




Molecular characterization of the serotonergic transporter from the cestode *Echinococcus granulosus*: pharmacology and potential role in the nervous system

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Abstract

Echinococcus granulosus, the etiological agent of human cystic echinococcosis (formerly known as hydatid disease), represents a serious worldwide public health problem with limited treatment options. The essential role played by the neuromuscular system in parasite survival and the relevance of serotonin (5-HT) in parasite movement and development make the serotonergic system an attractive source of drug targets. In this study, we cloned and sequenced a cDNA coding for the serotonin transporter from *E. granulosus* (EgSERT). Bioinformatic analyses suggest that EgSERT has twelve transmembrane domains with highly conserved ligand and ionic binding sites but a less conserved allosteric site compared with the human orthologue (HsSERT). Modeling studies also suggest a good degree of conservation of the overall structure compared with HsSERT. Functional and pharmacological studies performed on the cloned EgSERT confirm that this protein is indeed a serotonin transporter. EgSERT is specific for 5-HT and does not transport other neurotransmitters. Typical monoamine transport inhibitors also displayed inhibitory activities towards EgSERT, but with lower affinity than for the human SERT (HsSERT), suggesting a high divergence of the cestode transporter compared with HsSERT. In situ hybridization studies performed in the larval protoscolex stage suggest that EgSERT is located in discrete regions that are compatible with the major ganglia of the serotonergic nervous system. The pharmacological properties, the amino acidic substitutions at important functional regions compared with the HsSERT, and the putative role of EgSERT in the nervous system suggest that it could be an important target for pharmacological intervention.

Keywords Serotonin transporter · Cestodes · Neglected diseases · Drug target · Pharmacology · 5-HT

Introduction

Echinococcus granulosus is the etiological agent of human cystic echinococcosis (CE), a zoonotic disease of major medical importance. CE is among the 17 neglected tropical diseases (NTDs) prioritized by the World Health Organization (World Health 2012). This zoonotic disease is widely distributed throughout the world and represent a serious

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public health problem (Budke et al. 2009). Several species of the genus *Echinococcus* cause parasitic diseases in wildlife, domestic animals, and humans worldwide. The species belonging to the *E. granulosus* s. l. complex includes (besides others) *E. granulosus* sensu stricto and *E. canadensis*. The adult stage resides in the small intestine of the definitive host, and gravid proglottids release eggs that are passed in the feces and are infectious. After ingestion by an intermediate host, eggs hatch in the small intestine and release oncospheres that penetrate the intestine and migrate through the circulatory system. This latter larval stage migrates towards different organs, especially the liver and lungs, where the oncosphere develops into a hydatid cyst which enlarges and produces protoscoleces and daughter cysts. If ingested by humans, this can cause human CE (formerly hydatidosis) (Vuitton et al. 2020). The definitive host becomes infected by ingesting the cyst-containing organs of the infected intermediate host. After ingestion, the protoscoleces evaginate, attach to the intestinal mucosa, and develop into adult stages.

The anti-parasitic treatment against diseases caused by several platyhelminth species is currently based on the use of a limited number of approved anthelmintic drugs, such as albendazole (ABZ) for CE and praziquantel (PZQ) for adult stages (Budke et al. 2009; Garcia 2018). However, these compounds are not well tolerated by some patients (Horton 1997; Kyung et al. 2011; Lee et al. 2011), and it was shown that ABZ is ineffective in ~40% of CE cases (Gottstein et al. 2015; Hemphill et al. 2014). Given the limited availability of safe and efficacious anthelmintic drugs approved for treatment, the identification of new potential drug targets and novel anthelmintic drug candidates is urgently needed. The essential role played by the neuromuscular system in parasite survival makes it an attractive source of drug targets (McVeigh and Maule 2019). However, the understanding of the nervous system and the neuroactive substances of parasitic flatworms is a neglected area of research. Several neuroactive molecules have been described in flatworms, one of which is serotonin (5-hydroxytryptamine: 5-HT), a well-known neurotransmitter and hormone, which is present at high levels in the mammalian blood. It was observed that exogenous application of 5-HT onto larval or adult stages of the parasitic platyhelminths *Schistosoma mansoni* in culture causes a marked increase in their motility (Boyle et al. 2000; Hillman and Senft 1973; Pax et al. 1996). The same effect was observed also in cestodes including *E. granulosus* (Camicia et al. 2018, 2013) and *Mesocostoides vogae* (*Syn. Mesocostoides corti*) (Camicia et al. 2018, 2013). It was hypothesized that the hypermotility phenotype observed after serotonin addition can be attributed to an increase in the frequency of muscle contraction (Day et al. 1994; Pax et al. 1996) and, also, to an increase in carbohydrate metabolism (Mansour 1984; Pax et al. 1996; Rahman et al. 1985), which makes more energy available for movement. The fact

that the effects on motility are seen in intact animals that are not permeabilized suggests that 5-HT could be either acting through surface receptors and/or is taken up via a specific transporter and acting directly on receptors localized in the musculature or nerves that innervate them. Biochemical evidence obtained from studies performed in the cestode *Hymenolepis diminuta* suggests the existence of a sodium-dependent high-affinity serotonin transporter (Osloobi and Webb 1999). Their autoradiographic study suggests a sodium-dependent high-affinity serotonin transport system which is localized primarily in the serotonergic-like neurons of *H. diminuta*, also suggesting a possible recycling of neuronally released serotonin (Osloobi and Webb 1999).

The plasma membrane serotonin transporters (SERTs) are evolutionarily highly conserved transmembrane proteins which were first identified in the mammalian central nervous system (CNS), where they mediate reuptake of the monoamine across the presynaptic membrane. This protein is involved in the inactivation of the signal triggered by the binding of 5-HT to their cognate receptors by quickly removing it from the synaptic cleft. SERTs have been reported in the CNS of organisms from diverse species and also non-neuronal tissues that store 5-HT (Demchyshyn et al. 1994; Hoffman et al. 1998; Ranganathan et al. 2001). According to the transport classification database (TCDB), the serotonin transporters belong to the neurotransmitter:sodium symporter (NSS) family or the SLC6 family of solute carriers. Members of the NSS family catalyze uptake of a variety of neurotransmitters, amino acids, osmolytes, and related nitrogenous substances by a solute: Na⁺ symport mechanism (Rudnick et al. 2014). Some members of the NSS have a critical role in neurotransmission and are targets for psychostimulants, antidepressants, and other drugs. Structural information about serotonin transporters came first from the crystal structure of the bacterial homologue LeuT (Zhou et al. 2007), and several years later, the crystal structure of the mammalian homologue was also reported (Coleman et al. 2016). This type of transporter is characterized by a central orthosteric site (Coleman et al. 2016), an allosteric site (Aggarwal et al. 2021, 2019; Coleman et al. 2016; Kortagere et al. 2013; Navratna and Gouaux 2019; Plenge et al. 2012, 2021; Shi et al. 2008), and by highly conserved ionic sites (Coleman et al. 2016). The binding of neurotransmitter to the outward-open transporter triggers a series of conformational changes, in which the transporter isomerizes to an inward-open conformation and, subsequently, releases the neurotransmitter into the presynaptic neuron (reviewed in (Navratna and Gouaux 2019)). When SERT adopts an outward-open conformation, the protein exposes a cone-shaped extracellular vestibule to the aqueous solution, which contains residues that form the extracellular gate. When the extracellular gate is open, the intracellular gate

is closed, thus precluding direct access from the central ligand binding site to the intracellular solution (Coleman et al. 2016; Rudnick and Sandtner 2019). The translocation of serotonin involves the co-transport of sodium ions. In platyhelminths, the serotonin transporter homologue SmSERT, from the trematode species *Schistosoma mansoni*, was characterized in two separate studies, including one from our group, by cloning, sequencing, phylogeny, multiple sequence analyses, functional characterization, and transcriptomic studies (Fontana et al. 2009; Larsen et al. 2011; Patocka and Ribeiro 2007). In these studies, it was found that the recombinant schistosome transporter (SmSERT) mediates specific and saturable 5-HT transport. The heterologously expressed protein was inhibited by classic SERT blockers, and the same drugs also inhibited 5-HT uptake by intact schistosomula in culture, suggesting that SmSERT may be responsible for this transport. Efflux experiments reveal notably higher substrate selectivity for serotonin compared with their mammalian counterparts as only the endogenous substrate serotonin, but not exogenous substrates like amphetamine and MDMA, are transported by SmSERT (Fontana et al. 2009). In a later study (Ribeiro and Patocka 2013), confocal immunofluorescence studies using antibodies against SmSERT showed that this transporter is expressed predominantly in the nervous system both in adult worms and larvae (schistosomula). Co-localization experiments showed that the pattern of SmSERT expression was coincident with that of serotonin itself, suggesting that SmSERT is present in serotonergic neurons. *S. mansoni* schistosomula treated with SmSERT inhibitors or SmSERT-specific short-interfering RNAs (siRNAs) produced a strongly hyperactive phenotype. The siRNA effect correlated with a decrease in expression of the SmSERT when tested by real-time qPCR (Ribeiro and Patocka 2013). Later, a study from our group characterized a novel dopamine/norepinephrine transporter (SmDAT) gene transcript from the same parasite (Larsen et al. 2011). The study noted that the amphetamine was a much less potent efflux elicitor against SmDAT compared to the human DAT, a finding that could aid in the search for novel anti-schistosomal pharmacotherapy, highlighting the importance of characterizing neurotransmitter systems from these parasites. In a recent work from Herz and Brehm (Herz and Brehm 2021), the serotonin transporter orthologue from *Echinococcus multilocularis* was cloned and sequenced, and its role in development and utility as potential drug target was highlighted. However, no functional or pharmacological studies was performed in this study. In the present work, we have cloned, sequenced, bioinformatically analyzed, and functionally characterized the *E. granulosus* serotonin reuptake transporter. Its predicted structure, molecular evolution, and localization are also described. The pharmacological properties of

this transporter and its role in the nervous system suggest that it could be an important target for pharmacological intervention.

