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Two allelic isoforms of the serotonin transporter from *Schistosoma mansoni* display electrogenic transport and high selectivity for serotonin

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Abstract

The human blood fluke *Schistosoma mansoni* is the primary cause of schistosomiasis, a debilitating disease that affects 200 million individuals in over 70 countries. The biogenic amine serotonin is essential for the survival of the parasite and serotonergic proteins are potential novel drug targets for treating schistosomiasis.

Here we characterize two novel serotonin transporter gene transcripts, SmSERT-A and SmSERT-B, from *Schistosoma mansoni*. Southern blot analysis shows that the two mRNAs are the products of different alleles of a single SmSERT gene locus. The two SmSERT forms differ in three amino acid positions near the N-terminus of the protein. Both SmSERTs are expressed in the adult form and in the sporocyst form (infected snails) of the parasite, but are absent from all other stages of the parasite's complex life cycle.

Heterologous expression of the two cDNAs in mammalian cells resulted in saturable, sodium-dependent serotonin transport activity with an apparent affinity for serotonin comparable to that of the human serotonin transporter. Although the two SmSERTs are pharmacologically indistinguishable from each other, efflux experiments reveal notably higher substrate selectivity for serotonin compared with their mammalian counterparts. Several well-established substrates for human SERT including (±)MDMA, S-(+)-amphetamine, RU 24969, and m-CPP are not transported by SmSERTs, underscoring the higher selectivity of the schistosomal isoforms. Voltage clamp recordings of SmSERT substrate-elicited currents confirm the substrate selectivity observed in efflux experiments and suggest that it may be possible to exploit the electrogenic nature of SmSERT to screen for compounds that target the parasite *in vivo*.

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Keywords

Serotonin transporter; Parasite; Drug target; SERT; Allele; Gene expression

1. INTRODUCTION

Schistosoma mansoni is one of four major *Schistosoma* species that infect over 200 million people in tropical and subtropical regions worldwide (Ross et al., 2002). Schistosomes have a complex life cycle. Free-swimming cercariae penetrate human skin to gain access to its definitive host. After penetration the cercariae shed their tails and transform into schistosomulae, entering the vascular system and migrating via complex routes to their final locus in mesenteric veins where males and females mate, the females produce their eggs, and release them into the circulatory system. The eggs exit the host in feces and hatch on contact with water, releasing miracidia, which infect snails. The infected snails, bearing schistosomal sporocysts release cercariae into the water which, in turn, infect humans.

Schistosomiasis has serious health consequences such as acute nausea, diarrhea, dysentery, and fever. Most pathology is caused by a granulomatous reaction to the schistosomal eggs. The parasitic infections are also associated with malnutrition and impaired growth and development (Ross et al., 2002; Gryseels et al., 2006). Currently the drug of choice is praziquantel which cures 60 to 90 percent of infections, though it is not active on immature worms and eggs (Gryseels et al., 2006). Recent reports document an increase in worm resistance to praziquantel, thus underscoring the need for novel therapeutic strategies (Doenhoff and Pica-Mattoccia, 2006).

There is ample evidence that serotonin (5-hydroxytryptamine; 5-HT) is present in the flatworm nervous system, including that of schistosomes (Gustafsson, 1987; Ribeiro et al., 2005). Among parasitic flatworms, in particularly *S. mansoni*, serotonin is an important modulator of neuromuscular function and metabolism. Serotonin increases muscular activity in both human-(adult) and snail-host (sporocyst) stages of *S. mansoni* (Mansour, 1984; Boyle et al., 2000), and it also increases metabolic activity by stimulating glucose uptake, glycogen breakdown, and lactate excretion (Mansour, 1984; Rahman et al., 1985). Because serotonin is essential for the survival of the animal, serotonergic proteins are potential targets for novel drug treatments of schistosomiasis. (Mansour, 1984; Pax et al., 1996; Thompson et al., 1996).

Several reports have described a serotonin transporter-like activity in adult worms and sporocysts in the human parasite *Schistosoma mansoni* (Bennett and Bueding, 1973; Wood and Mansour, 1986; Boyle et al., 2003). The serotonin transporter (SERT) has been cloned from several mammalian species (Mortensen et al., 1999) and also from the nematode *Caenorabditis elegans* (Ranganathan et al., 2001). The SERT clears serotonin from the extracellular space using the sodium gradient as thermodynamic driving force (Torres et al., 2003) and is also the target for a major class of antidepressants (White et al., 2005).

In the present study we have cloned and pharmacologically characterized two novel isoforms of the serotonin transporter gene from *S. mansoni* by expressing the cDNA in mammalian cells and *Xenopus laevis* oocytes. We also show by RT-PCR that the transporter is expressed in only two of the distinct stages of the parasite life cycle. This transporter could be a potential novel therapeutic drug target against schistosomiasis.

2. METHODS

2.1. Parasites

An LE strain of *Schistosoma mansoni* is routinely maintained by passage through *Biomphalaria glabrata* snails and BALB/c mice. *B. glabrata*-infected snails were induced to shed cercariae by exposing them under artificial illumination in a 30°C water bath for 1h. Schistosomula were obtained by mechanical transformation of cercariae according to the procedure described by (Harrop and Wilson, 1993). Adult worms were recovered from mice previously infected with cercariae by perfusion of the livers and mesenteric veins. All animal experiments in Brazil were treated according to the guidelines for use of animals in research approved by the Ethics and Animal Experimentation committee of Ribeirão Preto School of Medicine, the University of São Paulo. Animal experiments carried out at BRI are covered by an IACUC-approved protocol and the animal program at BRI is AAALAC-accredited (#000779).

