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Dissection of an Allosteric Mechanism on the Serotonin Transporter: A Cross-Species Study

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ABSTRACT

The serotonin transporter (SERT), which belongs to a family of sodium/chloride-dependent transporters, is the major pharmacological target in the treatment of several clinical disorders, including depression and anxiety. Interaction with a low-affinity allosteric site on SERT modulates the ligand affinity at the high-affinity binding site. Serotonin (5-hydroxytryptamine) and certain SERT inhibitors possess affinity for both sites. In the present study, we report the characterization of a severely attenuated allosteric mechanism at the recently cloned chicken serotonin transporter (gSERT). A cross-species chimera study was performed, followed by species scanning mutagenesis. Residues important for the allosteric mechanism were mapped to the C-terminal part of SERT containing the transmembrane domains 10 to 12. We identified nine residues located in four distinct amino acid segments. The contribution of each segment and individual residues was investigated. Consequently, a gSERT mutant with a restored allosteric mechanism, as well as a human SERT (hSERT) mutant with a severely attenuated allosteric mechanism, was generated. The nine residues confer a functional allosteric mechanism for different combinations of ligands, suggesting that they contribute to a general allosteric mechanism at SERT. The finding of an allosteric mechanism at SERT is likely to be of physiological importance, in that serotonin was also found to act as an allosteric effector at duloxetine, RTI-55 and (*S*)-citalopram. Furthermore, the allosteric potency of 5-HT was found to be conserved for both hSERT and gSERT.

Serotonergic neurotransmission is modulated by clearance of serotonin (5-hydroxytryptamine; 5-HT). The clearance of 5-HT from the synaptic cleft is maintained by the serotonin transporter (SERT). The transporter therefore affects the magnitude and duration of the signaling and thus plays a key role in the spatio-temporal fine-tuning of serotonergic neurotransmission

SERT is a well established molecular target of both drugs of abuse (cocaine and amphetamines) and most high-affinity antidepressants. Multiple classes of antidepressants, including tricyclic antidepressants, 5-HT-selective reuptake inhibitors, and antidepressants with dual actions, are directed toward SERT. They enhance serotonergic neurotransmission by competitively inhibiting 5-HT reuptake with inhibitory constants in the low nanomolar range (Barker and Blakely, 1995; Owens et al., 1997; Tatsumi et al., 1997).

Dissociation of the tricyclic imipramine from platelet membranes is attenuated in the presence of 5-HT (Wennogle and Meyerson, 1982, 1985), suggesting that 5-HT acts at a site distinct from the imipramine binding site. Several high-affinity SERT inhibitors (citalopram, paroxetine, sertraline, imipramine) can also act as allosteric ligands (Plenge and Mellerup, 1985; Plenge et al., 1991). The affinity-modulating or allosteric site has been shown to be present at all the monoamine transporters (i.e., serotonin, dopamine, and norepinephrine transporters) (Plenge and Mellerup, 1997).

The interaction with the allosteric binding site is specific for SERT as supported by several findings. Strong effects on dissociation rates are exerted by only a subset of the drugs tested (Plenge et al., 1991; Chen et al., 2005a,b). The effect is stereo-selective in that some enantiomers have different potencies (Plenge et al., 1991). Species differences concerning the allosteric potency of specific drugs have been reported (Plenge et al., 1991). A species-scanning mutagenesis study comparing human and bovine SERT revealed that Met180, Tyr495, and Ser513 are important residues in both mediating the allosteric effect and contributing to high-affinity bind-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); SERT, serotonin transporter; gSERT, chicken serotonin transporter; hSERT, human serotonin transporter; PCR, polymerase chain reaction; RTI-55, 2β-carbomethoxy-3β-(4-iodophenyl)tropane; PBSCM, phosphate-buffered saline containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.2; TMD, transmembrane domain; wt, wild-type.

ing at the primary site (Mortensen et al., 2001; Chen et al., 2005a).

In the present study, we characterized the allosteric mechanism at the recently cloned chicken SERT (gSERT) (Larsen et al., 2004). The allosteric mechanism, as mediated by antidepressants, is severely attenuated at gSERT compared with hSERT; consequently, a species-scanning mutagenesis strategy was undertaken to identify key residues for a functional allosteric mechanism.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium, fetal bovine serum, trypsin, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). Cell culture flasks and 96-well plates were obtained from Nalge Nunc International (Naperville, IL). White 96-well culture plates, MicroScint-20 scintillation mixture, [^{3}H]5-HT (21.7 Ci/mmol), and [^{125}I]RTI-55 (2200 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{3}H](S)-Citalopram (85 Ci/mmol), [^{3}H]duloxetine (81 Ci/mmol), and (S)-citalopram were provided by H. Lundbeck A/S (Valby, Denmark). The QuikChange mutagenesis kit was obtained from Stratagene (La Jolla, CA), and Fugene-6 transfection reagent was obtained from Roche Molecular Biochemicals (Indianapolis, IN). The Wizard PureFection plasmid DNA purification system was from Promega (Madison, WI), and the ABI Prism BigDye terminator cycle sequencing ready reaction kit was from Applied Biosystems (Foster City, CA).

Construction of SERT Chimeras and Mutants. A PCR-based approach was chosen to generate rationally designed cross-species chimeras between hSERT and gSERT based on the method of Kirsch and Joly (1998). The coding region of hSERT was previously cloned from human placenta and inserted into the pcDNA3 vector, denoted hSERT. A region of the hSERT was amplified by PCR using primers complementary to hSERT in the 3′- end and complementary to gSERT in the 5′- end. The resulting fragment was gel-purified and subsequently used as a pair of complementary megaprimers following the QuikChange protocol (Stratagene). All chimeras were sequenced in their entirety to verify sequence switching points and to exclude PCR-generated errors. For the construction of chimeras 5 to 12, see Larsen et al. (2004). All megaprimers were generated from a hSERT template, and cross-species chimeras were subsequently generated, using appropriate chimera templates from Larsen et al. (2004). For primer sequences and chimera-constructs 1 to 17, see Larsen et al. (2004). Additional primers designed for this study are (5′ to 3′ direction): 24F, GTGGTGAAGCTGTTTGAAGAGTATGCCACG-GGGCCC; 25R, GCCCCGTGGCATA-CTCTTCAAACAGCTTCACCACATATG. The chimeras generated in this study were chimera 24, primers 24F-5R (modified template, chimera 6); chimera 25, primers 5F-25R (modified template, chimera 6); chimera 26, primers 17F-6R (modified template, chimera 5); chimera 27, primers 6F-16R (modified template, chimera 5); chimera 28, primers 5F-25R (modified template, chimera 16); chimera 29, primers 24F-5R (modified template, chimera 17); chimera 31, primers 5F-25R (modified template, chimera 17); chimera 32, primers 6F-16R (modified template, chimera 16). Chimeras with single, double, or triple mutations were generated using complementary primers containing the sequence for the desired mutation(s). All mutations were created using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's recommendations.

