Identification of a Serotonin Transporter and Serotonin Receptor in *Schistosoma mansoni*: A Step Toward Better Understanding the Serotonergic System

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Abstract

Among the top list of neglected tropical diseases sits schistosomiasis, a parasitic disease caused by the flatworm *Schistosoma*. The disease involves an intermediate snail host and the definitive human host with infection taking place through the skin in contaminated water, one of the reasons for its wide distribution. The diversity of the life cycle stages and the ability of the parasite to have adapted to quite different environments speak to the complexity of the organism. The underlying system that coordinates and controls all biological functions of the parasite is its nervous system. Signalling in the nervous system is accomplished by the release of neurotransmitters and signal transduction through specific receptors. Of the many neurotransmitters identified thus far in the parasite, serotonin (5-hydroxytryptamine: 5HT) is one of the most abundant. 5HT has been implicated in several biological functions in flatworms, including muscle contraction, motility and metabolism, but its mode of action is poorly understood. Here we provide molecular evidence for a serotonin-specific transporter (SERT) in *Schistosoma mansoni* as well as a serotonin specific receptor in the same organism. The transporter (SmSERT) was shown to share similar topology to SERTs from other organisms, namely 12 transmembrane regions with several predicted glycosylation sites. In a heterologous expression system, we show that it mediates the uptake of serotonin in a dose-dependent manner and is responsive to classical SERT inhibitors. The transporter is expressed in all life cycle stages tested (cercaria, schistosomulum, and adult) with upregulation occurring in the parasitic stages. Immunolocalization studies showed that the protein is expressed primarily in the nervous system of the parasite, both in central and peripheral neuronal structures. Using RNA interference (RNAi) to knockdown expression, we found strong increases in motility, suggesting that its primary function is in termination of serotonin signalling. The serotonin receptor (Sm5HTr) is the first as such cloned from parasitic flatworms. It shares homology with other 5HT receptors and most closely identifies with the 5HT7 class of receptors. Expression in mammalian cells found that it signals through upregulation of cAMP and not Ca^{2+}. Immunolocalization in adults and larvae found that the receptor is enriched in the central and

peripheral nervous system. Additional pharmacological assays using Sm5HTr agonists and antagonists suggest that the receptor may contribute to 5HT-induced stimulation of worm motility. Combined, these results show that there is a fully functional serotonergic system in the parasite *S. mansoni* and that these proteins play an important role in controlling signal transduction. The wide distribution of these proteins in the parasite nervous system and their apparent involvement in motor control makes them tempting targets for drug design.

Abrégé

Parmi la liste du haut des maladies tropicales négligées est assis la schistosomiase, une maladie parasitaire causée par le *Schistosoma*. La maladie implique un mollusque intermédiaires et hôte définitif de l'homme avec une infection en cours à travers la peau dans l'eau contaminée, l'une des raisons de sa large diffusion. La diversité des étapes du cycle de vie et de la capacité du parasite à avoir adapté à des environnements très différents parlent de la complexité de l'organisme. Le système sous-jacent qui coordonne et contrôle toutes les fonctions biologiques du parasite est son système nerveux. Signalisation dans le système nerveux est accompli par la libération des neurotransmetteurs et transduction du signal par récepteurs spécifiques. Parmi des nombreux neurotransmetteurs identifiés jusqu'à présent dans le parasite, la sérotonine (5-hydroxytryptamine : 5-HT) est l'un des plus abondants. 5HT a été impliqué dans plusieurs fonctions biologiques dans les vers plats, notamment contraction musculaire, la motilité et le métabolisme, mais son mode d'action est mal comprise. Ici nous fournir preuve moléculaire d'une transporteur de la sérotonine spécifique (SERT) au *Schistosoma mansoni* ainsi qu'un récepteur de la sérotonine spécifique dans le même organisme. Le transporteur (SmSERT) a été montré à partager la même topologie à SERT provenant d'autres organismes, à savoir 12 régions transmembranaire avec plusieurs sites de glycosylation prédits. Dans une expression hétérologue système, nous montrons qu'il négocie l'absorption de la sérotonine de manière dose-dépendante et est sensible aux inhibiteurs de SERT classiques. Le transporteur est exprimé dans toutes les étapes du cycle de vie testé (cercaria, schistosomula et adulte) avec regulation à la hausse survenant dans les stades parasitaires. Études immunolocalisation ont montré que la protéine est exprimée principalement dans le système nerveux du parasite, à la fois central et périphérique dans les structures neuronales. L'interférence ARN (ARNi) de rabattement d'expression, nous avons trouvé une forte augmentation de la motilité, suggérant que sa fonction principale est en cessation de signalisation de la sérotonine. Le récepteur de la sérotonine (Sm5htr) est le premier en tant que telle cloné à partir de vers plats parasitaires. Il partage homologie avec d'autres récepteurs 5HT et plus étroitement s'identifie à la classe des récepteurs 5-HT7. Expression dans des cellules de mammifères a constaté qu'il signale une

regulation positive de l'AMPc et non de Ca^{2+}. Immunolocalisation chez les adultes et les larves découvert que le récepteur est enrichi dans le système nerveux central et périphérique. D'autres essais pharmacologiques utilisant des agonistes et antagonistes suggèrent que le récepteur peut contribuer à la stimulation de la motilité du ver induite par la 5HT. Ensemble, ces résultats montrent qu'il existe un système sérotoninergique pleinement fonctionnel dans le parasite S. mansoni et que ces protéines jouent un rôle important dans le contrôle transduction du signal. La large diffusion de ces protéines dans le parasite système nerveux et leur implication apparente de commande de moteur fait d'eux des cibles tentantes dans la conception des médicaments.

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THESIS OFFICE STATEMENT

Candidates have the option, **subject to the approval of their Department**, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or clearly-duplicated text of the published paper(s) provided that these copies are bound as an integral part of the thesis.

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Additional material (procedural and design data, as well as description of the equipment used) must be provided where appropriate and in sufficient detail (*e.g.* in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

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STATEMENT OF CONTRIBUTIONS

The experimental work presented in this thesis was designed and performed by the author under the supervision of Dr. Paula Ribeiro, who provided financial support for the laboratory work and was involved in the experimental design, data presentation and pre-editing of the thesis and manuscripts.

STATEMENT OF ORIGINALITY

The following aspects presented in this thesis are considered original contributions to knowledge:

Manuscript I:

Here we provide the first evidence for a serotonin-specific transporter in *Schistosoma mansoni* (SmSERT). Sequence analysis shows that it has similar features to SERTs from other organisms, namely 12 transmembrane domains, intracellular facing N- and C-termini with several predicted glycosylation sites. Among the main differences in the parasite sequence are the longer N-terminal and a large insertion in the second extracellular loop (EL2) and a few non-conservative substitutions in key binding residues. Expression in mammalian cells shows that SmSERT is capable of saturable transport of serotonin with a K_i and V_{max} very similar to other characterized SERTs. SmSERT was specific to serotonin as all other biogenic amines tested showed no measurable transport. Incubation with known mammalian SERT inhibitors was able to block transport of serotonin but their potency was 10-fold less than in human SERT. Expression at the RNA level was detected in schistosomula, adults and cercariae, with lower concentrations in the cercaria. 5HT transport assays in cultured schistosomula showed similar pharmacological profile to that of recombinant SmSERT *in vitro*, suggesting that 5HT uptake may be mediated by this transporter. This was the first example of a SERT in parasitic flatworms and puts forth the idea that it may be responsible for 5HT recruitment *in vivo*.

Manuscript II:

Continuing from manuscript I, we pursue the investigation of SmSERT and its role in the parasite. Using a combination of immunofluorescence and RNA interference (RNAi), we tried to elucidate the biological function of the transporter. We show that antibodies raised against two peptides of the SmSERT are able to recognize a single band in Western blots from protein extracts from both adult worms and mammalian cells expressing SmSERT. Negative controls do

not show this band suggesting that it is specifically recognizing SmSERT. Immunofluorescence assays in larvae and adults show that the protein is localized primarily to the nervous system. Co-localization with serotonin showed that SmSERT colocalized with 5HT suggesting that it is expressed in serotonergic nerve fibres. In adults, we see the main nerve cords, transverse commissures and neurons innervating the oral sucker stained positively for SmSERT expression. There is also expression associated with the tubercules in adult males and the subtegumental nerve net both in adults and in the larvae. Behavioural assays using RNAi to silence expression of the SmSERT showed a robust 3-fold increase in motility, which correlated with significant decrease in SmSERT mRNA levels as determined by quantitative RT-qPCR. This increase in motility is similar to what is seen when untreated animals are incubated with either 5HT alone, or if animals are treated with serotonin-specific reuptake inhibitors (SSRIs). 5HT uptake assays in RNAi-silenced animals show a significant but modest 25% decrease in exogenous 5HT intake. These findings suggest that the SmSERT has a primary role of inactivating endogenous neuronal serotonin, thereby terminating serotonin-induced motor activity. It may also play a secondary role in the recruitment of exogenous (host-derived) 5HT.

Manuscript III:

Here we examine another important component of the serotonergic system, its receptors. This manuscript reports the first characterization of a serotonin receptor in *Schistosoma mansoni* (Sm5HTr). The gene was identified from the genome database cloned and the sequence confirmed. Sequence analysis showed that it shared the characteristics of the G protein-coupled receptors (GPCRs). When compared to other GPCRs, it most resembled serotonin receptors from other species, notably the 5HT7 receptors. 5HT7 receptors typically signal through the upregulation of cAMP and not Ca^{2+}. Activity was tested in mammalian cells first through measurement of intracellular Ca^{2+}. We found treatment with serotonin showed no changes in Ca^{2+} levels. We next measured intracellular cAMP levels following treatment with 5HT. We found that 5HT was able to increase cAMP in cells expressing the Sm5HTr, consistent with a 5HT7-like mechanism of signalling. Sm5HTr was also responsive to *o*-methyl-5HT but did not recognize other biogenic amines or amino acid precursors. The methylated form was a

strong agonist, having a Vmax higher than 5HT itself. Known mammalian Sm5HTr antagonists were tested for their ability to block 5HT activation. We found that all three drugs tested blocked 5HT-induced stimulation of cAMP. To correlate receptor activation with a behavioural response, motility assays were performed using the same agonists and antagonists as those shown *in vitro* to interact with Sm5HTr. 5HT and methyl-5HT showed a dose-dependent increase in motility with methyl-5HT being more potent. The antagonists were able to block this activation, suggesting that the changes in motility are a result of Sm5HTr activation or a 5HTr-like receptor being activated. Immunolocalization of the Sm5HTr was done to obtain a better idea of how the receptor might contribute to motor control. In both larvae and adults, we found that expression was localized to the nervous system. In adults, the main nerve cords and transverse commissures as well as peripheral nerve fibres were all positive for expression. There was no expression seen in the muscles of the parasite, suggesting that 5HT signalling is not done directly on muscle fibres. Activation of muscles by 5HT may be through the modulation of other biogenic amines or signalling molecules that directly affect muscle.

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List of Abbreviations

AC: adenylate cyclase

DAT: dopamine transporter

EL/ECL: extracellular loop

FITC: flurescein isothiocyanate

FLP: FMRFamide-like

GABA :y-aminobutyric acid

GAP: GTPase activating protein

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GPCR: G protein-coupled receptor

GRAF: glutamate, rhodopson, adhesion, frizzled/taste2 and secretin

HA: histamine

HBP: halogen binding pocket

HBSS: Hanks balanced salt solution

hSERT: human serotonin specific transporter

IBMX: 3-isobutyl-methylxanthine

ICL: intracellular loop

IP3: inositol triphosphate

LeuT: leucine transporter

LSD: lysergic acid diethylamide

MAPK: mitogen-activated protein kinase

MLCK: myosin light-chain kinase

NA: noradrenaline

NET: norepinepherine transporter

nNOS: neuronal nitric oxide synthase

NPF: neuropeptide F

NPY: neuropeptide Y

NSS: neurotransmitter sodium symporters

NT: neurotransmitter

OCT: organic cation transporter

PBS: phosphate buffer saline

PDE: phosphodiesterase

PFA: paraformaldehyde

PKA: protein kinase A

PMAT: plasma membrane monoamine transporter

PNS: peripheral neurotransmitter

PZQ: praziquantel

RNAi: RNA interference

RZ: retzius neurons

SCAMP2: secretory carrier membrane protein 2

SERT: serotonin specific transporter

siRNA: short interfering RNA

SLH: screw-like hyperkinesias

Sm5HTr: *Schistosoma mansoni* serotonin receptor

SmSERT: *Schistosoma mansoni* serotonin-specific transporter

SSRI: serotonin specific reuptake inhibitor

Syn1A: syntaxin 1A

TCA: tricyclic antidepressants

TM: transmembrane domain

TMH: transmembrane helices

TPH: tryptophan hydroxylase

TRITC: tetramethyl rhodamine isothiocyanate

VFM: venus flytrap model

Introduction

Neglected tropical diseases cause millions of deaths each year and as well as pose health risks to more than a billion people. The amount of resources that is devoted to their control and management runs in the billions of dollars (Hotez 2010). Schistosomiasis is estimated to infect over 200 million people worldwide, with the majority (>90%) of people residing in sub-Saharan Africa (Steinmann et al. 2006; Hotez and Kamath 2009). The disease is caused by the infection with one of five species of human parasites, namely *Schistosoma mansoni,S. haematobium, S. japonicum, S. mekongi* and *S. intercalatum*. Considering the pathology to the liver, bladder and kidneys (depending the species of schistosome), the chronic morbidities associated with anemia, impaired child development and inflammation, it is estimated that schistosomiasis results in 70 million DALYs (disability adjusted life years) lost each year (King and Dangerfield-Cha 2008). This places it above both malaria and tuberculosis for world burden. Deaths in Africa attributed to schistosomiasis are estimated to be around 300,000 (van der Werf et al. 2003). There is no vaccine for schistosomiasis and treatment relies heavily on a single drug, praziquantel (PZQ). PZQ is a relatively effective and inexpensive drug but it has some limitations. It is not effective against all forms of the parasite, with larval (schisosomula) stages being unaffected (Xiao et al. 1985; Sabah et al. 1986). Another problem with the use of PZQ is that its effectiveness relies on the immune system and, therefore, requires that populations have been exposed to the infection (Brindley and Sher 1987). Lastly, with the use of one single drug in mass treatment and control programs, there is the threat of resistance developing with some reports already surfacing (Melman et al. 2009). As such, there is a strong urgency to develop other chemotherapeutics for either treatment alone or co-administration with PZQ. To accomplish this, we must first acquire a better understanding of the biological processes and the proteins involved.

One area of focus in the development of novel anti-*Schistosoma*l drugs is the nervous system. The nervous system of worms holds a plethora of potential drug targets as well as targets that are currently being used for drug treatment (Hagel and Giusti 2010; Ribeiro and Geary 2010). The nervous system of schistosomes, in particular, plays an important role for the survival of the parasite. In more developed organisms, there is a coelomic cavity filled with circulating fluid which helps in the control and passage of signalling molecules. Schistosomes, however, lack both these features, which mean that they lack the capacity for classical endocrine signalling. Most signal transduction in these parasites occurs through the nervous system, which controls virtually every tissue in the body via a combination of synaptic and localized (paracrine) signalling. Unlike free-living flatworms, parasitic flatworms have developed systems that allow them to live within the host, namely sexual reproduction and the release of large number of eggs, ventral suckers or hooks that allow them to attach to the host, and a surface that is resistant to the host's defence system. All of these processes are important for the survival of the parasite and if disrupted, can mean death and an inability to continue the life cycle.

The signalling molecules that typically play a role in the nervous system are classified as neurotransmitters. These compounds can be divided into two major groups, the small-molecule neurotransmitters and the neuropeptides. Within the first group, there is another level of division where the biogenic amines, amino acids and other small molecules grouped. Overall, there are more than 50 neuroactive substances and more are being discovered every day. Within schistosomes, several neurotransmitters have been identified and work is ongoing to characterize their modes of action and biological relevance.

Serotonin (5-hydroxytryptamine: 5HT) is an important neurotransmitter and neurohormone in humans, controlling sleep, appetite, sex drive, and other behaviours. Serotonin is also an important neuroactive substance among invertebrate phyla, including flatworms. Serotonin along with acetylcholine, histamine, dopamine, glutamate, ϒ aminobutyric acid (GABA) and neuropeptides have been shown to be abundant in the nervous system of the parasite. Receptors for these molecules have been identified (Hamdan et al. 2002; Bentley et

al. 2004; Bentley et al. 2007; Taman and Ribeiro 2009; Taman and Ribeiro 2011) and show ligand specificity. For the most part, however, their modes of action are not well understood. One of these molecules that has garnered a lot of interest and yet still yields so many unanswered questions is 5HT.

Serotonin was first discovered in the nervous system of adult schistosomes and is present in larval stages as well (Bennett and Bueding 1971; Gustafsson 1987; Mair et al. 2000). 5HT has effects on several biological processes, namely motility (Mellin et al. 1983; Pax et al. 1984), glucose uptake and glycogen utilization (Rahman et al. 1985a; Rahman et al. 1985b), muscle fibre contraction (Day et al. 1994; Day et al. 1996) and adenylatecyclase dependent-signalling pathways (Kasschau and Mansour 1982b; Kasschau and Mansour 1982a; Estey and Mansour 1987). One early question that arose was how did the parasite obtain serotonin. Did it synthesize its own or did it recruit serotonin from the abundant supplies found in the host blood? Early work suggested that the parasite was capable of taking in exogenous serotonin (Bennett and Bueding 1973; Wood and Mansour 1986; Boyle et al. 2003) and this uptake was probably mediated by a surface transporter. Later evidence through the cloning of the first and rate limiting enzyme in the serotonin biosynthesis pathway, tryptophan hydroxylase (Hamdan and Ribeiro 1999), showed that the parasite has the enzymatic machinery to create its own 5HT. To reconcile these two lines of evidence, researchers speculated that schistosomes might take up exogenous (host-derived) serotonin to supplement internal stores, when endogenous production was insufficient (Hamdan and Ribeiro, 1999: Boyle, Hilyer et al. 2003) but the transporter responsible for this recruitment was never characterized at the molecular level. Besides the biosynthetic enzyme, no other proteins of the serotonergic system have been identified or shown to be important for the signalling or regulation of serotonin in the parasite.

The central hypothesis of this study is that schistosomes have a fully functional serotonergic system that includes a serotonin-specific transporter (SERT) and one or more serotonin receptors. This is supported by the aforementioned evidence of serotonin uptake in cultured worms, as well as the many effects of serotonin on worm physiology, which would require interaction with specific receptors. The first goal of this study was to determine

whether a SERT was present in the model parasite *S. mansoni* and to determine its role in 5HT transport and 5HT signalling. The second major goal was to identify serotonin receptor(s) and to elucidate their roles in serotonin signalling within the parasite. To test our hypothesis, a combination of cloning, pharmacological profiling, immunolocalization, RNA interference (RNAi) and behavioural assays were done.

Here we provide the first molecular evidence for a SERT-like transporter in *S. mansoni* and show that this protein plays an important role in the regulation of serotonin activity in the parasite (Manuscripts I and II). We follow up with the identification and functional analysis of a serotonin receptor in *S. mansoni* (Sm5HTr), the first to be discovered in any of the parasitic flatworms (Manuscript III). In the first chapter, we identified a short fragment that closely resembled the transmembrane region of the human SERT. Using cloning techniques, we cloned and expressed this protein in a heterologous system, showing that it is capable of mediating the active transport of 5HT (Manuscript I). In the following chapter, using antibodies targeting the protein, we localized the schistosome transporter in both larval and adult stages, showing that it is found primarily in the nervous system, both in the central nervous system (CNS) and periphery, including the peripheral subtegumental nerve plexus located just beneath the surface of the parasite. Using RNAi, we also show that the schistosome SERT is responsible for the transport of 5HT at least in larval stages and that it plays a major role in the termination of 5HT induced motility in larvae (Manuscript II). One of the receptors responsible for serotonin signalling in the parasite was subsequently cloned and characterized in a heterologous system (Manuscript III). Here we show that this receptor is specifically activated by 5HT, is localized to the nervous system and may be involved in the 5HT-induced motor activity in larvae. These studies give important new insight into the serotonergic signalling system in *S. mansoni* and may lead to the development of new therapeutics.

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CHAPTER I

LITERATURE REVIEW

A.*SCHISTOSOMA*

Schistosomes, also commonly known as blood flukes belong to the class trematoda and phylum platyhelminthes. This phylum comprises more than 18,000 species (Poulin and Morand 2000) of which the majority fall into the subclass of digenea. Digeneans have two distinctive features, namely a syncytial tegument and the presence of two suckers. The syncytial tegument is such that there are no junctions between cells and one continuous cytoplasm surrounds the entire animal. The suckers are positioned in the anterior portion of the worm, with the acetabulum or ventral sucker present on the ventral part of the body and the oral sucker surrounding the mouth. There are more than 45 families of digeneans of which one important group is the *Schistosoma*tidae. Unlike many of the other trematodes, schistosomes are dioecious, having either male or female reproductive organs. There are 14 genera of *Schistosoma*tidae of which 9 infect birds and the rest infect mammals and crocodiles. Of those that infect mammals, the best studied is the *Schistosoma* genus. There are currently 21 known species of *Schistosoma*, including 5 that can infect humans. The species infective to humans are *S. mansoni*, *S. japonicum*, *S. mekongi*, *S. haematobium*, and *S. intercalatum*. The most prevalent of all the species are *S. mansoni*, *S. japonicum*, and *S. haematobium*.

There are an estimated 210 million people that suffer from schistosomiasis with the majority residing in Africa. Of those, it is estimated that 93% people live in sub-Saharan Africa (Hotez and Kamath 2009). *S. Japonicum* is found mostly in Asia and causes hepato-intestinal schistosomiasis. Both *S. mekongi* and *S. intercalatum* are fairly rare and found in areas of Africa, and the Mekongi river basin as well as in Asia (Hoffmann and Dunne 2003). Infection with *S. haematobium* causes urinary schistosomiasis while infection with *S. mansoni* leads to hepatic and intestinal schistosomiasis. Infection with other species leads to the common 'swimmers itch' caused by the penetration of cercaria, however, no long term effects persist. The species that is responsible for the majority of human infections is *S. haematobium* with *S. mansoni* being second most prevalent (van der Werf et al. 2003).

A.1 Biology of Schistosomes – Life Cycle

The life cycle begins with the paired male and female worms releasing eggs either through the urine (*S. haematobium*) or through the feces (all other human *Schistosoma* species). Unlike all other trematodes, schistosomes are dioecious with separate sexes and pairing is absolutely necessary for propagation. The male holds the female in the gynaecophoric canal. This position allows for fertilization of the female eggs, and also assures the female is not swept away by the vascular flow as the male's ventral sucker is developed enough to hold them in place by clasping onto the vascular walls. Once the eggs are released from the human host, upon reaching fresh water, they hatch and release the miracidia. Miracidia can only survive for a short period of time in fresh water and need to find a snail host to continue development. They will penetrate the snail and begin a process of asexual multiplication. As the miracidium penetrates, it turns into a primary sporocyst and over the period of ~7-14 days, several secondary sporocysts are formed. The secondary sporocysts will migrate to the digestive glands where they will give rise to many thousands of cercariae. The cercariae will break free from the snail host into water and require a mammalian host in order to survive. There are several cues that attract cercariae and increase their chances of finding a suitable host. These range from chemicals released from human skin, shadows formed by bodies and turbulence in the water from moving objects. Once the cercariae have found a host, they will penetrate the skin and undergo several biological changes. Once inside the host, over a period of weeks, they will migrate through the skin into the vascular system, pass through the heart and lungs and finally reside either in the venous plexus (*S. haematobium*) or mesenteric veins (all other human species). Pairing occurs late in the development of the worm but it is hypothesized to take place before the males and females reach their final destination.

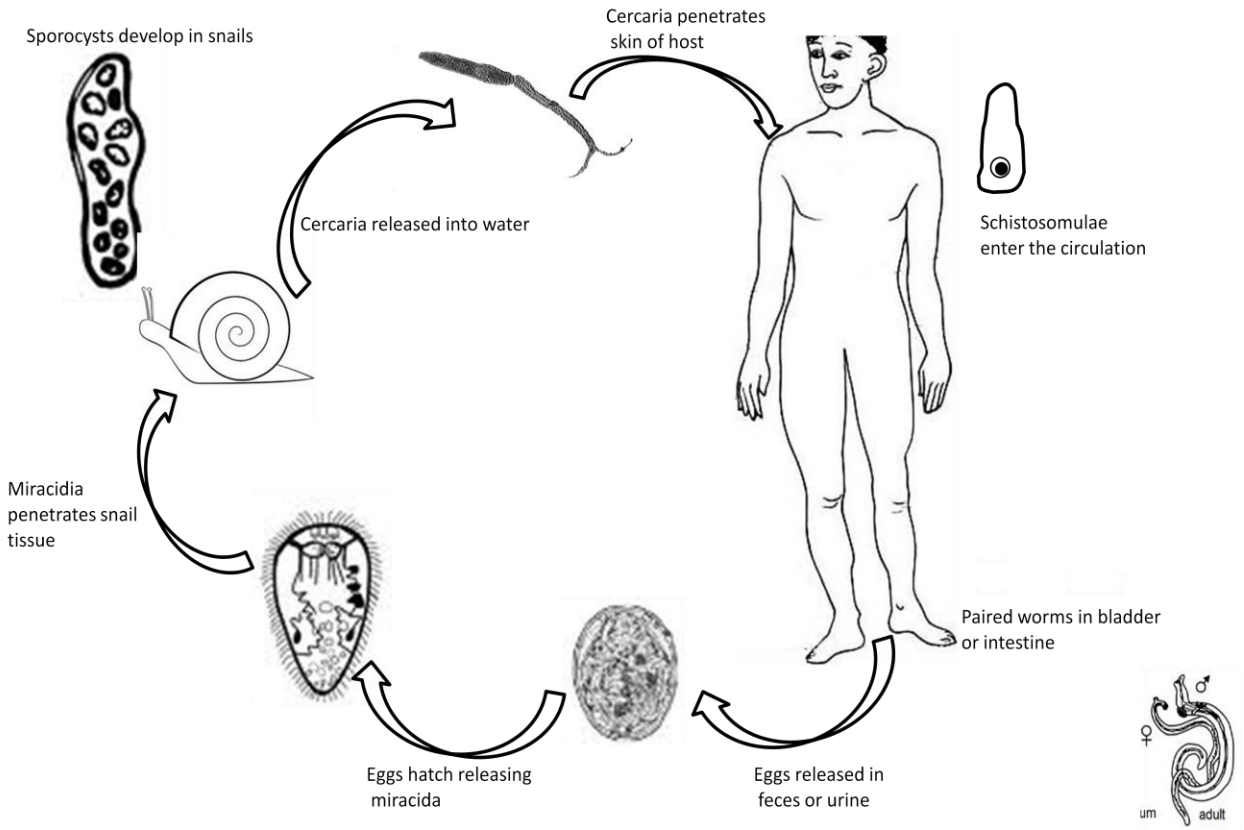

Figure 1. The life cycle of human schistosomes (*S. mansoni, S. japonicum, S. haematobium*)

A.2 Morphology

The morphology of the parasite differs slightly between species from their body structure to their eggs. The eggs of *S. mansoni* are ovoid in shape with a lateral prominent spine. The eggs of both *S. japonicum* and *S mekongi* are round with a reduced lateral spine. The eggs of *S. haematobium* and *S. intercalatum* are ovoid with prominent terminal spines. In terms of body shape and structure, male worms vary from 6-15 mm in length and 0.3-1.1 mm in width. In general, *S. japonicum* and *S. mekongi* are the longest with *S. mansoni* being the widest. The female worms are generally longer than the males but also narrower. They can range from 10-30 mm in length and 0.15-0.3 mm in width. The only case where we see the females shorter than the males is with *S. mekongi* (Gillespie 2001). The reproductive organs

also show some differences with the males having between 2-13 testes depending on the species and the females laying between 20-3500 eggs daily/female.

A.3 Schistosomiasis

There are three phases of schistosomiasis: schistosome dermatitis, acute schistosomiasis and chronic schistosomiasis. The first phase is associated with a rash following repeat exposure to cercariae, usually those of bird species. Acute schistosomiasis is most notably seen with infection with *S. japonicum* and occurs 4-8 weeks following infection. It is thought to correspond to the maturation of the worms and first round of egg deposition within the host. It is typically associated with an increase in circulating immune complexes and peripheral eosinophilia. For the most part, symptoms resolve themselves within a few weeks but the syndrome can be fatal. Chronic schistosomiasis is much more common than acute schistosomiasis and associated with egg-induced immune response, granuloma formation and fibrosis of the surrounding tissues. Both the adult worms and juvenile larvae are fairly non-immunogenic, however, the eggs are highly immunogenic and result in high levels of circulating immune response molecules. The disease begins with eggs becoming trapped in tissues and releasing antigens that result in the formation of granulomas involving T-cells, eosinophils and macrophages. Some of the more common locations are the bowel wall (*S. japonicum* and *S. mansoni*) which leads to bloody diarrhea. Chronic intestinal schistosomiasis can also result in acute appendicitis. Eggs that are swept back into the portal circulation can induce the formation of granulomas on the portal tracts. Hepatic disease is often seen in cases with heavy infections. Although hepatocellular function is retained, portal hypertension can ensue with splenomegaly and ascites formation. In the case of *S. haematobium*, egg retention in the urinary tract can result in bladder polyps and hematuria. People may only present symtoms months or years after the primary infection. The symptoms are influenced by several factors from burden of infection, location of egg deposition, to the extent of hepatosplenic and cardiopulmonary involvement. Typical symptoms for intestinal schistosomiasis are fatigue,

abdominal pain, dysentery and diarrhea. For urinary schistosomiasis, the symptoms include increased urinary frequency, painful urination and terminal hematuria.

Diagnosis is done using microscopic egg counts either with stool (*S. mansoni* and *S. japonicum*) or urine (*S. heamatobium*) or by immunological methods. Egg counts have been the gold standard for more than 40 years having 100% sensitivity for infections with at least 400 eggs per gram of feces and approximately 70% sensitivity for low to mild infections (Schneider and Fripp 1977; Creasey et al. 1982). There also exists an immunological test that reacts to the presence of circulating cathodic antigen (CCA) and which shows a similar sensitivity to egg counts (Legesse and Erko 2007; Ayele et al. 2008). Treatment of the disease is accomplished by taking one oral dose of praziquantel (PZQ). The mechanism of action of PZQ is yet to be fully understood, however gross effects on the physiology of the adult worm include rapid Ca^{2+} ion intake (Pax et al. 1978) possibly due to activation of voltage sensitive Ca^{2+} channels (Jeziorski and Greenberg 2006) and blebbing around the surface (Becker et al. 1980). This is the main drug of choice for curing schistosomiasis and as of yet, there are no signs of resistance, although several reports of lower sensitivity have emerged (Fallon and Doenhoff 1994; Stelma et al. 1995; Fallon et al. 1997). To compound the problem, it has been shown that PZQ has very low effect on immature worms (Sabah et al. 1986; Botros et al. 2005) requiring almost 30 times the dose required to kill adults. The two other compounds that have been tested as schistosomicides are oxamniquine and artemesinins. Oxamniquine's use has been relatively restricted to Brazil due to higher costs when compared to PZQ. Artemisinin derivatives are currently used for the treatment of malaria however they have been shown to be effective against several species of *Schistosoma* (Utzinger et al. 2000; N'Goran et al. 2003).

Research into the mechanisms of drug action, mass drug treatment and the implementation of control programs are all part of the ongoing fight against schistosomiasis. The fear of resistance is also leading to further research for new and novel drug targets.

A.4 Neurobiology

Schistosomes are acoelomate, lacking any circulatory system or classical endocrine system. This means that control of vital functions such as feeding, reproduction, and movement, is performed by the nervous system (Ribeiro et al. 2005). The basic layout for the nervous system of schistosomes is that of a ladder shape. The main components are two cerebral ganglia (paired), longitudinal cords and transverse commissures all of which are well developed. These elements comprise the central nervous system (CNS). From the main nerve cords sprout smaller nerve fibres and nerve plexuses that can be either motor or sensory in nature. These connect to the various organs and structures throughout the worm and comprise the peripheral nervous system (PNS). In the two suckers and sub-tegumental regions, both sensory and motor plexuses are found with differentiated nerve endings. As with other invertebrates, the axons are unmyelinated and the neuromuscular junctions are characterized by a high density of presynaptic vesicles. In the tegument, there can be found sensory nerve endings known as sensory papillae. These sensory papillae are exposed to the environment of the host and can serve as either chemo, mechano or photo reception (Gobert et al. 2003). Besides the nerve cords, there also exists nerve nets, known as plexuses. These are present throughout the PNS and can also be found in continuity with nerve cords. The control of muscle function and locomotion are important for the survival and propagation of the parasite. As a free living cercaria, it must locate and penetrate its host through swimming. Once within the host, the developing parasite must travel through the body until it reaches its final destination. Male and female worms pair and travel together against the flow of the circulatory system and clasp onto the walls throughout the lifespan of the worm. To accomplish this, control and coordination of the musculature is necessary. In both the cercaria and the adult worms, there are three types of muscle fibres that lie directly underneath the tegument. The three layers, in order from most topical, are circular, longitudinal and diagonal. Each type has its own function, with the circular being responsible for elongation, longitudinal for shortening and diagonal for bending laterally (Halton 2004).

A.5 Neurotransmitters

The communication among nerve fibres and subsequently to other cells is accomplished through the release of neurotransmitters (NTs). Neurotransmitters can be defined as chemicals that either modify or result in the transmission of impulses between synapses or neuromuscular connections. There are several chemicals that have been identified thus far as neurotransmitters and their classification relies on their chemical structures. Group A contains acetylcholine (ACh, choline ester), B contains biogenic amines including serotonin (5HT), dopamine (DA), noradrenaline (NA), adrenaline, the phenolamines (tyramine, octopamine) and histamine. Biogenic amines share a common protonated amine and are derived from the metabolism of amino acids, typically aromatic amino acids or histidine. Group C are the neuroactive amino acids, including γ-aminobutyric acid (GABA), acidic amino acids (glutamate, aspartate) and glycine. These can also be classified according to their function, as excitatory (glutamate and aspartate) or inhibitory (GABA and glycine). Group D consists of peptides such as enkephalins, endorphins, substance P, cholecystokinin, NPF/Y-like and FMRFamide-like . There are several other candidates for group D that have yet to be substantiated. . Finally, group E includes the purines adenosine and adenosine triphosphate (ATP). There are other substances that have recently been implicated as possible neurotransmitters and these are nitric oxide, steroids, and eicosanoids. It is not uncommon to join the first three groups (A, B, C) together as the small molecule or classical neurotransmitters, while group D would be considered the large molecule NTs (Webster 2001) . In schistosomes, there have been several NTs that have been identified and studied to determine their biological role. Within the small NTs, acetylcholine, serotonin, dopamine, histamine, glutamate, and noradrenaline have all been identified in schistosomes. Several neuropeptides have also been found to be active, namely NPF/Y-like and FMRFamide-like neuropeptides (Day et al. 1997; Moneypenny et al. 1997; Moneypenny et al. 2001; Humphries et al. 2004).

Most work on elucidating the role of neurotransmitters in schistosomes have followed the course of histo-immunolocalization, *in situ* and *in vivo* assays following exogenous exposure of drug, agonist or antagonist, or through the cloning and expression of proteins involved in the signalling and or synthesis pathway. Typically this has led to the classification of neurotransmitters as excitatory or inhibitory, with further roles attributed based on localization. Those that have been shown to have inhibitory effects are acetylcholine and dopamine.

One of the most extensively studied NTs in schistosomes is acetylcholine. Identification of acetylcholine esterase (AChE), the enzyme responsible for the degradation of acetylcholine, was the first indication of a cholinergic system. Through the use of antibodies targeting the enzyme, a pattern of expression was unveiled that localized to the CNS and PNS including the cerebral ganglia, major nerve cords and commissures, as well as innervations to all the major organs and muscles. Unlike its role in vertebrate systems as an excitatory neurotransmitter, ACh was shown to have an inhibitory effect in schistosomes. In whole worms and isolated muscle fibers, application of exogenous ACh led to a flaccid paralysis of the entire worm and inhibition of muscle contraction (Pax et al. 1984; Day et al. 1996). ACh may also play a role in the recruitment of glucose across the membrane (Camacho and Agnew 1995; Camacho et al. 1995; Jones et al. 2002). Evidence showed that exogenous ACh may regulate GLUT-$_1$-like transporters, which are present on the tegument and are responsible for glucose uptake.

Besides ACh, dopamine is another classical neurotransmitter identified in schistosomes that has shown inhibitory effects. It was identified in the CNS of *S. mansoni*, namely the main nerve cords and commissures (Gianutsos and Bennett 1977; Orido 1989). Exogenous application of dopamine caused muscle relaxation, leading to a lengthening of adult worms (Hillman and Senft 1973; Tomosky et al. 1974; Mellin et al. 1983) and near complete paralysis on larval stages of the parasite (El-Shehabi et al. 2012). Cloning of the rate-limiting biosynthetic enzyme tyrosine hydroxylase suggests that the parasite is capable of producing its own dopamine (Hamdan and Ribeiro 1998) and the recent discovery of a dopamine transporter in *S.*

mansoni (Larsen et al. 2011) provides further evidence for the existence of a functional dopaminergic system.

