

Exploring unbinding mechanism of drugs from SERT via molecular dynamics simulation and its implication in antidepressants

Xin-Guan Tan(谭新官), Xue-Feng Liu(刘雪峰), Ming-Hui Pang(庞铭慧), Yu-Qing Wang(王雨晴), and Yun-Jie Zhao(赵蕴杰)

Citation: Chin. Phys. B, 2023, 32 (**8**): 088702. DOI: 10.1088/1674-1056/acd687
Journal homepage: http://cpb.iphy.ac.cn; http://iopscience.iop.org/cpb

What follows is a list of articles you may be interested in

Coarse-grained simulations on interactions between spectrins and phase-separated lipid bilayers

Xuegui Lin(林雪桂), Xiaojie Chen(陈晓洁), and Qing Liang(梁清)
Chin. Phys. B, 2021, 30 (**6**): 068701. DOI: 10.1088/1674-1056/abf129

Protein-protein docking with interface residue restraints

Hao Li(李豪) and Sheng-You Huang(黄胜友)
Chin. Phys. B, 2021, 30 (**1**): 018703. DOI: 10.1088/1674-1056/abc14e

Molecular dynamics simulations of membrane deformation induced by amphiphilic helices of Epsin, Sar1p, and Arf1

Zhen-Lu Li(李振鲁)
Chin. Phys. B, 2018, 27 (**3**): 038703. DOI: 10.1088/1674-1056/27/3/038703

Protein-membrane interactions investigated with surface-induced fluorescence attenuation

Li Ma(马丽), Ying Li(李颖), Ming Li(李明), Shuxin Hu(胡书新)
Chin. Phys. B, 2017, 26 (**12**): 128708. DOI: 10.1088/1674-1056/26/12/128708

Exploring unbinding mechanism of drugs from SERT via molecular dynamics simulation and its implication in antidepressants

Xin-Guan Tan(谭新官)[1], Xue-Feng Liu(刘雪峰)[1,†], Ming-Hui Pang(庞铭慧)[1], Yu-Qing Wang(王雨晴)[1], and Yun-Jie Zhao(赵蕴杰)[2,‡]

[1] *College of Mathematics and Physics, Chengdu University of Technology, Chengdu* 610059, *China*
[2] *Institute of Biophysics and Department of Physics, Central China Normal University, Wuhan* 430079, *China*

(Received 27 February 2023; revised manuscript received 17 April 2023; accepted manuscript online 18 May 2023)

The human serotonin transporter (SERT) terminates neurotransmission by removing serotonin from the synaptic cleft, which is an essential process that plays an important role in depression. In addition to natural substrate serotonin, SERT is also the target of the abused drug cocaine and, clinically used antidepressants, escitalopram, and paroxetine. To date, few studies have attempted to investigate the unbinding mechanism underlying the orthosteric and allosteric modulation of SERT. In this article, the conserved property of the orthosteric and allosteric sites (S1 and S2) of SERT was revealed by combining the high resolutions of x-ray crystal structures and molecular dynamics (MD) simulations. The residues Tyr95 and Ser438 located within the S1 site, and Arg104 located within the S2 site in SERT illustrate conserved interactions (hydrogen bonds and hydrophobic interactions), as responses to selective serotonin reuptake inhibitors. Van der Waals interactions were keys to designing effective drugs inhibiting SERT and further, electrostatic interactions highlighted escitalopram as a potent antidepressant. We found that cocaine, escitalopram, and paroxetine, whether the S1 site or the S2 site, were more competitive. According to this potential of mean force (PMF) simulations, the new insights reveal the principles of competitive inhibitors that lengths of trails from central SERT to an opening were $\sim$ 18 Å for serotonin and $\sim$ 22 Å for the above-mentioned three drugs. Furthermore, the distance between the natural substrate serotonin and cocaine (or escitalopram) at the allosteric site was $\sim$ 3 Å. Thus, it can be inferred that the potent antidepressants tended to bind at deeper positions of the S1 or the S2 site of SERT in comparison to the substrate. Continuing exploring the processes of unbinding four ligands against the two target pockets of SERT, this study observed a broad pathway in which serotonin, cocaine, escitalopram (at the S1 site), and paroxetine all were pulled out to an opening between MT1b and MT6a, which may be helpful to understand the dissociation mechanism of antidepressants.

Keywords: human serotonin transporter (SERT), comprehensive molecular dynamics (MD) simulation, drug design, molecular mechanics/generalized Born surface area (MM/GBSA) method

PACS: 87.14.ep, 87.15.A–, 87.15.Cc, 87.15.K **DOI:** 10.1088/1674-1056/acd687

1. Introduction

Depression is listed by the World Health Organization as one of the world's leading disabilities, affecting over 280 million people.[1] Major depressive disorder is a serious mental health[2] condition with etiopathogenesis involving not only social and psychological factors,[3,4] but also genetic and biological elements.[5–7] The human serotonin (5HT) transporter (SERT) terminates neurotransmission by removing serotonin from the synaptic cleft, an essential process associated with depression.[8–10] In addition to transport substrate serotonin, SERT is also the target of abuse of the drug cocaine, and clinically used antidepressants, such as escitalopram and paroxetine.[11–17]

SERT belongs to the solute carrier 6 (SLC6) family, which includes neurotransmitter transporters such as dopamine transporter (DAT), γ-aminobutyric acid, norepinephrine and glycine.[18] In the mammalian brain, the expression of SLC6 neurotransmitter transporters plays a fundamental role in regulating neurotransmitter signaling via rapid reuptake of neurotransmitter molecules from the extracellular space into neurons and glial cells.[19,20] Cocaine is known to inhibit the activities of both SERT and DAT, which are homologous sequences.[16,21] Furthermore, cocaine has SLC6 neurotransmitter transporters as primary targets, which often leads to dependence and abuse. Three residues Tyr95,[22] Tyr175,[21] and Ser438[16,23] located within the orthosteric 5HT-binding pocket in SERT are key interaction locations responsible for conservation among biogenic amine neurotransmitter transporters and potential recognition to selective serotonin reuptake inhibitors such as escitalopram and paroxetine in the treatment of depression. However, the detailed mechanism of allosteric site of SERT is still unclear.

The highly conserved orthosteric substrate binding site (S1),[24] formed by transmembrane helices TM1, 3, 6, 8, and 10, is located in the center of SERT (Figs. 1(a) and 1(b)).[15,19,25–27] Based on the recently resolved x-ray structure of SERT, in addition to the S1 site, an allosteric site (S2) was found.[25,27,28] The S2 site is formed by an aromatic pocket positioned in the scaffold domain in the extracellular vestibule,

†Corresponding author. E-mail: liuxuefeng17@cdut.edu.cn
‡Corresponding author. E-mail: yjzhaowh@mail.ccnu.edu.cn

© 2023 Chinese Physical Society and IOP Publishing Ltd

connected to the central S1 site via a short tunnel. The allosteric modulators such as escitalopram targeting the S2 site of SERT have come into the market as a medication for depression, and the S2 site together with the S1 site are extremely promising drug targets for design of future novel inhibitors. Furthermore, the site (S3) and site (S4) of docking serotonin may have been, in theory, found by investigating the substrate-bound x-ray structures of the dopamine transporter.[29,30] The S3 site was adjacent to the S2 site, and the S4 site was further toward the extracellular region. In recent decades, a number of drugs with potential selectivity for inhibiting the activity of SERT to treat depression have been continued discovered, via computational and structural analyses within high-resolution protein structures.[8,12,14,27,30,31] But while providing new opportunities for drug discovery of SERT, the patients were still suffering serious side-effects and delayed functions due to drug abuse.[32–35] Previous studies have focused on binding the affinity of drugs at the orthosteric site or allosteric ite.[15,16,31,36] However, the lack of study of the unbinding mechanism of drugs from SERT limited our understanding of pharmacological implications of SERT inhibition.

