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The functional role of a serotonin transporter in *Schistosoma mansoni* elucidated through immunolocalization and RNA interference (RNAi)

Nicholas Patocka, Paula Ribeiro*

Institute of Parasitology, McGill University, Macdonald Campus, 21,111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec, Canada H9X 3V9

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ABSTRACT

Serotonin is an important neurotransmitter in both vertebrates and invertebrates. In the parasitic flatworm, *Schistosoma mansoni*, serotonin stimulates worm movement and potentiates muscle contraction. A specific serotonin transporter (SmSERT) was previously cloned from *S. mansoni* and characterized *in vitro*. Here we conduct a first investigation of the native protein in the worm so as to elucidate the biological role of SmSERT and to assess its drug targeting potential. Confocal immunofluorescence studies using specific antibodies determined that SmSERT is expressed predominantly in the nervous system both in adult worms and larvae (schistosomula). SmSERT immunoreactivity was detected in the main nerve cords of the central nervous system and the peripheral nerve plexus of the body wall in adult males and females, in apparent nerve endings of the male tubercles and possibly the male tegument. In the larvae, SmSERT localized mainly to the peripheral nerve plexus of the body wall. Co-localization experiments showed that the pattern of SmSERT expression coincides with that of serotonin itself, suggesting that SmSERT is present in serotonergic neurons. To test whether SmSERT is involved in the motor effects of serotonin, we treated *S. mansoni* schistosomula with SmSERT blockers or SmSERT-specific short-interfering RNAs (siRNAs) and then recorded larval motility, using a quantitative imaging assay. In both cases, the treatment produced a strongly hyperactive phenotype, corresponding to a ~3-fold increase in larval motility, roughly the same effect as treatment with an excess of exogenous serotonin. The siRNA effect correlated with a ≈50% decrease in expression of the SmSERT when tested by real-time qPCR. To test if SmSERT mediates transport of exogenous serotonin across the tegument, uptake assays were also performed in intact schistosomula treated with SmSERT siRNAs or an irrelevant siRNA. We found a significant but modest decrease (~25%) in serotonin uptake in the siRNA-suppressed larvae when compared to the negative controls. These results suggest that the SmSERT's function is primarily neuromuscular and may also play a secondary role in the uptake of exogenous (host-derived) serotonin.

1. Introduction

Schistosomiasis is a debilitating disease that afflicts over 200 million people worldwide. In the absence of a vaccine for any of the helminth infections, control relies exclusively on the use of chemotherapeutics. The disease is caused by infection with one of three major species of *Schistosoma*, the majority of cases (>80%) being attributed to *Schistosoma mansoni*. Infection begins when the free-living cercariae penetrate the skin of the host and over a period of 6–8 weeks will migrate throughout the system, eventually taking residence in the hepatic portal veins. The migration in the host, along with many other fundamental behaviors, are controlled in part by the worm's nervous system through a variety of neurotransmitters and associated proteins. Some of these signalling proteins are highly divergent compared to those of the host, which makes them exciting candidates for selective drug targeting.

Serotonin (5-hydroxytryptamine: 5HT) is one of the most abundant neuroactive substances in the schistosome nervous system. Immunolocalization studies in both larval and adult stages have shown that serotonin is present in the major nerve cords and transverse commissures that run the length of the body, as well as the peripheral nervous system, including the innervation of the suckers, the subtegumental and submuscular nerve nets of the body wall and numerous sensory nerve fibers that connect to the surface of the worm [1–4]. Serotonin is a well known neurotransmitter/neuromodulator of many animal phyla and it has important myoexcitatory effects in all the flatworms, including schistosomes. Evidence dating back to the 1970s has shown that serotonin stimulates worm movement when added exogenously to cultured *S. mansoni* sporocysts or adult worms [5–8], it potentiates muscle contraction in preparations of isolated muscle fibers [9] and it also stimulates glycogenolysis and glucose utilization

* Corresponding author. Tel.: +1 514 398 7607; fax: +1 514 398 7857.
E-mail address: paula.ribeiro@mcgill.ca (P. Ribeiro).

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in crude worm extracts [10,11], thus making more energy available for muscle contraction. How serotonin exerts its stimulatory effects remains unclear. In the mammalian nervous system, serotonergic signalling is accomplished through the activation of specific receptors, typically G protein-coupled receptors (GPCR), which are found on post-synaptic membranes and trigger changes in the levels of downstream effectors such as cAMP or Ca^{2+}. To terminate the signalling, serotonin is rapidly sequestered into neurons or adjacent glial cells by a specific serotonin transporter (SERT), whereupon the amine may be broken down enzymatically or recycled for later use. In schistosomes there has yet to be a receptor identified that can be linked to any of the behavioral or biochemical effects of serotonin. There are, however, at least two predicted serotonergic GPCRs in the *S. mansoni* genome, along with many of the proteins required for cAMP and Ca^{2+}-mediated signalling [12,13]. Moreover, other components of the serotonergic system have been identified, including the biosynthetic enzyme, tryptophan hydroxylase [14] and, more recently, a SERT-like serotonin transporter [15,16], suggesting that all major elements of serotonin biosynthesis, signalling and inactivation are conserved in the parasite.