2.2. Materials

[³H]-serotonin and [³H]-dopamine were obtained from NEN (Boston, MA, USA). Reagents for uptake and binding buffers, unlabelled serotonin, uptake inhibitors and substrates were from Sigma-RBI (St. Louis, MO, USA). (±)MDMA and S(+)-fenfluramine were generously provided by the NIDA Drug Supply System. Racemic citalopram was a gift from Lundbeck, Copenhagen, Denmark. Cell culture media, MEM, FCS, horse serum, penicillin/streptomycin, glutamine and D-PBS were from Invitrogen Life Technologies (Carlsbad, CA, USA). All other reagents were of analytical grade or better. The Generacer kit used for cloning was from Invitrogen Life Technologies (Carlsbad, CA, USA). The Phusion polymerase was from Finnzymes (Espoo, Finland).

2.3. Cloning procedures

Total RNA was isolated from adult *Schistosoma mansoni*. 5 µg of total RNA was used in the RNA ligase-mediated RACE protocol according to the manufacturer (Invitrogen, Carlsbad, CA, USA). PCR was performed using the following primers:

Schis5: 5'-TCTTATTAGCTGTTATTGGTTTTGCTG-3'

This sequence is found in the Institute for Genomic Research (TIGR)

Schistosoma mansoni whole genome shotgun project (Clone ID: SNDJF04 or 1264346).

Schis3: 5'-CCAAAGCCTGGTCCAAGTG-3'

This sequence is found in the Brazilian *Schistosoma mansoni* EST Genome Project (Clone ID: C709958).

The generated 850 bp fragment was cloned into pCR4Blunt-TOPO Vector (Invitrogen, Carlsbad, CA, USA) and sequenced using automated sequencing.

From this new sequence, *S. mansoni*-specific primers were constructed. For the 5'RACE reaction the following primers were used:

5'schis: 5'-GCACCTCCACCATTACGATAACATATAAATGG-3'

5'schis nested: 5'-CGCCAAATATTTCCAAGATCTACAGCAAAACC-3'

Together with

GeneRacer™ 5' Primer: 5'-CGACTGGAGCACGAGGACACTGA-3'

GeneRacer™ 5' Nested Primer: 5'-GGACACTGACATGGACTGAAGGAGTA-3'

For the 3'RACE the following primers were used:

3'schis: 5'-GGTCTAACATTAGAAGGTTTCATTAAGTGGTAT-3'

3'schis nested: 5'-GTAAAGTATTTCTGTTTGGAGTGATGCTGCAT-3'

Together with

GeneRacer™ 3' Primer: 5'-GCTGTCAACGATACGCTACGTAACG-3'

GeneRacer™ 3' Nested Primer: 5'-CGCTACGTAACGGCATGACAGTG-3'

Sequencing suggested that the start codon was not identified in the 5'RACE clone, as there was a full open reading frame. The most 5' sequence was used to BLAST the TIGR database and another clone was identified that included the upstream start codon (Clone ID: SMBUY43 or 1234180).

Full-length cDNA was constructed using the following primers:

SmATG: 5'-CCGCTCGAGCATGAATCATAATACACCTACAACCTTG-3' and

SmSTOP: 5'-GCTCTAGATTATAATTTATTTGTTTTGAATGTTTTTC-3'.

Finally the full-length isoforms were subcloned into pOTV (Sonders et al., 1997) for characterization in *Xenopus* oocytes, into pEGFP-N3 (Clontech, Mountain View, CA, USA) for expression in mammalian cells and into pEGFP-C2 (Clontech, Mountain View, CA, USA) for N-terminal tagging with GFP and expression in mammalian cells.

2.4. Southern blot analysis

For Southern blots, genomic DNA was isolated from adult worms. 10 µg of DNA was digested with BamHI, FokI, TaqI or NcoI (New England Biolabs, Ipswich, MA, USA), separated on a 0.7% agarose gel, transferred by capillary action to a nylon membrane (GE Healthcare Life Sciences, Piscataway, NJ, USA), and probed with smSERT-exon1 fragments labeled with ³²P (Prime-It II Random Primer labeling kit, Stratagene, La Jolla, CA, USA).

2.5. cDNA synthesis and PCR amplifications

Total RNA from different stages of *S. mansoni* was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. 3 µg of RNA from infected snails (sporocysts), uninfected snails (control), cercariae, schistosomulae, adult worms and eggs were reverse-transcribed using oligo (dt)₁₈ anchor primer and SuperScript II (Invitrogen, Carlsbad, CA, USA). Genomic DNA was extracted using the CTAB protocol (Winnepenninckx et al., 1993) from *S. mansoni* adult worms, from uninfected snails, and from snails infected with *S. mansoni*.

The specific primers to amplify serotonin transporter transcript were:

Sm300F 5'-CAAAAGGTAAATTACAACCAAATC-3'

Sm1R 5'-TTAAACAACCACTGCGCTGA-3'

The amplifications controls were: α -tubulin gene sequence (accession number: M80214.1) for *S. mansoni* and the proteasome subunit Rpn1 gene (accession number: CV042523) for *B. glabrata*. Several redundant or empty lanes were digitally removed from the gel image to simplify the image.

2.6. Cell surface biotinylation assay

Cell surface expression of the *S. mansoni* transporter constructs was assayed with modifications of the method described previously (Daniels and Amara, 1999). Briefly, COS-7 cells (ATCC, Manassas, VA, USA) were transfected with GFP-tagged versions of SERTs in 6-wells plates 48 hours before the biotinylation assay. The cells were incubated with 2 mg/ml sulfo-NHS-SS-biotin (Pierce, Rockford, IL, USA) for 40 min. The cell lysate was incubated with NetrAvidin Resin (Pierce, Rockford, IL, USA) for 2–3 hr at 4° C. The beads were washed three times with lysis buffer, twice with a high-salt wash buffer, and once with a no-salt wash buffer. Sample proteins were separated on SDS-PAGE gels and transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were probed for the expression of GFP-tagged proteins with polyclonal antibodies against GFP and HRP conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). Antibodies were visualized using a chemiluminescent imaging station (UVP, Upland, CA, USA)