One NT that is both inhibitory and excitatory in invertebrates is glutamate. In the case of *S. mansoni*, the available evidence suggests that glutamate acts at least in part as an excitatory transmitter with neuromuscular effects. Early work showed that exogenous application of glutamate on isolated muscle fibres elicited contractions, albeit not through a receptor (Miller et al. 1996). More recently, researchers have shown that application of glutamate receptor agonists on whole worms produced sustained muscle contractions leading to paralysis and this effect could be inhibited by classical antagonists of glutamate receptors (Mendonca-Silva et al. 2002). Further evidence for a signalling system came with the cloning of two glutamate receptors (Taman and Ribeiro 2011a; Taman and Ribeiro 2011b). One of these receptors was found to be localized along central and cerebral nerve cords and innervations to musculature, suggesting a potential role in neuromuscular control.

Another excitatory neurotransmitter, histamine (HA) was shown to have dose-dependent neuromuscular effects in worms. Addition of increasing amounts of exogenous HA led to an increase in motor activity. Furthermore, addition of antihistaminic compounds led to a paralysis of the worms that could be reversed through the addition of an excess of histamine (Ercoli et al. 1985). Immunolocalization studies using anti-HA antibodies identified extensive HA immunoreactivity in neuronal cell bodies and minor nerve cords of the subtegumental nerve plexus, oral suckers and the innervations of female reproductive organs (El-Shehabi and Ribeiro 2010). Recently, two histamine receptors were cloned and characterized from *S. mansoni* (El-Shehabi et al. 2009; El-Shehabi and Ribeiro 2010). One was located predominantly to the tegument while the other was found throughout the nervous system and subtegumental nervous plexus.

The class of neuropeptides currently contains 16 structurally distinct families; L/A/Iamides, PWamides, FMRFamide-like (FLP), neuropeptide F (NPF), and myomodulin-like to name a few. In schistosomes, genome analysis has gone on to show the presence of 41 possible peptides from across several families (McVeigh et al. 2009). Work on peptides from

the FLP family suggests that they act as excitatory NTs where those from the NPF family are able to cause a decrease in forskolin-induced cAMP accumulation (Day et al. 1994b; Day et al. 1997; Humphries et al. 2004). Recent work has also identified an I/Lamide that was present in the CNS and showed inhibitory effects on worm motility (McVeigh et al. 2011).

A.6 Schistosome Genome and RNA interference

With the publication of the *Schistosoma mansoni* genome in 2009 (Berriman et al. 2009) along with a plethora of transcriptome data now available (Felipe et al. 2003; Hoffmann and Dunne 2003; Oliveira 2007; van Hellemond et al. 2007), the potential for protein characterization has never been better. The link between characterization and biological function is another hurdle on its own that has benefited greatly from the discovery of RNA interference (RNAi). RNAi involves the transfection of the organism with double stranded RNA (dsRNA) or short interfering RNAs (siRNAs) resulting in the degradation of the target mRNA. RNAi in schistosomes has been shown to be a viable technique, targeting a variety of different genes (Correnti et al. 2005; Dinguirard and Yoshino 2006; Krautz-Peterson et al. 2007; Nabhan et al. 2007; Morales et al. 2008) and several participants of the RNAi machinery pathway have been identified in *S. mansoni* (Krautz-Peterson and Skelly 2008; Gomes et al. 2009). Although not completely mapped out, this technique offers a unique opportunity to validate predicted biological function for most genes.

B. Serotonin

Serotonin (5-hydroxytryptamine: 5HT) is the main focus of this thesis and it will be discussed here in more detail. 5HT is a ubiquitous biogenic amine of vertebrates and invertebrates phyla. In mammals, serotonin is found throughout the body in the blood, the brain, and the gut. It is synthesized from its precursor tryptophan with the enzyme tryptophan hyroxylase (TPH) and an aromatic L-amino acid decarboxylase (see fig.2). In humans, serotonin

has a wide range of physiological roles and has been linked to several psychological disorders. Serotonin can have both neurotransmitter-like effects and neurohormonal effects in the cardiovascular system. In the raphe nuclei, it stimulates parasympathetic and sympathetic pathways, leading to chronotropic and inotropic effects on the cardiovascular system. Its neurohormornal effects contribute to regulation of vasodilation and vasoconstriction, platelet aggregation and vascular tone (Azmitia et al. 1996). In the gastrointestinal tract, we find over 95% of the peripheral serotonin (Gershon 1999). Here, its role is controlling intestinal secretion, sensation, and peristalsis (Bulbring and Crema 1958; Bulbring and Lin 1958). Outside the gut, in the circulatory system, almost all serotonin is carried within platelet dense granules (Maurer-Spurej et al. 2004). In response to signals, serotonin is released where it can help in regulating thrombosis and hemostasis. Serotonergic neurons from the brainstem have well defined and organized projections in the cortical, limbic midbrain and hindbrain, however serotonin and serotonin receptors can be found throughout the brain. Due to the organization of the neurons and receptors, serotonin has been linked to several neuropsychological processes; mood, sleep, appetite, reward, anger, and sexuality (Roth et al. 2004; Airan et al. 2007; Canli and Lesch 2007). Besides these physiological roles, serotonin has been linked to several psychological diseases. Depression, eating disorders, anxiety and obsessive compulsive disorders are some of the clinical manifestations associated with serotonin deficiencies or deregulation (Bellivier et al. 1998; Lucki 1998; Mann et al. 2001).

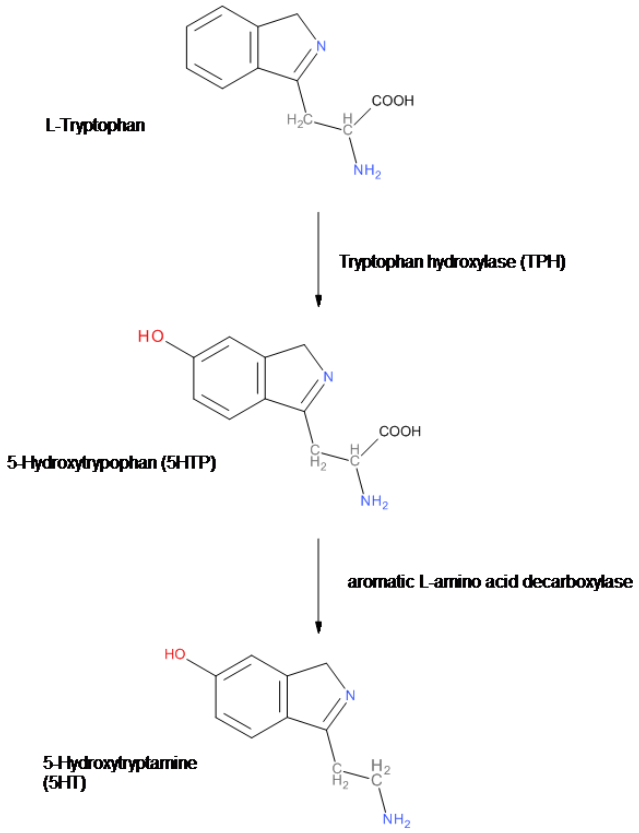

Figure 2. Serotonin synthesis pathway from its precursor tryptophan

B.1 Serotonin in invertebrates

In invertebrates, serotonin is one neurotransmitter that has garnered much interest for its behavioural effects. Molluscs, in particular, have a well-developed serotonergic system. In the gastropod *Aplysia*, serotonergic neurons of the cerebral ganglion have been shown to play a role in the modulation of feeding. Axons from these neurons innervate the buccal ganglion, the bucal mass and the lips of the organism (Kupfermann and Weiss 1981). Experiments showed that serotonin may act as both a neurotransmitter and neuromodulator in the action of feeding in the mollusc. Insufficient to cause a firing in the buccal ganglion on its own, serotonin acts in conjunction with another excitatory stimulus, increasing firing speed of an already firing buccal neuron. Its modulatory role occurs at the motor junction between the buccal motor neuron

and buccal muscle. It has been shown that serotonin both increases excitatory muscle junction potentials as well as stimulation of muscle adenylate cyclase and subsequent phosphorylation of muscle proteins (Kupfermann and Weiss 1981). Serotonin has also been shown play a role in defense arousal by activating the gill-and-siphon withdrawal reflex (Kandel 1981; Glanzman et al. 1989; Hawkins and Schacher 1989). Besides working on the sensory motor synapses for the defense mechanism, serotonin may also act on other sites within this system. Exogenously applied serotonin has been shown to increase the excitability of sensory neurons as well as an increase in the response of tactile stimulation of siphon sensory neurons (Klein et al. 1986).

In another gastropod mollusc, *Tritonia diomedia*, serotonin has been implicated in the escape response to predators. This response can be simulated by the injection of serotonin in the absence of contact with the predator and it can be inhibited by antagonists of serotonin activation (McClellan et al. 1994). Isolated brain preparations of *T. diomedia* also show stimulation of swimming neurons when incubated with serotonin. Several of the motor neurons that form the escape swimming circuitry have been shown to be serotonin-immunoreactive (Katz et al. 1994; McClellan et al. 1994) and have also been shown to play a neuromodulatory role. Serotonergic neurons enhance synaptic potentials produced in other neurons in this escape swimming circuitry (Katz et al. 1994; Katz and Frost 1995).

In annelids, there are 5-11 serotonin-containing neurons located mainly in the ventral ganglia, including the neuroendocrine retzius neurons (RZ) as well as other interneurons (Willard 1981; Glover and Kramer 1982; Nusbaum 1986; Nusbaum and Kristan 1986). Similar to molluscs, serotonin has been implicated in feeding behaviour in annelids. The first component in feeding is swimming toward a potential food source. Mechanosensory stimuli lead to activation of serotonergic interneurons, which in turn activate swim pattern generators (Nusbaum 1986; Nusbaum and Kristan 1986). The RZ neurons are also stimulated through mechanosensory stimulation leading to release of serotonin into the extraganglionic fluid. There are no direct connections of the RZ neurons with any swim-related neurons yet they are able to initiate swim motor output (Kristan and Nusbaum 1982). This suggests that they promote swim activation through neurohormonal release of serotonin. Serotonin has also

been shown to decrease the threshold current of non-serotonergic swim neurons required to elicit swim periods and decrease inhibitory swim interactions between swim motor neurons (Angstadt and Friesen 1993; Mangan et al. 1994).

Following swimming, the feeding phase begins with the activation of bite-like jaw movements, salivary secretion, pharyngeal paristalsis, mucus secretion and relaxation of the body wall (Lent 1984; Lent 1985). Application of exogenous 5HT was able to stimulate all of the aforementioned behaviours. Furthermore, hungry leeches have been shown to have higher levels of blood 5HT, which decrease following feeding (Lent et al. 1991).

In nematodes, there has been a large body of research using the model organism *Caernorhabditis elegans* to study neurotransmitters and neuronal signalling. 5HT has been shown to be synthesized in eight different neurons (Horvitz et al. 1982; Desai et al. 1988; McIntire et al. 1992; Loer and Kenyon 1993; Duerr et al. 1999; Sze et al. 2000). Behavioural roles of 5HT have been discovered through the use of mutants for genes involved in the 5HT synthesis pathway, or with the use of exogenous application of 5HT and 5HT drugs (agonists and antagonists). Exogenous treatment with 5HT was shown to have effects on locomotion, defecation, egg laying and pharyngeal pumping (Horvitz et al. 1982; Mendel et al. 1995; Segalat et al. 1995; Weinshenker et al. 1995; Waggoner et al. 1998; Sawin et al. 2000; Rogers et al. 2001; Niacaris and Avery 2003; Dernovici et al. 2007). 5HT has been shown to inhibit locomotion in response to food discovery of hungry worms, possibly with the aid of sensory endings within serotonergic neurons that end in the lumen of the pharynx, which has access to the outside environment (Sawin et al. 2000). Four 5HT receptors have been identified thus far in *C. elegans*, incuding three G protein-coupled receptors (GPCR) and one 5HT-gated chloride channel (Olde and McCombie 1997; Hamdan et al. 1999; Ranganathan et al. 2000; Hobson et al. 2003). The use of mutants for these receptors have helped to identify their roles in behaviour and to localize their expression either in vulval muscles (SER-1), pharyngeal muscles (SER-1, SER-4, SER-7) while MOD-1, the 5HT-gated channel has been implicated in modulating locomotion. Another roundworm that shows a well developed serotonergic system is *Ascaris suum*. It was shown that 5-HT stimulates cAMP levels, leading to activation of glycogen

phosphorylase and inactivation of glycogen synthase (Donahue et al. 1981a; Donahue et al. 1981b). 5HT also stimulates glycogenolysis in muscle (Donahue et al. 1982) and has been shown to produce a reversible paralysis when injected into muscle (Reinitz and Stretton 1996). Immunolocalization of 5HT showed it to be present in neurons in the pharynx and five neurons in the tail of the male adults (Brownlee et al. 1994; Johnson et al. 1996). More recently, two isoforms of a 5HT receptor were cloned from *A. suum* and expressed in mammalian cells. They both showed high affinity to 5HT although differed in their expression *in vivo* based on RT-PCR (Huang et al. 2002). In *Haemonchus contortus*, 5HT staining in both male and female worms was found in the amphids and pharynx. Four neurons had cell bodies that were consistently positive for 5HT, a pair anterior to the nerve ring with axons running to the lips of the worm and which also ran posterior forming a plexus which show many innervations running to the pharynx. In male worms, there was also staining in the posterior region with neuronal endings reaching the bursal sensory rays and ventral motor neurons connecting to the rimming axons (Rao et al. 2011). The effects of exogenously applied 5HT led to inhibition of worm movement, with a paralysis in the posterior region. A novel G protein-coupled receptor that shows high homology and affinity for 5HT was also cloned and characterized from *H. contortus* which showed different binding affinities then those from either *C. elegans* or from muscle and intestinal tissues from *A. suum* (Smith et al. 2003)

The study of 5HT in flatworms has taken on two main approaches, identifying the areas to which 5HT localizes and behavioural studies using 5HT, agonists and antagonists to measure and document changes in behaviour.

The localization of 5HT has been accomplished in several different species of flatworms. In *Fasciola hepatica*, 5HT was identified in both bipolar and multipolar cell bodies in the cerebral ganglia using immunoreactivity. Other important areas that showed positive staining for 5HT were elements of the CNS, namely processes of the neuropile, transverse commissures, longitudinal nerve cords as well as elements of the PNS, neural bodies innervating the musculature of the pharynx, nerve cords surrounding the ootype and sub-tegumental plexuses innervating the muscle. In *Echinostoma caproni*, 5HT was shown to be present in caudal nerve

fibres and cell bodies (Sebelova et al. 2004). It was also labelled in the CNS with large cell bodies near the pharynx, ventral sucker, oral sucker and excretory bladder. In free living planarians, immunolocalization of 5HT found it in cell bodies throughout the CNS in transverse commissures connecting the main nerve cords. There were also serotonergic cells around the brain neuropil, plexuses around the mouth opening and pharynx (Cebria 2008).

Behaviourally, 5HT has been shown in several different species of flatworm to be a neuro-excitatory NT, showing stimulatory effects for muscle contraction and upregulation of intra-signalling molecules. *Cryptocotyle lingua*, a parasitic flatworm of coastal fish, has been shown to have prolonged swimming following treatment with 5HT (Tolstenkov et al. 2010). In the turbellarian *Procerodes littoralis*, 5HT was able to induce muscle contractions in a dose dependent fashion, down to a concentration of .01 nM (Moneypenny et al. 2001). In the flatworm *Clonorchis sinensis*, 5HT was shown to have excitatory effects *in vitro* by stimulating muscle contraction (Shyu et al. 1998) where as in the rat tapeworm, *Hymenopolis diminuta*, 5HT showed both inhibitory and excitatory responses. Following treatment with 5HT, there was a measured decrease in scolex motility and an increase in strobila activity in all three regions, anterior, mid and posterior sections of the worm (Sukhdeo et al. 1984). In *Fasciola hepatica*, 5HT has been linked to motility and metabolism. Exogenous application of 5HT to cultured *Fasciola in vitro* increases parasite motility (Holmes and Fairweather 1984), activates both adenylate cyclase and protein kinase A (Abrahams et al. 1976; Gentleman et al. 1976) and stimulates carbohydrate metabolism (Mansour 1959; Mansour et al. 1960; Mansour 1962; Stone and Mansour 1967). In planaria, several documented behavioural changes occur following treatment with neuroactive substances, including headswinging, writhing, C-like position (CLP) and screw-like hyperkinesias (SLH) (Passarelli et al. 1999; Buttarelli et al. 2000; Raffa and Desai 2005). Treatment with 5HT showed significant increases in CLP, SLH and writhing. There was no significant difference in headswing behaviour compared to controls (Farrell et al. 2008). Besides the behavioural evidence for 5HT, there has also been molecular evidence for 5HT signalling at the molecular level. Four 5HT receptors were cloned which showed high sequence similarity to the human 5HT1A receptor (Saitoh et al. 1997). In the planaria *Dugesia japonica*, a serotonin receptor gene was isolated and mRNA was co-injected

into Xenopus oocytes with G alpha B-2 mRNA. Oocytes were responsive to serotonin but not other neurotransmitters, suggesting this to be a serotonin-specific receptor (Nishimura et al. 2009).

B.2 Serotonin in schistosomes

5HT is one of the most thoroughly investigated neurotransmitters in flatworms due to its widespread distribution. In schistosomes, immunologic staining has shown 5HT to be present throughout the central and peripheral nervous systems in both males and females. 5HT has been identified in the central nerve cords that extend from the cerebral ganglia along the length of the body as well as to the transverse commissures that connect them (Gustafsson 1987; Mair et al. 2000). 5HT is also present in nerve plexuses just below the musculature throughout the parenchyma on the ventral side in the male worms, the innervations of the ventral and oral suckers and the female reproductive system, namely the muscles of the ootype wall and ovo-vitelline duct. Behaviourally, 5HT has been shown to be strongly myoactive in schistosomes, eliciting a strong reaction in both intact animals as well as isolated muscle strips (Hillman and Senft 1973; Pax et al. 1981; Mellin et al. 1983; Pax et al. 1984; Boyle et al. 2000; Halton 2004). Of note, in experiments using muscle strips, 5HT did not initiate contractions by itself, but rather potentiated the myoactive effect of other neuroactive substances such as neuropeptides (Day et al. 1994a; Moneypenny et al. 2001). This suggests that 5HT may be acting as more of a neuromodulator rather than classical neurotransmitter, similar to the mode of action described earlier for molluscs and other invertebrates. Besides neuromodulation, 5HT also has important metabolic effects in schistosomes. In crude worm extracts, 5HT was able to increase carbohydrate metabolism, increasing energy supplies, which might contribute to the increase in muscle contraction (Mansour 1979; Pax et al. 1996). Although there exists many serotonergic nerves in both suckers and reproductive organs, there has yet to be any physiological function tied to 5HT in these tissues. Likewise for the plexuses that innervate the

tegument and the nerve endings that extend to the surface of the tubercules; they are rich in 5HT (Gustafsson 1987) but function of these serotonergic neurons is unknown.

Binding and signalling assays using various 5HT agonists have led to the hypothesis that there exists at least one 5HT receptor in schistosomes, which is positively linked to adenylate cyclase and cAMP (Mansour and Mansour 1989; Day et al. 1994a; Pax et al. 1996; Boyle et al. 2000). Following the publication of the *Schistosoma mansoni* and *japonicum* genome projects (Berriman et al. 2009; Y et al. 2009) , there has been an interest in identifying possible 5HT receptors using molecular tools. As of now, a BLAST search through the database reveals two predicted 5HT receptor sequences (Smp_126730 and Smp_148210). Both show high sequence homology to other known serotonin receptors from both vertebrates and invertebrates (see next section on structure). The two putatives fall into either the 5HT1 or 5HT7 subtype of serotonergic receptors although until recently, neither had had been characterized. As discussed later, one of the goals of this thesis is to determine if either sequence encoded a functional serotonin receptor.

Although present and biologically active, the question of how serotonin comes to be present in the worm and by what mechanism serotonin is inactivated still remain elusive. Two ever changing views have persisted throughout the years as new evidence lends itself to either side. Early work suggested that serotonin was obtained entirely from the host through active transport (Bennett and Bueding 1973). Later evidence through the cloning of the first enzyme in the synthesis pathway, tryptophan hydroxylase (TPH), provided the first evidence the parasite could manufacture its own 5HT (Hamdan and Ribeiro 1999). The second enzyme, an aromatic amino acid decarboxylase, is also present in the genome (Berriman et al. 2009) further suggesting the entire biosynthetic pathway is conserved in the parasite. Of note is the change in expression levels of these enzymes throughout the life cycle of the parasite. In free-living stages, such as cercaria, there is a marked upregulation of TPH where as in parasitic stages, TPH levels go down. This may have to do with the possible recruitment of 5HT from the host, which diminishes the need for endogenous production of the amine. Several authors reported the presence of a saturable serotonin transport system in schistosomes (Bennett and Bueding

1973; Catto and Ottesen 1979; Boyle et al. 2003). At the molecular level, the first serotonin transporter (SERT) from *S. mansoni* was cloned and shown to be able to transport serotonin specifically (see chapter 2). The transcript was inversely related to that of TPH, being highly expressed in parasitic stages and downregulated in free living stages. This suggests that the parasite may be taking advantage of the host serotonin, which is in ample supply in the surrounding environment. Its expression in free living stages suggests that it may also be playing a different role, probably to terminate serotonin signalling, typical of SERTs in neuronal systems (discussed later). Although its role remains unclear, it has been shown that treatment with SERT inhibitors has a negative effect on proper development of the parasite (Boyle and Yoshino 2005; Abdulla et al. 2009; Taft et al. 2010) suggesting that schistosomes require serotonin transport for growth and development.

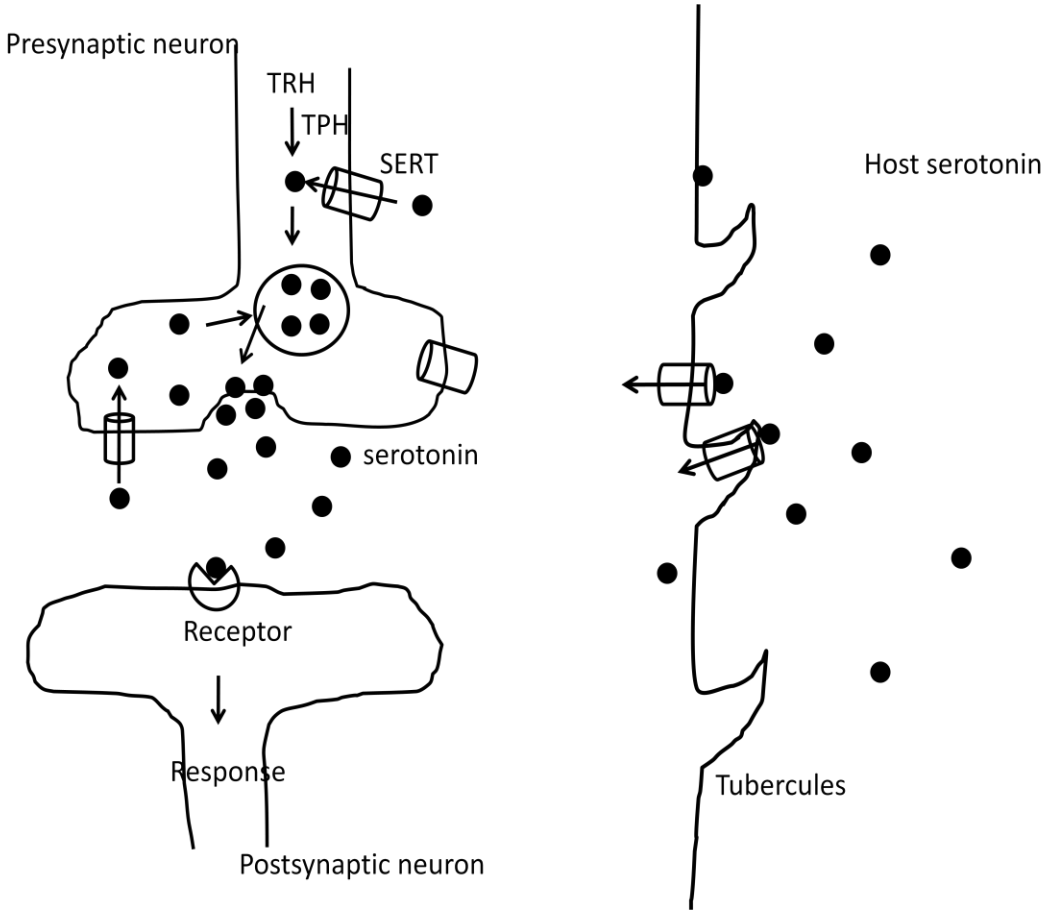

Figure 3. Proposed serotonergic signalling pathway in *S. mansoni.* Serotonin is synthesized in presynaptic serotonergic neurons and released from where they bind to receptors on postsynaptic membranes. Signal is terminated via reuptake of serotonin by a serotonin specific transporter (SERT). Host serotonin is recruited via the SERT located on or near the tegument (and possibly other transporters) to contribute to endogenous stores.

C. G protein-coupled receptors (GPCRs) and the mechanism of serotonin signalling

For most classical neurotransmitters, including serotonin, signalling is accomplished through the binding to G protein-coupled receptors (GPCRs). GPCRs are considered to be among the most diverse and largest protein families within the human genome. Due to their presence in plants, bacteria, yeast, invertebrates and vertebrates, it is believed they have an early evolutionary origin. Their role is primarily in the transduction of external stimuli into an internal signal. For neurotransmitters and hormones, it is estimated that approximately 80% signal through the activation of GPCRs (Birnbaumer et al. 1990). Given the large diversity of signals, GPCRs have been a major target of medications in the treatment of disease, comprising between 30-45% of pharmaceutical drug targets (Drews 2000; Hopkins and Groom 2002). In schistosomes, analysis of the genome predicts the encoding of at least 92 GPCRs (Berriman 2009) and possibly as many as 117 (Zamanian et al. 2011). A few of these have been cloned and characterized including two histamine receptors (Hamdan et al. 2002; El-Shehabi et al. 2009; El-Shehabi and Ribeiro 2010), a light sensitive rhodopsin receptor (Hoffmann et al. 2001), a surface-expressed Sm-7TM with no characterization (Pearson et al. 2007), two dopamine receptors (Taman and Ribeiro 2009; El-Shehabi et al. 2012) and two glutamate receptors (Taman and Ribeiro 2011a; Taman and Ribeiro 2011b). The receptors that are responsive to known neurotransmitters show distinct structural and pharmacological characteristics compared to known mammalian receptors, suggesting potentially important new drug targets to combat schistosomiasis.

In this section, we review the general structure and function of GPCRs. We will discuss their classification, their signalling mechanism and function in invertebrates with emphasis on flatworms and schistosomes.

C.1 GPCRs classification

Historically, GPCR classification consisted of 6 groups (A-F), with three being represented in mammalian species (Kolakowski 1994). However, recently, the human GPCRs have been reclassified into five different groups named GRAFs (Fredriksson et al. 2003). The acronym stands for glutamate, rhodopsin, adhesion, frizzled/taste2 and secretin. There exist some changes from the original classification system, namely to the adhesion receptors and the taste receptors, however for simplicity, the original system will be used here to discuss GPCR structure. The three largest families are family A, B, and C and will be discussed here in more detail. All three groups share a similar topology, containing 7 transmembrane helices (TMH), an extracellular N-terminus and a cytoplasmic facing C-terminus. The TMHs are connected by 3 intracellular (ICL) and 3 extracellular (ECL) loops that alternate throughout the protein. A nomenclature exists for the designation of conserved and important transmembrane amino acids, whereby the most conserved amino acid within each transmembrane region is arbitrarily assigned the position 50 and all other residues within the helix are numbered according to this reference residue (Ballesteros 1994; van Rhee and Jacobson 1996).

Family A receptors, also known as the rhodopsin-like receptors, have been the focus of much research in part due to the availability of detailed crystal structures (Palczewski et al. 2000; Teller et al. 2001; Okada et al. 2002; Cherezov et al. 2007; Hanson et al. 2008; Warne et al. 2008). The list of possible ligands for Family A GPCRs include photons of light, odorants and a variety of small molecules which include biogenic amines, adenosine, nucleotides, as well as lipid-like compounds. Peptides, molecules containing leucine rich repeat motifs and those that activate proteases have also been shown to activate family A receptors. Within the family, the

classification has been broken down based on the type of ligand. The opsins are distinct from other family A receptors in that a chromophore is covalently bound to the protein and activation is done through the absorption of light. The absorption of light changes the conformation of the chromophore which eventually becomes inactivated by the Schiff's base proton (Menon et al. 2001; Filipek et al. 2003). The receptors for odorants are believed to share a similar binding cleft as with the receptors for small molecules, which are buried within the 7TMs. There is also evidence through sequence analysis that residues in the second and seventh transmembrane domains, as well as residues on the second extracellular loop (ECL2) are important for odorant ligand binding (Man et al. 2004). The subgroup of peptide receptors forms the largest of the subtypes as they mediate important physiological processes of hormones, paracrines, peptides and neurotransmitters. For many peptide receptors, work has shown that peptide ligands interact directly with the N-terminus and the ECLs (Gether 2000). These would include the receptors for angiostatin II, neuropeptide Y (NPY), chemokines, arginine vasopressin/oxytocin, gonadotropin releasing hormone, TRH, complement factor 5a, and opioids to name a few (Gether 2000). Site-directed mutagenesis studies suggest that residues in TMH2, TMH3 and TMH5-7 interact with certain neuropeptides, for example the human Y_1 NPY (Sautel et al. 1996; Kannoa et al. 2001), the human delta-opioid (Valiquette et al. 1996) and human CXCR4 (Brelot et al. 2000).

The GPCRs specific for small molecules are all activated by the same mechanism. Molecular analysis has shown that the binding sites are located deep within the TM helices, in particular TMH3, TMH5, TMH6 and TMH7. The receptors studied thus far include receptors for biogenic amines (Strader et al. 1987; Strader et al. 1989; Liapakis et al. 2000; Lebon et al. 2011a; Lebon et al. 2011b; Rasmussen et al. 2011a), nucleotides (Jiang et al. 1997), melatonin (Kokkola et al. 2003) as well as several others. Residues in the ECL2 have also been shown to be important for binding of both agonists and antagonists in the adenosine receptor (Kim et al. 1996). It has been hypothesized that the ECL2 may enter into the binding crevice and forms a lid over the bound ligand in biogenic amine receptors. (For a summary of important residues see figure 2). The serotonin receptors fall into this group and will be discussed later.

Family B receptors all share an intermediate length N-terminus (~120a.a.) which along with the 3 ECLs, are all important points of contact with the ligand (Unson 2002). Two residues that have been shown to be crucial for binding are an aspartic acid in ECL1 and a basic amino acid just before the aspartate (Langer et al. 2003). Except for one receptor, all members of the family B receptors share these two residues. Within the family C receptors, we find the metabotropic glutamate receptors, the γ-aminobutyric acid (GABA) receptors, the Ca^{2+} receptors, taste receptors, basic amino acids receptors and some orphan receptors. Sequence analysis indicates that the N-terminus of the mGlu receptors has a Venus flytrap module (VFM) similar to bacterial periplasmic amino acid binding proteins (O'Hara et al. 1993). The binding region is formed by a cleft that separates two opposing lobes within the VFM and was confirmed using site directed mutagenesis in several receptors (O'Hara et al. 1993; Brauner-Osborne et al. 1999; Galvez et al. 2000). One of their unique characteristics is their constitutive dimerization as either homodimers or heterodimers. The crystal structure obtained in 2000 of the VFM (Kunishima et al. 2000) led to the proposed mechanism to explain the VFM dimer conformational changes, namely a resting state where only lobes I interact and an active state where both lobes I and II are interacting. The recent publication of the *S. mansoni* genome has allowed for rapid mining and sequence analysis for GPCRs. Work by one group (Zamanian et al. 2011) identified 117 potential GPCRs from all the families using a transmembrane oriented mining approach. Of note, they found 105 potential family A, two family B and two family C receptors. In support of these results, two receptors have been cloned from *S. mansoni* from the family C receptors (Taman and Ribeiro 2011a; Taman and Ribeiro 2011b) . Both of these receptors have the conserved venus flytrap module (VFM) however one of them is truncated and lacks the 7 TM region.

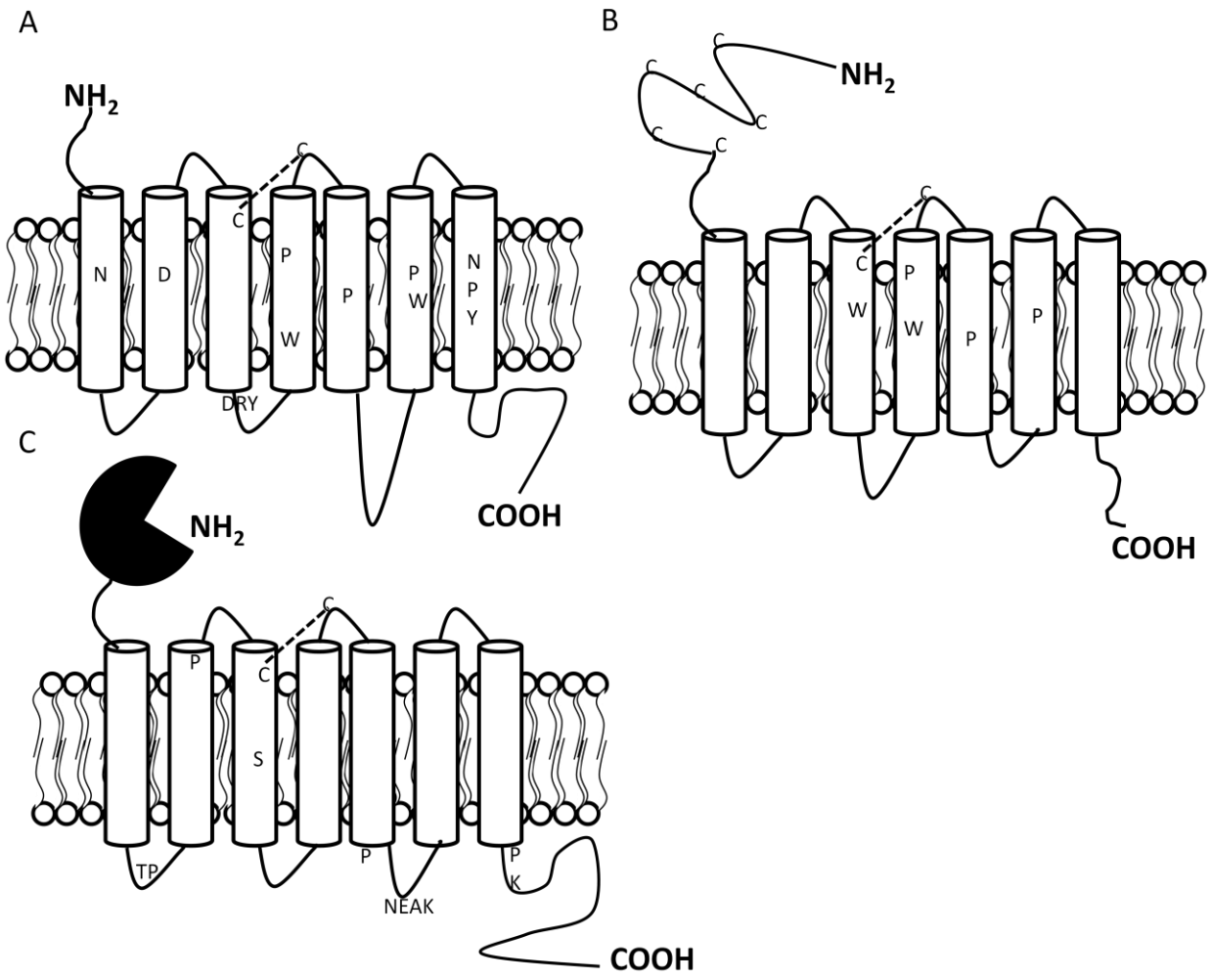

Figure 4. Topographic structure of the three major classes of GPCRs (A, B and C). Shown are some of the important residues for ligand binding and signalling and the conserved N-termini venus flytrap module (VFM) of family C receptors (modified from Gether 2000)

C.2 G-protein Signalling

GPCRs are transmembrane proteins that transmit their signal through the interaction with G proteins. G proteins can be classified into 2 main classes, heterotrimeric and small monomeric G proteins. Heterotrimeric G proteins consist of α, β, and y subunits. There are more than 25 unique G protein α subunits, 5 β and 12 y subunits identified to date (Cabrera-Vera et al. 2003). Within the heterotrimeric G proteins, there are 4 different families that have

been identified based on primary sequence similarities of their α subunits: G_s($G_s$and G_{olf}), G_i (G_{tr}, G_{tc}, G_g, G_{i1}, Gi_3, G_o, and G_z), G_q (G_q, G_{11}, G_{14} and $G_{15/16}$) and G_{12} (G_{12} and G_{13} ; (Cabrera-Vera et al. 2003). The activation/inactivation of all G-proteins follow the same cycle, which begins with the α subunit bound to GDP complexed with the βγ subunits in the inactive state. Upon activation of the receptor, a conformational change occurs which enhances the receptor's affinity for the α subunit and allows for the rapid release of the GDP. GTP replaces the GDP, which reduces the affinity of the α subunit for the βγ complex and results in dissociation of the heterotrimer. The subunits can now activate or inhibit downstream effector proteins such as adenylate cyclase (AC), phospholipase C-beta, tyrosine kinases, phosphodiesterase (PDE), GPCR kinases, ion channels, and molecules of the mitogen activated protein kinase pathway, which may lead to a number of cellular functions. Activation continues until GTP is hydrolyzed to GDP, after which the three subunits (α-GDP and βγ) reassociate to become inactive. There are also several GTPase activating proteins (GAPs) that can lead to GTP hydrolysis. Besides GTP hydrolysis, the signalling may be terminated by receptor desensitization, reuptake of ligand (neurotransmitter) or their precursor, and extracellular degradation by enzymatic processes (Bohm et al. 1997).

