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Interactions of antidepressants with the serotonin transporter: a contemporary molecular analysis

David L. Roman, Crystal C. Walline, Gustavo J. Rodriguez, Eric L. Barker*

Department of Medicinal Chemistry and Molecular Pharmacology, School of Pharmacy and Pharmacal Sciences, Purdue University, 575 Stadium Mall Drive, West Lafayette, IN 47907, USA

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Abstract

One of the most prevalent disorders in present society is depression. The development of treatments for this disorder, beginning with the tricyclic antidepressants and leading to the development of selective serotonin reuptake inhibitors, has focused on compounds that block the function of the serotonin transporter (SERT). In this paper, we have performed Comparative Molecular Field Analysis (CoMFA) using data generated from rat brain synaptosomes and heterologous expression systems expressing rat SERT. Using these models, we have described the molecular requirements for the interactions of antidepressants with SERTs. In addition, molecular studies were performed using chimeric human/*Drosophila* SERTs and SERT point mutants. These studies focused on identifying regions or discrete amino acids on SERT that may be responsible for recognizing antidepressants.

Keywords: Antidepressant; CoMFA (Comparative Molecular Field Analysis); *Drosophila*; 5-HT (5-hydroxytryptamine serotonin); SERT (serotonin transporter)

1. Introduction

The neuronal tone of the serotonergic system plays an important role in the determination of mood and other physiological processes such as sleep, libido, and appetite (Barker and Blakely, 1995; Zahniser and Doolen, 2001). Disruption of the finely regulated concentration of serotonin (5-hydroxytryptamine, 5-HT) at the cellular level appears to play a role in a host of psychiatric disorders, including depression and states of prolonged heightened anxiety such as obsessive compulsive disorder, eating disorders, and social phobias (Barker and Blakely, 1995). The most recently available statistical data from the National Institute of Mental Health, based on the 1998 United States census, indicates that 9.5% of the U.S. population aged 18 or older is afflicted by a diagnosable affective disorder (Mental Disorders in America, 2001). Perhaps even more striking is the statistic that 13.3% of the U.S. population suffers from one of the many subtypes of anxiety disorders. Clearly, in the United States and worldwide, the broadly categorized mental disorders of depression and anxiety are of major societal concern.

Most therapeutic agents for these disorders act by increasing the level of synaptic 5-HT, thus prolonging the downstream signaling effects of released neurotransmitter. The spatial and temporal actions of 5-HT are primarily determined by the action of the serotonin transporter (SERT), which removes released 5-HT from the extracellular space. The human SERT (hSERT) is a 630 amino acid protein with 12 putative membrane spanning helices and intracellular amino and carboxy termini (Ramamoorthy et al., 1993; Rudnick and Clark, 1993; Clark, 1997). This transporter is a member of the Na^+- and Cl^--dependent GAT/NET [GABA (gamma amino butyric acid)/Norepinephrine transporter] superfamily of transport proteins. Therapeutically, the blockade of this uptake process has been used to increase synaptic serotonin and alleviate the symptoms of depression and some anxiety disorders.

Historically, some of the earliest therapeutic agents for treating depression were the tricyclic antidepressants such as imipramine and amitriptyline (Fig. 1). This class of antidepressants has mixed activity, functioning to inhibit both SERT and NET. This mixed profile of transport inhibition

* Corresponding author. Tel.: +1-765-494-9940; fax: +1-765-494-1414.
E-mail address: ericb@pharmacy.purdue.edu (E.L. Barker).

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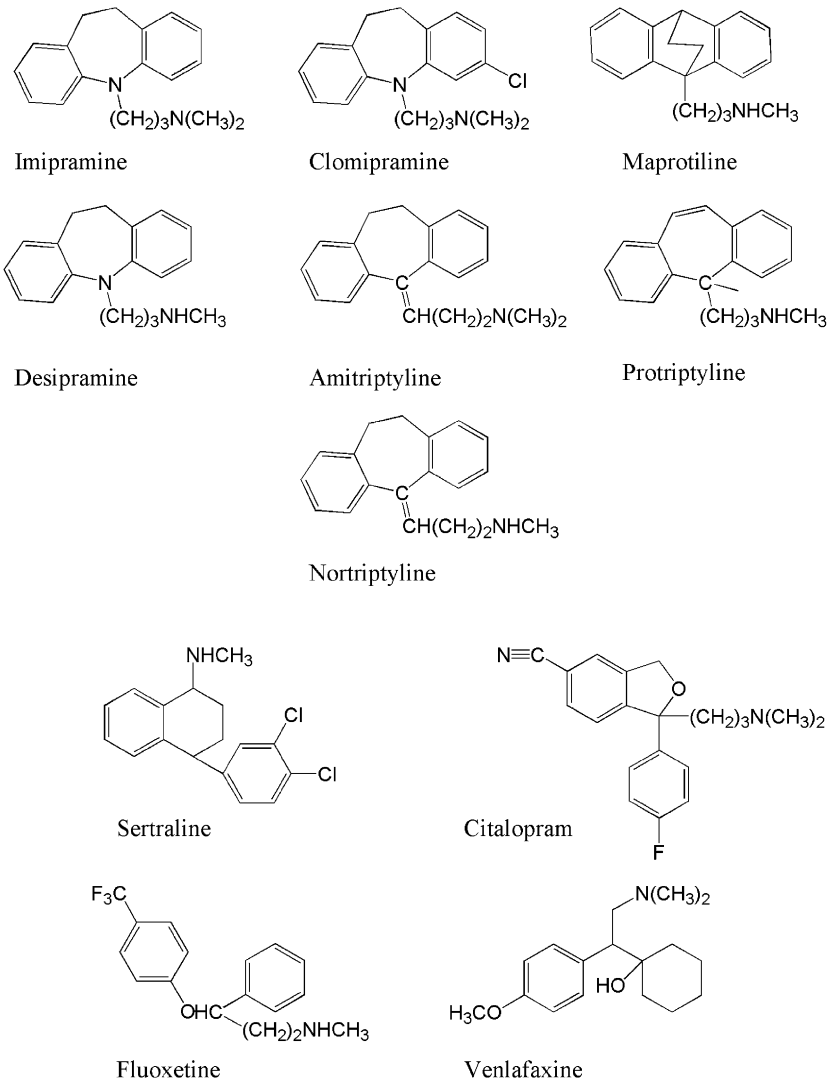

