribute to at least some of the conformational changes that occur during Na^+ translocation [63].

4. Conclusions

Modelling and site directed mutagenesis experiments will be complementary to each other in iterating towards a better understanding of the details underlying the structure and function of SERT and other members of the SNF superfamily. Even though this SERT model is crude, we believe that it may be useful as a tool to select reasonable candidates for site directed mutagenesis studies of SERT and the most closely related transporters. Such experiments could provide information that may be useful for further SERT model refinement, and at the same time validate the model. The present model of the human SERT is a first approximation working tool towards rational design of mutant experiments.

Co-ordinates of the SERT model are available from the authors upon request.

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