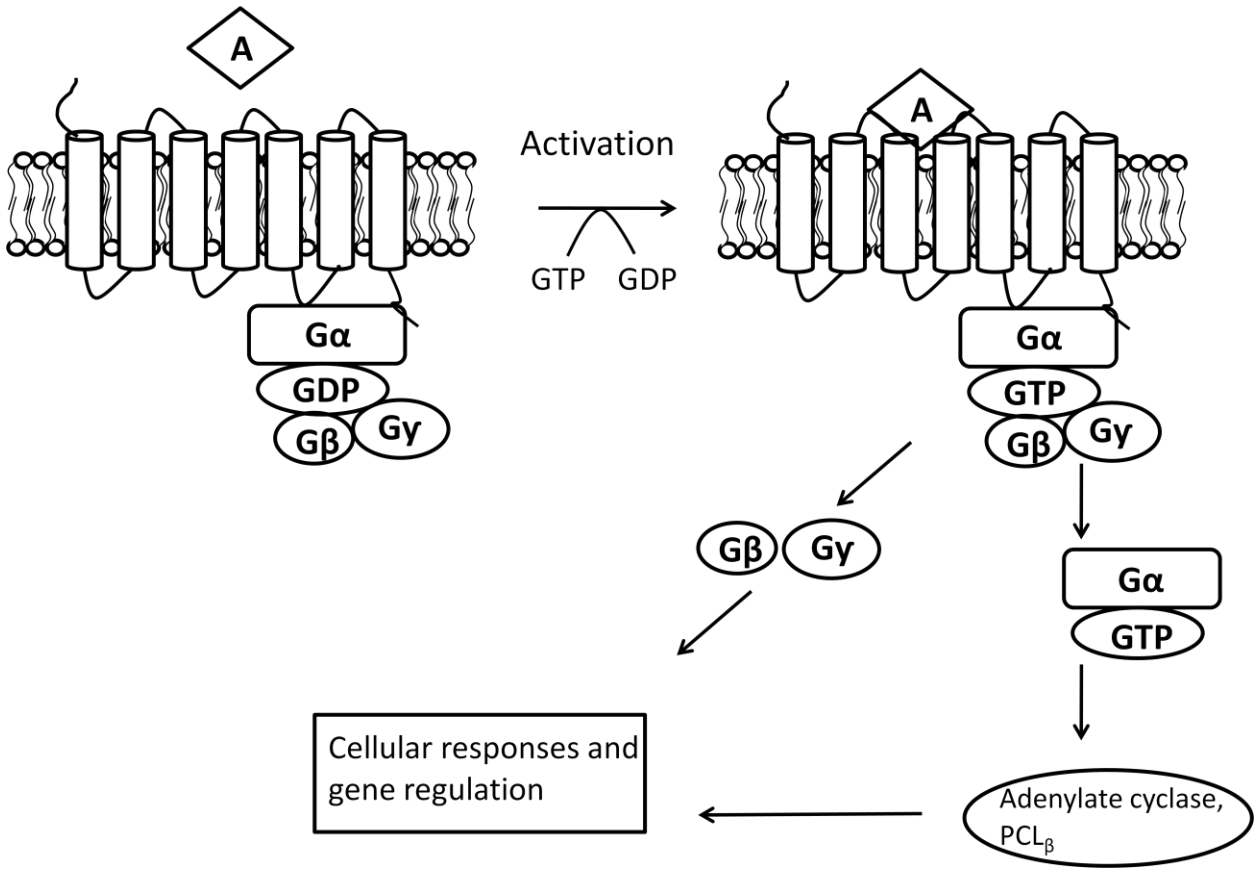

Figure 5. GPCR intracellular signalling pathway following ligand binding (modified from Lundstrom 2005). Ligand (A) binds to a GPCR leading to GDP being replaced with GTP and subsequent dissociation of G-proteins (Gα, Gβ and Gγ). Free G proteins lead to activation of effector proteins (adenylate cyclase and phospholipase C-β) which lead to activation of secondary signalling molecules.

C.3 Serotonin Receptors

The study of 5HT receptors began in the late 50s when Gaddum and colleagues showed that the effects of 5HT in the pig gut ileum could be blocked by either morphine or dibenzyline (Gaddum and Picarelli 1957). It was not for another twenty years that radio-labelled ligand studies were used to describe two classes of 5HT receptors based on their ability to bind [^{3}H]-5HT, [^{3}H]-spiperone, and [^{3}H]-LSD (Fillion et al. 1976; Fillion et al. 1977; Fillion et al. 1978; Fillion et al. 1979; Peroutka and Snyder 1979). Over the years, many 5HT receptors have been identified and a new classification system was developed to take into account the

pharmacology, structure and function of the receptors. Accordingly, there are 7 types of serotonin receptors of which 6 are family A GPCRs and one, 5HT-3 receptors are ligand-gated ion channels.

The different types of serotonergic GPCRs are classified on the basis of structure-binding properties and their coupling to second messengers. For 5HT-1 receptors, they are mostly linked to $G_{i/o}$, proteins, which show sensitivity to pertussis toxin and are negatively coupled to adenylate cyclase (AC). This in turn can lead to inhibition of cAMP-mediated signaling. There are five 5HT-1 receptors in mammals, with one of the group being re-classified to group 2 due to structural, operational and transductional similarities to that group. The five receptors vary in their location but are found primarily in the brain, with the 5HT-1a receptor also being found in the periphery. Interestingly, the 5HT-1b receptor has been located on non-5HT nerve terminals, suggesting that it may control release of other neurotransmitters. Within the 5HT-2 group, there are three types, 5HT-2a/b/c. They share approximately 45-50% sequence identity and are coupled to $G_{q/11}$ proteins. Activation through these receptors leads to an increase in intracellular Ca^{2+} and activation of calcium-dependent serine/threonine kinases (Pritchett et al. 1988; Julius et al. 1990; Stam et al. 1992; Watts 1996). In conjunction, these lead to activation of myosin light chains through an increase in myosin light-chain kinase (MLCK) activity and subsequent phosphorylation. However, in invertebrates, Ca^{2+} binds directly to light chains and then rapidly cycles cross-bridges creating the force necessary for contraction (Freeman 2000). In mammals these receptors are found either in the brain (5HT-2a/c) or in the pulmonary artery endothelial cells (5HT-2b). Contraction of smooth muscle, notably bronchial, uterine and urinary has been attributed to the 5HT2a receptor. As well, following 5HT treatment, platelet aggregation and increased capillary permeability is a result of 5HT2a activity. Of note is the 5HT2c receptor and its complex mRNA editing. In humans, there exists 5 different adenine deaminase editing sites which results in 24 different possible protein products (Burns et al. 1997; Fitzgerald et al. 1999; Price et al. 2001).

The 5HT-4/6/and 7 receptors all couple preferentially to G_s proteins that promote cAMP synthesis through activation of AC. cAMP stimulates protein phosphorylation by protein kinase

A, which can eventually lead to changes in gene expression and cellular responses. The 5HT-4 receptor has been identified in the brain, gastrointestinal tract and the heart (Bockaert et al. 1990; Kaumann et al. 1990; Ford et al. 1992). Although there is only one gene, at least 9 different splice variants have been reported with changes occurring at the C-terminal end (Claeysen et al. 1996; Blondel et al. 1998; Claeysen et al. 1999; Bender et al. 2000; Mialet et al. 2000; Vilaro et al. 2002), however there exists very little pharmacological differences between them. The 5HT-6 receptor mRNA has been identified primarily in the CNS, including the striatum, amygdala, hippocampus and cortex with no localization to peripheral tissues whereas the 5HT-7 receptor is expressed throughout the vascular system and in smooth muscle of the colon as well as the brain (To et al. 1995). Both are coupled to G_s proteins and stimulate cAMP formation (Bard et al. 1993; Lovenberg et al. 1993a; Lovenberg et al. 1993b; Adham et al. 1998). It has been suggested that the 5HT-7 receptor plays a role in sensory and affective processes, namely sleep, mood and circadian rhythmic activity. Alternative splicing produces 4 different isoforms although no distinct pharmacology has been shown between them (Jasper et al. 1997; Heidmann et al. 1998).

One additional type of serotonergic receptor, 5HT-5, has been reported in mammals. Humans have two isoforms, with the human 5HT-5a receptor showing inhibition of forskolin-stimulated cAMP production (Thomas et al. 2000) but their function and signalling mechanism are largely unknown.

Not all serotonin receptors are GPCRs. In vertebrates, the 5HT-3 receptors are ligand-gated ion channels of the cys-loop channel family. They are similar to the nicotinic acetylcholine, glycine or GABA-A receptors and share electrophysiological and structural patterns with this family (Hoyer 1990). There are 2 genes that encode 2 receptors, 5HT-3a and 5HT-3b and both are found on the central and peripheral neurons where due to opening of non-selective cation channels, they trigger rapid depolarization (Humphrey et al. 1993; Hoyer et al. 1994). Invertebrates also have serotonin gated ion channels, only in this case they appear to be inhibitory chloride channels. The best example is the MOD-1 receptor of *C. elegans*

(Ranganathan et al. 2000) which has plays a role in egg laying (Carnell et al. 2005) as well as motility in males.

C.4 Serotonergic GPCRs in invertebrates

Phylogenetic analyses suggest that 5HT GPCRs diverged first into three major classes (Gi-coupled, Gs-coupled and Gq-coupled) (Vernier et al. 1995; Tierney 2001). These later divided into the classes that we see now, however this is thought to have occurred before the evolution of vertebrates and invertebrates, suggesting that all three of the major classes should be present in invertebrates. However, independent differentiation of each subtype does make it difficult to classify invertebrate 5HT receptors according to vertebrate subtypes (Peroutka 1994; Tierney 2001). Receptors from class 1, 2 and 7 are some of the most common types found in invertebrates. In *C. elegans*, three GPCRs, one from each class 1, 2, and 7 have been cloned and shown to function in a similar fashion to mammalian receptors of the proposed subtype (Olde and McCombie 1997, Hamdan et al. 1999, Hobsen et al. 2003). In the molluscs *Lymnaea*, *Helisoma* and *Aplysia*, more than 6 receptors have been identified and classified as 5HT1/2/4/7 type receptors based on sequence homology and secondary signalling (Gerhardt et al. 1996; Angers et al. 1998; Barbas et al. 2002; Mapara et al. 2008; Nagakura et al. 2010). In *Ascaris*, a 5HT2-like receptor was been cloned and shown to be positively linked to Ca^{2+} and phosphatidylinositol turnover (Huang et al. 2002). In *Haemonchus*, a 5HT1-like receptor was cloned and produced a dose-dependent activation of cAMP when expressed heterologously in mammalian cells (Smith et al. 2003). Although similar in signalling, pharmacological profiling of several of these receptors have shown distinct affinities that differ from their mammalian counterparts. In *Drosophila*, there have been four serotonin receptors identified thus far (Witz et al. 1990; Saudou et al. 1992; Colas et al. 1995) with two showing homology with class 1 receptors and two to class 2 receptors. The 5HT2-like receptors are negatively linked to cAMP and one (5HT-dro2A) was found in motor neurons suggesting a role in motor control.

C.5 Structure of serotonergic GPCRs

The important regions for binding and activation of 5HT receptors have been elucidated using mutagenesis studies, protein modelling and by comparison with crystal structures of related GPCRs. What has been shown is that residues in the TM regions near the extracellular boundary are critical for binding of the amine. In TM3, there is a conserved aspartate (D3.32) that is critical for binding and proper protein targeting (Wang et al. 1993; Kristiansen et al. 2000). The side chain carboxylate of the aspartate serves as a counterion for the protonated amino group of serotonin and is therefore considered to be a core element of the receptor's binding pocket. There is also a D/ERY motif found at the end of TM3 that interacts with a glutamate in TM6 to stabilize the inactive form of the protein (Shapiro et al. 2000). The TM6 region has several residues that are important for both ligand and antagonist binding as well as may be involved in G protein interactions (Choudhary et al. 1993; Parker et al. 1996; Granas et al. 1998; Granas and Larhammar 1999; Manivet et al. 2002; Shapiro et al. 2002). The other two transmembrane domains that have been linked to ligand binding are the 5^{th}, and 7^{th} TM. In TM5, deletion of one of three serines or phenylalanines leads to a decrease or loss of binding of 5HT (Ho et al. 1992; Kao et al. 1992; Johnson et al. 1997; Granas et al. 1998; Shapiro et al. 2000). Similarly, in TM7, there are two asparagines that have been shown to be important for binding of ligand (Chanda et al. 1993; Glennon et al. 1996; Kuipers et al. 1997). Besides determining the binding site through mutagenesis, looking at how the protein changes conformation once it has become active has also helped in determining important regions and residues. Simulations using the 5HT-2a receptor sequence showed that binding was dependent on several of the above mentioned residues (Isberg et al. 2011). They also showed that following binding, there is a large inward shift in TM6, shifts of TM5 toward TM6 and TM3 toward TM5, reinforcing the required tightening of the triad TM3, TM5 and TM6. TM7 also shifts and this is believed to make room for TM6. The crystal structure of β2-adrenergic receptor-G_s protein complex was recently published and has confirmed certain aspects described above (Rasmussen et al. 2011b). Here we see similar shifts in the same TM regions, suggesting a similar binding pocket.

It has been shown that activation of GPCRs can occur as monomers, homodimers and heterodimers (Bouvier 2001; Devi 2001; Hansen and Sheikh 2004). For 5HT receptors, it has been shown that the 5HT1A receptor can form both homodimers and also heterodimers with 5HT7 receptors (Kobe et al. 2008; Woehler et al. 2009). Interactions with other receptors were shown to occur as well, namely with the adenosine (A2A) receptor (Lukasiewicz et al. 2007). Another important interaction occurs between the dopamine (D2) receptor and 5HT2a receptor. Evidence has shown co-expression in brain tissues as well as dimerization in *in vitro* experiments (Werkman et al. 2006; Lukasiewicz et al. 2010; Lukasiewicz et al. 2011). Lastly, the 5HT4 receptor was also shown to form homodimers in *in vitro* experiments (Berthouze et al. 2005). Co-expression with the β2-adrenergic receptor showed that they also formed heterodimers as well. In most of these studies, binding and pharmacology changed depending on the protein stoichiometry (monomer, dimer, heterodimer).

D. Serotonin Transporters (SERT)

As mentioned earlier, neuronal signalling is typically terminated either through the enzymatic degradation of neurotransmitter or through a transport system that removes the transmitter from the synaptic cleft into presynaptic neurons. The major mechanism for inactivation of serotonin is through reuptake via a sodium-dependent serotonin-specific transporter (SERT).

D.1 Structure of SERTs

Neurotransmitter sodium symporters (NSS) are crucial for the regulation and control of neurotransmitter activity. These transporters use the existing $Na^+/Cl^-/K^+$ electrochemical gradient to transport neurotransmitters across the plasma membrane (Masson et al. 1999;

Chen et al. 2004). The NSS transporters can be divided into two categories, the SLC1 and SLC6 transporters. The smaller group, the SLC1 transports glutamate (Kanai and Hediger 2004) whereas SLC6 transporters are involved in the transport of biogenic amines dopamine, noradrenaline and serotonin, and amino acids GABA and glycine (Chen et al. 2004). The members of the SLC6 group share several structural features that are important for proper function, including; 12 predicted transmembrane helices (TMH)(Guastella et al. 1990; Chen et al. 1998a), potential oligomerisation domains (Chang et al. 1998; Kilic and Rudnick 2000; Schmid et al. 2001; Veenhoff et al. 2002; Torres et al. 2003), several N-linked glycosylation sites in the EL2 (Klingenberg 1981; Tate and Blakely 1994; Torres et al. 2003), cytoplasmic facing N and C termini (Guastella et al. 1990; Chen et al. 1998a) and several phosphorylation sites (Sakai et al. 1997; Chang et al. 2001).

The mechanism of transport is proposed to begin with the binding of Na^+, Cl^- and 5HT in a 1:1:1 stoichiometry. This triggers a conformational change, which closes the binding site from the extracellular surface and reveals it to the cytoplasmic side (Nelson and Rudnick 1979). Here the ions and substrate dissociate and a K^+ ion is bound. The protein undergoes another conformational shift, this time releasing the K^+ ion into the extracellular domain (Rudnick and Nelson 1978; Talvenheimo et al. 1983; Rudnick 1998) and is thus now able to repeat the process. A major advance in helping to understand the transport process was the high resolution structure of the LeuT from *Aquifex aeolicus*, a leucine transporter that is Na^+ dependent and has provided a framework for understanding the differences in structure and transport (Yamashita et al. 2005). Of note is the degree of sequence similarity within the ion binding sites between the LeuT and other SCL6 transporters (Yamashita et al. 2005). The LeuT transporter binds 2 Na^+ ions for every molecule of leucine being transported. The four residues that have contact with the first bound Na^+ (A22, N27, T254 and N286) are highly conserved among eukaryotic SLC6 transporters, suggesting a common mechanism of Na^+ transport (Yamashita et al. 2005; Beuming et al. 2006). Similarly, those residues that have contact with the second Na^+, namely G20, V23, A351 and S355 in TMH1 and TMH8 are also well conserved within SLC6 transporters with the exception of A351. This has raised several questions about the second Na^+ ion and its function in transport. It does not appear to be transported and may

be involved instead in structural stabilization of the protein (Yamashita et al. 2005; Beuming et al. 2006). These studies are also beginning to elucidate the role of chloride in SCL6 type transporters. Although transport in the LeuT is Cl^- independent, a Cl^- ion was observed bound between TMH3, EL2 and TMH4, where it is believed to play a role in the conformational change required for leucine transport. Similarly, work using NET-SERT chimeras suggested that these undergo structural changes during transport, which are initiated by the binding of Cl^- (Stephan et al. 1997; Smicun et al. 1999). More recent work has mapped out the possible binding site of the Cl^- ion to residues Y121 (TMH2), S336 (TMH6), N368 and S372 (TMH7) of the LeuT which are conserved across several transporters from the group (Forrest et al. 2007; Zomot et al. 2007). Work by Erreger et al (2008) showed that binding of Cl^- ions helps in the conformational changes of SERTs from the open-extracellular to open-intracellular and back to the open-extracellularly orientation (Erreger et al. 2008).

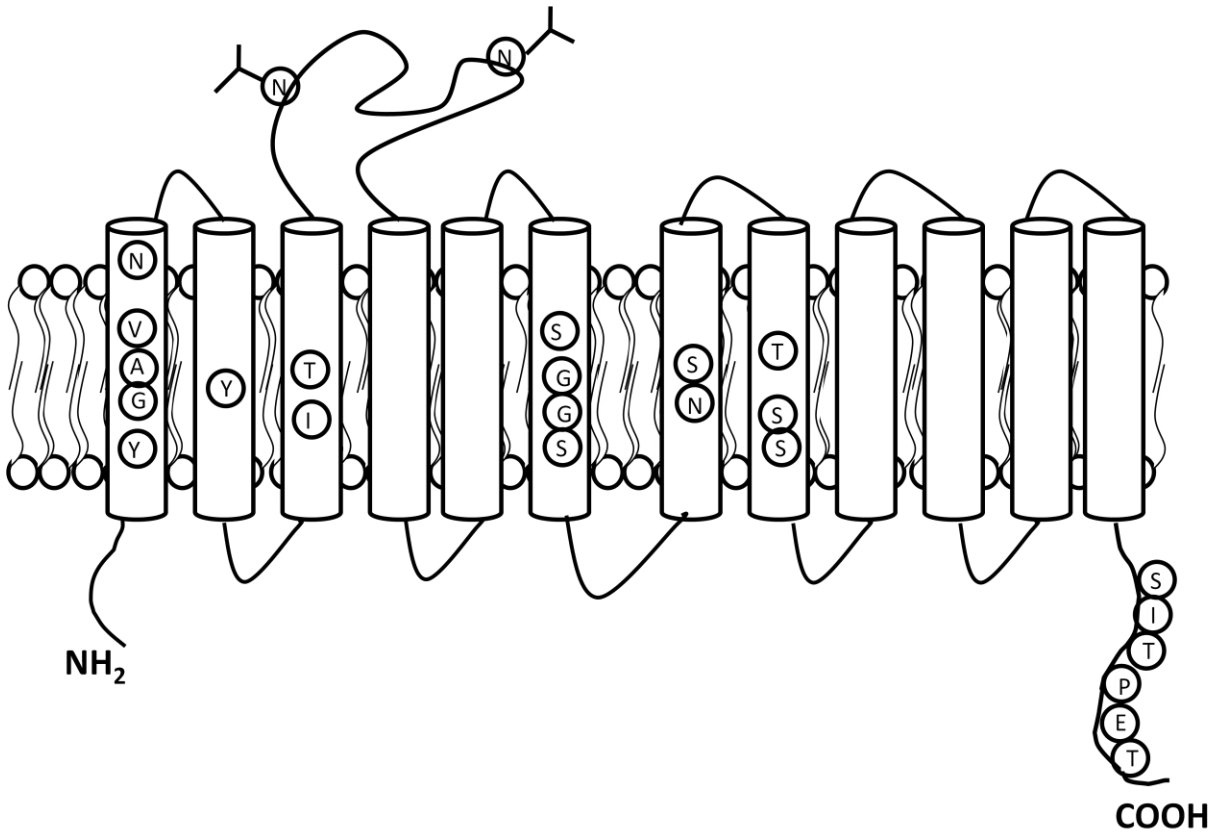

Figure 6. Topology of SERT. SERTs share a common organization consisting of 12 TMH, intracellular facing termini and severa glycosylation sites in EL2. Shown are several important residues for ion and ligand binding, membrane trafficking and protein-protein interaction.

Besides the role of Na^+ and Cl^-, a great deal of the research on SERTs over the past 10 years has focused on the structure of the amine binding site and related pharmacology (Chen et al. 1997; Barker et al. 1999; Adkins et al. 2001; Henry et al. 2003; Celik et al. 2008). These studies have shown that residues in TMH1, 3 and 8 are important for ligand binding (see fig.6). The binding site for inhibitors has been suggested to be different from the substrate site but mutually exclusive, in that binding of the inhibitor will render the substrate binding site unavailable. Radioligand-binding studies, combined with site-directed mutagenesis have revealed several important residues in TMH1, 3, 5, 8, 10, and 12 to be important specifically for binding of antidepressants, which are classical inhibitors of mammalian SERTs (Barker et al. 1994; Barker and Blakely 1996; Barker et al. 1998; Adkins et al. 2001; Mortensen et al. 2001;

Henry et al. 2006; Neubauer et al. 2006; Severinsen et al. 2008). Work using tricyclic antidepressants that co-crystallized with LeuT showed that they share the same binding site made from residues R30, Q34, F235, A319, F320, D401 and D404, located just above the leucine binding site (Singh et al. 2007) of which R30, F325 and A319 are low to highly conserved in the human SERT. Mutagenesis studies of SERTs found residues in EL4 are also important for desipramine (a tricyclic antidepressant) binding (Talvenheimo et al. 1979; Apparsundaram et al. 2008). Using the same approach with serotonin-specific reuptake inhibitors (SSRI), they found a conserved interaction with a halogen binding pocket consisting of residues L25, G26, L29, R30, Y108, I111 and F253 which, are all conserved among the monoamine transporters except for one residue in hSERT (Zhou et al. 2009). Another method has been the use of cysteine replacement which identified residues that have important functional roles for the transporter. Glycines at positions 338 and 342 in TMH6 in the human SERT (Horschitz et al. 2008) are part of the glycophorin A motif (GxxxG), found in many transporter proteins. Mutation of these residues led to failure to reach the plasma membrane, inability to form dimers and subsequent loss of 5HT uptake. The C-terminus has also been shown to be important for the proper functioning with deletion mutants losing their ability to transport 5HT (Larsen et al. 2006). Nobukuni and colleagues showed that deletion of the C-terminus led to decreased SERT trafficking to the plasma membrane and protein lacking glycosylation (Nobukuni et al. 2009) Although the LeuT has helped propel the research of how SLC6 transporters function and the identification of important residues, there is still much work needed to determine the dynamic changes that occur during transport and how the residues that are somewhat distant from the binding pocket may affect protein conformation.

D.2 SERT expression and regulation

SERT expression has been studied extensively and has been described in central nervous and peripheral nervous systems from several different organisms, both vertebrate and invertebrate (Hoffman et al. 1991; Demchyshyn et al. 1994; Chang et al. 1996; Chen et al.

1998b; Jafari et al. 2011). Outside the nervous system, SERTs have also been characterized in several nonneuronal cells. For example, in mammals, SERTs are expressed in blood platelets (Brenner et al. 2007), pulmonary endothelial cells (Lee and Fanburg 1986), placental syncytiotrophoblasts (Ramamoorthy et al. 1993) and intestinal cells (Gill et al. 2008). SERTs themselves have been found to interact with several different proteins that influence their trafficking, recycling, or expression. Bauman et al. (2000) described the first such protein, the serine/threonine phosphotase PP2A, that has been found to form complexes with active, membrane bound SERTs (Bauman et al. 2000). The plasma membrane SNARE protein syntaxin 1A(Syn1A) has been shown to associate with SERT and that it may dictate whether or not SERT transport contributes to membrane excitability through either electrogenic transport (Syn1A absence) or electroneutral transport (presence)(Haase et al. 2001; Quick 2003). The scaffolding protein Hic-5 was found in associations with SERT in platelet cells where it is hypothesized to stabilize catalytic inactivation (Carneiro et al. 2002). Another protein that may contribute to decreased SERT activity is secretory carrier membrane protein 2 (SCAMP2). It was found to interact with the amino terminus, and colocalizes with SERT resulting in a decrease in V_{max} when co-expressed (Carneiro et al. 2002; Muller et al. 2006). Two other proteins that have been linked to rapid decreases in SERT expression are neuronal nitric oxide synthase (nNOS) and α2 adrenergic receptor (Ansah et al. 2003; Chanrion et al. 2007)

On the other hand, proteins have been identified that increase SERT activity or expression. When the adenosine A2 receptor is activated (A2AR), there is an increase in 5HT transport capacity (Miller and Hoffman 1994; Zhu et al. 2004; Zhu et al. 2007). The same group using A2AR knockout mice showed that SERT is also stimulated through a PKG-linked pathway. Evidence for p38 MAPK in enhancing NET activity led to the discovery that it also enhances 5HT affinity (Zhu et al. 2005), which may explain why inflammatory cytokines like interleukin-1β that activate p38 MAPK can result in enhanced SERT activity (Zhu et al. 2006).

Based on what is known about neurotransmitter paracrine signalling, neurotransmitters diffuse through the extracellular matrix to their targets (Fuxe 1991; Fuxe et al. 2007). As such, neurotransmitters come into contact with many different proteins and synapses, not

necessarily specifically serotonergic. Although SERTs bind and transport serotonin preferentially, they can transport other biogenic amines just as other transporters can bind and transport serotonin, albeit to a decreased efficiency. In a study involving DAT knockout mice, it was shown that dopamine was removed from the striatum, suggesting that SERTs were transporting dopamine. The SSRI fluoxetine was able to ablate this effect and resulted in increased levels of dopamine (Shen et al. 2004; Kannari et al. 2006). Similar experiments were done with knockout mice for NET, which showed that norepinephrine was still being accumulated in regions of the brain, although at levels less than 50% of normal. This effect was abolished following incubation with citalopram, a SERT inhibitor (Vizi et al. 2004).

Looking at the other side of the coin, 5HT can itself be transported by carriers other than SERTs. For more than 4 decades, research has shown that both the NET and the DAT are capable of 5HT uptake (Burgen and Iversen 1965; Shaskan and Snyder 1970; Jackson and Wightman 1995; Daws et al. 1998; Pan et al. 2001; Callaghan et al. 2005; Daws et al. 2005; Zhou et al. 2005). Besides the monoamine transporters DAT and NET, there are two other classes of transporters that have recently been shown to transport 5HT; the organic cation transporters (OCTs) and plasma membrane monoamine transporter (PMAT). There are 3 OCTs that have been cloned thus far from humans and they are highly expressed in the brain. They show a high capacity to transport 5HT and have been shown to have increased expression in tissues that either lack or have decreased expression of SERT (Busch et al. 1996; Chen et al. 2001; Schmitt et al. 2003; Baganz et al. 2008). PMAT is not a homologue of any of the other monoamine transporters discussed thus far, but has fairly comparable transport efficiency to SERT for serotonin (Engel et al. 2004). It is also found in areas that overlap with SERT and OCTs, suggesting that all three may play an important role in the homeostasis of serotonin.

D.3 SERTs in invertebrates

The study of SERTs in invertebrates is still relatively young. SERTs have been identified in three invertebrates (not including schistosomes) with only two being cloned and

characterized. In *D. melanogaster*, a serotonin transporter (dSERT) has been cloned and shown to share pharmacological sensitivities of mammalian catecholamine transporters (Corey et al. 1994). The dSERT was shown to be expressed in the nervous system, specifically to serotonergic neurons, although in some areas, it is uncoupled from synaptic serotonin recycling or storage (Giang et al. 2011). In the nematode *C. elegans*, a SERT (MOD-5) has been shown to be able to transport 5HT in a heterologous system and confer 5HT hypersensitivity when absent in worms (Ranganathan et al. 2001). MOD-5 has been linked to several behaviours, through assays in null mutants. Hyperenhanced slowing, increases in egg-laying and a decrease in fat content were all observed in MOD-5 mutants, consistent with an excess of synaptic 5HT (Ranganathan et al. 2001; Srinivasan et al. 2008).

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CHAPTER II (MANUSCRIPT I)

Characterization of a serotonin transporter in the parasitic flatworm *Schistosoma mansoni*: Cloning, expression and functional analysis

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Abstract

The biogenic amine serotonin (5-hydroxytryptamine: 5HT) is a widely distributed neuroactive substance of vertebrates and invertebrates. Among parasitic flatworms, in particularly the bloodfluke, *Schistosoma mansoni*, 5HT is an important modulator of neuromuscular function and metabolism. Previous work has shown that schistosomes take up 5HT from host blood via a carrier mediated mechanism. This transport is thought to contribute to the control of schistosome motility in the bloodstream and is essential for survival of the parasite. Here we provide the first molecular evidence for the existence of a 5HT transporter in *S. mansoni*. A cDNA showing high homology with plasma membrane serotonin transporters (SERT) from other species was cloned and characterized by heterologous expression in cultured HEK293 cells. Functional studies showed that the recombinant schistosome transporter (SmSERT) mediates specific and saturable [^{3}H]-5HT transport with a K_t =1.30 ± 0.05 µM. The heterologously expressed protein was inhibited by classic SERT blockers (clomipramine, fluoxetine, citalopram) and the same drugs also inhibited [^{3}H]-5HT uptake by intact schistosomula in culture, suggesting that SmSERT may be responsible for this transport. Conventional (end-point) and real-time quantitative RT-PCR analyses determined that SmSERT is expressed both in the free-living stage (cercaria) and parasitic forms of *S. mansoni* but the expression level is significantly higher in the parasites. These results suggest that SmSERT is upregulated following cercarial transformation, possibly to mediate the recruitment of exogenous 5HT from the host.

Keywords: Serotonin; Transporter; SERT; Biogenic amines; Neurotransmitter; *Schistosoma mansoni*

Introduction

The flatworm *Schistosoma mansoni* is a blood dwelling parasite and one of the major causes of schistosomiasis, a debilitating disease that afflicts over 200 million people worldwide. A free-living larva (cercaria) infects humans by direct penetration through the skin and is transformed into an immature parasitic form (schistosomula) that rapidly enters the circulation and begins to develop into sexually mature worms. During this development, the young parasite undergoes a complex migration through the heart and lungs towards the mesenteric veins of the hepatic portal system, where the adult worms typically reside. This migration is vital to schistosome survival and is coordinated by the parasite's own neuromuscular system, as well as host-derived signals that modulate motility. One such signal is serotonin (5-hydroxytryptamine: 5HT), a well known neurotransmitter and hormone, which is present at high levels in mammalian blood. Studies dating back to the 1970s and 80s have shown that exogenous application of 5HT onto schistosomes in culture causes a marked increase in parasite motility (1-3). The stimulation is due to an increase in the frequency of muscle contraction (3, 4) and also an increase in carbohydrate metabolism (3, 5, 6), which makes more energy available for movement. The effects on motility are seen in intact animals that are not permeabilized, suggesting 5HT is either acting through surface receptors, or is taken internally via a specific transporter and acting directly on the musculature.

Plasma membrane serotonin transporters (SERT) were first identified in the mammalian central nervous system (CNS), where they mediate re-uptake of the amine across the presynaptic membrane. This is part of a mechanism designed to inactivate 5HT by quickly removing it from the synaptic cleft. SERTs have since been reported in the CNS of other organisms and also non-neuronal tissues that store 5HT (7-9). Serotonin transporters have not yet been cloned from any of the parasitic worms, including *S. mansoni*. There is, however, good biochemical evidence to suggest that a SERT-like mechanism of transport is conserved in these organisms. A concentrative, sodium-dependent serotonin transport system was reported in *S. mansoni* and the cestode, *Hymenolepis diminuta*. Moreover, this transport was blocked by treatment with antidepressant drugs, such as fluoxetine (Prozac) (10-14), which are classic SERT inhibitors (15).

Here we report the cloning, functional characterization and developmental expression analysis of a *S. mansoni* cDNA (SmSERT) that is homologous with previously cloned SERTs from other species. The results provide the first molecular evidence for the existence of a 5HT transporter in schistosomes and further suggest that SmSERT mediates the intake of exogenous 5HT *in vivo*.

Materials and Methods

Parasites. The Puerto Rican strain of Schistosoma mansoni was used throughout these experiments. Infected snails (Biomphalaria glabrata) were obtained from the Biomedical Research Institute (Bethesda, MD) and were induced to shed S. mansoni cercaria by exposure to artificial light for 1 hr at room temperature. Schistosomula were obtained by in vitro transformation of cercaria, using the mechanical tail-shearing method (16). To obtain adult parasites, CD1 female mice were infected with cercaria by active penetration through the skin. Approximately 7-8 weeks post-infection the mice were sacrificed and the adult worms were collected by perfusion of the livers and mesenteric veins, as described (16).

Cloning of SmSERT. A first analysis of schistosome Expressed Sequence Tags (EST) available (www.genedb.org/genedb/smansoni/) identified two overlapping SERT-like ESTs, one belonging to S. mansoni (Sm26427) and the other to the closely related species, S. japonicum (BU710860). To amplify these sequences, we extracted total RNA from adult S. mansoni worms using TRIzol (Invitrogen), as per the recommendations of the manufacturer. The RNA was oligo-dT reverse-transcribed, according to standard procedures, and the resulting cDNA was

subsequently used in a PCR reaction with sequence-specific primers targeting the S. mansoni and S. japonicum ESTs. This produced a 655bp partial SERT-like sequence, corresponding roughly to the predicted mid region of the protein's open reading frame. The remaining 3' and 5' ends were subsequently obtained by RACE (rapid amplification of cDNA ends) procedures with the use of commercial kits (Invitrogen). For 3' RACE, aliquots of total RNA (3.5 μg) were reverse-transcribed, using an oligo-dT anchor primer supplied by the kit and the resulting cDNA was used for standard PCR with a gene-specific primer (5'GTCTCTGCTTAATGGCTGTATTTAC 3') and a reverse primer targeting the anchor region. A second round of PCR was then done using a nested gene-specific primer (5'CTGGTATTAAATACTATCTTAC 3') and the same anchor primer. For 5' RACE, adult S. mansoni total RNA (3.5 μg) was reverse-transcribed using a gene-specific primer (5' CAAATAGCTCACGTCTACCAG 3') and the resulting first strand cDNA was modified by the addition of an anchor sequence to the 5'end, as described in the kit protocol (5'RACE, Invitrogen). The 5'end tailed cDNA was used as a template for PCR with a gene-specific reverse primer (5'GAAACACTGTCCATGATTGCTG 3') and a forward primer targeting the anchor sequence at the 5'end. This was followed by a second round of PCR using an internal gene specific reverse primer (5'TAACATAAGGCAAAGTAGCAG 3') and the same forward anchor primer. 3' and 5' RACE products were cloned into vector pGEM-T (Promega) and confirmed by DNA sequencing of two separate clones. Finally, to obtain the full length SmSERT, we designed primers targeting the predicted start and stop codons (forward primer: 5'ATGAGTGTATCAAGTCAAAAATTCA '3; reverse primer: 5'TTATAATTTATTTGTTTTGAATG '3) and amplified the cDNA directly from oligo-dT reverse-transcribed total S. mansoni RNA. Once again, products were cloned into pGEM-T and at least two separate clones were sequenced.