Cell Culture and Expression of SERTs in COS-1 Cells. COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37°C and 5% CO_2 in a humidified atmosphere.

For transfections, 0.2 μg of plasmid and 0.4 μl of Fugene-6 (Roche Molecular Biochemicals) were used per cm^2 of plating area. Appropriate amounts of plasmid and Fugene-6 were mixed with Dulbecco's modified Eagle's medium according to the manufacturer's recommendations. COS-1 cells were trypsinized and suspended in growth media, and the plasmid/Fugene6 mixture was added, followed by dispensing into growth plates.

Membrane Preparations. Cells were transfected as described and plated at 35% confluence in 150-mm dishes. Cells were grown for 64 h, and before harvesting, dishes were rinsed in PBS. Cells were harvested with a cell scraper in buffer 1 (50 mM Tris base, 150 mM NaCl, and 20 mM EDTA, pH 7.4). After centrifugation (3000g at 4°C for 10 min), cells were suspended and homogenized with an IKA Ultra-Turrax (IKA Works, Inc., Wilmington, NC) for 20 s in buffer 1. Membranes were pelleted by ultracentrifugation (12,000g at 4°C for 15 min), and homogenization was repeated in buffer 1. Finally, after ultracentrifugation (12,000g, 4°C, 15 min) membranes were resuspended in buffer 3 (50 mM Tris-base, 120 mM NaCl, and 5 mM KCl, pH 7.4) and stored at −80°C.

Determination of Dissociation Rates. Dissociation rates were determined on membrane preparations from transiently transfected COS-1 cells. The time kinetic of dissociation of the SERT-radioligand complex was measured after extensive dilution of unbound radioligand or by excess addition of a displacer. Affinity changes revealed in such experiments are due to noncompetitive mechanisms as opposed to competitive mechanisms, which are measured in equilibrium assays. At first, a SERT–[^{125}I]RTI-55 complex was formed in buffer 3 during a 60-min incubation at 4°C. The radioligand was present at a concentration 10 times the K_d value. The time kinetic of dissociation was followed by adding 10 μl of complex solution to 250 μl of buffer 3 containing (S)-citalopram in 96-well plates and incubating subsequently for increasing time intervals at RT. Eight increasing concentrations of (S)-citalopram were examined, and variation in concentrations ranged from no effect on dissociation to nearly complete stabilization of the SERT–[^{125}I]RTI-55 complex (Chen et al., 2005a). Identical procedures were followed using [^{3}H](S)-citalopram or [^{3}H]duloxetine as radioligand. Reactions were terminated by filtration through GF/C glass-fiber filters (Unifilter; PerkinElmer), preincubated with 40 μl of 0.5% polyethylenimine, using a PerkinElmer cell harvester, and subsequently washed three times with water. Filters were soaked in 40 μl of Microscint-20 scintillation liquid (PerkinElmer). Bound radioactivity was determined by direct counting of plates using a PerkinElmer microplate scintillation counter. Assays were carried out in duplicate. Dissociation curves were obtained by plotting residual binding versus time of dissociation. As the complex dissociates according to first-order kinetics, the dissociation rates, k_t values, were determined from the first-order equation $B = B_0\exp(k_t)$ and were consequently obtained as the slopes of log-transformed plots of residual binding, B, versus time of dissociation. B_0 indicates initial amount of complex. The allosteric potency is determined as the drug concentration that retards the dissociation rate by 50% compared with dissociation in buffer (i.e., the EC_{50} value). Because the dissociation curves remain monophasic in the presence of an allosteric modulator, and because the maximum effect is almost complete inhibition of dissociation, the EC_{50} value corresponds to half-maximal occupancy of the allosteric site (i.e., the K_d value for binding of the allosteric modulator). EC_{50} values were calculated from concentration-effect curves of normalized dissociation rates versus log-drug concentrations and are given as mean values ± 95% confidential intervals (GraphPad Prism).

Binding Assays. Radioligand binding experiments were used for determining the equilibrium dissociation constant K_d. A membrane suspension (5 μg/well) was dispensed into a 96-well plate, and buffer 3, containing increasing concentrations of [^{3}H](S)-citalopram or [^{125}I]RTI-55 (diluted with unlabeled RTI-55), was added to each well. Membranes were incubated at room temperature for 60 min and transferred to a 96-well glass fiber filter plate and washed as described under *Determination of Dissociation Rates*. Nonspecific binding was determined by adding imipramine to a final concentration of 5 μM.

Membrane Surface Expression. The level of surface membrane expression of the SERT mutants were determined using a whole-cell binding assay as described by Zhu et al. (2004a,b). The transfected cells were grown for 48 h in a 96-well plate, and washed with ice-cold phosphate-buffered saline containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.2 (PBSCM) before incubation. The 96-well plate was placed on ice and the cells were preincubated 5 min with PBSCM or PBSCM containing 10 μM paroxetine or 200 μM 5-HT, respectively. PBSCM containing [^{125}I]RTI-55 was added to a final concentration of 20 nM. After 45 min of incubation, the cells were washed twice with ice-cold PBSCM. Subsequently, 40 μl of Microscint 20 scintillation liquid (PerkinElmer Life and Analytical Sciences) was added to each well. Bound radioactivity was determined by direct counting of plates using a PerkinElmer microplate scintillation counter. [^{125}I]RTI-55 and paroxetine are membrane permeable and preincubation with paroxetine blocks the total whole cell [^{125}I]RTI-55 binding sites. 5-HT is not membrane permeable at 4°C, and preincubation thus blocks all external [^{125}I]RTI-55 binding sites. The unspecific binding was determined by measuring the amount of [^{125}I]RTI-55 bound after preincubation with paroxetine. Surface expression levels were calculated as the fraction of [^{125}I]RTI-55 binding sites that were blocked by 5-HT. The surface expression level of each construct was normalized to hSERT WT surface expression level. The C-terminal truncated hSERT (1–613) was used as a negative control for surface expression, because it has been shown to have highly diminished surface expression but conserved affinity for RTI-55 (M. B. Larsen, A. W. Fjordbak, and O. Wiborg, manuscript submitted for publication).