In this study, we focused the mechanisms of each ligand interaction with SERT, and investigated the pathway of substrate and drugs dissociated from the S1/S2 site amid to provide spectrums of the molecular events involved in SERT. The high-resolution x-ray structure of SERT[27] resolved by Coleman *et al.* in 2016 was used as the initial structure for comprehensive molecular dynamics (MD) simulation. We investigated the molecular mechanism of the unbinding of substrate serotonin and the abuse drug cocaine and two antidepressants (escitalopram and paroxetine) (Fig. 1(c)) against the S1 site or S2 site of SERT. Serotonin, cocaine, and escitalopram each cooperatively bind to the S1 and S2 sites of SERT, while paroxetine only binds to the S1 site of SERT. Some interesting difference and similarity in the potential of mean force (PMF) simulations between SERT-serotonin, SERT-cocaine, SERT-escitalopram and SERT-paroxetine unbinding processes have been revealed. Based on our results, it can be concluded that the other three ligands, for either the S1 or the S2 site, were much more favorable than the original substrate serotonin, and the distance between serotonin and both cocaine and escitalopram at the allosteric site was ~ 3 Å, and the pathway of escitalopram dissociated from the S1 site may be an alternative. Our work may aid in the future further development of conformational selective inhibitors for the treatment of depression.

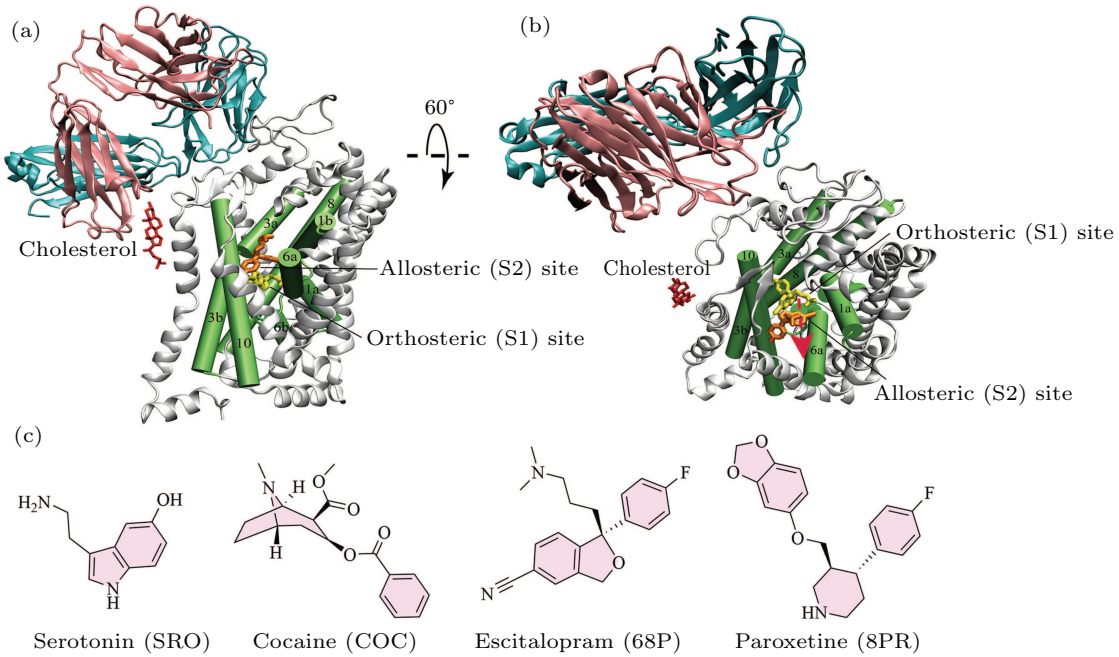


Fig. 1. Protein and ligand structures and related information used in this study. (a) and (b) Two target pockets of human SERT orthosteric (S1) site (yellow stick) and allosteric (S2) site (orange stick) are confirmed in x-ray crystal structure. The human SERT and two 8B6 antibody molecules are colored in pink, cyan, and white in cartoon representations, respectively. MT1, MT3, MT6, and MT10 are shown as green column, and cholesterol molecule is represented as red stick. Red arrow indicates the direction of the transit channel, from the intracellular to extracellular. (c) Chemical structures of the four ligands including their molecular names and non-standard names in PDB.

2. Materials and methods

2.1. Systems preparation

Four simulation complexes were modeled. The complexes escitalopram-bound and paroxetine-bound were directly obtained from x-ray structures of SERT (PDB codes: 5I73 and 5I6X),[27] escitalopram simultaneously binding at S1 site and S2 site but paroxetine only at S1 site. Serotonin and cocaine (extracted from PDB code: 6HIQ,[37] 1Q72[38] respectively) were docked into the S1 site and S2 site of SERT (PDB code: 5I73) in MOE[39] software: (i) Putting into SERT-escitalopram and dealing the structure with default databases. (ii) Selecting escitalopram atoms as the docking

site and using wall constraint. (iii) 300 placement poses following 5 refinement poses. The RMSDs of the re-docked and co-crystalized ligand approximately were 1.60 Å (serotonin bound S1), 1.87 Å (serotonin bound S2), 1.07 Å (cocaine bound S1), and 0.95 Å (cocaine bound S2), respectively. The results is rational. The cholesterol (CLR) molecule contained in the experimental structure was reserved in consideration of its probable role in maintaining SERT's conformation (Figs. 1(a) and 1(b)). Three mutations: T110A, I291A, and T439S in the crystal structure of SERT ware mutated back to its native state using the mutagenesis tool in PyMOL. CHARMM-GUI was used to build the model of protein inserted into DOPC lipid bilayer (see Fig. S2 in supporting information). 128×128 phospholipid molecules (DOPC lipid) were arranged in the X and Y axes (XY dimension ratio was 1) and solvated with KCl (0.07 M), resulting in a box with approximate dimensions of 106 Å$\times$106 Å$\times$ 150 Å that contained around 1.5×10^5 atoms.

2.2. Conventional molecular dynamics simulation and hydrogen bonds calculation

For each structure, energy-minimization was performed by using the Sander module of Amber 18 program[40–44] via a combined use of a 4000-step steepest descent calculation followed by a 36000-step conjugate gradient optimization, with a convergence criterion of 0.01 kcal·mol^{-1}·Å^{-2}, and the non-bonded cutoff distance was set to 10.0 Å. MD simulation was performed by using the Sander module of the Amber18 program package. SERT protein, DOPC liquids, K^+ and Cl^- ions, and TIP3P water were described using AMBER ff14SB,[45] Lipid 14,[46] and monovalent ion parameters for TIP3P water, respectively. The parameters of serotonin, cocaine, escitalopram, paroxetine, and cholesterol were generated by Antechamber using GAFF2 force fields.[47] The partial charges of atoms of the ligands were calculated by using the restrained electrostatic potential-fitting (RESP) protocol implemented in the Multiwfn program,[48] following the electrostatic potential (ESP) calculation at ab initio B3LYP/6-311+G(2d.p) level using Gaussian 09 program.[49] The electrostatic potential involved in the analyses was evaluated by Multiwfn based on the highly effective algorithm.[50] Sodium counter ions (Na^+) were added to neutralize the solvated system. The solvated system was gradually heated to 298.15 K by the weak-coupling method[51] and equilibrated for 150 ps. During the MD simulations, a 10.0-Å non-bonded interaction cutoff was used and the non-bonded list was updated every 25 steps. The motion for the mass center of the system was removed every 1000 steps. The particle-mesh Ewald (PME) method[52] was used to treat long-range electrostatic interactions. The lengths of covalent bonds involving hydrogen atoms were fixed with the SHAKE algorithm,[53] enabling the use of a 2-fs time step to numerically integrate the equations of motion. Finally, the production MD was kept running for 100 ns with a periodic boundary condition in the NPT ensemble at $T = 298.15$ K with Berendsen temperature coupling, and at $P = 1$ atm (1 atm = 1.01325×10^5 Pa) with isotropic molecule-based scaling.[54]