Material and methods

Ethics statement

Experiments involving the use of experimental animals were carried out according to protocols approved by the Comité Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL), Facultad de Medicina, Universidad de Buenos Aires, Argentina (protocol “Farmacología, localización y función de receptores acoplados a proteínas G (GPCRs) y de canales de calcio de *Echinococcus granulosus* y otros parásitos cestodos como posibles blancos de drogas antiparasitarias” CD N° 2542/2019). Cyst puncture was performed following the approved protocol by the same institution (protocol “Punción de quistes hidatídicos de infecciones naturales” CD N° 3723/2014).

Parasite material

E. granulosus s.s. protoscoleces were obtained under sterile conditions by needle aspiration of hepatic hydatid cysts of ovine origin, provided by abattoirs from Buenos Aires, Córdoba, and Santa Fe provinces, Argentina. The livers used for parasite extraction were from animals that were not specifically used for this study, and all the material obtained was processed as part of the normal work of the abattoir. Samples from animals at the abattoir were collected under consent from local authorities. Protoscolex viability was assessed using the eosin exclusion test after three washes with phosphate buffered saline (PBS, 2.7 mM KCl, 1.2 mM KH_2PO_4 , 138 mM NaCl, 8.1 mM Na_2HPO_4 , pH 7.4), with 50 $\mu\text{g}/\text{ml}$ of gentamicin to remove cyst wall debris, and only protoscoleces showing more than 95% viability were used. A major portion of the protoscoleces was used for cDNA synthesis and in situ hybridization; the remaining protoscoleces were used for species/genotype determination by sequencing a fragment of the mitochondrial cytochrome c oxidase subunit 1 (CO1), as previously described (Cucher et al. 2011). The resulting species and genotype of all protoscoleces used in this work were from *E. granulosus* G1 (sheep) genotype.

Bioinformatic analysis

The selection of the sequence for cloning of *E. granulosus* serotonin transporter (EgSERT) was based on bioinformatic analysis done by our group previously to this work (Camici et al. 2013). Transmembrane segments were predicted using the topology prediction server HMMTOP (<http://www.hmmtop.org/>).

enzim.hu/hmmtop/index.php), and bidimensional models of receptors were generated using Protter (<http://www.enzim.hu/hmmtop/index.php>). Analyses of critical residues for serotonin transporter binding and phylogenetic characterization were performed using vertebrate and invertebrate sequences from the neurotransmitter:sodium symporter (NSS) family (TC number 2.A.22) since high scores (very low *E*-values) for this family were found in the conserved domain database (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The sequences were aligned using ClustalW program from the ExPasy proteomic package (<https://embnet.vital-it.ch/software/ClustalW.html>). For the search of best hits, BLAST searches were performed in WormBase ParaSite (<https://parasite.wormbase.org/index.html>), and only sequences with at least 70% of amino acid identity were chosen for the alignment. Only some gene models were chosen for the alignment, which also gave the best alignments by visual inspection in multiple sequence alignments. All sequences used for the alignment are listed in Supplementary Table 2.

A phylogenetic tree was built according to the neighbor-joining method using the best tree mode available in phylogeny (<https://www.phylogeny.fr/>) and was verified by bootstrap analysis with 500 replicates. All sequences used for construction of the phylogenetic tree are listed in Supplementary Table 2. With the exception of the cestode sequences, the rest of the sequences used for multiple sequence alignments and phylogenetic groupings were from functionally tested transporters.

Homology modeling

For molecular modeling studies, protein domains of the *E. granulosus* serotonin transporter (EgSERT) studied in this work were screened against PFAM databases using Pfam-Scan (Finn et al. 2006). Protein structure model was performed using PHYRE2 (Kelley et al. 2015) and SWISS-MODEL (Arnold et al. 2006; Benkert et al. 2008; Biasini et al. 2014). The available human serotonin transporter (HsSERT) crystal structure (PDB IDs: 5i71) was used as template for modeling. The molecular visualization of models and figures generated in this work was performed using the software PyMOL version 2.0.4 (<https://pymol.org>). The structure model obtained was validated calculating several parameters such as ERRAT (Colovos and Yeates 1993) (<https://servicesn.mbi.ucla.edu/ERRAT/>), QMEN and Ramachandran plots that were calculated using Structure Assessment Tool of SWISS-MODEL (<https://swissmodel.expasy.org/assess>), and RMSD and TM-align (Zhang and Skolnick 2005) (<https://zhanglab.ccmb.med.umich.edu/TM-align/>) which were used to compare the structure models to human SERT (HsSERT). Finally, structural comparisons

were performed to identify relevant and conserved residues in the ligand and ion binding sites.

Levels of EgSERT expression

The transcriptional expression levels (in RPKM or reads per kilobase per million reads) for the serotonin transporter gene model EG_05393 in oncosphere, protoscolex, cyst, and adult stages from *E. granulosus* s.s. (G1 genotype) were from Zheng et al. (2013).

RNA extraction and cDNA synthesis

Total RNA from *E. granulosus* (G1) was extracted from protoscolexes that were crushed under liquid nitrogen and processed using Trizol reagent (Invitrogen). The RNA obtained was treated with RNase-Free DNase (Fermentas), ethanol precipitated, and reverse transcribed using SuperScript™ III reverse transcriptase (RT) (Invitrogen) and oligodT primer.

Amplification and cloning of a cDNA coding for the serotonin transporter

The gene model EgrG_000391300 from *E. granulosus*, coding for the serotonin transporter, was first identified in Camicia et al. (2013) by using reciprocal BLAST searches. The gene model was retrieved from the WormBase ParaSite database and used for primer design. The primers used for the amplification of the cDNA coding for the EgSERT were egsertf 5'-ACAGCTAGCATGCAACAAATAACCATCG-3' and egsertR 5'-ATAGCTAGCCTATCCCTCGTCTCCTTC-3. The cycling parameters were 94 °C for 3' (initial denaturalization); then 94 °C for 45'', 45 °C for 45'', and 72 °C for 2' (repeated 5 times); then 94 °C for 45'', 55 °C for 45'', and 72 °C for 2' (repeated 30 times); and finally, 72 °C for 10' (final extension). PCR reactions were performed using the High-Fidelity Polymerase (cat #K0192, Thermo Scientific) and the cDNA synthesized in the previous section as a template. Amplification products were visualized by agarose gel electrophoresis and GelRed staining, and the bands of interest were extracted from the gel using the QIAquick® Gel Extraction Kit (Qiagen), used for a non-templated adenine adding or A-tailing procedure employing a nonproofreading DNA polymerase (Pegasus, Embiotech), and finally cloned into the TOPO TA Vector (Invitrogen). The recombinant plasmids were used for *Escherichia coli* (DH5α) transformation, and the transformed bacteria were grown in LB with ampicillin and kanamycin. The selected colonies were then used for plasmid purification using the GeneJET Plasmid Miniprep Kit (Fermentas) and sequencing using an Applied Biosystems Big Dye terminator kit (Applied Biosystems) on an ABI 377 automated DNA sequencer.

Each clone was sequenced twelve times with forward and reverse primers (six for each one) in order to avoid error in the sequence obtained. The sequence obtained is identical to the sequence deposited in database (<https://www.ncbi.nlm.nih.gov/genbank/>) under the accession code XP_024350969.

Finally, the full-length EgSERT was subcloned into pcDNA3.1(+)-N-eGFP (GenScript, NJ, USA) for N-terminal tagging with GFP and expression in mammalian cells.

Whole mount in situ hybridization (WISH) and co-localization studies

Protoscolexes were first activated by treating them with pepsin under acidic conditions and subsequently with sodium taurocholate as previously described (Ritler et al. 2017). Then, parasite material was fixed and permeabilized as described elsewhere (Koziol et al. 2013).

We generated probes for EgSERT (XP_024350969) by reverse transcription-PCR (RT-PCR) using the Q5® High-Fidelity DNA Polymerase (cat #M0491S, New England Biolabs) and the cDNA synthesized in the previous section as a template. The primers used for RT-PCR were *egsertf4* 5'-ACAGCTAGCATGGGCGGACTGCCACTGTTC-3' and *egsert2* 5'-ATAGCTAGCCTAATCTCCTTCGGACTGTGTATC-3'. The amplicon (a 1680-bp fragment of the coding region of the transcript) was cloned into the pGEM-T vector (Promega, USA), the orientation of the insert was confirmed by Sanger sequencing, and subsequently used for in vitro transcription of digoxigenin-labeled antisense and sense probes, essentially as previously described (Koziol et al. 2014). The protocol for WMISH was performed as previously described for *E. multilocularis* (Koziol et al. 2014). Negative controls with sense probes did not result in any signal. All samples were analyzed using a ZEISS Axio Observer Z1, LSM 800 confocal microscope (Institut Pasteur de Montevideo). For co-localizations studies, the protocol of Koziol (Koziol et al. 2013) was followed after in situ procedure, using a FMRFamide rabbit polyclonal antibody (Immunostar, catalog number # 20,291, diluted 1:100).

Chemicals, cell culture media, and radiolabeled substrates

Non-radiolabeled serotonin and compounds fluoxetine, citalopram, desipramine, paroxetine, cocaine, and amphetamine were purchased from MilliporeSigma (Billerica, MA, USA).

D-PBS was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Culture media for cell lines, including Dulbecco's Modified Eagle's Medium (DMEM) with glucose, fetal bovine serum, and penicillin/streptomycin, were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Transfection reagent TransIT-LT1 was from Mirus Bio LLC (Madison, WI, USA).

Radiolabeled substrates [³H]-dopamine (53.6 Ci/mmol), [³H]-serotonin (28.2 Ci/mmol), [³H]-norepinephrine (14.9 Ci/mmol), [³H]-glutamic acid (51.1 Ci/mmol), [³H]-glycine (48.7 Ci/mmol), and [³H]-GABA (92.1 Ci/mmol) were purchased from PerkinElmer (Boston, MA, USA).

Scintillation fluid (Ecolite) was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Cell culture and DNA transfection

COS-7 cells (ATCC, Manassas, VA) were maintained in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin in a humidified incubator with 5% CO₂ at 37 °C. Subconfluent COS-7 cells were transiently transfected with 0.5 µg of plasmid DNA per well using TransIT-LT1 transfection reagent (Mirus Bio LLC, Madison, WI, USA) and plated at a density of 50,000 cells/well, and uptake experiments were performed 48 h later. Transfection with empty vector pcDNA3.1(+)-N-eGFP was used to control for the level of endogenous uptake of radiolabeled substrate in each experimental condition.

