A Study of the Schistosoma mansoni Nervous System: Characterization of Catecholamine and Phenolamine Signaling

by

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List of Abbreviations

ATP: Adenosine Triphosphate

PZQ: Praziquantel

BA GPCR: Biogenic Amine G-Protein Coupled Receptor

SmGPR: *Schistosoma mansoni* G-Protein Coupled Receptor

LNC: Lateral Nerve Chord

VNC: Ventral Nerve Chord

DNC: Dorsal Nerve Chord

CNS: Central Nervous System

PNS: Peripheral Nervous System

Cerebral Ganglial: CG

5HT: Serotonin

OA: Octopamine

TA: Tyramine

AA: Amino Acid

HA: Histamine

EPN: Epininine

DA: Dopamine

SmTH: *Schistosoma mansoni* Tyrosine Hydroxylase

SmTBH: *Schistosoma mansoni* Tyramine Beta-Hydroxylase

cAMP: Cyclic Adenosine Monophosphate

IP3: Innositol Triphosphate

qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction

dsRNA: Double stranded Ribonucleic Acid

RNAi: Ribonucleic Acid Interference

Abstract

Schistosomiasis is a debilitating and painful chronic disease that affects the visceral organs and negatively affects the growth and development of school-aged children. The disease persists in regions of poverty in 78 countries in tropical and subtropical regions, and is associated with significant morbidity and mortality. Schistosomiasis is a "neglected" disease of poverty found in regions without access to clean freshwater. The causative agents of schistosomiasis, trematodes of the species *Schistosoma*, have a complex life cycle that includes a water-borne snail vector host and a definitive mammalian host, as well as parasitic and non-parasitic stages. Preceding the chronic stage of infection, an initial 4-6 week acute stage is associated with the migrating larvae, preceding their transformation to adult-stage parasites. The parasite evades the immune response throughout the commencment and establishment of infection, and it has been reported that infection can persist for up to 40 years. The migration of larvae and the persistence of infection in the host are governed by the interaction of schistosome neurotransmitters with their associated receptors, and neuromuscular control of parasite motility. To target the schistosome nervous system for drug development, it must be further studied, as relatively little is known about the parasite's neurobiology. An ideal treatment for schistosomiasis would specifically target the parasite without adversely affecting host biology. Thus, in the work described here, we identify phenolamine signaling in *Schistosoma mansoni*, which has previously been identified in virtually all invertebrate phyla, but was not known to be present in schistosomes. The identification of this signaling pathway in schistosomes is a landmark discovery in the field of schistosome research, as this signaling is absent in vertebrates, including humans. Octopamine (OA) signaling, therefore, represents a unique signaling pathway that can be specifically targeted to cure schistosomiasis. We used confocal immunolocalization to determine that the phenolamine OA is widespread in the peripheral and central nervous system of the parasite (PNS and CNS), and that it is found in the female reproductive structures, in larvae and in the developing schistosome embryo, demonstrating its importance to schistosome neurobiology. The localization of OA to peripheral neurons

innervating musculature suggests a role in neuromuscular control for OA. To verify a role in neuromuscular control, we performed motility studies of phenolamines and related catecholamines. We demonstrated concentration-dependent effects of OA, its precursor tyramine (TA) and its metabolite synephrine (SE) on stimulation of motility and lengthening of the parasite. We also confirm concentration-dependent effects on length and motility for the related catecholamine dopamine (DA) in schistosomula. Having measured effects on motility for these tyrosine derivatives, we tested synthetic compounds on schistosomula *in vitro*, and identified a putative antagonist as well as an agonist of OA-signaling in schistosomes, and determined concentration-dependent effects for a known DA antagonist. Next, to validate these effects, we used RNAi to downregulate expression of OA and DA-related signaling genes and measured changes in neuromuscular control. We observed changes in frequency of body movements and in length, posture and coordination in both larval and adult-stage parasites. Finally, we performed a preliminary characterization of two OA signaling proteins, the OA receptor Smp_150180 and the OA-synthesizing enzyme *S. mansoni* tyramine beta-hydroxylase (SmTBH, Smp_243830). Our unprecedented identification of phenolamine signaling in schistosomes provides new insight into the neurobiology of schistosomes. The characterization of phenolamine and catecholamine signaling in *S. mansoni* serves not only to highlight their importance to schistosome neurobiology, but also to identify these signaling pathways as drug targets for schistosomicide discovery and development to treat this debilitating disease.

Abrégé

La schistosomiase est une maladie chronique débilitante et douloureuse qui affecte les organes viscéraux et affecte négativement la croissance et le développement des enfants d'âge scolaire. La maladie persiste dans les régions de pauvreté et dans 78 pays de régions tropicales et subtropicales ; elle est associée à une morbidité et une mortalité significative. La schistosomiase est une maladie «négligée» de la pauvreté qui se rencontre dans les régions qui n'ont pas accès à l'eau potable propre. Les agents pathogènes de la schistosomiase, les trématodes de l'espèce *Schistosoma*, ont un cycle de vie complexe qui comprend un hôte vecteur, les escargots d'eau, et un hôte mammifère définitif, ainsi que des phases parasitaires et non parasitaires. Avant la phase chronique de l'infection, une phase aiguë initiale de 4-6 semaines est associée aux larves migrantes, avant leur transformation en parasites adultes. Le parasite élude la réponse immunitaire tout au long de l'établissement de l'infection. Il a été rapporté que l'infection peut persister jusqu'à 40 ans. La migration des larves et la persistance de l'infection chez l'hôte sont réglementés par l'interaction des neurotransmetteurs schistosomiques et leurs récepteurs associés, et du contrôle neuromusculaire de la motilité parasitaire. Le ciblage du système nerveux du schistosome à des fins thérapeutiques doit être davantage étudié, car la neurobiologie du parasite est peu connue. Un traitement idéal de la schistosomiase ciblerait spécifiquement le parasite sans nuire à la biologie de l'hôte. Nos travaux de recherche ont identifié une signalisation phénolamine chez *Schistosoma mansoni*, signalisation qui a été identifié dans pratiquement tous les «phyla» invertébrés, mais qu'on ne savait pas présent chez *Schistosoma*. L'identification de ce type de signalisation chez les schistosomes est une découverte importante dans le domaine, car cette signalisation est absente chez les vertébrés, y compris les humains. La signalisation OA représente donc un type de signalisation unique qui peut être spécifiquement ciblée pour guérir la schistosomiase. Nous avons utilisé l'immunolocalisation confocale pour déterminer que la phénolamine octopamine (OA) est répandue dans le système nerveux périphérique et central du parasite (SNP et SNC), et qu'elle se trouve dans les structures reproductrices femelles, les larves et l'embryon du

schistosome ; ce qui démontre son importance en neurobiologie des schistosomes. La localisation de l'OA sur les neurones périphériques innervant la musculature suggère un rôle dans le contrôle neuromusculaire de l'OA. Pour valider cette hypothèse, nous avons effectué des études de motilité des phénolamines et des catécholamines qui sont apparentées. Nous avons démontré des effets dépendant à la concentration de l'OA, de son précurseur tyramine (TA) et de son métabolite synéphrine (SE) sur la stimulation de la motilité et l'allongement du parasite. Nous confirmons également des effets dépendant à la concentration sur la longueur et la motilité de la catécholamine dopamine (DA) des schistosomulums. Après avoir mesuré les effets sur la motilité de ces dérivés de tyrosine, nous avons testé des composés synthétiques sur des schistosomules *in vitro,* et identifié un antagoniste putatif ainsi qu'un agoniste de la signalisation OA. Nous avons également déterminé des effets dépendant à la concentration par un antagoniste DA connu. Ensuite, pour valider ces interactions, nous avons utilisé l'ARNi pour réguler négativement l'expression des gènes de signalisation liés à l'OA et à la DA et mesuré les changements du contrôle neuromusculaire. Nous avons observé des changements dans la fréquence des mouvements du corps et dans la longueur, la posture et la coordination chez les larves et les parasites adultes. Enfin, nous avons effectué une caractérisation préliminaire de deux protéines de signalisation OA, le récepteur Smp_150180 et l'enzyme de synthèse *S. mansoni* tyramine bêta-hydroxylase (SmTBH). Notre identification sans précédent de la signalisation phénolamine chez *Schistosoma* représente une nouvelle découverte en neurobiologie des schistosomes. La caractérisation de la phénolamine et de la signalisation de la catécholamine chez *S. mansoni* permet non seulement de mettre en évidence leur importance à la neurobiologie des schistosomes, mais aussi d'identifier ces types de signalisation comme cibles thérapeutiques potentielles au développement d’un schistosomicide afin d’enrayer cette maladie débilitante.

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Thesis Office Statement

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The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rational and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.

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 judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent**; supervisors must attest to the accuracy of such claims at the Ph.D. Oral defense. Since the task of examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

Statement of Contributions

All experimental work presented in this thesis was performed by the author under the supervision of Dr. Paula Ribeiro. Dr. Ribeiro was also involved in the experimental design, presentation of the data, and in the pre-editing of the thesis and manuscripts, and provided funding for the research described. The phenotypic screen of schistosomula, described in the first manuscript, was performed under the direction of Dr. Ribeiro and Dr. Conor R. Caffrey. Schistosomula used in the pharmacological screen were prepared by Brian M. Suzuki at the University of California, San Francisco. Steven Chen and Dr. Michelle Arkin designed the imaging setup at the University of California, San Francisco, which was used to obtain images from the screen. These images were converted to videos using ImageMagick with the assistance of Steven Chen. Dr. Mostafa Zamanian identified the homologues in *Dugesia tigrina* of *Schistosoma mansoni* proteins which were cloned by the author.

Statement of Originality

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

Chapter II

In this study, we identified the invertebrate neurotransmitter octopamine (OA) in the *Schistosoma mansoni* nervous system and determined its effects, as well as those of structurally related compounds, on length and motility. Confocal immunolocalization identified OA in the parasitic life stages of *S. mansoni* in both the central and peripheral nervous systems (CNS and PNS). The presence of OA in neuronal tissue was confirmed by the use of an antibody against the neuronal marker synapsin. Localization of OA to the peripheral nerve net, which typically innervates body-wall muscle, suggests a neuromuscular role for OA. This identification of OA in the nervous system represents the first time this neurotransmitter has been identified in schistosomes. To verify a possible role for OA in muscle control, schistosomula were incubated with OA and related biogenic amines *in vitro*, and changes in length and motility were quantified and determined to be concentration-dependent. Together, these data identify OA in schistosomes for the first time and suggest a role for OA and related tyrosine derivatives in motor control.

Chapter III

In the second manuscript, we used bioinformatics analysis to identify genes involved in tyrosine derivative signaling, with a focus on OA-related signaling genes. We targeted known and putative tyrosine derivative signaling genes via Ribonucleic Acid interference (RNAi) to determine effects on neuromuscular control. We downregulated the expression of 8 genes in both larval and adult stage parasites and quantified effects on motility, length, posture and coordination. We observed significant changes in motor control for several of these genes, including *Schistosoma mansoni* tyramine beta-hydroxylase (SmTBH) which putatively synthesizes OA, and the putative OA receptor Smp_150180. This RNAi screen represents a thorough

investigation of phenotypes associated with tyrosine derivative-related signaling genes, highlights the importance of these signaling genes in schistosome motor control and identifies the proteins encoded by these genes as targets for development of schistosomicides.

Chapter IV

In chapter IV, we cloned cDNAs encoding 6 full-length BA GPCRs from *S. mansoni*, which can potentially be de-orphanized to identify an OA-signaling receptor. We also cloned cDNAs encoding 3 proteins from the free-living flatworm *Dugesia tigrina*, which are homologues of tyrosine derivative signaling genes targeted via RNAi in schistosomes in the first manuscript of this thesis. The cloned homologues in *D. tigrina* will be targeted by RNAi to determine whether this organism can serve as a model in the study of schistosomes. Next, we heterologously expressed one of the cloned BA GPCRs, Smp_150180, in mammalian cells and determined that OA can signal through this receptor, as opposed to other tyrosine derivatives. Finally, we cloned and recombinantly expressed SmTBH in bacterial cells and determined that it can synthesize OA from its precursor tyramine (TA). This finding is the first indication that schistosomes can synthesize OA endogenously. While the work detailed in this chapter represents preliminary data, these experiments highlight SmTBH and Smp_150180 as putative drug targets.

Introduction

Rationale

Schistosoma mansoni is one of the main causative agents of schistosomiasis, also known as bilharzia, Katayama fever and snail fever. Schistosomiasis, discovered in 1853 (Bilharz, 1853), is a disease that affects over 250 million people worldwide (Fenwick et al., 2003). More than 800 million people are estimated to be at risk of contracting infection. *S. mansoni* is a digenetic (having both male and female forms) trematode (flatworm). Infection comprises an acute and chronic stage. The acute stage lasts 4-6 weeks and coincides with the larval schistosomula stage migrating through the skin and blood vessels. The chronic stage corresponds to egg production by adult parasites.

One aspect of schistosome biology that holds much potential for drug targeting is the schistosomal nervous system. The nervous system is central to worm survival because it is involved in virtually all aspects of worm function, including migration, feeding, maturation, egg-laying and host penetration. This marked dependence of the worm on the nervous system is, in part, due to its lack of an endocrine system. Consequently, most schistosome signaling functions are performed via its neuronal system.

Biogenic amines (BAs) are the largest class of small classical neurotransmitters and play a central role in the control of parasite motility (Pax et al., 1984; Day et al., 1994). BAs can be obtained from the human host by the parasite via BA-specific transporters in the parasite tegument, or they can be synthesized endogenously via BA synthesis enzymes. BAs typically signal through G protein-coupled receptors (GPCRs). The *S. mansoni* genome has been sequenced and annotated (Protasio et al., 2012, Berriman et al., 2009) and proteins identified *in silico* have been predicted or determined to be involved in transport, synthesis or signaling of schistosome BAs (Ribeiro et al., 2012). BA GPCRs share little homology with human orthologs, marking the potential of BA signaling proteins as anti-schistosomal drug targets. Here, we explore the role of BAs, particularly invertebrate-specific phenolamines and the structurally-related catecholamines, and their respective signaling proteins in *S. mansoni.* These BAs, which

are derived from tyrosine, have received little attention, but we show here that they play an important role in schistosome biology, particularly schistosome motor function. We focus on identification and functional studies of BAs of interest, including dopamine (DA) and octopamine (OA), along with their respective signaling receptors and biosynthetic enzymes. Obtaining a better understanding of the role of BAs in the parasite will provide important information on the schistosome nervous system and give insight into which of these pathways are most vital to parasite survival and would serve as ideal targets for drug design.