2.7. Transport assays in COS-7 cells

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum supplemented with penicillin and streptomycin in a humidified atmosphere with 5% CO₂ at 37°C. Uptake experiments were performed two days after transfecting with Eugene6 reagent (Roche) and plating the cells in 96 wells-plates. Using an automatic plate washer, the cells were washed with room temperature phosphate buffered saline containing 0.1 mM CaCl₂ and 1 mM MgCl₂. The cells were pre-incubated for 5 min with varying concentrations of the inhibitors or substrates at room temperature. The uptake assays were initiated by addition of 100 nM tritiated substrate and incubated for 10 min. Uptake was terminated by two washes and cells were solubilized in Optiphase scintillation cocktail (Perkin Elmer, Waltham, MA, USA) and counted on a Wallac 1450 microbeta liquid scintillation counter (Perkin Elmer, Waltham, MA, USA). To determine $V_{\max}$ and K_M for serotonin uptake in the human and *S. mansoni* transporters a mix of unlabeled serotonin and [3H]-serotonin (in an 80 μ l volume, 0.15 μ M to 20 μ M, 95% unlabeled and 5% labeled compound) was added and incubated for 10 min at room temperature. All assays were done in quadruplicate. Assuming Michaelis-Menten kinetics the data were analyzed using non-linear regression using GraphPad Prism version 3.02 for Windows (Graphpad software, La Jolla, CA, USA). $V_{\max}$ and K_M are given as mean $\pm$ SD of three independent experiments.

2.8. Calculation of IC₅₀ and EC₅₀ for the effects of the inhibitors and substrates

To determine the effects of inhibitors and substrates on serotonin uptake COS-7 cells were pre-incubated with various concentrations of substrates and inhibitors for 10 minutes prior to the addition of 100 nM tritiated serotonin. The concentration-curves generated were fitted to a Hill equation by non-linear regression analysis using GraphPad Prism version 3.02 for Windows (Graphpad software, La Jolla, CA, USA). IC₅₀'s are given as mean $\pm$ Standard deviation of three or four independent experiments. Statistical significance was assessed using Student *t*-test or two-way ANOVA. A value of $p < 0.05$ was considered to be significant.

2.9. Efflux experiment in COS-7 cells

COS-7 cells were maintained under the same conditions as above. Efflux experiments were performed two days after cell transfection and plating of the cells in 24 wells-plates. Cells were washed with room temperature phosphate buffered saline containing 0.1 mM CaCl₂ and 1 mM

MgCl₂ and then loaded with 100 nM [³H]-serotonin for 1 hour or longer. After pre-loading cells were washed twice with buffer and then incubated for 10 min with substrates or the inhibitor fluoxetine at room temperature. The concentrations of substrates and inhibitor used were high enough to inhibit uptake mediated by both hSERT, SmA and SmB (according to table 2): 5 μM serotonin, 20 μM tryptamine, 320 μM (±)MDMA, 300 μM S(+)-amphetamine, 180 μM RU 24969, 60 μM m-1-(3-Chlorophenyl)piperazine dihydrochloride (m-CPP), and 50 μM fluoxetine. After the incubation the supernatant was collected, liquid scintillation added and counted (Beckman LS-6500 Multi-purpose Scintillation Counter). The cells were solubilized in a solution of 0.1% SDS and 0.1M NaOH, liquid scintillation was added and tubes were counted as above. All assays were done in quadruplicate.

The statistical analyses used consider the percentage of [³H]-serotonin efflux among different treatments as the dependent variable. To analyze this the total amount of radioactivity in the samples was calculate by adding counts of supernatant plus cell lysis, the supernatant counts were then divided by the total and multiplied by 100 to yield the percentage of efflux.

Bar graphics and statistics (*t*-student test comparing vehicle and treated cells) was performed using GraphPad Prism version 4.0 for Windows (Graph Pad software, La Jolla, CA, USA).

2.10. Determination of substrate specificity in oocytes

Synthetic RNA was transcribed *in vitro* using the mMessage mMachine kit (Applied Biosystems/Ambion, Austin TX, USA) and injected into defolliculated stage V–VI *Xenopus* oocytes. Oocytes were incubated at 17°C for three to 10 days before being studied for [³H] serotonin uptake or for SmSERT-mediated currents under voltage-clamp. Electrophysiological recordings were made using two-electrode voltage clamp according to procedures previously described (Sonders et al., 1997). To determine V_{max} and K_M for serotonin uptake various concentrations of a mix of unlabeled serotonin and [³H]-serotonin was added and incubated for 100 to 300 s. Nonspecific uptake was defined by parallel treatment of water-injected oocytes. All assays were done in quadruplicate. Assuming Michaelis-Menten kinetics the data were analyzed using non-linear regression using GraphPad Prism version 3.02 for Windows (Graphpad software, La Jolla, CA, USA).

3. RESULTS

3.1. Molecular cloning of serotonin transporter from *Schistosoma mansoni*

Several reports have characterized the physiological functions of serotonin transporter-like activity in adult worms and sporocysts in the human parasite *Schistosoma mansoni* (Bennett and Bueding, 1973; Wood and Mansour, 1986; Boyle et al., 2003) and we were motivated to search for sequences homologous to mammalian serotonin transporters in both the recently completed Brazilian *S. mansoni* EST database (Verjovski-Almeida et al., 2003) and in the *S. mansoni* genome project at TIGR and the Sanger Institute. Based on several sequences within these databases and using a conventional PCR based cloning strategy we generated a 2200 bp fragment that included a full open reading frame encoding a protein with a size of 735 amino acids (Fig. 1). Recently the sequence of a 208 amino acid peptide from the related organism *Schistosoma japonicum* has been deposited in Genbank (Accession number: AAW26870) that has high sequence similarity to the SmSERT N-termini identified in this study: it contains strong sequence homology through the first 40 residues and also in the last 50 residues that encode transmembrane segments I and II. Transmembrane sequences are particularly well-conserved among members of the neurotransmitter-sodium-symporter (NSS) family. Therefore the *S. japonicum* peptide supports the suggestion that we have cloned the complete versions of SmSERT and identified the actual start codon. During the course of this study the cloning of a serotonin transporter from *S. mansoni* was reported (Accession number:

EF061308) (Patocka and Ribeiro, 2007). This sequence is similar to the sequences reported here but most significantly it is missing 78 amino acids in the N-terminus. Otherwise it is identical to smSERT-B and most likely the product of the same gene. This finding could suggest that several different N-terminal variants of the transporter exist.