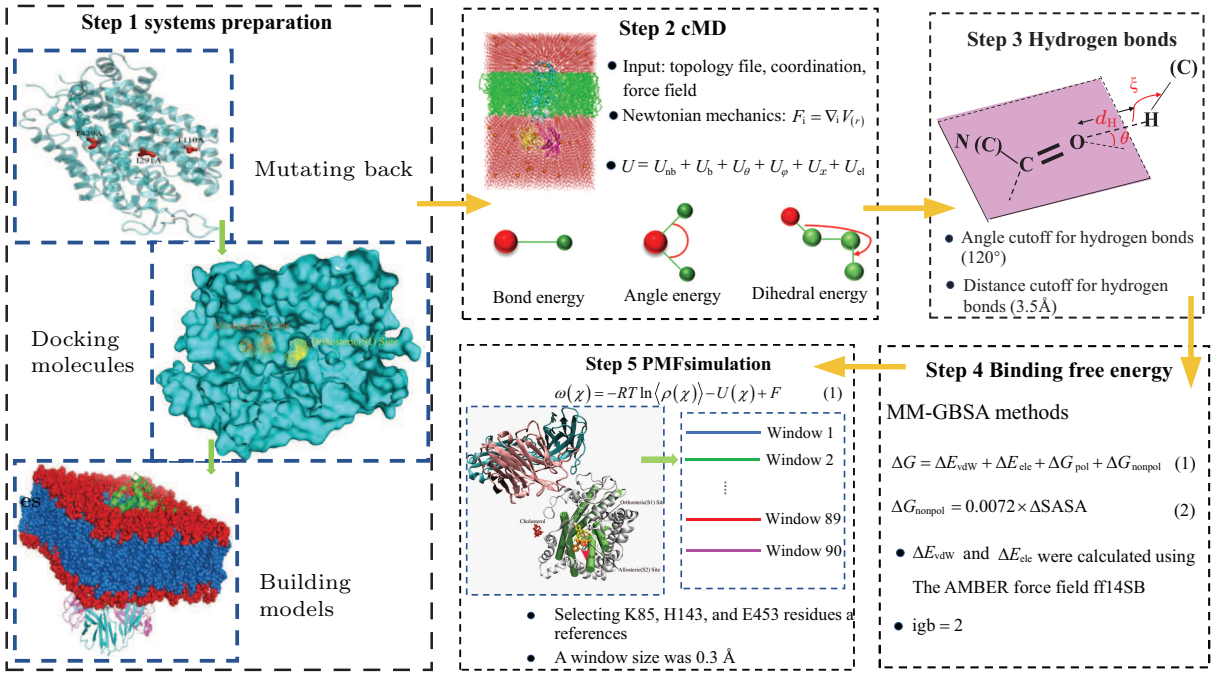


Fig. 2. The workflow of our work. Firstly, three mutations of SERT are mutated back to its native state using the mutagenesis tool in PyMOL, and then serotonin molecules or cocaine are docked into two target pockets (S1 site and S2 site) of SERT in MOE, and next CHARMM-GUI is employed to insert four complexes into DOPC lipid bilayer. Then, the conventional MD is performed for each structure. Next, hydrogen bonds analyses are employed for the interactions of substrate and three drugs to SERT after systems have been stable. Next, the binding free energy is calculated with Molecular mechanics/generalized Born surface area (MM/GBSA) method in thermo-dynamics at the equilibrium states. Finally, the PMF simulation is carried out by using umbrella-sampling MD simulation.

In the energy-minimization and heating procedures all of the ligands, protein and lipid atoms were restrained by a harmonic potential with a force constant of 2000 kcal·mol^{-1}·Å^{-2}. The following NPT equilibration simulation divided into 6 steps: (i) 100 ps, all of the ligand, protein and lipid atoms were restrained; (ii) 100 ps, the atoms of the protein were restrained; (iii) 100 ps, the mainchain atoms of the protein were restrained; (iv) 100 ps, the backbone atoms of the protein were restrained; (v) 100 ps, the C_{alpha} atoms of the protein were restrained; (vi) 100 ps, none of the systems was restrained. The harmonic potential used in equilibration simulation was the same as that used in energy-minimization and heating procedures.

Hydrogen bonds analyses were based on the production simulation trajectory. The criterion for forming a hydrogen bond was that the distance between the heavy atoms was no larger than 3.5 Å and the angle between the acceptor, hydrogen, and donor atoms was no smaller than 120°. Thus, the snapshots inconsistent with the cutoff protocol would be discarded in hydrogen bonds calculation. The workflow of our work was showed as Fig. 2.

2.3. MM/GBSA method

MM/GBSA method[55] was employed to calculate the binding free energy (ΔG) between SERT and each ligand. The method has been successfully applied to rank the relative binding free energy of small molecules in membrane proteins.[15,56–60] In this work, using the 100 snapshots extracted from the last 70-ns equilibrium MD trajectory, ΔG was estimated as below:

$$\Delta G = \Delta E_{\mathrm{vdW}} + \Delta E_{\mathrm{ele}} + \Delta G_{\mathrm{pol}} + \Delta G_{\mathrm{nonpol}}. \quad (1)$$

The terms ΔE_{vdW} and ΔE_{ele} represent the van der Waals and electrostatic interaction energies in the gas phase, respectively. ΔG_{pol} and $\Delta G_{\mathrm{nonpol}}$ are the polar and nonpolar solvation energies, respectively. ΔE_{vdW} and ΔE_{ele} were calculated using the AMBER force field ff14SB.[36] The free energy of polar solvation (ΔG_{pol}) was calculated by the modified GB model (igb = 2), and solute and solvent dielectric constants were set to 2 and 80, respectively. The free energy of nonpolar solvation ($\Delta G_{\mathrm{nonpol}}$) was calculated by $\Delta G_{\mathrm{nonpol}} = 0.0072 \times \Delta\mathrm{SASA}$, and ΔSASA was estimated by the DOPC method with 1.4-Å probe radii.

2.4. Steered molecular dynamics simulation

In order to explore the free energy profiles for each complex, the potential of mean force (PMF) simulation was carried out by using umbrella-sampling[61] MD simulation. The classic PMF definition[62] can be represented by a function of reaction coordinate as below:

$$\omega(\chi) = -RT \ln \langle \rho(\chi) \rangle - U(\chi) + F. \quad (2)$$

In which $\rho(\chi)$ is the probability density along the reaction coordinate χ, R is the gas constant, T is the simulation temperature, $U(\chi)$ is the biasing potential applied in the umbrella-sampling MD simulation, and F is the normalization constant. According to this approach, the reaction coordinate is usually divided into different regions, *i.e.*, windows, and each window is sampled separately. A biasing (umbrella) potential, *i.e.*, $U(\chi)$ is applied for each window in order to obtain nearly uniform sampling of the potential energy surface. In the present study, the reaction coordinate was defined as the distance from the mass center of the non-hydrogen atoms of each ligand to the mass center of the C_{alpha} atoms of several residues of SERT. The total number of windows was 90, with a window size of 0.3 Å. The biasing force constant applied in different windows of umbrella-sampling was 10.0 kcal·mol^{-1}·Å^{-2}. For each umbrella-sampling window, the initial complex structure was selected from the last snapshot of the PMF simulations of the previous window. The selected structure for each window was first equilibrated for 200 ps and then kept running for 800 ps for production sampling. The frequency for data collection was set to 1 fs, which was the same as that of the time step of the umbrella-sampling MD. After the umbrella-sampling MD simulations for all windows were completed, the data collected from separate simulation windows were combined along the reaction coordinate. These data were then used to calculate the PMF for the whole binding process with the weighed histogram analysis method (WHAM)[63,64] using the code developed by Alan Grossfield (http://membrane.urmc.rochester.edu/fipage_id=126).