Heterologous expression studies. For functional expression and pharmacological analyses of SmSERT, the full-length cDNA was cloned into the pCI-Neo vector (Promega) (pCI-neo-SmSERT) and expressed in mammalian cells. HEK293 cells were routinely cultured in Dulbecco's Modified Essential Media (DMEM) supplemented with 20mM HEPES and 10% heat inactivated fetal calf serum. For transfection, HEK293 cells were seeded in 24 well plates ($1x10^5$ cells/well) and allowed to grow overnight. The following day, cells were transiently transfected with pCI-Neo-SmSERT or empty vector for the mock control. Transfections were performed with the use of Fugene 6 (Roche), according to the recommendations of the manufacturer. Two days post transfection, cells were washed twice in warm TB (10mM Hepes pH 7.5, 150mM NaCl, 2mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$) and incubated in DMEM (400µl /well) containing $2.22x10^6$ DPM / well 5-hydroxy[G-^{3}H]tryptamine trifluroacetate (98.0 Ci/mmol) and sufficient unlabeled 5HT to produce the desired final concentration. Incubations were carried out for 1 hour at room temperature, unless indicated otherwise. Following incubation, cells were washed three times in cold TB and lysed by addition of 2% SDS. Incorporated radioactivity was then measured using a liquid scintillation counter. Nonspecific uptake was assessed by running parallel experiments with cells transfected with empty pCI-Neo plasmid. For measurements of transport constant (K_t) and V_{max} values, SmSERT-transfected and control cells were incubated in various amounts of 5-hydroxy[G-^{3}H]tryptamine trifluroacetate (0.05 – 3 µM) and transport was measured as above. The kinetic parameters were obtained by nonlinear curve-fitting analyses of saturation curves, using the GraphPad Prism v.4.0 software package. Inhibition assays were done with a constant amount (0.1 µM) of 5-hydroxy[G-^{3}H]tryptamine trifluroacetate and variable concentrations of test inhibitor over a range of 10^{-12} M – 10^{-4} M. Inhibition curves were fitted with a one-site competition equation (GraphPad Prism v.4.0) to obtain IC_{50} values and the corresponding inhibition constants (K_i) were calculated from the IC_{50}, according to the method of Cheng and Prusoff (17). The pharmacological agents used in this study (5HT, fluoxetine, dopamine, histamine, norepinephrine, citalopram hydrobromide, clomipramine hydrochloride) were purchased from Tocris Biosciences and Sigma Aldrich.

To monitor expression of SmSERT at the protein level we produced a second pCI-Neo construct in which SmSERT was fused at the C-terminal end to a FLAG epitope (DYKDDDDK), using standard PCR methods. HEK293 cells transfected with pCI-neo-SmSERT-FLAG or empty vector were tested 48 hr post-transfection by *in situ* immunofluorescence, as described previously (18, 19). Briefly, the cells were fixed and permeabilized by treatment with ice-cold methanol (10 min at –20°C), incubated with anti-FLAG M2 monoclonal antibody (10µg / ml in PBS) (Sigma) for 1 hr at 4°C, washed with phosphate buffered saline (PBS) and then incubated for 1 hr at 4°C with a goat anti-mouse IgG antibody conjugated to FITC (fluorescein isothiocyanate) (Sigma; 1:300 dilution in PBS). Cells were washed again in PBS and finally examined with a Nikon Optiphot-2 fluorescence microscope equipped with a FITC filter.

5HT transport in cultured schistosomula. Measurements of [3H]5HT uptake in parasites was done using 4-6 day old schistosomula. In vitro transformed schistosomula (16) were cultured in Opti-MEM (GIBCO) supplemented with 4% serum and 0.25 µg/ml fungizone and 100 µg/ml streptomycin, and 100 units/ml penicillin in 24 well plates at approximately 200 animals/well. Fresh media was added every 3 days and animals were monitored daily by microscopic examination. Schistosomula can be maintained in culture under these conditions for at least 3 weeks with minimal loss of viability. After 4-6 days, animals were placed in 1.5ml eppendorf tubes, washed twice in PBS and incubated with 0.1 µM [3H]5HT (containing 2.22×10^6 DPM/well) in serum-free Opti-MEM. Incubations were carried out at room temperature for up to 1 hour either in the presence or absence of a SERT inhibitor added at a concentration of 10^{-4} M. After incubation, the larvae were washed three times in PBS, solubilized in 1% SDS and then radioassayed to measure [3H]5HT incorporation (11).

SmSERT expression analyses: End-point and quantitative RT-PCR. Total RNA was extracted from cercaria, 4-6 day schistosomula and adult parasites using either TRIzol (Invitrogen) or the RNA micro extraction kit (QIAGEN), as per the recommendations of the manufacturer. The RNA was quantitated by spectrophotometry with the use of a NanoDrop instrument (Wilmington, USA) and equal amounts (approximately 300-500 ng) of RNA from the various developmental stages were used for reverse-transcription (RT). The RT was performed with oligo-dT primer and Superscript III reverse transcriptase (Invitrogen), according to standard protocols and the resulting cDNA was used for PCR. For end-point PCR analysis, we amplified a partial SmSERT fragment (635bp) with primers 5'GATTTCCATTTATATGTTATCG 3' (forward) and 5'TAACATAAGGCAAAGTAGCAG3' (reverse) according to the following protocol: 30 cycles each of 94^0C/30s, 52^0C/ 45s and 72^0C /30s. The same cDNA was PCR amplified with primers targeting the *S. mansoni* house-keeping gene GAPDH (Accession #: M92359) to standardize expression. The GAPDH primers (5'GTTGATCTGACATGTAGGTTAG3' forward and 5'ACTAATTTCACGAAGTTGTTG 3' reverse) were designed to amplify a 206bp product, using the same protocol as above except the annealing temperature was 54^0C. PCR products were analyzed by agarose gel electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide. For the quantitative PCR (qPCR) analysis the cDNA was amplified with the Quantitect SYBR Green PCR kit (Qiagen) in a Rotor-Gene RG3000 instrument (Corbett Research). Primers for qPCR were designed using Oligo (MBI) and the settings were adjusted to the highest possible stringency to generate ≈150-200 bp amplicons, as recommended. The primers selected for SmSERT (Forward: 5'GTGGACTACCTTTATTTTATC 3' and Reverse: 5'CATATAGAACAGTGCCCATGC 3') amplified a product of 178bp. As an endogenous house-keeping reference for qPCR we used primers targeting *S. mansoni* α-Tubulin (Accession # M80214) (Forward: 5'TCGTGGTGATGTTGTCCCCAAG 3' and

Reverse: 5'TCGGCTATTGCGGTTGTATTAC 3'; product size of 201bp). Preliminary validation experiments demonstrated that the amplification efficiencies of SmSERT and the internal reference (α-tubulin) were approximately equal. Reaction conditions were as described in the SYBR green kit and the cycling protocol was as follows: 45 cycles of 95°C for 25 s, 58°C for 30 s, and 72°C for 40 s. The generation of specific PCR products was confirmed by melting curve

analysis and agarose gel electrophoresis. Quantitation of relative differences in expression were finally calculated using the comparative $\Delta\Delta C_T$ method (20).

Other methods. SmSERT homologues were identified by BLAST analysis of the NCBI sequence database with an E value cutoff of $3e^{-165}$. ClustalW sequence alignments were done with MacVector v.8.0 and a phylogenetic tree was constructed using the Neighbor-Joining method. The dataset was bootstrapped using 1000 replicates. Statistical comparisons were done either with a student t-test or a one-way ANOVA followed by a Tukey pairwise comparison, as required. A $P \leq 0.05$ was considered statistically significant. Protein concentrations were determined with a Lowry assay, using a commercial kit (BioRad), according to the manufacturer's protocol.

Results

Cloning and sequence analysis of SmSERT. An analysis of the *S. mansoni* sequence database identified a short EST that resembled SERT-encoding sequences from other species. This sequence was first PCR amplified directly from oligo-dT reverse transcribed adult *S. mansoni* RNA, using sequence specific primers. A second overlapping SERT-like EST was detected in the *S. japonicum* database and the corresponding *S. mansoni* sequence was also amplified by RT-PCR, using primers based on the *S. japonicum* EST. The remaining 5' and 3' ends were subsequently obtained by RACE procedures. The final sequence included a single continuous open reading frame (ORF) of 1974 bp followed by 773bp of 3' untranslated region and a short polyA tail at the 3'end. The predicted coding sequence was finally cloned by RT-PCR and confirmed by DNA sequencing (Accession#: EF061308). During the course of this study, a putative 5HT transporter cDNA was posted in GenBank (Accession# DQ220811; unpublished). This cDNA is identical to ours except for an additional 234 bp of predicted coding sequence at

the 5' end, which would add 78 amino acid residues at the N-terminus. We were unable to detect this additional sequence in any of the 5'RACE products that were tested (a total of 3 products were sequenced) and attempts to amplify the longer ORF by RT-PCR were also unsuccessful. Moreover, the protein encoded by the shorter 1974 bp ORF is similar in length to SERTs cloned from other species. Thus we conclude that the sequence reported here is the predominant form of SmSERT, though there may be a longer variant of this transporter that could not be detected in this study.

SmSERT encodes a protein with a predicted molecular weight of 73687da. Hydropathy analysis indicated the presence of 12 transmembrane regions with both N- and C- termini being retained in the cytosol, a topological organization conserved in all the Na^+/Cl^- dependent neurotransmitter carriers, including SERTs. The dendogram in Fig. 1 shows the genetic relationship of SmSERT to other SERT sequences and closely related biogenic amine transporters. The schistosome transporter clusters with members of the SERT family and shares high sequence homology with both vertebrate and invertebrate SERTs, including rat (65% similarity), human (64% similarity), Drosophila (62% similarity) and C. elegans (58% similarity). A ClustalW alignment of SmSERT with selected homologues (Fig. 2) shows high conservation of amino acid sequence (>75% similarity) within the predicted transmembrane regions, whereas the ends and loop regions are more divergent. Of note is the exceptionally large extracellular loop between transmembrane region 3 and 4 (EL2); SmSERT has an insertion of 25 amino acids that is not present in any other organism. This loop has several unique N-glycosylation sites, whereas those conserved in other species are not retained within the *S. mansoni* sequence. Several key amino acid residues of the predicted binding pocket are present in SmSERT, notably residues of TM1 (D^{84}), TM3 (Y^{157}), TM6 (F^{346}, F^{352}) and TM8 (S^{448}, G^{452}) (21). Additional residues near TM2 (F^{103}, Y^{107}, M^{110}), TM4 (F^{274}, Y^{278}) TM5 (Y^{300}), TM10 (Y^{525}) and TM11 (F^{557}, I^{561}) are thought to contribute to antagonist binding (22) and are also conserved in SmSERT. Not all predicted binding sites are conserved, however. We detected substitutions at two key positions of the predicted binding pocket, $Y \rightarrow F^{81}$ (hSERT position Y^{95}) and $I \rightarrow T^{158}$ (hSERT position I^{172}) of TM1 and TM3, respectively (23). Other potentially important substitutions include $F \rightarrow G$ (TM11,

position G^{560}) and Y→H near the extracellular boundary of TM10 (position H^{504}). Both these residues have been implicated in antagonist binding (22).

Functional expression analysis of SmSERT. To determine if the cloned cDNA encoded a functional transporter, the full-length SmSERT was transiently expressed in cultured HEK293 cells for subsequent measurements of [3H]5HT uptake activity. In preliminary separate experiments, the SmSERT plasmid construct was modified to introduce a C-terminal FLAG epitope, which was subsequently targeted with an anti-FLAG antibody to monitor expression of the fusion protein. HEK293 cells transfected with this modified construct were tested by in situ immunofluorescence analyses with anti-FLAG. The results revealed significant immunofluorescence in the cells transfected with SmSERT-FLAG but not the mock control transfected with plasmid only (data not shown), indicating that the transporter was expressed in the heterologous cell environment. This was confirmed by subsequent measurements of transport activity. As shown in Fig.3, the accumulation of [3H]5HT in the SmSERT-transfected cells was both concentration-dependent and saturable with an estimated transport constant (Kt) of 1.30 ± 0.05 μM and Vmax of 6.84 ± 0.35 nmol of 5HT/hr/105cells. By comparison, the control cells transfected with empty plasmid exhibited only background absorption, which increased linearly over the concentration range tested. In subsequent studies, we measured transport activity in SmSERT-transfected cells treated with various amounts of three classic SERT inhibitors, fluoxetine, citalopram and clomipramine. The results (Fig. 4) show that the [3H]5HT uptake was inhibited by all three test drugs in a concentration-dependent manner. Fluoxetine and clomipramine exhibited approximately the same potency, with Ki values of 78.6

± 17 nM and 69.8 ± 13.8 nM, respectively. Citalopram was less potent than the other two drugs (Ki = 155 ± 45 nM) but the difference in Ki was not statistically different (one way ANOVA at P< 0.05) (Fig. 4). To test for specificity, we repeated the transport assays in the presence of structurally related biogenic amines, including dopamine, noradrenaline and histamine. None of these amines was able to inhibit [3H]5HT transport in the transfected cells up to concentrations of 10-4M, suggesting that SmSERT is selective for 5HT.

Transport studies in cultured schistosomula. To compare the drug profile of the recombinant SmSERT with that of 5HT transport in vivo, we measured [3H]5HT transport in cultured schistosomula in the presence of the same SERT blockers. S. mansoni schistosomula has been previously shown to take up exogenous 5HT from the medium (10, 14) and this was confirmed here. The results (Fig 5) show that intact parasites can incorporate [3H]5HT in a time-dependent fashion. In addition, this accumulation of [3H]5HT is sensitive to 10^{-4}M fluoxetine, as shown by the reduction in uptake in the presence of inhibitor. The transport is also significantly inhibited by treatment with 10^{-4}M clomipramine, 10^{-4}M citalopram and an excess of unlabeled 5HT (10^{-4} M), whereas other biogenic amines (histamine, dopamine, and noradrenaline) had no significant effect (student t-test, P<0.05) at the same concentration, consistent with the profile of SmSERT (Fig. 5).

RT-PCR analyses. Expression levels of SmSERT were measured at the mRNA level in different developmental stages of *S. mansoni*, using both conventional (end-point) and real-

time quantitative RT-PCR analyses. Three stages were examined, including the free-living infective stage (cercaria), in vitro transformed 5-day old schistosomula and adult worms. An initial comparison of cercaria and adults by conventional RT-PCR showed an apparent upregulation of SmSERT in the adult worms. Fig. 6 shows a typical gel analysis of the PCR products after standardization relative to a constitutively expressed control gene from S, mansoni (GAPDH) (24). Based on densitometry analysis, the intensity of the SmSERT PCR product is visibly higher in the adults than cercaria, whereas no such difference can be seen in the GAPDH control amplified from the same cDNA samples. No GAPDH or SmSERT PCR products were detected in the minus reverse transcription control (not shown), thus ruling out the possibility of genomic DNA contamination. To quantitate this apparent up-regulation we repeated the analysis with real-time qPCR and broadened the study to examine the schistosomula as well. Fold-change in SmSERT expression levels were calculated relative to the cercarial stage and after normalization to a housekeeping gene (S. mansoni α -tubulin (24, 25)), according to the $\Delta\Delta$Ct method (20). GAPDH and α-tubulin are both widely used as endogenous standards for qPCR analysis (24, 25). The results showed that SmSERT mRNA expression levels in the adults and schistosomula were significantly increased 4.2 ± 1.1 fold (n=9) and 5.3 ± 2.0 fold (n=10), respectively, compared to the cercaria (Fig. 6) (Student t-test; P =0.05). Combined with the gel analysis, the results suggest that SmSERT is moderately upregulated following the transition from free-living to parasitic stage.

Discussion

5HT is one of the more ubiquitous neuroactive agents among animal phyla and it is particularly important in flatworms. There is ample evidence that 5HT is present in the flatworm nervous

system, including that of schistosomes (26, 27) and is biologically active in these organisms. When applied onto isolated flatworm muscle fibers, 5HT stimulates the frequency of contractions and increases muscle tone, possibly through modulation of neuromuscular transmission (1-3, 4, 28). In addition, 5HT added onto crude worm extracts markedly stimulates glycogenolysis and activates phosphofructokinase, suggesting a role in the control of carbohydrate metabolism (6, 12, 29). These effects are thought to be mediated by multiple 5HT receptors (2-4, 5, 30-32) though none has yet been cloned or characterized at the molecular level. Whereas the importance of 5HT as an endogenous signal is well demonstrated, it is less clear how the parasites respond to exogenous (host-derived) 5HT. As mentioned earlier, intact schistosomes treated with 5HT show a very pronounced increase in motor activity (1, 2) and the same is true of other parasitic flatworms that live in 5HT-rich environments, such as *H. diminuta* (28). Since the worms lack a well-developed alimentary tract to ingest 5HT, the question arises of how the exogenous amine is able to elicit this effect on motor activity. It has long been suggested that exogenous 5HT is taken up via a surface carrier (10-14) and therefore could be interacting with internal receptors located on the musculature. Our results support this hypothesis by demonstrating the presence of a 5HT transporter in *S. mansoni*. The recombinant protein was found to be selective for 5HT and, importantly, was inhibited by the same classic SERT antagonists that also blocked intake of ^{3}H-5HT by the intact parasites in culture. This suggests that SmSERT mediates the recruitment of exogenous 5HT and, as such, could play a critical role in the parasite's response to host 5HT *in vivo*.

Why schistosomes take up 5HT from the host is not clear. Earlier studies had proposed that the parasites were unable to synthesize the amine and therefore relied upon the host for an exogenous supply (5). More recently, however, it has been determined that schistosomes have the necessary enzymes for 5HT biosynthesis and are able to produce it internally (33). It is possible that additional 5HT is needed to supplement endogenous stores when biosynthetic activity in the parasite is low. Alternatively, the intake of host 5HT is part of a signaling mechanism used by the parasite to modulate its motility in the bloodstream. This is supported by evidence that some flatworms guide their migration in the host by responding to changes in

5HT concentration (34). SmSERT could play an important role in this process by controlling the intake of amine in response to variable concentrations in the host.

The results of the RT-PCR analysis showed that the transporter was expressed both in the free-living (cercaria) and parasitic stages (schistosomula and adults) but the level of expression was higher in the parasites. This increase was seen even in young (5 day old) schistosomula, suggesting that SmSERT was upregulated during or shortly after the process of cercarial transformation. A possible explanation for the increase is that more transporter is needed to take in 5HT from the host in the parasitic forms, whereas no such activity would be required in the free-living (freshwater) cercaria. On the other hand, the fact that SmSERT was present in the cercaria, albeit at lower level, suggests that the transporter has other function(s), which are common to both free-living and parasitic stages. One potential function is the inactivation and/or salvage of endogenous 5HT. Across phylogeny, SERTs are used to remove 5HT from extracellular spaces, either to inactivate or store the amine. This is particularly prevalent in the mammalian CNS where plasma membrane SERTs rapidly sequester extracellular 5HT into presynaptic neurons (or glial cells) as a way of terminating the signal (7-9). Parasitic flatworms also have a neuronal 5HT uptake system (13) and there is evidence for storage of 5HT in a variety of tissues (13), which could be mediated by a SERT-like transporter. Thus we postulate that SmSERT has a dual function in schistosomes, acting on the surface to recruit host 5HT and also internally to inactivate/ store the amine as part of the parasite's endogenous 5HT system.

SmSERT is the first example of a monoamine transporter in the so-called "lower" invertebrates including flatworms and coelenterates. The sequence shares many characteristics of the SERT family, notably the predicted topology of 12 highly conserved transmembrane regions, and many of the residues previously implicated in substrate binding are also conserved in SmSERT. When expressed in HEK293 cells, the schistosome protein exhibited about the same 5HT transport kinetics as other members of this family, with a K_t of about 1.3 µM (8, 35, 36). The drug profile, on the other hand, was different from the mammalian prototype. Although SmSERT was sensitive to inhibition by classic SERT blockers their potencies were between 11- and 120-fold lower than those reported for human and mouse SERTs (23), suggesting the

affinity for these drugs was generally low. The relative order of drug potency was also different; human SERT has ≈5-fold higher affinity for citalopram than fluoxetine (23) whereas SmSERT exhibited about the same K_i for both drugs. This suggests there are important differences in the binding sites of these transporters despite the overall similarity of the sequences.

Two noteworthy differences between the *S. mansoni* and human SERTs can be seen in TM1 and TM3. Recent studies of human hSERT identified TM1 and TM3 as principal helices of the transporter's binding pocket, with two residues, Y^{95} (TM1) and I^{172} (TM3), serving as primary antagonist binding sites (23). Mutations of these residues decreased the affinity for SERT blockers (antidepressants) between 30- and nearly 1000-fold, the greatest decrease occurring with citalopram. In contrast, the mutations had no measurable effect on the affinity for 5HT itself, suggesting these two residues contribute only to antagonist binding (23). In *S. mansoni*, Y^{95} and I^{172} are replaced with a phenylalanine (F^{81}) and a threonine (T^{158}), respectively. This may explain why SmSERT has generally lower affinity for antidepressants and why there is less of a preference for citalopram. Aside from these two residues, we noted numerous differences in the vicinity of previously identified binding domains, for example in TM 10, 11 and 12. TM10, in particular, has been implicated in antagonist binding (22) and is poorly conserved in *S. mansoni* (see Fig. 2). Moreover, SmSERT has a very distinctive insertion in its EL2 segment that could influence substrate interactions with neighboring binding sites, as well as the translocation of 5HT across the membrane (37).

Schistosomiasis remains a major health problem worldwide and the strategies available for treatment are limited. Neurotransmitter transporters, SERTs in particular, hold great potential for drug targeting, as can be seen from the many successes of mammalian neuropharmacology. The results reported here suggest it may be possible to exploit unique structural and pharmacological properties of SmSERT to identify a selective anti-schistosomal agent. The functional relevance of these distinctive SmSERT features deserves further investigation.

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Figures

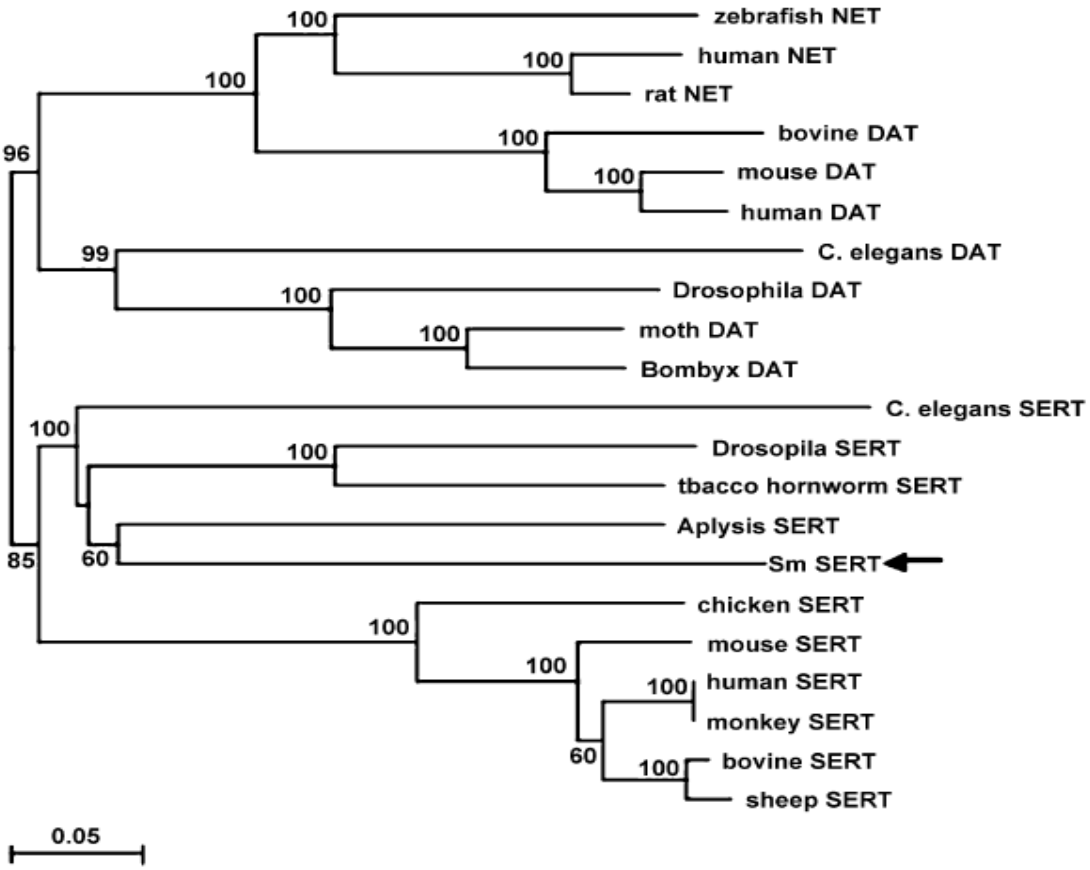


Figure 1 Dendogram analysis of SmSERT and other biogenic amine transporters. Amino acid sequences of serotonin (SERT), dopamine (DAT) and norepinephrine (NET) plasma membrane transporters were aligned using the ClustalW method (MacVector 8.0) and a neighbor-joining phylogenetic tree was constructed from the multiple sequence alignment. The numbers at branch points are bootstrap values and the lengths of branches are proportional to the genetic distance between sequences. Sequence sources are as follows: SmSERT (GenBank accession number: EF061308), human SERT (NP_001036.1), mouse SERT (Q60857), monkey SERT (Q9MYX0), bovine SERT (AAG01287.1), sheep SERT (AAG01287.1), chicken SERT (AY573844.1), *C. elegans* SERT (AF385631), *Drosophila* SERT (NP_523846.2), tobacco hornworm SERT (*Manduca sexta*, AAN59781.1), *Aplysia* SERT (AAK94482), moth DAT (AAZ17654.1), *Bombyx* DAT (NP_001037362.1), bovine DAT (P27922), *C. elegans* DAT (Q03614), mouse DAT (AAF85795), *Drosophila* DAT (AAF76882), human DAT (AAC50179), zebrafish NET (XP_694138), rat NET (CAA73665), human NET (NP_001034).

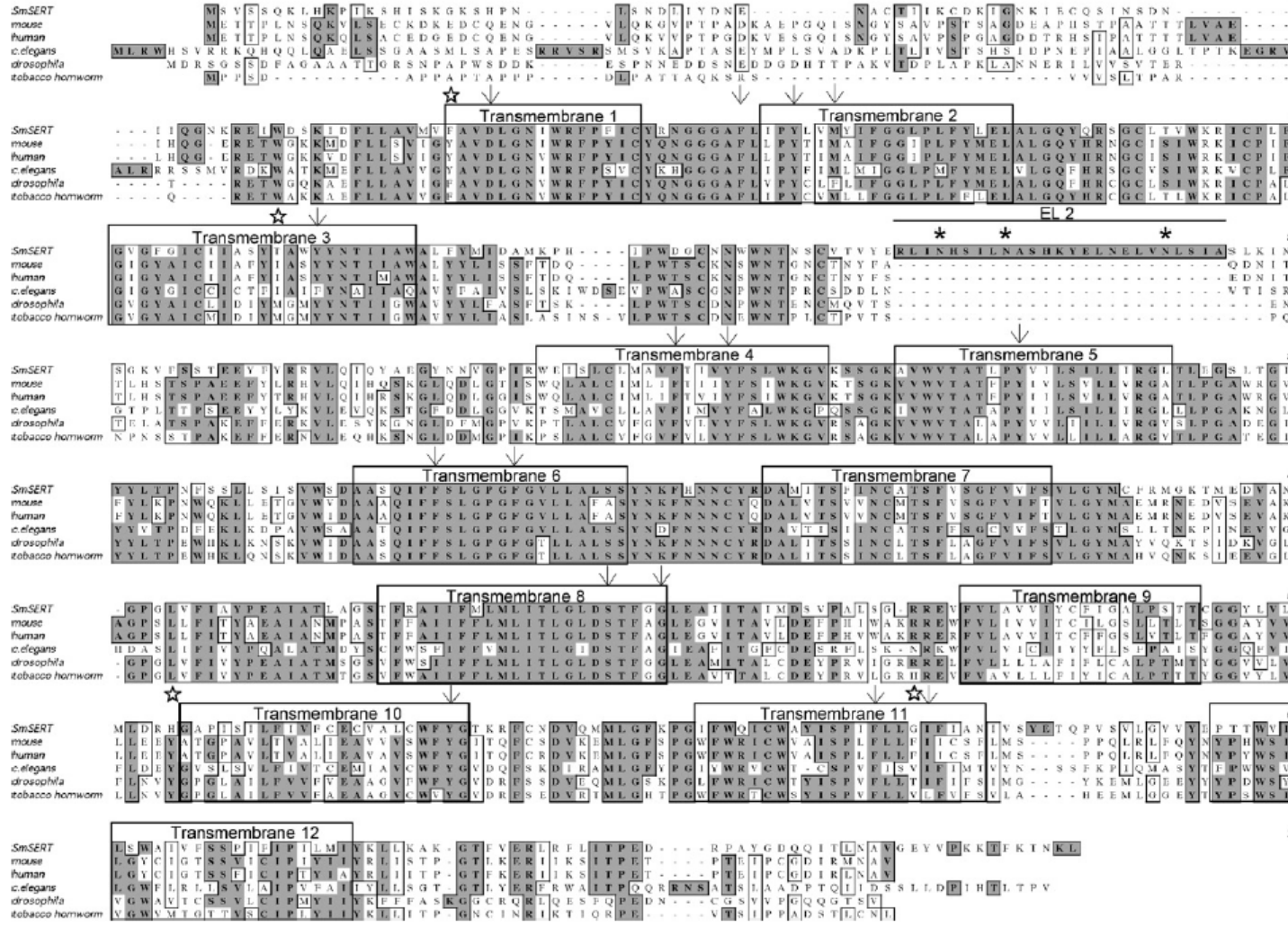


Figure 2. Sequence analysis of SmSERT. Sequence alignment using ClustalW method)Macvector 8.0) of SmSERT with SERTs of other organisms. Areas shaded in grey denote conserved amino acids. Predicted transmembrane regions are marked. The arrows indicate conserved binding sites whereas the stars identify important amino acid substitutions, as discussed in the text. SERT sequence accession numbers can be found in Figure 1.

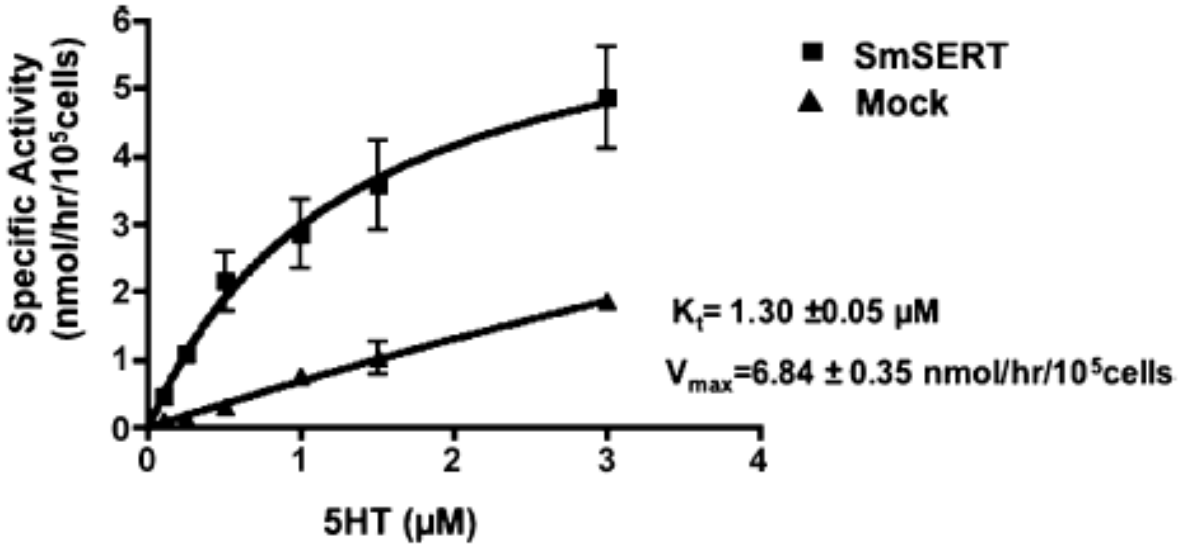
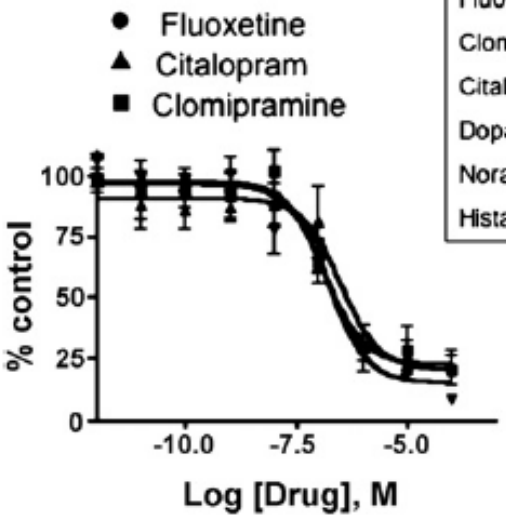


Figure 3. Saturation kinetics of SmSERT-mediated uptake of [^{3}H]5HT in HEK293 cells. HEK293 transiently expressing SmSERT were incubated with increasing [^{3}H]5HT concentrations for 60 minutes at room temperature. After incubation, cells were washed, lysed and radioassayed to measure incorporation of [^{3}H]5HT. Non-specific uptake was done using parallel transfections with parent plasmid lacking the SmSERT. Each data point is the mean ± SEM of at least three separate experiments done in duplicates.



Drug	SmSERT (K_i) nM	hSERT (K_i) nM
Fluoxetine	78.6 ± 17.0	6.7 ± 1.2
Clomipramine	69.8 ± 13.8	2.3 ± 0.4
Citalopram	155 ± 45	1.3 ± 0.9
Dopamine	> 10,000	> 10,000
Noradrenaline	> 10,000	> 10,000
Histamine	> 10,000	> 10,000

Figure 4. Pharmacological profile of recombinant SmSERT expressed in HEK293 cells. HEK293 cells expressing SmSERT were incubated with a constant amount of [^{3}H]5HT and various concentration of test inhibitor or no added drug (control). Data are normalized relative to the untreated control sample and are expressed as the means ± SEM of three to four separate experiments, each in duplicate. K_i values were calculated from the corresponding inhibition curves by non-linear regression curve-fitting analysis (GraphPad Prism v.4.0). K_i values for human hSERT were obtained from Henry *et al* (23).