We identified two different SmSERT isoforms that differed in three amino acids located in the protein's predicted intracellular N-terminal. In isoform A, residue 99 is a leucine, residue 100 is a glutamine, and 118 is a valine. The corresponding residues in isoform B are serine, histidine and isoleucine (Fig. 1). Several independent clones were sequenced to eliminate the possibility of PCR artifacts and both sequences have been deposited in Genbank (Accession no: **DQ220811** (A) and **DQ159205** (B)). A scan of the genomic sequences deposited in the genome databases of TIGR and the Sanger Institute covering this region identifies 5 independent shotgun reads and reveals the existence of both the A (4 reads: e.g. shisto8151 from the Sanger Institute) and B (1 read: SNDJF04TR from the Institute for Genomic Research (TIGR)) isoforms in the parasite genome. Searching the nearly completed *Schistosoma mansoni* genome project (<http://www.genedb.org/genedb/smansoni/>) reveals an approximately 55 kbp gene consisting of 13 exons (Fig. 2A). Because of the incompleteness of the genome assembly, a few ambiguities still remain around exon 8 and from the genomic data we can not rule out the possible existence of additional exons between exon 8 and 9. Otherwise, our full length cDNA clone aligns completely with the genomic sequences. Comparing the sequence of the genomic sequences containing the polymorphic region suggest that the two isoforms are a product of the same gene as they show almost complete conservation outside the polymorphic exon region, extending several hundred bases into the intronic region (Fig. 2C). To further confirm that only one SERT gene exists in the parasite we employed Southern blot analysis. A probe spanning exon 1 that detects both isoforms A and B hybridized to single bands in BamHI, NcoI and TaqI digested genomic DNA from adult worms. The sizes of the bands correlated well with what could be predicted from the genomic assembly (Fig. 2A and 2B). Because the B variant contains a unique FokI restriction enzyme site, this enzyme can be used to investigate the existence of B in the genome. Indeed we could detect both A (~1500 bp) and B (~750 bp) alleles.

The sequence reveals a protein with the typical structure of sodium/chloride dependent neurotransmitter transporters (neurotransmitter: sodium symporters, <http://www.tcdb.org/tcdb/index.php?tc=2.A.22>). It has an unusually long 150 residue intracellular N-terminal compared to the human SERT (85 amino acids) and the large second extracellular loop of SmSERT is also extended by about 20 amino acids compared to all other cloned serotonin transporters. Like the serotonin transporters of *C. elegans* (Ranganathan et al., 2001) and *Manduca sexta* (Sandhu et al., 2002) it does not contain the same glycosylation sites that have been found in the mammalian carriers, but does contain other potential sites for glycosylation suggesting it could become glycosylated. Biotinylation with subsequent immunoblotting of SmSERT-expressing COS-7 cells also revealed that the molecular size of the surface expressed SmSERTs (~120 kDA compared to a theoretical molecular weight of ~105 kDA (for GFP-tagged SmSERTs)) is consistent with posttranslational modifications such as glycosylation (Fig. 3A).

3.2. SmSERT-A and B expression during the life cycle of the *S. mansoni*

In order to evaluate the presence of SmSERT transcripts in cercariae, schistosomulae, adult worms, eggs, and the sporocyst life stage found in infected snails, RT-PCR was performed using specific primers as described in material and methods. As shown in Fig. 4 significant levels of the SmSERT were only detected in the adult worms and in sporocysts—the two stages of the parasite life cycle when it resides in host organisms (human and snail, Fig. 4, Lanes B and J). As a control for *S. mansoni* RNA, primers for the *S. mansoni* α -tubulin gene amplified a product from reverse-transcribed RNA extracted from all life stages (Fig. 4, lanes A, C, E,

G, and I). As control for snail RNA, primers for *B. glabrata* proteasome subunit Rpn1 were employed (Fig. 4, lane K). The study by Patocka and Ribeiro (Patocka and Ribeiro, 2007) additionally found low levels of SERT expressed by cercaria.

3.3. Functional characterization of SmSERT-A and B in mammalian cells

To identify the substrates of the cloned transporter, we expressed the two different isoforms SmSERT-A and B heterogeneously in COS-7 cells, but initially found no functional monoamine uptake. Because Patocka and Ribeiro (Patocka and Ribeiro, 2007) reported functional uptake by their shorter version, we also tested the similar shorter versions we initially cloned, but found no functional uptake in these versions. We believe the reason for this discrepancy may be attributed to differences in expression systems and the fact that the low uptake reported by Patocka (Patocka and Ribeiro, 2007) was measured following prolonged (60 min) incubation periods.

We also constructed versions of the cloned transporter that were fused with GFP at their N-termini and this tagging ameliorated the expression of SmSERT isoforms, yielding functional transporters that could be pharmacologically and biochemically characterized. GFP-transporter fusion proteins of several carriers in the NSS family have been shown to function normally when compared to wild type transporters (Schmid et al., 2001). Uptake experiments verified that the cloned transporter is a serotonin transporter as both isoforms, SmSERT-A and B, transport tritiated serotonin but not dopamine or norepinephrine (data not shown). The transport was found to be Na⁺-dependent as uptake experiments carried out using choline chloride as a replacement for sodium chloride in the buffer showed uptake similar to the background (data not shown). Kinetic analyses of serotonin uptake by hSERT, SmSERT-A and SmSERT-B (Fig. 3B) revealed no differences in apparent affinity (K_T) for serotonin between the two isoforms (A: $2.3 \pm 0.9 \mu\text{M}$ and B: $2.2 \pm 1.1 \mu\text{M}$) or between SmSERT isoforms and hSERT human SERT ($3.2 \pm 0.4 \mu\text{M}$).