Fig. 1. Structures of the antidepressants used in this study. Tricyclic antidepressants include imipramine, clomipramine, maprotiline, desipramine, amitriptyline, protriptyline and nortriptyline. Selective serotonin reuptake inhibitors include sertraline, citalopram, and fluoxetine, while venlafaxine has mixed actions at both SERT and NET.

yields an effective treatment option for those suffering from anxiety and depression. However, the adverse effects of the tricyclic antidepressants can be quite pronounced and, thus, their use has diminished in recent years as newer, more selective agents became available. The synthesis and characterization of fluoxetine as a specific, selective serotonin reuptake inhibitor led to the development of a new class of compounds for the treatment of depression (Fig. 1) (Wong et al., 1974, 1975). The specificity of fluoxetine for SERT vs. NET is almost 150-fold in rat brain synaptosome preparations (Robertson et al., 1988) and even more pronounced when comparing cloned hSERT to cloned hNET (0.9 vs. 777 nM) (Owens et al., 1997). Selective serotonin reuptake inhibitors are, to date, the most potent and selective drugs available for inhibiting the action of SERT.

In addition to having selectivity for SERT as opposed to the other biogenic amine neurotransmitter transporters, selective serotonin reuptake inhibitors also show selectivity for species variants of SERT. The human and other cloned species variants of SERTs exhibit markedly different sensitivities to antidepressants, as well as to other drug classes that inhibit the reuptake of serotonin (Barker et al., 1994, 1998; Demchyshyn et al., 1994; Adkins et al., 2001; Rodriguez et al., 2003). These species-specific properties have been exploited in molecular studies seeking to identify domains of SERT possessing determinants for antidepressant recognition (Barker and Blakely, 1996; Barker et al., 1998).

In this paper, we will describe the structure–activity relationships of antidepressants for inhibiting the uptake of serotonin in rat brain synaptosome preparations and cloned rat SERT (rSERT). The use of molecular modeling and Comparative Molecular Field Analysis (CoMFA) provides insight into the structural features of these drugs that contribute to the specificity and potency for their respective

targets. In particular, the role of the common amine group and free aromatic substituents will be a focus of this analysis. In addition to an exploration of antidepressant structure–activity relationships, we investigated potential mechanisms for the recognition of antidepressants by SERT at the molecular level. hSERT and *Drosophila* SERT (dSERT) were used to compare species variant sensitivity to a subset of antidepressants. These studies, coupled with experiments using a chimeric human/*Drosophila* SERT and SERT point mutants, provide insight into the location of putative sites of interaction for antidepressants with the transporter.

2. Materials and methods

2.1. Construction of the $H^{1-281}D^{282-476}H^{477-638}$ chimera

This chimera was generated as previously described (Rodriguez et al., 2003). Briefly, it is a full-length 638 amino acid transporter constructed from amino acids 1–281 and 477–638 with identity from hSERT and residues 282–476 with identity from dSERT. The resulting chimera was cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) and stably transfected into HEK-293 (Human Embryonic Kidney) cells using Lipofectamine 2000 (Invitrogen) as per manufacturer's protocol.

2.2. Construction of S375A and P418S hSERT point mutants

The hSERT mutants S375A and P418S were generated using the Quickchange® mutagenesis kit (Stratagene, La Jolla, CA) as per manufacturer's provided protocols. The oligonucleotides used for the mutations are as follows: S375A-Sense: 5′-GGTGAACTGCATGACAAG-CTTCGTTGCGGGATTTGTC-3′, S375A-Antisense: 5′-GACAAATCCCGCAACGAAGCTTGTCATGCAGTT-CACC-3′, P418S-Sense: 5′-GCGATAGCCAACATGT-CAGCGTCAACTTTCTTTGCC-3′, P418S-Antisense: 5′-GGCAAAGAAAGTTGACGCTGACATGTTGGC-TATCGC-3′, Bacterial colonies screened to be positive for the mutation were sequenced and subcloned into pBluescript parental vector before being maxiprepped (Qiagen, Valencia, CA) and used in uptake experiments.

2.3. [^{3}H]5-HT uptake assays in HEK-293 cells stably expressing SERTs

HEK-293 cells stably expressing either the hSERT or dSERT were generous gifts from Dr. Randy D. Blakely, Vanderbilt University. All cells were maintained in Dulbecco's modified Eagle's medium with 10% dialyzed fetal bovine serum supplemented with 2 mM glutamine, 1% penicillin/streptomycin (10,000 U/ml), and 600 mg/l geneticin (G-418 sulfate) in a humidified 5% CO_2 incubator at 37 °C.

Uptake assays were performed on cell suspensions in 96-well microtube racks. Confluent cells in 150-mm tissue culture dishes were washed with phosphate-buffered saline (PBS), scraped, and resuspended in Krebs–Ringer–HEPES (KRH) buffer supplemented with D-glucose (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 10 mM HEPES, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.8 g/l D-glucose, pH 7.4). Total protein concentration was determined using the Bradford assay (Bradford, 1976). Cell suspensions were diluted in KRH to yield a concentration of 0.25 mg/ml total protein. Four hundred microliters (100 μg) of the cell suspension was added to each tube in a 96-well microtube rack. Drugs were diluted serially in KRH buffer, and 50-μl aliquots of increasing concentrations of drug were added to triplicate microtubes. Cells were preincubated with drug at 37 °C for 10 min. [^{3}H]5-HT (~ 110 Ci/mmol) was diluted to yield a final assay concentration of 20 nM in KRH buffer supplemented with 100 μM L-ascorbic acid and 100 μM pargyline. Fifty microliters of the [^{3}H]5-HT solution was added to each microtube, and uptake was allowed to proceed at 37 °C for 10 min. Transport was terminated via vacuum filtration onto 96-well GF/B filter plates (presoaked with 0.3% polyethyleneimine) using a Packard Filtermate 96-well harvester (Meriden, CT). Nonspecific uptake was determined using 10 μM cocaine. The filter plates were dried at ambient temperature overnight. The next day, scintillation cocktail (Microscint O, Packard Instrument) was added prior to counting in a Packard TopCount NXT to determine accumulated [^{3}H]5-HT. IC_{50} values were determined using the nonlinear regression routine in GraphPad Prism v.3.0 (GraphPad Software, San Diego, CA), and K_i values were calculated using the Cheng–Prusoff equation (Cheng and Prusoff, 1973). Experiments were performed in triplicate and repeated in at least three separate assays.