Initial analysis of substrate specificity

For an initial examination of the substrate selectivity of the transporter from *E. granulosus* (which we refer to as serotonin transporter, or EgSERT), COS-7 cells were transfected with plasmids containing the following DNAs: human dopamine transporter (HsDAT), human serotonin transporter (HsSERT), human glycine transporters (subtypes HsGlyT1 and HsGlyT2), human glutamate transporters (subtypes EAAT1, EAAT2, and EAAT3), and human GABA transporters (HsGAT-1 and HsGAT-3), as well as empty vector pcDNA3.1(+)-N-eGFP for obtaining the background. EgSERT was also transfected side by side along with each transporter for comparison. Forty-eight h later, cells were washed with PBS with 1 mM CaCl₂ and 0.1 M MgCl₂ (PBS-CM, for glycine, glutamate, and GABA transporter assays) or PBS-CM with the addition of 5 mM RO 41-0960 (a catechol-O-methyl transferase inhibitor) and 100 mM ascorbic acid (PBS-CM-RA), for preventing dopamine and serotonin degradation, in their respective transporter assays. Uptake assays were initiated by the addition of the appropriate radiolabeled substrate (dopamine, serotonin, glycine, glutamate, or GABA) at 50 nM (final concentration), diluted in PBS-CM or PBS-CM-RA buffers as appropriate. After 10 min incubation at room temperature, uptake was terminated by removal of solution, followed by two washes with buffer and lysis with 1% SDS/0.1 M NaOH. Cells were placed on a rocker for 20 min then transferred to scintillation

vials containing 3 mL of scintillation fluid. Radioactivity was quantified in a scintillation counter Tri-Carb 2800 TB (PerkinElmer, Boston, MA, USA).

Saturation analyses of serotonin uptake

Cells transfected with either HsSERT, EgSERT, or empty vector pcDNA3.1(+)-N-eGFP (for the background) were washed with PBS-CM-RA, and uptake reactions were initiated by the addition of varying concentrations (15 nM–10 μ M), in triplicate, of a mixture of non-radiolabeled and radiolabeled serotonin (97.5:2.5%). After 10 min, uptake reactions were terminated, and radioactivity was counted as above.

Dose–response assays

Inhibitors were screened for activity against HsSERT and EgSERT. COS-7 cells transfected with HsSERT or EgSERT (or empty vector for obtaining the background) were washed with PBS-CM-RA and incubated for 10 min at room temperature with various concentrations of the fluoxetine, citalopram, desipramine, paroxetine, amphetamine, or cocaine (0.1–200 μ M). Uptake reactions were initiated by the addition of 50-nM radiolabeled serotonin. After 10 min, uptake was terminated, and radioactivity was counted as above.

Data and statistical analysis

For the assays in this study, the nonspecific transport (background) obtained from cells transfected with empty vector was subtracted from the total. The signal-to-noise ratio observed across the assays was between 1 and 3%. Previous characterization of HsSERT and current characterization of EgSERT have demonstrated that the uptake rate measured under these conditions was linear for the time period (10 min) of the assay (not shown). All data analysis was performed using GraphPad Prism version 8.4.3 for Windows.

Initial substrate selectivity

Statistical significance was assessed using *t*-test comparing empty vector–transfected cells with transporter–transfected cells. Values were significantly different when $p < 0.05$.

Calculations of the kinetic parameters of transport

Nonspecific uptake (from COS-7 cells transfected with empty vector pcDNA3.1(+)-N-eGFP) was subtracted, and the data were analyzed assuming Michaelis–Menten kinetics. Values of the maximum efficiency of transport by the transporters (V_{\max}) and the affinity for the substrate (K_m) were determined from at least three independent assays.

Statistical analysis on the values of V_{\max} and K_m , obtained in each experiment, was performed using Student's *t*-test for paired data. Values were significantly different when $p < 0.05$.

Calculations of IC₅₀

Dose–response curves were generated considering IC₅₀ as the concentration of compound resulting in 50% of the maximum observed inhibition. Data were fitted to a dose–response curve by nonlinear regression analysis and normalized to the percentage of control (vehicle) for better visualization and comparison among compounds. IC₅₀ values are given as means \pm SEM of four independent assays for the effects of each inhibitor on each of the transporters.

Results

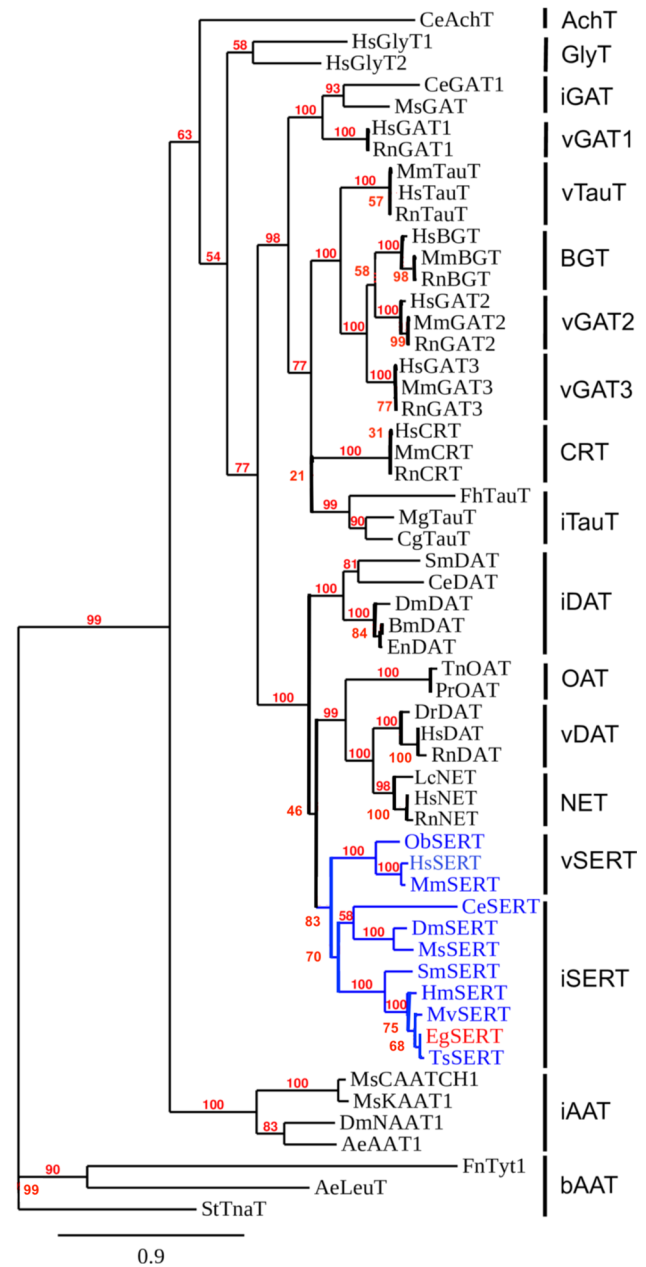
Bioinformatic analysis

In a previous work (Camicia et al. 2013), we have identified a gene called EgrG_000391300 from the GeneDB database (<https://www.genedb.org/#/species/Egranulosus>) by reciprocal BLAST searches. The sequence finally obtained by cloning and sequencing is identical to that found in the database (GenBank accession number XP_024350969.1, (Zheng et al. 2013)). The gene has fourteen exons and thirteen introns according to the information retrieved from the WormBase ParaSite database (Home 2020).

The nucleotide sequence has 2001 base pairs and encodes a 666 amino acids long protein with an amino acid identity of 48.94% with HsSERT (UniProt code, P31645.1), its human orthologue (Coleman et al. 2016). The bioinformatics analyses suggest twelve transmembrane segments with intracellular amino terminal and carboxyterminal ends (Supplementary Fig. S1), which are characteristic of eukaryotic members of the neurotransmitter:sodium symporter family (NSS). The theoretical isoelectric point (pI) was of 7.99, and the predicted molecular weight was of 74.9 kDa. The amino acid sequence obtained here seems to be almost identical to its orthologue from *E. multilocularis* (Herz and Brehm 2021), but some residue substitutions were observed between both sequences. The *E. granulosus* sequence is 26 amino acids longer at the amino terminal end than the *E. multilocularis* version. The best orthologue from *E. canadensis* is the gene EcG7_01618 with 98.6% of identity with the *E. granulosus* version characterized here. BLAST searches have identified other hypothetical orthologues of EgSERT in other cestode species like *H. microstoma*, *M. vogae*, and *T. solium*, and these sequences were named HmSERT, MvSERT, and TsSERT, respectively.