These apparent affinities are comparable to those obtained in serotonin uptake experiments on living parasites. In sporocysts, the apparent affinity for serotonin was found to be $1.4 \mu\text{M}$ (Boyle et al., 2003). In adult worms male and female parasites showed an apparent affinity for serotonin uptake of $1.73 \mu\text{M}$ and $1.46 \mu\text{M}$, respectively (Wood and Mansour, 1986).

The V_{max} was found to be significantly diminished for the SmSERTs with a for SmSERT-A of $1.8 \pm 1.4 \text{ pmol/min}$ and for SmSERT-B of $1.4 \pm 1.1 V_{\text{max}} \text{ pmol/min}$ compared to the V_{max} for human SERT of $2.7 \pm 0.1 \text{ pmol/min}$. To investigate the cause of the relatively low V_{max} found for the SmSERTs we carried out surface biotinylation assay (Fig. 3A) and found the level of surface expressed SmSERTs was lower than for human SERT.

3.4. Pharmacological characterization of inhibitors and substrates on SmSERT-A and SmSERT-B compared to human SERT

We investigated the pharmacology of the two SmSERT isoforms and compared their sensitivity to inhibitors to that of hSERT. Table 1 shows the IC_{50} values for inhibiting serotonin uptake by GFP-hSERT, GFP-SmSERT-A and GFP-SmSERT-B expressed in COS-7 cells for the following compounds: 6-Nitroquipazine, clomipramine, desipramine, chlorpromazine, imipramine, fluoxetine, methiothepin, paroxetine, citalopram, cocaine and RTI-55. All of the drugs exhibited identical potencies for the two SmSERT isoforms however they were all weaker inhibitors of [³H]-serotonin uptake by SmSERTs than by hSERT with the exceptions of chlorpromazine, and methiothepin, which were more potent at SmSERTs (Table 1). Some striking differences between close congeners were observed in species specificity: for example, the potency difference versus SmSERTs as compared with hSERT was 60-fold lower for RTI-55 but only 8-fold lower for its analog cocaine. Clomipramine was 15-fold whereas its

congeners desipramine and imipramine were only two-fold and three-fold less potent versus SmSERTs, respectively. 6-Nitroquipazine had an exceptional potency on both human and parasite serotonin transporters, also being only two-fold less potent at SmSERTs. Most of the IC₅₀ values we measured with cloned SmSERT-B (and A) are comparable with those found in studies characterizing [³H]-serotonin uptake in *S. mansoni* sporocysts, though paroxetine, citalopram, cocaine and RTI-55 were not examined in that study (Boyle et al., 2003). The one exception is imipramine, which we find to be 10-fold less potent than what was found in the sporocyst. Table 2 shows IC₅₀ values for the putative substrates of serotonin transporters, (±)MDMA, tryptamine, S-(+)-amphetamine, RU 24969, and m-CPP for inhibiting uptake of serotonin by GFP-hSERT, GFP-SmSERT-A and GFP-SmSERT-B expressed in COS-7 cells. All of these drugs had substantially higher affinity for the human serotonin transporter than for the SmSERTs.

An inhibition assay does not distinguish if a compound is an inhibitor or a competitive substrate of the transporter and we performed efflux (reverse transport) experiments to further investigate if the above compounds are actual substrates of the transporter. In these experiments the efflux of preloaded serotonin is induced by compounds that are substrates of the carrier. Fig. 5 shows such efflux experiments on GFP-hSERT (Fig. 5A), GFP-SmSERT-A (Fig. 5B), and GFP-SmSERT-B (Fig. 5C) expressed in COS-7 cells. All of the compounds tested were able to elicit serotonin efflux by the human hSERT (serotonin, tryptamine, (±)MDMA, S-(+)-amphetamine, RU 24969, and m-CPP) and the inhibitor fluoxetine did not elicit efflux. On the other hand, only non-labeled serotonin and tryptamine were able to elicit [³H]-serotonin efflux from the parasite transporters, SmSERT-A and SmSERT-B.

3.5. Identification of transport-associated currents by SmSERT in *Xenopus* oocytes

Injection of *Xenopus* oocytes with cRNAs encoding non GFP-tagged versions of the two SmSERT isoforms led to functional expression of the transporters assessed by uptake of [³H]-serotonin and transport-associated currents (Fig. 6). The apparent affinities of SmSERT isoforms for [³H]-serotonin transport were approximately 3 μM (Fig. 6B), in good agreement with the observed *K_m* values for the GFP-fused SmSERTs expressed in COS cells. Transport-associated currents in multiple batches (n=17) of oocytes voltage-clamped at -60mV displayed modest amplitudes on the order of 20 nA. In contrast to mammalian SERTs, voltage jump experiments with SmSERT-expressing oocytes provided no evidence of steady-state leak or transient currents sensitive to serotonin or to the non-substrate inhibitors fluoxetine, citalopram, or cocaine (data not shown). The A and B isoforms were indistinguishable with respect to all the electrophysiological properties examined (data not shown).

Application of serotonin to oocytes expressing either SmSERT isoform elicited concentration-dependent inward currents (Fig. 6A). These currents were abolished by substitution of Na⁺ in the superfusion buffer with the monovalent cation choline (data not shown), illustrating a general property of all serotonin transporters (Mager et al., 1994). Also similar to other SERTs, the currents elicited by serotonin were reversibly abolished by co-incubation with the serotonin transporter inhibitor fluoxetine (Fig. 6A). The K_{0.5} of serotonin for eliciting currents was approximately 0.3 μM (isoform A, 260 nM, n=2; isoform B, 342 nM, n=3). As reported for the rat SERT (Mager et al., 1994), serotonin transport exhibited a biphasic concentration-dependence where current amplitudes decline at substrate concentrations greater than 5 μM (Fig. 6B). Confirming the result from the efflux experiments that tryptamine is a substrate of SmSERTs we found that tryptamine like serotonin elicits clear transport associated currents by SmSERTs (Fig. 6A).