SERTs belong to the superfamily of sodium-dependent, solute carrier (SLC6) plasma membrane transporters that also include carriers for dopamine and noradrenaline neurotransmitters. They have a characteristic topology consisting of 12 transmembrane domains and intracellular N- and C-termini [17,18]. The *S. mansoni* SERT (SmSERT) shares this classical topology and many of the key residues for solute binding are also conserved. There are at least two variants of this protein in *S. mansoni*, which are derived from the same gene. The two isoforms differ by 78 amino acids at the N-terminal end but are otherwise identical and are both active when expressed heterologously in mammalian cells [15,16]. Transport assays showed that the SmSERT has high affinity for serotonin and responds well to classical selective serotonin reuptake inhibitors (SSRI) that normally target SERTs, including fluoxetine (Prozac) and tricyclic antidepressants. However the relative potencies of these drugs toward the schistosome transporter differed substantially from their known effects on mammalian SERTs, an indication of the great evolutionary distance between the host and parasite proteins. Moreover, SSRIs were found to have potent anti-schistosomal effects in two recent drug screens [19,20], suggesting that SmSERT could be a good candidate for selective drug targeting.

Although SmSERT has been well characterized *in vitro*, very little is known about the biological function of the native protein, where it is expressed in the worm or how it contributes to the control of serotonin activity. SmSERT could have more than one function depending on where it is expressed. If present in serotonin neurons or glial-like storage sites, SmSERT would be expected to mediate serotonin reuptake and inactivation, similar to what has been reported in the mammalian CNS [17,18]. On the other hand, if SmSERT is present on the parasite surface, it could be used to transport exogenous (host-derived) serotonin across the tegument. The latter possibility is of considerable interest because schistosomes live in a serotonin-rich environment (*i.e.* blood) and there is compelling biochemical evidence that the worm can take up exogenous serotonin *via* a saturable transport system [21–23]. Moreover the uptake of exogenous serotonin was reduced when the worms were treated with classical SERT inhibitors, suggesting the mode of transport was through a SERT-like transporter. Researchers have speculated that schistosomes downregulate their own serotonin biosynthesis once they invade the host [14,23] and exogenous recruitment may be required to supplement internal stores of serotonin when endogenous production is insufficient. However, the importance of this transport activity has never been elucidated, nor do we know if SmSERT is the carrier involved.

Here we conduct a first investigation of the tissue expression pattern and biological role of SmSERT in *S. mansoni*. The results suggest that SmSERT is unlikely to play a major role in the tegumental transport of exogenous serotonin. Instead, the evidence identifies SmSERT predominantly as a neuronal transporter and a key player in the serotonergic control of parasite motility.

2. Materials and methods

2.1. Parasites

A Puerto Rican strain of *Schistosoma mansoni* was used for all experiments in this study. *Biomphalaria glabrata* snails infected with *S. mansoni* were kindly provided by Dr. F. Lewis, Biomedical Research Institute (BRI) and BEI Resources, USA. Snails were cultured for 6–8 weeks, after which cercarial shedding was induced by exposure to light for 2 h at room temperature. The cercariae were transformed to schistosomula *in vitro*, using the mechanical shearing method [24–27] and cultured in Opti-MEM (Gibco) supplemented with 4% serum, 0.25 μg/ml fungizone, 100 μg/ml streptomycin and 100 units/ml penicillin in 24 well plates at approximately 200 animals/well. Adult worms were obtained by infecting CD1 female mice with cercariae by active penetration. 7–8 weeks post infection, mice were sacrificed and adult worms were collected by perfusion of livers and mesenteric veins, as described [28,29].

2.2. Antibody production

A polyclonal anti-SmSERT antibody was produced in rabbits against two synthetic peptides (Twenty first Century Biochemicals, Malboro, USA). Peptide 1 (CPEDRPAYGDQQITLNA) was located in the C-terminal region of SmSERT, and peptide 2 (CLNAVGEYVPKKTFKTNKL) was located just following peptide 1. Both peptides were conjugated to ovalbumin as a carrier and tested for specificity using BLAST analysis against the Schistosome Genome Database and the general protein database at NCBI. Recognition of the peptides was tested using an ELISA test and the antiserum was found to be of high titer. The IgG fraction specific to SmSERT was purified using peptide conjugated beads (Sigma), according to standard procedures, and the eluted fractions were tested again by ELISA. The anti-5HT antibody was a commercial monoclonal antibody (Abcam) raised in rat and used for localization of serotonin.

2.3. Western blot analysis

The affinity-purified anti-SmSERT antibody was tested by western blot analysis against the recombinant protein expressed in HEK 293 cells. SmSERT was previously cloned in our lab [15] and shown to be functional in HEK 293 cells. For western blotting, the full length SmSERT was cloned into the pCRUZ expression vector (Santa Cruz Biotechnology) to express a fusion protein that contained an N-terminal GFP tag. HEK 293 cells were seeded in 10 cm culture dishes at a density of 1.5×10^6 cells/dish. Cells were allowed to grow overnight at 37 °C 5%CO_2 and then transfected with 3 μg of either pCRUZ-SmSERT or empty plasmid (mock-transfected control), using Fugene6 transfecting agent, following the manufacture's recommendations. The cells were grown for 48 h after which the cells were lysed and a solubilized membrane fraction was prepared using a commercial kit (ProteoExtract® Native Membrane Protein Extraction Kit, Calbiochem), as described in the kit protocol. Protein was quantified using Lowry assays (Sigma) and samples were loaded on 4–12% Tris–glycine gels (Invitrogen) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Blots were probed using affinity-purified anti-SmSERT antibodies (1:1000 dilution) and HRP-conjugated goat anti-rabbit secondary