Molecular Pharmacology Fast Forward. Published on January 24, 2006 as doi:10.1124/mol.105.018507
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MOL Manuscript # 18507

DISSECTION OF AN ALLOSTERIC MECHANISM ON THE SEROTONIN TRANSPORTER: A CROSS-SPECIES STUDY

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Running title "Mapping an allosteric mechanism on the serotonin transporter"

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Number of text pages: 17

Number of references 22

Number of figures 8

Words in abstract 224

Words in introduction 362

Words in discussion 1331

ABBREVIATIONS USED

5-HT, 5-hydroxytryptamine; PBS, phosphate buffered saline; SERT, serotonin transporter (gSERT, Chicken SERT; hSERT, human SERT); TMD, transmembrane domain;

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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-HT) 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 10-12. 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 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 as 5-HT 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.

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Serotonergic neurotransmission is modulated by clearance of serotonin (5-hydroxytryptamine or 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 signalling 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 towards 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;Wennogle and Meyerson, 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. the 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 only exerted by a subset of the drugs tested (Plenge *et al.*, 1991;Chen *et al.*, 2005c;Chen *et al.*, 2005b). The effect is stereo selective as 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 binding at the primary site (Mortensen *et al.*, 2001;Chen *et al.*, 2005a).

In the present study we characterize the allosteric mechanism at the recently cloned chicken SERT (gSERT) (Larsen *et al.*, 2004). The allosteric mechanism, as mediated by antidepressants, is

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severely attenuated at gSERT compared to hSERT, and consequently a species-scanning mutagenesis strategy was undertaken in order to identify key residues for a functional allosteric mechanism.

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Materials and Methods

Materials - Dulbecco's modified Eagle's medium, fetal bovine serum, trypsin, and penicillin/streptomycin were purchased from Invitrogen. Cell culture flasks and 96-well plates were obtained from NUNC, white 96-well culture plates and MicroScint-20 scintillation mixture from Packard and [^{3}H]5-HT (21.7 Ci/mmol) and [^{125}I]RTI-55 (2200 Ci/mmol) from PerkinElmer Life Sciences. [^{3}H]-S-citalopram (85 Ci/mmol) and [^{3}H]-duloxetine (81 Ci/mmol) were provided by H. Lundbeck A/S (Valby, Denmark). The QuikChange mutagenesis kit was obtained from Stratagene and Fugene-6 transfection reagent from Roche Molecular Biochemicals. The Wizard PureFection plasmid DNA purification system was from Promega, and the ABI Prism BigDye terminator cycle sequencing ready reaction kit was from PerkinElmer Life Sciences. S-citalopram was provided by H. Lundbeck A/S.

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 by (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 exclude PCR-generated errors. For the construction of chimeras 5-12; see (Larsen *et al.*, 2004). All megaprimers were generated from a hSERT template, and subsequently cross-species chimeras were generated, using appropriate chimera templates from (Larsen *et al.*, 2004). For primer sequences and chimera-constructs 1-17, 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 are: 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 –

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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 Quickchange 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 dispension into growth plates.

Membrane preparations - Cells were transfected as described and plated at 35% confluency in 150 mm dishes. Cells were grown for 64 h, and prior to harvesting, dishes were rinsed in PBS. Cells were harvested with a cell scraper in buffer 1 (50 mM Tris-base, 150 mM NaCl, 20 mM EDTA, pH 7.4). After centrifugation (3000 *g* at 4°C for 10 min), cells were suspended and homogenized with an IKA Ultra-Turrax from Rose Scientific Ltd (Edmonton, Alberta, Canada) for 20 s in buffer 1. Membranes were pelleted by ultracentrifugation (12 000 *g* at 4°C for 15 min), and homogenization was repeated in buffer 1. Finally, after ultracentrifugation (12000 g, 4°C, 15 min) membranes were resuspended in buffer 3 (50 mM Tris-base, 120 mM NaCl, 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 non-competitive mechanisms as opposed to competitive mechanisms, which are measured in equilibrium assays. Initially a SERT-[^{125}I]-RTI-55 complex was formed in buffer 3 during a 60 min incubation at 4°C. The radioligand was