Comparison of substrate-elicited currents with uptake of [³H]-serotonin in the same batches of oocytes provides insight into the relationship between transport and charge movements. The ratio of maximal current (*I_{max}*) by the charges moved (*V_{max}* * Faraday constant) indicates that

1.9 charges move per serotonin molecule transported (Q/J_{max}). As has been noted for other serotonin transporters, the Q/J_{max} ratio of the SmSERT isoforms indicates that more charges move across the membrane than can be accounted for by a fixed transport stoichiometry of $1\text{Na}^+ : 1\text{Cl}^- : 1\text{Serotonin}$ moving in and 1K^+ moving out. Moreover, the disparity between $K_{0.5}$ of serotonin for eliciting currents ($0.3\text{ }\mu\text{M}$) and K_T for uptake ($3.0\text{ }\mu\text{M}$) implies that the ratio will be considerably larger at lower serotonin concentrations (Fig. 6B). This apparent uncoupling between the ability of substrates to elicit currents and to be transported has been noted for other electrogenic neurotransmitter transporters including the *Drosophila* SERT (Beckman and Quick, 2001), rat dopamine transporter (Ingram et al., 2002), and the human glutamate transporter EAAT4 (Fairman et al., 1998).

4. DISCUSSION

The two *Schistosoma mansoni* SERT isoforms reported in this study have amino acid sequences highly similar to other cloned SERTs (Fig. 1). They have a much longer N-terminus compared to most homologs in other species, but this region is generally the least conserved between the members of the serotonin transporter family. Genomic sequence and southern blot analysis suggest that the two isoforms are products of different alleles (Fig. 2) and that only a single SERT gene exists in the parasite. We were unable to detect any functional and pharmacological difference between the two isoforms and the previously reported shorter SmSERT (Patocka and Ribeiro, 2007) appear functionally very similar. Both versions generate currents with highly similar, if not identical properties (Data not shown). The difference in primary sequence between smSERT-A and B lies within the N-terminus and this is also the region that is absent in the previously reported SERT. The function of the N-terminus of SERT and other NSS is not completely understood, but it has been found to interact with a number of other proteins and to regulate transporter function (Mortensen and Amara, 2003; Khoshbouei et al., 2004; Steiner et al., 2008). These data suggest that proteins that interact with the SmSERTs in an isoform specific manner could be important for regulating the localization and trafficking of the transporters in their native environment. Interestingly, a search using the Scansite search engine (<http://scansite.mit.edu>) (Obenauer et al., 2003) identified a B isoform specific binding site for 14-3-3 proteins. 14-3-3 proteins have previously been found to interact with the N-terminus of the related human norepinephrine transporter (Sung et al., 2005).

SmSERT was expressed well in *Xenopus* oocytes with or without a GFP tag at the N-terminus. However, in mammalian cells expression was only obtained with the GFP-containing construct, suggesting that mammalian cells may lack factors required for efficient translation and surface expression of the SmSERT. Other *S. mansoni* transporters have been successfully expressed and functionally characterized in *Xenopus* oocytes (Skelly et al., 1994) suggesting this expression system may have more in common with the endogenous environment of the parasite.

Because serotonin is essential for the survival of the *S. mansoni* parasite through its regulation of neuromuscular function and metabolism (Mansour, 1984; Rahman et al., 1985; Boyle et al., 2000), molecules that interact with and modulate the function of serotonergic proteins such as smSERT could have therapeutic interest. Furthermore it has been suggested that the parasite when residing in its human and snail host could be scavenging serotonin through an active transport system from the host and blocking this source of serotonin could be detrimental to the survival of the parasite. Interestingly it has indeed recently been shown that the treatment of the sporocyst snail stage with a specific SERT inhibitor fluoxetine prevents the production of daughter sporocysts (Boyle and Yoshino, 2005). Most drugs showed lower potency against the SmSERTs compared to human SERT. The two SmSERTs display nearly identical pharmacological sensitivities to both inhibitors and substrates.

Only the two antipsychotic drugs chlorpromazine and methiothepine were more potent for the parasites transporters than for hSERT. Chlorpromazine is derived from phenothiazine and clinically used as an anti-psychotic agent, that elicit its antipsychotic and antiemetic action via interference with central dopaminergic pathways in the brain (Abidi and Bhaskara, 2003). Methiothepin is an aryl-piperazine and a non-specific dopamine and serotonin receptor antagonist with antipsychotic properties (Domino, 1999). It was used very briefly clinically and had some serious side effects. Although both of these drugs show higher affinity for SmSERT over human SERT, their potencies for inhibiting the transporter is rather low compared to their nanomolar affinities to the dopamine and serotonin receptors. Thus, their potential use as therapeutic agents against schistosomiasis would be limited, but does provide an avenue for the development of drugs with higher affinity for SmSERT and lower affinity for human transporter and receptor targets.

The difference in inhibitor sensitivity between SERT homologs from different species have proven useful for the identification of amino acid residues within SERT involved in the interaction between inhibitors and SERT. Several of these positions that have been implicated in inhibitor interaction are different when comparing human and SmSERT and could therefore cause the lower affinity for the inhibitors used. Residue Tyr-95 in human SERT (Phe-159 in SmSERT) was in one study found to be important for citalopram and mazindol binding (Barker et al., 1998). At the equivalent position in SmSERT is a phenylalanine and this is identical to the residue found in *Drosophila* SERT that displays less sensitivity to inhibitors. Similarly at other positions where specific amino acids have been found to be important for inhibitor interaction such as the isoleucine at human SERT position 172 (Thr-236 in SmSERT) (Larsen et al., 2004; Henry et al., 2006), at the phenylalanine at human SERT position 586 (Pro-677 in SmSERT) (Barker and Blakely, 1996) and the methionine at human position 180 (Ile-244 in SmSERT) (Mortensen et al., 2001) the corresponding amino acid in SmSERT is different and could cause less affinity towards the inhibitors.