2.4. [^{3}H]5-HT uptake assays in HeLa cells transiently expressing hSERT point mutants

HeLa cells were transfected using the vaccinia virus T7 (VVT7) method as previously described (Fuerst et al., 1986; Blakely et al., 1991b; Barker et al., 1994). Briefly, HeLa cells were plated at a density of 10^5 cells per well in 24-well tissue culture plates 18 h prior to transfection. At the time of transfection, cells were washed with PBS, and then VVT7 (10 pfu/cell) diluted in 100 μl OptiMEM®/0.1% β-mercaptoethanol was added to each well. Infection proceeded for 30 min at 37 °C. Then, 300 ng of hSERT, dSERT, S375A, or P418S cDNA (premixed with Lipofectin transfection reagent as per manufacturer's protocol) was added to each well. Transfection with the vector pBluescript was used to define nonspecific uptake. Transfected cells were assayed 6–8 h post-transfection.

At the time of assay, transfection media was aspirated and cells were washed with 0.5 ml KRH buffer supplemented with 1.8 g/l D-glucose (KRH/glucose). Four hundred microliters of KRH/glucose was then added to each well.

Fifty microliters of inhibitor (or buffer to determine total or nonspecific uptake) was then added to each well and cells were incubated for 10 min in a 37 °C incubator. [^{3}H]5-HT (~ 110 Ci/mmol) was diluted to yield a final assay concentration of 20 nM in KRH buffer supplemented with 100 μM L-ascorbic acid and 100 μM pargyline. 50 μl of the [^{3}H]5-HT solution was added to each well, and uptake was allowed to proceed at 37 °C for 10 min. Following incubation, cells were washed three times with ice-cold KRH to terminate uptake. Scintillation cocktail was added (Microscint 20, Packard Instrument), plates were shaken overnight, and accumulated tritium was determined using a Packard Topcount NXT.

2.5. Molecular modeling and CoMFA analysis of antidepressant interactions with SERT

Computational molecular modeling studies were performed using the SYBYL v.6.8 software package (Tripos, St. Louis, MO) on a Silicon Graphics O2 workstation (Mountain View, CA) running IRIX v.6.5. The antidepressant analogs were modeled using library fragments in SYBYL. Atom types were automatically assigned by SYBYL and then manually checked. The compounds were subjected to a grid search (0–360°, 10° increments) to find the lowest energy conformation for all rotatable bonds where the possibility of unfavorable torsions existed. Compounds in this conformation were then minimized using the conjugate gradient minimization method with no initial simplex minimization, a gradient value of 0.05, and 10,000 maximum iterations. Each set of compounds was aligned, compiled into a molecular spreadsheet, and pK_i values inserted as explicit data. CoMFA data was generated using the Tripos Standard CoMFA field class with distance dielectric, no smoothing, and a steric and electrostatic cutoff of 30.0 kcal/mol. Cross-validated partial least squares (PLS) analysis was validated using the leave-one-out option. The number of optimal components was determined and a CoMFA map generated.

2.5.1. CoMFA analysis of imipramine analogs assayed using rat brain synaptosomes

Data for this analysis were taken from a previously published report by Horn and Trace (1974). For this analysis, 12 compounds based on the core structure of imipramine were modeled in SYBYL using library fragments and minimized as indicated above. The molecules were aligned using their minimized structures and the common tricyclic fused ring substructure. Following alignment and subsequent PLS analysis, three components were found to be suitable, with a $q^2 = 0.530$.

2.5.2. CoMFA analyses of antidepressants assayed using heterologously expressed rat SERT

Data for these analyses were taken from a previously published report by Barker et al. (1994). For the first analysis, seven compounds (imipramine, clomipramine, desipramine, amitriptyline, protriptyline, nortriptyline, and maprotiline) based on the core structure of imipramine were modeled in SYBYL using library fragments and minimized as indicated above. The molecules were aligned using their minimized structures, the two flanking aromatic rings of the fused system, and the amine of the alkylamine side chain. Following alignment and PLS analysis, two components were utilized, with a $q^2 = 0.388$. For the second analysis, 11 antidepressants (imipramine, clomipramine, desipramine, amitriptyline, protriptyline, nortriptyline, paroxetine, citalopram, maprotiline, fluoxetine, and sertraline) with much more diverse structures were modeled in SYBYL using library fragments and minimized as indicated above. The molecules were aligned using their minimized structures and the two flanking aromatic rings of the fused system. For those compounds in the set lacking the fused ring system, the equivalent positions (either aromatic or cyclohexyl) were locked in a distance constraint to match the rest of the compound set as closely as possible, while still maintaining their local energy minima. Following alignment and PLS analysis, three components were utilized, with a $q^2 = 0.551$.

2.5.3. CoMFA analysis of fluoxetine and related compounds assayed using rat brain synaptosome preparations

Data for this analysis were taken from a previously published report by Robertson et al. (1988). For this analysis, 10 compounds based on the core structure of fluoxetine were modeled using SYBYL library fragments and minimized. Molecules were aligned using the amine functional group and the distal (phenoxy) aromatic ring on fluoxetine. Following alignment and PLS analysis, three components were determined to be optimal, with a $q^2 = 0.597$.

2.6. Materials

Dulbecco's modified Eagle's medium, pargyline, citalopram, and L-ascorbic acid were purchased from Sigma (St. Louis, MO). Dialyzed fetal bovine serum was purchased from Hyclone (Logan, UT). Trypsin, L-glutamine, penicillin/streptomycin, and geneticin were from Life Technologies (Grand Island, NY). Bradford Protein Assay Dye Concentrate was from Bio-Rad Laboratories (Hercules, CA). Cell culture flasks and 150-mm dishes were from Falcon/B-D Labware (Mountain View, CA). 96-well microtube racks and tubes were purchased from Marsh Bio Products (Rochester, NY). [^{3}H]5-HT trifluoroacetate (~ 110 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). Cocaine was purchased from Research Biochemical International (Natick, MA). Venlafaxine HCl was a gift from Wyeth (Monmouth Junction, NJ), and sertraline was a gift from Pfizer, (Groton, CT). All other chemicals were purchased from Sigma or Fisher Scientific (Pittsburg, PA) and were of the highest grade available. Integrated DNA

Technologies (Coralville, IA) synthesized the mutagenic oligonucleotides, and dye-termination sequencing was performed by the University of Michigan Medical Center core facility (Ann Arbor, MI).