3. Results and discussion

3.1. Different binding modes and critical residues

Hydrogen bonds are crucial for modeling protein folding, structure prediction, and complex formation,[65,66] which revealed the interaction patterns between SERT and ligands (serotonin, cocaine, escitalopram, and paroxetine). The root-mean-square deviation (RMSD) of the Cα atoms of SERT (Fig. 3) showed that the RMSD of SERT-escitalopram system was markedly higher than that of the binding original substrate serotonin system, compared with cocaine and paroxetine. This suggested that escitalopram enable to cause SERT structural instability, thereby inhibiting its activities. All the systems were stable after about 30 ns during simulations, and therefore we chose the 30 ns–100 ns simulation trajectory for hydrogen bonds analyses.[67,68]

For the S1 site, one stable hydrogen bond was formed for each complex. The N atom of the amine group of serotonin and hydroxyl oxygen of the Ser438 side chain formed a hydrogen bond with occupancy of 82.45%, and that of the Tyr (95 and 175) side chain form a hydrogen bond with occupancy of ∼ 22.19% (Table S1). The carbonyl oxygen of

cocaine benzoyl ester group and the oxygen atom of the phenol group of Tyr176 side chain formed a hydrogen bond with occupancy of 61.20%, and hydroxyl oxygen of the Ser438 side chain formed a hydrogen bond with occupancy of 11.86% (Table S3). The N atom connected to the benzofuran group of escitalopram and hydroxyl oxygen of the Ser438 side chain (the same donor as that for serotonin) formed a hydrogen bond with occupancy of 78.36% (Table S5). Finally, The N atom of the piperidine group of paroxetine and the oxygen atom of the Tyr95 main chain formed a hydrogen bond with occupancy of 99.52% (Table S7). This occupancy was the highest (approximating to 100%) among the above four hydrogen bonds, and the F atom connected to the benzene ring of paroxetine and hydroxyl oxygen of the Tyr175 side chain formed a hydrogen bond with occupancy of 8.43% (Table S7). Overall, the residues Tyr (95 and 175)[21,22] and Ser438[16,23] were the primary determinants of recognizing compounds by forming hydrogen bonds, and the hydrogen bonds with Tyr176 may be responsible for selective recognition of cocaine towards the S1 site of SERT.

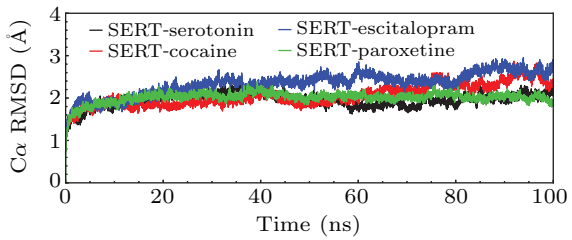


Fig. 3. The root-mean-square deviation (RMSD) of Cα atoms of SERT. the RMSD of SERT-escitalopram system is markedly higher than that of the binding original substrate serotonin system, compared with cocaine and paroxetine, and the systems all are stable after about 30 ns during the simulations.

In general, hydrogen bonds for the S2 site were much weaker (with much lower occupancy) than those for the S1 site, irrespective of the three complexes. Interestingly, serotonin, cocaine and escitalopram formed a relatively stable hydrogen bond with the residue Arg104 of SERT, with occupancies of 25.63% (Table S2), 7.39% (Table S4), and 64.77% (Table S6), respectively.

Moreover, LigPlot[69] was applied to predict hydrophobic interactions between SERT and the natural substrate or the above-mentioned drugs. All the compounds were stabilized in the pockets of SERT because of the surrounding interactional residues, which may reveal the mechanism of how compounds were captured in SERT. For example, Serotonin bounded at the S1 site was lodged between the hydrophobic residue groups of Tyr95, Asp98, Ile172, Tyr176, Gly338, and Ser438 (Fig. S2(a)), and, Tyr95 and Ser438 was able to form hydrogen bonds with serotonin. Those binding modes and behaviors were consistent with the previous experimental results.[16] All the residues also enlarged the hydrophobic interactions between cocaine, escitalopram and paroxetine, and SERT (Fig. S2); It is noted that the drug compounds have much more hydrophobic residues than the substrate serotonin in both S1 and S2 sites (Figs. S2 and S3), which indicates that cocaine, escitalopram, and paroxetine are more competitive. At the S2 site, the residues of hydrophobic interactions for each molecule were nearly equivalent to that of the S1 site. However, a part of hydrophobic residues (Arg104, Ala331, and Phe335) were the primary determinants of recognizing between the substrate and drugs (Fig. S3). Interestingly, the residues Arg104 at the S2 site of SERT enabled producing hydrogen bonds among the compounds too.

In general, hydrophobic interactions are critical in the cocaine binding to SERT compared with another two antidepressants, which involved about 13 hydrophobic residues at each binding site. The residues Tyr176 and Ser438 at the S1 site, and Arg104 at the S2 site were critical residues for cocaine, whose interactions included hydrophobic interactions and hydrogen bonds. The two interactions make essential contributions to protein stability, thus it suggested that the residues Tyr176 and Ser438 at the S1 site, and Arg104 at the S2 site of SERT play a key role in the abused cocaine interacting with SERT. For antidepressant escitalopram, about 13 and 10 hydrophobic residues at the S1 and S2 sites respectively, provided stronger hydrophobic interactions while it simultaneously binds to the S1 and S2 sites of SERT, and the key residues Ser438 and Arg104 can form hydrogen bonds and hydrophobic interactions. For paroxetine, the residues Tyr75 contributed to those two interactions.

Our molecular dynamics simulations show the residues Tyr95 and Ser438 could form hydrogen bonds and hydrophobic interactions with antidepressants or substrate. Meanwhile, the residues Arg104 illustrate conserved interactions for serotonin, cocaine, and escitalopram. A previous study found that the hydrophobic compounds are mainly recognized by transport proteins in the lipid phase of the membranes.[70] Thus, our computational study suggests that the hydrophobic residues at the S1 (Tyr95, Asp98, Ile172, Tyr176, Gly338, and Ser438) and S2 (Arg104, Ala331, and Phe335) sites are critical for the development of antidepressants.

3.2. Binding free energies at the equilibrium states

The binding free energy is generally used in drug design to characterize the binding strength of drugs. To further explore the interactions between the four studied ligands and SERT, the binding free energies (ΔG) of the newly designed four different complexes were estimated by the MM/GBSA method, based on the MD equilibrium trajectory (30 ns–100 ns). As shown in Table 1, the ΔG values of serotonin, cocaine, escitalopram and paroxetine at the S1 site were 28.38 kcal/mol, −46.42 kcal/mol, −51.47 kcal/mol, and −51.34 kcal/mol, respectively. Further, the ΔG values of serotonin, cocaine, escitalopram at the S2 site were −18.88 kcal/mol, −39.60 kcal/mol, and −38.76 kcal/mol, respectively.

Table 1. Calculated and experimental binding free energies of studied substrate/drugs to targets.

Substrate/drug	Target	ΔG_{cale} (kcal/mol)[a]	ΔG_{exp} (kcal/mol)[b]	K_i (nM)[b]
Serotonin	SERT (S1)[c]	–28.38	–6.62	14.2 μM[16]
	SERT (S2)[d]	–18.88	–5.90	48 μM
Cocaine	SERT (S1)[c]	–46.42		–[f]
	SERT (S2)[d]	–39.60		
Escitalopram	SERT (S1)[c]	–51.47	–10.23	32[e][15]
	SERT (S2)[d]	–38.76	–7.16	5800 (IC_{50})
Paroxetine	SERT (S1)[c]	–51.34	–10.40	24

[a]Estimated MM/GBSA binding free energy. [b]Experimental binding free energy (ΔG_{exp}) based on K_i or IC_{50} values using $\Delta G = RT\ln(K_i)$ or $\Delta G \approx RT\ln(IC_{50})$ (where $R = 8.314$ J/(mol·K), $T = 310$ K, IC_{50}: half maximal inhibitory concentration).[31,71] [c]Substrate/drug binding to the orthosteric (S1) site of SERT. [d]Substrate/drug binding to the allosteric (S2) site of SERT. [e]The S2 site is empty, [f]No experimental data reported.