Central hypotheses

Bioinformatics analyses (Ribeiro et al., 2012; Protasio et al., 2012) suggest that phenolamine signaling is present in *S. mansoni*. The central hypotheses of this thesis are:

1) The phenolamine OA is present in *S. mansoni.*

2) OA and structurally-related tyrosine derivatives, phenolamines and catecholamines, play an important role in schistosome motor control.

(3) Antagonists and/or agonists of tyrosine derivative signaling can interfere with schistosome neurobiology.

Objectives

The aims of this thesis were to (1) detect OA in *S. mansoni* using confocal immunolocalization and (2) to determine the role of OA and related tyrosine derivatives in motor control of schistosomes using pharmacological studies, reverse genetics and functional expression studies. The specific objectives of this thesis were:

- Detect OA in *S. mansoni*.
- Determine the effects of OA and related tyrosine derivatives on schistosomes.
- Identify antagonists and/or agonists of tyrosine derivative signaling in *S. mansoni*.

- Determine if tyrosine derivative signaling pathways are involved in motor control of schistosomes.
- Clone and characterize cDNAs encoding proteins involved in tyrosine derivative signaling in *S. mansoni* to verify a role in phenolamine signaling.
- Clone cDNAs encoding proteins involved in tyrosine derivative signaling in *Dugesia tigrina*, a potential flatworm model organism.

We used techniques in immunolocalization, RNAi (ribonucleic acid interference), heterologous cell expression and recombinant expression as well as *in vitro* drug screens on parasites in culture. Using these techniques, we gained insight into the function of these tyrosine derivatives and their associated proteins in schistosomes at the molecular level and in the context of the whole organism.

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CHAPTER I: LITERATURE REVIEW

1 Schistosoma mansoni

1.1 Epidemiology

The digenetic trematode parasite *Schistosoma mansoni* is one of the main infectious agents causing schistosomiasis (also called "snail fever" or Bilharzia). Approximately 250 million people are infected with the parasite (Fenwick et al., 2003; Knudsen et al., 2005; Berriman et al., 2009; Taman and Ribeiro, 2009; WHO, Fact Sheet No. 115), and more than 800 million people are at risk of contracting infection; > 280,000 schistosomiasis-caused deaths occur per annum (Steinmann et al., 2006). Other schistosome species that infect humans are *S. haematobium, S. japonicum, S. mekongi and S. intercalatum*. *S. mansoni* infections occur in Africa, South America, the Caribbean, and the Middle East. Schistosomiasis is a major health concern in more than 50 countries and is the leading cause of hepatic fibrosis in the world (Doenhoff et al., 2008).

Schistosomiasis transmission has been reported in 78 countries and schistosomiasis is deemed endemic in 52 countries with moderate to high transmission rates, signaling the need for preventative chemotherapy (WHO, Fact Sheet No. 115). The WHO reports that in 2014, more than 250 million people were in need of prophylactic treatment (WHO, Fact Sheet No. 115). That year, 61.6 million people were treated for schistosomiasis (WHO, Fact Sheet No. 115).

Chronic schistosomiasis is debilitating, particularly in rural areas of poverty in the developing world. Schistosomiasis infection is linked to disability and can perpetuate poverty (King and Dangerfield-Cha, 2008) in infected individuals. In the developing world, neglected tropical diseases such as schistosomiasis present a constant risk of infection. Schistosomiasis can persist over the course of a lifetime (Ukwandu and Nmorsi, 2004; de Vlas et al., 2004; Danso-Appiah et al., 2004; King and Dangerfield-Cha, 2008). Infection is often compounded with other parasitic infections in endemic areas, and contributes to anemia and malnutrition in infected individuals (King et al., 2005; King and Dangerfield-Cha, 2008).

In endemic areas, symptoms of infection often go unnoticed, as symptoms common to these areas are mistaken for the norm (King and Dangerfield-Cha, 2008). Detection limits due to the level of sensitivity of detection methods mean that infection can be missed (Katz et al., 1972; Carneiro et al., 2012), leading to underestimation of infection (Ebrahim et al., 1997; Alarcon et al., 1999; Wilson et al., 2006).

1.2 The Life Cycle and Ultrastructure of *S. mansoni*

S. mansoni eggs are released into the water with the feces of the human definitive host. Miracidia are surrounded by a mucoid substance, encased in the shell of the egg (Watts and Boyd, 1950). This juvenile form of the parasite is shaped like an ellipse, pointed at its ends, has few germ cells in the lower portion of its body and, in its anterior, papillae, including cephalic and lateral papillae, from which it emits secretions (Watts and Boyd, 1950). Miracidia have a nerve center and a simple gut. The oral opening of the gut is located at its anterior end (Watts and Boyd, 1950). Once hatched in water, due to the decrease in salt concentration and exposure to light, miracidia swim rapidly with their many cilia (Watts and Boyd, 1950). The miracidial form of the parasite has similar dimensions to the egg, measuring approximately 165 x 65 µm. Eggs typically measure approximately 145 x 55 µm (Faust and Meleney, 1924; Watts and Boyd, 1950). Once released into water, miracidia infect the intermediate snail host, *Biomphalaria glabrata* (Boissier et al., 1999), subsequently transforming into a mother sporocyst. The mother sporocyst reproduces asexually, producing several daughter sporocysts, which develop into thousands of cercariae that are released into water (Wesenberg-Lund, 1934).

Cercariae, composed of a body and forked tail, are the infective stage of *S. mansoni*. Once released from the snail host following exposure to light, cercariae swim until they find a host (Samuelson and Caulfield, 1985). Arginine and ceramides released from host skin serve as chemo-attractants and facilitate infection (Haas et al., 2002; Haas et al., 2008). Cercariae measure approximately 120 x 25 µm (Samuelson and Caulfield, 1985) and contain a digestive,

excretory and nervous system, as well as a pair of secretory glands, all within two layers of muscle (Stirewalt, 1974; Samuelson and Caulfield, 1982, 1985). The cercarial tegument is a syncytium composed of a single unit-size membrane with a single phospholipid bilayer at its surface, which connects to cell bodies beneath the muscle (Samuelson, and Caulfield. 1982, 1985).

Cercariae first attach to the host via their highly muscularized and innervated ventral sucker, or acetabulum (Cousin et al., 1995). This ventral sucker surrounds the acetabular glands and is surrounded by both circular and longitudinal muscle types (Cousin et al., 1995). This highly innervated structure has sensory papillae at the surface tegument, which are highly innervated by dendritic nerve fibers (Cousin et al., 1995) and point to a tactosensory function in cercariae. The tegument morphology of cercariae differs slightly from that of adults and contains spines and granules (Cousin et al., 1995).

Three pairs of postacetabular glands are located posterior to the acetabulum and play a role in cercarial attachment (Lewert et al., 1966). Postacetabular gland secretions are enzyme-stimulating, promote host adhesion and protect the parasite (Lewert et al., 1966). Preacetabular glands are said to have proteolytic activity and aid in host penetration (Stirewalt, 1959; Gordon and Griffith, 1951; Cousin et al., 1995). Cercariae penetrate the skin of the human host and shed the tail to become schistosomula (Samuelson and Caulfield, 1985; Knudsen et al., 2005). The change from a cercaria to a schistosomulum produces a life stage that is morphologically distinct from the cercaria and from the adult stage. When schistosomula penetrate the host skin, they release mucoid, enzyme and amino-acid-containing secretions and shed the majority of their outer glycocalyx layer (Stirewalt, 1963; Kemp, 1970), while forming microvilli on their surface (Samuelson and Caulfield, 1985). The glycolax is acidic, immunogenic and composed of a dense mesh-like material made up of 15-30 nm thick fibrils which cover the underlying spines (Samuelson and Caulfield, 1985).

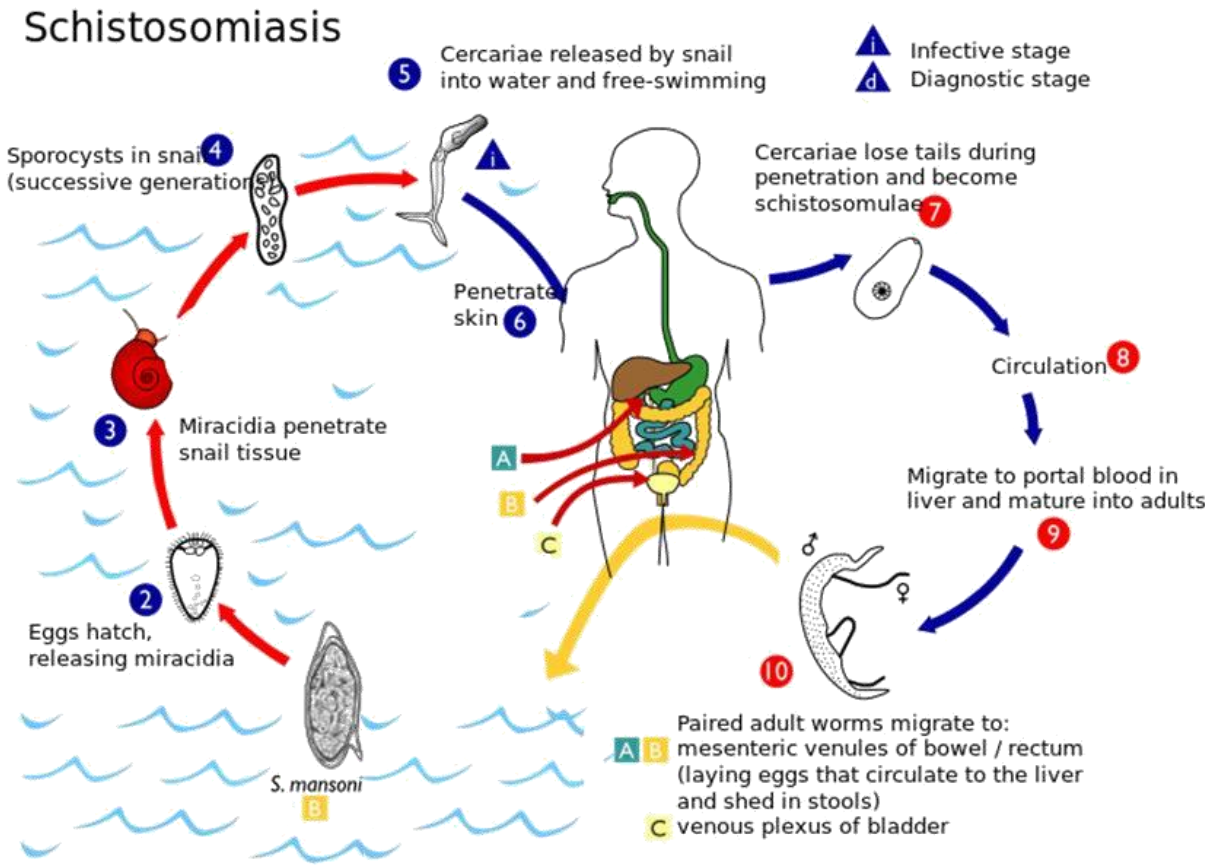
The process of penetration of host skin by schistosomula takes up to 3 days. During the first 4 hours of infection, schistosomula migrate through the epidermis of the skin, and remain

in the outermost layers (Yi-Xun et al., 2002). By 8 hours from the start of infection, shistosomulae reach the deeper layers of the epidermis and lie horizontal to the basal lamina of the skin (Yi-Xun et al., 2002). At 12-16 hours following commencement of infection, schistosomula begin to penetrate the skin basement membrane (Yi-Xun et al., 2002). At the 24 hour time-point, schistosomula begin to penetrate the dermis, and by 48 hours, they typically travel deeper into the dermis for up to 24 hours and reach the blood vessels of the dermis 72 hours following the start of infection (Yi-Xun et al., 2002).

The schistosomulum makes its way through the blood to the hepatoportal system where it matures into the adult stage (LoVerde, 1998). The tegument of the adult worm contains tetraspanins, which are distantly related to host tetraspanin. Schistosome Tetraspanin-2 has been designated a potential vaccine target, due to its membrane expression and relatively low homology to the host protein (Schulte et al., 2013).

Adult male and female schistosomes couple and migrate together to the mesenteric venules, against blood flow (Schramm and Haas, 2010) where the female releases approximately 300 eggs per day (Moore and Sandground, 1956). Coupling of male and female adult schistosomes is required for female development as well as maintenance of its maturity (Popiel et al., 1984; Basch, 1990; Ribeiro-Paes and Rodrigues, 1997). The thinner female sits in the larger male's gynaecophoric canal, at the male ventral side (Southgate et al., 1998; Mair et al., 2000; Morand and Muller-Graf, 2000; Neves et al., 2005). Adults not coupled, as is the case with single-sex infections, produce smaller, immature worms (Khalil and Mansour, 1995; Kunz, 2001). Although half of the eggs layed by the female are released with the feces, the other half typically becomes lodged in host tissue. These eggs are the primary cause of host pathology, because they release antigen and result in the formation of granulomas and fibrous tissue. A quarter of the eggs produced are transported to liver sinusoides via the blood stream and cause granulomas in the liver (Dunne and Doenhoff, 1983; Schramm and Haas, 2010). Pathologies that occur in a schistosomiasis infection include hypertension, colon obstruction, hepatospleenomegaly, hepatic fibrosis, ascites, esophageal varices and gastrointestinal tract

tearing (Warren, 1978; Coutinho, 2004). It has been suggested that schistosomes can live in the human host for up to 40 years.



Modified from: http://www.cdc.gov/parasites/schistosomiasis/biology.html

Figure 1. The life cycle of *S. mansoni*. Eggs are released in feces of the human host into freshwater, and hatched miracidia infect *Biomphalaria glabrata* snail intermediate hosts. Cercariae are released into water and infect the definitive human host. Schistosomula complete the migration in the human host in 6-7 weeks post infection, migrate to the portal vein, develop into the male and female adult stages of the parasite, and subsequently migrate to the mesenteric venules (B), where females lay approximately 300 eggs per day, which is the primary cause of host pathology.