Results from efflux experiments suggest that SmSERTs are more selective for serotonin than human SERT, because fewer compounds elicit efflux of previously loaded serotonin from SmSERTs as compared with the human counterpart. A similar observation has been made with the *drosophila* SERT, which also appears more selective for serotonin than human SERT. This observation is consistent with the assertion that the human SERT is a more promiscuous transporter with respect to substrate recognition (Rodriguez et al., 2003). A comparison of *Schistosoma*, *Drosophila* and human SERTs could be used to determine structurally features of the transporters involved in substrate recognition and selectivity. Since we tested relatively few drugs on SmSERT and yet they exhibit quite distinct profiles at *Schistosoma* and human SERTs, it seems possible that SmSERT-selective substrates might be identified that could serve as a novel avenue of anti-schistosomal pharmacotherapy. The discovery of substrate induced currents by SmSERT expressed in *Xenopus* oocytes could also open up a method to screen large numbers of potential SmSERT substrates.

The source of serotonin for the parasite is not completely understood but the parasite has the capability to synthesize endogenous serotonin as its genome contains tryptophan hydroxylase (Hamdan and Ribeiro, 1999), the rate limiting enzyme responsible for serotonin synthesis. However, it has been shown that the expression levels of tryptophan hydroxylase are down regulated in the adult worm, which is strictly parasitic, and upregulated in the free living larval stage (cercaria), suggesting that the worm in its parasitic stages depend more on host serotonin potentially acquired via active transport (Boyle et al., 2003). Our RT-PCR expression results supports this idea as we only find SmSERT expressed to a detectable level in the strictly parasitic adult and sporocyst stages of the animal. The findings that both adult worms and sporocysts (Bennett and Bueding, 1973; Wood and Mansour, 1986; Boyle et al., 2003) accumulate exogenous [³H]-serotonin when they are cultured *in vitro* indicate that SmSERT

is expressed at the organism's surface. In fact the serotonin transporter has been identified in an analysis of the surface tegument of adult parasite worms (Skelly and Alan, 2006). Obviously this expression suggests the possibility that schistosomes can reduce a metabolic cost by scavenging circulating serotonin from their hosts: However, it also poses the more interesting question of whether serotonin serves as a signal from the host that regulates parasite biology and their interactions with the host. Insofar as SmSERT resembles other mammalian and insect SERTs in being electrogenic, either or both substrate transport and substrate-elicited ion flux could subserve a signaling function of SmSERT. It is intriguing to note that in both mammalian and SmSERTs serotonin elicits currents at a 10-fold lower concentration than it is transported. Another explanation for the observed expression of SmSERT when the parasite is residing in one of its two hosts could be that the parasite at these two more developed stages requires a more precise regulation of extra-cellular serotonin within the parasite to modulate serotonergic signaling, much like is the function of its mammalian SERT counterparts.

In conclusion we here report the isolation of the gene for the serotonin transporter from the human parasite *Schistosoma mansoni*. The biogenic monoamine serotonin is essential for the survival of the animal (Mansour, 1984), and serotonergic proteins are potential targets for novel drug treatments of schistosomiasis. Considering that the current drug of choice praziquantel only cures 60 to 90 percent of patients and there are reports of worm resistance to praziquantel, novel therapeutic strategies are necessary. The SmSERT could represent a novel target for therapeutic intervention. Treatment of mother sporocysts with serotonin transporter inhibitors reduces the production of daughter sporocysts by depleting endogenous stores of serotonin (Boyle and Yoshino, 2005) and limiting the availability of serotonin to the parasite could be a novel therapeutic strategy against schistosomiasis. Thus the cloning and characterization of SmSERT function could facilitate development of novel drugs to treat schistosomiasis.

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Fig. 1. Alignment of SmSERT-A and SmSERT-B and other monoamine transporters ceSERT (*C. elegans* SERT), hSERT (human SERT), ceDAT (*C. elegans* dopamine transporter (DAT)), hDAT (human DAT), and hNET (human norepinephrine transporter). Black shading denotes residues that are completely conserved. Dark grey and light grey shading denotes residues that are at least 80% and 60% conserved, respectively.

Fig. 2. SmSERT is encoded by a single copy gene with at least two alleles, A and B

A: Graphical representation of the SmSERT gene spanning at least 13 exons. The A/B polymorphism is located in exon 1. The approximate locations of the restriction enzyme cleavage sites used for the southern blot in panel B are noted. B: Southern blot of genomic DNA isolated from adult worms, digested with the noted restriction enzymes and hybridized with a radiolabeled probe encompassing exon 1. In the FokI digest arrows point to faint bands representing the DNA fragments corresponding to the undigested A allele (upper arrow) and the digested B allele (lower arrow). C: Alignment of two, raw whole genome shotgun, sequences from the TIGR and Sanger genome projects.

Fig. 3. Expression of SmSERTs in mammalian COS-7 cells

A: Immunoblot of surface biotinylated proteins from COS-7 cells expressing GFP-hSERT, GFP-SmSERT-A or GFP-SmSERT-B detected using a GFP antibody. B: Kinetic analysis of serotonin uptake in COS-7 cells expressing GFP-hSERT ($\blacktriangledown$), GFP-SmSERT-A ($\square$) or GFP-SmSERT-B ($\triangle$). Data from three independent experiments using eight substrate concentrations in six replicates generated values of $3.2 \pm 0.4 \mu\text{M}$ for the K_M and $2.7 \pm 0.1 \text{ pmol/min}$ for the V_{max} for GFP-hSERT; $2.3 \pm 0.9 \mu\text{M}$ for the K_M and $1.8 \pm 1.4 \text{ pmol/min}$ for GFP-SmSERT-A and $2.2 \pm 1.1 \mu\text{M}$ for the K_M and $1.4 \pm 1.1 \text{ pmol/min}$ for GFP-SmSERT-B.