After only considering the comparison of relative values (without absolute values taken into accout), the trend of the MM/GBSA results was consistent with the previous studies and the trend of experimental values. The results suggest in thermodynamics at the equilibrium states, the other three ligands (cocaine, escitalopram, and paroxetine) were much more favorable for the S1 site than the original substrate serotonin. The same holds true for the S2 site (in the experiment, whether paroxetine can bind the S2 site was not found). In general, the S1 site was much more favorable than the S2 site, for each ligand.

For a more detailed understanding of the interaction mechanisms between SERT and the four ligands, we further analyzed the effects of the energy component of ΔG in each system. As shown in Fig. 4, the cocaine-binding affinity's main contribution was van der Waals force (ΔE_{vdW}), binding at the S1 site (−42.53 kcal/mol) and S2 (−39.47 kcal/mol) (Table S8), compared the original substrate serotonin, and the solvation free energy (ΔG_{sol}) was beneficial to cocaine competing for the target binding site of SERT. Between among bindings of the three drugs with SERT, the electrostatic interactions for escitalopram have very significant advantages. Overall, van der Waals interactions have fundamental roles in associations of cocaine, escitalopram, and paroxetine with SERT, whether the S1 site or S2. Accordingly, in future drug designs towards SERT van der Waals interactions between inhibitors and SERT would be keys for the successful development of inhibitors, and enhancing the electrostatic interaction of drugs may be a potential strategy for developing potent drugs.

Based on previous studies, for Drosophila dopamine transporter (dDAT), another important monoamine transporter in the neurotransmitter sodium symporter (NSS) family and closely related to SERT,[72] cocaine (a drug of abuse) obviously hads higher competitiveness than its original substrate dopamine. In this respect, SERT and DAT showed the similar behavior for cocaine binding.

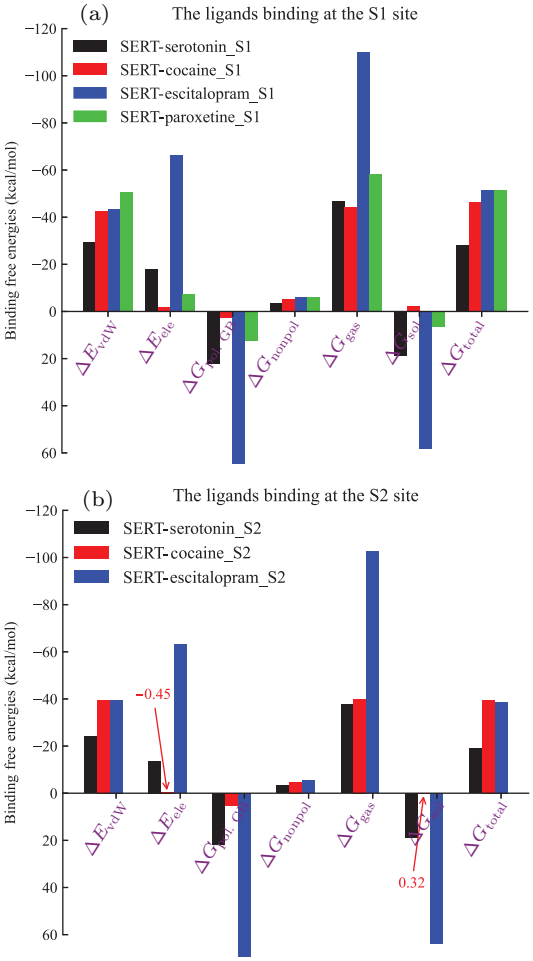


Fig. 4. The energy component of binding free energy (MM/GBSA method). ΔE_{vdW} and ΔE_{ele} indicate the electrostatic and van der Waals interactions in the gas phase, respectively, and ΔG_{gas} is the gas-phase energy (ΔE_{vdW} and ΔE_{ele}). The solvation free energy (ΔG_{sol}) can divide to polar (ΔG_{pol}) and non-polar (ΔG_{nonpol}). ΔG_{total} denotes the total binding free energies. (a) ΔE_{vdW} was favorable for associations of cocaine, escitalopram and paroxetine with SERT and ΔG_{sol} was beneficial to cocaine competing for the target binding site of SERT. (b) Between among bindings of the three drugs with SERT, ΔE_{ele} for escitalopram have very significant advantages. ΔE_{ele} and ΔG_{sol} of SERT-cocaine were −0.45 kcal/mol and 0.32 kcal/mol, respectively.

3.3. Potential of mean forces along the unbinding pathways

To explore inhibitors interactions with the corresponding target protein in kinetics along the unbinding pathways and to

further understanding the mechanisms of each ligand studied in this work with SERT, we performed PMF simulation. The PMF results coincided perfectly well with the trend of our previous MM/GBSA results. As shown in Fig. 5(a), among the four ligands, the PMF of serotonin dissociated from the S1 site was the lowest (60.81 kcal/mol). As a drug of abuse, cocaine had a higher PMF of 74.56 kcal/mol. The PMFs for the two clinically used antidepressants, escitalopram and paroxetine, were 132.66 kcal/mol and 93.35 kcal/mol, respectively. Therefore, we can conclude that escitalopram was the most competitive for binding to SERT, with a PMF nearly twice of that for the substrate serotonin. In addition, our result for escitalopram was quite consistent with the result of 136.23 kcal/mol (when the S2 site is occupied by a second escitalopram) from the previous steered MD study by Zhu *et al.*; however, it was much higher than their result of 111.97 kcal/mol (when the S2 site was empty), though we adopted a different method (including model, protocol, parameters, *etc.*) compared to theirs methods.

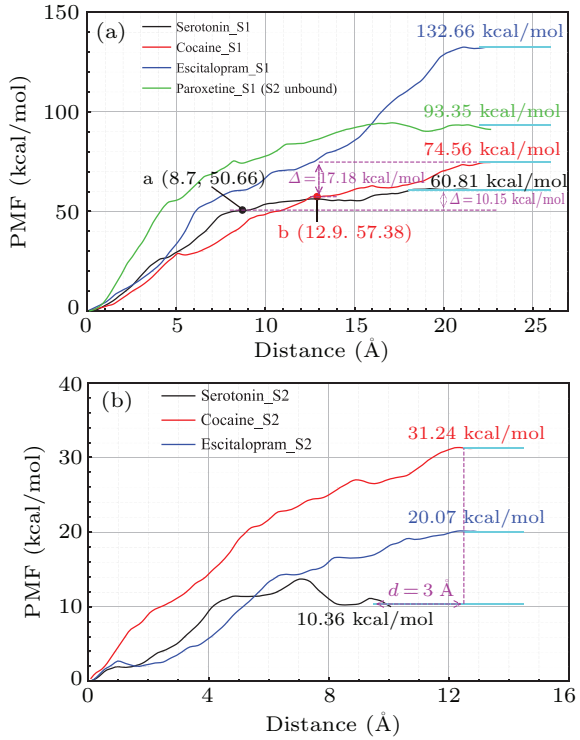


Fig. 5. Potentials of mean force (PMF) profiles of substrate/drugs dissociated from S1 and S2 sites of SERT. The cyan solid line represents that the substrate/drugs have escaped the protein via the channel. (a) The value of escitalopram dissociated from the S1 site (132.66 kcal/mol) was higher than that of paroxetine (93.35 kcal/mol), cocaine (74.56 kcal/mol), and serotonin (60.81 kcal/mol). The position of serotonin and cocaine dissociated from S1 site to S2 site are represented a black and red circles with a and b representations, respectively. the value of serotonin and cocaine dissociated from the S2 site to away from protein was 10.15 kcal/mol and 17.18 kcal/mol respectively. (b) The PMF value of cocaine dissociated from the S2 site (31.24 kcal/mol) was higher than that of escitalopram (20.07 kcal/mol) and serotonin (10.36 kcal/mol). The length of the path to dissociate from the S2 site of cocaine was equal to that of escitalopram, and both of them was $\sim$ 3 Å lagther than that of serotonin.