3. Results and discussion

3.1. Three-dimensional quantitative structure–activity relationships of tricyclic imipramine derivatives at rat brain synaptosomes

Studies conducted by Horn and Trace in the 1970s led to much of what we now know about the structural features of drugs that act to inhibit 5-HT uptake (Horn, 1973; Horn et al., 1973; Horn and Trace, 1974). We have performed a contemporary re-analysis of this early structure–activity relationship data. Due to observed species differences in the way SERTs recognize many of these drugs, we have focused our comparisons based on rSERT data, from early work in synaptosomes to more recent studies using cloned rSERT in a heterologous expression system. Using the data of Horn and Trace (1974), the K_i values for 12 tricyclic antidepressants were used to generate a Comparative Molecular Field Analysis. The CoMFA map provides a powerful, graphical model of determinants of high-affinity interactions of drugs with SERT. In the absence of any definitive structural information for SERT, one of the few methods in which to examine quantitatively ligand-binding properties is using CoMFA. CoMFA analyses are performed in silico using Silicon Graphics workstations and the SYBYL software package (Tripos, St. Louis, MO). In the analysis, compounds with a similar substructure, such as the fused tricyclic rings of the imipramine derivatives, are sketched and their minimum energy conformations determined. Once the compounds in the training set are compiled into a database, the group is stacked or aligned on top of each other in a virtual energy grid. The aligned molecules are then tabulated with their respective K_i values, and the CoMFA subroutine is executed. The CoMFA program maps regions of favorable and unfavorable electrostatic and steric interactions as colored geometric fields onto the energy grid that the molecules have been aligned. Through thorough inspection, it is possible to analyze each atom's position in a drug molecule, and determine if that position plays a role in dictating higher or lower affinity based upon the steric and electrostatic properties of substitutions at that position. Thus, if given a large enough set of compounds with a similar core structure, one can predict how subtle changes in the molecule's structure will affect affinity.

In these original studies, rat brain synaptosomes were prepared and subsequently treated with inhibitors in the presence of radiolabeled 5-HT (Horn and Trace, 1974). These data were tabulated and published with an analysis of the structural features that provided higher or lower affinity for inhibiting the transport process. Our CoMFA map (Fig. 2A) for tricyclic antidepressants inhibiting the uptake of 5-HT into synaptosomes (Horn and Trace, 1974) provides a graphical representation of how these compounds may be interacting with SERT. Several distinct regions on the map provide information about molecular features favored for higher affinity interactions. The green region of the figure shows an area of the molecule where steric bulk is somewhat favored for higher potency. Imipramine, the parent compound, exhibits a K_i value of approximately 285 nM, whereas 3-chloroimipramine, which places a chlorine substitution in the 3-position (as indicated in Fig. 2A), exhibits a K_i value of 56 nM. The blue region located near the alkylamine chain indicates that hydrogen bond donors and slight positive charge are accepted at this position, but there is a steric limitation, as shown in yellow. This yellow area correlates to Horn and Trace's (1974) observation that α- or β-substitution on the side chain reduced potency. The red regions indicate areas where negative charge and hydrogen bond acceptors are favored, or positive charge and hydrogen bond donors are disfavored. The character of the amine, as either secondary or tertiary, affects the favorability of the drug's interaction with SERT. The secondary amine, as a hydrogen bond donor, is less favored as compared to the tertiary amine, yielding the red fields. The dual red regions indicate the location of the substitutions on the amine. Since the amine is tethered to the fused tricyclic system through the variable length carbon chain, the relative locations of the red field on the map vary as a function of the chain length, hence, two red fields are observed. As observed in the original publication, the potency of this group of compounds is greatly and adversely affected by varying the carbon chain length that, in turn, alters the position of the amine (Horn and Trace, 1974). For instance, imipramine, a three-carbon chain length species, exhibits a K_i value approximately 10-fold lower than either the two- or four-carbon chain length derivative (285 vs. 2450 and 1980 nM, respectively). Changing the length of the chain alters the distance between the fused ring system and the amine, and this distance change results in the dual red fields toward the bottom of the CoMFA map.

3.2. Three-dimensional quantitative structure activity relationships of tricyclic antidepressants at the cloned rSERT

Since the cloning of several SERT species variants in the 1990s (Blakely et al., 1991a; Hoffman et al., 1991; Ramamoorthy et al., 1993; Corey et al., 1994; Demchyshyn et al., 1994; Padbury et al., 1997), more refined studies have been undertaken in order to understand SERT function, and more specifically, how ligands interact with SERT. Our next analysis was based upon data from a study by Barker et al. (1994), focused on differences between SERT species variants in recognizing antidepressants and other ligands. An initial CoMFA map (Fig. 2B) was generated using a small subset of K_i values for tricyclic antidepressants at the cloned rSERT (Barker et al., 1994). Whereas the small set of seven

compounds (imipramine, clomipramine, desipramine, amitriptyline, protriptyline, nortriptyline, and maprotiline) does not provide for an exhaustive CoMFA analysis, it does provide an opportunity to compare directly the data from rat synaptosomes (Fig. 2A) with similar compounds at the cloned rSERT. The CoMFA map generated from the data reported by Barker et al. (1994) (Fig. 2B) showed remarkable similarity to the map constructed from the rat brain synaptosome data reported by Horn and Trace (1974). Specifically, the two red fields near the bottom of the map in Fig. 2B, indicating where negative charge and hydrogen bond acceptors are favored, match closely to the two red fields in Fig. 2A (note that the bottom blue field in Fig. 2B overlaps, to a large extent, a red field at approximately the same position). In addition, the blue region near the side chain amine correlates between the two maps, again indicating parallels between the rat synaptosome and cloned rSERT data. The minimal green field in the cloned rSERT data (Fig. 2B) correlates to the larger green field surrounding the 3-position of the aromatic ring from the synaptosome data (Fig. 2A), indicating the favorability of steric bulk at this position. One notable feature that appears in the CoMFA map for the cloned rSERT data (Fig. 2B), but is absent in the rat synaptosome data (Fig. 2A), is the presence of a red field near the 3- and 4-position of the aromatic ring. This red field indicates that negative charge (such as from the chlorine substitution on clomipramine) is favorable for higher affinity recognition of the drug by SERT.