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present at a concentration 10 times the K_d value. The time kinetic of dissociation was followed by adding 10 µL complex solution to 250 µL 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-fibre filters (Unifilter, Perkin Elmer Life Sciences), preincubated with 40 µl 0,5 % polyethyleneimine, using a Packard Bell cell harvester, and subsequently washed three times with water. Filters were soaked in 40 µl Microscint 20 scintillation liquid (Pachard Bell). Bound radioactivity was determined by direct counting of plates using a Packard Bell microplate scintillation counter. Assays were carried out in duplicate. Dissociation curves were obtained by plotting residual binding vs. 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 (eqn 1):

$$B = B_0 \exp(k_t) \qquad (1)$$

and were consequently obtained as the slopes of log-transformed plots of residual binding, *B*, vs. 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 to dissociation in buffer, i.e. the EC_{50} value. As the dissociation curves remain monophasic in the presence of an allosteric modulator, and as 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 vs. 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 unlabelled RTI-55), was added to each well. Membranes were incubated at room temperature for 60

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min and transferred to a 96-well glass fiber filter plate and washed as described above. Non-specific 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* (Zhu *et al.*, 2004b; Zhu *et al.*, 2004a). The transfected cells were grown for 48 h in a 96-well plate, and washed with ice cold PBSCM prior to 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 incubation the cells were washed twice with ice cold PBSCM. Subsequent 40 μl of Microscint 20 scintillation liquid (Pachard Bell) was added to each well. Bound radioactivity was determined by direct counting of plates using a Packard Bell 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, as it has been shown to have highly diminished surface expression but conserved affinity for RTI-55 (M. B. Larsen; A. W. Fjordbak, O. Wiborg, manuscript submitted for publication).

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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 analogue 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 as this high-affinity inhibitor also was shown to possess high allosteric potency. 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 μM 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 which are not involved in the binding of RTI-55 at the primary binding site as the affinity for this drug is similar for the two species.

We performed a chimera-study in order 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) 1-6 in gSERT were exchanged by the corresponding region in hSERT (chimera K8), and TMDs 7-12 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 9-10 (chimera K5) or TMDs 11-12 (chimera K6), did not improve the gSERT allosteric mechanism (Table 1). These findings provided evidence that at least two distinct segments, in TMDs 9-10 and TMDs 11-12, respectively, are important for the allosteric mechanism.

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Furthermore, we found that a hSERT-construct with TMDs 9-12 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 TM-domains containing at least one TM domain from TMDs 9-10 and TMDs 11-12 respectively. The different constructs are presented in Figure 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 TMDs 10, 11 and 12, respectively 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 10-12 region is shown in Figure 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 neighbouring 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 Figure 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-

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domain to restore a functional allosteric mechanism. As 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 comprised by 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 to hSERT wt and chimera K24. This was also the case when determining the EC_{50} values of the chimeras for R-citalopram; [^{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 the introduction of hSERT identities at the four segments in gSERT to cause an enhancement of the allosteric mechanism compared to hSERT wt. Interestingly, 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 two-fold increase in the EC_{50} value compared to 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 to 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. Due to 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

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mM S-citalopram in the dissociation buffer has a deleterious effect upon the dissociation and 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 to 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 when comparing to 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 & 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 comprised of one subset of residues which also affect the affinity at the primary binding site, and one 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 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).

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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 at most caused a six-fold increase in the EC_{50} value compared to 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 e.g. Ala505 caused a six-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 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. Initially, 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}=208.2 μM (Table 5). Although a 40-fold attenuation, the affinity was not completely reversed to that of 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 out of four. Interestingly, 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 five-fold. However, no single residue is indispensable for a functional allosteric mechanism (Table 5).

In order 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)

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for a representative subset of the chimeras (Table 2, 4 and 5). The determined values were found to be similar when comparing the constructs tested. Finally, in order 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-terminal truncated hSERT construct; hSERT (1-613). From a biotinylation assay this construct is known to have highly diminished plasma membrane expression (M. B. Larsen; A. W. Fjordbak, 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 when comparing to 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;Wennogle and Meyerson, 1985;Plenge *et al.*, 1991;Chen *et al.*, 2005b). We tested the 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 μM-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 due to a high off-rate for the radioligand.