Fig. 1 Phylogenetic tree of bacterial, invertebrate, and vertebrate members of the neurotransmitter:sodium symporter (NSS) family of transporters. Protein sequences were aligned with ClustalW, and a rooted neighbor-joining best tree was constructed from the multiple sequence alignment. Included in the alignment are the serotonin transporter from *E. granulosus* (EgSERT, labeled in red) characterized here and representative examples of the major classes of the neurotransmitter:sodium symporter (NSS) family of transporters in vertebrate (labeled with the prefix v in the groupings at the right) and invertebrate representatives (labeled with the prefix i in the groupings at the right), including the cation-amino acid transporter/channel from *M. sexta* (MsCAATCH1); the invertebrate neutral amino acid transporter from *M. sexta* (MsKAAT1); the sodium-dependent nutrient amino acid transporter 1 from *D. melanogaster* (DmNAAT1); the cation-dependent nutrient amino acid transporter from *A. aegypti* (AeAAT1); the sodium-dependent acetylcholine transporter from *C. elegans* (CeAchT); the sodium- and chloride-dependent betaine/GABA transporters from *M. musculus*, *R. norvegicus*, and *H. sapiens* (MmBGT, RnBGT, and HsBGT1); the sodium- and chloride-dependent glycine transporters 1 and 2 from *H. sapiens* (HsGlyT1 and HsGlyT2); the sodium- and chloride-dependent creatine transporters from *M. musculus*, *H. sapiens*, and *R. norvegicus* (MmCRT, HsCRT, and RnCRT); the sodium- and chloride-dependent GABA transporter 1 from the vertebrate species *R. norvegicus* and *H. sapiens* (RnGAT1 and HsGAT1); the sodium- and chloride-dependent GAT transporters from the invertebrate species *C. elegans* and *M. sexta* (CeGAT1 and MsGAT); the sodium- and chloride-dependent GABA transporter 2 from the vertebrate species *M. musculus*, *R. norvegicus*, and *H. sapiens* (MmGAT2, RnGAT2, and HsGAT2); the sodium- and chloride-dependent GABA transporter 3 from the vertebrate species *M. musculus*, *R. norvegicus*, and *H. sapiens* (MmGAT3, RnGAT3, and HsGAT3); the high-affinity octopamine transporters from *T. ni* and *P. rapae* (TnOAT and PrOAT); the sodium-dependent dopamine transporters from *C. elegans*, *S. mansoni*, *D. melanogaster*, *B. mori*, *E. noyesi*, *D. rerio*, *H. sapiens*, and *R. norvegicus* (CeDAT, SmDAT, DmDAT, BmDAT, EnDAT, DrDAT); the sodium- and chloride-dependent taurine transporters from *M. musculus*, *R. norvegicus*, *H. sapiens*, *M. galloprovincialis*, *Crassostrea gigas*, and *F. hepatica* (MmTauT, RnTauT, HsTauT, MgTauT, CgTauT, and FhTauT); the sodium- and chloride-dependent serotonin transporters from *H. sapiens*, *M. musculus*, *O. beta*, *D. melanogaster*, *S. mansoni*, *M. sexta*, *C. elegans*, *H. microstoma*, *M. vogae*, and *T. solium* (HsSERT, MmSERT, ObSERT, DmSERT, SmSERT, MsSERT, CeSERT, HmSERT, MvSERT, and TsSERT, labeled in blue); and the sodium-dependent noradrenaline transporters from *L. catsebeianus*, *H. sapiens*, and *R. norvegicus* (LcNAT, HsNET, RnNET). Prokaryotic representatives of the family were also included (labeled with the prefix b in the groupings at the right), including the TnaT transporter from *S. thermophilum* (StTnaT), the leucine transporter from *A. aeolicus* (AeLeuT), and the tyrosine transporter from *F. nucleatum* (FnTyt1). Serotonin transporters are shown in blue. Sequences were all obtained from the general database available at the National Center for Biotechnology Information (NCBI) site, the UniProt database (<https://www.uniprot.org/proteomes/>), and the WormBase Parasite (). The numbers in red at branch points are bootstrap values. The length of the branches is proportional to the genetic distance between sequences (see scale bar)

The multiple sequence alignment of the sequence obtained here with other neurotransmitter transporters from invertebrate and human species shows similarities at key residues with serotonin transporters but also in some instances with dopamine or noradrenaline transporters (Supplementary Fig. S1).



We next performed phylogenetic studies to analyze the identity of the sequence obtained. We studied the phylogenetic position of the EgSERT in a phylogenetic tree composed with different members of the neurotransmitter:sodium symporter (NSS) family (TC N° 2.A.22) or the SLC6 family. The phylogenetic tree shows that serotonin transporters form a group of transporters made of vertebrate and invertebrate subgroups that are well separated from dopamine or norepinephrine transporters (Fig. 1). Figure 1 clearly shows that the sequence analyzed here from *E. granulosus* (EgSERT) grouped inside the invertebrate group of serotonin transporters, with the serotonin transporter from the platyhelminth species *M. vogae*, *T. solium*, *H. microstoma*, and *S. mansoni*

(Fontana et al. 2009) being the closest orthologues. More distant invertebrate orthologues of SERT are the serotonin transporter from the insect species *M. sexta* and *D. melanogaster* and the transporter from the model nematode, *C. elegans*. The vertebrate sequences seem to form a separate subgroup of serotonin transporters (Fig. 1).

Homology modeling of EgSERT

Homology modeling was performed to determine important structural features of EgSERT, and the X-ray structure of the human serotonin transporter complexed with (S)-citalopram at the central site was used as a template for this task (PDB: 5I71). Ramachandran plots for this model showed that 93.75% of residues were in favored regions, while 99.75% of residues were in allowed regions, and only 1.25% of residues were in outlier regions (Supplementary Fig. S2). In addition, an ERRAT value of 92.6829 and QMEN value closed to -4.0 were calculated for the homology structure model, indicating a good quality. RMSD value was 0.23, while TM-align value was 0.99372 suggesting the same fold for EgSERT with respect to HsSERT and a good

superposition between these serotonin transporters (Supplementary Table 1). Figure 2A shows the cestode (pink) and human (blue) receptors overlapped suggesting a high level of similarity at the structural level. The structural studies also suggest, as it was shown for the mammalian version of the transporter, the existence of a central binding and an allosteric site located halfway across the membrane (Fig. 2B and C). Regarding the central binding site, the models suggest that residues phenylalanine at position 80 and aspartate at position 83 in the *E. granulosus* model could perform similar binding functions to tyrosine 95 and aspartate 98 in the mammalian version and could interact with the tertiary amine of the citalopram molecule (Fig. 2B). The serine 336 in the mammalian receptor could perform a similar function to serine 340 in the invertebrate receptor which is also proximal to the tertiary amine of the citalopram (Fig. 2B). The functions performed by residues isoleucine in 172 and tyrosine 176 in the mammalian orthologue could be performed by threonine 157 and tyrosine 161, respectively, in the invertebrate receptor, which seems to be oriented towards the 4-fluorophenyl group of the drug (Fig. 2B). The positions and orientations of the residues phenylalanine 335 and

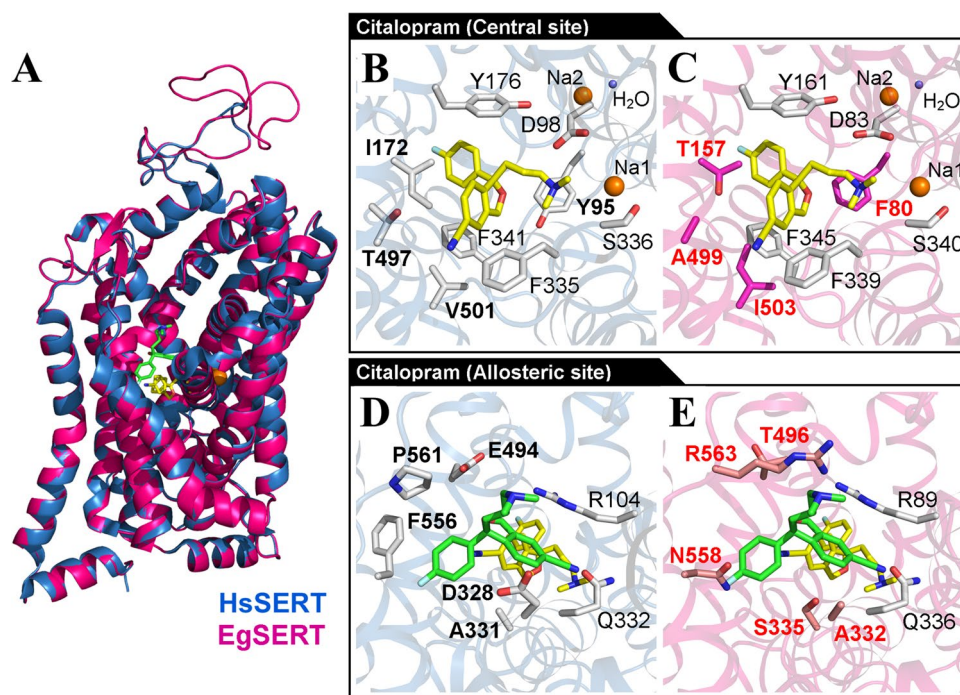


Fig. 2 Architecture of the *Echinococcus granulosus* serotonin transporter (EgSERT) compared to the human serotonin transporter (HsSERT). **A** Superposition of the crystallized HsSERT structure (PDB ID: 5I73; blue) and the EgSERT structure homology model (pink) viewed parallel to the membrane. Both structures are shown as cartoons. The (S)-citalopram molecules at the central and allosteric sites are shown as sticks in yellow and green, respectively. Sodium ions are shown as spheres in orange. **B** and **C** Interactions of (S)-citalopram (yellow) at the central binding site of **B** HsSERT and **C**

EgSERT. **D** and **E** Interactions of (S)-citalopram (green) at the allosteric site of **D** HsSERT and **E** EgSERT. In these last panels, a few atoms of (S)-citalopram at the central site (yellow) are visible below the (S)-citalopram molecule bound to the allosteric site (green). Residues in close proximity to (S)-citalopram molecules at the central and allosteric sites are shown as sticks. Note the substitutions of some residues involved in the interactions of (S)-citalopram at the central and allosteric sites in EgSERT (labeled in bold red) with respect to residues in HsSERT (labeled in bold black)

phenylalanine 341 seem to be identical to the phenylalanine residues 339 and 345 in the cestode transporter. However, the threonine residue at position 497 and the valine residue at position 501 seem to be replaced by the residues alanine 499 and isoleucine at positions 503 of the cestode sequence (Fig. 2B). The four later residues mentioned seem to interact with the center of the citalopram molecule (Fig. 2B). The ionic binding sites seem to be completely conserved (Supplementary Fig. S3). The modeling studies have shown that the orientations and positions of the residues conforming the sodium binding sites 1 and 2 and the chloride binding site are absolutely conserved (Fig. 2C and Supplementary Fig. S3). Regarding the allosteric site, we observed a lower level of conservation of residues that could be involved in allosteric function (Fig. 2D and E). Of the seven residues previously described (Fig. 2D), only two were conserved (Fig. 2E). For example, arginine at position 104 and glutamine 332 seem to be conserved at positions 89 and 336, respectively, in the cestode receptor. The rest of the residues, for example, aspartate at 328, alanine at 331, glutamate at 494, phenylalanine at 556, and finally, proline 561, seems to be replaced by alanine at 332, serine at 335, threonine at 496, glutamine 558, and arginine at 563, respectively, in the cestode receptor (Fig. 2E).