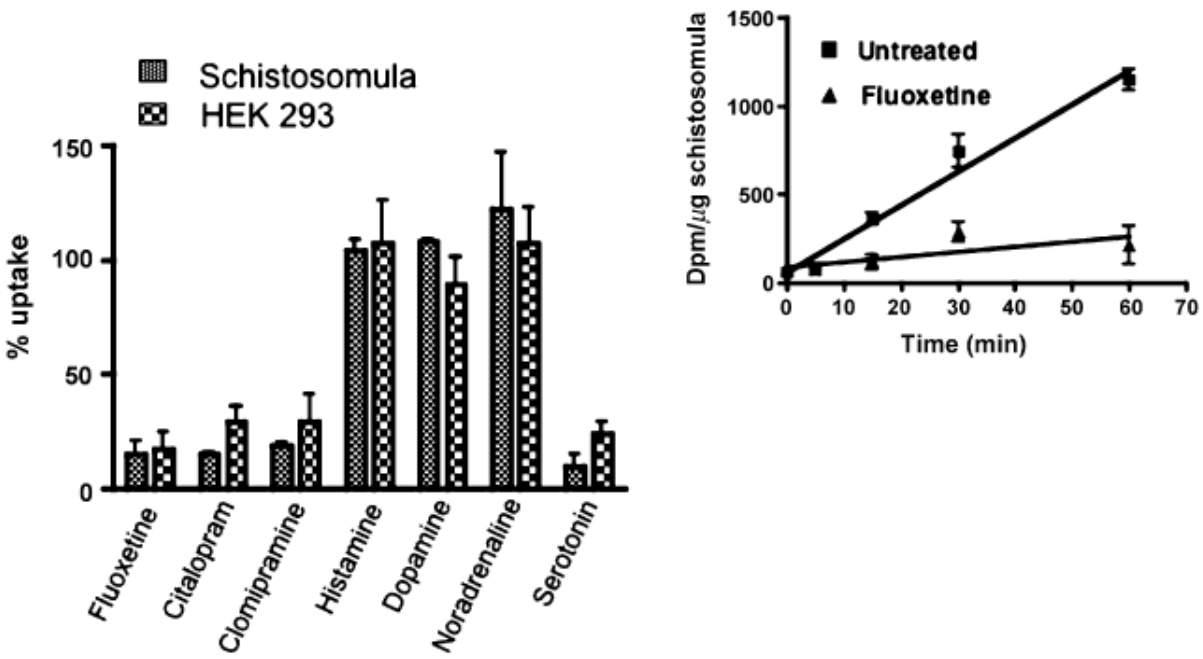


Figure 5. Comparison of [^{3}H]5HT uptake in schistosomula and transfected HEK293 cells. Five day old schistosomula (hashed bars) were incubated with [3H]5HT for 60 minutes at room temperature either in the presence of test drugs (each at 10^{-4}M) or untreated. Following incubation, animals were washed in ice-cold PBS and lysed for radioactive counting. HEK293 cells expressing recombinant SmSERT (checkered bars) were harvested 48 hours post transfection, incubated with [3H]5HT for 60 minutes under the same conditions as above, washed and radioassayed. Data shown were normalized relative to the untreated control samples and are the mean of three experiments done in duplicate. (Inset) Time course of [3H]5HT intake by 5-day old schistosomula. Parasites were incubated with [3H]5HT as above either in the presence or absence of fluoxetine and the incubations were stopped at the indicated times by washing with ice-cold PBS. Values represent the means of three experiments done in duplicate.

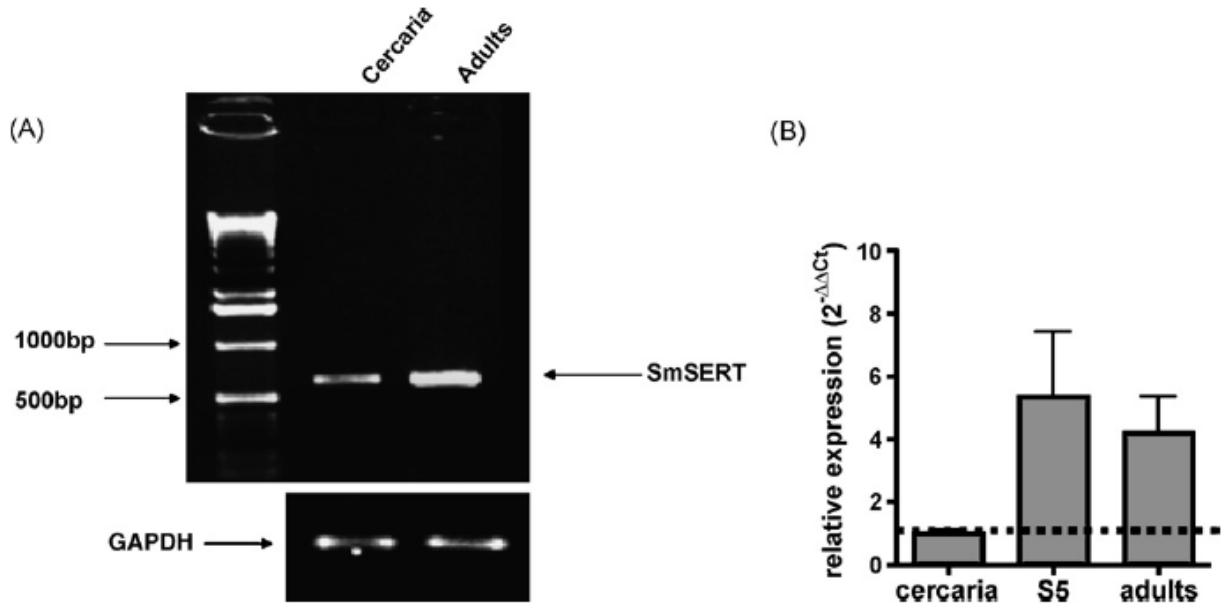


Figure 6. developmental expression of SmSERT in *S.mansoni*. (A) RT-PCR was performed on total RNA extracted from two different developmental stages of *S. mansoni* (cercaria and adult) and was standardized by simultaneous amplification of an internal housekeeping control gene (GAPDH). The PCR products were resolved by agarose gel electrophoresis and visualized by staining with ethidium bromide. A typical amplification of SmSERT (635bp) and GAPDH (206bp) in the two stages is shown. No products were seen in parallel negative control reactions in which reverse transcriptase was omitted (not shown). (B) Samples were subsequently analyzed by real-time RT-PCR to quantitate the difference in SmSERT expression levels between the two stages. The data were calculated according to the comparative ΔΔCt method (20) and are shown as the fold-change in SmSERT expression relative to the cercaria. The data are the means and SEM of 9-10 experiments. S5, 5 day old *in vitro* transformed schistosomula.

Connecting Statement I

In the previous manuscript, we reported the presence of a serotonin specific transporter (SmSERT) in *Schistosoma mansoni*. The sequence for the SmSERT is similar to other known serotonin transporters with the exception of a slightly longer N-terminal and a large insertion in the second extracellular loop. Expression in mammalian cells showed it is capable of saturable transport with an EC_{50} = 1.30 ±0.05 µM, a figure close to that of human SERT. A preliminary pharmacological profile showed that typical antagonists of hSERT are also capable to inhibiting the transport of 5HT by SmSERT. The transporter is expressed in all life cycle stages tested, both parasitic and free living, suggesting it may have a role other than exogenous recruitment. In the next chapter, we describe the biological investigation of the SmSERT through immunolocalization using a peptide derived antibody and behavioural assays following knockdown with RNAi.

Chapter III (manuscript II)

The Functional Role of the SmSERT in *S. mansoni* Elucidated through Immunolocalization and RNAi

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Abstract

Serotonin (5-hydroxyryptamine: 5HT) is an important neurotransmitter in both vertebrates and invertebrates. In the parasitic flatworm, *Schistosoma mansoni*, serotonin stimulates muscle contraction, motility and metabolic activity. A serotonin-specific transporter (SmSERT) was previously cloned from *S. mansoni* and shown to be active when expressed heterogeneously *in vitro*. Here we report a first investigation of the native transporter in cultured parasites, using a combination of immunolocalization techniques, pharmacological tools and RNA interference (RNAi). In the adult stages, we saw that the protein was expressed in the central nervous system (CNS) localizing to the main nerve cords running the length of the body and transverse commissures. Significant SmSERT expression was also observed in the subtegumental nerve net located just beneath the surface of the worm and in the innervations of the suckers. In schistosomula, SmSERT immunoreactivity was enriched in the peripheral nerve net and transverse commissures. Using an anti-5HT antibody, we saw co-localization with serotonin throughout the length of the body suggesting that SmSERT is found in serotonergic neurons. In order to determine the biological role of the SmSERT, quantitative motility assays were performed in cultured schistosomula using classical SERT inhibitors or short interfering RNAs (siRNAs) targeting the SmSERT. Treatment with SmSERT blockers or SmSERT-specific siRNAs resulted in a ≈ threefold increase in 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 using RT-qPCR. In order to test the hypothesis that SmSERT transports exogenous serotonin across the tegument, uptake assays were subsequently performed on intact siRNA-treated schistosomula. We found a modest decrease (~25%) in serotonin uptake when compared to negative controls. These results suggest that the SmSERT's function is primarly neuromuscular and may also play a secondary role in the uptake of exogenous (host-derived) serotonin.

Introduction

Schistosomiasis is a debilitating disease that afflicts over 200 million people worldwide. In the absence of a vaccine for any helminth infection, control relies exclusively on the use of chemotherapeutics. The disease is caused by infection with one of three major species of *Schistosoma* with the majority (>80%) attributed to *Schistosoma mansoni*. Infection begins when the free-living cercaria penetrates 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. Some of the key components of the biology of the parasite that are important to its survival are the migration through the host, pairing of the adult worms, feeding and release of eggs. These are controlled in part by the worm's nervous system through a variety of neurotransmitters and associated signalling proteins. Some of these signalling systems are highly divergent compared to those of the host which makes them exciting targets for new selective therapeutics. Neuroactive substances that have been shown to be important in these systems include the biogenic amines, acetylcholine and neuropeptides among others. The biogenic amines constitute a family of structurally related cationic monoamines, which are derived from the metabolism of amino acids and function broadly as neurotransmitters and/or modulators virtually in all animal phyla. Schistosomes have at least three of these amines within their nervous system, dopamine (Gianutsos and Bennett 1977), histamine (El-Shehabi and Ribeiro 2010) and the most abundant of the three, serotonin.

Serotonin (5-hydroxytryptamine; 5-HT), has been shown to play a key role in both the neuromuscular and metabolic systems of schistosomes. 5-HT stimulates worm movement when added exogenously to cultured sporocysts or adult worms (Hillman and Senft 1973; Pax et al. 1984; Boyle et al. 2000), it potentiates muscle contraction in preparations of isolated muscle fibres (Day et al. 1994) and it also stimulates glycogenolysis and glucose utilization in crude worm extracts (Rahman et al. 1985b; Rahman et al. 1985a), all suggesting an excitatory

mode of action. Immunostaining in both larval and adult stages against this biogenic amine has shown that it 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 innervations of the suckers, the subtegumental and submuscular nerve nets and numerous sensory nerve fibers that connect to the surface of the worm (Bennett et al. 1969; Gustafsson 1987; Skuce et al. 1990; Mair et al. 2000). How serotonin exerts its stimulatory effects remains unclear. In mammals, serotonin signalling is accomplished through the binding and activation of specific receptors found on post-synaptic membranes leading to downstream effectors, typically cAMP or Ca^{2+}, being increased or decreased. Not only does serotonin act on neurons through intracellular signalling, it can also modulate the neuronal network that underlies locomotion (Brustein et al. 2003). Serotonin itself is either broken down by a monoamine oxidase or can be recycled by a specific serotonin transporter (SERT) located on the presynaptic membrane. SERTs have also been located in peripheral cells in the body where they function to sequester (and thereby inactivate) serotonin. In schistosomes there has yet to be a receptor identified that can be linked to any of the behavioural or biochemical responses to exogenous serotonin. However other components of serotonin signalling have been identified including the biosynthetic enzyme, tryptophan hydroxylase (Hamdan and Ribeiro 1999) and more recently, a SERT-like serotonin transporter (SmSERT)(Patocka and Ribeiro 2007; Fontana et al. 2009).

Sequence analysis of the *S. mansoni* SERT (SmSERT) showed that it shares a classical SERT topology with 12 transmembrane domains and intracellular N- and C-terminal ends. The level of conservation is high within the transmembrane domains and key residues for binding are also well conserved. Expression of the SmSERT was previously examined in several life stages and found to be upregulated in parasitic stages, schistosomula and adults, compared to the free living cercaria. There are at least two variants of this protein derived from the same gene. The two isoforms differ by 78 amino acids at the intracellular N-terminal end but are otherwise identical and are both active when expressed heterogolously in mammalian cells (Fonatana and Sonders et al. 2009). Transport assays showed that the SmSERT has a similar affinity for serotonin as human SERT (hSERT) and also responded to classical hSERT antagonists, including fluoxetine (Prozac) and tricyclic antidepressants. Some of these drugs were found to

have potent anti-*Schistosoma*l effects in two recent drug screens (Abdulla et al. 2009; Taft et al. 2010) suggesting that SmSERT may be a suitable candidate for drug targeting.

Previous evidence suggests that schistosomes are capable of taking up serotonin from the extracellular medium via a saturable transport system, which is believed to be located on the tegument. This has been shown through transport assays and active accumulation of 5HT both in adult worms and larvae (Bennett and Bueding 1973; Wood and Mansour 1986; Boyle et al. 2003). Researchers have speculated that a transporter is required to obtain exogenous serotonin when endogenous production is insufficient. *S. mansoni* has the enzymatic machinery for *de novo* serotonin biosynthesis but one of the enzymes was found to be downregulated in the parasitic stages compared to cercaria (Hamdan and Ribeiro 1999), suggesting that the parasite may need to supplement its endogenous stores with host-derived serotonin via a surface transporter. Recent evidence has shown that larvae treated with serotonin specific reuptake inhibitors (SSRIs), which are classical SERT blockers showed pronounced motor phenotypes (Abdulla, Ruel et al. 2009) and abnormal development (Taft, Norante et al. 2010), suggesting that the SmSERT plays an important role in the control of endogenous serotonin signalling.

Here we show for the first time the localization of the SmSERT in both adult and larval stages to the CNS and PNS including the subtegumental plexus. We also show evidence suggesting that it plays a role in serotonin inactivation and the control of movement through the use of RNA interference (RNAi).

Materials and Methods

Parasites. A Puerto Rican strain of *Schistosoma mansoni* was used for all experiments in this study. *Biomphalria glabrata* snails infected with *Schistosoma mansoni* were kindly provided by Dr. F. Lewis, BEI Resources, USA. Snails were cultured for 6-8 weeks after which cercarial shedding was induced by exposure to light for 2hr at room temperature. The cercariae were transformed to schistosomula using the mechanical shearing method (Ramalho-Pinto et al.

1974; Basch 1981; Gold and Flescher 2000) and cultured using Opti-MEM (Gibco) supplemented with 4% serum and 0.25µg/ml fungizone and 100µg/ml streptomycin and 100units/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 (Smithers and Terry 1965; Carneiro and Lopes 1986).

Antibody production. A polyclonal 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, 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 procedure and the eluted fractions were tested using an ELISA assay.

The anti-5HT antibody was a commercial monoclonal antibody raised in rat and used as an internal control for permeabilization and localization of serotonin.

4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) was used to stain for cell nuclei in adult worms. Samples were incubated following secondary antibody treatment using manufacturer recommended conditions.

Western Blot Analysis. The affinity-purified anti-SmSERT antibody was tested by Western blot analysis against the recombinant protein expressed in HEK 293 cells. The full length SmSERT was previously cloned in our lab (Patocka and Ribeiro, 2007) 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 $1.5x10^6$ cells/dish. Cells were allowed to grow overnight at 37^0C 5%CO_2 and then transfected

with 3 µg of either pCRUZ-SmSERT or empty plasmid using Fugene6 transfecting agent following the manufacture's recommendations. The cells were grown for 48h 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 (Invitrogen) gels 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 antibodies (1:20, 000) (Calbiochem). To verify the specificity of the antibody, the blots were also probed with anti-GFP primary antibody, targeting the GFP fusion tag (1:750) followed by HRP-conjugated goat anti-rabbit secondary antibodies (1:20,000). In subsequent studies, Western blots were performed directly on *S. mansoni* tissue extracts to determine if the anti-SmSERT antibody could recognize the native transporter. Adult *S. mansoni* male and female worms were obtained from infected mice and a crude membrane protein fraction was prepared using the same ProteoExtract kit. Aliquots of solubilized membranes were resolved by gel electrophoresis, electroblotted and probed with anti-SmSERT antibody followed by an HRP-conjugated secondary antibody, as described above. As negative controls, the blots were probed with anti-SmSERT antibody that was preadsorbed with an excess of both peptide antigens (0.25 mg/ml of each peptide).

Immunofluoresence Microscopy. Freshly obtained adult worms or *in vitro* transformed schistosomula were washed in PBS and fixed for 4hrs in 4% PFA at 4^0C with end-over-end rotation. Following fixation, the animals were washed three times for 5 minutes in 100 mM glycine in PBS and blocked overnight in antibody diluent (0.1%Tween-20, 0.5%BSA in PBS). Animals were then incubated with peptide purified anti-SmSERT antibody (1:25) or anti-5HT antibody (1:200) in the same antibody diluent for 2-3 days at 4^0C. Animals were washed three times, each for 5 minutes, prior to incubation with appropriate secondary antibodies conjugated to FITC, Alexa Fluor 568 or Alexa Fluor 594 (1:1000) for 2 additional days at 4^0C. During the incubation with secondary antibody, DAPI stain was added (1:750) for the second day. Samples were washed 3 times with PBS and mounted for visualization. Slides were viewed using either a Bio-Rad Radiance 2100 confocal scanning microscope equipped with a

Nikon E800 fluorescence microscope for confocal image acquisition and the Lasersharp 2000 analyzing software package or the Zeiss LSM 710 confocal microscope with Zen 2010 image software package (Carl Zeiss Inc., Canada). For co-labeling of SmSERT and serotonin, the confocal images were acquired using a sequential acquisition mode and two separate lasers, Argon 488 nm and either HeNe 543 nm or HeNe 594 nm. Filter sets were optimized to eliminate fluorescence spectral overlap and bleed-through artefacts. Controls used include anti-SmSERT preadsorbed with 0.25 mg/ml of each peptide and preimmune serum (not shown).

RNA Interference (RNAi). Short interfering RNAs (siRNA) targeting the SmSERT were generated using the Ambion Silencer Cocktail kit. Primers were designed to amplify a short and unique region of the SmSERT carrying T7 promoters on both ends. The primer sequences were: forward 5'-GTGGACTACCTTTATTTTATC-3' and reverse 5'-CATATAGAACAGTGCCCATGC. The PCR product was analyzed on a 1.3% agarose gel and purified using ethanol precipitation. The product was used as a template for the transcription of dsRNA and subsequently digested into siRNAs *in vitro* using RNAse III following the kit protocol (RNAse III, Ambion). Transfection of schistosomula were performed as previously described (Nabhan et al. 2007) using the siPORT lipid transfection agent (Ambion) and 50nM SmSERT or 50nM of scrambled siRNA negative control (Ambion). Animals were cultured for 6-8 days before analysis for behavioral changes and harvested for qRT-PCR.

RNA preparation and qRT-PCR. RNA was obtained from siRNA treated schistosomula and controls using the micro RNA extraction kit (QIAGEN), following manufacturer's recommendations, with changes made to the lysis step (Nabhan et al. 2007). Schistosomula were transferred to lysis buffer (supplied by kit) and sonicated for 1 minute (6 pulses of 10s) before continuation with protocol. RNA was quantitated with a Nanodrop ND1000 spectrophotometer (Wilmington,USA) and approximately 100-500 ng of total RNA was used for reverse-transcription (RT) reactions. The RT was performed according to standard protocols in a 20µl reaction using 200U M-MLV reverse transcriptase (Invitrogen) and oligodT primer. As negative controls for this step, a no-RT reaction was performed to rule out possible contamination with genomic DNA. Real-time qPCR was subsequently performed using the

Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) in 25µl reactions. Primers were designed to amplify a 250bp region in the SmSERT and were as follows: forward 5'-GTGGACTACCTTTATTTTATC-3' and reverse 5'-CATATAGAACAGTGCCCATGC-3'. As a standard internal control for expression, primers targeting the *S. mansoni* α-tubulin gene (Accession S79195) were designed to amplify a product of 201bp. The primers were; forward 5'-TCGTGGTGATGTTGTCCCCAAG-3' and reverse 5'-TCGGCTATTGCGGTTGTATTAC-3'. The qPCR was performed in a Rotor-Gene RG3000 instrument (Corbbert Research, Australia) with the following cycling conditions: 50^0C/2min, 95^0C/2min, followed by 40 cycles of 94^0C/15s, 54.5^0C/30s, and 72^0C for 30sec. Samples were also tested for non-specific knockdown using primers against an irrevelant schisotosome gene (Smp_176310), this time using GAPDH as an internal housekeeping reference. Primers for Smp_176310 were: forward 5'-TCAGCTCGATCTACGAATC-3' and reverse 5'-CTAGCTGATCTGACGTCAC-3' and for GAPDH (Accession # M92359) forward 5'-GTTGATCTGACATGTAGGTTAG-3' and reverse 5'-ACTAATTTCACGAAGTTGTTG-3' with the following cycling conditions: 50^0C/2min, 95^0C/2min, followed by 40 cycles of 94^0C/20s, 56^0C/20s, and 72^0C for 20sec. Primer validation curves were generated for each primer set to insure that the amplification efficiencies of the target (SmSERT or Smp 176310) and the corresponding housekeeping gene (α-tubulin and GAPDH, respectively) were similar. Expression data were normalized relative to the housekeeping gene and then to the scrambled siRNA control, according to the comparative delta delta Ct method (Livak and Schmittgen 2001). All qPCR data are derived from a minimum of two experiments each in duplicate.

Behavioural Assays. Animals that were transfected with either siRNA targeting the SmSERT or non-specific scrambled control were monitored for behavioural changes, using a previously described motility assay (El-Shehabi et al. 2012). Schistosomula were cultured for 6 days in 24-well dishes and from each well, 3 digital recordings of 45 seconds were done using a compound microscope (Nikon, SMZ1500) equipped with a digital video camera (QICAM Fast 1394, mon 12 bit, Qimaging) and SimplePCI version 5.2 (Compix Inc.) for image acquisition. Briefly, the software will make an ellipse of best fit around the parasite and will measure the major axis of the ellipse as an approximation of body length for each frame of the video (~3 frames/sec).

Changes in the major axis proved to be the most consistent indicator for motion and were used to determine the frequency of movements during the observation period as described (El-Shehabi et al. 2012). Differences in axis length from one frame to the next were calculated and if proved to be larger than 10%, either by a shortening or elongation, they were counted as a movement. Each data point is the mean and SEM of at least 3 separate experiments from which approximately 12 animals per experiment were analyzed.

Transport assays. Measurements of [^{3}H]serotonin were done as previously described (Patocka and Ribeiro 2007). Briefly, *in vitro* transformed schistosomulae were trasfected with either 50nM siRNA targeting the SmSERT or a scrambled non-specific siRNA as above and cultured in Opti-MEM (GIBCO) supplemented with 4% serum and 0.25 µg/ml fungizone and 100µ/ml streptomycin and 100units/ml penicillin. 6 days following treatment, animals were washed three times in PBS and resuspended in culture media without serum. [^{3}H]5HT was added to a final concentration of 0.1 µM(containing 2.22x10^6 DPM/well) and allowed to sit at room temperature for 1 hour. Animals were then washed twice in PBS, solubilized using 1% SDS and radioassayed to measure [^{3}H]5HT incorporation (Boyle et al. 2003). Transport data are derived from 3 experiments, each containing approximately 200 animals per treatment.

Biotinylation. Adult worms were incubated in RPMI 11640 culture medium (GIBCO) and examined under the microscope to remove visibly damaged parasites. Intact male and female worms were biotinylated as previously described (Braschi and Wilson 2006; Taman and Ribeiro 2011). In brief, the worms were washed in Hanks' balanced salt solution (HBSS) (Invitrogen) and incubated with EZ-Link© sulfo-NHS-LC Biotin (Pierce) at 1.5 mg/ml in HBSS for 30min at 4^{0}C with gentle agitation. The reaction was terminated by washing the worms in RPMI 1640 (Invitrogen). Membrane proteins were isolated as previously described and the biotinylated proteins were further purified using a Streptavidin conjugated resin (Pierce) according to manufacturer's recommendations. Approximately 40 µg of proteins were incubated with resin for 1 hour at room temperature followed by centrifugation at 500xg for 1 minute to remove unbound proteins. The resin was washed 5 times with PBS and bound biotinylated proteins were recovered by heating the sample in 1x SDS-PAGE sample buffer for 5min at 90^{0}C. Aliquots

of biotinylated and total (unlabelled) membrane proteins were resolved by SDS-PAGE and blotted onto PVDF membrane. Membranes were probed with both streptavidin-horseradish peroxidase conjugate (Amersham) or anti-SmSERT antibody as described above.

Other methods. Protein concentrations were performed using the Lowry method using a commercial kit (BioRad). Statistical analysis of qPCR expression data, behavioural and transport assays was done with the Student t-test . A $p<0.05$ was considered significant.

Results

Antibody production and Western blot analysis. Based on previous research (Patocka and Ribeiro 2007; Berriman et al. 2009; Fontana et al. 2009; Protasio et al. 2012) it has been shown that there is one gene coding for the *S. mansoni* serotonin transporter (SmSERT) and 2 allelic forms of the protein of 74kDA and 83kDA. The two proteins are identical except for 78 amino acids at the N-terminal end, which are lacking in the shorter species. Both papers showed this protein was able to mediate the uptake of serotonin in both a dose- and time-dependent manner, suggesting saturable transport. In order to determine the location of the protein in the parasite, antibodies targeting two distinct peptides, common in both forms of the protein, were designed and used to raise antibodies in rabbits. The two peptides are located in the C-terminal end of the protein (Fig. 1). Immunogenic and sequence blast analyses identified this location as the best position for peptide synthesis. Antibodies were purified using peptide-coupled beads and tested against the peptide antigens to determine titer efficiency. Purified antibodies were able to detect both peptides at concentrations of 1:5000 or higher.

In order to confirm specificity of the antibody, western blot analysis was performed on membrane extracts prepared from adults worms. Antigen-preadsorbed antibody was used as a negative control to verify specificity of the signal. The result showed a single broad band at approximately 100-110kDA in blots treated with anti-SmSERT but not the preadsorbed antibody control (Fig. 2A), suggesting that the band is specific. The size of the band is larger than the estimated mass of either SmSERT variant (74kDA or 83kDa) but this may be due to

glycosylation, since there are several predicted glycosylation sites in both variants. We note also that the pI of the shorter SmSERT variant is quite high (9.1), which could interfere with normal migration of the protein on the gel. For these reasons, and given that the two isoforms are similar in size, we cannot predict if the western positive band corresponds to the shorter or longer variant, or possibly both.

In order to further verify the specificity of the antibody, western blot analyses were repeated using recombinant SmSERT expressed from mammalian cells. Membrane extracts were prepared from HEK 293 cells that expressed an N-terminal GFP-SmSERT fusion and western blots were performed using both anti-SmSERT and anti-GFP antibodies. The two antibodies recognized a major immunoractive band of the same size, which corresponds to the SmSERT-GFP fusion protein. A smaller immunoreactive band is also seen in both blots and is presumed to be a proteolytic fragment (Fig. 2B). Neither band could be detected in mock control cells transfected with empty plasmid. Based on these results, we conclude that the anti-SmSERT antibody is specific and recognizes both native and recombinant forms of the protein.

Immunolocalization. We wanted to localize the protein in the parasite in order to gain a better understanding of its biological role. Previous work from other research groups and immunofluorescence assays in our lab have demonstrated that serotonin is present throughout the nervous system of the parasite and is located to both main nerve cords of the CNS and peripheral nerve cords. Staining has also shown that the peripheral serotonergic neurons extend to the surface of the parasites, localizing to the tubercules in the adult worms (Gustafsson 1987). To test how this expression pattern compares to that of the transporter, adult worms were probed with affinity-purified anti-SmSERT antibody and visualized by confocal microscopy. The results detected extensive labeling of the nervous system both in males and females. Punctate fluorescence was observed in the major nerve cords and transverse commissures along the length of the body (Fig. 3A), a pattern similar to that observed with serotonin itself. In males we detected significant immunoreactivity in the tubercles (Fig.3B) and in the subtegumental nerve net located beneath the surface of the worm

(Fig. 3C). No visible fluorescence was observed in the controls, including an antigen-preadsorbed antibody control (Fig. 3D) or controls in which primary antibody was omitted. In order to determine whether or not these were serotonergic nerve fibers, co-localization was performed with monoclonal anti-5HT antibody (Fig.4). We found a high degree of co-localization suggesting that the SmSERT is expressed in serotonin-containing neurons.

Schistosomula were used for confocal microscopy following incubation with antibodies targeting the SmSERT. We found that the expression of the protein was localized to the peripheral nervous system throughout the body (Fig. 5A, B, C). We see a punctate pattern that form rings around the body of the parasite. This is just below the surface of the parasite and most likely represents elements of the peripheral nerve net. Negative controls (Fig. 5D) show no positive staining suggesting the signal is specific to the SmSERT.

RNAi and Behavioural Assays. To help identify the function of the protein, we began by treating the parasite with two classical inhibitors, fluoxetine and clomipramine, which were previously shown to inhibit the schistosomula SmSERT when expressed *in vitro* (Patocka and Ribeiro, 2007). The pharmacological studies was performed on cultured schistosomula, using a video camera and imaging software, as described recently (El-Shehabi, 2012). In order to quantify larval movement, 3 recordings per treatment were taken each for 45 seconds and a minimum of 12 worms were analyzed per recording. The frequency of movements was quantified by measuring changes in body length from one frame to another, either a shortening or elongation (Fig. 6A). We found that larvae treated with a transport inhibitor showed approximately a 3 fold increase in frequency of movements compared to the untreated controls, an effect comparable to that caused by exogenous application of serotonin (Fig. 6B).

In order to confirm that these results were due to a specific decrease in SmSERT activity, we used RNAi to knockdown SmSERT expression. Schistosomula were transfected with siRNAs targeting the SmSERT or a non-specific scrambled siRNA and monitored for up to 8 days. The effect of RNAi was tested first by RT-qPCR the results showed approximately a 50% decrease in

expression of the SmSERT when compared with control animals treated with scrambled siRNA. To confirm specificity, we also amplified an unrelated sequence (Smp_176310, predicted nicotinic receptor) from the same cDNA and found no significant change in expression (Fig.6A). Once knockdown was confirmed at the RNA level, larval motility assays were performed under the same conditions. 6 day old animals treated with either a non-specific scrambled siRNA or one against the SmSERT were filmed and quantified for frequency of movements. We saw that there was a significant increase (~3 fold) in motility in those animals treated with siRNA against the SmSERT compared to control.

Transport assays. To gain a better understanding of the role of SmSERT on transport of exogenous 5HT, we measured incorporation of [^{3}H]serotonin in cultured schistosomula following treatment with SmSERT-specific siRNAs. Schistosomula were treated as previously described for 6 days after which they were washed and incubated in culture media in the absence of serum. [^{3}H]5HT was added to the media and incubated for 1 hour. Media was removed and washed with PBS to remove any bound [^{3}H]5HT and solubilized using 1% SDS. Lysates were radio assayed and changes in incorporation were normalized to negative controls. We found approximately 25% knockdown in incorporation of radio-labeled serotonin compared to controls.

Biotin labeling and western blot analysis. To test for localization of the protein to the surface, live adults were labeled with biotin as previously described (Braschi and Wilson, 2006). Adult male and female adult worms were separated and examine under a microscope prior to labeling. Worms showing no visible signs of damage were kept for biotin labeling. Membrane extracts were prepared from biotinylated worms and applied onto a streptavidin column to purify biotin labeled proteins. Purified proteins were loaded on a SDS acrylamide gel for western blot analysis with anti-SmSERT antibody. The results show a single immunoreactive band in the biotinylated fraction (Fig.8). The band is of the same size as in the unlabelled (non-biotinylated) membrane extract and could not be seen in the preadsorbed antibody control.

Discussion

Serotonin has been studied in *S. mansoni* for more than 40 years and has been shown to be an essential biogenic amine for the survival of the parasite. As such, understanding the mechanism of serotonin action, inactivation and synthesis, is crucial to developing drug therapies that can target this system. The synthesis of serotonin is accomplished through a two-step enzymatic pathway that begins with the conversion of tryptophan to 5-hydroxytrytophan. This reaction is catalyzed by tryptophan hydroxylase and has been both identified and characterized in *S. mansoni* (Hamdan and Ribeiro 1999). The second enzyme in the pathway is a nonselective aromatic amino acid decarboxylase (AAAD) that converts 5-hydroxytryptophan to serotonin. This enzyme has not yet been characterized through biochemical methods but there is an AAAD homologue in the genome (Berriman et al. 2009; Protasio et al. 2012), suggesting the entire biosynthetic pathway is conserved in the parasite. Besides the enzymes, the genome database encodes at least two possible serotonin receptors. One of these receptors has been shown to be active, specific to serotonin, and present in the neuronal system of the parasite in several developmental stages (see Chapter IV, unpublished results). Other important components of the serotonergic system are the transporters, including SmSERT and also vesicular transporters, which sequester cytosolic serotonin into vesicles for subsequent release. There are at least two predicted amine-like vesicular transporters in the *S. mansoni* genome (Berriman et al. 2009; Protasio et al. 2012) but it is unknown if either is selective for serotonin.

It has been suggested by several authors that the parasite takes in serotonin from its environment in a saturable manner (Bennett and Bueding 1973; Wood and Mansour 1986; Boyle et al. 2003) and that SmSERT could play a role in this transport (Patocka and Ribeiro, 2007; Fontana et al. 2009). However, there was no direct evidence that SmSERT was responsible for this exogenous recruitment, nor did they determine if the transporter played a role in serotonin storage and inactivation. In other systems, SERTs mediate the reuptake of endogenously released serotonin either into presynaptic terminals or non-neuronal storage sites, thus terminating the signalling. Neither of these biological roles has been linked to the schistosomes SmSERT. Here we propose that the SmSERT is responsible for both serotonin

inactivation in the nervous system and that it facilitates the uptake of exogenous 5HT from the surrounding environment.

In order to gain a better understanding of the functional role of the SmSERT, immunolocalization experiments were performed. A specific anti-SmSERT antibody was raised in rabbits, affinity-purified and tested first by western blot analysis. There have been two variants of the SmSERT identified in *S. mansoni* with differences being found in the N-terminal region (Patocka and Ribeiro 2007; Fontana et al. 2009). The two variants differ in length by approximately 78aa, resulting in a ≈10kDa difference, but are otherwise identical. Antibodies were raised against two C-terminal peptides that are common to both variants of the SmSERT but show no significant homology to other *S. mansoni*, *B.glabrata* or mammalian proteins as verified through blast analysis. Western blots of crude worm extracts identified one predominant band at approximately 100kDa, higher than the predicted sizes of either variant. Several glycosylation sites exist in the second extracellular loop which may account for the larger than expected size. In addition, SERTs are known to form functional dimers as well as to interact with other proteins (Jess et al. 1996; Horschitz et al. 2003). The size of the immunoreactive band is too small to be a homodimer, however it does not eliminate the possibility of other protein-protein interactions that resist denaturation in the SDS environment of the gel. Samples were treated with DTT suggesting that if there are protein-protein interactions, they are unlikely to involve disulfide linkages. Finally, as noted earlier, it is possible the larger than expected size is due to aberrant migration of the SmSERT on the gel, possibly due to the high pI of the protein or its hydrophobicity. Prebsorbed antiserum was used to detect non-specific binding. We did not see western positive bands in blots treated with peptide-preabsorbed antibody suggesting the band was specific. To further confirm the specificity of the antibody, membrane extracts were prepared from HEK 293 cells expressing SmSERT fused to GFP. Cells were transiently transfected with the SmSERT and membranes were purified following the same protocol as that used for adult worms. Following western blot analysis, we see a major band of the expected size both with anti-SmSERT and anti-GFP antibodies. Negative controls using preabsorbed antibody or mock transfected cells show no

band confirming that the antibody is specific for SmSERT. Due to the larger than expected size, it is impossible to tell which of the two variants is actually expressed in the parasite.

In subsequent studies, immunofluorescence assays were performed in both adult and larval life stages with peptide purified anti-SmSERT, following established protocols (Mair et al. 2000; El-Shehabi et al. 2009). In the adults, we observed distinct staining to the central nervous system, along the main nerve cords that run the length of the worm and connecting tranverse commissures. SERTs are typically found near or in the same region as serotonin, especially in the context of the nervous system. In order to determine if the transporter is expressed in serotonergic neurons, co-localization experiments were performed using both anti-SmSERT and a commercial anti-serotonin antibody. We observed that the two co-localized in the main nerve cords and in some of the peripheral nerve fibers that branch out. We did not see complete co-localization, which was to be expected as serotonin is more widely distributed within the neuron and may also travel some distance from its site of release. In adult males, besides the CNS, we see SmSERT immunoreactivity in the innervations of the suckers near the surface of the parasite, both in the tubercules and in the peripheral nerve net just below the surface. Previous work has shown that the tubercules are important sensory structures of the worm and have serotonergic neurons that connect toward the peripheral nerve net and ultimately the CNS (Gustafsson 1987). Our results suggest that the SmSERT is associated with this peripheral serotonergic system. In the larvae, we see further evidence that SmSERT is present in the nervous system. There was a distinct staining pattern seen in larvae, consisting of multiple parrale nerve fibers, which are varicose in appearance and run just beneath the surface of the worm throughout the body. These resemble arrangement of nerve fibers in the peripheral nerve net of planarians (Cebria 2008) and are presumed to represent an early developments stage of the schistosome's PNS.