Molecular Pharmacology Fast Forward. Published on January 24, 2006 as DOI: 10.1124/mol.105.018507
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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 10-12 and confer a functional allosteric mechanism for S-citalopram; [^{125}I]-RTI-55 and S-citalopram; [^{3}H]-S-citalopram, as presented in this paper, 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 only affect the allosteric mechanism. The data presented in this paper 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 10-12 have indicated important structural-functional relationships in the region. Rudnick and coworkers (Keller *et al.*, 2004) performed cysteine scanning analysis of the extracellular loop (EL) between TMD 9 to 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 methanethio-sulfonates, or alternatively they are sterically inaccessible to the reagents. The neighbouring 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 in the vicinity of 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 was depending 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. As monoamine transporters are expected 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 reversely 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 coworkers thus observed that paroxetine allosterically increases the dissociation rate for imipramine (Plenge and Mellerup, 1985). 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).

Alternatively, 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;Hastrup *et al.*, 2003). Consequently, interface regions for monoamine transporters may differ compared to LeuTAa but involves most likely 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, thereby 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

Molecular Pharmacology Fast Forward. Published on January 24, 2006 as DOI: 10.1124/mol.105.018507
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induce a decrease in the dissociation rate as suggested by Plenge and coworkers (Plenge and Mellerup, 1997). Our finding that essential residues for the allosteric mechanism reside in a potential interface region distant from the primary binding site does not, however, 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 μM-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 in a similar manner can accumulate locally to levels where 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 when compared to hSERT, the EC_{50} of 5-HT; [^{3}H]-duloxetine was found to be in the low μM-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, despite that 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 have previously been suggested (Kilic and Rudnick, 2000).

ACKNOWLEDGEMENTS

We thank Bente Ladegaard for skillful technical assistance.

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Footnote: This study was supported by the Lundbeck Foundation.

Molecular Pharmacology Fast Forward. Published on January 24, 2006 as DOI: 10.1124/mol.105.018507
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FIGURE LEGEND

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 in "Experimental Procedures", the off-rate of bound radioligand K_{obs} is normalized with K_0 (off-rate in the absence of allosteric modulation) and plotted vs. log S-citalopram concentrations to obtain EC_{50} values. In the gSERT chimera K24, the TMDs 10-12 have been replaced by the corresponding hSERT fragment.

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 grey, 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 grey were mapped to the allosteric mechanism.

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

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 mut.) denote gSERT (D209A, V212I, I626F).

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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).

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 "Background". The normalization was performed in order to correct for different off-rates of the radioligands, and thus describe the relative contribution of 133 μM 5-HT upon the radioligands used in this assay. The dissociation buffer contained 200 μM fluoxetine in order to prevent re-association of the radioligand. B_0 indicates initial amount of bound radioligand and B_t indicates residual binding vs. time of dissociation.

Fig. 8. Structure of the bacterial leucine transporter (LeuTAa). The transporter is shown as a homodimer, seen from above. The TM domains 10-12 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).

Molecular Pharmacology Fast Forward. Published on January 24, 2006 as DOI: 10.1124/mol.105.018507
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Table 1

Characterization of the allosteric mechanism for gSERT-hSERT chimeras

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

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Table 2

Characterization of the allosteric mechanism for gSERT mutants

	EC_{50} (μM)	K_d (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

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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)

Molecular Pharmacology Fast Forward. Published on January 24, 2006 as DOI: 10.1124/mol.105.018507
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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} (μM)	K_d (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)	

Molecular Pharmacology Fast Forward. Published on January 24, 2006 as DOI: 10.1124/mol.105.018507
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Table 5