Transcriptomic analysis of EgSERT in several larval stages

We analyzed the transcript levels for EgSERT on each parasite stage based on the transcriptomic and genomic data from Zheng (Zheng et al. 2013). The graphical representation of the data (Fig. 3) shows the highest levels of mRNA for EgSERT at the protoscolex stage. The next stage is the cyst stage, which resulted in an intermediate level between protoscolex and adult stages. The latter stage has the lowest detectable levels of transcript for the transporter. Finally, the oncosphere stage has no detectable levels of transcript for EgSERT.

Pharmacological assays

We first investigated the substrate specificity of the *E. granulosus* transporter, by examining the transport of several neurotransmitter substrates by their respective human transporters, side by side with the parasite transporter. For these assays, we performed dopamine uptake (mediated by HsDAT), serotonin uptake (mediated by HsSERT), glycine uptake (mediated by HsGlyT1 and HsGlyT2), glutamate uptake (mediated by EAAT1, EAAT2, and EAAT3), and GABA uptake (mediated by HsGAT-1 and HsGAT-3) in transfected COS-7 cells. Figure 4 suggests that EgSERT is a serotonin transporter (as indicated by significantly higher accumulation of radiolabeled serotonin compared with

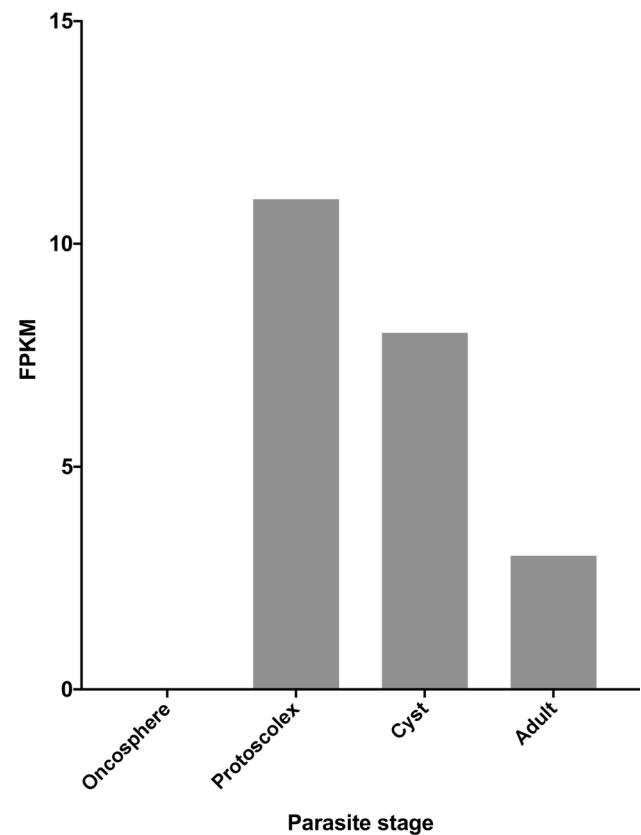


Fig. 3 Transcriptomic levels of EgSERT in several life cycle stages. Transcript levels of EgSERT transcript (from Zheng et al. (2013)) measured in FPKM (fragments per kilobase of transcript per million mapped reads) in oncosphere, protoscolex, cyst, and adult stages from *E. granulosus*

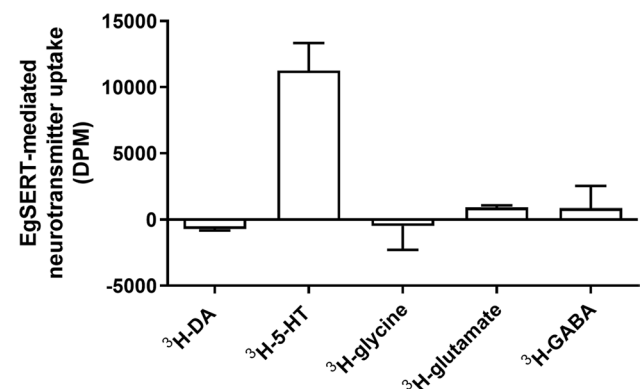


Fig. 4 Functional characterization: substrate specificity of EgSERT. COS-7 cells plated in 24-well plates were transfected with several transporters and pcDNA3.1(+)-N-eGFP (empty vector for obtaining background) and EgSERT and examined for their respective neurotransmitter transport. Graph is representative of at least four independent experiments performed for each neurotransmitter uptake. Positive controls for other neurotransmitter transporters including glutamate, dopamine, glycine, and GABA transporters are shown in Supplementary Fig. S4

background) (** $p=0.002$, t -test, not shown) and that this transporter does not transport dopamine, glutamate, GABA, or glycine (as indicated by no difference in accumulation of respective radiolabeled compounds compared with background). Since we did not observe dopamine uptake mediated by EgSERT, we can also eliminate the possibility that it transports noradrenaline, as dopamine is efficiently taken up by all known noradrenaline transporters (NETs (Buck and Amara 1994)). Positive controls for other neurotransmitter transporters demonstrated significant accumulation of their respective substrates (Supplementary Fig. S4). Taken together, this confirms that this transporter takes up serotonin. From this point, we designated this transporter EgSERT (for *E. granulosus* serotonin transporter).

Then, we proceeded to characterize the kinetic properties of this transporter, in comparison with the human serotonin transporter (HsSERT). Figure 5 shows that the V_{\max} and K_m values for HsSERT were 1800 ± 65 fmol/min/well and 1.5 ± 0.5 μM , respectively, whereas for EgSERT, we obtained V_{\max} and K_m values of 615 ± 33 fmol/min/well and 2.2 ± 0.8 μM , respectively. These data indicate that V_{\max} of EgSERT is lower than that of the human SERT (** $p < 0.001$, t -test), meaning that the parasite transporter

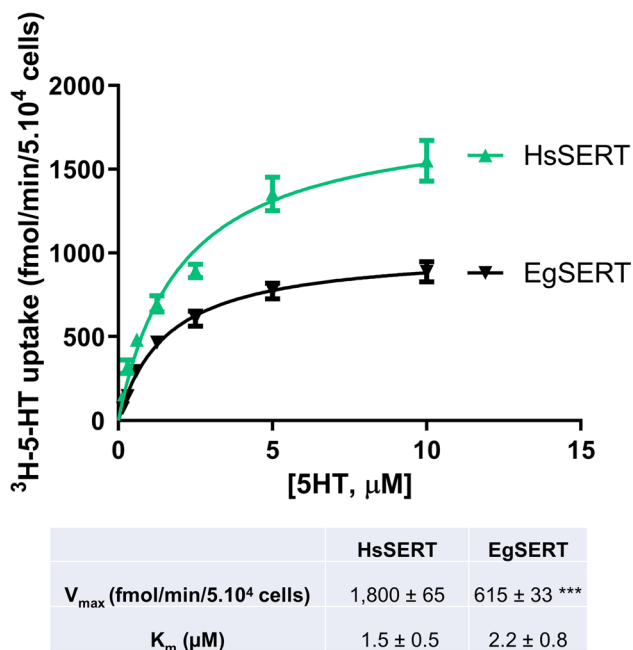


Fig. 5 Kinetic parameters of EgSERT, compared to HsSERT. This graph is representative of an average of four independent experiments. Data were fitted to the Michaelis–Menten equation using nonlinear regression. The V_{\max} and K_m values were determined to be 1800 ± 65 fmol/min/well and 1.5 ± 0.5 μM , respectively, for HsSERT, whereas for EgSERT, they were 615 ± 33 fmol/min/well and 2.2 ± 0.8 μM , respectively. V_{\max} of EgSERT is lower than that of HsSERT (** $p < 0.001$, t -test), and no statistical significance was found in K_m values using t -test

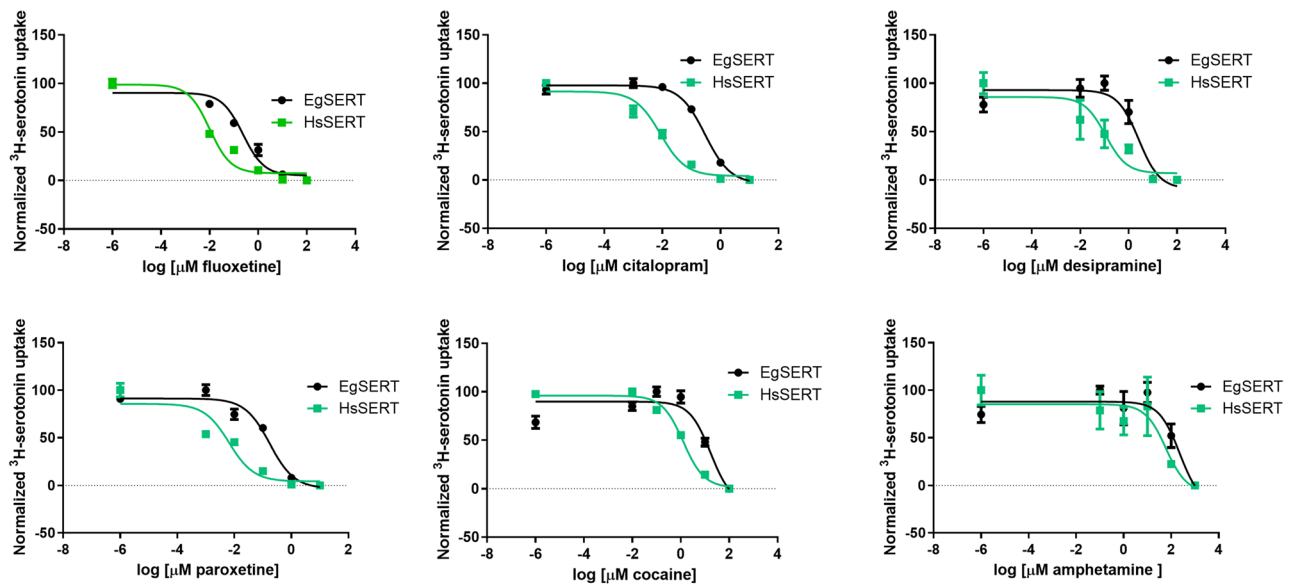
is not as efficient or not expressed at similar levels. However, the K_m values are similar, suggesting no significant change for the apparent affinity for serotonin, when compared to the human orthologue.