SERTs' main function in mammals is the reuptake of serotonin into presynatpic neurons and inactivation of the amine. Our immunolocalization analysis of the SmSERT in both adults and larvae show that it is localized mainly to the nervous system, which is consistent with this

mode of action. Serotonin has been shown to be an excitatory neurotransmitter in schistosomes, increasing motility following exogenous application. To determine if the SmSERT plays a role in the serotonin signalling pathway, we performed RNAi assays and tested several SSRIs looking for changes in motility of larvae. Incubation of larvae with drugs that have been shown to inhibit SmSERT activity led to increases in motility (≈2-3 fold), as did knockdown of SmSERT expression. These results are consistent with the role of a SERT inactivating serotonin in the nervous system. If serotonin is activating receptors that lead to increased motility, a decrease or blockage of SmSERT would be expected to cause hyperactivity as a result of elevated serotonin in the extrasynaptic space. The results support this idea and show that the SmSERT is important in the serotonin signalling pathway that influences motility.

It has been suggested that schistosomes can take up exogenous serotonin from the host via a surface transporter and that this serotonin is important for maintaining endogenous stores of the amine. In order to address this possibility, immunolocalization and transport assays were done on adult and larval parasites. In both life stages, we saw little evidence that SmSERT was expressed on the tegument. In males, there was staining specific to SmSERT in discrete regions of the tubercules; however, it is difficult to determine whether this was associated with serotonergic nerve endings within the tubercles, or whether SmSERT was present in the tegument itself where it could play a role in uptake of exogenous serotonin. The surface biotinylation experiments confirmed SmSERT is present at or near the surface but they can not distinguish between the tegument and neuronal elements in this region. In the larvae, we saw only staining to the nervous system and not to the tegument. These animals were 4 days old so it is possible that the SmSERT is not found on the tegument at this age and may only be expressed on the tegument later in its development. It is also possible that it is expressed in low amounts and we were not able to detect it on the surface.

Transport studies in RNAi-supressed larvae showed that a 50% reduction in SmSERT mRNA expression led to only a ~25% decrease in uptake of exogenous serotonin. This modest effect on transport activity could be due to insufficient RNAi silencing at the protein level. Alternatively, it is possible that SmSERT plays only a minor role in the surface transport of

serotonin, a possibility consistent with the apparent absence of protein in the larval tegument. One possible explanation for the continued uptake of serotonin despite its decreased expression is the presence of other transporters. The *S.mansoni* genome encodes at least one homologue of the OCT-1/2 transporter, a non-selective organic cation transporter that is capable of carrying monoamine transmitters, including serotonin (Busch et al. 1996; Busch et al. 1998). If present on the tegument, it could take in exogenous serorotonin, which would then be rapidly sequestered into serotonergic neurons by SmSERT. It is important to note that although there was a decrease in serotonin uptake in RNAi-treated animals, the endogenous stores of 5HT did not seem to suffer as the animals still showed hyperactivity and no apparent loss of viability, even after 6 days of treatment. Whether this is due to increase in expression of other transporters or because the endogenous stores are not effected by exogenous uptake still needs to be determined.

In conclusion, here we have shown for the first time the presence of the SmSERT to both the central nervous system as well as peripheral nervous system in both adult and larval stages of *S. mansoni*. We showed that its primary role is neuromuscular in that it controls serotonin induced motility. A possible secondary role is the uptake of exogenous serotonin from the environment, although to what end, it is unclear. Further research is needed to elucidate the contribution of host-derived serotonin to endogenous levels and to serotonergic signalling in the parasite.

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Figures

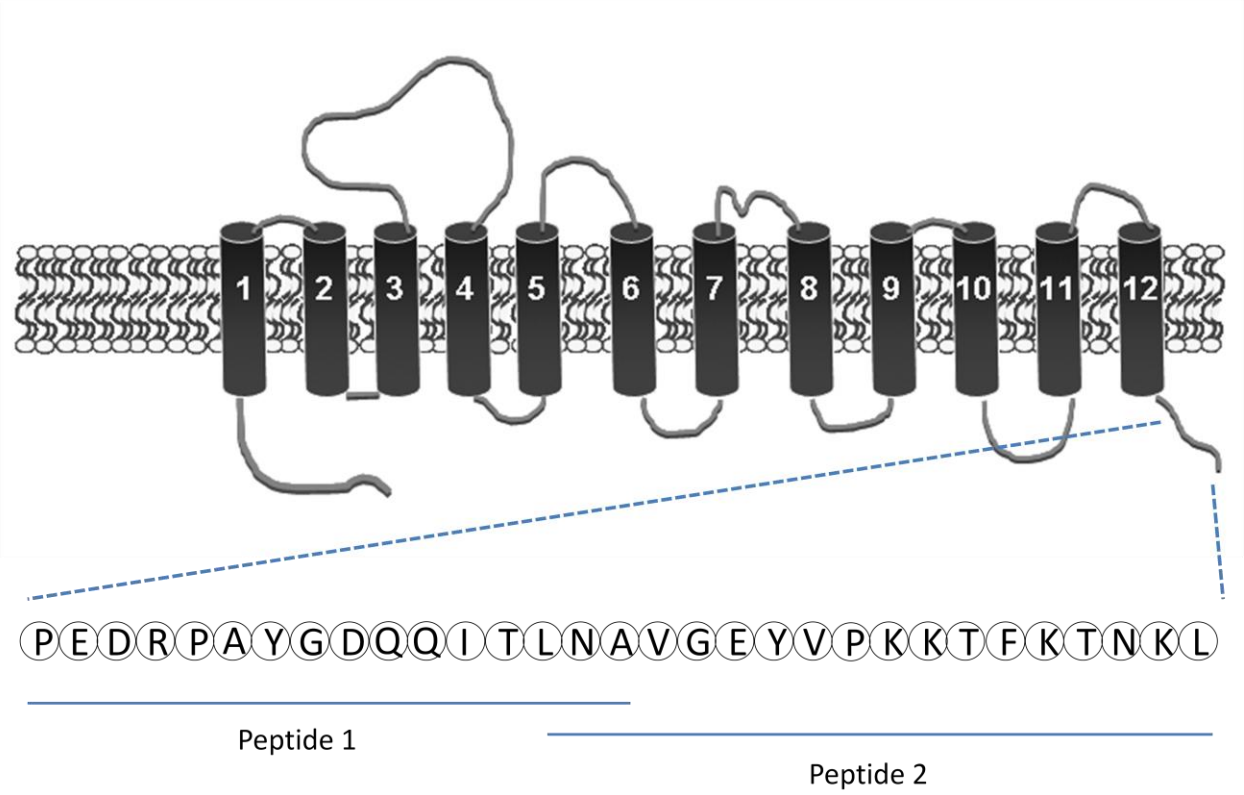

Figure 1. Schematic representation of the SmSERT with the location and amino acid sequence of the peptides used for antibody production

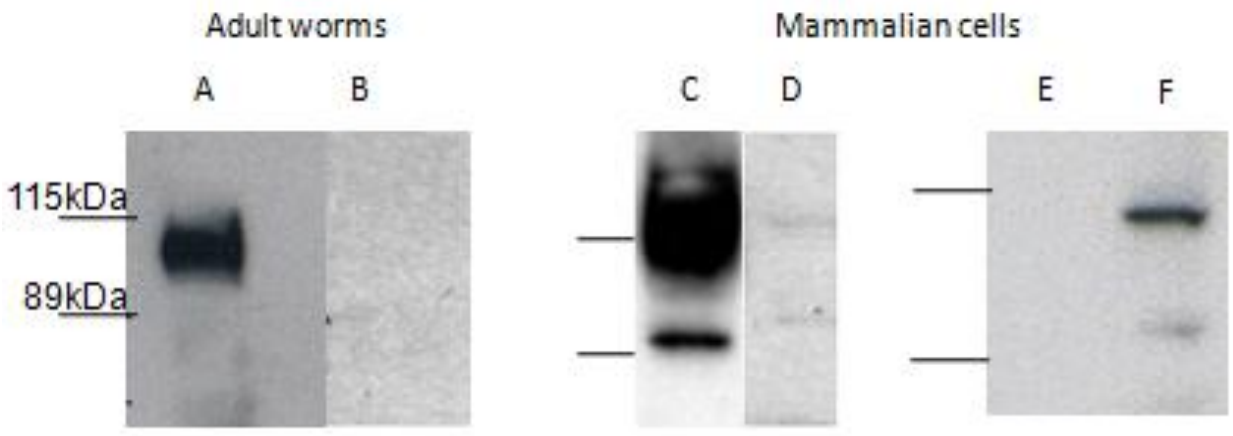

Figure 2. Western Blot analysis using the Sm5HTr peptide purified antibody (Lanes A, C, D), anti-5HTr antibody preadsorbed with excess antigen (0.25ug/ml each peptide)(Lane B) or anti-GFP antibody (lanes E and F). Protein extracts are from adult worms (Lanes A and B), mammalian cells expressing the GFP-SmSERT fusion protein (Lanes C and F) or mammalian cells transfected with a mock plasmid (D and E)

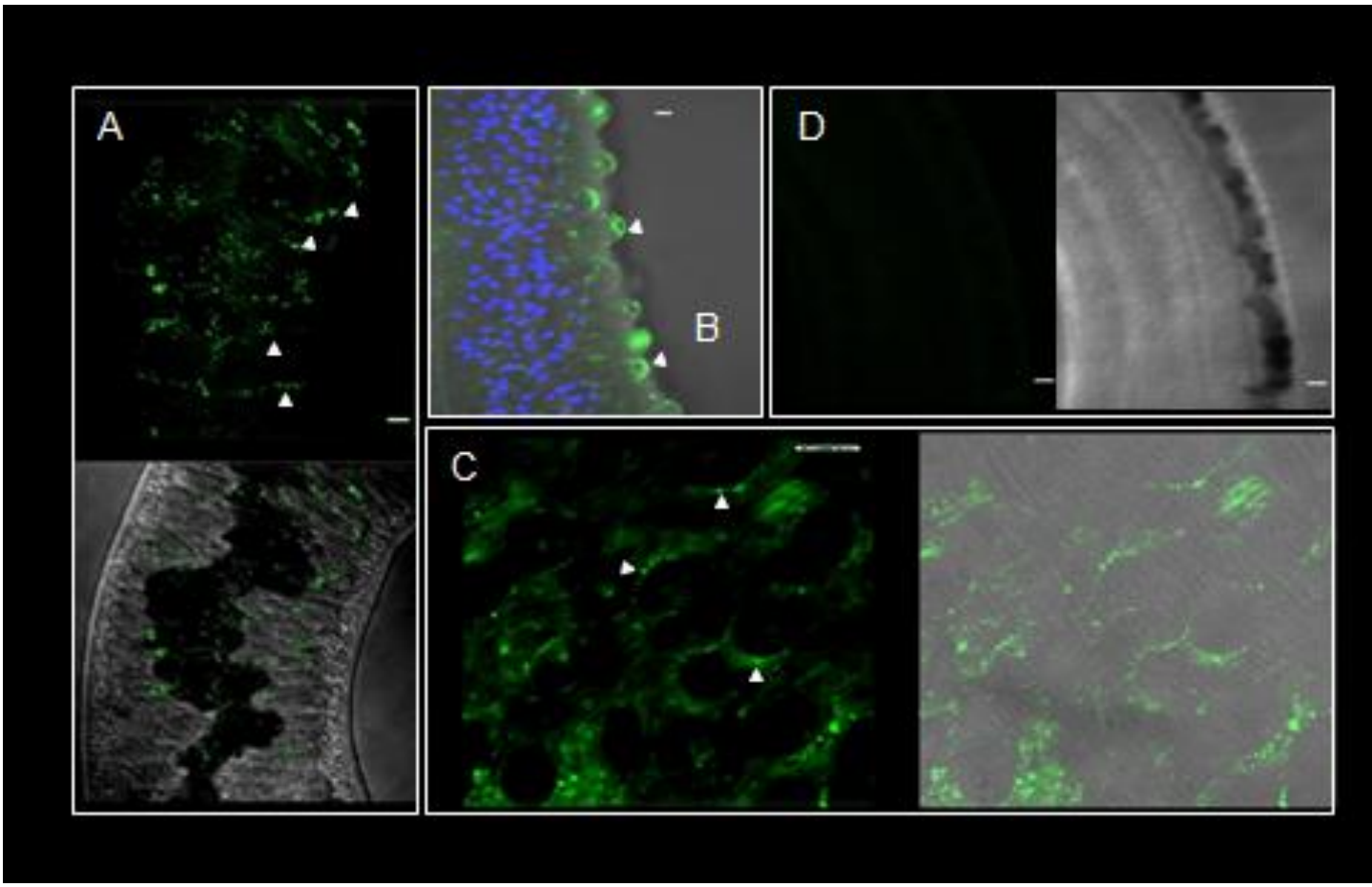

Figure 3. Confocal scanning microscopy of adult worms using either anti-SmSERT antibody (A ,B and C) or anti-SmSERT preadsorbed with excess antigen (D).(A) Female adult worm probed with anti-SmSERT showing expression in main nerve cords and transverse commissures. Adult male worms were probed with anti-SmSERT antibody in the presence of DAPI to visualize nuclei (B). Positive SmSERT staining was observed in the tubercules (green arrowheads) and the syncytial tegument, which lacks DAPI-labelled nuclei (B). At higher magnification, SmSERT expression (green) could be seen in the peripheral nerve net just beneath the surface (C). Numerous fine immunoreactive nerve fibers were detected in this region, many showing varicosities (arrowheads). (D) Male adult worms probed with preadsorbed negative control. Scale bars represent 25μm

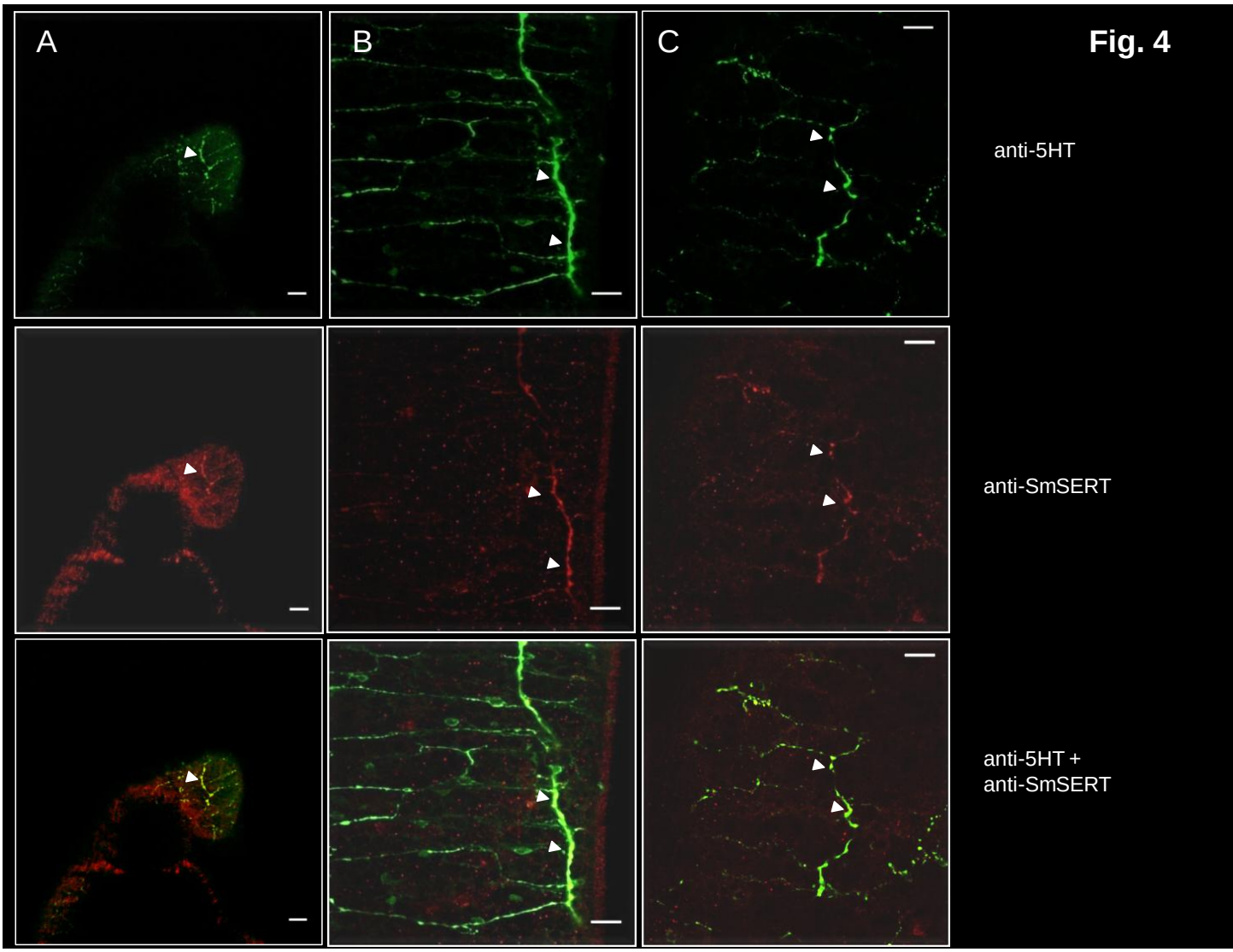

Figure 4. Immunolocalization of SmSERT co-localized with serotonin (5HT). Adult male and female worms were incubated with anti-5HT and anti-SmSERT. (A) Male oral sucker showing expression of the SmSERT (red) and 5HT (green) in the innervations of the suckers. Bright yellow fluorescence denotes areas where SmSERT co-localizes with serotonin (anti-SmSERT, anti-5HT overlay). (B and C) Female and male worms showing presence of the SmSERT in the central nervous system, main nerve cords and transverse commissures. Co-localization with 5HT shows these to be serotonergic neurons. Arrow heads indicate serotonergic varicosities and areas of co-localization (where applicable). Scale bars represent 25µm.

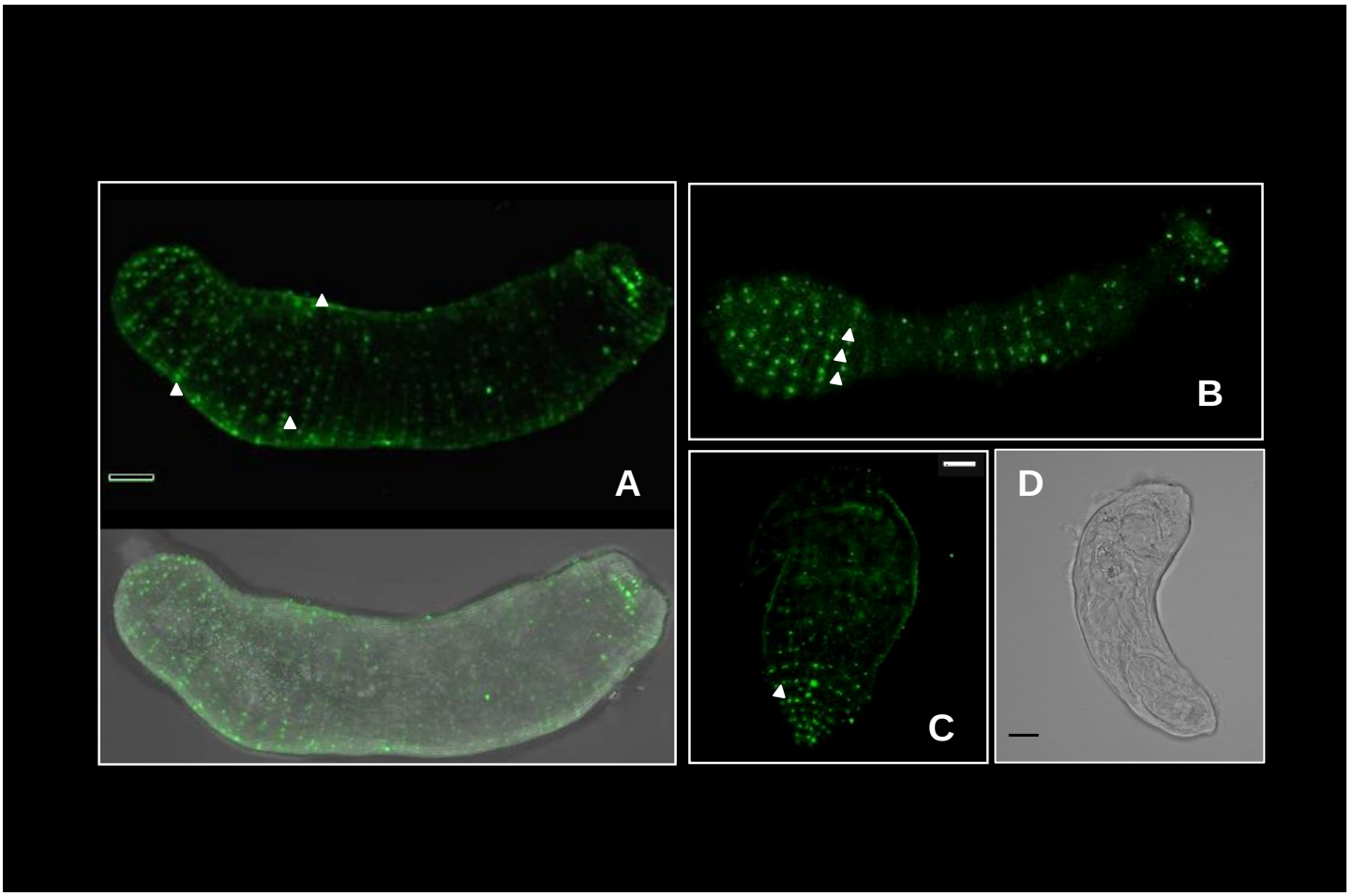

Figure 5. Immunolocalization of SmSERT in schistosomula. Newly transformed schistosomula were treated with anti-SmSERT and visualized with confocal microscopy (A, B and C). Expression pattern is localized to the peripheral nerve net just below the surface of the parasite throughout the body. (D) Larva treated with anti-SmSERT preadsorbed with excess antigen. Scale bars represent 25um.

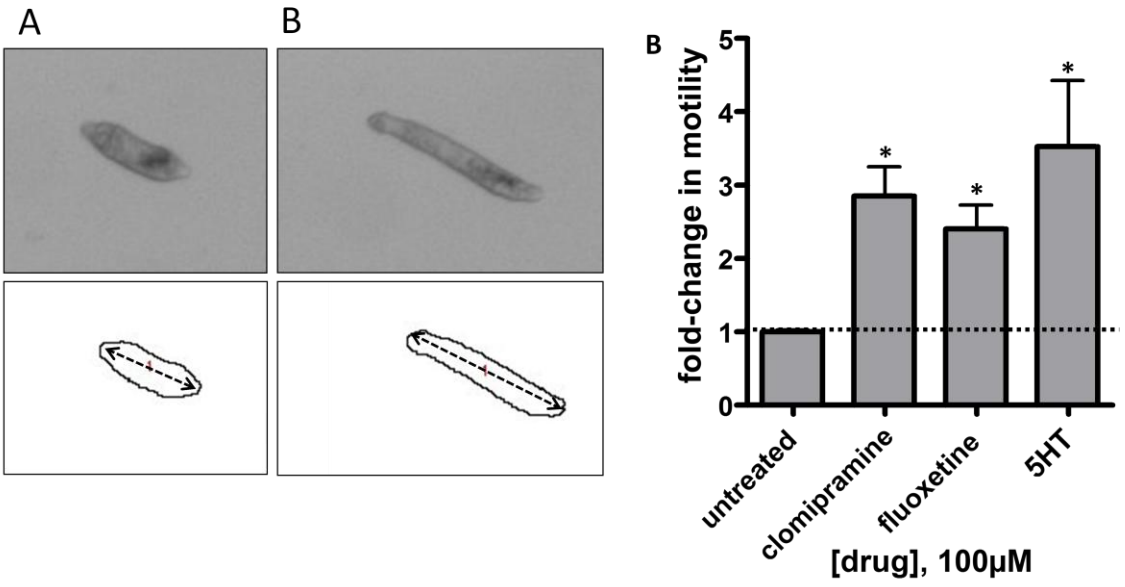

Figure 6. Motility assay of larval schistosomula treated with SmSERT antagonists. (A and B) Video recordings of 6 day old larvae were analyzed for changes in the major axis from one frame to the next. Changes greater than 10% from one frame to the next were considered a movement and the frequency of movements was measured for each animal over a 45 second observation period. (B) Parasites treated with 5HT, SmSERT inhibitors or vehicle and motility was recorded as above. The data were normalized relative to the untreated control and each data point represents the average and S.E.M. of at least 36 animals taken over 3 experiments. Statistical differences were determined using Student's t-test with $p<0.05$ being significant.

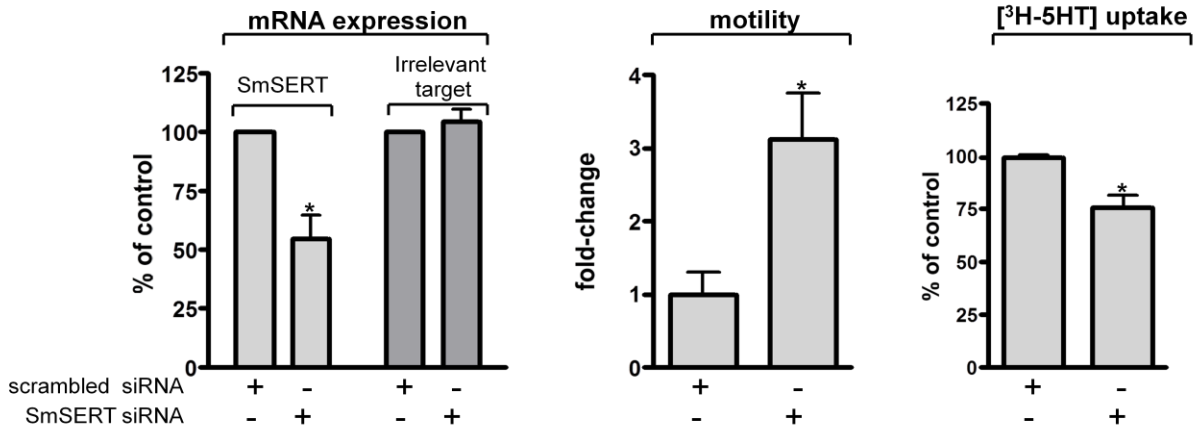

Figure 7. RNA interference assays on schistosomula. Larvae were treated with siRNA targeting the SmSERT or a scrambled non-specific negative control and cultured for 6 days. Quantitivate RT-PCR was performed on treated animals and showed a 50% decrease in expression when compared to controls (A). No off target effects were seen as measured by simultaneous qPCR amplification of an irrelevant target gene. Motility assays were done on RNAi treated schistosomula, as previously described. A 3-fold increase in motility was seen in animals who were treated with siRNA targeting the SmSERT compared to the scrambled siRNA control (B). [^{3}H]-5HT uptake experiments were done on animals treated with siRNAs. Apprimately 25% decrease in uptake of [^{3}H]- 5HT was seen compared to animals treated with scrambled siRNA. All measurements are an average and SEM of at least 3 experiments, each performed with at least 36 animals. Statistical differences were determined using Student's t-test with a $p<0.05$ being significant.

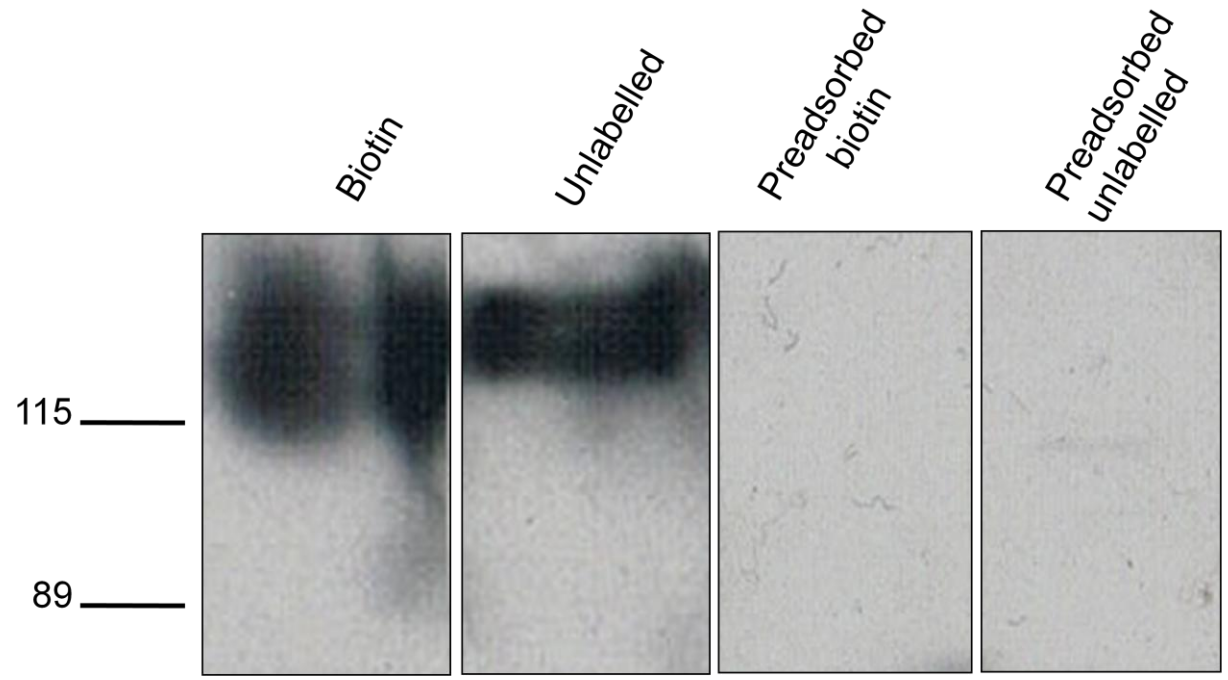

Figure 8. Western blot analysis of biotinylated membrane extracts with SmSERT antibody. Male adult worms were biotinylated and membrane extracts were purified and membrane labelled protein separated using streptavidin labelled beads. Biotin labelled proteins were run on SDS PAGE gel and probed with either anti-SmSERT or antigen-preadsorbed antibody (preadsorbed biotin). Total (non-biotinylated) membrane was run along side as a positive control (unlabelled) with the corresponding negative control included (preadsorbed unlabelled).

CHAPTER IV (manuscript III)

Cloning, characterization and immunolocalization of a serotonin-like receptor (Sm5HTr) in *S. mansoni*

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Abstract

Serotonin (5-hydroxytryptamine: 5HT) is a ubiquitous neurotransmitter, found in vertebrates and invertebrates throughout the animal kingdom. In schistosomes, immunofluorescence targeting 5HT has shown it to be present in the central and peripheral nervous systems in several life stages. Behaviourally, serotonin has been shown to play an important neuroexcitatory role, effecting muscle contraction, motility and metabolism however its mode of action has yet to be elucidated. Here we show for the first time, a novel serotonin receptor (Sm5HTr) from *Schistosoma mansoni*. Sequence analysis shows that it belongs to the G protein-coupled receptor (GPCR) superfamily and is most closely related to serotonergic-5HT7 type receptors. The full length protein was cloned, expressed in HEK 293 cells and shown to be activated specifically by serotonin and *o*-methylserotonin, with no other biogenic amine showing significant activation. Activation of the receptor caused an increase in intracellular cAMP but not Ca^{2+} suggesting that Sm5HTr is likely to interact with a G_s type protein. Classical mammalian serotonin receptor agonists and antagonists were used to obtain a rough pharmacological profile of the recombinant receptor. Treatment with agonists DPAT and buspirone showed no change in cAMP levels. Pre-treatment with aminergic antagonists, chlorpromazine, mianserin and cyproheptadine were all able to abolish the effect of 5HT. In other studies we tested the effects of the same receptor agonists/antagonists on parasite motility *in vitro*. The results showed that serotonin and methylserotonin both caused a significant increase in motility and this effect was blocked by addition of Sm5HTr antagonists. Immunolocalization of the protein in the parasite was done using affinity purified antibodies targeting the Sm5HTr. The protein was detected in the central nervous system both in adult worms and larvae (schistosomula). There is also significant expression in the innervations of the suckers and the subtegumental nerve plexus in adult males and females. Taken together these results identify Sm5HTr as an important protein of the parasite's neuromuscular system and suggests potential role in the control of motility.

Introduction

Schistosomes belong to the phylum platyhelminths and reside in the intravascular system of the host. They affect more than 200 million people worldwide with a death toll of over 250,000 per year (King et al. 2005; Koukounari et al. 2007; King and Dangerfield-Cha 2008). The three species responsible for the majority of people suffering from the disease are *Schistosoma mansoni, S. hematobium, and S. japonicum*. The disease is caused by the adult paired worms releasing eggs that become trapped in surrounding tissues, notably the liver and spleen. Schistosomes can live for several years within the host resulting in a chronic disease state. In order to survive and continue its lifecycle, the parasite must travel both as a free-living larva as well as navigate through the body of two hosts. Proper migration is critical for its continued propagation and development. The coordination of muscle function, movement and sensory inputs required for successful navigation are all controlled by the parasite nervous system.

Flatworms have a well developed nervous system and a rich diversity of neuroactive signaling molecules, including several families of neuropeptides and numerous small-molecule (classical) neurotransmitters. The two most studied neurotransmitters are 5-hydroxytryptamine (serotonin) and acetylcholine. The former has been shown to stimulate worm motility in a variety of flatworm species, including *S. mansoni* (Mellin et al. 1983; Pax et al. 1984; Boyle et al. 2000). This effect is associated with an increase in muscle fiber contractions (Day et al. 1994; Day et al. 1996), increased glucose uptake and glycogen utilization (Rahman et al. 1985b; Rahman et al. 1985a) and stimulation of adenylate cyclase-dependent signalling pathways (Kasschau and Mansour 1982b; Kasschau and Mansour 1982a; Estey and Mansour 1987; Estey and Mansour 1988). In the molluscan larval stages of *S. mansoni*, exposure of mother sporocysts to 5HT led to an increase in the release of daughter sporocysts, which was hypothesized to be a downstream effect of an increase in muscle contraction rate (Bayne and Grevelding 2003). Immunocytochemical work (Bennett et al. 1969; Gustafsson 1987; Skuce et al. 1990; Mair et al. 2000) showed serotonin to be present

throughout the central nervous system (CNS) as well as the peripheral nervous system (PNS) of *S. mansoni* adults and larvae. More recent molecular cloning studies have shown that a serotonin transporter and the serotonin biosynthetic enzyme, tryptophan hydroxylase (TPH) are both present and functional in schistosomes (Hamdan and Ribeiro 1999; Patocka and Ribeiro 2007; Fontana et al. 2009) but no serotonin receptor has yet been characterized in this parasite.

Neurotransmitters exert their effects by interacting with specific receptors followed by activation, inhibition, or modulation of downstream targets. Some of the most common signalling receptors in the nervous system are G protein-coupled receptors (GPCRs). GPCRs are among the most ubiquitous and highly diverse family of proteins. Crystal structure analysis of the prototype rhodopsin has shown that GPCRs have 7 transmembrane domains, an extracellular N-termini and intracellular C-termini (Palczewski et al. 2000). Signaling through GPCRs can be activated by a wide variety of different molecules be it proteins, amino acids, light or small organic molecules. The signal itself is transmitted by a trimeric G protein consisting of Gα,Gβ, and Gγ subunits that associate with the receptors. There are 18 different human α subunits, 5 different β subunits and 11 different γ subunits that have been discovered (Hermans 2003; Wong 2003). In humans, it is estimated that there are 800 GPCRs (Fredriksson et al. 2003) of which more than 700 are predicted to fall into the type A (or rhodopsin-like receptors). Within the type A class, we find the GPCRs that signal through serotonin activation.

Serotonin-specific GPCRs have been well studied and analyzed in a variety of species, from planaria to man. The study of 5HT specific receptors dates back more than 30 years (Fillion et al. 1976) and has led to the classification of 7 types of 5HT receptors following established nomenclature (Humphrey et al. 1993). Within the 7 classes there are more than 10 subtypes identified to date. The basis for the seven classes is pharmacology, signal transduction mechanism and structure. With the exception of 5HT3 and the nematode MOD1 receptors, which are ligand-gated ion channels (Hoyer 1990; Ranganathan et al. 2000), all other known serotonergic receptors belong to the GPCR superfamily. Two classes of receptors, 5HT1 and 5HT5 are typically linked to $G_{i/o}$ proteins which couple negatively to adenylate cyclase and

may lead to inhibition of firing. There are 5 subtypes classified as 5HT1 receptors (A-F, the C was reclassified as 5HT2C and two subtypes of 5HT5 (A and B).The second major group of serotonergic receptors, 5HT2, couple preferentially to $G_{q/11}$ which lead to increases in inositol triphosphate (IP3) and cytosolic Ca^{2+}. There are 3 subtypes included in this group (5HT2A-C) following the inclusion of the 5HT1C receptor. Three additional classes of serotonergic GPCRs, 5HT4, 5HT6 and 5HT7, preferentially couple to G_s and stimulate cAMP production through activation of adenylate cyclase. In turn, cAMP stimulates protein kinase A (PKA), which modulates the activity of a variety of downstream effectors, such as ion channels, metabolic enzymes and the cAMP response element binding protein (CREB) to name a few. Recent evidence suggests cAMP may also act directly on particular cellular signalling proteins, such as Epac guanine-exchange proteins (Emery and Eiden 2012). All of these targets have potentially different downstream effects and can lead to changes in calcium ion flux, membrane excitability, or other cellular processes. Despite the diversity of 5HT receptors, there are additional processes that lead to changes in the receptors already found. Alternative splicing and mRNA editing of some 5HT receptors can lead to changes in tissue location and/or differences in G protein coupling (Gerald et al. 1995; Burns et al. 1997; Jasper et al. 1997; Blondel et al. 1998; Heidmann et al. 1998; Bender et al. 2000; Pindon et al. 2002). Oligomerization is another tool that is used to influence function of the receptor. It has been shown that 5HT1A, 5HT2C and 5HT4 receptors can form both homo and hetero-dimers and that depending on the oligomeric state, the pharmacology can differ (Lee et al. 2000; Lukasiewicz et al. 2011; Pellissier et al. 2011). With so many classes of 5HT receptors coupling to many G proteins and possible modifications in sequence, it is no wonder that serotonin is still being studied and yielding new insights in how it can control behavior, not to mention as a target in treating disease.