Characterization of the allosteric mechanism for hSERT mutants

	EC_{50} (μM)	K_d (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

Figure 1

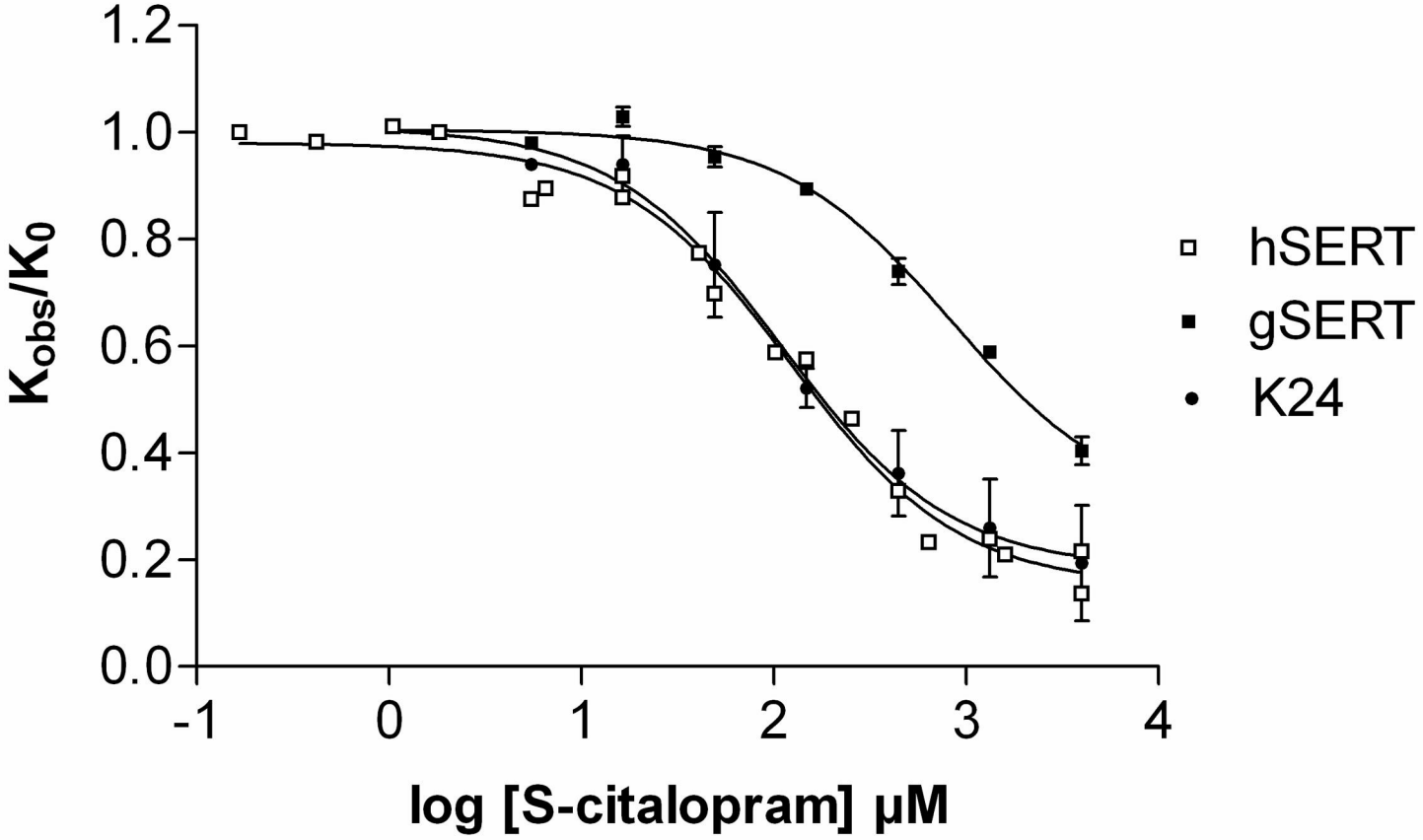

Figure 2

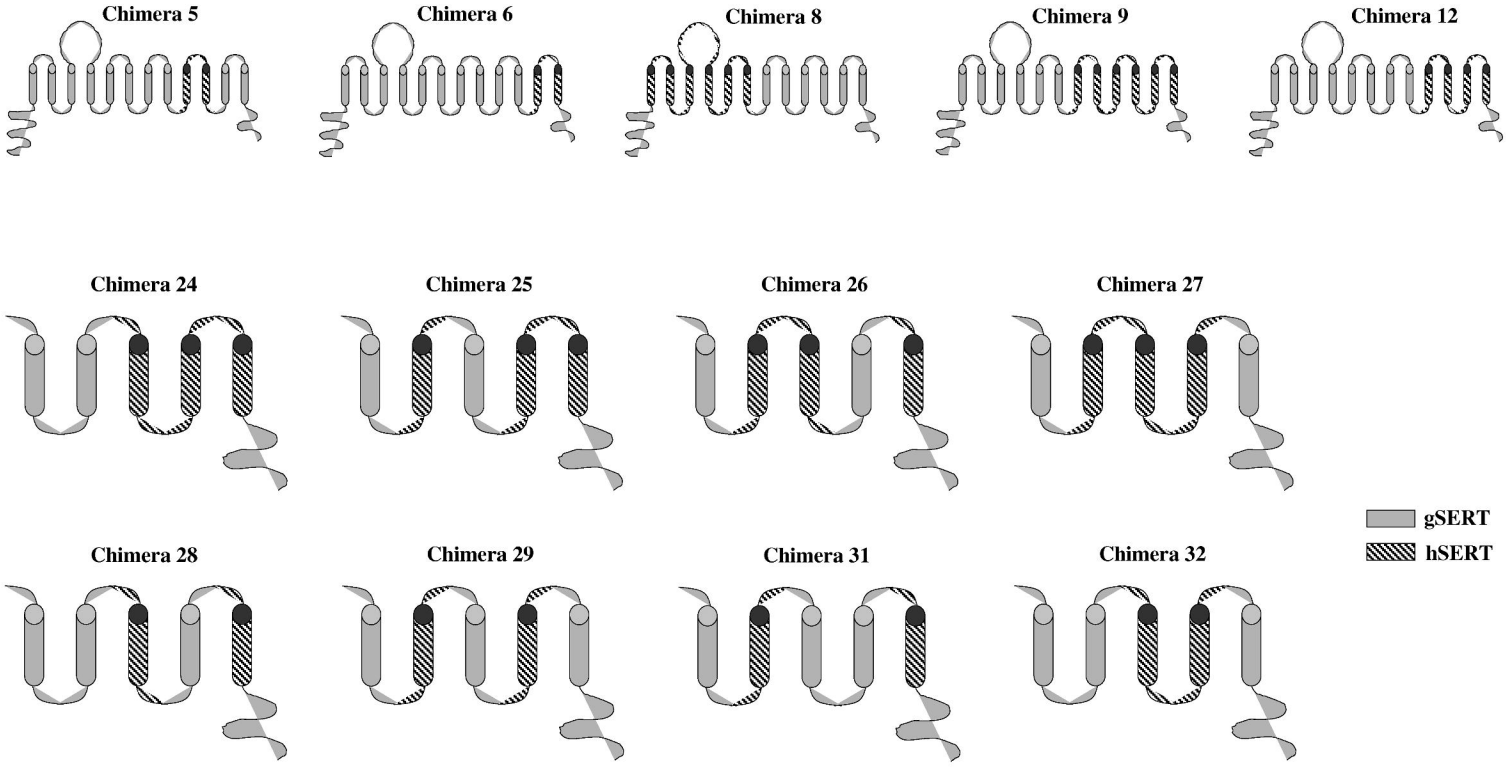

Figure 3


gSERT 474 FPHVWSKRREFFVLGLIIICFLGSLATLTFGGAYVVKLFEEYATGPAVLTVVFLEAVAV 552
hSERT 454 FPHVWAKRRERFVLAVVITCFFGSLVTLTFGGAYVVKLLEEYATGPAVLTVALIEAVAV 512
gSERT 553 AWFYGITQFCNDVKEMLGFAPGWYWRVCWVAISPIFLLFVTCSFLSNPPELRLFDYNYP 611
hSERT 513 SWFYGITQFCRDVKEMLGFSPGWFWRICWVAISPLFLLFIICSFLMSPPQLRLFQYNYP 571
gSERT 612 YWTTVVGYCIGTSSIICIPIYMAYRLIITPGTLKERILKSITPETATEIPFGDIRMNAV 670
hSERT 572 YWSIILGYCIGTSSFICIPTYIAYRLIITPGTFKERIIKSITPETPTEIPCGDIRLNAV 630