On the one hand, whether in kinetics along the unbinding pathways or in thermodynamics at the equilibrium states, for each ligand the PMF from the S2 site was much lower than that from the S1 site, which was reasonable in consideration of the relative positions of the two sites in SERT. On the other hand, limited to comparation only between the three ligands (serotonin, cocaine, and escitalopram), the S2 site unbonded paroxetine, the situation was similar and different to that for the S1 site. Firstly, serotonin has the lowest PMF of 10.36 kcal/mol, among the three ligands. Secondly, our result of 20.07 kcal/mol for escitalopram is extremely in accordance with the result of 17.25 kcal/mol (when the S1 site is occupied by a second escitalopram) from the previous steered MD study by F Zhu *et al.*, while much lower than their result of 33.72 kcal/mol (when the S1 site is empty). thirdly, the PMF value of cocaine dissociated from the S2 site (31.24 kcal/mol) was higher than that of escitalopram (20.07 kcal/mol) and serotonin (10.36 kcal/mol), as shown in Fig. 5(b).

In this article, based on the knowledge of structure of SERT and the mothed of umbrella-sampling all the steered MD of four systems were performed selecting the same three (K85, H143, and E453, PDB code: 5I73) residues as the reference for the reaction coordinate. The pathways of ligands dissociated from the S1 site or S2 site were further investigated to gain some insights into the unbinding process. Based on the statistics of PMF windows counts (from the point at the binding site to the point of the absolute dissociation state which corresponds to the final platform of the PMF profile), the length for the dissociation pathway of serotonin from the S1 binding site (about 18 Å) was slightly shorter than that of other three ligands (cocaine, escitalopram, and paroxetine), which was $\sim$ 22 Å. This observation indicates that cocaine, escitalopram, and paroxetine bound more deeply to the S1 site than serotonin. However, the length of dissociation pathway for cocaine or escitalopram from SERT of the S2 site is slightly longer than that of serotonin ($\sim$ 9.5 Å), which is about 12.5 Å. Therefore, for binding the S2 site, cocaine or escitalopram is farther to the protein surface than serotonin. Investigating the structures of each window frame by frame with PMF values was to better understand unbinding processes of the four ligands. we found that the distances of serotonin and cocaine at the S1 site pulled to the S2 site were 8.7 Å (29th window) and 12.9 Å (43rd window), respectively (Fig. 5(a)), while escitalopram bounded at the S1 site moved directly and did not pass through the S2 site. Based on the above analysis, we predict that the lengths of pathways from the orthosteric site of SERT to an opening were $\sim$ 18 Å for serotonin and $\sim$ 22 Å for cocaine, escitalopram and paroxetine. The experimental size of SERT is $\sim$ 55 Å[73] and the above speculations are valid. Similarly, it was presumed that the distances of pathways from the S2 site to the opening were $\sim$ 9.5 Å for serotonin and $\sim$ 12.5 Å for cocaine and escitalopram, respec-

tively. The distance between serotonin and both cocaine and escitalopram at the allosteric site was ∼ 3 Å (Fig. 5(b)). But the PMF value of cocaine (initially at the S1 site) dissociated from the S2 site (17.18 kcal/mol) was still higher than that of serotonin (10.15 kcal/mol) (Fig. 5(a)). Overall, the abused cocaine, whether at the S1 site or S2 site, has a stronger binding ability beyond substrate serotonin. Experiment result shows that the fluorophenyl group of escitalopram is positioned 1.5 Å deeper into subsite B of the S1 site of SERT compared with the benzodioxol of paroxetine.[27] Thus, it can be inferred that the potent antidepressants have the tendency to bind at deeper positions of the S1 site or S2 site of SERT in comparison to the substrate.

3.4. Structural analysis and dissociative process of PMF simulations

During PMF simulations of the four complexes, SERT was always maintains stable and it underwent slight conformational changes; therefore, SERT could be a perfect background for further investigation of the ligands' behaviors including movement relative to the protein. Thus, we investigated the intermediate snapshots of each system during our PMF simulations (a–f in Figs. S4(a)–S4(d)). The unbinding pathways of the four ligands, except escitalopram at the orthosteric site, were similar; *i.e.*, each ligand exported from the channel gradually with each step choosing the broadest space, which validates the reasonability of our definition for PMF reaction coordinates. Along the unbinding pathway, significant conformational changes (rotations) occurred for each ligand to fit different protein environment. Surprisedly, escitalopram at the S1 site was different from the other molecules, in the departure from the channel directly pulled out SERT along the MT8 shielding the S2 site (Fig. 6(a)) (a–f in Fig. S4(c)). Serotonin, cocaine, paroxetine, and escitalopram (at the S2 site) were all via a broad pathway from the orthosteric site of

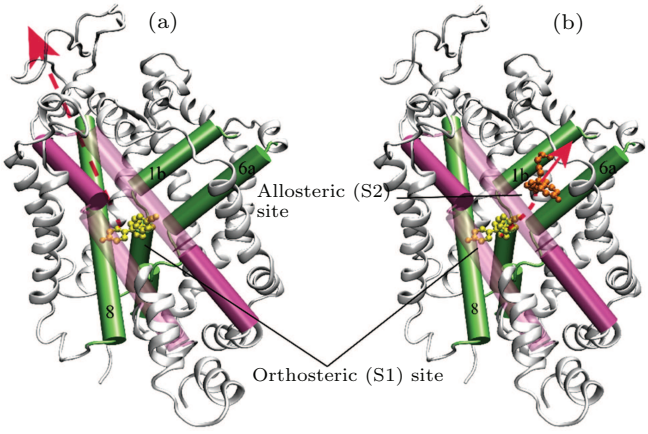


Fig. 6. Two unbinding pathways in this work. (a) escitalopram at S1 site dissociated from SERT directly along the MT8 shielding S2 site. (b) There is a broad pathway where serotonin, cocaine, paroxetine, and escitalopram (S2 bound) are all pulled towards a similar opening between MT1b and MT6a, whether at the S1 site or initially S2.

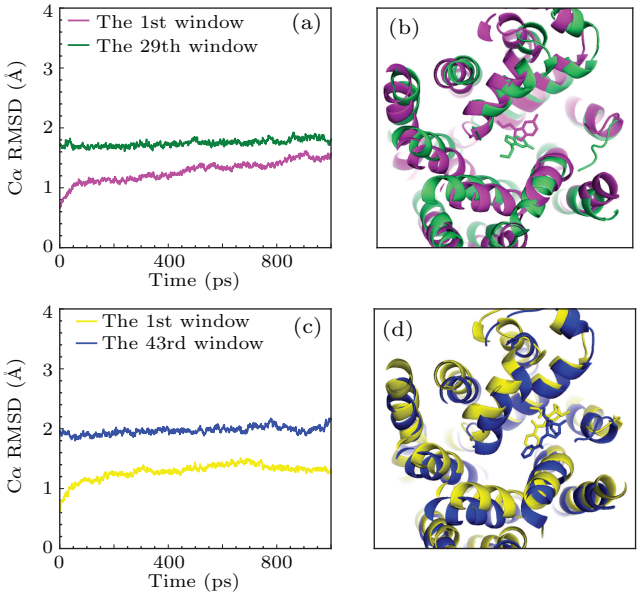


Fig. 7. Structure superposition at the S2 site and the RMSDs of the protein Cα in corresponding windows. (a)–(b) SERT-serotonin system. the structures of 0 ns (the 1st window) and 29 ns (the 29th window) were colored in magenta and green, respectively. (c)–(d) SERT-cocaine system. The structures of 0 ns and 43 ns (the 43rd window) were colored in yellow and blue, respectively. In the 1st window RMSDs of the protein Cα both increased slightly and kept stable in the 29th window (1.75 Å) and 43rd (1.97 Å). Based on the RMSDs and compared the initial structures, protein both of all underwent slight conformational changes and the conformations of ligands occurred as an angle flip.