1.3 The Male and Female *S. mansoni* Reproductive Systems

Female schistosomes have pluricellular vitelline glands, which produce the egg shell, and resemble a cluster of grapes, each having an irregularly-sized nucleus (Neves et al., 2005; Erasmus, 1973). The ovary is composed of oocytes, hexagonal germ cells, in cord-shaped cell bundles, each cell having a central nucleus and each at various stages of maturation. As cells in the ovary mature, they "unbundle" and detach (Neves et al., 2005). Oocytes are fertilized following their release from the ovary and pass through the oviduct until they reach the ootype where the egg shell is formed (Neves et al., 2005). Typically, a single zygote with a completely formed egg shell occupies the uterus at a time. The uterus joins to the seminal receptacle, with already-stored sperm (Neves et al., 2005).

Following egg-laying, embryos contained within the egg shell undergoe mitosis and pass through 8 stages of embryogenesis (Jurberg et al., 2009). As the embryo matures, it occupies a larger portion of the egg, grows and elongates. In stage 5 embryos, organogenesis begins and the neural mass, the brain, is formed. Organ formation continues through stages 5 to 7, and the neurons of the neural mass undergoe condensation in stage 7 (Jurberg et al., 2009). Stage 8 represents the final stage of embryogenesis, at which point the embryo reaches maturity and the developed miracidium subsequently hatches. Mature embryos are associated with muscle contraction, cilia and the beating of flame-cells.

In male worms, the testicular lobes are located at the anterior of the body and are filled withspermatozoa and spermatid, at various stages of development (Otubanjo, 1980). Each testicular lobe is arranged linearly and from each lobe extends a vas efferens, which joins the lobe to a neighboring lobe and by which the testes connect to the vas deferens. The vas deferens joins to the seminal vesicle, a wider, spherical portion of the duct (Otubanjo, 1980). The seminal vesicle is a storage site for sperm. The duct ultimately connects to the cirrus, positioned directly below the ventral sucker, which releases sperm (Otubanjo, 1980).

1.4 Immune Effects of Schistosomiasis Infection

It is thought that the best stage to clear a schistosome infection from the host is the larval, pre-adult stage (Wilson, 1987). Once the adult stage is established, the worms persist, and are not easily cleared by the host immune system (Agnew et al., 1993). A successful vaccine against schistosomiasis has yet to be developed, and it is thought that schistosome evasion of the immune system is a barrier to vaccine development (Terry and Smithers, 1975; Kolata, 1985; Damian, 1997). The different stages of the parasite have developed mechanisms to evade the host immune response.

Proteases, including elastase, secreted from the acetabular glands of the cercariae during penetration of host skin, facilitate quick infection of the host, and evasion of the immune system (Hockley and McLaren, 1973; McLaren and Hockley, 1977; Whitfield et al., 2003).

Schistosomula up-regulate Fas and FasL expression in CD4+ and CD8+ T-lymphocytes to promote T-cell apoptosis (Chen et al., 2002, Carneiro-Santos et al., 2000; Estaquier et al., 1997; Fallon et al., 1998; DosReis and Barcinski, 2001; Remoue et al., 2001) and desensitize T-cells to schistosome excretory-secretory (ES) products (Vieira et al., 1986; Colley et al., 1977; Colley et al., 1979; Estaquier et al., 1997; Fallon et al., 1998; Kumar and Ramaswamy, 1999; Carneiro-Santos et al., 2000). ES products also cause regulatory cytokine IL-10 production (Ramaswamy et al., 2000; Yi-Xun et al., 2002), production of IL-1-ra, which dampens IL -1β, an inflammatory cytokine (Ramaswamy et al., 1995) as well as a significant decrease in inflammatory cytokine IL-1α production (Yi-Xun et al., 2002), consistent with an anti-inflammatory and immunomodulatory role of schistosomula on host skin.

Adult schistosomes also express, on their tegument, proteins or receptors that bind to components from the host and mask the parasite from the immune response, including transforming growth factor beta (TGFbeta) (Davies et al., 1998; Forrester et al., 2004) and IgG (Rasmussen and Kemp, 1987).

Immune effects of the schistosome-infected host largely correspond to acute stage infection, the clinical stage of schistosomiasis (Ottensen et al., 1978; Nash et al., 1983; Malaquias et al., 1997).

The shift to a TH2 (T-helper cell 2) response is observed at 6 weeks following infection by cercariae, when the female adult parasite produces eggs, typically 300 per day (Grzych et al., 1991; Pearce et al., 1991; Wynn et al., 1993; Araujo et al., 1996; Pearce and MacDonald, 2002; Forrester and Pearce, 2006). This immune shift is accompanied by an inflammation of the liver and intestine at the site of egg release, where eggs become lodged in host tissue (Grzych et al., 1991; Pearce and MacDonald, 2002). Severe morbidity typically develops in 10% of infections (Kheir et al., 1999; Forrester and Pearce, 2006).

The passage of eggs through the endothelial barrier results in eosinophilia, granuloma formation, IgE production, consistent with a TH2 response (Doenhoff, 1997; Pearce and MacDonald, 2002; Wilson et al., 2006). Granulomas associated with high egg loads, can lead to formation of fibrotic tissue leading to pathologies such as hepatospleenomegaly and ascites (Pearce and Macdonald, 2002; Schramm and Haas, 2010).

It is believed that the shift from a primarily TH1- to TH2-mediated immune response, which corresponds with the start of egg production and granulomatous tissue formation at 6 weeks following infection (Stadecker et al., 1990; de Andres et al., 1997; Kaplan et al., 1998; Rumbley and Phillips; 1999; Fallon et al., 2000; Rumbley et al., 2001) is largely due to schistosome egg-caused TH1-mediated cell death (Estaquier et al., 1997; Fallon et al., 1998; Rumbley et al., 1998). Chronic schistosomiasis usually occurs with the formation of the complete granuloma, at 8 weeks following infection (Pearce et al., 1991; Boros and Lucaks, 1992; Boros, 1999; Lundy et al., 2001), and the associated release of interleukins IL-3, IL4, as well as IgE, producing a TH2-type inflammatory response (Pearce and MacDonald, 2002; Pearce et al., 2004; Schramm and Haas, 2010).

1.5 Schistosomiasis Treatment

There is currently mainly only one drug, praziquantel (PZQ), used in treatment of schistosomiasis, which has been in use for the past 35 years (Fenwick et al., 2003; Hagan et al., 2004). Other anti-schistosomals include oxamniquine, which can elicit resistance in schistosomes (Rogers and Bueding, 1971; Cioli et al., 1993), and mefloquine, which is used primarily in treatment of malaria (Keiser et al., 2009; Manneck et al., 2011; Amany et al., 2014; Fahmy et al., 2014). Since the 1970s, praziquantel (PZQ) has been the mainstay of schistosomiasis treatment (Andrews et al., 1983; Kumari and Allan, 2005; Abdul-Ghani et al., 2009; Cioli et al., 2012). While PZQ is effective against all schistosome species, the drug has its limitations (Cioli et al., 2012). While the drug is effective against the adult worm, it is ineffective against the immature juvenile stage (Xiao et al., 1985; Sabah et al., 1986). PZQ is most effective at 6-7 weeks post infection, when the parasite develops into the adult stage. PZQ is administered at 40mg/kg of body weight (Kumari and Allan, 2005). Following administration of the first dose of PZQ, 60-90% of schistosomal infections are typically cured (Kumari and Allan, 2005). If the infection is not resolved following administration of the first treatment, a second dose is administered 6-8 weeks later, to target any newly developed adult worms, which had not reached maturity at the time of the first treatment (Cioli, 2000).

Large-scale treatments with PZQ are used as a control measure for schistosomiasis (World Health Organization (WHO) Fact Sheet No. 115). The fact that a single drug compound is used in treatment of a disease, which infects more than 250 million people worldwide raises concerns for the development of resistance (Chitsulo et al., 2000; Doehnoff et al., 2008; Melman et al., 2009). Given the widespread use of the drug and the lack of an available alternative, there are concerns for the development of resistance (Doenhoff and Mattoccia, 2006; Melman et al., 2009; Doenhoff et al., 2008). While resistance to PZQ at the population level has not, to date, been reported (Cioli et al., 2012), there have been incidences of schistosomiasis reported, which did not respond to treatment (Cioli, 2000; Cioli et al., 2012). In the laboratory, it is possible to obtain a strain with decreased PZQ sensitivity (Cioli et al., 2012)

and to select for a resistant strain (Cioli, 2000). These reports suggest resistance is indeed possible and it is therefore important to seek out drug targets for treatment against the parasite.

The mechanism of action of PZQ is believed to be caused by binding to the beta-subunit of Ca++ channels, causing an influx of Ca++, leading to loss of muscle control, flacid paralysis in the parasite and allowing for elimination of the parasite from the host (Greenberg, 2005). PZQ has also been associated with inhibition of the influx of Ca++ into neuronal cells via activation of the alpha subunit (Cupit and Cunningham, 2015). Recently, it was shown that treatment of immature worms with cytocholasin-D, which causes an influx of Ca++, did not kill the parasite, indicating that PZQ may have another mode of action, which is not limited to the influx of Ca++ associated with its affect on Ca++ channels (Cioli et al., 2012). PZQ has also been associated with vacuolation, blebbing and damage of tegumental and subtegumental structures in adult parasites (William et al., 2001). This damage is associated with exposing surface antigens, allowing recognition of these sites and clearance of the parasite by the host immune system (Xiao et al., 2009; Cupit and Cunningham, 2015). Reduced susceptibility to PZQ has been associated with an increase in p-glycoprotein expression, which is associated with efflux of PZQ from the cell (Messerli et al., 2009). A recent study found that in planarians, a free-living flatworm, PZQ affected the flatworm's regenerative polarity, a result that was also Ca++-dependent and supports the notion of a Ca++-dependent mechanism of PZQ action, but does not rule out other potential mechanisms and a broader spectrum of activity (Chan et al., 2014). In planarians, PZQ has also been associated with a decrease in the influx of Ca++ into neuronal cells, mediated through activation of the Ca_v1A subunit of Ca++ channels (Zhang et al., 2011; Cupit and Cunningham, 2015). This effect of PZQ suggests that its mechanism of action may, in part, be mediated through the nervous system.

2 The Schistosomal Nervous System

2.1 The Importance of the Nervous System to Schistosome Biology

The schistosomal nervous system is central to the survival of the worm because it is responsible for migration, feeding, maturation, egg-laying, as well as host penetration. This marked dependence of the worm on the neuronal system is due in part to the lack of an endocrine system. Consequently, most schistosome signaling functions are performed via the nervous system.

The organization of the schistosome nervous system has primarily been determined by techniques in immunocytochemistry (Gustafsson, 1987, Collins et al., 2011,) and in part by electrophysiology (Fetterer et al., 1977). The understanding of the schistosome nervous system is largely based on what has been described in other flatworms (Cousin and Dorsey, 1990, Halton and Maule, 2004) and the immunohistochemical mapping of the nervous system in the cercaria stage of the parasite (Collins et al., 2011).

2.2 Organization of the Schistosome Nervous System

Schistosomes have a diverse set of neurotransmitters and receptors. Most of these receptors were found to have little or no sequence homology with human receptors (El-Shehabi and Ribeiro, 2010; El-Shehabi et al., 2012; Ribeiro et al., 2012), making them ideal drug targets. Being an acoelomate, schistosomes lack a body cavity and therefore, also lack an endocrine system (Ribeiro and Geary, 2010). The majority of the worm's signaling functions are mediated by the nervous system. The importance of this system in the parasite, as well as the success of drug compounds targeting the nervous system in other helminths, namely nematodes, and pesticides, targeting the invertebrate nervous system (Hollingworth and Murdoch, 1980; Gole et al., 1983; Martin., 1992; Raymond and Sattelle, 2002; Bloomquist, 2003; Keifer and Firestone, 2007; Boulin et al., 2008; Ribeiro and Geary, 2010; Wolstenholme, 2011; Blenau et al., 2012), highlight its potential as a drug target for schistosomiasis treatment.

The schistosome central nervous system (CNS) is made up of the brain, located in the head region of the parasite, as well as paired longitudinal nerve cords, cross-linked with transverse commissures along the length of the worm (Hyman, 1951, Halton and Maule, 2004; Collins et al., 2011). The peripheral nervous system (PNS) is made up of a set of smaller nerve cords and plexuses, which are outsourced to all the major bodily structures of the worm (Halton and Gustaffson, 1996; Halton, 2004; Halton and Maule, 2004). Such structures include the somatic musculature, the tegument, the oral and ventral suckers, the reproductive organs, and the alimentary tract (**Figure 1**) (Halton and Gustaffson, 1996; Halton, 2004; Halton and Maule, 2004; Ribeiro and Geary, 2010).

The brain is composed of cerebral ganglia, a bi-lobed structure made up of a dense neuropile consisting of axons and dendrites, joined by a ring commissure (Halton and Maule, 2004). In platyhelminths, the CNS connects to a peripheral nervous system (PNS) made up of fine nerve fibres and nerve plexuses.

The oral and ventral (acetabulum) suckers of the adult parasite are highly innervated, likely due to their roles in feeding and attachment. The nervous system controls the muscles of the suckers and allow for efferent sensory function of nerve endings at the sucker surface. Cerebral ganglia are positioned above the acetabulum, below the anterior muscle, overlapping with the oesophagus, the ducts of the acetabulum, near the bifid cecum in cercariae (Cousin and Dorsey, 1991).