Here we show for the first time that a GPCR-like protein cloned from *S. mansoni* is responsive and specific to serotonin in its activation of intracellular cAMP. The schistosome receptor has distinct pharmacology compared to serotonin receptors from other species and, when localized through confocal miscroscopy, is present in the neuronal system of both larval stages and adult worms. Motility assays showed that drugs that inhibit activity of this receptor

also show an effect on schistosome movement, suggesting a role for the receptor in muscle control.

Materials and Methods

Neurochemicals. o-methyl-serotonin, buspirone, (R)-(+)-8-Hydroxy-DPAT hydrobromide, mianserin hydrochloride, cyproheptadine hydrochloride sesquihydrate, chlorpromazine hydrochloride were purchased from Sigma Aldrich.

Parasites. For all experiments in this study, a Puerto Rican strain of *S. mansoni* was used. Sporocyst-infected *Biomphalaria glabrata* snails were obtained from the Biomedical Research Institute (Bethesda, MD, USA). Cercarial shedding was performed 6 weeks post infection as previously described (Lewis et al. 1986; Lewis et al. 2001). Cercariae were obtained and mechanically transformed to obtain larval schistosomula (Basch 1981) and cultured under previously optimized conditions (El-Shehabi et al. 2009) To obtain adult worms, female CD1 mice were infected with approximately 150 cercaria/mouse and worms were harvested 6-8 weeks post-infection by portal perfusion.

Cloning of Sm5HTr and phylogenetic analysis. A BLAST analysis of the *Schistosoma mansoni* genome database revealed a predicted coding sequence (Smp_126730) that showed characteristics of family A GPCRs and shared high sequence homology with known serotonin receptors from other species. The sequence was thus termed Sm5HTr and cloned from reverse transcribed *S. mansoni* cDNA. In brief, RNA was isolated using TRIZOL® (Invitrogen) and reverse transcribed using MMLV reverse transcriptase (Invitrogen) using Oligo-$(dt)_{12\text{-}18}$ primer following manufacturer's recommendations. The resulting cDNA was used as a template to amplify the open reading frame (ORF) with sequence specific primers. The primer sequences targeting the beginning and end of the sequence are as follows: sense primer (5'-ATGACAATCTCACAATTGG);

antisense (5'-TCATCTTTCATCCGTTTGACC). The resulting product was cloned into p-GEMT-easy vector (Promega) and the sequence was confirmed for at least 2 different clones.

ClustalW protein sequence alignments and the construction of a phylogenetic tree were performed using MacVector 7.1.1 (Accelrys, Inc). Trees were built according to Neighbour-Joining method using the Best Tree mode available in MacVector and were verified by bootstrap analysis with 1000 replicates.

Expression of Sm5HTr in mammalian cells and Immunofluorescence assays.

The full length Sm5HTr cDNA was engineered for expression by PCR, introducing a Kozak sequence for optimal translation efficiency in mammalian cells (Kozak 1987) followed by a FLAG fusion tag (DYKDDDDK) at the receptors N-terminal end. The modified cDNA was cloned into the expression vector pCI-neo between the XbaI and NotI sites and verified by sequencing. HEK 293 cells were seeded in six-well plates and transfected with either empty plasmid or pCI-neo-Sm5HTr using Fugene6 (Roche) following the manufacturer's protocols. 48h-72h post-transfection, the media was removed and cells were tested for expression using an anti-FLAG antibody (Sigma). Briefly, cells were washed 3 times in PBS and fixed on cover slips for 15min at room temperature using 4% paraformaldehyde (PFA). Cells were washed with PBS containing 100mM glycine and then permeabilized with PBS containing 0.1% triton-X 100 for 4min at room temperature. Cells were washed and incubated with anti-FLAG antibody in PBS (1:100) for 1.5 hours at room temperature. They were then washed and incubated in PBS with FITC conjugated anti-mouse IgG secondary antibody (1:700) for 1 hour. Cells were mounted and visualized using a Nikon S1200 series microscope.

Functional Analysis of Sm5HTr. HEK 293 cells were cultured in HEPES-buffered Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and transfected with 3µg plasmid/dish with pCI-Neo-Sm5HTr using Fugene6 transfecting agent, following the manufacturer's recommendations. Negative controls were similarly transfected

with empty plasmid (mock). As a positive control, we transfected HEK 293 with a plasmid (pCI-Neo-5HT2ce) that expresses a previously described *Caenorhabditis elegans* serotonin receptor (Hamdan et al., 1999). 48h post transfection, cells were harvested and seeded in 96-well plates at a density of 100,000 cells/well and cultured overnight before testing for receptor activity. Since the majority of GPCRs signal through either changes in calcium or cAMP, we tested for activity using both types of signaling assays. Changes in intracellular calcium were measured with a FLIPR Calcium Assaykit (Molecular Devices), as previously described (Xie et al., 2005). Briefly, transfected cells were washed once and incubated in calcium dye reagent for 1hr at 37^0C. Following incubation, cells were placed in a FlexStation plate fluorometer and were injected with either 5HT or vehicle alone. Fluorescence readings were done prior to injection and every 1.8s for a total of 60s/well. Functional responses were measured as peak fluorescence after subtraction of baseline as described (Xie el al., 2005). Data represent averages of 2 replicates, each done in duplicates. To test for receptor activation through changes in cAMP, the transfected cells were similarly seeded in 96 well plates at a density of 100,000 cells/well and allowed to sit overnight at 37^0C. The next day they were used for cAMP assays with the CatchPoint™Cyclic-AMP Fluorescent Assay kit (Molecular Devices) following the manufacturer's recommendations. Briefly, cells were washed once in Kreb's-Ringer Bicarbonate buffer supplemented with glucose and were subsequently treated with 0.75mM 3-isobutyl-l-methylxanthine (IBMX) prepared in the same buffer for 10 minutes at room temperature. Cells were washed again in buffer and treated with 20 µM forskolin and test substances at 10^{-4}M (unless otherwise noted) for 10 minutes at 37^0C. Antagonists were tested in the presence of 10^{-4}M serotonin. Following incubation, cells were washed, and lysed. Aliquots of the resulting cell lysates were tested for cAMP by a competitive immunoassay using rabbit anti-cAMP antibody followed by Horseradish Peroxidase (HRP)-cAMP conjugate and a fluorescent substrate, as per the kit protocol. Fluorescence was measured in a FlexStation micro plate reader set at λ_{em}=485nm and λ_{ex}= 554nm. Quantitative amounts were calculated from a standard curve of known amounts of cAMP (provided with kit). Each assay was performed in duplicates or triplicates and was replicated through at least three independent experiments.

Antibody production and western blot analyses. A polyclonal anti-Sm5HTr antibody was produced in rabbits against two synthetic peptides (Twenty first Century Biochemicals). Peptide 1 (CKAREYDKRLNSYSS) was located in the third intracellular loop, while peptide 2 (CKRQSIVISSPYTRND) is located in the C-terminal tail. 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 Sm5HTr was subsequently purified using peptide conjugated beads (Sigma), according to standard procedures and the eluted fractions were tested again using an ELISA assay. For western blot analyses, we extracted membrane proteins from adult *S. mansoni* (males and females) using the ProteoExtract Native Membrane Protein Extraction kit (EMD Millipore Bioscience,as described in the kit protocol). Aliquots of membrane proteins were resolved on a 4-12% gradient Tris-Glycine precast gel (Invitrogen) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Blots were probed using affinity purified anti-Sm5HTr antibody (1:1000 dilution) followed by HRP-labeled goat anti-rabbit IgG antibody (1:25,000)(Calbiochem). Negative controls were performed with an excess of combined peptide antigen (0.25mg/ml of each peptide).

Confocal Microscopy. Freshly recovered adult worms and newly transformed schistosomula (1 day old) were fixed in 4% paraformaldehyde for 4hours at 4^0C. Following fixation, samples were washed three times in antibody diluents (1x PBS pH 7.4, 1% bovine serum albumin, 0.5% triton X-100, 0.1% sodium azide) and incubated for 3 days with affinity purified anti-Sm5HTr (1:125) dilution with end/over end rotation at 4^0C (Mair et al. 2000; El-Shehabi et al. 2009). Samples were then washed three times for 10 minutes each and incubated for 2 additional days with fluorescein isothiocyanate (FITC)-labeled goat anti rabbit antibody (1:400 dilution) and 200 µg/ml phalloidin-tetramethyl rhodamine isothiocyanate (TRITC, Sigma) to label the musculature. Animals were washed overnight with antibody diluent,

then mounted with anti-quenching mounting media (Sigma) and visualized using the BIO-RAD RADIANCE 2100 confocal laser scanning microscope equipped with a Nikon E800 fluorescence microscope for confocal image acquisition and LASERSHARP 2000 analyzing software package. Controls used include anti-Sm5HTr preabsorbed with 0.25 mg/ml of each peptide antigen and secondary antibody alone.

Behavioural Assays. *In vitro* transformed schistosomula were treated with test substance or vehicle in Opti-MEM (Gibco) supplemented with 0.25 µg/ml fungizone and 100 µg/ml streptomycin and 100 units/ml penicillin for up to 10 minutes. After incubation, parasite motility was recorded using a compound microscope (Nikon, SMZ1500) equipped with a digital video camera (QICAM Fast 1394, mon 12 bit, Qimaging) and SimplePCI version 5.2 (Compix Inc.) for image acquisition. Images were obtained at a rate of ≈3 frames/ s for the tree consecutive periods of 45 seconds each and the data were analyzed with ImageJ software (version 1.41, NIH, USA). Briefly, we used the software to draw an ellipse of best fit around each parasite and an estimate of body length was obtained by measuring the major axis of each ellipse for each frame of the video. Changes in the major axis proved to be the most consistent measure of motion and were used to determine the frequency of length changes during the observation period. The difference in axis length from on frame to the next was calculated and if proved to be larger than 10%, it was counted as a movement, which could be either an increase (elongation) or decrease (shortening) ; changes ≤ 10% were disregarded. Approximately 12 animals were monitored per treatment and each treatment was replicated at least 3 times.

Other methods. Protein concentration was determined by Lowry or Bradford protein assay, using commercial kits from BioRad. Indirect ELISA was performed according to standard methods in 96-well plates coated with Sm5HTr peptides (1 µg/well) and serial dilutions (1:100,000-1:500) of rabbit Sm5HTr antiserum, pre-immune or peptide purified Sm5HTr antiserum followed by HRP-labeled goat anti-rabbit IgG at 1:2000 dilutions. Statistical analyses

of receptor activity were done using the Prism software package for Macintosh (v3.0a, Graphpad Software, San Diego, CA, USA.

Results

Cloning and sequence analysis of Sm5HTr. Smp_126730 was cloned using the predicted coding sequence from the online *S. mansoni* genome database (www.genedb.org/Homepage/Smansoni). The sequence was verified by sequence analysis of three separate clones and found to be identical to the predicted coding sequence. The full length sequence is 1393bp (463aa) with a molecular weight of 52kDa and a pI. of 9.98. Sequence analysis revealed the protein to have a typical GPCR topology consisting of 7 transmembrane domains, an extracellular N-terminal end and intracellular C-terminus. The extracellular N-terminus carries putative N-glycosylation sites in the 1st extracellular loop, which are characteristic of biogenic amine GPCRs. Sequence homology analysis suggests this receptor is distantly related to the 5HT7 class of serotonin receptors (Fig. 1). The proteins with highest homology are from the planarian *Dugesia japonica* with all three proposed serotonin receptors (1, 4, and 7) having greater than 58% sequence similarity and 44% sequence identity.

The Sm5HTr protein contains several key residues that are characteristic of aminergic GPCRs and more specifically serotonin receptors. Of the 33 amino acids shown to be conserved among 5HT GPCRs in other species (Kroeze et al. 2002), the Sm5HTr shows 100% conservation. The highly conserved N (1.50) of transmembrane (TM) 1 is present as are 2 other residues in the first intracellular loop, namely L1.63 and P2.38. Both these residues mark the transition to the next transmembrane domain. Four amino acids in transmembrane 2 (S2.45, D2.50, V2.57 and P2.59) are conserved as well as the leucine in position 2.46, which is found in most 5HT receptors. The first extracellular loop contains glycines at both termini, important for flexibility and the highly conserved tryptophan in the middle of the loop is also present. All 7 conserved residues of TM3 are present in the schistosome receptor including the DRY motif at the cytosolic interface of TM3, a feature shared among all rhodopsin-like receptors. Also present is

the canonical TM3 aspartate at position 3.32 (D3.32), which serves as a principal binding site for the protonated amino group of serotonin (Strader et al. 1987). Two residues that are highly conserved in 5HT receptors within the second intracellular loop (IL2) are a proline at position 3.57 followed by a hydrophobic residue at position 3.58, which in the case of Sm5HTr is a leucine. The tryptophan residue at position 4.50 is conserved as is the tyrosine at position 4.38 and the PP motif at the end of the transmembrane 4 region. The only conserved residue in the second extracellular loop (EL2) is cysteine 5.25 which is hypothesized to form disulfide bridges with a similarly conserved cysteine from EL1. There are 3 residues in TM5 that are highly conserved, including F5.47 and P5.50 as well as a tyrosine toward the end of the membrane region at position 5.58. The two most conserved transmembrane regions are the 6^{th} and 7^{th}. Here there are 12 residues that are conserved, F6.44, W6.48, P6.50, F6.51, F6.52 and W7.40, G7.42, Y7.43, S7.46, N7.49, P7.50, Y7.53. Lastly, there is a conserved predicted palmitoylation site at position C7.70 that is also present in the Sm5HTr.

Expression of Sm5HTr and cAMP-induced accumulation in HEK 293 cells.

Serotonin signaling through GPCRs can function either through change in Ca^{2+} or through the up or down regulation of cAMP. In order to test for activity and to identify the possible secondary signalling molecules, Sm5HTr was expressed in HEK 293 cells. Cells were transiently transfected with Sm5HTr and probed using an anti-FLAG (m2) antibody to confirm expression and proper trafficking of the protein (Fig. 2). We first tested if Sm5HTr was able to signal through Ca^{2+}. Cells were transfected with Sm5HTr or empty plasmid (mock) and seeded in 96 well plates for Ca^{2+} assays 48hr post-transfection. As a positive control for the assay, the *C. elegans* SER-1 GPCR was transfected alongside the Sm5HTr receptor as it has been shown to signal through the elevation of intracellular calcium (Hamdan et al, 1999; Xie et al, 2005). Cells were washed and incubated with a calcium dye for 1hr followed by treatment with 10^{-4}M serotonin. Treatment was performed in a FlexStation plate fluorometer and fluorescence measurements were taken throughout the assay (Fig 2c). As expected, the positive control SER-1 receptor showed a spike in fluorescence, typical of an influx of intracellular calcium (Xie et al. 2005). In contrast, those cells expressing Sm5HTr and the mock trasnfected control showed no change in signal.

The second signaling pathway occurs through changes in cAMP level, typical of receptors that couple through Gs or Gi proteins. Here HEK 293 cells were transiently transfected with Sm5HTr and 48h post transfection seeded for cAMP assay. Cells were washed and treated with 3-isobutyl-l-methylxan- thine (IBMX), a cAMP phosphodiesterase inhibitor and forskolin, a cAMP stimulator. Cells were treated with 10^{-4}M serotonin and incubated for 1hr at room termperature. Cells were lysed and loaded on 96well plates, which were pre-treated with anti-cAMP antibody. Plates were read in a FlexStation plate fluorometer and total amounts of cAMP were calculated based on standard curve. Cells expressing Sm5HTr showed an increase in intracellular cAMP when compared with mock-transfected cells. The experiment was repeated with varying concentrations of 5HT (Fig 2f) and we found that the response was dose dependent with an EC_{50} value of 61.7nM In order to test for specificity toward 5HT, several other naturally occurring biogenic amines and related metabolites were tested to see if they could produce any changes in cAMP (Fig.3A). None of the other biogenic amines tested were able to produce a significant change on cAMP levels when compared to mock cells, suggesting that the Sm5HTr is sensitive only to serotonin. To obtain a first pharmacological profile, several known mammalian serotonin receptor agonists and antagonists were used to determine if they could either stimulate cAMP activity or inhibit 5HT activation of the receptor. Of the three agonists tested, the only one that was able to produce a significant activation was o-methylserotonin (Fig.3A). Neither buspirone nor DPAT, two common agonists of mammalian serotonin receptors, was able to induce any changes. Further analysis of the o-methylserotonin response showed that the methylated derivative produced stronger activation than serotonin itself when tested at the same concentration (Fig.3A) and the effect was dose-dependent with an EC_{50} of 98.5nM (Fig.3B). To search for antagonists, cells expressing Sm5HTr were treated with a variety of test drugs in the presence of 10^{-4}M 5HT. The analysis showed that the two common serotonergic antagonists, mianserin and cyproheptadine and a drug of mixed serotonergic/dopaminergic specificity, chlorpromazine, were all able to significantly inhibit activation of Sm5HTr by serotonin (Fig.3A)

Behavioural Assays. Those substances shown to interact with Sm5HTr *in vitro* were subsequently tested on cultured parasites to search for possible effects on motility. Four to eight-day old *in vitro* transformed schistosomula were washed 3 times to remove residual serum and incubated in Opti-MEM for 30 minutes to acclimate samples. Animals were then treated with serotonin or o-methylserotonin, each at 10^{-4}M to 10^{-8}M. Animals were incubated for 10 minutes and movement was recorded for 45s at a rate of 3 frames / second. In order to quantitate movement, an ellipse of best fit was traced around each animal. The major axis was measured for each frame and the difference from frame to the next was calculated over the observation period. If a difference of greater than 10% was found, it was counted as a body movement, which could be either lengthening or shortening (El-Shehabi et al, 2012). Data were obtained from 3 separate trials, each with a minimum of 10-12 animals. Animals that were incubated with serotonin exhibited a significant increase ($P < 0.05$) in movement of approximately 2-3 fold over controls when treated with 10^{-4}M 5HT (Fig .5A). A significant but smaller stimulation (≈50%) was also observed at 10^{-5}M. Since we saw that methylated serotonin was able to stimulate activation of the receptor *in vitro*, we decided to test its ability to stimulate larval motility. Animals were treated as previously mentioned using either methyl-serotonin or vehicle alone. Again, animals that were treated with methyl-serotonin were more active than the controls, and the stimulation was dose-dependent (Fig5B). Methyl-5HT was more potent than the unmethylated form, causing significant stimulation of movement at 10^{-6}M and about 4- to 5- fold increase in activity at concentrations above 10^{-5}M. To test if Sm5HTr antagonists were able to block the response to serotonin, schistosomula were pre-treated with 10^{-4}M mianserin or cyproheptadine, followed by addition of 10^{-4}M serotonin. After 5min incubation, animals were recorded and analyzed for frequency of movements using the same imaging assay. What we saw was consistent with the results *in vitro* in that the effect of serotonin was abolished at 10^{-4}M with both drugs (Fig.6). This suggests that the drug effects on larval motility could be mediated, at least in part, by the Sm5HTr receptor.

Immunolocalization. A polyclonal anti-Sm5HTr peptide antibody was raised in rabbits, affinity-purified and verified by ELISA. Western blot analyses (fig.7) detected a major immunoreactive band of about 80kDa in preparations of solubilized adult *S. mansoni*

membranes. The band was shown to be specific since it was eliminated when the antibody was preadsorbed with the peptide antigens, or when it was replaced with preimmune serum. The western positive band is larger than the expected size of Sm5HTr but this may be due to glycosylation of the receptor, a common feature among GPCRs, or the exceptionally high pI value of the protein (pI ≈9.98, which would be expected to produce aberrant migration on the SDS-PAGE gel. The tissue localization of Sm5HTr was subsequently examined both in larval and adult stages of the parasite by confocal microscopy using affinity-purified anti-Sm5HTr antibody followed by an FITC-conjugated secondary antibody. In some experiments, we also stained with phalloidin in order to visualize the musculature. In both developmental stages of the parasite, the receptor was seen to be localized entirely to the nervous system (Fig. 6). Sm5HTr immunoreactivity was detected in the developing cerebral ganglia and nerve cords of newly transformed schistosomula (Fig.6 A-C) and in the CNS of adult parasites along the main nerve cords and transverse commissures (Fig. 6E, G). No significant labelling could be seen in any of the negative controls tested, including peptide preadsorbed antibody controls (Fig.6D, F). Besides the CNS, we observed significant expression of the Sm5HTr in the peripheral nervous system of adult worms. There was diffuse but extensive staining seen immediately beneath the tegument both in the males and females, particularly in the midbody of the worm (Fig.6H). A closer examination of this region revealed an extensive network or immunoreactive and interconnecting nerve fibers, which can be seen forming a “fishnet” pattern, consistent with that of the subtegumental nerve plexus in flatworms (Cebria 2008). Some of these fine nerve fibers appear to be interspersed with the phalloidin-stained body wall muscles but there is no visible co-localization of Sm5HTr immunoreactivity with phalloidin (Fig.6), suggesting that the receptor is probably associated with neuronal structures rather than the musculature per se. Immunoreactivity was also observed in the peripheral nerve net of the ventral sucker and again it was not localized to the musculature (Fig.6).

Discussion

Serotonin has been extensively studied in schistosomes for more than 40 years (Bennett and Bueding 1971). In other helminths, serotonin has been shown to be present in the nervous system and to have numerous biological activities. In *Schmidtea mediterranea*, 5HT has been localized to neurons throughout the CNS as well as transverse commissures. Staining for 5HT was also present in the subepidermal nerve net around the oral opening (Cebria 2008). In *C. elegans,* it has been shown to be present in neurosecretory NSM cells in the pharynx as well is in the hermaphrodite specific neurons (Desai et al. 1988). In *Ascaris suum*, homologous cells to those in *C. elegans*, namely the NSM in the pharynx also contain high levels of 5HT (Johnson et al. 1996) and the same is true of motor neurons in the tail region. It has been shown that nerve cords innervating the wall musculature and responsible for locomotion were positive for 5HT immunostaining (Chaudhuri and Donahue 1989). In the parasitic nematode *Haemonchus contortus*, serotonin has been localized to several different areas throughout the worm (Rao et al. 2011) including amphidial and pharyngeal neurons and, in males only, ray sensory neurons. Further evidence for 5HT signalling in *H. contortus* came from the cloning of the first GPCR showing high specificity for 5HT sensitivity to known 5HT agonists and antagonists (Smith et al. 2003). In schistosomes, the role of 5HT is equally important and diverse. Here we have shown for the first time a novel receptor that shows specificity for serotonin and provide evidence suggesting it may play a role in motor control.

Analysis of the protein structure for the Sm5HTr found a typical heptahelical topology of a GPCR. There also existed sequence patterns similar to other known 5HT receptors, notably the presence of a relatively short extracellular amino end, an intracellular carboxy tail, and a large 3rd intracellular loop. Many of the conserved residues of serotonin receptors are present in Sm5HTr, including core residues of the predicted binding site such as D3.32. Phylogenetic analysis with other known serotonin receptors from both vertebrate and invertebrates show that the Sm5HTr clusters with the 5HT7 class of receptors. It shows highest sequence similarity and identity with the planarian receptors from *Dugesia* and overall greater than 50% sequence similarity with the human 5HT-7 receptor.

Expression of the Sm5HTr in HEK 293 cells produced a functional receptor that was responsive to serotonin. Initially we tested for receptor activity using calcium signalling assays and found no change in intracellular Ca^{2+} levels upon incubation with 5HT. Functional expression studies were repeated using cAMP assays and determined that the receptor responds to serotonin by elevating intracellular cAMP levels. Research on the human 5HT7 receptor showed that it also stimulates intracellular cAMP by binding to a G_s protein (Bard et al. 1993; Lovenberg et al. 1993a; Lovenberg et al. 1993b; Adham et al. 1998). Other biogenic amines and amino acid precursors were also tested to determine if this receptor was specific for serotonin or showed sensitivity to other substrates. Results suggest that this receptor is indeed specific for serotonin as no other drug was able to elicit a response. The EC_{50} for 5HT is in the nanomolar range, which is similar to those found in vertebrate 5HT7 receptors, albeit slightly less sensitive (Witz et al. 1990; Jasper et al. 1997; Obosi et al. 1997)

To obtain a preliminary pharmacological profile of the receptor, experiments were repeated in the presence of known mammalian agonists and antagonists. Among the agonists, o-methyl-serotonin, DPAT and buspirone were tested in the absence of serotonin in order to determine if they could activate Sm5HTr. The only drug that showed a difference from the controls was the methylated form of serotonin. Research has shown that o-methylserotonin is able to act as an agonist for both human 5HT1A and 5HT2A type receptors (Ismaiel, Titeler et al. 1990) as well as for the *Drosophila* 5HT7 receptor (Witz, Amlaiky et al. 1990) and the same appears to be true for Sm5HTr. Interestingly, the methylated derivative produced a stronger activation of the receptor than serotonin itself, leading to nearly 3-fold increase in cAMP compared to < 2-fold for the natural ligand. This apparent preference for the methylated derivative is consistent with the behaviour of two other biogenic amine receptors of *S. mansoni*. The dopaminergic SmGPCR3 receptor (Taman and Ribeiro 2009) and histaminergic SmGPCR2 (El-Shehabi and Ribeiro 2010) are both more responsive to methylated forms of the respective amines than the natural transmitters. It is unknown at the present if serotonin and other biogenic amines are methylated in the parasite or if this activity is biologically relevant. The other agonists tested have been examined in other systems with varying effects. In the Drosophila 5HT7 receptor, DPAT was able to elicit a small response but at less than half the

efficacy of serotonin. Buspirone has been shown to be a partial agonist of 5HT1 receptors, but not 5HT7 which may explain the lack of activity of the drug toward the schistosome receptor. Several classical aminergic antagonists were tested for their ability to inhibit cAMP signalling, including mianserin, cyproheptadine and chlorpromazine. Each has been shown to inhibit 5HT7-mediated accumulation of intracellular cAMP in mammals (Jasper et al. 1997) and they also inhibited the recombinant schistosome receptor.

As a first step towards elucidation of function, we questioned whether Sm5HTr might be involved in the previously described effects of serotonin on parasite motility. Serotonin is known to stimulate schistosome motility when added exogenously onto cultured parasites. This was first observed in adult worms (Willcockson and Hillman 1984) and more recently in sporocysts as well (Boyle et al. 2000). In both cases, the stimulation was blocked by the co-application of classical serotonergic antagonists, suggesting this was a receptor-mediated event. To investigate the possible involvement of Sm5HTr, we repeated these pharmacological studies using a more quantitative motility assay (El-Shehabi et al. 2012) and the same agonists and antagonists that were shown to interact with the recombinant receptor *in vitro*. Schistosomula were selected for these studies because they express Sm5HTr and are better suited for quantitative measurements of movement than adult worms. Animals were first treated with various concentrations of 5HT, o-methylserotonin or vehicle and the frequency of movements was recorded for each animal. We saw that both agonists produced similar motor phenotypes, which were characterized by a dose-dependent increase in movement. Once again, the methylated form of 5HT produced a more robust response than the natural transmitter, similar to what was observed in the *in vitro* expression studies. When analyzed for dose dependency, we saw that the methylated form has an effect down to a concentration of 10^{-6}M whereas for 5HT the effect was lost below 10^{-5}M. In order to confirm that the response was due to 5HT signalling through a receptor, Sm5HTr antagonists mianserin and cyproheptadine were used to determine if the behavioural effect could be abolished. Larvae were pre-treated with drug followed by an incubation with 5HT and measured for body wall movements as before. We saw a significant decrease in the frequency of movements compared to samples treated with 5HT alone, suggesting that both of these drugs were able to

inhibit 5HT-induced signalling. Based on this limited survey, we conclude that Sm5HTr is likely to play a role in motor control and the drug effects are mediated, at least in part, by interactions with this receptor.

Continuing the investigation of biological function, we used a specific peptide antibody and confocal microscopy to examine the tissue localization of the receptor in the worm. Sm5HTr was found entirely in the nervous system both in adult worms and larvae. A closer examination of adult worms detected significant Sm5HTr immunoreactivity in both males and females and throughout the nervous system, including the main longitudinal nerve cords, transverse commissures and the peripheral nerve net. In order to determine if the receptor was localized on muscle fibres, phalloidin was used as a counter stain. We saw no co-localization of the two, suggesting that the effects of 5HT through this receptor target neurons and not muscle fibres themselves.

The apparent absence of this receptor in the musculature and yet the overwhelming evidence that serotonin produces changes in motility suggest that serotonin may be acting on more than one receptor, or that there are neuromodulatory effects being transmitted through Sm5HTr. Neuromodulation in invertebrates is well documented, specifically in influencing muscle contraction and movement. In *C. elegans*, for example, serotonin controls locomotion indirectly by modulating the release and/or activity of neuromuscular transmitters (Horvitz,Chalfie et al. 1982, Mendel, Korswagen et al. 1995, Segalat, Elkes et al. 1995, Sawin, Ranganathan et al. 2000). We propose a similar mechanism here in schistosomes, in that although not present in the musculature, serotonin is activating the Sm5HTr to modulate other neurotransmitters resulting in increased motility. Previous work by Day et al (1994) support an indirect mode of action for serotonin. They showed that serotonin itself was not sufficient to elicit muscle contraction in preparations of muscle fibers of *S. mansoni*, but rather potentiated the effects of other neuromuscular transmitters (Day, Bennett et al. 1994). Moreover, serotonin stimulates glycogenolysis and glucose utilization in schistosomes (Rahman et al. 1985b; Rahman et al. 1985a) , which could also stimulate movement indirectly by making more energy available for muscle contraction.

Male schistosomes exhibited significant Sm5HTr immunoreactivity in the innervation of the ventral sucker, again with no apparent co-localization with phalloidin-stained muscle fibers. The ventral sucker is a key organ for the survival of the parasite and for the continuation of the life cycle. Adult males are required to latch onto the walls of the veins and grasp against the flow of the blood for the duration of copulation. This is accomplished primarily through the ventral sucker. It has been shown that the acetabulum has high levels of biogenic amine transmitters and at least three other biogenic amine receptors besides Sm5HTr, two of which are activated by histamine (El-Shehabi, Vermeire et al. 2009; El-Shehabi and Ribeiro 2010) and another by dopamine (El-Shehabi, Taman et al. 2012) . This suggests that control of the sucker is highly coordinated, with several transmitter systems playing an important role.

In both sexes, we saw positive staining for Sm5HTr to the area just below the tegument. The expression pattern in this region resembles the "fishnet"-like structure of the subepidermal nerve plexus in planaria (Cebria 2008). In flatworms, there exists both a subtegumental nerve plexus and a submuscular nerve plexus, each with its own function. Co-staining with phalloidin showed that the Sm5HTr immunoreactivity was located above (peripherally) to the musculature, suggesting that it is in the subtegumental region. The subtegumental nerve plexus may play an important role in neuronal signalling to the tegument and to receive and coordinate signals received from the environment (Gustafsson 1987). The tegument is a fairly complex organ that is yet to be fully understood. It is essential for the survival of the parasite by protecting against the host immune responses. It helps in the recruitment of nutrients from the environment and also acts as a sensory organ for the parasite to navigate through its surroundings. It has been shown that there are serotonergic sensory neurons that extend to the surface of the parasite (Gustafson 1987). The function of these neurons is not known but one can hypothesize that their function is related to environmental cues and signal transduction. A serotonin receptor expressed in this region could play an important role in relaying the signal to the brain and controlling parasite behaviour.

In summary this study provides the first molecular evidence for a functional serotonin receptor that mediates neuronal signalling and may be responsible for the myoexcitatory

effects of serotonin in schistosomes. Sequence analyses have predicted only two possible 5HT receptors (Berriman et al. 2009; Protasio et al. 2012) in *S. mansoni*. The lack of any other receptors offers a unique opportunity to exploit a system that has been shown to be very important for the survival and development of the parasite (Abdulla et al. 2009; Taft et al. 2010). Targeting a protein within the neuromuscular system which may lead to a disruption of movement are successful strategies already in use in nematode chemotherapeutics. The paralysis of filarial worms with ivermectin through glutamate-gated-chloride channels is one such success story (Burkhart 2000). The Sm5HTr could prove to be a valuable target in schistosomes for drug design.

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Figures

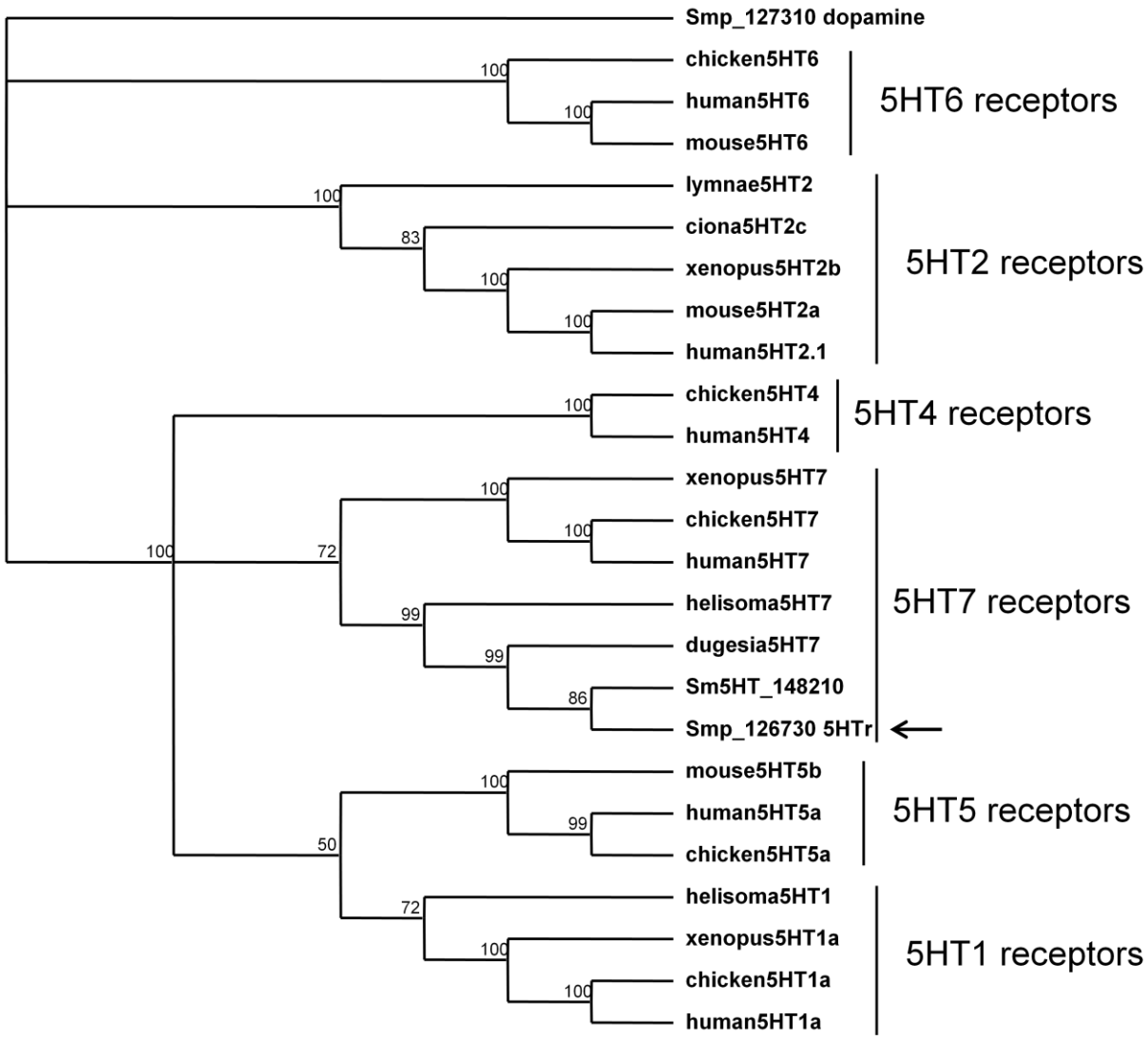

Figure 1. Phylogentic Tree of serotonin receptors. The predicted Sm5htr sequence was analyzed using using Macvector 9.0 phylogenetic analyses with other known or predicted serotonin receptors of different types. The accession numbers for the sequences are (in order shown):, chicken 5ht4 XP_414481.2, human 5ht4 NP_000861.1, chicken 5ht6 NP_001166911.1, human 5ht 6 NP_000862.1, mouse 5ht6 NP_067333.1, lymnae 5ht2 AAC16969.1, Ciona 5ht2 XP_00215339.1, xenopus 5ht2 NP_001082744., human 5ht2.1NP_000612., mouse 5ht2a NP_766400., mouse 5ht5b NP_034613.2,

chicken 5ht5a XP_426970.1, human 5ht5a NP_076917.1, helisoma 5ht1 AAQ95277.1, xenopus 5ht1a NP_001079299.1, human 5ht1a NP_000515.2, chicken 5ht1a NP_001163999.1, xenopus 5ht7 NP_001079253.1, human 5ht7 NP_062873.1, chicken 5ht7 NP_001167605.1, helisoma 5ht7 AAQ84306.1, dugesia 5ht7 BAI44327.1, Smp5htr SMP_126730, SMP_148210, SMP_127310.