SERT to an opening between MT1b and MT6a (Fig. 6(b)) (a–f in Figs. S4(a), S4(b), and S4(d)). The pathway is consistent with experiments.[16,73] Since serotonin and cocaine were dissociated from a similar opening, the previous presumption is rational that distance between serotonin and cocaine (or escitalopram) at the allosteric site was ∼ 3 Å.

In order to better understand the PMF values difference between serotonin or cocaine dissociated from the S1 site to the S2 site then leaving the channel and directly from the S2 site away, the corresponding structures were superimposed and analyzed the corresponding RMSDs of the protein Cα, when both ligands were at the S2 site during the PMF simulations. In the 1st window when the ligands began to be pulled away from the S1 site along the direction of intracellular to extracellular, the both RMSDs of 1st window increased slightly. Finally, it were constant as the ligands reached the S2 site, in the 29th window (1.75 Å) and the 43rd window (1.97 Å), respectively (Figs. 7(a) and 7(c)). The variation of RMSDs were reasonable. Further, as substrate uptake into cells from the extracellular solution to the primary substrate site the transporter in an open-to-out conformation.[74] In theory, the observations may provide slight verification that the molecular activity phenomenon that conformation transitions between the outward-open to the occluded state.[74,75] Based on the RMSDs and compared the initial structures, protein both of all underwent slight conformational changes (Figs. 7(b) and 7(d)). Similarly, when ligands were pulled to the S2 site, the conformations of

ligands occurred as an angle flip. Thus, considerations of variations between the conformational comparisons of structures of proteins and ligands could interpret the differences above values in our steered molecular dynamics simulations. In summary, we could conclude that the abused drug cocaine was more competitive than the natural substrate serotonin, whether at the S1 site or the S2 site, when SERT drive to reuptake.

4. Conclusions

In this work, combing all-atom MD simulations and the high spatial of x-ray crystal structures probed into the molecular mechanism of substrate serotonin and drug of abused cocaine and two antidepressants (escitalopram and paroxetine) against the S1 or S2 sites in human SERT. For serotonin, cocaine, and escitalopram, the hydrogen bonds for the S2 site were much weaker than those for the S1 site. Nevertheless, the residues of hydrophobic interactions were nearly equivalent to that of the S1 site. Moreover, the insights revealed the mechanism of how compounds were captured in SERT. The conserved residues Tyr95 and Ser438 located within the S1 site in SERT and Arg104 located within the S2 site in SERT not only contributed to hydrophobic interactions but also hydrogen bonds while recognizing selective serotonin reuptake inhibitors. The hydrophobic residues at the S1 (Tyr95, Asp98, Ile172, Tyr176, Gly338, and Ser438) and S2 (Arg104, Ala331, and Phe335) sites are critical for the development of antidepressants. In kinetics along the unbinding pathways and thermodynamics at the equilibrium states, cocaine, escitalopram and paroxetine (S2 unbound), whether at the S1 or the S2, are much more favorable than the natural substrate serotonin and the inhibitory effect of drugs is more potent at the S1 site. Further, van der Waals interactions were beneficial to the affinities of the three drugs compared substrate serotonin and electrostatic interactions were primary contributions for escitalopram. We predicted that distances of pathways from the S1 site to an opening, for serotonin and the three-mentioned drugs, were $\sim$ 18 Å and $\sim$ 22 Å, respectively. Furthermore, the distances between serotonin and both cocaine and escitalopram at the allosteric site were $\sim$ 3 Å, which indicates that drugs positioned deeper into the pocket of SERT compared with the natural substrate may be a potential candidate for the treatment of depression in the future. Continuing exploring the processes of unbinding four ligands against the two target pockets of SERT, we observed a broad pathway from the S1 site of SERT to the opening between MT1b and MT6a in the dissociation process of substrate and drugs, which may be helpful to understand the dissociation mechanism of drugs, and maybe, in theory, provide slight proofs for the molecular activity phenomenon that conformation transitions between the outward-open to the occluded state. Our work would be beneficial for further studies of better inhibitors to treat depression.

Acknowledgements

Project supported by the National Natural Science Foundation of China (Grant Nos. 11904036 and 12175081) and Fundamental Research Funds for the Central Universities (Grant No. CCNU22QNOO4).

References

[1] Sepanlou S G, Safiri S, Bisignano C, Ikuta K S, Merat S, Saberifiroozi M, Poustchi H, Tsoi D, Colombara D V and Abdoli A 2020 *Lancet Gastroenterol. Hepatol.* **5** 17
[2] Coccaro E F 2019 *N. Engl. J. Med.* **381** 980
[3] Recourt K, de Boer P, Zuiker R, Luthringer R, Kent J, van der Ark P, Van Hove I, van Gerven J, Jacobs G and van Nueten L 2019 *Transl. Psychiatry* **9** 110
[4] Saltiel P F and Silvershein D I 2015 *Neuropsychiatr. Dis. Treat.* **11** 875
[5] Krishnan V and Nestler E J 2008 *Nature* **455** 894
[6] Kupfer D J, Frank E and Phillips M L 2012 *Lancet* **379** 1045
[7] Caspi A, Sugden K, Moffitt T E, Taylor A, Craig I W, Harrington H, McClay J, Mill J, Martin J and Braithwaite A 2003 *Science* **301** 386
[8] Fuller R W and Wong D T 1990 *Ann. N. Y. Acad. Sci.* **600** 68
[9] Murphy D L, Lerner A, Rudnick G and Lesch K P 2004 *Mol. Interv.* **4** 109
[10] Chen N H, Reith M E A and Quick M W 2004 *Pflügers Archiv* **447** 519
[11] Sitte H H and Freissmuth M 2015 *Trends Pharmacol. Sci.* **36** 41
[12] Niello M, Gradisch R, Loland C J, Stockner T and Sitte H H 2020 *Trends Pharmacol. Sci.* **41** 446
[13] Rothman R B, Partilla J S, Baumann M H, Lightfoot-Siordia C and Blough B E 2012 *J. Pharmacol. Exp. Ther.* **341** 251
[14] Hasenhuetl P S, Bhat S, Freissmuth M and Sandtner W 2019 *Mol. Pharmacol.* **95** 303
[15] Xue W, Fu T, Deng S, Yang F, Yang J and Zhu F 2022 *ACS Chem. Neurosci.* **13** 340
[16] Yang D and Gouaux E 2021 *Science Advances* **7**
[17] Chan M C, Selvam B, Young H J, Procko E and Shukla D 2022 *Biophys. J.* **121** 715
[18] Gether U, Andersen P H, Larsson O M and Schousboe A 2006 *Trends Pharmacol. Sci.* **27** 375
[19] Kristensen A S, Andersen J, Jørgensen T N, Sørensen L, Eriksen J, Loland C J, Strømgaard K and Gether U 2011 *Pharmacol. Rev.* **63** 585
[20] Hahn M and Blakely R 2002 *Pharmacogenomics J.* **2** 217
[21] Severinsen K, Koldsø H, Thorup K A V, Schjøth-Eskesen C, Møller P T, Wiborg O, Jensen H H, Sinning S and Schiøtt B 2014 *Mol. Pharmacol.* **85** 208
[22] Henry L K, Field J R, Adkins E M, Parnas M L, Vaughan R A, Zou M F, Newman A H and Blakely R D 2006 *J. Biol. Chem.* **281** 2012
[23] Andersen J, Taboureau O, Hansen K B, Olsen L, Egebjerg J, Strømgaard K and Kristensen A S 2009 *J. Biol. Chem.* **284** 10276
[24] Coleman J A and Gouaux E 2018 *Nat. Struct. Mol. Biol.* **25** 170
[25] Gradisch R, Szöllősi D, Niello M, Lazzarin E, Sitte H H and Stockner T 2022 *J. Biol. Chem.* **298**
[26] Yamashita A, Singh S K, Kawate T, Jin Y and Gouaux E 2005 *Nature* **437** 215
[27] Coleman J A, Green E M and Gouaux E 2016 *Nature* **532** 334
[28] Zhu R, Sinwel D, Hasenhuetl P S, Saha K, Kumar V, Zhang P, Rankl C, Holy M, Sucic S and Kudlacek O 2016 *Angew. Chem.* **128** 1751
[29] Jørgensen A M and Topiol S 2008 *Chem. Biol. Drug Des.* **72** 265
[30] Topiol S, Bang-Andersen B, Sanchez C and Bøgesø K P 2016 *Bioorg. Med. Chem. Lett.* **26** 5058
[31] Zhang Y, Zheng G, Fu T, Hong J, Li F, Yao X, Xue W and Zhu F 2020 *Phys. Chem. Chem. Phys.* **22** 5132
[32] Millan M J 2009 *Neurotherapeutics* **6** 53
[33] Stuivenga M, Giltay E J, Cools O, Roosens L, Neels H and Sabbe B 2019 *Expert Opin. Pharmacother.* **20** 251
[34] Crits-Christoph P, Siqueland L, Blaine J, Frank A, Luborsky L, Onken L S, Muenz L R, Thase M E, Weiss R D and Gastfriend D R 1999 *Arch. Gen. Psychiatry* **56** 493
[35] Lambert N M, McLeod M and Schenk S 2006 *Addiction* **101** 713