A CoMFA map (Fig. 2C) was generated using cloned rSERT K_i values for the following compounds: imipramine, clomipramine, desipramine, amitriptyline, protriptyline, nortriptyline, paroxetine, citalopram, maprotiline, fluoxetine, and sertraline. We fully recognize that these compounds are not true derivatives of one another, nor do they align in a perfect fashion when performing molecular modeling. How-

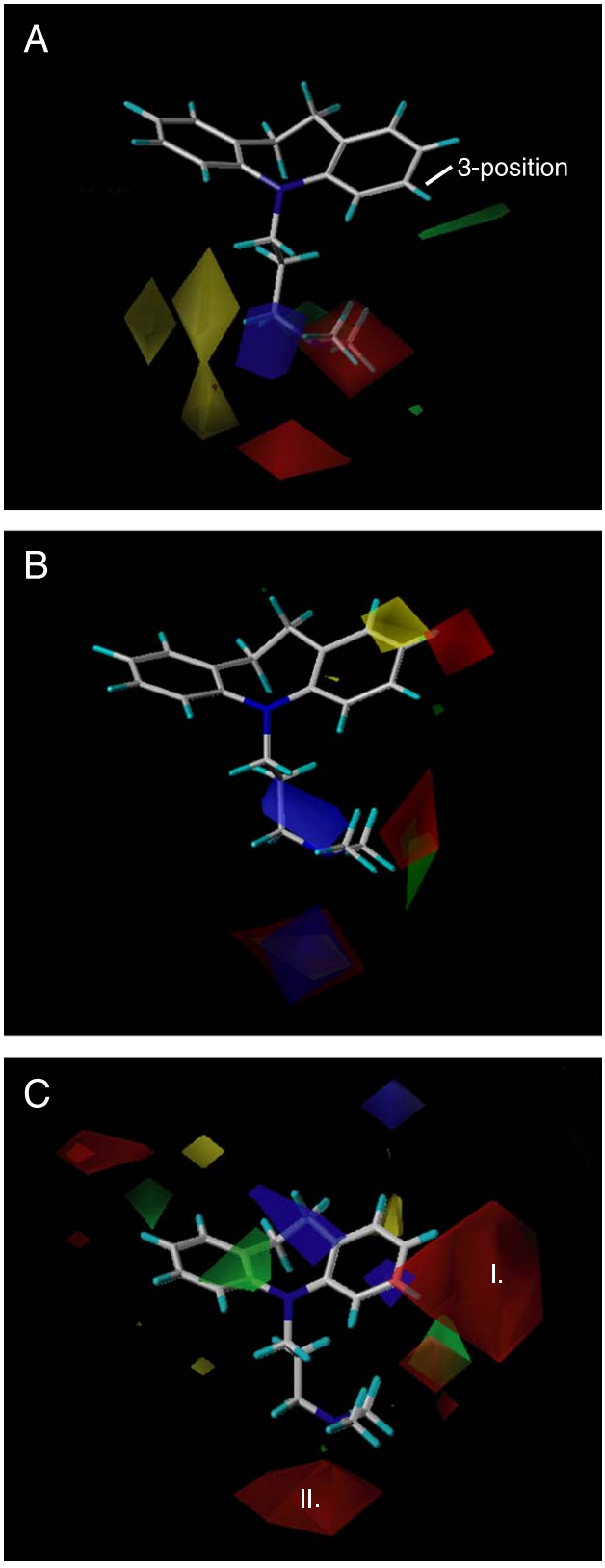

Fig. 2. Comparative Molecular Field Analysis (CoMFA) maps of antidepressants modeled using data obtained from rat brain synaptosomes (A) and cloned rSERT (B and C). Imipramine is modeled within each field for directional orientation. Panel A depicts the CoMFA map generated with the data from 12 imipramine derivatives assayed at rat brain synaptosomes as reported by Horn and Trace (1974). Panel B depicts the CoMFA map generated with the data from seven tricyclic antidepressants (imipramine, clomipramine, desipramine, amitriptyline, protriptyline, nortriptyline, and maprotiline) assayed at heterologously expressed rSERT as reported by Barker et al. (1994). Panel C depicts the CoMFA map generated with a larger group of compounds from the report by Barker et al. (1994), including the following 11 compounds: imipramine, clomipramine, desipramine, amitriptyline, protriptyline, nortriptyline, paroxetine, citalopram, maprotiline, fluoxetine, and sertraline. The compounds for the map in panel C include selective serotonin reuptake inhibitors and as in panel B, the drugs were assayed at cloned rSERT in a heterologous expression system. For each panel, colored fields represent characteristics of the ligand that favorably or unfavorably affect high-affinity interactions. Steric contours are shown in green and yellow, whereas electrostatic contours are shown in blue and red. Green fields indicate molecular regions where steric bulk is favored for high-affinity interactions, whereas regions indicated by yellow are areas in which steric bulk is disfavored for high-affinity interactions. Blue fields indicate regions of the molecule where more positive charge and hydrogen bond donors are favored, or negative charge and hydrogen bond acceptors are disfavored for high-affinity interactions. Red fields indicate regions where negatively charged substitutents and hydrogen bond acceptors are favored, or positive charge and hydrogen bond donors are disfavored.

ever, for the purpose of understanding how antidepressants may be interacting with SERT globally, CoMFA methods can provide us with important structural information about how SERT may recognize these drugs. Due to the variability in structure and the imperfect alignment, the CoMFA map in Fig. 2C contains outlying points of color indicating smaller contributions by one, or a small subset, of the molecules assayed. These regions may provide some insight into how an individual molecule is recognized, but we will focus on the regions providing the major contributions to high-affinity interactions with rSERT, as these should provide evidence for the most crucial determinants of recognition.

The two red regions on the CoMFA map have been labeled "I" and "II" for clarity. Both of these regions are areas where negative charge and hydrogen bond acceptors are favored, or positive charge and hydrogen bond donors are disfavored. Red region I is focused around the 3- and 4-position of the aromatic ring. This area of favorable negative charge is again demonstrated by comparing the potency of imipramine with clomipramine (3-chloroimipramine) where the K_i values are 46 and 3.9 nM, respectively. The addition of the electronegative chlorine promotes high-affinity interaction with SERT. Also, directly below this red region is a small green area homologous to the green field observed in Fig. 2A surrounding the 3-position of the aromatic ring suggesting steric bulk is favorable for high-affinity interaction. The red region II indicates negative charge and hydrogen bond acceptors are favored. This result could indicate that the side chain amine provides higher affinity interaction when it is in a state to accept a hydrogen bond. In contrast to the CoMFA map of imipramine analogs alone (Fig. 2A), these latter data do not include compounds with variable length side chains or side chain substitutions (Fig. 2C), and this provides explanation for the lack of yellow areas around the alkylamine side chain.