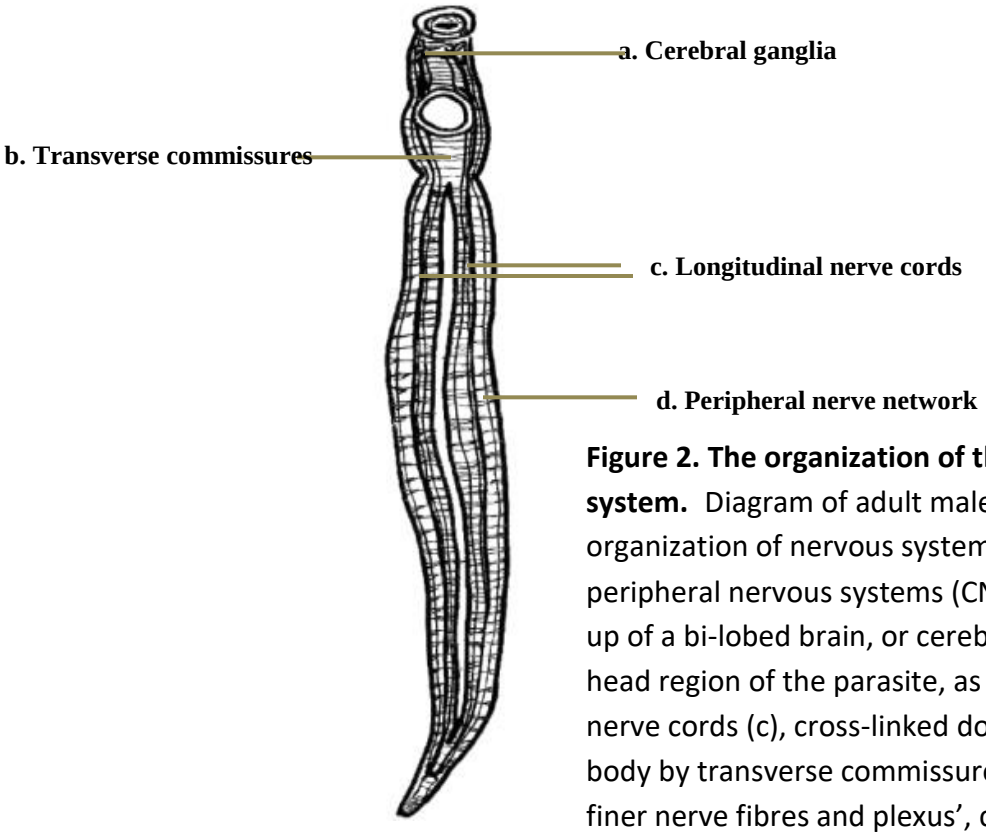


Figure 2. The organization of the schistosome nervous system. Diagram of adult male *S. mansoni*, demonstrating organization of nervous system, composed of central and peripheral nervous systems (CNS and PNS). The CNS is made up of a bi-lobed brain, or cerebral ganglia (a), located in the head region of the parasite, as well as paired longitudinal nerve cords (c), cross-linked down the length of the worm body by transverse commissures (b). The PNS is made up of finer nerve fibres and plexus', closely associated with the CNS, typically innervating organs and muscle (d).

The highly coordinated movement of the cercarial tail is required for swimming and host location for infection (Mair et al., 2003) and has its own unique neuromuscular innervation. The tail has a ventral and dorsal nerve trunk (Cousin and Dorsey, 1991, Pearson, 1961, Nutman, 1974). The CNS is composed of 12 evenly spaced longitudinal nerve cords (Cousin and Dorsey, 1991), which extend from the cerebral ganglia with pairs of dorsal, ventral and lateral nerve cords extending anteriorly and posteriorly from each lobe. The anterior cords extend to the head region of the animal, joining in a single neuropile or ganglion. Few nerve fibers extend across the body-tail junction (Cousin and Dorsey, 1991).

Invertebrates are said to lack, or have incompletely formed, dendritic spines, the membranous protusions on dendrites, at pre-synaptic terminals, which are common storage sites for synaptic

vesicles (Giachello et al., 2012). A common feature of invertebrate neurons are varicosities, which are also found in vertebrate neurons. Varicosities are protrusions on neurons, which vary in organelle content, varying in density, mitochondria and endoplasmic reticulum content and participate in synaptogenesis and synapses (Ahmari et al., 2000; Giachello et al., 2012). Varicosities are said to develop from growth cones, which have come into contact with a post-synaptic terminals or long axons (Giachello et al., 2012). These varicosities can also form by the splitting of pre-existing varicosities, or following neuronal advancement (Giachello et al., 2012). Synapsin is believed to have a role in varicosity formation. The formation of varicosities is preceded by a clustering of synaptic vesicles at discrete regions along the neuron, from which varicosities forms. Varicosities allow a single neuron to form synaptic connections with more than one neuron and increases the signaling capacity of neurons (Giachello et al., 2012).

2.3. Neuron Ultrastructure

Flatworm neurons are unmyelinated and are rich in mitochondria, vesicles and granules, as described by Dixon and Mercer (1965) in the trematode *Fasciola hepatica*. Perikarya, neuronal cell bodies, were determined to surround the neuronal cell bodies. Surrounding the perikarya nucleus, the neuronal cytoplasm contains endoplasmic reticulum (ER), mitochondria, RNA particles and Type I and II vesicles, identified based on their electron density and size (Dixon and Mercer, 1965).

In schistosomes, neuromuscular junctions and synapses are similar to what is observed in other animals, where these is a close proximity of plasma membranes at these sites, with an asymetric distribution of vesicles (Cousin and Dorsey, 1991). Cell bodies of the central ganglia in cercariae are considered to be irregularly shaped, having heterochromatic nuclei and less cytoplasm than those of other neuronal cell bodies. The nerve fibers of the neuropile of the ganglion are unsheathed, filamentous, with mitochondria and microtubules and are vesicle-rich (Cousin and Dorsey, 1991).

Nerve fibers in cercariae have various organizations. Synaptic sharing can occur, where two nerve fibers can share a presynaptic terminal. A single nerve fiber may participate in synaptic sharing with more than one nerve fiber resulting in the formation of a synaptic network. Alternatively, more than one synapse may branch off a nerve fiber at a discrete site on the fiber or the nerve fibers may be aligned in parallel, where only one of the membranes contains the dense material along the inner side of the nerve fiber, indicating a synapse between parallel nerve fibers. This organization is reflective of a complex and highly branched neuronal network.

2.4 Synapsin in Schistosomes and other Invertebrates

Synapsin is used as a neuronal marker in this study and therefore I will provide a brief overview of how synapsin functions in the nervous system. Synapsin plays a role in tethering synaptic vesicles in a pre-synaptic nerve terminal (Bloom et al, 2003) and was one of the first actin-binding neuronal proteins to be identified (De Camilli et al, 1983). Synapsin is enriched in the nervous system at the surface of synaptic vesicles (De Camilli et al., 1983; Greengard, 1987; Ueda and Benfenati et al., 1990) at sites where there is a reserve pool of synaptic vesicles (Leitinger et al., 2004) and where synapses occur. Release of these synaptic vesicles is calcium-dependent and follows phosphorylation of synaptic proteins with a subsequent mobilization of synaptic vesicles (Chi et al., 2001). Synapsin dissociates from reserve pools of synaptic vesicles following tetanic stimulation, a sustained, high frequency firing of action potentials (tetanus), usually resulting in a synapse, and depletion of the synaptic vesicle reserve pool (Greengard et al., 1993). Synapsin is required for post-tetanic potentiation (PTP), where synaptic vesicles readily migrate to the pre-synaptic terminal, following depletion of the vesicle reserve pool, such that chemical synapses can ensue (Greengard et al., 1993; Humeau et al., 2001).

Synapsin is also involved in the maturation of a synapse, as has been described in the mollusc *H. pomatia*. In *H. pomatia*, synapsin localization to sites where a synapse will form

preceding even the bringing in proximity of pre- and post-synaptic cells (Cibelli et al., 1996). This localization of synapsin means that synapsin is not restricted to pre-synaptic terminals.

Another instance of localization of synapsin, not solely restricted to pre-synaptic terminals is following the formation of a chemical synapse, when synapsin redistributes within the pre-synaptic cell, primarily to varicosities proximal to the post-synaptic cell (Cibelli et al., 1996; Humeau et al., 2001).

To date, one putative synapsin protein has been identified in the schistosome genome (locus tag: Smp_120640).

Synapsin is an ideal neuronal marker as it is neuron-specific, found throughout the nervous system and, in schistosomes, has already been immunolabeled in the cercariae, demonstrating that it can be labeled in schistosomes (Sudhof, et al., 1989; Kao, et al.; 1999; Cesca, et al., 2010; Humeau et al., 2011; Collins et al., 2011).

2.5 The Role of the Nervous System in Sensation

There is evidence in schistosomes and other flatworms of the role of the nervous system in sensory functions. Surface innervation of sensory organs and the surface membrane is suggestive of efferent sensory input to sense the worm environment, as has been described in flatworms (Dixon and Mercer, 1965).

In *S. mansoni* cercariae, dendritic processes connected to neurons innervate sensory papillae at the surface are also indicative of a role in sensation in schistosomes (Gordon et al., 1934; Vercammen-Granjean, 1951; Wagner, 1961; Richard 1968, 1971; Morris and Threadgold, 1967; Smith et al., 1969; Robson and Erasmus, 1970; Matricon-Gondran, 1971; Dorsey and Stirewalt, 1971; Morris, 1971; Nuttman, 1971; Short and Cartreet, 1973; Short and Gagne, 1975; Short and Kuntz, 1976; Cousin and Dorsey, 1987; Cousin and Dorsey, 1991).

2.6 *S. mansoni* Neurochemistry

Schistosomes have a diverse set of neurotransmitters and receptors. Most of these receptors were found to have little or no sequence homology with human receptors (El-Shehabi and Ribeiro, 2010; El-Shehabi et al., 2012; Ribeiro et al., 2012), making them ideal drug targets. The nervous system is under the control of both peptidergic transmitters, small classical transmitters, as well as small molecule neurotransmitters, including nitric oxide (Kohn et al., 2001). Some of these classical transmitters include acetylcholine (Ach), glutamate and the biogenic amines (BAs). The peptidergic neurotransmitters include the NPY/F-Like and FMRFamide-Like neuropeptides (FLPs or FarPs), which have been identified in flatworm species (Maule et al., 1993; Maule et al., 1994; Johnston et al., 1995; Johnston et al., 1996; Mair et al., 2000; Humphries et al., 2004; Ribeiro and Geary, 2010), including schistosomes (Humphries et al., 2004; McVeigh et al., 2009). More recently, helminth neuropeptides, named I/Lamides, were discovered and one of these neuropeptides characterized in *S. mansoni* and is said to modulate muscular activity (Gustafsson, 1987; McVeigh et al., 2011). In invertebrates, FMRF peptides are typically involved in neuromodulation (Schaefer et al., 1985; Wikgren, 1986) and have been identified in all classes of flatworms (Gustafsson and Wikgren, 1985; Wikgren, 1986).

Here we will focus our attention primarily on the BAs, which are most relevant to this study.

3 Biogenic amines

BAs are the largest subset of classical transmitters and are derived from aromatic amino acids (AAs), or histidine. BAs, like other neurotransmitters, require specific enzymes for synthesis and metabolism (Hillman and Senft, 1973). In schistosomes, these enzymes have been extensively studied for catecholamines, as well as serotonin and acetylcholine and were generally shown to resemble the vertebrate forms of the enzymes (Hillman and Senft, 1973). In flatworms, BAs typically play a role in metabolism, motility and survival of the parasite (Ribeiro and Geary, 2010).

3.1 BA GPCR Signaling

Most BA receptors identified to date, of both invertebrate and vertebrate species, belong to the superfamily of GPCRs (G protein-coupled receptors). GPCRs are the largest superfamily of cell surface transmembrane receptors. GPCRs are of particular interest in schistosomes not only to explore their role in BA signaling but because GPCRs themselves are proven drug targets. Approximately 30-50% of all pharmaceutical drug targets are GPCRs.

There are 6 classes of GPCRs (A-F), the largest of these being classes A, B and C (Davies et al., 2008). GPCR classification is based on the length of the N-terminal sequence, as well as the presence of key residues, which are involved in receptor function. Class A, the rhodopsin-like GPCR receptors, contains ~ 90% of all GPCRs (Fotiadis et al., 2006; Palczewski et al., 2000) including the BA GPCRs. These receptors share common residues at the cytoplasmic end of TM3 (E/DRY) and TM6 (NPXXY) (Rosenbaum et al., 2009).

All GPCR receptors have 7 transmembrane (TM) domains, which are arranged in a counterclockwise orientation (Schertler et al., 1993; Ji et al., 1998; Congreve and Marshall, 2010). The receptor has 3 extracellular and 3 intracellular loops (Gudermann, 1995; Palczewski et al., 2000). The N-terminal segment extends out of the cell membrane and is located in the cell cytosol, while the C-terminal segment extends into the cell and is located in the cell cytoplasm. The third intracellular loop (IL3) is the most variable and the largest of the loops. The IL3 is highly flexible and is involved in interactions with G proteins (Ghanouni et al., 2001; Congreve and Marshall, 2010).

Upon binding of a ligand to the GPCR, the receptor will undergo a conformational change, resulting in the activation of a trimeric G-protein (G α, G ß, and G γ subunit), which is located at the inner surface of the cell. The G α subunit dissociates from the G ß and γ subunits and subsequently binds an effector protein and a signaling cascade ensues whereby second messengers are activated.

GPCRs signal through second messengers, following so-called “first messenger” stimulation, by an exogenous, typically hydrophilic ligand, which include neurotransmitters,

such as the BAs. Second messengers include cAMP, cGMP, diacyl glycerol (DAG), inositol triphosphate (IP3), and Ca++ (Kimmel and Eisen, 1988; Bouvier, 1990; McKnight, 1991; Berridge, 1993; Morel et al., 2003). Common second messenger effects include an inhibition or activation of adenylate cyclase - leading to changes in cytosolic cAMP - and an increase in intracellular Ca++.

Second messenger signaling can result in changes in cell metabolism, apoptosis, development, gene expression, development and exocytosis. In invertebrates, cAMP has been associated with increased neuronal excitability (Lee, 2015). Phosphodiesterases (PDEs) can negatively regulate cAMP levels in the cell (Lee et al., 2016). cAMP stimulates PKA, which phosphorylates proteins in a signal cascade, including other kinases, transcriptional factors, and ion channels. Long-term effects, including transcriptional and translational changes, typically involve cAMP Response Element Binding Protein (CREB). PDEs (accession Numbers: CCD81051, CCD77807) and adenylate cyclases (ACs) (accession Numbers: CCD82246, CCD82242, CAY18716, CCD58799 and CCD58798) have been identified *in silico* in *S. mansoni* (Protasio et al., 2012).

BAs can be synthesized endogenously by the parasite or taken up from the host blood in which the parasite resides. Transporters located on the pre-synaptic terminal serve to transport the BA out of the synaptic junction to prevent further signaling. Following re-uptake, the BA can be recycled further, once again sequestered to a synaptic vesicle, pending release following a subsequent neuronal impulse, or degraded, to prevent unchecked stimulation of receptors at the post-synaptic terminal.