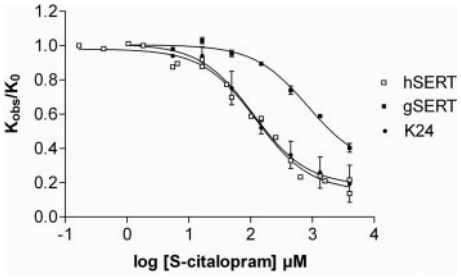

Fig. 1. Comparison of gSERT and hSERT allosteric site for (*S*)-citalopram–[^{125}I]RTI-55. COS-1 cells were transfected with wild-type hSERT, gSERT, or chimera K24 and assayed for dissociation of bound [^{125}I]RTI-55 with (*S*)-citalopram present in the dissociation buffer. As described under *Materials and Methods*, the off-rate of bound radioligand K_{obs} is normalized with K_0 (off rate in the absence of allosteric modulation) and plotted versus log (*S*)-citalopram concentrations to obtain EC_{50} values. In the gSERT chimera K24, TMDs 10 to 12 have been replaced by the corresponding hSERT fragment.

Results

Characterization and Mapping of an (*S*)-Citalopram–[^{125}I]RTI-55 Allosteric Mechanism on hSERT and gSERT. To investigate the presence of an allosteric mechanism on gSERT, we used the cocaine analog RTI-55 as radioligand at the primary binding site because this ligand has a similar affinity for both species (Larsen et al., 2004). (*S*)-Citalopram was used as the allosteric effector because this high-affinity inhibitor was found to possess high allosteric potency against RTI-55. A concentration-dependent attenuation of the dissociation rate of [^{125}I]RTI-55 from gSERT was observed when adding (*S*)-citalopram to the dissociation buffer (Fig. 1). The modulation of [^{125}I]RTI-55 dissociation, using (*S*)-citalopram as an allosteric effector, will be denoted "(*S*)-citalopram–[^{125}I]RTI-55" henceforward.

The EC_{50} for (*S*)-citalopram–[^{125}I]RTI-55 was 123.7 and 836.4 μM for hSERT and gSERT, respectively (Table 1). Thus, a 7-fold difference in allosteric potency of (*S*)-citalopram at [^{125}I]RTI-55 was observed between the two species. This difference is likely to be attributed by residues that are not involved in the binding of RTI-55 at the primary binding site because the affinity for this drug is similar for the two species.

We performed a chimera study to map residues determining this species difference at the allosteric mechanism. To investigate whether the residues determining the allosteric mechanism were located in the N- or C-terminal part of SERT, transmembrane domains (TMDs) 1 to 6 in gSERT were exchanged by the corresponding region in hSERT (chimera K8), and TMDs 7 to 12 in gSERT were replaced by the corresponding hSERT sequences in chimera K9 (Fig. 2). Table 1 shows that chimera K9 possesses a hSERT allosteric mechanism, whereas chimera K8 carries the gSERT phenotype, thus mapping the allosteric mechanism to the C-terminal part of SERT.

Further mapping using different constructs (Fig. 2) showed that chimera K12 (i.e., TMDs 9–12 replaced with the corresponding hSERT region) has a hSERT allosteric mechanism, whereas replacing TMDs 9 to 10 (chimera K5) or TMDs 11 to 12 (chimera K6) did not improve the gSERT allosteric mechanism (Table 1). These findings provided evidence that at least two distinct segments, in TMDs 9 to 10 and TMDs 11 to

TABLE 1
Characterization of the allosteric mechanism for gSERT-hSERT chimeras

Name of Chimera	EC_{50}	Sequence Switch Points in Chimeras (Using gSERT Numbering)
	μM	
hSERT WT	123.7 (94–163)	
gSERT WT	836.4 (588–1190)	
K5	1624 (558–4727)	g1–488 h489–571g572–670
K6	797.2 (458–1387)	g1–571 h572–643g644–670
K8	1048 (444–2473)	g1–118 h119–393g394–670
K9	120.2 (87–166)	g1–393 h394–643g644–670
K12	90 (59–139)	g1–488 h489–643g644–670
K24	111.7 (58–217)	g1–532 h533–643g644–670
K25	384.1 (274–539)	g1–488 h489–532g533–571 h572–643g644–670
K26	621.6 (440–879)	g1–488 h489–571g572–604 h605643g644–670
K27	339.6 (186–622)	g1–488 h489–604g605–670
K28	524.1 (305–901)	g1–532 h533–571g572–604 h605–644g645–670
K29	531.7 (360–786)	g1–488 h489–532g533–571 h572–604g605–670
K31	680.7 (545–850)	g1–488 h489–532g533–604 h605–643g644–670
K32	N.D.	g1–532 h533–604g605–670

N.D., not determined.

12, respectively, are important for the allosteric mechanism. Furthermore, we found that a hSERT-construct with TMDs 9 to 12 replaced by the corresponding gSERT region, possessed an allosteric site with gSERT WT affinity (data not shown).

Motivated by these observations, we constructed a series of chimeras consisting of all combinations of TMDs containing at least one TMD from TMDs 9 to 10 and TMDs 11 to 12, respectively. The different constructs are presented in Fig. 2. Of all constructs tested, only chimera K24 possessed a hSERT allosteric mechanism, whereas the remaining constructs contained a gSERT allosteric mechanism, except chimera K25 with an intermediate EC_{50} at 384 μM (Table 1). These data showed that hSERT TMD 9 is not needed to restore the allosteric mechanism, whereas the remaining TMDs 10, 11, and 12 were all indispensable for a fully functional allosteric mechanism. Our data implied that at least three distinct subdomains in each TMD, 10, 11, and 12, determine the allosteric mechanism.

To identify distinct amino acids essential for the allosteric mechanism, we focused on four segments positioned in the upper part of the TMDs (i.e., the ALI/VFL segment in TMD 10, the II/VT and MS/SN segments in TMD 11, and the SI/TT segment in TMD 12). The alignment of hSERT and gSERT in the TMD 10-to-12 region is shown in Fig. 3. gSERT identities were introduced in each segment in chimera K24 to identify residues that attenuate the allosteric mechanism of this chimera. All four segmental modifications of chimera K24 caused at least a 3-fold attenuation of the allosteric mechanism (Table 2). The most profound effects were seen with the II/VT (TMD 11) and SI/TT (TMD 12) modifications and to a lesser extent for the ALI/VFL (TMD 10) and MS/SN (TMD 11) segments. None of the mutations caused a complete switch to gSERT affinity. The neighboring residues to the TMD 12 segment (IL/VV) were found not to affect the allosteric mechanism (data not shown).