[36] Larsen M A B, Plenge P, Andersen J, Eildal J N, Kristensen A S, Bøgesø K P, Gether U, Strømgaard K, Bang-Andersen B and Loland C J 2016 *Br. J. Pharmacol.* **173** 925

[37] Polovinkin L, Hassaine G, Perot J, Neumann E, Jensen A A, Lefebvre S N, Corringer P J, Neyton J, Chipot C and Dehez F 2018 *Nature* **563** 275

[38] Pozharski E, Moulin A, Hewagama A, Shanafelt A B, Petsko G A and Ringe D 2005 *J. Mol. Biol.* **349** 570

[39] Vilar S, Cozza G and Moro S 2008 *Curr. Top. Med. Chem.* **8** 1555

[40] Pearlman D A, Case D A, Caldwell J W, Ross W S, Cheatham III T E, DeBolt S, Ferguson D, Seibel G and Kollman P 1995 *Comput. Phys. Commun.* **91** 1

[41] Case D A, Cheatham III T E, Darden T, Gohlke H, Luo R, Merz Jr K M, Onufriev A, Simmerling C, Wang B and Woods R J 2005 *J. Comput. Chem.* **26** 1668

[42] Frank M, Gutbrod P, Hassayoun C and von Der Lieth C-W 2003 *J. Mol. Model.* **9** 308

[43] Karplus M 2003 *Biopolymers* **68** 350

[44] Adcock S A and McCammon J A 2006 *Chem. Rev.* **106** 1589

[45] Maier J A, Martinez C, Kasavajhala K, Wickstrom L, Hauser K E and Simmerling C 2015 *J. Chem. Theory Comput.* **11** 3696

[46] Dickson C J, Madej B D, Skjevik Å A, Betz R M, Teigen K, Gould I R and Walker R C 2014 *J. Chem. Theory Comput.* **10** 865

[47] He X, Man V H, Yang W, Lee T S and Wang J 2020 *J. Chem. Phys.* **153** 114502

[48] Lu T and Chen F 2012 *J. Comput. Chem.* **33** 580

[49] Frisch M, Trucks G, Schlegel H, Scuseria G, Robb M, Cheeseman J, Scalmani G, Barone V, Mennucci B and Petersson G 2009 Gaussian Inc, Wallingford

[50] Zhang J and Lu T 2021 *Phys. Chem. Chem. Phys.* **23** 20323

[51] Morishita T 2000 *J. Chem. Phys.* **113** 2976

[52] Toukmaji A, Sagui C, Board J and Darden T 2000 *J. Chem. Phys.* **113** 10913

[53] Ryckaert J-P, Ciccotti G and Berendsen H J 1977 *J. Comput. Phys.* **23** 327

[54] Berendsen H J, Postma J v, Van Gunsteren W F, DiNola A and Haak J R 1984 *J. Chem. Phys.* **81** 3684

[55] Kollman P A, Massova I, Reyes C, Kuhn B, Huo S, Chong L, Lee M, Lee T, Duan Y and Wang W 2000 *Acc. Chem. Res.* **33** 889

[56] Fu T, Zheng G, Tu G, Yang F, Chen Y, Yao X, Li X, Xue W and Zhu F 2018 *ACS Chem. Neurosci.* **9** 1492

[57] Xue W, Wang P, Li B, Li Y, Xu X, Yang F, Yao X, Chen Y Z, Xu F and Zhu F 2016 *Phys. Chem. Chem. Phys.* **18** 3260

[58] Xue W, Wang P, Tu G, Yang F, Zheng G, Li X, Li X, Chen Y, Yao X and Zhu F 2018 *Phys. Chem. Chem. Phys.* **20** 6606

[59] Zheng G, Xue W, Wang P, Yang F, Li B, Li X, Li Y, Yao X and Zhu F 2016 *Sci. Rep.* **6** 26883

[60] Wang P, Fu T, Zhang X, Yang F, Zheng G, Xue W, Chen Y, Yao X and Zhu F 2017 *Biochim. Biophys. Acta* **1861**

[61] Torrie G M and Valleau J P 1977 *J. Comput. Phys.* **23** 187

[62] Kirkwood J G 1935 *J. Chem. Phys.* **3** 300

[63] Berneche S and Roux B 2001 *Nature* **414** 73

[64] Roux B 1995 *Comput. Phys. Commun.* **91** 275

[65] García A E and Sanbonmatsu K Y 2002 *Proc. Natl. Acad. Sci.* **99** 2782

[66] Pace C N, Fu H, Fryar K L, Landua J, Trevino S R, Shirley B A, Hendricks M M, Iimura S, Gajiwala K and Scholtz J M 2011 *J. Mol. Biol.* **408** 514

[67] Ning S, Zeng C, Zeng C and Zhao Y 2021 *Biophys. J.* **120** 5158

[68] Wang H, Song L, Zhou T, Zeng C, Jia Y and Zhao Y 2020 *Phys. Chem. Chem. Phys.* **22** 25474

[69] Wallace A C, Laskowski R A and Thornton J M 1995 *Protein Eng. Des. Sel.* **8** 127

[70] Sarkadi B and Szakács G 2010 *Nat. Rev. Drug Discovery* **9** 897

[71] Huang X, Zhao X, Zheng F and Zhan C G 2012 *J. Phys. Chem. B* **116** 3361

[72] Wang K H, Penmatsa A and Gouaux E 2015 *Nature* **521** 322

[73] Coleman J A, Yang D, Zhao Z, Wen P C, Yoshioka C, Tajkhorshid E and Gouaux E 2019 *Nature* **569** 141

[74] Singh S K, Piscitelli C L, Yamashita A and Gouaux E 2008 *Science* **322** 1655

[75] Krishnamurthy H and Gouaux E 2012 *Nature* **481** 469