3.2 Conformational activation of GPCRs

Little is known about the nature of the conformational changes by which GPCRs are converted to their active states (Wess et al., 2008). The TM regions, which are most prominently involved in ligand binding and conformational activation are TM 3, 5, 6 and 7 (van Rhee and Jacobson, 1996). According to current models, the binding of ligand disrupts inter-

helical interactions among these TM regions. Subsequently, a new set of interactions will result, enabling the receptor to change from its previous conformation to one that is more energetically favorable. One such interaction includes that which occurs between Ser3.36 (using Ballesteros-Weinstein nomenclature of GPCRs (Ballesteros and Weinstein, 1995; van Rhee and Jacobson, 1996) of TM3 and Cys7.42 of TM7 (Wess et al., 2008). In BA receptors, this interaction occurs directly below the ligand binding domain, where the side chain of the negatively charged carboxylate of Asp3.32 in TM3 will interact with the protonated amino moiety of the BA. This ligand-receptor interaction is thought to be aided by a "cage" of aromatic AAs formed by TMs 3, 6 and 7. AAs involved in cage formation include Tyr3.33, Tyr6.51, Tyr7.39 and Tyr7.43. It is believed that the cation-π interactions between these tyrosine residues, serve to stabilize interaction with ligand (Wess et al., 2008). This model of ligand-receptor interaction and subsequent conformational activation is based on analyses of mammalian BA receptors, particularly the human β2-adrenergic receptor, which has been crystallized (Lefkowitz et al., 2008; Rasmussen et al., 2007). It is unknown if the same model applies to BA receptors of schistosomes.

The following is an overview of the most prevalent BAs in schistosomes as documented in literature, as well as our primary BAs of interest, the catecholamines and phenolamines.

4 Serotonin

Serotonin (5-hydroxytryptamine: 5HT), derived from tryptophan, is one of the most widely distributed neuroactive substances in the animal kingdom (Hutson et al., 1986; Weiger, 1997; Horner et al., 1997; Loverde, 1998, Carre -Pierrat et al., 2006; Roberts, 2011; Hen, 1993; Curran and Chalasani, 2012). 5HT was first identified in adult schistosomes in 1969 (Bennett et al., 1969; Catto and Ottenson, 1979). 5HT has also been identified in other human-infecting schistosome species, *S. haematobium* and *S. japonicum* (Chou et al., 1972).

5HT is widely distributed in the flatworm nervous system and typically plays a role in muscle control and movement (Boissier et al., 1999; Kumari et al., 2005; Ribeiro and Geary,

2010). Schistosomes show a sustained response to 5HT, which, in vitro, can be sustained for more than 2 hours following exposure (Hillman and Senft, 1973). Concentrations of 1µM 5HT can stimulate glycolysis and glucose uptake in adult worms (Nimmo-Smith and Raison, 1968; Catto and Ottenson, 1979).

Schistosomula contain one fifth of the serotonin levels contained in adult stage male parasites (Catto and Ottenson, 1979). 5HT was first detected histochemically in adult schistosomes by Bennett and Bueding (Bennett and Bueding, 1969). 5HT was identified in the commissure, in the head region, proximal to the main longitudinal nerve chords, as well as in small granules in the worm parenchyma. Many of the early neuro-pharmacological findings regarding schistosomes were observed through the study of 5HT.

4.1 Synthesis of Serotonin by *S. mansoni*

There is evidence of a serotonin synthesis pathway in the parasite. In 1971, Bennett and Bueding showed that preincubation of adult schistosomes with the 5HT precursor, 5-hydroxytryptophan, in the presence of the monoamine oxidase (MOA) inhibitor iproniazid, which prevents metabolism of 5HT, caused a ten-fold increase in 5HT levels in the parasite, along with an increased number of 5HT-containing structures identified in the parasite by histochemical fluorescence (Bennett and Bueding, 1971).

S. mansoni tryptophan hydroxylase (SmTPH) the rate limiting enzyme in 5HT synthesis, which synthesizes 5-hydroxy-L-tryptophan, precursor to 5HT, from L-tryptophan has been cloned from *S. mansoni* and characterized (Hamdan and Ribeiro, 1999), providing further evidence of a 5HT synthesis system in the parasite. Semi-quantitative PCR revealed that SmTPH is 2.5 times more expressed at the RNA level in cercariae compared to in the adult parasite, suggesting an important role for 5HT in this early infective stage. It was also shown that SmTPH activity is inhibited by low DA concentrations and by competition for cofactor BH4 (tetrahydrobiopterin) which both TH, the rate limiting enzyme of DA synthesis, and TPH, use.

This inhibition by DA and BH4 is thought to be biologically relevant in a nervous system where predominantly DA or 5HT neurons interact (Kumer and Vrana, 1996 , Kaufman and Ribeiro, 1996, Hamdan and Ribeiro, 1999), and may serve a modulatory role of DA on 5HT signaling.

An amino acid decarboxylase (AADC) has been identified *in silico* in *S. mansoni* (locus tag: Smp_171580) (Berriman et al., 2009; Ribeiro et al., 2012), which shares homology with the human homologue and is predicted to perform the decarboxylation reactions that synthesizes 5HT from 5-hydroxytryptophan (5-HTP), as well as dopamine (DA) from L-DOPA (Ribeiro et al., 2012).

4.2 The Role of 5HT in Schistosome Motility

In the literature, 5HT has been described as having an excitatory effect on schistosomes. In adult schistosomes, acetylcholine (ACh), contrary to the effects of 5HT, has an inhibitory effect on parasite activity, causing flacid paralysis when parasites are incubated with ACh (Pax et al., 1984; Macdonald et al., 2014). As an example of the role in 5HT on stimulation of motility, the stimulation of schistosome motility by anticholinergic signaling compoundscan be inhibited by administration of anti-serotonergic compounds, including bromolysergic acid and diethylamide (Tomosky et al., 1974). Conversely, prior depletion of 5HT levels in the adult parasite by administration of vesicular monoamine transporter (VMAT) inhibitor reserpine, prevents stimulation by 5HT (Tomosky et al., 1974).

On muscle strip preparations of adult *S. mansoni*, 5HT was shown to cause rythmic contractile activity, having effects on both circular and longitudinal muscle (Pax et al., 1984). These effects were blocked by 5HT antagonist, metergoline. Administration of serotonin to schistosomes in culture causes an increase in motility up to ten-fold in magnitude, depending on concentration (Bennett et al., 1969; Bennett and Bueding 1971; Bennett and Bueding, 1973; Hillman and Senft, 1973; Barker et al., 1966; Tomosk y et al., 1974; Fetterer et al., 1977; Chou et al., 1972; Meuleman et al., 1980; Mellin et al., 1983; Semeyn et al., 1982; Boyle and Yoshino, 2005) and causes an ondulating movement in the body as well as movement of the oral sucker

(Hillman and Senft, 1973). For adult worms having a high basal level of activity, the magnitude of stimulation by 5HT is not as pronounced as in those worms not moving as much following removal from the mouse host (Hillman and Senft, 1973), indicating that there is a maximal threshold of activity, stimulated by 5HT, and that variation in baseline-level motility can mean more pronounced motility changes, for worms having low baseline activity.

In studies on the contractility of isolated adult *S. mansoni* muscle fibers, it was demonstrated that 5HT potentiates muscle contraction (Day et al., 1994). Muscle fibers contracted following exposure to potassium ions (K+) in a concentration-dependent manner, in the presence of 5HT (Day et al., 1994). Electrochemical activity associated with 5HT-induced action potentials, has also been measured. The most pronounced stimulation by 5HT observed by electrochemical detection occurred at 100 µM and anti-serotonergic metergoline and cyproheptadine caused concentration-dependent inhibition of schistosome motor activity (Mellin et al., 1983; Semeyn et al., 1982). p-chlorophenylethyamine (p-CPEA), which, in mammalian cells, causes a release of 5HT from the synapse (Hwang and Woert, 1979) also had a stimulatory effect on schistosome motility.

5HT-induced metabolic effects on schistosomes include increases in glucose usage (Fetterer et al., 1977; Rahman et al., 1985) to supplement this enhanced schistosomal activity, similarly to in *F. hepatica* (Mansour et al., 1960), marked by an increase in adenylyl cyclase activity in the adult and schistosomulum worm stages.

Administration of 5HT to homogenates of adult schistosomes causes adenylate cyclase stimulation (Higashi et al., 1973; Catto and Ottenson, 1979).

5HT was shown to induce adenylate cyclase in the parasite (Estey and Mansour, 1987). Activation of adenylate cyclase by 5HT was verified in both larval (cercariae and schistosomula) and adult stages of development (Estey and Mansour, 1987). Early-stage schistosomula are not as responsive as older schistosomula (Kasschau and Mansour, 1982). 5HT responsiveness in schistosomula develops within 4 days post transformation in culture (Estey and Mansour,

1987; Estey and Mansour, 1987). Antagonists of 5HT signaling, including 2-Bromo-LSD, methiothepin, metergoline and less potently, haloperidol, inhibit the effects of 5HT on adenylate cyclase, at 100µM 5HT (Estey and Mansour, 1987).

4.4 5HT Transport in *S. mansoni*

5HT uptake in schistosomes is potently inhibited by serotonin reuptake inhibitor fluoexetine and by the Na+/K+-ATPase inhibitor ouabain (Wong et al., 1975; Catto and Ottenson, 1979). 5HT uptake was demonstrated to be concentration-dependent and exhaustible, indicating a dependence on transporters for 5HT uptake (Catto and Ottenson, 1979). A schistosome transporter, capable of taking up 5HT, was thus presumed to be present on the worm surface. Also, as studies of exogenously applied 5HT modulate schistosome motility, suggesting that 5HT is taken up from the host environment (Patocka and Ribeiro, 2007, Patocka and Ribeiro, 2013). An SmSERT, which is the only 5HT transporter encoded in the *S. mansoni* genome, has been cloned and characterized (Patocka and Ribeiro, 2007; Patocka and Ribeiro, 2013). In schistosomula, RNAi inhibiton of SmSERT with a corresponding decrease in expression by approximately 50% causes a marked hypermotile effect on larvae, consistent with the expected effects of causing an increase in extracellular 5HT in the parasite, prolonging the activity of 5HT on serotonin receptors and serotonin signaling. Also, incubation of schistosomula with classical SERT inhibitors clomipramine, citalopram and fluoxetine inhibited 5HT uptake, suggesting that the SmSERT is present on the surface of schistosomula (Patocka and Ribeiro, 2007). Fluoxetine and chlomipramine cause an increase in motility of approximately 3 fold, similar to the effect observed with the addition of 5HT alone. Conversely, immunolocalization studies of schistosomula and adult-stage parasites indicates that this receptor is not expressed on the tegument, but rather, is closely associated with the nervous system, and, in adult parasites, in tubercles associated with the nervous system (Patocka and Ribeiro, 2013). SmSERT co-immunolabels with 5HT in CNS nerve cords as well as in regions of the PNS. This co-localization is notable in varicosities. SmSERT localization at these sites suggests release of 5HT to adjacent

nerve fibers with subsequent re-uptake for storage or destruction by SmSERT. This co-labelling of transporter and neurotransmitter follows the mammalian cell paradigm, which sees these SERTs in areas of neurotransmitter release for subsequent storage. Also, in RNAi studies where SmSERT was downregulated in schistosomula, only a slight hypermotile effect was observed. It is therefore speculated that while SmSERT may account for a portion of 5HT uptake from the environment, another mechanism, such as a non-selective carrier may be involved in 5HT uptake (Patocka and Ribeiro, 2013; Ribeiro and Patocka, 2013).

4. 5 Serotonin Receptors in *S. mansoni*

A serotonin receptor, Sm5HTR (Smp_126730) was recently cloned and characterized from *S. mansoni* (Patocka et al., 2014). The receptor is selectively activated by 5HT and signals through second messenger cAMP. Classical serotonergic antagonists, including anti-serotonergic mianserin (Hiripi et al., 1994) and cyproheptadine abrogate this serotonergic response. Serotonin derivative o-methyl-serotonin strongly activates Sm5HTR and serotonin agonists, buspiperone, 7-(Dipropylamino)-5,6,7,8-tetrahydronaphthalen-1-ol (DPAT) and tryptamine also activated receptor activity, though not to the same extent as 5HT and its methylated derivative. Another predicted schistosome 5HTR (Smp_197700) has been identified *in silico* (Patocka et al., 2014), and *in silico* analysis of the schistosome genome identifies ~117 GPCRs (Zamanian et al., 2011), only few of which have been characterized (Hamdan et al., 2002; El-Shehabi and Ribeiro; 2010; Taman and Ribeiro, 2009; El-Shehabi et al., 2012; Patocka et al., 2014; Macdonald et al., 2015). Given the importance of 5HT to parasite motility, the large number of predicted GPCRs present in the schistosome genome suggests that more 5HTRs (as well as other BA GPCRs) are likely present in the parasite.

5 Catecholamines

5.1 Catecholamine Synthesis and Degradation

The catecholamines have a catechol (dihydroxylphenyl) ring and are derived from tyrosine. Catecholamines include dopamine, epinephrine, norepinephrine (Moore and Bloom, 1978) and epinine (EPN), which is a metabolite of dopamine. After 5HT, catecholamines are the most widely distributed BAs in the animal kingdom (Ribeiro et al., 2005). Tyrosine hydroxylase (smTH), the rate-limiting enzyme in catecholamine synthesis, which synthesizes L-DOPA (3, 4-dihydroxy phenylalanine) from tyrosine, was previously cloned from *S. mansoni* (accession number: AAC62256) and characterized (Hamdan and Ribeiro, 1998), indicating that the parasite can perform endogenous synthesis of DA (Hamdan and Ribeiro, 1998). Recombinantly expressed SmTH from *E. coli*, which has a predicted size of ~54 kDa, has a specific activity of 0.78 umol/min/mg, as determined using a tritiated water release assay (Nagatsu et al., 1964; Reinhard et al., 1986; Ribeiro et al., 1991; Hamdan and Ribeiro, 1998). SmTH shares up to 61% homology with THs from other species. SmTH shares the four cysteine residues, as well as a fifth cysteine residue (Cys140), which, in other species, are important active sites (Nagatsu and Ichinose, 1991; Ramsey et la., 1995; Kaufman and Ribeiro, 1996; Kumer and Vrana, 1996), and in SmTH, are located at AA positions 212, 274, 292 and 343, respectively. SmTH also contains the glutamate and two histidine iron-binding sites required for iorn cofactor binding (Ramsey et al., 1995; Goodwill et al., 1997; Hamdan and Ribeiro, 1998). Following L-DOPA synthesis, the enzyme amino acid decarboxylase (AADC) synthesizes DA from L-DOPA via a decarboxylation reaction. Catecholamines can be degraded by MAO, and subsequently, catechol-O-methyltransferase (COMT), which inactivates catecholamines by incorporation of a methyl group. Degradation of DA by MAO produces 3,4-dihydroxyphenylacetic acid (DOPAC), which is subsequently methylated to become homovanillic acid by COMT, and methylation of DA by COMT produces 3-methoyxytyramine (3-MT), which can subsequently be degraded to homovanillic acid by MAO (van Praag, 1978; Post et al., 1982 ; Ebinger et al., 1987; Jaques et al., 2011; Andrzej et al., 2012).