The identification of the four segments involved in the allosteric mechanism prompted us to determine whether they solely constitute the allosteric mechanism, or if additional residues remained to be identified. We therefore constructed three chimeras as presented in Fig. 4. The three chimeras were all derived from chimera K24 and contained gSERT TMDs 10, 11, or 12, respectively. Subsequently, hSERT identities in the segments described above were introduced in the gSERT TMDs. The purpose of these constructs was to determine the minimal requirement of each TMD-domain to restore a functional allosteric mechanism. Because the MS/SN mutation caused a partial loss of function of chimera K24, the TMD 11 chimera (K28) was constructed to contain both segments. All three chimeras possessed hSERT-like properties with respect to the allosteric mechanism (Table 2). We therefore conclude that the four segments, comprising a total of nine amino acids, account for the difference in the allosteric mechanism for (*S*)-citalopram–[^{125}I]RTI-55 between hSERT and gSERT.

The chimeras K28 (VT/II SN/MS) and K32 (TT/SI) possess a more sensitive allosteric mechanism compared with hSERT wt and chimera K24. This was also the case when determining the EC_{50} values of the chimeras for (*R*)-citalopram–

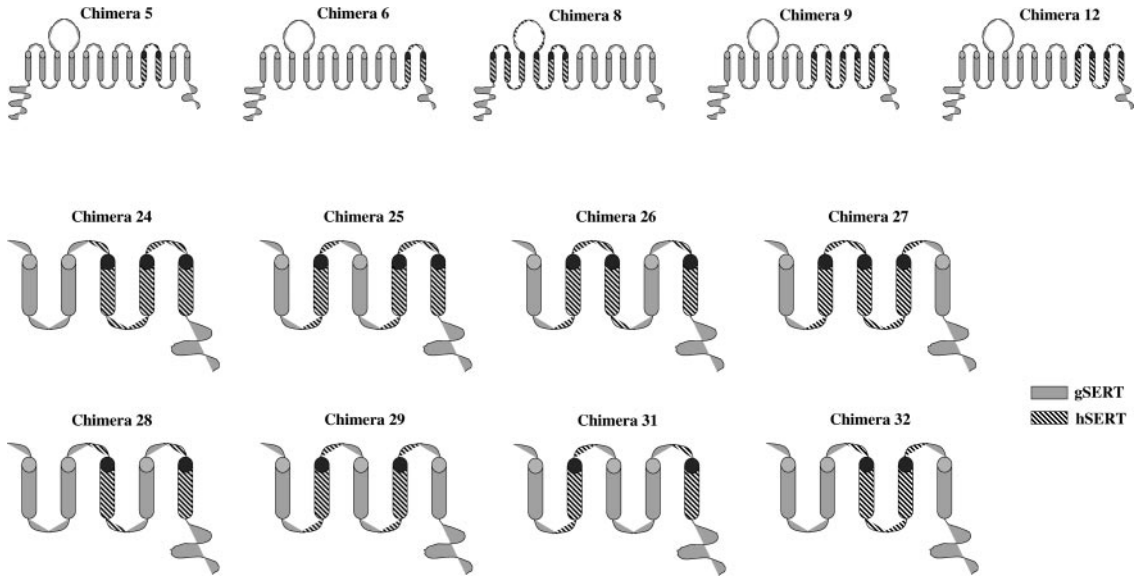

Fig. 2. A schematic representation of the gSERT/hSERT chimeras used in this study. The vertical bars represent transmembrane domains, each connected with intracellular or extracellular loops. Parts of gSERT were exchanged for the corresponding parts of hSERT. The gSERT part of the chimera is shown in gray, and the hSERT part is shown in a hatched pattern.

Fig. 3. Amino acid sequence alignment of the C-terminal part of gSERT and hSERT. Identical residues are shown in black. Putative transmembrane domains are underlined. Residues shaded in gray were mapped to the allosteric mechanism.

[^{125}I]RTI-55 and imipramine–[^{125}I]RTI-55 (data not shown), suggesting the sensitive allosteric mechanism to be a general property of these chimeras. Finally, the four segments in gSERT were substituted with the corresponding hSERT identities and the construct was denoted gSERT (ALI II MS SI). The EC_{50} value was estimated to be 33.2 μM for (*S*)-citalopram–[^{125}I]RTI-55, suggesting that the introduction of hSERT identities at the four segments in gSERT causes an enhancement of the allosteric mechanism compared with hSERT wt. It is noteworthy that the decreased EC_{50} value for gSERT (ALI II MS SI) was similar to the values found for chimeras K28 (VT/II SN/MS) and K32 (TT/SI) respectively (Table 2).

To identify the contribution of the individual residues in the four segments, we performed single reverse mutations at each position in gSERT (ALI II MS SI). This scanning revealed that a single reverse mutation at most caused a 2-fold increase in the EC_{50} value compared with gSERT (ALI II MS SI) (Table 3) and consequently no single residue is indispensable for a functional allosteric mechanism.

Characterization and Mapping of an (*S*)-Citalopram–[^{3}H](*S*)-Citalopram Allosteric Mechanism on hSERT and gSERT. The affinity of (*S*)-citalopram at the primary binding site in gSERT is highly decreased compared with hSERT (Larsen et al., 2004). We therefore decided to examine the allosteric effect of (*S*)-citalopram at gSERT and to determine the EC_{50} for (*S*)-citalopram–[^{3}H](*S*)-citalopram. Because of a high off-rate of [^{3}H](*S*)-citalopram from the primary binding site at gSERT, we performed the dissociation assay at 0°C. We have previously shown that the potency of (*S*)-citalopram with respect to the allosteric mechanism is temperature-independent, allowing direct comparison of data from assays performed at different temperatures (Chen et al., 2005a). We found that a concentration higher than 5 mM (*S*)-citalopram in the dissociation buffer has a deleterious effect upon the dissociation; as a result, we were unable to obtain an accurate determination of the EC_{50} value. Consequently, the EC_{50} value for the gSERT allosteric mechanism was approximated to 3.4 mM. Thus the allosteric mechanism at gSERT for (*S*)-citalopram–[^{3}H](*S*)-citalopram was found to be severely attenuated compared with hSERT.

The pharmacological characterization and subsequent mapping of gSERT performed by (Larsen et al., 2004) revealed three key residues highly contributing to the primary binding site of (*S*)-citalopram. When introducing hSERT identities at position Asp209, Val212, and Ile626, the affinity was restored compared with hSERT. The corresponding positions of the three residues in hSERT are Ala169, Ile172, and Phe586. To investigate whether this restoration of the primary binding site for (*S*)-citalopram also restores the allosteric potency for (*S*)-citalopram–[^{3}H](*S*)-citalopram, we characterized the allosteric mechanism of gSERT (D209A, V212I, I626F). We found that the sensitivity of the allosteric mechanism increased, but was far from restored to the hSERT wild type sensitivity (Fig. 5 and Table 4). Thus, the restoration of the primary binding site was not accompanied by a similar restoration of the allosteric site. These results implied that the allosteric mechanism is composed of one subset of residues that also affect the affinity at the primary binding site and one subset apparently acting independently of the primary binding site.