Overall, this analysis has shown that some of the seminal data for SERT and antidepressant structure–activity relationships developed in rat brain synaptosomes correlates closely with later studies utilizing the cloned rSERT. In consideration of the species differences observed in SERT species variants, we also performed a CoMFA analysis using the same compounds used to generate Fig. 2C and data from the hSERT (Barker et al., 1994). Interestingly, the CoMFA maps for the hSERT and rSERT data appeared to be almost identical (data not shown). The similarity of the CoMFA maps is not surprising, because, although the absolute potencies of the compounds vary between hSERT and rSERT, the rank order of potency for the compounds does not change.

3.3. Evaluation of fluoxetine derivatives at rat brain synaptosomes

Many commercial successes in the development of drugs to treat depression came with the marketing of the selective serotonin reuptake inhibitors. Released in 1988, Prozac® (fluoxetine hydrochloride, Eli Lilly and Co.) became the most prescribed drug in the United States market in only two years (Stokes and Holtz, 1997). The development of fluoxetine by researchers at Eli Lilly research laboratories resulted in the selective serotonin reuptake inhibitor class of antidepressants (Wong et al., 1974, 1975; Robertson et al., 1988).

Our next analysis used data from a previously published report by Robertson et al. (1988). This report focused on the structure of fluoxetine and the in vitro inhibition of serotonin uptake in crude rat brain synaptosomes. We used this data and developed a contemporary re-analysis using modern computational methods. The CoMFA analyses (Fig. 3A and B) are presented to give an overall picture of the features of selective serotonin reuptake inhibitors that make them both very potent and very specific for SERTs. Fig. 3A is a CoMFA map with the molecule fluoxetine displayed for orientation. Fig. 3B depicts the same CoMFA fields, but instead displays nisoxetine, a norepinephrine reuptake inhibitor, which is based on the fluoxetine core structure. A prominent blue field, indicating a region where positive charge and hydrogen bond donors are favored, is common to both CoMFA maps (Fig. 3A and B). The blue region is localized to the amine on the selective serotonin reuptake inhibitors, indicating that this molecular feature is vital for high-affinity recognition by SERT. The most striking components on the two maps are the green and yellow regions that flank the aromatic ring towards the bottom of the figure. The green fields indicate regions where steric bulk is favorable for high-affinity interaction with SERT, and yellow fields indicate regions where steric bulk is disfavored. When modeled with fluoxetine (Fig. 3A), the aromatic ring lies in the same plane as the green regions, indicating favorable interactions. However, when nisoxetine's interactions with SERT were modeled (Fig. 3B), the plane of the aromatic ring lies within the yellow regions, indicating the unfavorability of this geometry. The presence of the methyl ether on the aromatic ring of nisoxetine (and tomoxetine as described below) effectively causes the aromatic ring to rotate into a less favorable conformation (demonstrated by K_i values of 70 vs. 2000 nM, respectively). Interestingly, when examining norepinephrine transport inhibition, the potencies are virtually reversed (10 nM at NET vs. >10,000 nM at SERT), indicating a mechanism for specificity of serotonin transporters for fluoxetine and norepinephrine transporters for nisoxetine based upon the orientation of the aromatic ring. The orientation of the aromatic ring has also been examined in a previous three-dimensional quantitative structure–activity relationship study by Wellsow et al. (2002), and our model correlates closely with respect to the regions of favored and disfavored steric bulk surrounding the aromatic ring.

Another compound that induces this conformational change in the highly favored fluoxetine geometry to a lower affinity geometry is tomoxetine, which places a methyl substituent in the same position as the methyl ether on

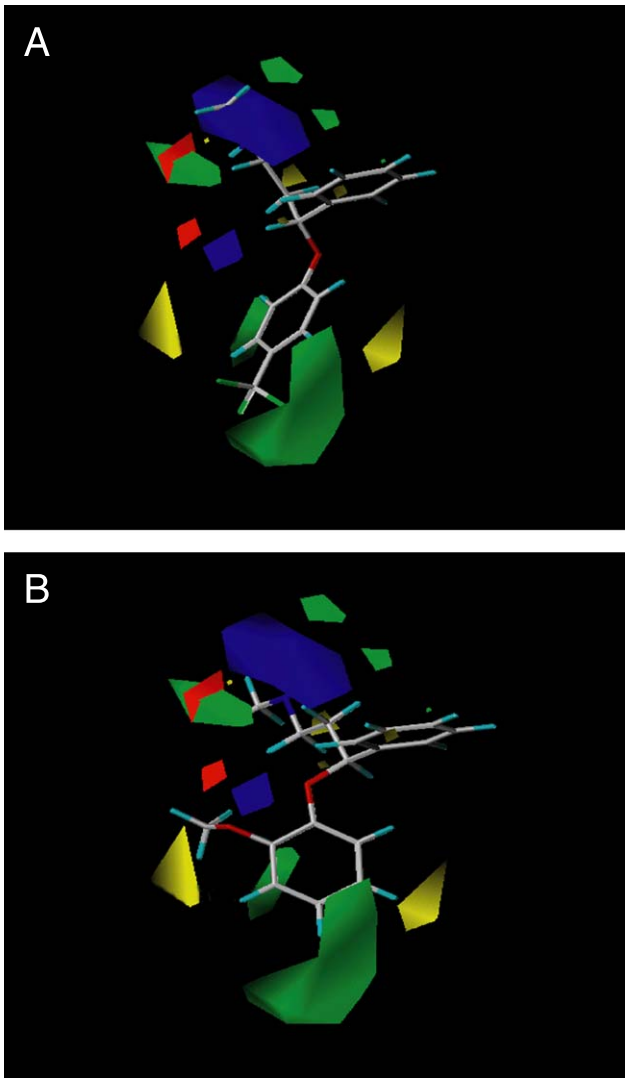

Fig. 3. Comparative Molecular Field Analysis (CoMFA) of 12 selective serotonin reuptake inhibitors modeled using data generated at rat brain synaptosomes by Robertson et al. (1988). Fluoxetine is depicted in panel A and nisoxetine in panel B, for orientation of the map. For each panel, colored fields represent characteristics of the ligand that favorably or unfavorably affect high-affinity interactions. Steric contours are shown in green and yellow, whereas electrostatic contours are shown in blue and red. Green fields indicate molecular regions where steric bulk is favored for high-affinity binding, whereas regions indicated by yellow are areas in which steric bulk is disfavored for high-affinity interactions. Blue fields indicate regions of the molecule where more positive charge and hydrogen bond donors are favored, and negative charge and hydrogen bond acceptors are disfavored for high-affinity interactions. Red fields indicate regions where negatively charged substitutents and hydrogen bond acceptors are favored, or positive charge and hydrogen bond donors are disfavored.