Figure 4

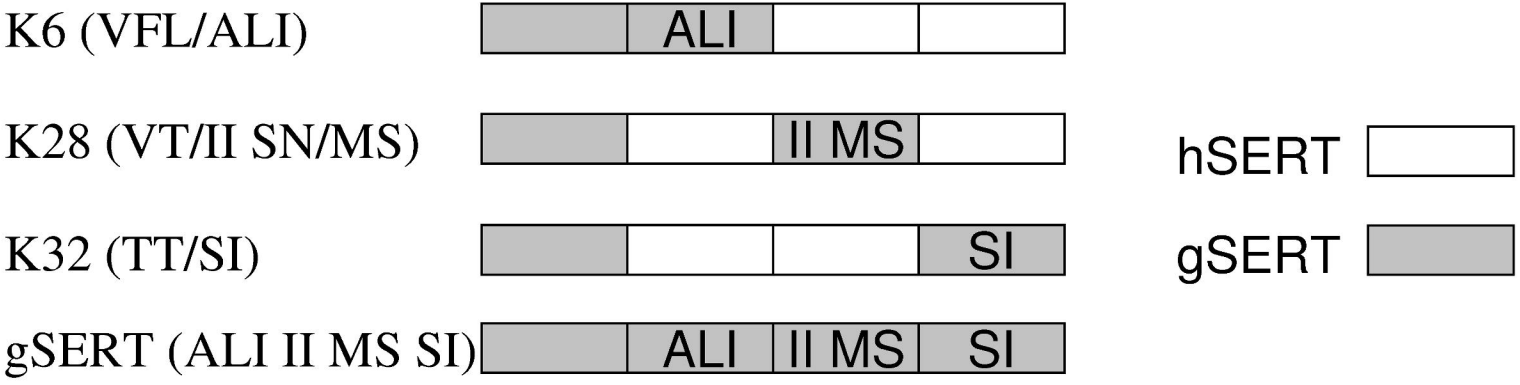
K6 (VFL/ALI)
ALI
K28 (VT/II SN/MS)
II MS
K32 (TT/SI)
SI
gSERT (ALI II MS SI)
ALI
II MS
SI
hSERT
gSERT