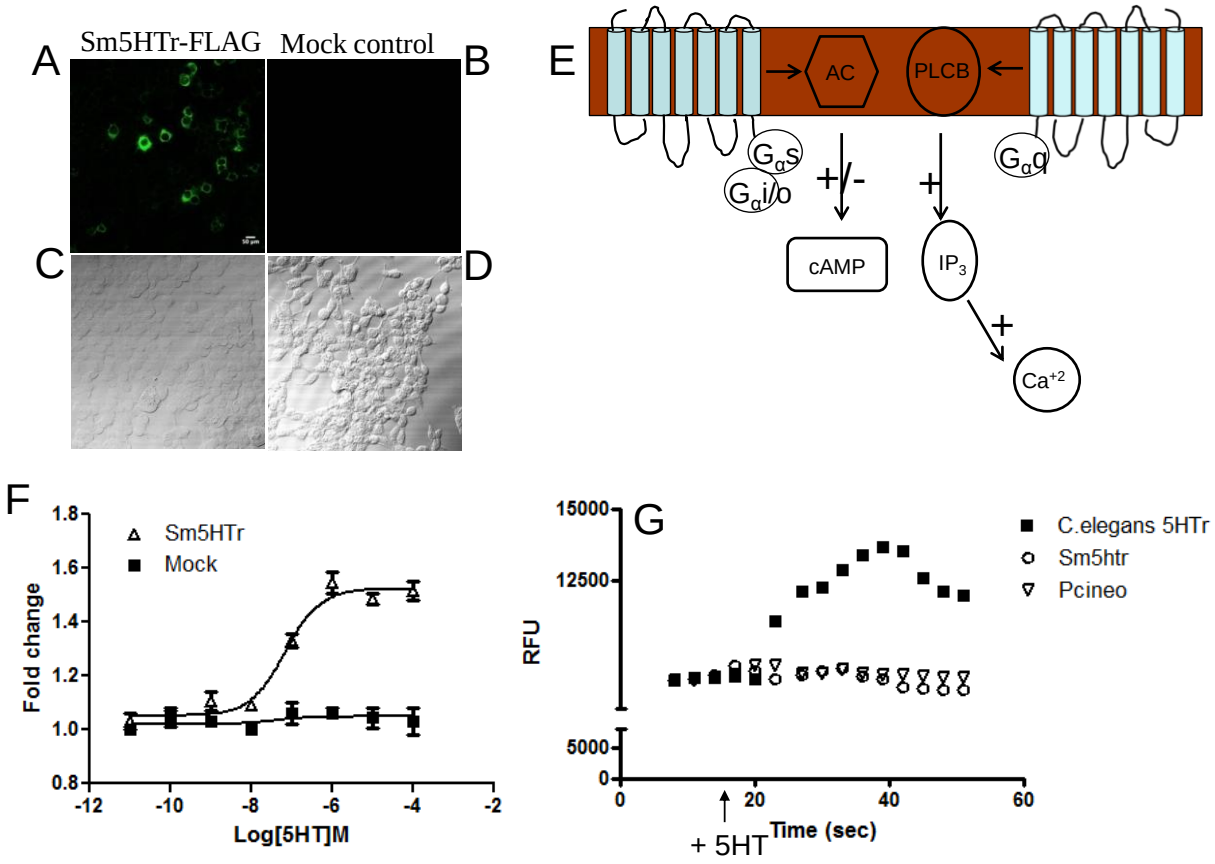

Figure 2. Mammalian expression of Sm5HTr-like receptor. HEK 293 cells were transfected with Sm5HTr-FLAG (A and C) or mock control (B and D) and probed with anti-FLAG. (E) Schematic of typical GPCR activation pathway. Receptors can go on to effect intracellular stores of Ca^{2+} or cAMP following binding of ligand. Cells were tested for activation to 5HT either through measuring relative fluorescent units (RFU) intracellular Ca^{2+} levels(G) or by accumulation of intracellular cAMP (F) following treatment of 5HT. Positive control for the Ca^{2+} assay was the *C.elegans* 5HTr.

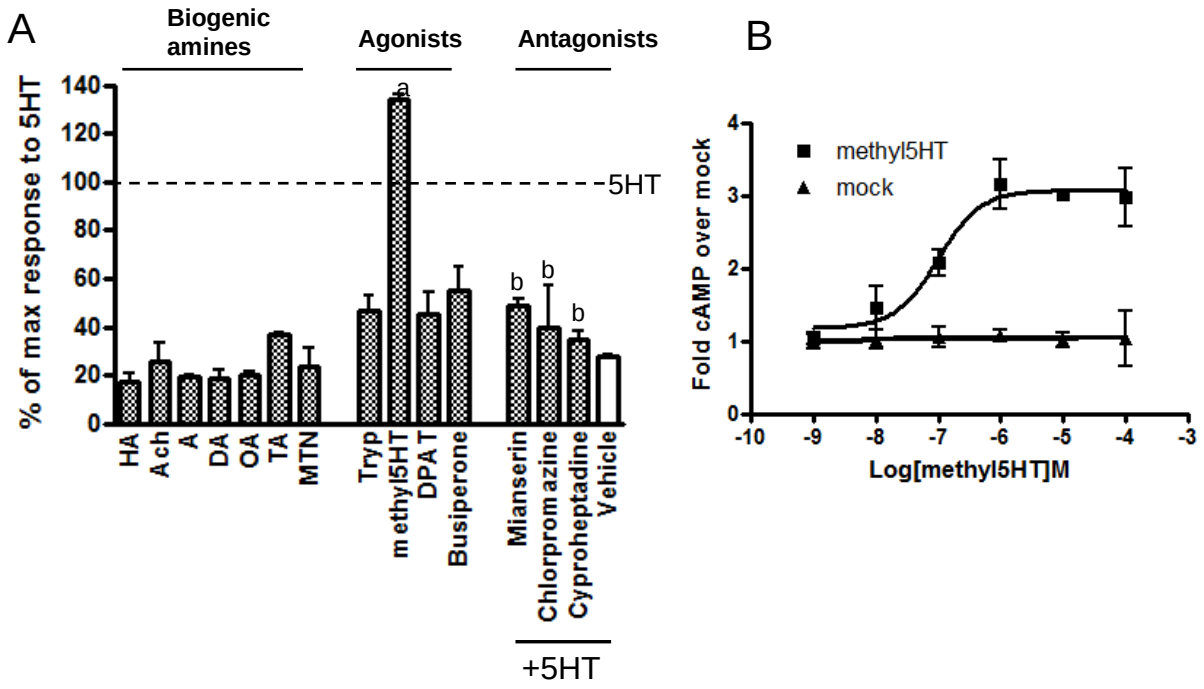

Figure 3. cAMP assay measuring sensitivity of Sm5HTr to other compounds. HEK 293 cells transfected with Sm5HTr were incubated with 10^{-4}M of various drugs and tested for intracellular cAMP levels. In the case of antagonists, cells were incubated with drug and 10^{-4}M 5HT. Drugs included histamine (HA), achetylcholine (ACH), dopamine (DA), octopamine (OA), tyrosine (TA), metanephrine (MTN), tryptamine (Tryp), o-methylserotonin (methyl5HT), buspirone, (R)-(+)-8-Hydroxy-DPAT hydrobromide (DPAT), mianserin, chlorpromazine, cyproheptadene. Mock cells are cells transfected with empty plasmid, treated with vehicle alone. (^{a}) significantly different from vehicle-treated control at P< 0.05; (^{b}) Significantly different from 5HT-induced stimulation at P<0.05. (B) Dose response curve of o-methylserotonin in HEK 293 cells expressing Sm5HTr. HEK 293 cells were transfected with Sm5HTr and incubated with varying amounts of o-methylserotonin. The data are normalized relative to mock transfected cells. EC_{50} was determined to be 98.5 nM.

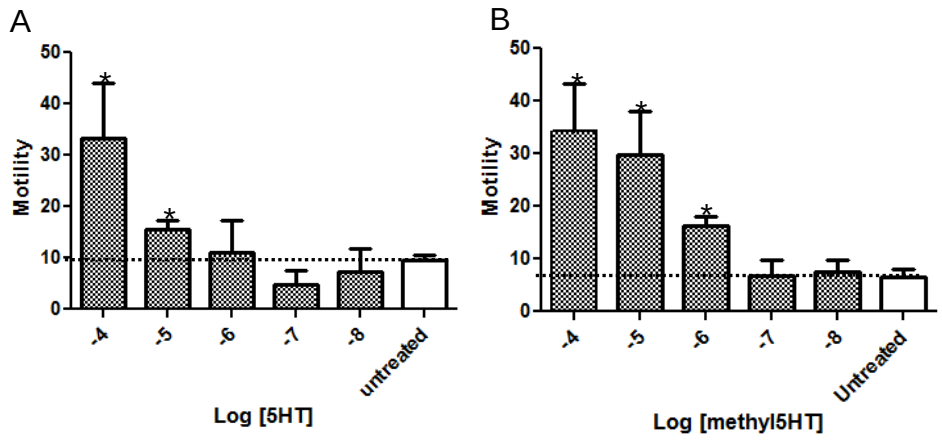

Figure 4. Behavioural assays of 8 day old schisotosomula. Larvae were harvested and allowed to grow for 8 days. Animals were then treated with varying amounts of either 5HT (A) or methyl5HT (B) and filmed over a period of 45 seconds. Analytical software was used to calculate the number of body contractions throughout the time filmed (frame x frame). A minimum of 10-12 animals for each trial and a total of 3 trials were performed. Treatment at 10^{-4}M and 10^{-5}M for 5HT and 10^{-4}M to 10^{-6}M for methyl5HT were both significantly different than controls (t-test, $p<.05$)

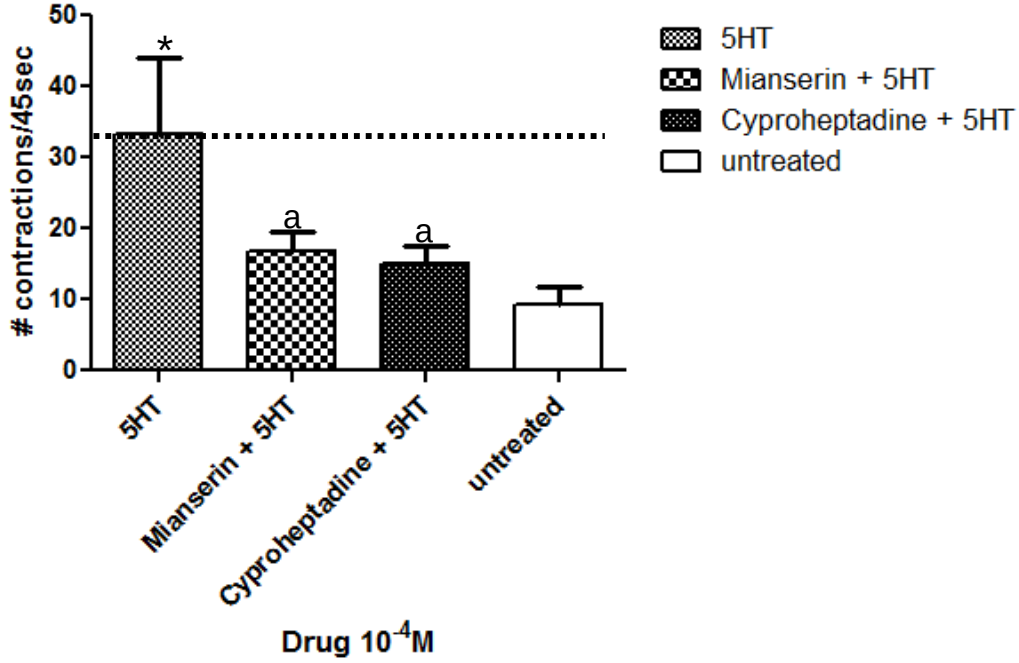

Figure 5. Behavioural studies of schistosomula. 8 day old parasites were incubated with 5HT antagonists, mianserin or cyprohepdatine for 10 minutes followed by treatment with 10^{-4}M serotonin. Animals were video recorded for 45 seconds and analyzed for muscle contraction. A miminum of 10 animals were used per trial with 3 trials per drug. Both drug treatments were significantly different from 5HT alone (ttest, two-way ANOVA, p≤.05)

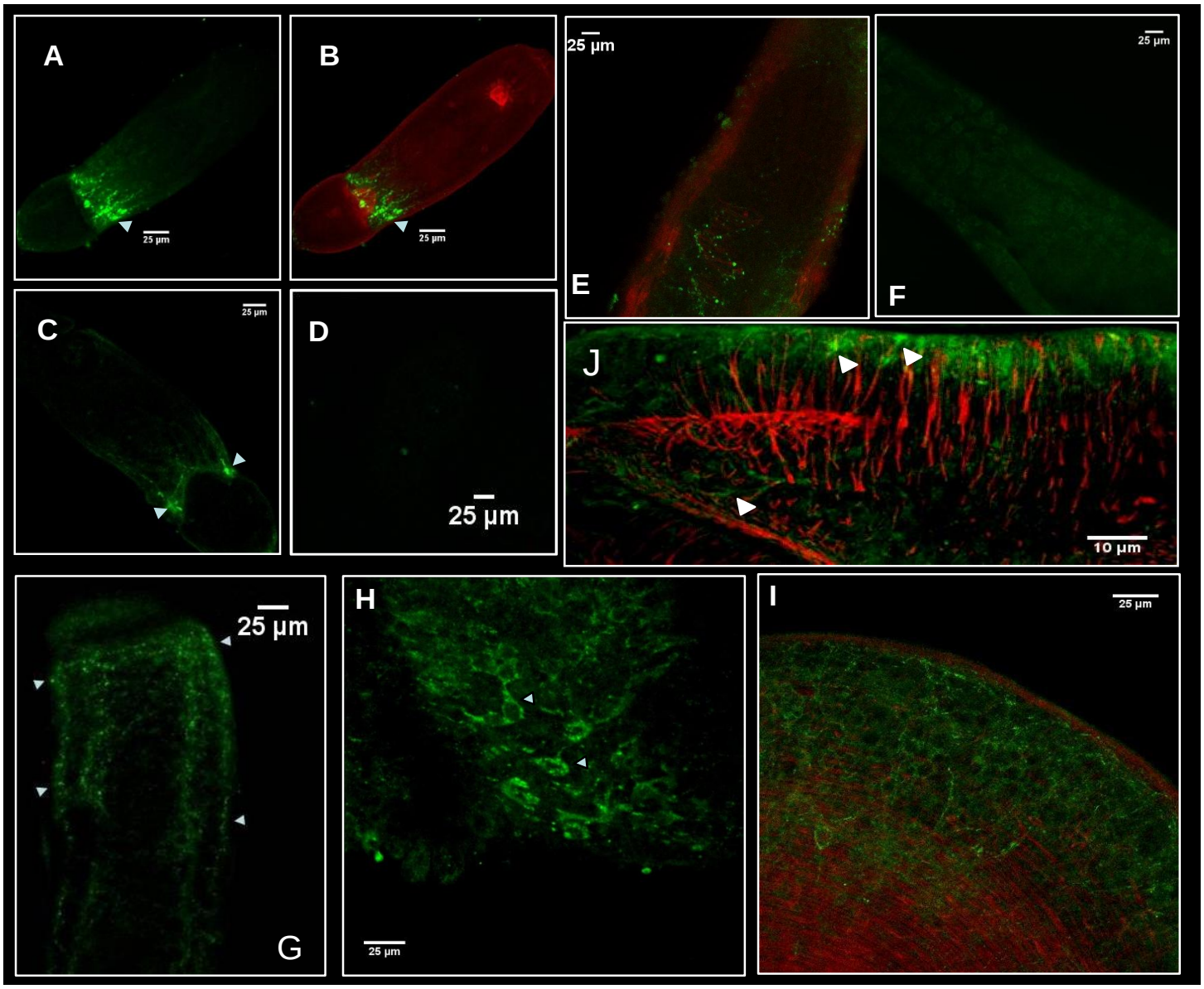

Figure 6. Confocal microscopy against Sm5htr in larval and adult *S. mansoni*. Larval parasites were grown in culture media for 4 days prior to fixation in 4% PFA. Adults were obtained from CD1 infected mouse. Animals were incubated with antibody for 2 days and probed with anti-rabbit-FITC conjugated secondary for an additional 2 days then visualized using confocal microscopy. A-C, larva stained against Sm5HTr, with phaloidin (B) or preabsorbed (D). Localization to the main nerve cords, developing cerebral ganglia as well as peripheral nerve fibres Adult male and female worms showing staining to central nerve fibers and transverse commissures (E and G). Additional localization to subtegumental varicosities of the ventral sucker (J) and to the peripheral nerve net (H and I). Preadsorbed negative controls were run to eliminate non-specific staining (F). Arrows indicate immunopositive nerve fibers located in the CNS or PNS.

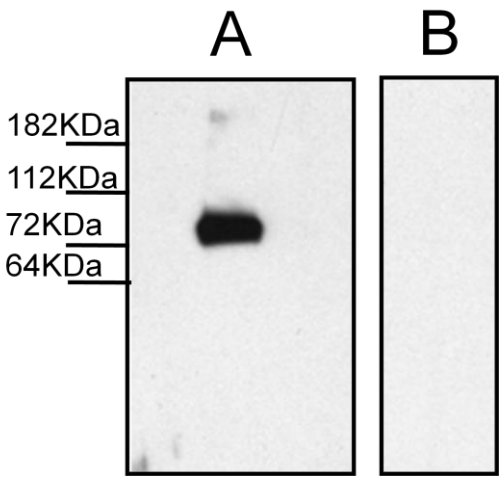

Figure 7. Western Blot analysis of Sm5HTr. Western blots were performed on solubilized membrane proteins obtained from adult *S. mansoni w*orms. Proteins were resolved on a 4-12% gradient SDS-PAGE gel prior to immunoblotting with peptide purified anti-5HTr antibody (A) or antigen pre-absorbed antibody control (B).

Chapter V

Discussion and Conclusions

Schistosomes are remarkably well adapted parasites. They are unique among the platyhelminthes in that they are dioecious. They survive in two very different hosts, the intermediate snail host and the definitive human host. The non-parasitic stage is equally impressive in that it must locate and find a suitable host in a very narrow time frame before it becomes non-infective. The basic understanding of parasite biology has been advanced in large part with the recent publication of the genome. The annotation of signalling proteins and cell-surface receptors, including GPCRs, has led to the discovery of several important proteins and a better understanding of parasite biology.

In this thesis, we take advantage of this new wealth of genomic information to undertake the first comprehensive analysis of the schistosome serotonergic system. We describe the cloning and characterization of a novel serotonin-activated GPCR and a serotonin transporter from *S.mansoni*. Serotonin has been identified in the nervous system of schistosomes and has been shown to affect both motility and metabolic systems of the worm. Our goal was to identify the proteins involved in serotonin signalling in the parasite and to link these proteins with specific behavioural phenotypes.

There are two full-length serotonin receptors (Sm5HTr) encoded in the *S.mansoni* genome and one serotonin transporter (SmSERT) although neither had been identified at the beginning of this thesis. In the first manuscript, we describe the first cloning and characterization of the SmSERT and show evidence for its expression and potential role in the recruitment of 5HT from the environment.

In mammalian systems, serotonin transporters typically have two functions; the reuptake of serotonin into presynaptic terminals in the nervous system, and the storage of serotonin in peripheral (non-neuronal) cells. In both cases, the SERT is used to remove 5HT from the extracellular space so as to terminate the signalling and in some cases, to recycle the amine for later use. In schistosomes, it has been

suggested that serotonin is taken in from the surrounding environment, specifically from the host, in a carrier-dependent manner (Catto and Ottesen 1979; Boyle et al. 2003). These studies went on to show that the uptake of exogenous 5HT could be blocked by known inhibitors of mammalian SERTs, suggesting that this process was mediated by a serotonin-specific transporter.

Cloning of this transporter showed that it shared similar topological structures, namely an intracellular amino terminus, 12 transmembrane regions and an intracellular C-terminal end. Expression analysis determined that SmSERT is present in all life cycle stages tested with increased expression in parasitic stages. Sequence analysis of the protein showed that it has a large insert in the 3rd extracellular loop (EL3) that is unique to schistotosomes. ClustalW alignment using other cloned transporters from both vertebrates and invertebrates showed a much shorter EL3 when compared to the *S. mansoni* protein.

Functional assays showed that the transporter was able to facilitate the uptake of serotonin when expressed in mammalian cells. Further analysis showed that the recombinant SmSERT was selective for serotonin and was susceptible to inhibition by classical mammalian SERT inhibitors. Transport assays in cultured schistosomula mirrored the results found in the heterologous system. Serotonin was taken up by intact schistosomula in a dose-dependent manner and the addition of SERT inhibitors was able to block this transport.

These results provided the first molecular evidence for the presence of a serotonin-specific transporter in the parasite. More recently, results from another group showed that there may be two allelic isoforms of the protein which are derived from the same gene. The second species has an additional 78 amino residues at the N-terminal end but is otherwise identical to our sequence. Functional assays of the longer variant (Fontana et al. 2009) suggest the two isoforms have similar affinity for serotonin and they are both sensitive to mammalian SERT inhibitors.

We wanted to further explore the role of SmSERT in the parasite using a combination of immunofluorescence assays and RNAi. As noted earlier, one of the main functions of SERTs in other systems is the reuptake of serotonin in synaptic clefts, which terminates activation of 5HT receptors on post synaptic membranes. We found that SmSERT is expressed primarily in the nervous system of both adults and larvae, as would be expected of a transporter involved in synaptic reuptake. When colocalized with serotonin, we see that there is a high degree of overlap between the two signals. This is consistent

with the idea that SmSERT sequesters serotonin into serotonergic neurons, presumably to terminate 5HT signalling and also for storage /recycling of the amine. We see expression of the SmSERT in the main nerve cords and transverse commissures. There is also expression in the PNS in both sexes, with nerve fibres extending from the main cords towards the periphery. Expression is found in the oral sucker, again with co-localization with serotonin. This implies that serotonin signalling may be important for the control of the sucker musculature and therefore feeding for the parasite.

In larvae, we localized the SmSERT to the PNS throughout the body. The expression is present around the entire body of the parasite in a bead-like pattern. This pattern is reminiscent of the neuronal architecture of planarian (Cebria 2008). As a model organism for *S. mansoni*, it is not surprising that we see similar structural features in both organisms, especially when comparing the larval schistosome and free-living planaria. Comparison of genomes and proteomes from the two organisms show high degrees of similarity suggesting a conservation of biological functions (Bocchinfuso et al. 2012). In adult worms, we see significant SmSERT expression in the periphery, with staining present not on the surface but in the subtegumental layers and tubercles. This suggests that serotonin may be playing an important role in the neuro-sensory processes related to the subtegumental region. It may also be playing a role in the recruitment of exogenous serotonin, bringing it from the tegument to internal storage cells for signalling.

RNAi studies were done in larvae to determine if a decrease in SmSERT expression led to any measurable phenotype, as well as to determine its role in exogenous transport of serotonin. Larvae were used because they are more amenable to siRNA transfection than the adults and the ease of *in vitro* cultivation. Transfection of siRNAs targeting the SmSERT led to a significant but only partial decrease in mRNA expression levels (~50%). The lack of off-target effects in the qPCR suggested the RNAi silencing was specific and any phenotypic change was due to decreased expression of SmSERT. Viability assays showed no significant change between controls and down-regulated animals. Initially, visual examination suggested an increase in the motility of SmSERT siRNA treated larvae. A quantitative assay was subsequently developed to confirm these changes. These studies showed a robust ≈3-fold increase in motility in the RNAi-suppressed animals when compared to controls. This suggests that SmSERT has an important role in the control of muscle function and movement. The hyperactive RNAi phenotype was not surprising since serotonin is strongly myoexcitatory. If serotonin is inactivated through SERT-mediated

reuptake, as postulated, we would expect the RNAi to cause an accumulation of serotonin in the extrasynaptic space, leading to increased activation of serotonin pathways and, ultimately, hyperactivity.

Early studies of serotonin in schistosomes reported that the parasites obtained their serotonin solely from the host via a tegumental transport system. This model was later refuted by studies showing that the parasite has the enzymatic machinery to synthesize serotonin endogenously (Hamdan and Ribeiro 1999). Nonetheless, we and others have also shown that serotonin can be taken up from the environment in larvae and therefore we wanted to evaluate how big a role the SmSERT played in this process. Larvae treated with siRNAs against the SmSERT were used for [^{3}H]-5HT transport assays. We found that although there was 50% knockdown at the RNA level, there was only a 25% decrease in transport activity. This could be due to insufficient silencing at the protein level. Alternatively, it suggests to us that the role of SmSERT is primarily for inactivating serotonin in neuronal pathways rather than recruitment of exogenous serotonin. The predominant localization of the SmSERT to the CNS and PNS with little expression on the tegument further supports the idea that recruitment of exogenous 5HT via SmSERT is secondary to neuronal sequestration.

In order to better understand the serotonin signaling pathway, we analysed the *S. mansoni* genome for any possible serotonin receptor genes. Initially, there were two sequences that showed high sequence similarity to GPCRs and 5HTrs in particular (Berriman et al. 2009). Sequence analysis showed that one contained all 7 transmembrane regions typical of GPCRs, while the other was missing TM7 and was believed to be incomplete by our group. Following the most recent re-annotation of the genome (Protasio et al. 2012), another putative serotonin receptor was identified, which appears to be complete, thus suggesting there are at least two full-length 5HTrs in this parasite. Cloning and expression of one of these full length receptors showed that it was specific to serotonin and not responsive to any other biogenic amine. Of the 7 known types of receptors, Sm5HTr most closely clustered with the type 7 receptors. Like other 5HT-7 receptors, the Sm5HTr signalled through activation of the cAMP signalling pathway rather than the Ca^{2+} signalling pathway. Of note, none of the known mammalian serotonin agonists tested were able to elicit reactions with the exception of methyl-serotonin. Consistent among invertebrates, methylated serotonin has been shown to be an agonist for 5HT-7 receptors from *Drosophila* and X*enopus* (Witz et al. 1990; Nelson et al. 1995). In schistosomes, methyl-serotonin is also myoexcitatory with exogenous

application leading to a potentiating of muscle contraction in isolated muscle fibres from adult worms (Day et al. 1994). Methyl-serotonin is not the only methylated biogenic amine shown to be active in the parasite. Two other biogenic amine receptors were shown to be activated by methylated forms of the amine when expressed *in vitro* (Taman and Ribeiro 2009; El-Shehabi and Ribeiro 2010). Known mammalian serotonin antagonists were able to block activation of Sm5HTr by serotonin providing more evidence that this is a serotonin specific receptor.

In order to get a better idea of the role of the Sm5HTr, immunofluorescence assays were done with phalloidin as a counter stain. We found that Sm5HTr stained predominantly to the nervous system in both larvae and adult worms. In the larvae, we found that it was present in the developing cerebral ganglia and main nerve cords. In the adults, we saw both CNS and PNS structures stain positive for Sm5HTr. In the female worms, we saw the lateral nerve cords in the posterior region as well as in the midsection of the worm. There was also staining of transverse commissures and nerve fibres extending toward the surface. When co-stained with phalloidin, we saw that serotonin was not present within the musculature, as there was no visible co-localization of the two signals. There was also significant innervation in the ventral sucker and elements of the peripheral system. We see structures reminiscent of the subtegumental nerve net as seen in planaria.

We wanted to confirm the role of serotonin in eliciting a muscular response by developing a quantitative assay measuring changes in motility. We found we were able to measure changes in length over time giving us a motility rate. We were able to confirm that both serotonin and methyl-serotonin were indeed able to elicit a change in motility. The drugs used to block activity of our receptor *in vitro* were used *in vivo* to test whether they were able to block the serotonin induced changes in motility. Both drugs tested, mianserin and cyproheptadine, were equally effective at abolishing serotonin induced motility.

The absence of Sm5HTr expression in the musculature and the evident link between serotonin and motility suggest that the effect serotonin is having on the parasite is not through direct activation of muscle fibres. Neuromodulation is a common method of neuronal control for both vertebrates and invertebrates and serotonin is a well known neuromodulator. In rats it has been shown that serotonin can enhance the transmission of GABAergic neurons (Tokarski et al. 2011) through activation of a 5HT7 receptor. Similarly in mice, it was shown that activation of 5HT7 receptors can enhance glutamate (AMPA) receptor-mediated

synaptic transmission. In a recent review, it was reported that in the mammalian system, 5HT receptors are expressed on presynaptic membranes of cholinergic, dopaminergic, GABAergic and noradrenergic, enabling serotonin to modulate release of a variety of neurotransmitters (Fink and Gothert 2007). We know that neuromodulation occurs in invertebrates as well, with serotonin showing similar capabilities of potentiating other neurotransmitter circuits (Horvitz et al. 1982; Mendel et al. 1995; Marinesco and Carew 2002). Here we propose a similar role for serotonin. Serotonin is present throughout the nervous system of the worms in all life stages implying it has an important role in the parasite. Database mining suggests that at least 2 full-length GPCRs exist, however Sm5HTr is present in nerve tissue only. Moreover, we see measurable effects of serotonin, serotonin receptor agonists, as well as SSRIs on parasite motility. The conclusion that can be drawn from this is that any effects that serotonin is having on the musculature must be in the context of modulating activity of other neurotransmitters or downstream signalling factors (See Fig.1 below). Several other neurotransmitter systems have been identified in schistosomes and shown to play important roles in excitatory neurotransmission. Neuropeptides and glutamate, for example are strongly myoexcitatory (Ribeiro and Geary 2010) and could be targeted by serotonin to indirectly stimulate parasite motility. However, we cannot rule out the possibility that the other, as yet unidentified serotonergic GPCR of *S. Mansoni* is located in the musculature and is responsible for the motor effects of 5HT.

The presence of Sm5HTr to the subtegumental nerve net suggests that it may play a role in host-parasite interactions. There is very little known about how the parasite is able to navigate through its host to reach its final location. In the cestode *H.diminuta*, it has been shown that migration in the host is directly influenced by serotonin levels in the surrounding environment (Mettrick and Cho 1981). Another example of a parasitic worm having a relationship with host serotonin is with the nematode *Nippostrongylus*. Infection leads to increases in 5HT receptor expression (~2fold) in the gut and subsequently increased smooth muscle contractility as a result of downstream signalling molecules (Zhao et al. 2006). It has also been shown that infection can lead to decreases in host tissue serotonin levels (Grosclaude et al. 1998). These results show that host serotonin plays an important role in the host-parasite interaction and may also be as important for schistosome development in adults.

The schistosome nervous system is well developed but still not fully understood. Recent reviews discuss the wide distribution of the neurotransmitters and receptors present in the parasite, but there

exists little information on how they interact with each other or the host (Ribeiro and Geary 2010; Ribeiro et al. 2012). Here we have shown that a complete serotonergic signalling pathway is present in this parasite. The enzyme necessary to synthesize serotonin, the receptor needed to transduce a signal, and transporter to inactivate and store serotonin are all present. In parallel with serotonin expression, we see their presence in the CNS and PNS of the organism. Moreover, the abundance of these proteins near the surface in the sub-tegumental nerve net, the tubercules and possibly the tegument itself, all suggest that serotonin must play an important role in host-parasite interaction.

Finally, we propose that this system is a potential target for anti-*Schistosoma*l drug development. Recent medium-throughput drug screens have identified SERT and 5HTr inhibitors among the most potent disruptors of larval motor activity and development (Abdulla et al. 2009; Taft et al. 2010). The presence of only one other full length receptor identified thus far, and the lack of any other SmSERT gene, further suggests that these are important parasite proteins and that any disruption of activity is likely to be detrimental to the health of the parasite. Having access to these proteins in recombinant form, as described in this thesis, will benefit the fight against schistosomiasis by aiding future drug screens and potentially accelerating the discovery of future therapeutics.

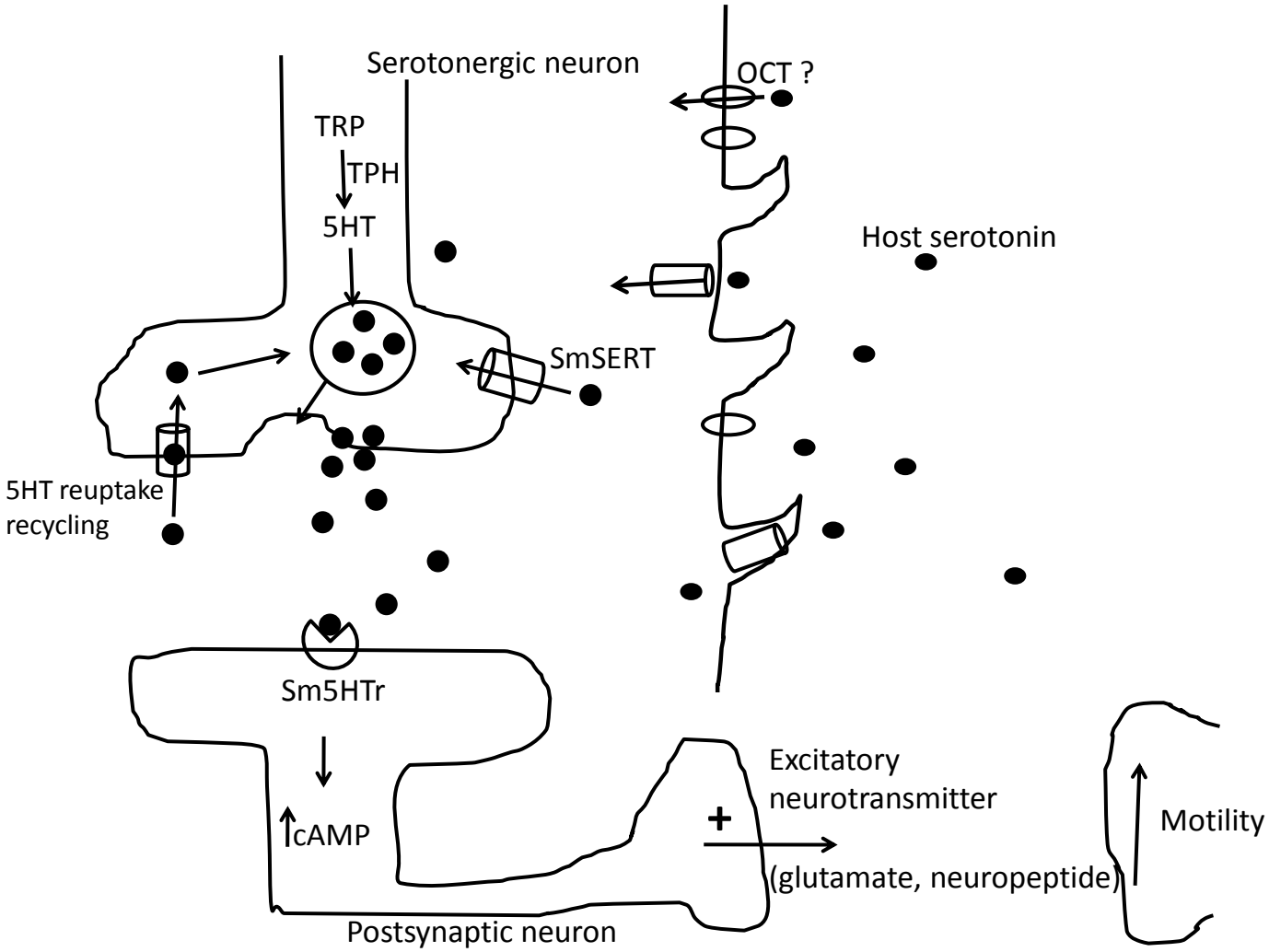

Figure 1 Proposed signalling pathway for serotonin in *S. mansoni*. Serotonin is synthesized in serotonergic neurons and released into synaptic clefts where it binds to Sm5HTr on postsynaptic membranes. Activation of cAMP leads to release or excitatory neurotransmitters resulting in increased motility.

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