5.2 The Effect of Catecholamines on Intact Schistosomes and in Muscle Preparations

When applied exogenously to *S. mansoni*, dopamine as well as norepinephrine (NE) and epinephrine (EPN), were shown to cause lengthening of the worm, indicative of an inhibitory effect of these BAs on the worm somatic musculature (Ribeiro and Geary, 2010, Tomosky et al., 1974). In muscle preparations of adult *S. mansoni*, DA causes relaxation of circular muscle, decreasing muscle tone. While a net relaxation effect was observed, this effect was determined to be biphasic, with an initial tension, followed by a decrease in muscle tension (Pax et al., 1984). DA also causes a relaxation of longitudinal muscle, and DA antagonist spiroperidol inhibits DA-induced relaxation of circular and longitudinal muscle (Pax et al., 1984). When applied directly onto three day-old schistosomula in culture, DA decreases parasite motility in a concentration-dependent manner, over a concentration range of 0.1-100 µM (El-Shehabi et al., 2012). Other studies have shown that incubation of adult worms with DA or its metabolites, norepinephrine and epinephrinere, results in an increase in worm length (Mellin et al., 1983), associated with a relaxation of longitudinal muscle. DA administered to adult parasites causes a decrease in frequency of electric potentials in nerve cells, though no change in motility is observed, suggesting an inhibitory role for DA (Semeyn et al., 1982). DA receptor-specific antagonist apomorphine also, similarly, causes a lengthening effect. This lengthening effect is more pronounced following incubation with of DA and DA antagonist apomorphine, than with NE, EPN, and NE and EPN antagonists (alpha and beta adrenergic blockers), which fail to produce this lengthening effect indicating that this effect can be more specifically attributed to DA signaling.

Recent advances in parasite genomics have confirmed the existence of a DA signaling system in schistosomes. Besides the biosynthetic enzyme described earlier (SmTH), *S. mansoni* has a specific dopamine transporter (Larsen et al., 2011) and two dopamine receptors SmD2 (Gene ID: FJ985986) and SmGPR3 (Smp_043290) have been cloned and characterized.

5.3 SmDAT

A schistosome dopamine transporter, SmDAT (Smp_156320) was cloned from *S. mansoni* and characterized via heterologous expressed in mammalian cells (Larsen et al., 2011). SmDAT shows specificity for DA, where, in uptake assays NE and 5HT were transported at less than 10% of DA uptake, at a concentration of 100 µM. Despite the specificity of the transporter for DA, its pharmacological profile more closely resembles that of the human NE transporter (hNET) than the human DA transporter (hDAT). SmDAT inhibitors desipramine and nisoxetine have lower IC50 values than for the hDAT (desipramine IC50 for SmDAT: 1.41 µM, compared 42.9 µM with hDAT and 0.114 µM nisoxetine IC50 for SmDAT compared to 0.862 µM for hDAT).

Expression of the SmDAT was confirmed at the RNA level by qPCR in sporocyst (from parasite infected snails), schistosomula and adult, miracidia and egg stages but cercaria SmDAT is missing the exon encoding the eighth TM region of the SmDAT. This difference in life stage expression reflects a recurring theme in schistosomiasis research, which sees a stage-specific fluctuation in gene expression and, for SmDAT, switching of gene isoform expression, demonstrating the adaptability of the parasite, in the context of host infection.

5.4 SmGPR3

SmGPR-3 belongs to a family of novel "amine-like" G Protein-Coupled Receptors (named SmGPRs), which appear to be unique to parasitic flatworms. When expressed heterologously in *Saccharomyces cerevisiae*, SmGPR3 exhibited strong specificity for dopamine and the related DA metabolite, epinine, compared to other biogenic amines. Yeast-expressed SmGPR-3 was shown to have an atypical pharmacological profile, which does not resemble that of mammalian DA receptors. This unusual pharmacological profile is exemplified by the pronounced agonist activity of spiperone, a classical mammalian D2 type dopamine receptor antagonist, which caused a two-fold stimulation of SmGPR-3 activity compared to DA alone. Also, the classical DA antagonist clozapine has no significant effect on receptor activity. On the other hand, SmGPR3 is potently inhibited by compounds that do not typically act as dopamine

antagonists, for example promethazine (an antihistaminic drug), mianserin (mixed anti-serotonergic and anti-adrenergic), and the anti-histaminic/ serotonergic drug, cyproheptadine, the latter producing > 70% inhibition of SmGPR3.

The atypical pharmacological profile of SmGPR3 may be explained by its divergent AA sequence, which has < 40% identity with mammalian DA receptors (El-Shehabi et al., 2012). As mentioned earlier, SmGPR-3 belongs to a novel clade of "amine-like" receptors, which are distantly related to BA GPCRs from other species (El-Shehabi and Ribeiro, 2010; El-Shehabi et al., 2012). A particularly unusual feature of these receptors is the lack of a highly conserved binding residue of TM3 ($D^{3.32}$), which normaly anchors the protonated amino group of the BA ligand (Ribeiro and Geary, 2010). While, unlike other SmGPRs, SmGPR-3 does have the conserved $D^{3.32}$ residue, it lacks another important ligand-binding residue ($S^{5.46}$) and homology modelling suggests that two of the conserved serine residues of TM5, ($S^{5.42}$ and $S^{5.43}$), which typically interact with the hydroxyl group of DA's catechol ring, are not involved in ligand interaction (El-Shehabi et al., 2012).

Immunolocalization studies revealed that SmGPR3 is abundantly expressed in *S. mansoni*, both adult worms and larvae. In adults, SmGPR-3 showed widespread labelling in the CNS, including cerebral ganglia and major longitudinal nerve cords, as well as the smaller nerve fibers of the PNS innervating the caecum, the body wall musculature, the male testes and tegument. In schistosomula, SmGPR-3 immunolocalized primarily to longitudinal nerve cords and transverse commisures of the CNS (El-Shehabi et al., 2012). These studies suggest an important role for SmGPR3 in nervous system function as well as reproduction (testes innervation) and feeding (ventral sucker innervation) and highlights the importance of DA signaling in the parasite (El-Shehabi et al., 2012). Moreover, the widespread expression of SmGPR3, combined with the receptor's unusual pharmacology and divergent structure indicate strong potential for drug targeting (El-Shehabi et al., 2012).

5.5 SmD2

SmD2 is a homologue of mammalian and invertebrate dopaminergic type 2 receptors. SmD2 is specifically activated by DA in a concentration-dependent manner when expressed heterologously either in yeast (5x10^-8M to 5x10^-4M) or mammalian cells (HEK293) (EC50: 1.7x10^-7 M) (Taman and Ribeiro 2009). In mammalian cells, SmD2 signals through stimulation of second messenger cAMP (Taman and Ribeiro 2009). This is surprisingly different from the conventional signaling mechanism of D2 receptors, which typically inhibit adenylate cyclase and thereby decrease cAMP levels. The most potent inhibitors of SmD2 tested are: apomorphine > chlorpromazine > and spiperone, all classical antagonists of DA signaling.

Immunolocalization studies detected SmD2 in cercariae, schistosomula and adult schistosomes, the most pronounced expression occurring in the larval stages (Taman and Ribeiro, 2009). SmD2 immunolocalized to somatic musculature in both circular and longitudinal muscles in all three life stages. In cercariae, SmD2 localized to the acetabulum of schistosomula, the host penetration glands and, in adults, the muscular lining of the intestinal caecum and associated ducts, suggesting a role in attachment, feeding and infectivity.

7 Histamine

7.1 The Role of HA in Schistosome Motility

Histamine (HA), or 2,4-Imidazolyl ethylamine, is an imidazolamine (Alvarez, 2009) that is synthesized via the decarboxylation of histidine by the enzyme histidine decarboxylase (HDC). HA has been detected in several flatworms, including *S. mansoni* (Ercoli et al., 1985; El-Shehabi and Ribeiro, 2010). Though it is unknown if *S. mansoni* can synthesize HA (Catto, 1981), sequencing of the schistosome genome (Berriman et al., 2009; Protasio et al., 2012) has revealed two decarboxylase-like genes in the *S. mansoni* genome (locus tags: Smp_130860 and Smp_135230), which share homology with both HDC, as well as tyrosine decarboxylase (TDC), the enzyme which catalyzes the synthesis of biogenic amine, tyramine (TA), from tyrosine, in other species (Ribeiro et al., 2012). The identification of these HDC-like genes in the

schistosome genome suggests that *S. mansoni* may synthesize HA endogenously. Alternatively, HA may be taken up by diffusion across the tegument, as in the rat tapeworm *H. diminuta*, where HA was shown to be taken up by the worm by simple diffusion (Yonge and Webb, 1992). HA is abundantly found in the shistosome peripheral neuronal plexuses, which are outsourced to the body wall musculature (El-Shehabi and Ribeiro, 2010, El-Shehabi et al., 2009). HA was shown to cause a concentration-dependent increase in motility when applied exogenously to cultured S. mansoni larvae. Moreover the larvae were paralyzed when treated with anti-histaminergic drug, promethazine, an effect that was reversed with subsequent incubation with HA (Ercoli et al., 1985; Ribeiro et al., 2005). These findings indicate that HA has a role in parasite motility.

7.2 HA-Selective Receptors in *S. mansoni*

Bioinformatics analysis did not detect any homologues of mammalian HA receptors. However some SmGPRs, the aforementioned "amine-like" GPCRs of *S. mansoni* are responsive to HA when expressed *in vitro*. Two of these novel receptors, in particular, SmGPR-1 (formerly SmGPCR) (Hamdan et al., 2002; El-Shehabi et al., 2009) and SmGPR-2 (El-Shehabi and Ribeiro, 2010), were both determined to be HA-selective. Like other members of the SmGPR family, these two receptors lack the signature D3.32 ligand-binding residue of BA GPCRs, which is replaced with an asparagine in *S. mansoni*. As mentioned earlier, the aspartate D3.32 plays a key role in the interaction between ligand and receptor. Typically, the carboxylate group of the aspartate side chain will interact with the protonated amino moiety of the BA. The presence of the asparagine residue, a polar uncharged AA, at this site, points to an alternative mode of ligand interaction for the smGPRs, including these HA receptors.

HA-selective receptors SmGPR-1 (Hamdan et al., 2002; El-Shehabi et al., 2009) and SmGPR-2 (El-Shehabi and Ribeiro, 2010) have been cloned and characterized, further demonstrating that histamine signaling is present in the parasite. Mammalian HA GPCRs are classified into four subtypes, H1-H4 (Shahid et al., 2010) based on their structure,

pharmacological profile and signaling properties. SmGPR-1 shares some structural similarities with the H1 receptors (Hamdan et al., 2002), while SmGPR-2 shares only ~30% homology with all other BA GPCRs ~ (El-Shehabi and Ribeiro, 2010). Both SmGPRs differ in residues which are typically conserved in HA GPCRs (Hamdan et al., 2002). SmGPR-1 signals through second messenger intracellular Ca++ production. SmGPR-2 is more potently activated by the methylated derivative of HA, 1-methylhistamine (El-Shehabi and Ribeiro, 2010), than by HA. The pharmalogical profile of SmGPR-2 was determined to be uncommon, as compared to that of mammalian HA receptors (El-Shehabi and Ribeiro, 2010). The receptor is selectively inhibited by the antihistaminic promethazine and, unexpectedly, by classical antagonists of DA and 5HT signaling, flupenthixol and buspirone (El-Shehabi and Ribeiro, 2010). SmGPR-2 is expressed in the PNS, primarily in the subtegumental neuronal plexus that innervates the body wall musculature of adults and schistosomula (El-Shehabi and Ribeiro, 2010). The distinct structural properties of these HA receptors, and the localization of SmGPR-2 highlight the divergence of schistosome BA GPCRs from those of the mammalian host, their role in schistosome motility, and their potential for drug targeting.

8 Octopamine

Octopamine (OA), derived from tyramine (TA), is the invertebrate equivalent of noradrenaline. OA has been identified virtually in every invertebrate phylum, including arthropods, molluscs, nematodes and the free-living flatworms *Dugesia japonica* (Nishimura et al., 2008) and *Schmidtea mediterranea* (Rangiah and Palakodeti, 2013). Prior to the findings of this thesis, OA has never been identified or characterized in schistosomes. OA signaling is largely absent in mammals and OA signaling could prove to be an attractive drug target in schistosomes. OA, along with its precursor TA, are said to be the only non-peptide neurotransmitters that are exclusive to invertebrates. OA signaling in invertebrates is said to be the invertebrate counterpart to the adrenergic signaling system of vertebrates (Roeder, 1999; Blenau and Baumann, 2001). Although OA can be identified in “trace” amounts in the

mammalian brain, and is thus referred to as a “trace amine”, there is no evidence of OA signaling in mammals. OA differs from NA in its lack of a 3-hydroxyl group in the phenolic ring.