nisoxetine. Again, the geometry of the aromatic ring is shifted from the favored plane to the disfavored plane (with respect to SERT potency). The K_i values for tomoxetine at SERT and NET are 1500 and 4 nM, respectively. These subtle changes in the substitutions on the aromatic ring of fluoxetine induce dramatic changes in pharmacology. Both the potency of the compound, as well as the biogenic amine neurotransmitter transporter specificity, switch in an almost orthogonal manner in accordance with the orientation of the free aromatic ring. The strong dependence on the conformation of the aromatic ring for potent interactions with SERT leads to the question, what regions or discrete amino acid on SERT interact with this aromatic ring? For example, some features that might be identified include aromatic amino acids on SERT that can provide favorable pi–pi stacking interactions with the aromatic ring of the antidepressants. A more detailed discussion of potential molecular determinants of antidepressant recognition by SERT will be discussed below.

3.4. Regions of SERT containing determinants of antidepressant recognition

Previous studies by our laboratory identified transmembrane domains V–IX as being important for both substrate (Rodriguez et al., 2003) and psychostimulant (Roman et al., unpublished observation) recognition. The key to these studies was the construction of a chimeric SERT that contained amino acids 1–281 and 477–638 of hSERT identity and amino acids 282–476 of dSERT identity (Fig. 4A). To investigate potential domains of SERT involved with antidepressant recognition, we performed a series of [^{3}H]5-HT uptake inhibition experiments using this chimera and a set of antidepressants. As discussed earlier, the antidepressants have been shown to have species selectivity (Barker et al., 1994, 1998; Demchyshyn et al., 1994), and our experiments confirmed this phenomena (Table 1). Consistent with previous results, each antidepressant was approximately 100-fold less potent at dSERT as compared to hSERT. A critical examination of the chimeric SERT data revealed a shift in potency for each antidepressant towards dSERT-like potency, but in many cases this shift was attenuated. For example, the most potent compound at hSERT, sertraline, exhibited a K_i value of 3.6 nM at hSERT, 390 nM at dSERT, and 150 nM at the $H^{1-281}D^{282-476}H^{477-638}$ chimera. Similar comparisons can be drawn for each of the other compounds except for desipramine and venlafaxine. These two compounds are relatively non-potent at dSERT and the $H^{1-281}D^{282-476}H^{477-638}$ chimera, while having modest potency at hSERT.

These experiments suggest that some determinants for the interaction of antidepressants with SERTs exist within transmembrane domains V–IX. The decreased potency of each compound when transmembrane domains V–IX are switched to dSERT character in a parental hSERT construct indicate the location of some points of interaction likely to be within this region. However, the shift in potency is not a complete shift toward dSERT-like pharmacology. This finding indicates that regions or amino acids outside of the central transmembrane domain V–IX region most likely also play a significant role in recognizing antidepressants. Previous reports have identified discrete amino acids, such as D98 (Barker et al., 1999), Y95 (Barker et al., 1998), and

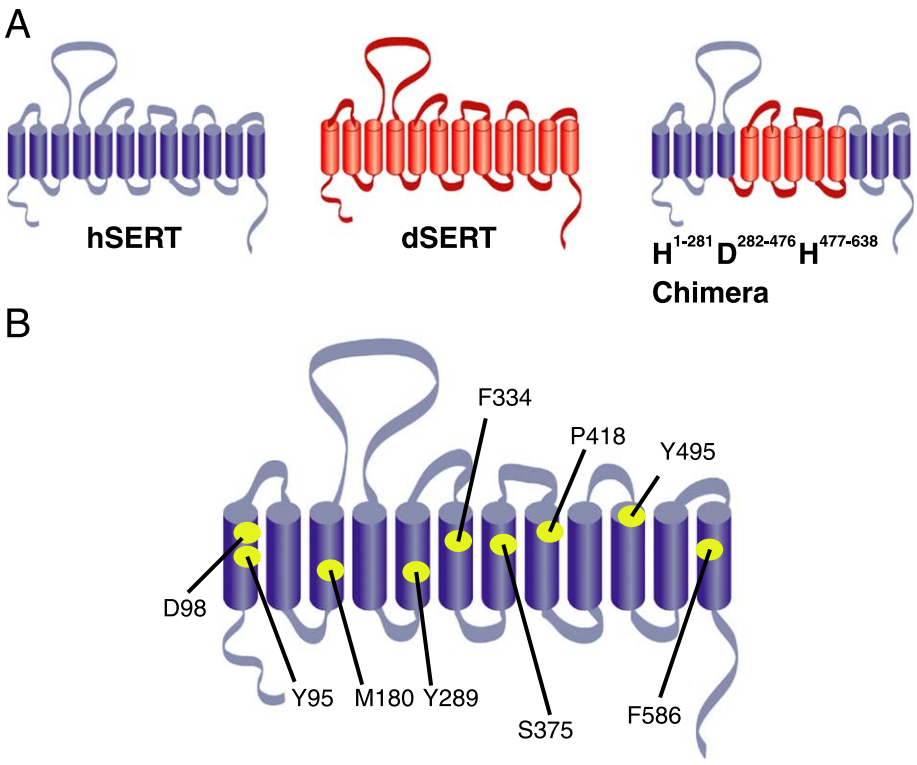

Fig. 4. (A) Diagram of wild-type hSERT, dSERT, and the $H^{1-281}D^{282-476}H^{477-638}$ chimeric transporter. The chimeric transporter was constructed as described in Materials and methods, with hSERT sequence indicated in blue and dSERT sequence indicated in red. (B) Representative model of SERT with amino acids implicated as sites of interaction with antidepressants (Barker and Blakely, 1996; Barker et al., 1998, 1999; Fang et al., 1998; Danek Burgess and Justice, 1999; Mortensen et al., 2001; Paczkowski and Bryan-Lluka, 2001, 2002; Roubert et al., 2001).