As outlined previously, we identified four segments accounting for the gSERT/hSERT species differences in the allosteric mechanism for (*S*)-citalopram–[^{125}I]RTI-55. Subsequently, we attempted to investigate whether these segments furthermore contribute to the allosteric site for (*S*)-citalopram–[^{3}H](*S*)-citalopram and therefore introduced the

TABLE 2
Characterization of the allosteric mechanism for gSERT mutants

	EC_{50}	K_d
	μM	nM
Loss of function mutants		
K24 (ALI/VFL)	333.3 (217–512)	2.3
K24 (II/VT)	496.6 (243–1010)	3.2
K24 (MS/SN)	225.4 (193–263)	N.D.
K24 (SI/TT)	464.0 (345–625)	N.D.
Gain of function mutants		
K6 (VFL/ALI)	119.0 (75–188)	2.4
K28 (VT/II SN/MS)	57.2 (47–70)	N.D.
K32 (TT)	49.4 (40–61)	N.D.
gSERT (ALI II MS SI)	33.4 (22–50)	3.7
hSERT WT	123.7 (94–163)	3.8
K24	111.7 (58–217)	2.0
gSERT WT	836.4 (588–1190)	1.3

N.D., not determined.

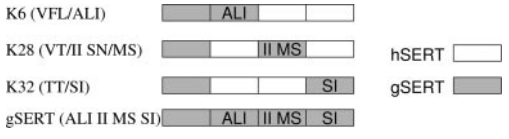

Fig. 4. A schematic representation of TMDs 9 to 12 of gSERT/hSERT chimeras, where different amino acid segments have been exchanged. The vertical bars represent TMD 9 to 12 in the C-terminal part of SERT. The gSERT TMDs are shown in gray, and the hSERT TMDs are shown in white. The amino acid substitutions are represented as letters denoting the identity of the introduced residue.

TABLE 3
Characterization of the allosteric mechanism for single reverse mutations of gSERT (ALI II MS SI)

Mutant	EC_{50}
	μM
gSERT ALI II MS SI	33.4 (22–50)
gSERT VLI II MS SI	38.0 (24–61)
gSERT AFI II MS SI	35.5 (19–65)
gSERT ALL II MS SI	61.6 (38–100)
gSERT ALI VI MS SI	52.2 (35–79)
gSERT ALI IT MS SI	43.9 (33–59)
gSERT ALI II SS SI	51.6 (36–74)
gSERT ALI II MN SI	85.7 (62–118)
gSERT ALI II MS TI	23.6 (18–32)
gSERT ALI II MS ST	65.2 (47–87)

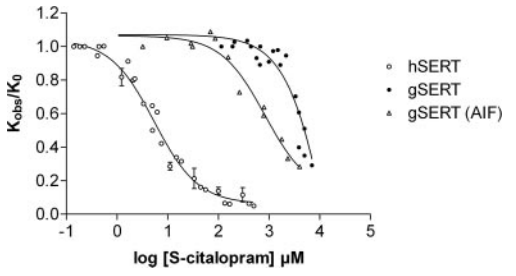

Fig. 5. Comparison of gSERT and hSERT allosteric site for (*S*)-citalopram–[^{3}H](*S*)-citalopram. COS-1 cells were transfected with wild-type hSERT, gSERT, or gSERT (D209A, V212I, I626F) and assayed for dissociation of bound [^{3}H](*S*)-citalopram with (*S*)-citalopram present in the dissociation buffer. The gSERT (triple mutation) denote gSERT (D209A, V212I, I626F).

four segments into gSERT (D209A, V212I, I626F). The EC_{50} was subsequently found to be 5.0 μM and thus similar to hSERT wt. We conclude that the four identified segments additionally can confer a functional allosteric mechanism for (*S*)-citalopram–[^{3}H](*S*)-citalopram similar to hSERT wt. This makes them more likely to contribute to a general allosteric mechanism, which was further supported by the finding that gSERT (ALI II MS SI) also possesses a restored allosteric mechanism for *R*-citalopram–[^{125}I]RTI-55 and imipramine–[^{125}I]RTI-55 (data not shown).

To investigate the contribution from each amino acid in the four segments, we made a series of mutations derived from gSERT (D209A, V212I, I626F) (ALI II MS SI) including conversion of each single amino acid to gSERT identity. The purpose was to determine whether the residues involved in the allosteric interaction for (*S*)-citalopram–[^{125}I]RTI-55, as described above, differed from the residues involved in the allosteric interaction for (*S*)-citalopram–[^{3}H](*S*)-citalopram. The scanning revealed that a single reverse mutation caused at most a 6-fold increase in the EC_{50} value compared with gSERT (D209A, V212I, I626F) (ALI II MS SI) (Table 4). Comparison of the magnitude of changes in EC_{50} values for the individual mutations showed some accordance with the findings for (*S*)-citalopram–[^{125}I]RTI-55 (Table 3). Although mutation of Ala505, for example, caused a 6-fold change in the EC_{50}-value for (*S*)-citalopram–[^{3}H](*S*)-citalopram, the EC_{50}-value for (*S*)-citalopram–[^{125}I]RTI-55 was unaffected, suggesting that the contribution from the individual residues to depend on the ligands used.

We subsequently used the data obtained from the gSERT study to investigate the role of the four segments in hSERT. First, we constructed a hSERT mutant denoted hSERT (VFL VT SN TT) with all four segments converted to the gSERT identities. The construct revealed an allosteric mechanism for (*S*)-citalopram–[^{3}H](*S*)-citalopram with an EC_{50} of 208.2 μM (Table 5). Although a 40-fold attenuation, the affinity was not completely reversed to that of gSERT (D209A, V212I, I626F), and we therefore determined the contribution from each separate segment. Table 5 shows that reversion of each segment to gSERT identity caused a slight attenuation, particularly regarding ALI/VFL and SI/TT. Subsequently, we made constructs containing two or three segments of four. It is noteworthy that VFL-TT, VT-SN-TT, and VFL-VT-TT caused attenuation close to the EC_{50} of gSERT (D209A, V212I, I626F). All other combinations caused intermediate attenuation (Table 5). To investigate the contribution from each single amino acid in the four segments, we made nine hSERT point mutations with each amino acid converted to the gSERT identity. Similar to the findings from the previous single mutation scanning of the nine residues, several residues caused the EC_{50} to increase up to 5-fold. However, no single residue is indispensable for a functional allosteric mechanism (Table 5).