Octopamine was first identified in the posterior salivary glands of the octopus (Erspamer and Boretti, 1951) and acts as a neurotransmitter (Robertson and Juorio, 1976, Orchard and Loughton, 1981), neuromodulator (Axelrod and Saavedra, 1977; O’Shea and Evans, 1979; Orchard, 1982; Evans, 1985; Saavedra, 1989) and/or neurohormone in a variety of invertebrate species. In the 1970s, there was a resurgence of interest in this neurotransmitter, when it was found to have a role in the physiology of cockroaches and other insects (Nathanson and Greengard, 1973; Roeder, 1999). OA plays a role in locomotion, motivation and behaviour (Hoyle, 1986; Sombati and Hoyle, 1984; Mulloney et al., 1987; Kyriakides and McCrohan, 1989; Bacon et al., 1995; Roeder et al., 1998; Verlinden et al., 2010), feeding, ‘fight or flight’ response, female reproduction (Lee et al. 2003; Monastirioti 2003), learning and memory (Dudai *et al*., 1987; Menzel *et al*., 1988; Hammer, 1993; Hammer and Menzel, 1998), wakefulness (Crocker et al., 2010; Sombati and Hoyle, 1984) and olfaction (Arakawa et al. 1990; Saudou et al. 1990; Robb et al., 1994). There are similarities in phenolamine signaling between insect species, such as honeybee and locust OA signaling, as exhibited by their similar pharmacological profiles, distribution to optic lobes and mushroom bodies and their physiological functions.

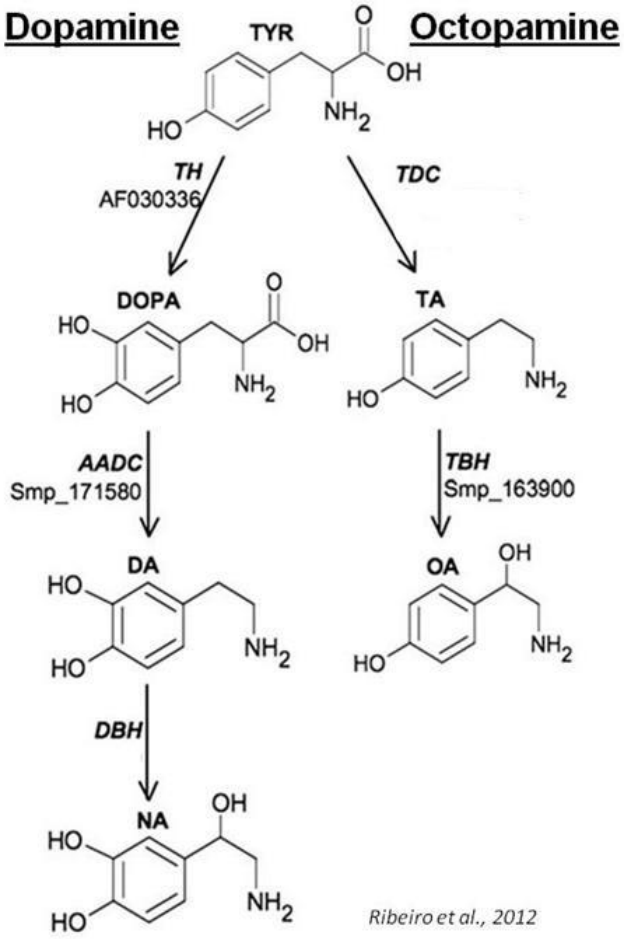


Figure 3. Octopamine (OA) is synthesized from tyramine (TA) via the hydroxylation of the beta carbon of OA's precursor TA. Synthesis pathways for tyrosine derivative OA, of the phenolamine structural class of amines, and DA, of the catecholamine structural class of amines are indicated, and schistosome homologues of enzymes involved in catecholamine and phenolamine signaling pathways are indicated (Ribeiro et al., 2012). Homologues include *Schistosoma mansoni* tyramine beta-hydroxylase (SmTBH) (locus tag Smp_243830), amino acid decarboxylase (SmAADC, Smp_171580) and tyrosine hydroxylase (SmTH) (GenBank accession number: AF030336), indicated along the arrows. OA is the invertebrate equivalent of noradrenaline (NA) (from Ribeiro et al., 2012).

OA has been found to be most abundant in the octopus *O. vulgaris*, the first invertebrate in which OA was identified, particularly in the salivary gland where it was determined to have a concentration of 1,310,000 ng/g of tissue (Saavedra, 1974). In *C. elegans*, the total tissue contains approximately 130ng/g (Horvitz et al., 1982), approximately 10 000 times less than in the octopus salivary glands (Saavedra, 1974 ; Dymond and Evans, 1979).

8.1 Octopamine Synthesis

The neurotransmitter OA is typically synthesized by hydroxylation of the beta carbon of TA by the enzyme tyramine beta hydroxylase (TBH) (Stuart et al., 1974; Wallace, 1975; Walker and Kurkut, 1978; Monastirioti et al., 1996; Herman et al., 2006; Nishimura et al., 2008), which shares homology with mammalian dopamine beta hydroxylase (DBH) (Klinman, 2006) as well as peptidylglycine α-hydroxylating monooxygenase (PHM). Non-traditional OA synthesis occurs in the locust *L. migratoria,* which can also synthesize the active isoform of OA, *m*-OA, from dopamine via dehydration in a salvage pathway (David et al., 1981; Farooqui, 2012). Invertebrate TBH typically shares ~39% identity and 55% homology with mammalian DBH. PHM shares a ~32% sequence identity with DBH (Southan and Kruse, 1989). The structure and function of DBH and PHM have been extensively studied, and shed insight into the structure - function of these copper enzymes (Prigge et al., 2000). DBH is found in the chromaffin granules of the adrenal medulla, in both membrane-bound and soluble forms (Winkler and Carmichael, 1982 ; Winkler et al., 1986; Winkler et al., 1987; Phillips and Pryde, 1987) and in blood. DBH tetramerizes (Rush and Geffen, 1980; Kapoor et al., 2011) and has a molecular weight of ~290 kDa (Kaufman, 1966). DBH and TBH typically synthesize noradrenaline and OA respectively. TBH and DBH activity has been detected by measuring conversion of TA to OA (Menniti et al., 1986), with subsequent detection of OA derivative P-hydroxybenzaldehyde, following oxidation with sodium periodate (NaIO4) (Nagatsu and Udenfriend, 1972; Nagatsu, 1972), or by detecting conversion of a radioisotope-labelled substrate (Huysse et al., 1988).

The copper hydroxylases use ascorbate as a cofactor, which acts as an electron donor to copper (Friedman and Kaufman, 1965; Levin et al., 1969). The enzyme first binds ascorbate, which acts as the first substrate for the enzyme, before the second substrate, such as DA or TA, can be bound (Rush and Geffen, 1980; Stewart and Klinman, 1990). Following binding of DA or TA, the substrate is oxidized by Cu+ in the presence of molecular oxygen, resulting in production of a water molecule and the intended product: noradrenaline or OA (Kaufman et al., 1968; Friedman and Kaufman, 1966).

A TBH (SmTBH, locus tag: Smp_243830) has been identified *in silico* in *S. mansoni*, suggesting that the organism can synthesize OA (**Figure 3**, Ribeiro et al., 2012).

8.2 Degradation of Octopamine

In insects, OA and other monoamines are said to be primarily targeted for enzymatic degradation by N-acetylation, which is said to be the most important step in OA inactivation (Dewhurst et al., 1972; Hayashi et al., 1977; Evans, 1977; Isaac et al., 1990; Wierenga and Hollingworth, 1990; Kempton et al., 1992; Martin and Downer, 1989, Downer et al., 1988). Following N-acetylation, conjugases degrade OA.

Inhibitors of N-acetyltransferase (NAT) include insecticides and known inhibitors of OA signaling and demonstrate the "druggability" of NATs. Inhibitors that are catechol and phenol group derivatives retaining the 4-hydroxyl group on the phenol common to OA were determined to be more potent inhibitors of N-acteyltransferase activity on OA than the non-4-hydroxyl group-containing counterpart. By that same token, the N-acetyltransferases in *S. mansoni* could be potential targets for drug development. NCBI lists 5 putative *S. mansoni* NATs (accession numbers: CCD76354, CCD76356, CCD76356, CCD76194 and CCD76195) and 4 NAT-related proteins (CCD78499, CCD78500, CCD75995) (Protasio et al., 2012). While NATs also target other biogenic amines including 5HT and DA, the presence of putative NATs in *S. mansoni* represent an opportunity for specific targeting of schistosome NATs, to potentially identify inhibitors, which would not adversely affect host biology.

Alternatively to N-acetylation, OA can be converted to synephrine via N-methylation by phenylethanolamine-*N*-methyl-transferase (PNMT), though synephrine is not typically referred to as a degradation product, because it typically has a higher affinity for OA receptors (OARs) than OA itself.

Monoamine Oxidase (MAO), which typically degrades BAs in mammals, converts the amine to an aldehyde, with removal of amino group and then to oxidizes the aldehyde to carboxylic acid. While MAO can also degrade OA, this is uncommon, and has primarly been reported to occur in insect malpighian tubules. Given the uncommon degradation of OA by MAO, is not likely involved in OA degradation in schistosomes.

Another rare invertebrate pathway for OA degradation has been observed in *L. polyphemus*, the horseshoe crab (Battelle et al., 1988) and involves the gamma-glutylation of OA to produce gamma-glutamyl OA, identified exclusively in the CNS and eye, which is thought to have a biological function in the crab optic system in modulating light sensitivity.

8.3 Octopamine Transporters in Invertebrates

The first evidence of a re-uptake system for OA was demonstrated by identification of Na+-dependent reuptake in a nerve cord in the cockroach *P. americana* (Evans, 1977). OA inactivation typically occurs by extracellular enzymes (Hayashi et al., 1977) or by transporter-mediated reuptake into storage sites, either nerve terminals (Scavone et al., 1994) or glial cells surrounding these nerve terminals (Orkand and Kravitz, 1971; Evans, 1974; Iversen and Kelly, 1975; Evans and O'Shea, 1977; Livingstone et al., 1980; Evans, 1981; Sombati and Hoyle, 1984; Hoyle, 1986; Stevenson and Kutsch, 1986; Heinzel, 1988).

Biogenic amine transporters are primarily of the SLC6 (solute carrier 6) sodium-dependent symport transporter clade, which localize to the plasma membrane (Ribeiro and Patocka, 2013). SLC6 transporters are both Na+ and Cl- dependent (Roeder, 1999; Ribeiro and Patocka, 2013). SLC6 transporters have 12 transmembrane (TM) regions with intracellular N- and C- terminal domains. OATs are said to be derived from a common ancestor of vertebrate

noradrenaline transporters (NATs) (Caveney et al., 2006, Ribeiro and Patocka, 2013). In the schistosome genome, a putative OAT has been identified (locus tag: Smp_193800) (Caveney et al., 2006, Ribeiro and Patocka, 2013), though this gene does not clade with other OATs (Ribeiro and Patocka, 2013). Smp_193800 shares similarity with an uncharacterized *C. sinensis* gene and is thought to be a different type of OAT, if at all (Ribeiro and Patocka, 2013), which would need to be determined by heterologous expression and characterization to confirm its specificity.

Cocaine is an inhibitor of OA re-uptake by OA transporters that occurs naturally in coca plants to protect against insect feeding (Nathanson et al., 1993). Other amines that compete for OAT-mediated OA uptake include TA, DA, 2-Phenylethylamine and L-noradrenaline (Evans, 1977).

8.4 Octopamine Receptor Classification and Identification in Invertebrates

Octopamine receptors belong to the GPCR superfamily of receptors (Roeder, 1999; Blenau and Baumann, 2001) and are believed to be good targets as they are invertebrate-specific (Roeder, 1999), and their targeting would not likely adversely affect host biology. OA receptors have been most extensively studied in insects, and therefore, insect OA receptors serve as a model for studying OA receptor function in other invertebrates (Degen et al., 2000). Accordingly, characterization of OA receptors is largely based on their study in insect species.

Initial octopamine receptor classification was based on their identification in *Drosophila melanogaster* in the early 1990s (Evans and Robb 1993; Roeder et al. 1995; Roeder 1999; Han et al. 1998; Balfanz et al. 2005; Maqueira et al. 2005; Evans and Maquiera, 2005). This classification was later amended to indicate parallel signaling mechanisms between OA GPCRs and mammalian adrenergic receptors and to account for the discovery of receptors more potently activated by TA than OA (Von Nickisch-Rosenegk et al. 1996; Balfanz et al., 2005).

OA receptors include β-adrenergic receptor-like OctβR (formerly OA2) receptors, α-adrenergic receptor-like OctαR (formerly OA1) receptors, which share similar pharmacological profiles to β- and α-adrenergic receptors, respectively (Evans, 1981; Evans and Maqueira,

2005), as well as TyrR, TA receptors (Nagaya et al., 2002; Roeder et al., 2003). Not all OA receptors fit this classification. In the *Lymnaea stagnalis* snail, OA GPCR Lym OA2 induces Cl- ion uptake, rather than acting through second messenger fluctuations in cAMP or Ca++ levels, as do the three OA receptor classes.

OctαR receptors share sequence homology with vertebrate alpha-adrenergic receptors, and, like alpha-adrenergic receptors signal through an increase in intracellular Ca++ production (Evans and Robb 1993; Roeder et al. 1995; Roeder 1999).

OctβR receptors share sequence homology with vertebrate beta-adrenergic receptors, including a short third intracellular loop (IL3) (Evans and Maqueira, 2005) and, like beta-adrenergic receptors signal through an increase in cAMP production (Evans and Robb 1993; Roeder et al. 1995; Roeder 1999; Pierce et al., 2002; Chang et al., 2012). OctβR receptors are the most extensively studied OA receptors to date (Roeder, 1992; Roeder and Nathanson 1993), and have been localized to distinct locations in invertebrate tissue. OctβR receptors found in insect CNS tissue (formerly OCT2c receptors) are thought to be the targets of insecticides (Roeder, 1992; Degen et al., 2000).

Tyr R receptors (Blenau et al. 2000; von Nickisch-Rosenegk et al. 1996; Grosmaitre and Jacquin-Joly 2001; Ono and Yoshikawa 2004) signal through a decrease in cAMP production (von Nickisch-Rosenegk et al. 1996). The concept of TA-specific signaling in invertebrates is further supported by the discovery of neurons that express TA and not OA (Saraswati et al. 2003; Cole et al. 2005).