We were then interested in evaluating how typical monoamine transport inhibitors behave in EgSERT-mediated serotonin uptake assays. Dose–response assays (Fig. 6) demonstrated that fluoxetine, a selective serotonin reuptake inhibitor (SSRI), displayed an IC_{50} for HsSERT of ~ 37 nM, with tenfold less affinity for EgSERT. Citalopram (also a SSRI) displayed an IC_{50} for HsSERT of ~ 4 nM, with 90-fold less affinity for EgSERT. Desipramine, a tricyclic antidepressant (TCA) selective norepinephrine reuptake inhibitor (SNRI), which is also a weak HsSERT inhibitor and has $\alpha 1$ -blocking, antihistamine, and anticholinergic effects, had an IC_{50} for HsSERT of ~ 200 nM, with sevenfold lower affinity for EgSERT. Paroxetine, a SSRI with an IC_{50} for HsSERT of ~ 4 nM, showed 30-fold less affinity for EgSERT. Cocaine, a non-selective inhibitor of DAT, SERT, and NET, had an IC_{50} for HsSERT of ~ 1 μM , with \sim tenfold less affinity for EgSERT. Amphetamine, an inhibitor of DAT and NET and a weak inhibitor SERT, displayed an IC_{50} for HsSERT of ~ 47 μM and a threefold less affinity for EgSERT.

Whole mount in situ hybridization localization (WMISH) of the messenger for EgSERT in protoscolexes

We next studied the localization of the mRNA coding for the putative transporter EgSERT in protoscolexes of *E. granulosus* by WMISH (Fig. 7). The localization of *E. granulosus* EgSERT-positive cells is coincident with the location of each ganglion observed during descriptions of the serotonergic system in protoscolexes (Fig. 7B and D) (Camicia et al. 2013; Koziol et al. 2013). The EgSERT-positive cells were positioned in regions that could correspond to the lateral ganglion, the posterior lateral ganglion, the lateral nerve cords, and the medial nerve cords (Fig. 7D). In this report, the rostellar ganglia and the ganglia from the anterior ring were not observed. We do not observe positive cells for EgSERT using a sense probe for the same region of the transporter, suggesting that the signal obtained is specific (Fig. 7F).

To explore further the potential role of this transporter in the nervous system, co-localization studies were performed using an antibody against the neuropeptide FMRFamide, a well-studied neurotransmitter. The co-localization studies using confocal scanning laser microscope show a complementary pattern of staining in which the FMRFamide staining appears to represent axons of neuronal cells and the EgSERT-positive cells seem to be the somas of close neuronal cells (Fig. 7H and J). As in the previous images, no



Compound	HsSERT - IC ₅₀ +/- SEM (nM)	EgSERT - IC ₅₀ +/- SEM (nM)	Ratio EgSERT/HsSERT
Fluoxetine	37 ± 27	284 ± 44	7.6
Citalopram	4 ± 2	351 ± 39	87
Desipramine	185 ± 93	1334 ± 650	7
Paroxetine	4 ± 2	120 ± 27	30
Cocaine	1,400 ± 100	16,000 ± 5,000	11
Amphetamine	47,000 ± 11,000	133,000 ± 49,000	3

Fig. 6 Dose–response curves of several typical inhibitors mediated by HsSERT and EgSERT. IC₅₀ values are indicated in the tables for both transporters. Graphs are representative of at least four independent

experiments performed for each compound. The data was fitted using nonlinear regression, and results are normalized to control levels (vehicle, no inhibitor) in each group

signal for EgSERT was observed for protoscolecids obtained with the sense probe (Fig. 7L).

Discussion

This work presents for the first time a comprehensive study of a neurotransmitter transporter performed in a cestode parasite. The multiple sequence alignment, phylogeny, modeling, and functional studies presented here strongly suggest that the transporter identified here is indeed a serotonin transporter which belongs to the neurotransmitter:sodium symporter (NSS) family (TC N° 2.A.22) (Saier et al. 2014). The phylogenetic analysis grouped this transporter with other serotonin transporters from invertebrates. The prediction of additional orthologues for this transporter in other cestode species (i.e., EmSERT, HmSERT, MvSERT, and TsSERT), which are also grouped with EgSERT as invertebrate serotonin transporters in phylogenetic analyses, suggests that the function played by this transporter could be highly conserved in cestodes, probably playing an essential function in the parasite physiology.

The comparative pharmacology data suggest that EgSERT has lower affinity for all inhibitors examined, with the biggest difference being in the activity of citalopram, with a 90-fold decrease in the potency of the compound towards inhibition of serotonin uptake. This difference is lower than the affinity difference of the *S. mansoni* orthologue, SmSERT, compared with the affinity of HsSERT, that showed an eightfold decrease in affinity (Fontana et al. 2009). Another important difference was paroxetine, with a 30-fold decrease in the affinity of EgSERT compared with HsSERT. This difference was one order of magnitude lower when SmSERT is compared to HsSERT that was 4.5-fold lower (Fontana et al. 2009). The results obtained with citalopram and paroxetine suggest that EgSERT could have a higher degree of pharmacological divergence with the HsSERT than the SmSERT, compared with the mammalian transporter. This high difference in citalopram sensitivity between HsSERT and EgSERT could be attributed to some differences observed between both transporters in the citalopram binding site. One remarkable difference observed between both sequences was the replacement of tyrosine by phenylalanine at position 95 (position 80 in

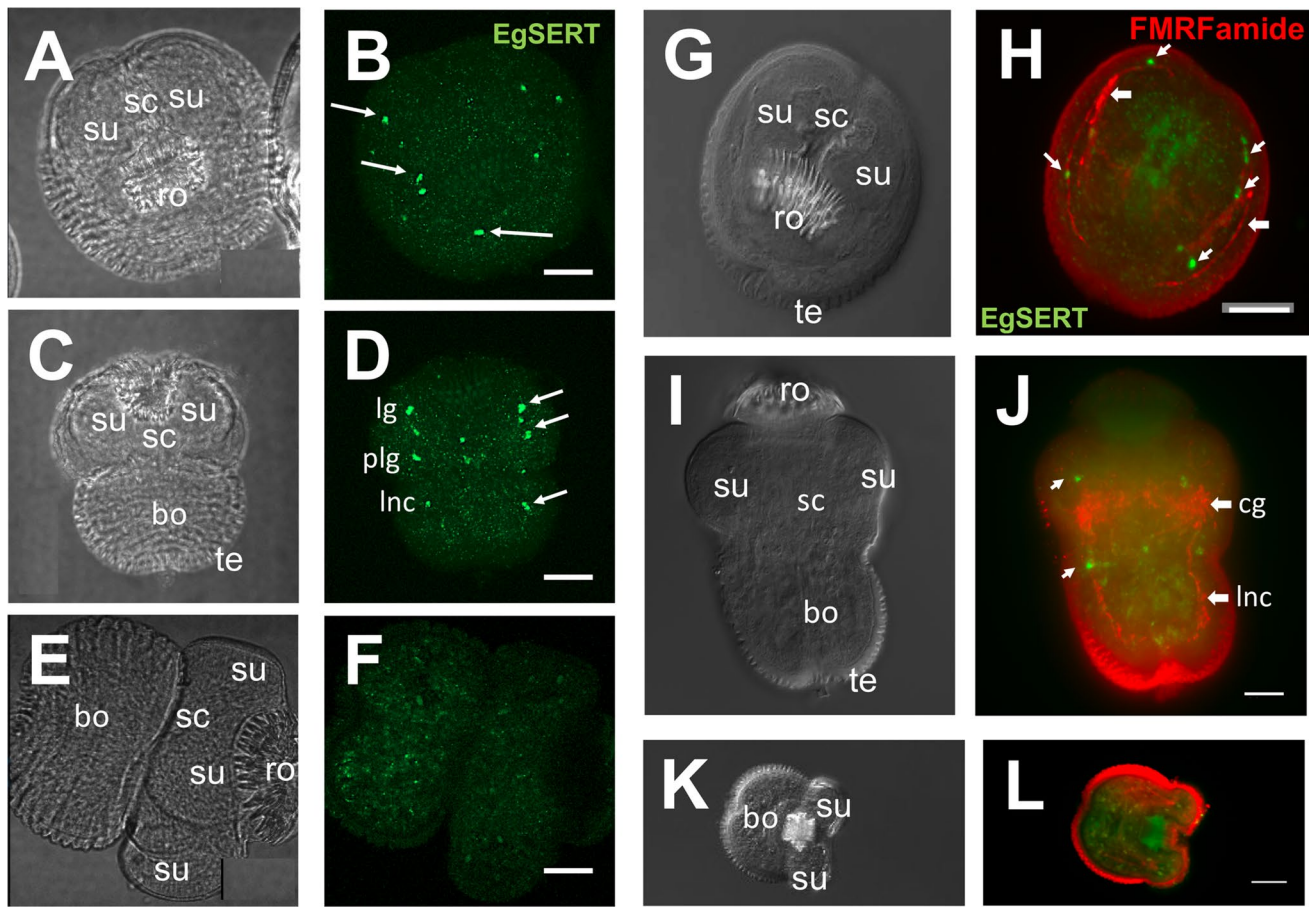


Fig. 7 EgSERT transporter localization in worm tissues of *Echinococcus granulosus* by confocal scanning laser microscopy, immunohistochemistry, and in situ hybridization techniques. **A to F** Localization of EgSERT by in situ hybridization. **A** Phase contrast of the invaginated protoscolex shown in **B**. **B** Invaginated protoscolex stained with an antisense probe complementary to EgSERT (green). **C** Phase contrast of the evaginated protoscolex shown in **D**. **D** Evaginated protoscolex stained with the same probe as in **B** (green). **E** Phase contrast of the evaginated protoscolex shown in **F**. **F** Evaginated protoscolex stained with the sense probe for EgSERT (negative control). **G to L** Co-localization of the messenger coding for EgSERT and the FMRFamide neuropeptide protein by in situ hybridization (in green) and immunohistochemistry (in red). **G** Phase contrast of the invaginated protoscolex shown in **H**. **H** Co-localization of the mRNA coding for EgSERT (green) and the FMRFamide protein (red) in the invaginated protoscolex. **I** Phase contrast of the evaginated protoscolex shown in **J**. **J** Co-localization of the mRNA coding for EgSERT (green) and the FMRFamide protein (red) in the evaginated protoscolex. **K** Phase contrast of the evaginated protoscolex shown in **L**. **L** Evaginated protoscolex stained with the sense probe for egstert (negative control) showing only the staining for the FMRFamide protein (red). Thin arrows show serotonergic ganglia and thick arrows show FMRFamide staining. For the phase contrast images, the tegument (te), body (bo), and scolex (sc) regions with sucker (su) and the rostellum (ro) were marked. For the fluorescent image in panel **D**, the lateral ganglia (lg), posterior lateral ganglia (plg), and lateral nerve cord-associated ganglia (lnc) were marked. For the fluorescent image in panel **J**, the cerebral ganglia (cg) and the lateral nerve cord (lnc) were marked. Scale bar 20 μ M