To assess whether the introduced mutations affected the affinity in the primary binding site for RTI-55 and (*S*)-citalopram, we determined the equilibrium dissociation constant (K_d) for a representative subset of the chimeras (Tables 2, 4, and 5). The determined values were found to be similar among the constructs tested. Finally, to assess whether the introduced mutations caused intracellular retention of the transporter, we compared the relative amount of SERT expressed at the plasma membrane for a representative subset of constructs. The constructs hSERT and chimera K6 (VFL/ALI) were selected as having a functional allosteric mechanism, and the constructs gSERT (D209A, V212I, I626F) and hSERT (VT SN TT) were selected as having an attenuated allosteric mechanism. As a negative control for surface expression, we used a C-terminally truncated hSERT construct, hSERT (1–613). A biotinylation assay revealed this construct to have highly diminished plasma membrane expression (M. B. Larsen, A. W. Fjordbak, and O. Wiborg, manuscript submitted for publication). The relative amount of plasma membrane expression was found to be similar for the constructs tested, whereas hSERT (1–613) has a 77% lower expression level compared with hSERT WT (Fig. 6).

5-HT as an Allosteric Effector. 5-HT has previously been found to act as an allosteric effector at a number of SERT high-affinity ligands (Wennogle and Meyerson, 1982, 1985; Plenge et al., 1991; Chen et al., 2005b). We tested the

TABLE 4
Characterization of the allosteric mechanism for single reverse mutations of gSERT (D209A, V212I, I626F) (ALI II MS SI)
gSERT (D209A, V212I, I626F) is denoted g(AIF).

	EC_{50}	K_d
	μM	*nM*
hSERT WT	7.0 (5.8–8.4)	6.7 (5.4–8.0)
gSERT WT	3425 (1910–5387)	N.D.
g(AIF)	831 (407–1695)	10.2 (8.2–11.9)
g(AIF) (ALI II MS SI)	5.0 (3.0–8.3)	5.4 (2.0–8.7)
g(AIF) VLI II MS SI	36.6 (21–63)	
g(AIF) AFI II MS SI	4.9 (3–9)	
g(AIF) ALL II MS SI	18.4 (11–32)	
g(AIF) ALI VI MS SI	8.2 (5–14)	
g(AIF) ALI IT MS SI	28.5 (20–41)	
g(AIF) ALI II SS SI	2.9 (2.1–4.1)	
g(AIF) ALI II MN SI	13.3 (10–18)	
g(AIF) ALI II MS TI	8.2 (4.4–15)	
g(AIF) ALI II MS ST	15.9 (11–22)	

N.D., not determined.

TABLE 5
Characterization of the allosteric mechanism for hSERT mutants

	EC_{50}	K_d
	μM	*nM*
hSERT WT	7.0 (5.8–8.4)	6.7 (5.4–8.0)
hSERT ALI/VFL	45.2 (32–64)	N.D.
hSERT II/VT	23.0 (19–27)	5.1 (3.9–6.3)
hSERT MS/SN	15.4 (14–17)	4.6 (3.9–5.2)
hSERT SI/TT	35.3 (26–48)	6.8 (5.7–7.9)
hSERT VFL-II-SN-TT	216.3 (126–372)	11.3 (8.3–14.4)
hSERT ALI-VT-SN-TT	396.5 (257–613)	7.5 (4.3–10.8)
hSERT VFL-VT-SN-SI	193.0 (146–255)	6.6 (3.3–10.0)
hSERT VFL-VT-MS-TT	532.3 (358–792)	N.D.
hSERT VFL-VT-SN-TT	208.2 (169–256)	16.9 (12.4–21.3)
hSERT VFL-II-SN-SI	213.5 (176–259)	10.2 (6.7–13.7)
hSERT ALI-VT-MS-TT	207.7 (120–359)	15.2 (12.3–17.7)
hSERT ALI-II-SN-TT	47.1 (39–56)	10.8 (8.4–13.2)
hSERT VFL-II-MS-TT	483.8 (369–634)	12.5 (6.5–18.5)
hSERT VFL-VT-MS-SI	113.6 (89–145)	14.1 (10.5–17.8)
hSERT A505V	34.9 (24–51)	N.D.
hSERT L506F	33.2 (20–56)	N.D.
hSERT I507I	6.2 (5.2–7.3)	N.D.
hSERT I552V	7.3 (5.8–9.4)	N.D.
hSERT I553T	21.4 (14.6–31.4)	N.D.
hSERT M558S	7.6 (6.5–8.8)	N.D.
hSERT S559N	16.1 (11.6–22.4)	N.D.
hSERT S574T	5.8 (4.3–7.8)	N.D.
hSERT I575T	23.4 (19.7–27.9)	N.D.

N.D., not determined.

potency of 133 μM 5-HT at the dissociation of [^{3}H](*S*)-citalopram, [^{125}I]RTI-55, and [^{3}H]duloxetine, and we found that 5-HT possessed high allosteric potency for [^{3}H]duloxetine, intermediate potency for [^{125}I]RTI-55, and minor effect at [^{3}H](*S*)-citalopram (Fig. 7). Thus 5-HT acts as an allosteric modulator in the micromolar range against duloxetine and RTI-55. Subsequently, the EC_{50} for 5-HT–[^{3}H]duloxetine was determined to 70.3 μM (49–101) and 18.2 μM (11–30) for hSERT and gSERT, respectively. The EC_{50} for 5-HT–[^{125}I]RTI-55 was determined to 139.7 μM (104–189) and 191.6 μM (129–286) for hSERT and gSERT, respectively. To prevent reassociation of radioligands, 200 μM fluoxetine was added in these assays. Fluoxetine is known not to possess any allosteric effect (Chen et al., 2005a). The gSERT [^{3}H]duloxetine dissociation assay was performed at 0°C because of a high off rate for the radioligand.