Fig. 4. SmSERT is expressed exclusively in the parasitic lifestages of the parasite developmental cycle

RT-PCR assessing SmSERT mRNA expression throughout different developmental stages of the *S. mansoni* life cycle. Approximately 3 µg of total RNA from adult worms, schistosomula, eggs, cercariae, infected snails (sporocysts) and non infected snails (control without infection), were reverse transcribed and amplified by PCR using SmSERT, Smαtubulin and snail *B. glabrata* proteasome subunit primers.

A: cDNA of adult worm amplified with Smαtubulin primers.

B: cDNA of adult worm amplified with SmSERT primers.

C: cDNA of schistosomulus (mechanic transformation for 3 hours) amplified with Smαtubulin primers.

D: cDNA of schistosomulus (mechanic transformation for 3 hours) amplified with SmSERT primers.

E: cDNA of eggs amplified with Smαtubulin primers.

F: cDNA of eggs amplified with SmSERT primers.

G: cDNA of cercariae amplified with Smαtubulin primers.

H: cDNA of cercariae amplified with SmSERT primers.

I: cDNA of infected snail (45 days) amplified with Smαtubulin primers.

J: cDNA of infected snail (45 days) amplified with SmSERT primers.

K: cDNA of non-infected snail amplified with *B. glabrata* proteasome subunit Rpn1 primers.

L: cDNA of non-infected snail amplified with SmSERT primers.

Fig. 5. Substrate efflux from human and parasite SERTs

Efflux experiments in GFP-hSERT (A), GFP-SmSERT-A (B), and GFP-SmSERT-B (C) transfected COS-7 cells after pre-loading with 100 nM $[^3\text{H}]$ -serotonin. Concentrations of substrates used: 5 μM serotonin, 20 μM tryptamine, 320 μM ($\pm$)MDMA, 300 μM S-(+) amphetamine, 180 μM RU 24969 and 60 μM m-CPP) and 50 μM inhibitor fluoxetine. All assays were done in quadruplicate. The graphic represents the average of 4 experiments for each condition. *t*-student test comparing vehicle and treated cells showed that serotonin and tryptamine were significantly different in comparison with vehicle for efflux of GFP-SmSERT-A (B) and GFP-SmSERT-B (C) (** $p < 0.05$, *** $p < 0.001$).

Fig. 6. SmSERT-mediated currents and transport in *Xenopus* oocytes

A: Traces of membrane currents in representative voltage-clamped oocyte expressing SmSERT-A illustrates that inward currents (downward deflections) elicited by superfusion of serotonin (5HT) are concentration dependent and strongly diminished by 1 μ M fluoxetine. Tryptamine (Tpm) also elicits clear transport associated currents. Numbers indicate concentrations of serotonin and tryptamine in μ M. Panel B compares the concentration dependences for serotonin in eliciting currents in voltage-clamped oocytes (left ordinate – open circles) and in uptake assays (right ordinate – closed circles) performed in parallel in a batch of oocytes expressing SmSERT-B. Serotonin has roughly 10-fold higher potency for eliciting currents (0.3 μ M) than for being transported (3.0 μ M) ($K_{0.5} < K_T$). Current data in panel B represent pooled data from three oocytes.

Table 1

Inhibitory constants (IC_{50}) for serotonin transport inhibitors in *S. mansoni*, and human serotonin transporters (SmSERT-A, SmSERT-B, and hSERT).

Drug	IC ₅₀ (μM)		
	hSERT	SmSERT-A	SmSERT-B
6-Nitroquipazine	0.002±0.0004	0.004±0.002 ^a	0.004±0.002 ^b
Clomipramine	0.009±0.005	0.15±0.11 ^c	0.2±0.1 ^c
Desipramine	0.6±0.09	1.1±0.3 ^a	1.5±0.4
Chlorpromazine	1.15±0.4	0.2±0.1 ^b	0.3±0.2 ^a
Imipramine	0.09±0.03	0.3±0.1 ^a	0.3±0.2 ^b
Fluoxetine	0.1±0.09	0.2±0.1	0.2±0.1
Methiothepin	6.7±4.1	1.9±0.7 ^a	2.2±0.7 ^a
Paroxetine	0.02±0.009	0.09±0.07 ^c	0.1±0.1 ^c
Citalopram	0.03±0.01	0.25±0.1 ^c	0.19±0.13 ^c
Cocaine	4.7±3.4	37.6±16 ^b	35.4±7.6 ^c
RTI-55	0.01±0.01	0.6±0.4 ^b	0.6±0.4 ^b

Uptake was initiated by addition of 100 nM [³H]serotonin and incubated for 10 min. IC_{50} values were calculated from the average of three or four experiments performed in six replicates. Determinations of significance were made using Student's *t* test (^a: $P<0.05$, ^b: $P<0.01$, ^c: $P<0.001$). SmSERT-A and SmSERT-B were compared to hSERT

Table 2**IC₅₀**

for serotonin transporter substrates by hSERT, SmSERT-A and SmSERT-B.

Drug	IC ₅₀ (μM)		
	hSERT	SmSERT-A	SmSERT-B
(±)MDMA	2.7±0.5	319.6±162 ^a	230±23 ^c
Tryptamine	1.4±0.1	19.6±3.6 ^c	19.3±4.4 ^c
S(+)-Amphetamine	27.5±3	300±27 ^c	241±54 ^b
RU 24969	8.5±0.9	180±63 ^c	165±65 ^c
m-CPP	1.8±0.3	58±4 ^c	60±3 ^c

Putative substrates were mixed with 50 nM [³H]-serotonin and added simultaneously to the cells and incubated for 10 min. IC₅₀ values were calculated from the average of three or four experiments performed in six replicates. Determinations of significance were made using Student's *t* test (^a: *P*<0.05, ^b: *P*<0.01, ^c: *P*<0.001). SmSERT-A and SmSERT-B were compared to hSERT.