EgSERT). In a previous work by Andersen and coworkers (Andersen et al. 2010), it was found that the substitution of Y95F induces only a modest loss of potency (fourfold), but removal of the aromatic moiety has pronounced a negative effect on K_i suggesting that the aromatic moiety of Tyr-95, rather than the hydroxyl group, is important for citalopram inhibition. Another important difference was the replacement of the isoleucine in position 172 (position 157 in EgSERT) by threonine. This residue was reported as particularly relevant in citalopram binding (Andersen et al. 2010). The same authors reported that replacement

of isoleucine by methionine decreased K_i of citalopram by almost three orders of magnitude (Andersen et al. 2010). They hypothesized that the hydrophobic side chain of isoleucine 172 mediates a direct hydrophobic interaction between HsSERT and citalopram. This hypothesis could be extended to EgSERT to explain why the change of isoleucine by threonine in position 157 of EgSERT (which seems to perform a similar function to isoleucine 172 of HsSERT providing a more polar side chain) results in a lower affinity of citalopram to the cestode transporter compared to the human orthologue.

Paroxetine, the highest-affinity and one of the most selective SERT inhibitors known, displayed also an extremely reduced affinity by EgSERT compared with the human counterpart (Davis et al. 2016). The low affinity observed by this inhibitor could be attributed at least in part to the presence of the polar threonine (position 157 of EgSERT) in a critical position (homologous to 172 in HsSERT) that usually is reserved for non-polar and aliphatic side chain (Davis et al. 2016). The non-polar benzodioxol group could have some degree of repulsion with the polar hydroxyl group of threonine (Davis et al. 2016). Other important residues are threonine at position 497 (499 of EgSERT) and valine at position 501 (503 of EgSERT). The substitution of any of these residues was shown to affect paroxetine binding (Davis et al. 2016), specifically at the fluorophenyl region of the inhibitor. Regarding the allosteric site, we observed a low degree of conservation of most of residues involved in allosteric function, and we speculate if the cestode transporter could or could not display allosteric behavior like the human orthologue does. The high degree of protein divergence compared with the mammalian counterpart could also imply a pharmacological divergence, a topic which deserves further investigations.

In previous works of our group (Camicia et al. 2013), it was observed that citalopram at high concentrations (100 μM) has strong inhibitory effect in motility and development of protoscolexes of *E. granulosus* (Camicia et al. 2013; Herz and Brehm 2021). This observed effect in motility could be attributed to the inhibition of the EgSERT given that we can see an almost complete inhibition of serotonin transport at around 1 μM of citalopram (IC_{50} of 350 nM, Fig. 7). At 100 μM citalopram, the high 5-HT concentration in the synaptic cleft resulting from the complete EgSERT inhibition could lead to serotonin receptor overstimulation. This could cause the inhibition in motility by receptor desensitization or worm paralysis by neuromuscular overstimulation.

The citalopram effects on worm motility and development at high concentrations are also in line with studies performed with the trematode *S. mansoni* in which exogenously added citalopram (50 μM) had a detrimental effect on miracidial transformation rates (Taft et al. 2010).

Previous studies from Herz and Brehm (2021) have shown that exogenous serotonin induces metacystode vesicle formation and cell proliferation from primary cells of *E. multilocularis* metacystodes in a dose-dependent manner. The same authors have shown that paroxetine (100 μM), a well-known serotonin reuptake inhibitor, caused significant metacystode vesicle damage and cell viability reduction of primary cell culture. It is intriguing to hypothesize that paroxetine could inhibit EmSERT at this concentration given that according to our results its closest orthologue, EgSERT, has an IC_{50} around 120 nM

for paroxetine. These results strongly suggest that serotonin transport is crucial for the integrity of *E. multilocularis* metacystode vesicles and the viability of primary cells. Extrapolating these results to *E. granulosus*, we suggest that EgSERT inhibition could be directly valid for treatment of CE. In fact, we think that it could be of great interest to explore the effects of SSRIs studied here (and not only paroxetine) in the development of the metacystode from *Echinococcus* spp. species. Moreover, we expect that the effects on development could be correlated with the effect of SSRIs on transporter inhibition: a more potent transporter inhibition will cause stronger effects on development. These latter experiments could unveil the role of serotonin as a morphogen and the role of EgSERT as a key player in their non-neuronal and developmental effects. Taken together, these studies suggest that clinically used SERT inhibitors including SSRIs and TCAs target the parasite EgSERT and could therefore have therapeutic relevance for treating human CE.

Transcriptomic analysis showed that this transporter protein is highly expressed in the protoscolex larval stage but also in other stages from this parasite. We notice some similarities but also some differences between transcript levels of EgSERT and EmSERT. As it can be seen for EgSERT, the protoscolex is also the stage of *E. multilocularis* with higher expression of EmSERT (Herz and Brehm 2021). However, the metacystode stage has similar expression levels of EmSERT compared with the adult stage of *E. multilocularis*. This is a clear difference with *E. granulosus*, in which we can see a comparatively higher expression of EgSERT in the metacystode stage over the adult stage. The reason for such differences is unknown. Transport function could be important during the early setting of the larvae in the intestine of the definitive host. We hypothesize that the protoscolex larval stage needs the host's serotonin as a signal to move and navigate towards the Lieberkühn's cryptae to finally locate and firmly establish in the intestine of the final host. However, high levels of expression can also be seen in the metacystode and adult stages. The levels of transcript expression on these stages suggests that EgSERT could also be a target for CE and/or for definitive host treatment.

The localization of the messenger RNA coding for EgSERT suggests a major role of this transporter in the nervous system of the parasite. A similar pattern of staining was reported in Herz (Herz and Brehm 2021) working with *E. multilocularis*, in which discrete staining was observed in several of the major ganglia described in the serotonergic system. In *S. mansoni*, immunolocalization studies performed in the larval schistosomula stage showed a pattern of varicose immunoreactive fibers, forming what appeared to be concentric rings around the body of the parasite and located just below the surface (Patočka and Ribeiro 2013). In cestodes, it would be of interest to analyze the pattern of

EgSERT localization compared with serotonin localization in the protoscolex stage in whole mount studies.

One unresolved question is related to the fact that it is not known if the effects of serotonin on motility and development are mediated directly by receptors located on the surface of the worm or by internal receptors sensing serotonin transported from the surface by transporters located in the surface tegument (Patocka and Ribeiro 2013). The strong signals of EgSERT observed in internal ganglia suggest that EgERT is not superficial and its role is more related to neuronal function; however, more experiments like surface biotinylation, tegument proteomic analyses, or immunolocalization of non-permeabilized worms should be performed to answer this very interesting open question. Alternatively, the discovery and localization of new receptors from cestodes can provide another clue to answer this question.

Overall, the findings described here suggest that EgSERT transporter is a serotonin transporter with a major role in motor control and development and also an interesting potential target for drug therapy against CE.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00436-022-07466-y>.

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Declarations

Conflict of interest The authors declare no competing interests.