Discussion

In the present study, we identified nine SERT residues that confer the species difference of the allosteric mechanism between hSERT and gSERT. The residues reside in the SERT C-terminal region containing the transmembrane domains 10 to 12 and confer a functional allosteric mechanism for (*S*)-citalopram–[^{125}I]RTI-55 and (*S*)-citalopram–[^{3}H](*S*)-citalopram, as presented herein, and furthermore for (*R*)-citalopram–[^{125}I]RTI-55 and imipramine–[^{125}I]RTI-55 (data not shown). This indicates that the residues are important for a general allosteric mechanism at SERT. Residues that affect both the primary binding site for (*S*)-citalopram and the allosteric mechanism have been identified previously (Chen et al., 2005a), whereas residues identified in the present study affect only the allosteric mechanism. The data presented in this article provide evidence that the allosteric mechanism is constituted by distinct segments. This is demonstrated by introducing hSERT segments into the gSERT triple mutant (D209A, V212I, I626F), which has a hSERT WT affinity at the primary site but a very weak allosteric mechanism. When introducing the allosteric segments into gSERT (D209A, V212I, I626F), the allosteric mechanism is restored. The allosteric segments reported in the present study may be part of an allosteric binding site distinct from the primary binding site or they may be residues mediating conformational changes induced by the allosteric effector.

The identified residues are all strongly conserved among mammalian SERTs, and Leu506 in the ALI-segment is highly conserved among all monoamine transporters. In mammalian NET and DAT, the residues corresponding to the II- and SI-segments are conserved between species as VV and AN, respectively. The position of highly conserved residues in the close vicinity of the segments may be of functional importance for the allosteric mechanism as well. The ALI-segment is thus flanked by conserved Leu502, Glu508, and Ala509. The II-segment is flanked by Phe548, Leu549, and Leu550.

Although the identity or position of the identified residues provides no obvious clues to the allosteric mechanism of action, previous studies of TMD 10 to 12 have indicated important structural-functional relationships in the region. Keller et al. (2004) performed cysteine scanning analysis of the extracellular loop (EL) between TMD 9 and TMD 10 and included the ALI-segment in TMD 10. Substitution of Ala505, Leu506, and Ile507 with cysteines does not alter 5-HT uptake kinetic. Furthermore, Ala505, Leu506, and Ile507 are insensitive to modification by methane-thiosulfonates, or they are sterically inaccessible to the reagents. The neighboring Thr503 and Glu508 are highly conserved among monoamine transporters, and cysteine substitution of these residues caused a severe attenuation of 5-HT-transport. Mutation of Ser559, within the MS segment, to alanine was not found to affect 5-HT uptake or surface expression. Mutation of Ser545 near the II-segment affects transport as well as imipramine affinity (Sur et al., 1997).

The species difference between hSERT and the bovine SERT for the affinity of (*S*)-citalopram and the enantiomer (*R*)-citalopram has previously been mapped to three residues (Mortensen et al., 2001). The three residues, Met180, Tyr495, and Ser513, were also important for mediating the allosteric effect of (*S*)-citalopram (Chen et al., 2005a); however, they only partially accounted for the species differences, indicating that additional residues take part in constituting the allosteric mechanism.

The introduction of single reverse mutations provided no indication of residues having an indispensable role for the allosteric mechanism. Although residues causing a severe attenuation of the allosteric mechanism were identified, the contribution of each residue to the allosteric mechanism depended on the SERT species as well as on the nature of the ligands occupying the primary and allosteric binding sites.

The allosteric effectors identified so far share the ability to bind to the primary binding site with affinities considerably higher than for the allosteric site. This raises the possibility that affinity for the primary binding site is a prerequisite for the ability to act as an allosteric modulator, suggesting that the two binding sites partially overlap.

To explain the allosteric mechanism, communication between binding sites on different subunits in an oligomer can be envisaged. Because monoamine transporters are expected

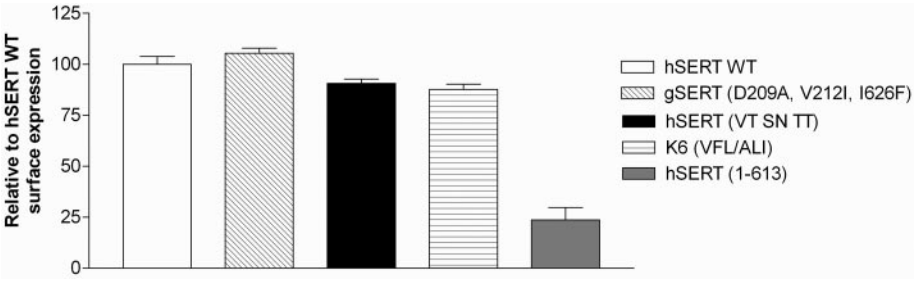

Fig. 6. Comparison of plasma membrane expression of SERT mutants. COS-1 cells were transfected with wild-type hSERT, hSERT (VT SN TT), gSERT (D209A, V212I, I626F), or chimera K6 (VFL/ALI) and assayed for the fraction of SERT expressed at the plasma membrane. Nonspecific binding was determined as [^{125}I]RTI-55 binding after preincubation with paroxetine. Data are given as mean values ± S.E.M of the fraction of plasma membrane SERT normalized to hSERT WT, 100 ± 3.9; hSERT (VT SN TT), 90 ± 2.1; gSERT (D209A, V212I, I626F), 105 ± 2.5; chimera K6 (VFL/ALI), 87.6 ± 2.6; and hSERT (1–613), 23.0 ± 6.0 (n = 3–5).

to assemble into homo-oligomeric complexes, the binding of high-affinity ligands to the primary binding site at one subunit may be speculated to perturb neighbor subunits, thus leaving the (*S*)-citalopram binding site on the second subunit in a low-affinity state. Consequently, (*S*)-citalopram may at high concentrations activate the low-affinity allosteric binding site and, conversely, induce perturbation of the occupied high-affinity primary binding site at the first subunit. This perturbation may induce either a positive or negative modulatory allosteric effect. Plenge and Mellerup (1985) thus observed that paroxetine allosterically increases the dissociation rate for imipramine. The lack of correlation between the affinities for the primary and the allosteric binding sites may indicate that the conformational changes induced at the low-affinity binding site have different effects upon the binding potential for individual ligands. Thus certain high-affinity ligands for the primary binding site (e.g., fluoxetine and RTI-55) do not act as allosteric effectors (Chen et al., 2005b).

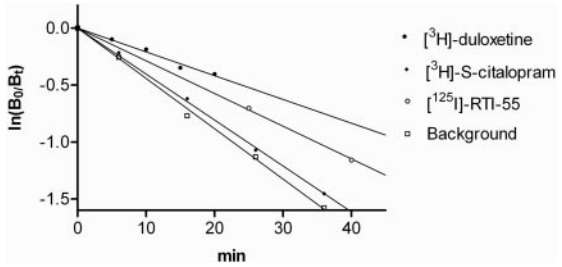

Fig. 7. 5-HT attenuates dissociation of bound radioligand. COS-1 cells were transfected with hSERT WT and assayed for dissociation of bound [^{3}H](*S*)-citalopram, [^{3}H]duloxetine, or [^{125}I]RTI-55 in the presence (or absence) of 133 μM 5-HT in the dissociation buffer. The dissociation of [^{3}H]duloxetine and [^{125}I]RTI-55 was normalized to the dissociation of [^{3}H](*S*)-citalopram in the absence of 5-HT, denoted as "Background". The normalization was performed to correct for different off-rates of the radioligands and thus describes the relative contribution of 133 μM 5-HT upon the radioligands used in this assay. The dissociation buffer contained 200 μM fluoxetine to prevent reassociation of the radioligand. B_0 indicates initial amount of bound radioligand, and B_t indicates residual binding versus time of dissociation.