Figure 5

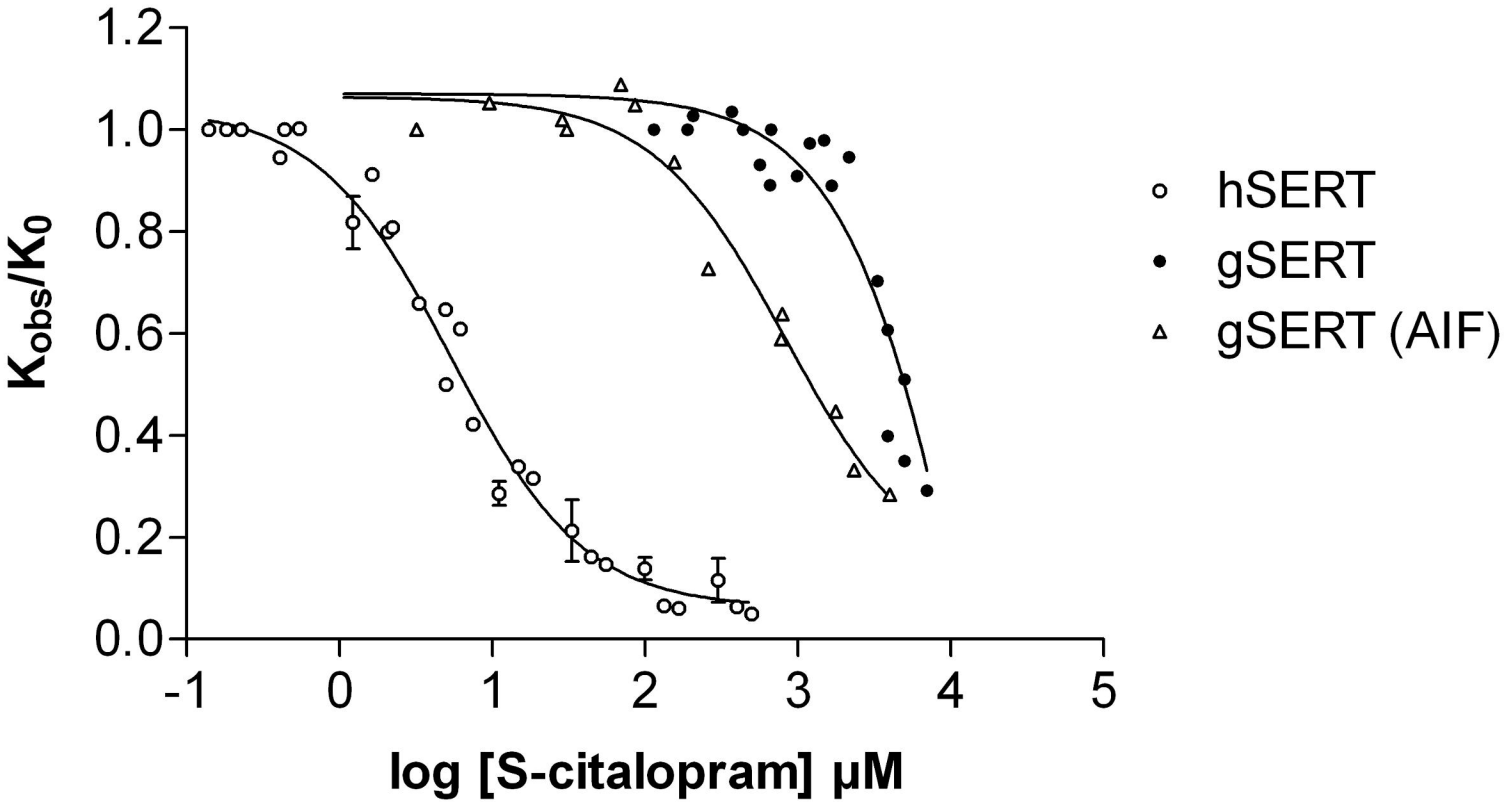

Figure 6

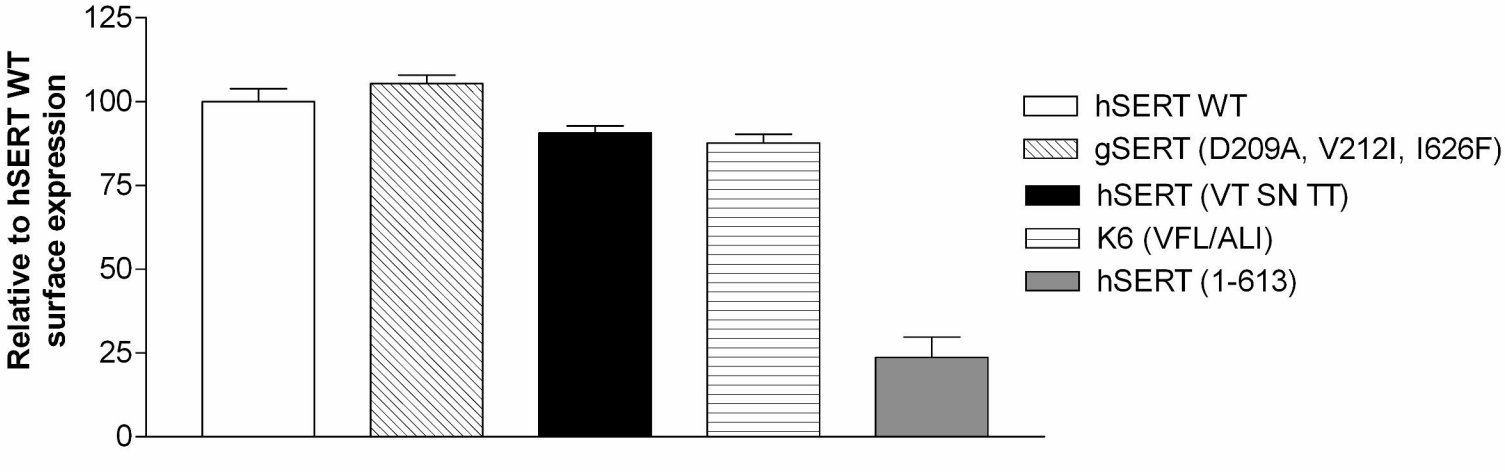
Relative to hSERT WT surface expression
125
100
75
50
25
0
hSERT WT
gSERT (D209A, V212I, I626F)
hSERT (VT SN TT)
K6 (VFL/ALI)
hSERT (1-613)