References

- Aggarwal S, Liu X, Rice C, Menell P, Clark PJ, Papparoidamis N, Xiao YC, Salvino JM, Fontana ACK, España RA, Kortagere S, Mortensen OV (2019) Identification of a novel allosteric modulator of the human dopamine transporter. *ACS Chem Neurosci* 10:3718–3730
- Aggarwal S, Cheng MH, Salvino JM, Bahar I, Mortensen OV (2021) Functional characterization of the dopaminergic psychostimulant sydnocarb as an allosteric modulator of the human dopamine transporter. *Biomedicines* 9
- Andersen J, Olsen L, Hansen KB, Taboureau O, Jorgensen FS, Jorgensen AM, Bang-Andersen B, Egebjerg J, Stromgaard K, Kristensen AS (2010) Mutational mapping and modeling of the binding site for (S)-citalopram in the human serotonin transporter. *J Biol Chem* 285:2051–2063
- Arnold K, Bordoli L, Kopp J, Schwede T (2006) The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* 22:195–201
- Benkert P, Tosatto SC, Schomburg D (2008) QMEAN: a comprehensive scoring function for model quality assessment. *Proteins* 71:261–277
- Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Gallo Cassarino T, Bertoni M, Bordoli L, Schwede T (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res* 42:W252–258
- Boyle JP, Zaide JV, Yoshino TP (2000) Schistosoma mansoni: effects of serotonin and serotonin receptor antagonists on motility and length of primary sporocysts in vitro. *Exp Parasitol* 94:217–226
- Buck KJ, Amara SG (1994) Chimeric dopamine-norepinephrine transporters delineate structural domains influencing selectivity for catecholamines and 1-methyl-4-phenylpyridinium. *Proc Natl Acad Sci USA* 91:12584–12588
- Budke CM, White AC Jr, Garcia HH (2009) Zoonotic larval cestode infections: neglected, neglected tropical diseases? *PLoS Neglect Trop Dis* 3:e319
- Camicia F, Herz M, Prada LC, Kamenetzky L, Simonetta SH, Cucher MA, Bianchi JI, Fernández C, Brehm K, Rosenzvit MC (2013) The nervous and pre-nervous roles of serotonin in *Echinococcus* spp. *Int J Parasitol* 43:647–659
- Camicia F, Celentano AM, Johns ME, Chan JD, Maldonado L, Vaca H, Di Siervi N, Kamentzky L, Gamo AM, Ortega-Gutierrez S, Martin-Fontecha M, Davio C, Marchant JS, Rosenzvit MC (2018) Unique pharmacological properties of serotonergic G-protein coupled receptors from cestodes. *PLoS Neglect Trop Dis* 12:e0006267
- Coleman JA, Green EM, Gouaux E (2016) X-ray structures and mechanism of the human serotonin transporter. *Nature* 532:334–339
- Colovos C, Yeates TO (1993) Verification of protein structures: patterns of nonbonded atomic interactions. *Protein Sci* 2:1511–1519
- Cucher M, Prada L, Mourglia-Ettlin G, Dematteis S, Camicia F, Asurmendi S, Rosenzvit M (2011) Identification of *Echinococcus granulosus* microRNAs and their expression in different life cycle stages and parasite genotypes. *Int J Parasitol* 41:439–448
- Davis BA, Nagarajan A, Forrest LR, Singh SK (2016) Mechanism of paroxetine (Paxil) inhibition of the serotonin transporter. *Sci Rep* 6:23789
- Day TA, Bennett JL, Pax RA (1994) Serotonin and its requirement for maintenance of contractility in muscle fibres isolated from *Schistosoma mansoni*. *Parasitology* 108(Pt 4):425–432
- Demchshyn LL, Pristupa ZB, Sugamori KS, Barker EL, Blakely RD, Wolfgang WJ, Forte MA, Niznik HB (1994) Cloning, expression, and localization of a chloride-facilitated, cocaine-sensitive serotonin transporter from *Drosophila melanogaster*. *Proc Natl Acad Sci USA* 91:5158–5162
- Finn RD, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, Lassmann T, Moxon S, Marshall M, Khanna A, Durbin R, Eddy SR, Sonnhammer EL, Bateman A (2006) Pfam: clans, web tools and services. *Nucleic Acids Res* 34:D247–251
- Fontana ACK, Sonders MS, Pereira-Junior OS, Knight M, Javitch JA, Rodrigues V, Amara SG, Mortensen OV (2009) Two allelic isoforms of the serotonin transporter from *Schistosoma mansoni* display electrogenic transport and high selectivity for serotonin. *Eur J Pharmacol* 616:48–57
- Garcia HH (2018) Neurocysticercosis. *Neurol Clin* 36:851–864
- Gottstein B, Stojkovic M, Vuitton DA, Millon L, Marcinkute A, Deplazes P (2015) Threat of alveolar echinococcosis to public health—a challenge for Europe. *Trends Parasitol* 31:407–412
- Hemphill A, Stadelmann B, Rufener R, Spiliotis M, Boubaker G, Müller J, Müller N, Gorgas D, Gottstein B (2014) Treatment of echinococcosis: albendazole and mebendazole – what else? *Parasite* 21:70
- Herz M, Brehm K (2021) Serotonin stimulates *Echinococcus multilocularis* larval development. *Parasit Vectors* 14:14
- Hillman GR, Senft AW (1973) Schistosome motility measurements: response to drugs. *J Pharmacol Exp Ther* 185:177–184

- Hoffman BJ, Hansson SR, Mezey E, Palkovits M (1998) Localization and dynamic regulation of biogenic amine transporters in the mammalian central nervous system. *Front Neuroendocrinol* 19:187–231
- Home, W.P., 2020. *Echinococcus canadensis*, pp. The cestode *Echinococcus canadensis* belongs to the complex *Echinococcus granulosus sensu lato*. This parasite is a member of Cyclophyllidea which comprise the majority of tapeworms that are of medical importance. Adult *E. canadensis* parasitise the small intestines of dogs and other canids. The larval stage is one of the causative agents of the serious and life-threatening human disease cystic echinococcosis. *E. canadensis* has a worldwide distribution
- Horton RJ (1997) Albendazole in treatment of human cystic echinococcosis: 12 years of experience. *Acta Trop* 64:79–93
- Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ (2015) The Phyre2 web portal for protein modeling, prediction and analysis. *Nat Protoc* 10:845–858
- Kortagere S, Fontana AC, Rose DR, Mortensen OV (2013) Identification of an allosteric modulator of the serotonin transporter with novel mechanism of action. *Neuropharmacology* 72:282–290
- Kozioł U, Krohne G, Brehm K (2013) Anatomy and development of the larval nervous system in *Echinococcus multilocularis*. *Front Zool* 10:24
- Kozioł U, Rauschendorfer T, Zanon Rodriguez L, Krohne G, Brehm K (2014) The unique stem cell system of the immortal larva of the human parasite *Echinococcus multilocularis*. *EvoDevo* 5:10
- Kyung SY, Cho YK, Kim YJ, Park JW, Jeong SH, Lee JI, Sung YM, Lee SP (2011) A paragonimiasis patient with allergic reaction to praziquantel and resistance to triclabendazole: successful treatment after desensitization to praziquantel. *Korean J Parasitol* 49:73–77
- Larsen MB, Fontana AC, Magalhaes LG, Rodrigues V, Mortensen OV (2011) A catecholamine transporter from the human parasite *Schistosoma mansoni* with low affinity for psychostimulants. *Mol Biochem Parasitol* 177:35–41
- Lee JM, Lim HS, Hong ST (2011) Hypersensitive reaction to praziquantel in a clonorchiasis patient. *Korean J Parasitol* 49:273–275
- Mansour TE (1984) Serotonin receptors in parasitic worms. *Adv Parasitol* 23:1–36
- McVeigh P, Maule AG (2019) Can CRISPR help in the fight against parasitic worms? *eLife* 8
- Navratna V, Gouaux E (2019) Insights into the mechanism and pharmacology of neurotransmitter sodium symporters. *Curr Opin Struct Biol* 54:161–170
- Osloobi N, Webb RA (1999) Localization of a sodium-dependent high-affinity serotonin transporter and recruitment of exogenous serotonin by the cestode *Hymenolepis diminuta*: an autoradiographic and immunohistochemical study. *Can J Zool* 77:1265–1277
- Patocka N, Ribeiro P (2007) Characterization of a serotonin transporter in the parasitic flatworm, *Schistosoma mansoni*: cloning, expression and functional analysis. *Mol Biochem Parasitol* 154:125–133
- Patocka N, Ribeiro P (2013) The functional role of a serotonin transporter in *Schistosoma mansoni* elucidated through immunolocalization and RNA interference (RNAi). *Mol Biochem Parasitol* 187:32–42
- Pax RA, Day TA, Miller CL, Bennett JL (1996) Neuromuscular physiology and pharmacology of parasitic flatworms. *Parasitology* 113(Suppl):S83–96
- Plenge P, Shi L, Beuming T, Te J, Newman AH, Weinstein H, Gether U, Loland CJ (2012) Steric hindrance mutagenesis in the conserved extracellular vestibule impedes allosteric binding of antidepressants to the serotonin transporter. *J Biol Chem* 287:39316–39326
- Plenge P, Yang D, Salomon K, Laursen L, Kalenderoglou IE, Newman AH, Gouaux E, Coleman JA, Loland CJ (2021) The antidepressant drug vilazodone is an allosteric inhibitor of the serotonin transporter. *Nat Commun* 12:5063
- Rahman MS, Mettrick DF, Podesta RB (1985) *Schistosoma mansoni*: effects of in vitro serotonin (5-HT) on aerobic and anaerobic carbohydrate metabolism. *Exp Parasitol* 60:10–17
- Ranganathan R, Sawin ER, Trent C, Horvitz HR (2001) Mutations in the *Caenorhabditis elegans* serotonin reuptake transporter MOD-5 reveal serotonin-dependent and -independent activities of fluoxetine. *J Neurosci* 21:5871–5884
- Ribeiro P, Patocka N (2013) Neurotransmitter transporters in schistosomes: structure, function and prospects for drug discovery. *Parasitol Int* 62:629–638
- Ritler D, Rufener R, Sager H, Bouvier J, Hemphill A, Lundstrom-Stadelmann B (2017) Development of a movement-based in vitro screening assay for the identification of new anti-cestodal compounds. *PLoS Negl Trop Dis* 11:e0005618
- Rudnick G, Sandtner W (2019) Serotonin transport in the 21st century. *J Gen Physiol* 151:1248–1264
- Rudnick G, Krämer R, Blakely RD, Murphy DL, Verrey F (2014) The SLC6 transporters: perspectives on structure, functions, regulation, and models for transporter dysfunction. *Pflugers Arch* 466:25–42
- Saier MH Jr, Reddy VS, Tamang DG, Vastermark A (2014) The transporter classification database. *Nucleic Acids Res* 42:D251–258
- Shi L, Quick M, Zhao Y, Weinstein H, Javitch JA (2008) The mechanism of a neurotransmitter: sodium symporter–inward release of Na⁺ and substrate is triggered by substrate in a second binding site. *Mol Cell* 30:667–677
- Taft AS, Norante FA, Yoshino TP (2010) The identification of inhibitors of *Schistosoma mansoni* miracidial transformation by incorporating a medium-throughput small-molecule screen. *Exp Parasitol* 125:84–94
- Vuitton DA, McManus DP, Rogan MT, Romig T, Gottstein B, Naidich A, Tuxun T, Wen H, Menezes da Silva A (2020) International consensus on terminology to be used in the field of echinococcoses. *Parasite* 27:41
- World Health, O. (2012) Accelerating work to overcome the global impact of neglected tropical diseases: a roadmap for implementation: executive summary. World Health Organization, Geneva
- Zhang Y, Skolnick J (2005) TM-align: a protein structure alignment algorithm based on the TM-score. *Nucleic Acids Res* 33:2302–2309
- Zheng H, Zhang W, Zhang L, Zhang Z, Li J, Lu G, Zhu Y, Wang Y, Huang Y, Liu J, Kang H, Chen J, Wang L, Chen A, Yu S, Gao Z, Jin L, Gu W, Wang Z, Zhao L, Shi B, Wen H, Lin R, Jones MK, Brejova B, Vinar T, Zhao G, McManus DP, Chen Z, Zhou Y, Wang S (2013) The genome of the hydatid tapeworm *Echinococcus granulosus*. *Nat Genet* 45:1168–1175
- Zhou Z, Zhen J, Karpowich NK, Goetz RM, Law CJ, Reith ME, Wang DN (2007) LeuT-desipramine structure reveals how antidepressants block neurotransmitter reuptake. *Science* 317:1390–1393

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