F586 (Barker and Blakely, 1996), as playing critical roles for the recognition of specific antidepressants by SERT. The complete shift in desipramine and venlafaxine potency at the chimera might suggest that these antidepressants have distinct contact sites that play a major role in their recognition and that these sites are within the transmembrane domain V–IX regions. It is likely that the interaction of these critical residues, as well as other unidentified points of interaction within the transmembrane domain V–IX regions, provide a site or sites through which SERT recognizes these compounds with high-affinity. Our studies correlate with previous reports of transmembrane domains V–VII of the norepinephrine transporter as being important for the recognition of tricyclic antidepressants (Buck and Amara, 1995).

Table 1
K_i values for inhibition of [^{3}H]5-HT uptake in stably transfected HEK-293 cells

	K_i (nM)		
	hSERT	dSERT	$H^{1-281}D^{282-476}H^{477-638}$
Sertraline	3.6 ± 0.4	390 ± 98	150 ± 37
Citalopram	6.7 ± 1.7	110 ± 30	73 ± 27
Imipramine	6.9 ± 2.7	230 ± 17	130 ± 6.0
Fluoxetine	17 ± 7.0	350 ± 40	270 ± 69
Desipramine	220 ± 90	900 ± 18	860 ± 32
Venlafaxine	310 ± 200	28,000 ± 5600	32,000 ± 11,000

[^{3}H]5-HT uptake experiments performed using stably transfected HEK-293 cells. Values are the mean ± S.E.M. for at least three independent experiments performed in triplicate.

3.5. Amino acids implicated in providing high-affinity interactions of antidepressants with SERT

Within the transmembrane domain V–IX regions, several amino acids have been implicated in antidepressant recognition (Fig. 4B) (Paczkowski and Bryan-Lluka, 2001, 2002; Roubert et al., 2001). Of these amino acids, two residues (S375 and P418) represent positions where the hSERT and dSERT sequences diverge. S375 is a position that has been identified as being important for the recognition of substrates and antidepressants by NET (Fang et al., 1998; Danek Burgess and Justice, 1999). This serine in transmembrane domain VII has also been identified as a critical residue for the function of SERTs (Kamdar et al., 2001; Penado et al., 1998). As a point of sequence divergence between hSERT and dSERT, we considered the possibility that S375 might play a role in the observed species differences for antidepressant recognition. We employed species-scanning mutagenesis whereby the identity from dSERT (an alanine) was substituted into the equivalent position of hSERT (S375). Interestingly, the hSERT S375A mutant did not show any

Table 2
K_i values for inhibition of [^{3}H]5-HT uptake in transiently transfected HeLa cells

	K_i (nM)		
	hSERT	P418S	S375A
Sertraline	0.7 ± 0.3	0.3 ± 0.1	0.8 ± 0.4
Imipramine	4.1 ± 1.6	2.1 ± 0.7	3.5 ± 1.6
Fluoxetine	9.3 ± 2.7	5.4 ± 1.9	13 ± 1.2
Citalopram	9.7 ± 3.7	12 ± 9.0	5.0 ± 2.4
Venlafaxine	22 ± 4.0	18 ± 7.2	7.2 ± 1.3
Desipramine	84 ± 47	39 ± 10	50 ± 14

[^{3}H]5-HT uptake experiments performed using transiently transfected HeLa cells. Values are the mean ± S.E.M. for at least three independent experiments performed in triplicate.

statistically significant differences in K_i values for the antidepressants as compared to the parental hSERT (Table 2), indicating that this residue may not play a major role in the recognition of these antidepressants. However, it is important to note that the level of [^{3}H]5-HT uptake at the hSERT S375A mutant was only approximately 25% of wild-type. Cell surface binding experiments confirmed a lower expression of hSERT S375A as compared to wild-type hSERT (data not shown).

The other species divergent position, P418, was also converted to dSERT identity (serine). This residue, equivalent to S399 in hNET, is located in transmembrane domain VIII near the extracellular face of the helix. P418 is interesting in that it is a proline residue in the hSERT, but a serine at the equivalent position in hNET and dSERT. The presence of the ''helix-breaking'' proline located within a putative helix provides a unique residue to exploit using mutagenesis. Other studies (Roubert et al., 2001) have shown that this residue is critical for the recognition of tricyclic antidepressants by hNET, providing another facet of interest for this residue. However, the hSERT P418S mutant did not show significant divergence from the parental hSERT in recognizing any of the antidepressants assayed (Table 2). Cell surface binding and transport levels indicated that hSERT P418S expression is virtually identical to wild-type hSERT (data not shown). One explanation for this finding is that the interaction with antidepressants provided by the proline in hSERT is maintained even when the residue is mutated to a serine. More likely, however, the mutation of the proline to a serine maintains intermolecular or intrahelical interactions that provide access to high-affinity recognition sites for these antidepressants. These findings suggest that P418 (SERT) or S399 (NET) could play a role in the overall conformation of the transporter, as opposed to serving as a direct contact site for antidepressants.

4. Conclusions

The cloning of SERT provided scientists with a target for the development of antidepressants. For our analyses, we have drawn from reports published long before the molecular cloning and characterization of this protein. The initial reports of structure–activity relationships for both the tricyclic antidepressants, as well as the selective serotonin reuptake inhibitors, have been used to perform a contemporary re-analysis of several seminal findings. The modern computer modeling approach that we used to analyze data from rat brain synaptosomes and cloned rSERT revealed close correlations between the original and current studies. We have also shown the importance of the positional orientation of the selective serotonin reuptake inhibitors when interacting with SERTs. The specific orientation of the aromatic ring provides both high-affinity and specificity for the selective serotonin reuptake inhibitors, which have become widely prescribed for those two very reasons: potency and specificity. Finally, we have performed experiments using chimeric SERT and SERT point mutants in order to develop a better understanding of how the protein interacts with antidepressants. Although the point mutants did not suggest critical points for recognition, the data from the cross-species chimera hint at the presence of multiple areas of SERT being involved with the recognition of antidepressants. Future studies will focus on further exploration of transmembrane domains V–IX to identify specific residues involved with high-affinity recognition of antidepressants, as well as guiding our efforts to understand how the transporter functions as a unit to recognize both tricyclic antidepressants and selective serotonin reuptake inhibitors.

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