On the other hand, potential conformational changes induced by activation of the high-affinity binding site may be suggested to have no allosteric impact on the affinity at the low-affinity binding site. In such a model, the residues identified in our study may be lining, or connecting to, a distorted version of the primary binding site either at a different subunit in an asymmetric oligomer or as part of a subunit interface, thereby mediating conformational changes. This suggestion is supported by the finding that TMD 11 and 12 are part of an oligomerization interface (Just et al., 2004) and further substantiated by a recent determination of the crystal structure of a bacterial homolog to monoamine transporters (Yamashita et al., 2005). The bacterial leucine transporter (LeuTAa) was crystallized as a dimer, and the structure reveals that the interface is formed by EL 2, TMD 9, and TMD 12. Monoamine transporters have a much larger EL 2 and were furthermore suggested to form quaternary assemblies (Hastrup et al., 2001, 2003). Consequently, interface regions for monoamine transporters may differ compared with LeuTAa but probably involves at least C-terminal TMDs. The corresponding positions in LeuTAa of the allosteric segments identified in the present study indicate that the segments are located at the protein surface distant to the primary binding site, which is embedded in the interior of the structure (Fig. 8). Consequently, the identified allosteric residues may be located either at subunit interfaces, or connected to these, and therefore may be capable of transmitting signals on the bound/unbound status of binding sites between subunits.

Finally the allosteric site may be explained in terms of a low-affinity interaction of allosteric ligands with the solvent exposed parts of an occupied primary binding site. This interaction may cause the allosteric effector to impose a steric hindrance for dissociation of the high-affinity ligand and thus induce a decrease in the dissociation rate, as suggested by Plenge and Mellerup (1997). Our finding that essential residues for the allosteric mechanism reside in a potential inter-

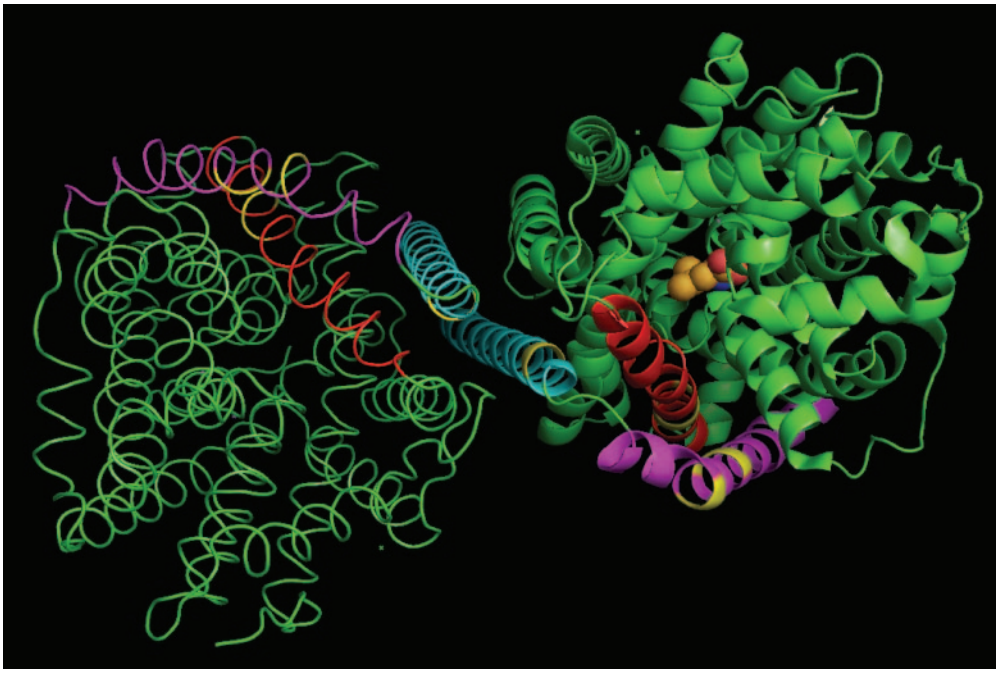

Fig. 8. Structure of the bacterial leucine transporter (LeuTAa). The transporter is shown as a homodimer, seen from above. TMDs 10, 11, and 12 are shown in red, purple, and blue, respectively. The positions of the allosteric segments are indicated in yellow. The leucine substrate, situated in the right subunit of the dimer, is shown in orange. The individual positions of the segments are based on the alignment presented in Yamashita et al. (2005).

face region distant from the primary binding site, however, does not support this model.

The allosteric potency of 5-HT on dissociation of [^{3}H]duloxetine and [^{125}I]RTI-55 was found to be in the micromolar range. Presynaptic SERTs may be transiently exposed to 5-HT concentrations as high as 6 mM (Bunin and Wightman, 1998). This suggests that 5-HT can modulate duloxetine and RTI-55 under physiological conditions and even raises the possibility that 5-HT can act as an autoregulating allosteric effector modulating the transporter function in vivo. It has recently been reported that the antidepressants fluoxetine and desipramine can accumulate locally at $5HT_A$ receptors localized to lipid rafts in the plasma membrane (Eisensamer et al., 2005). SERT also localizes to lipid rafts in the plasma membrane (Magnani et al., 2004). This raises the possibility that (*S*)-citalopram can, in a similar manner, accumulate locally to levels at which an allosteric effect can be exerted.

Although gSERT has a weak allosteric effect for (*S*)-citalopram–[^{125}I]RTI-55 and (*S*)-citalopram–[^{3}H](*S*)-citalopram compared with hSERT, the EC_{50} of 5-HT–[^{3}H]duloxetine was found to be in the micromolar range for both species. Thus, the allosteric mechanism for 5-HT, and hence the potential autoregulating role of 5-HT, seems to be conserved between the two species, although (*S*)-citalopram is a weak allosteric modulator at gSERT. A possible functional implication of the 5-HT-induced conformational changes between subunits in the SERT oligomer has previously been suggested (Kilic and Rudnick, 2000).

Acknowledgments

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