Figure 7

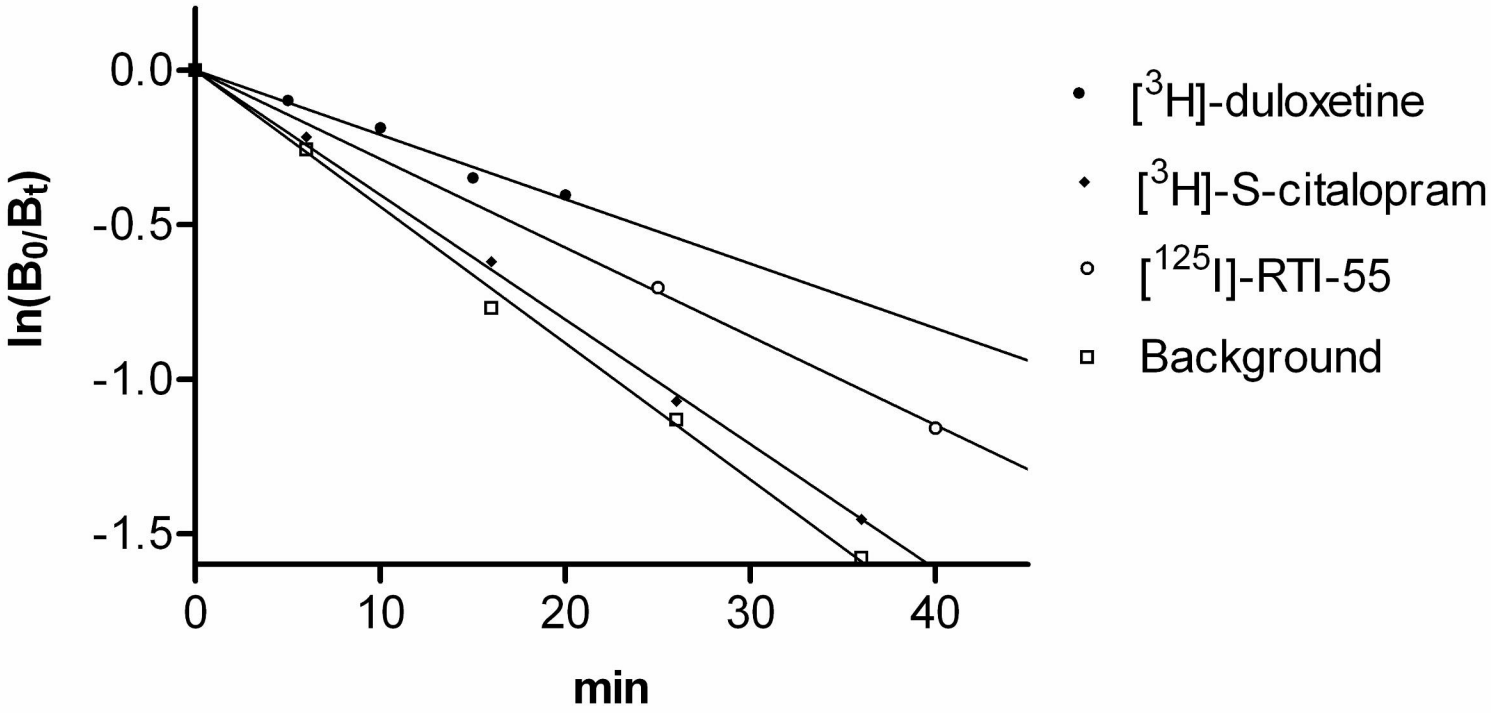

Figure 8