8.5 OA in Helminth Species

In helminth model *C. elegans*, OA has been shown to play a role in motility (Petrascheck et al., 2007), behaviour (Horvitz et al., 1982), fat metabolism (Horvitz et al., 1982; Noble et al., 2013; Mills et al., 2012; Suo et al., 2009), to regulate worm lifespan (Suo et al., 2009), and to have an inhibitory effect on egg laying and pharyngeal pumping (Evans and O'Shea, 1978 ; Horvitz et al., 1982; Alkema et al., 2005; Chase and Koelle, 2007). OA is thought to act more predominantly in

C. elegans adults than in juvenile stages and is described as a physiological antagonist of 5HT (Horvitz et al., 1982). The counteracting effects of OA and 5HT are also described in other invertebrates, including locusts (Evans and O'Shea, 1978; Horvitz et al., 1982) and lobsters (Livingstone et al., 1980; Horvitz et al., 1982). *C. elegans* has a TBH (TBH-1) that is expressed in the RIC interneuron pair in the worm head (Alkema et al., 2005; Chase and Koelle, 2007; Noble et al., 2013). *C. elegans* also has a tyrosine decarboxylase (TDC-1), which synthesizes TA from tyrosine, and is expressed in motorneurons, indicating a neuromuscular role for TA (Rex et al., 2004; Alkema et al., 2005; Chase and Koelle, 2007).

While the role of OA in planarians, has not, to our knowledge, previously been described, the close juxtaposition of DA and OA neurons in planarians suggests that OA may share a similar function with DA (Nishimura et al., 2008), which is involved in learning (Best and Rubinstein, 1962; Shafer and Corman, 1963; Barron et al., 2010), locomotion, and behaviour (Nishimura et al., 2007). Phenolamines OA and TA have been tested in experiments to determine a possible role in determining regeneration polarity in amputated planarians, and were determined not to have an effect on polarity (Chan et al., 2014). Identification of OA in *Dugesia* is of particular interest given its genetic similarity to schistosomes. *D.japonica* tyramine β-hydroxylase (DjTBH) was cloned and characterized. DjTBH is expressed in the ventral nerve chords (VNCs) in a beads-on-a-string pattern and dorsal and ventral portions of the *D. japonica* brain. Confocal immunolocalization reveals that DjTBH is localized to neuronal structures including cell bodies, neuronal axons and dendritic processes. One of two cloned *Dugesia japonica* amino acid decarboxylases, DjAACB, has been speculated to be involved in OA synthesis, synthesizing TA from tyrosine (Nishimura et al., 2006). Alternatively, it is also possible that this AADC may synthesize of 5HT from 5-HTP (Nishimura et al., 2006). The presence of the DjTBH and a putative OA-synthesizing AADC in planarians demonstrates that flatworms are capable of endogenous synthesis of OA. Also, it has been determined that during regeneration of the planarian head, the ratio of neurons containing OA synthesis enzyme DjTBH, to neurons containing DA synthesis enzyme *Dugesia japonica* tyrosine hydroxylase

(DjTH) remains constant (Takeda et al., 2009). This rigid maintenance of the proportion of neurons containing OA-synthesizing enzyme expression points to an important role for OA in planarian neurobiology (Takeda et al., 2009).

Among the parasitic flatworms, the best evidence of an OA system comes from studies of the cestode *Hymenolepis diminuta*, where OA was detected (~2.63pg/g of protein) in the anterior region and large amounts of OA were produced, following incubation of tissue extracts with OA precursor TA (up to ~64% conversion following 12h incubation, compared to ~6.2% conversion to NA) (Ribeiro and Webb, 1983). These studies suggest that cestodes are capable of endogenous OA synthesis, though no biosynthetic enzymes have yet been identified. A few limited studies of schistosomes have shown that the OA precursor, TA, has a stimulatory effect on adult schistosome muscle activity at 100µM, following a ~1 hour incubation (Barker et al., 1966), whereas OA itself had no effect on motility or parasite length in adult worms (Mellin et al., 1983). NE, applied exogenously to worms at 1mM, also had no effect on adult *S. mansoni* (Hillman and Senft, 1973).

8.6 Agonists and Antagonists of OA Signaling

Antagonists and agonists of OA receptors have been identified by determining pharmacological profiles of OA receptors *in vitro*, particularly mollusc OCTbetaR receptors (Blais et al., 2010). Some agonists of OA receptors include naphazoline, clonidine, tolazoline, beta-adrenergic agonist isoproterenol, OA metabolite synephrine, AC-6, demethylchlordimeform (DCDM) and adrenaline (Roeder et al., 2003). Antagonists of OA receptors include anti-serotonergic mianserin, cyproheptadine, phentolamine (a classical alpha-adrenergic receptor antagonist), metoclopramide, promethazine (a classical HA antagonist), beta blocker propranolol, chlorpromazine, epinastine, yohimbine, phentolamine and lysergic acid diethylamide (LSD) (Evans, 1980; Hiripi et al., 1994; Roeder et al., 2003; Finger et al., 2009; Blais et al., 2010). OA signaling stimulation can also occur through an inhibition of OA metabolizing

enzyme NAT, as has been reported for OA receptor agonists DCDM and naphazoline (Martin and Downer, 1989).

While OA receptors are sometimes capable of responding to catecholamines adrenaline and noradrenaline, their pharmacological profiles typically differ from β-adrenergic receptors, where classical β-adrenergic antagonist, propranolol and agonist isoproterenol typically show weak antagonist and agonist activity. Also, antagonistic activity by promethazine, a classical HA receptor antagonist demonstrates this difference in pharmacological profile, highlighting the potential for OA receptors in drug targeting. Previously, incubation of schistosomes with promethazine was shown to cause paralysis of *S. mansoni* (Ercoli et al., 1985). While this effect was attributed to an antagonistic effect on HA signaling, this does not rule out that promethazine may also have an antagonistic effect on OA receptors, which may contribute to this paralysis. It is thought that the small amounts of adrenaline and noadrenaline, which have been identified in the insect nervous system, may also act through OA receptors (Evans, 1980).

Given the importance of OA and TA to invertebrate biology, and their absence in mammals, this is regarded as a promising neural system for drug targeting, particularly with respect to insecticides (Degen et al., 2000). In nature, cocaine, a potentiator of OA signaling, is produced by the leaves of the coca plant *E. coca* as a natural insecticide (Cohen, 1993). Cocaine causes concentration-dependent tremors, rearing, walk-off behaviour and killing of *M. sexta* larvae on cocaine-sprayed tomato plants. OA and to a lesser extent DA, with lower concentration preparations of cocaine protect tomato plant leaves from insect feeding (Nathanson et al., 1993). Potentiators of OA signaling make for ideal insecticides as they interfere with invertebrate behaviour and motor function, and as such, have been exploited pharmacologically.

The most potent synthetic agonists of OA signaling are the formamidines (Harmar and Horn, 1977; Osborne, 1996) and the phenyliminoimidazolidines (PIIs). The formamidines include N-Demethylchlordimeform (DCDM), chlordimeform (CDM), and and amitraz (Palmer et al., 1971; Harrison et al., 1972; Harrison et al., 1973; Baker et al., 1973; Baker, 1975; Evans and

Gee, 1980; Hirashima et al., 2003; Chen et al., 2007; Pohanish, 2015), which are said to act by potentiating OA signaling, via stimulation of OctβRs and inhibition of NAT (Evans and Gee, 1980, Hollingworth and Murdoch, 1980; Kinnamon et al., 1984; Dudai et al., 1987). Structurally, DCDM resembles the phenolamines, particularly OA metabolite SE (Murdoch and Hollingworth, 1980). PIIs include NC-5, one of the most potent OA agonists reported (Osborne, 1996), and the analog NC-7, which are structurally related to the insecticides naphazoline and tolazoline (Nathanson, 1985; Osborne, 1996). Insecticides that are potentiators of OA signaling cause neuronal excitation via stimulation at octopaminergic synapses resulting in tremors, convulsions, continuous flight, increased locomotor activity, uncoordination and changes in feeding and mating behaviour (Evans and Gee, 1980, Yu, 2014).

Given the success of insecticides in targeting octopaminergic signaling, and the absence of phenolamine signaling in the mammalian host, phenolamine signaling, if present in *S. mansoni*, could be specifically targeted to interrupt schistosome motor function and cure infection. Thus, in the first manuscript of this thesis, we will determine whether phenolamines are present in schistosomes, with a focus on the parasitic stages, and determine whether phenolamines have a function in schistosomes.

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CHAPTER II (Manuscript I)

Octopamine Signaling in *Schistosoma mansoni*: Immunolocalization of octopamine (OA) and effects on parasite motility

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(In preparation)

ABSTRACT

Schistosoma mansoni is one of the causative agents of schistosomiasis, a neglected tropical disease that infects more than 250 million people worldwide. Praziquantel has been the mainstay of schistosomiasis treatment for the past 35 years, despite its inefficacy against the juvenile stages of the parasite, and reports of resistant strains obtained from infected individuals. The dependence on a single therapeutic drug to treat such a widespread disease raises concerns for the development of resistance and highlights the need for the development of novel antischistosomals. The nervous system of parasitic worms is an effective target for drug discovery but it remains poorly understood in schistosomes. Here, we report the labelling of *S. mansoni* schistosomula and adult worms with neuronal marker synapsin, providing new insight into the organization of the nervous system in the parasitic stages. Next, we show for the first time that the schistosome nervous system contains high levels of octopamine (OA), a neurotransmitter that is invertebrate-specific, and typically plays a role in neuromuscular signaling and control of movement. Co-immunolocalization studies of OA with synapsin demonstrate localization of OA to the central nervous system (CNS) and most abundantly, the peripheral nervous system (PNS), including innervation of the somatic body wall muscles and sensory neuronal processes. To test for a possible role in motor control, we performed motility studies on schistosomula *in vitro*, to determine the effect of OA and related tyrosine derivatives, including the catecholamine dopamine (DA) and determine concentration-dependent effects on motility and length. We also screened 28 synthetic modulators of tyrosine derivative signaling, to identify a possible agonist or antagonist of OA signaling. We observed concentration-dependent motor-stimulatory and lengthening effects of OA, its precursor tyramine (TA) and its metabolite synephrine (SE). Finally, we showed that two drugs with effects on OA receptors, propranolol, and carvedilol, both have robust, concentration-dependent effects on schistosome motility.

Keywords: *Schistosoma mansoni*, synapsin, nervous system, octopamine, dopamine, neuromuscular

1. Introduction

The digenetic trematode *Schistosoma mansoni* is one of the main causative agents of the Neglected Tropical Disease (NTD), schistosomiasis, also called bilharzia or "snail fever". Schistosomiasis infects more than 250 million people worldwide (Fenwick et al., 2003; Knudsen et al., 2005, WHO, Fact Sheet No. 115), with more than 800 million people at risk of contracting infection (King and Dangerfield-Cha, 2008), and resulting in more than 280,000 deaths per annum (Fenwick et al., 2003; Knudsen et al., 2005; Steinmann et al., 2006; Doenhoff et al., 2008). *S. mansoni* infection has been reported in 78 countries, in tropical and subtropical regions including Africa, South America, the Middle East and the Caribbean. Schistosomiasis has been deemed endemic with moderate to high transmission rates in 52 countries (WHO Fact Sheet No. 115). A single drug compound, praziquantel (PZQ), has been used in schistosomiasis treatment for the past 35 years, despite the fact that it is ineffective against the juvenile stages of the parasite (Sabah et al., 1986; Cioli and Pica-Mattoccia, 2003). Also, while resistance to PZQ at the population level has not yet been reported (Cioli et al., 2012), there have been reports of schistosomiasis cases that did not respond to treatment (Cioli, 2000). Moreover, it is possible to derive a laboratory strain with decreased sensitivity (Cioli, 2012 et al., 2012) and resistance to PZQ (Cioli, 2000; Couto et al., 2011), raising concerns for the development of resistance to PZQ (Doenhoff and Mattoccia, 2006; Melman et al., 2009; Doenhoff et al., 2008). The dependence on a single drug compound and the lack of PZQ efficacy against the larval parasitic stages (Sabah et al., 1986), highlights the importance of developing novel antischistosomals. In other helminths, the nervous system has proven to be a successful drug target. Two such examples are the anthelminthcs ivermectin (IVM) and levamisole (Robertson and Martin, 2007; Kaminsky et al, 2008; Gutman et al., 2010; Wolstenhome, 2011), which exert their effects by acting on proteins involved in neuronal signaling, leading to paralysis (Geerts et

al., 1989; Gill et al., 1991; Geary et al., 1993) and elimination of the parasite from the host. The schistosomicide, metrifonate, which targets *S. haematobium* acetylcholinesterase is a further example of the potential of the helminth nervous system as a target for therapeutic drugs (Bueding et al., 1972; Bloom, 1981 ; Pakaski and Kasa, 2003). Pesticides, such as formamidines and imidazolidines, which inhibit function of invertebrate neurotransmitter octopamine (OA) (Hollingworth, 1980; Nathanson and Kaugars, 1989), all act by disrupting nervous system function.

Being an acoelomate, schistosomes lack a body cavity and a circulating fluid for endocrine signaling (Ribeiro and Geary, 2010). Thus, the majority of the worm's signaling functions are mediated through the nervous system. The nervous system is involved in such biological processes as migration through the host (Crabtree and Wilson, 1980), feeding and egg-laying (Maule et al., 2005), which are central to schistosome infectivity and its associated morbidity and mortality. The importance of this system in the parasite, as well as the success of therapeutics targeting the nervous system in other helminths (Gole et al. 1983; Downer et al. 1985; Martin et al., 1991; Raymond and Sattelle, 2002; Bloomquist, 2003; Keifer and Firestone, 2007; Boulin et al., 2008; Ribeiro and Geary, 2010; Wolstenholme, 2011; Blenau et al., 2012), highlight the potential of neuronal proteins as targets for schistosomiasis treatment.

The organization of the schistosome nervous system has been determined primarily by comparisons with other flatworms (Fairweather et al., 1988; Skuce et al., 1990; Halton et al., 1991; Maule et al., 1992; Brennan et al., 1993 ; Brownlee et al., 1994 ; Halton and Maule, 2004) and a few limited studies of *S. mansoni* adults (Bennett and Bueding, 1971; Gustafsson, 1987) and cercariae (Cousin and Dorsey, 1991; Collins et al., 2011). These studies show that schistosomes share the typical orthogonal neuroanatomy of flatworms. The central nervous system (CNS) is made up of a brain, composed of cerebral ganglia, a bilobed structure made up of a dense axon-rich neuropile, joined by a ring commissure (Halton and Maule, 2004). The brain, in turn, connects to multiple longitudinal nerve cords (Cousin and Dorsey, 1991), which extend from the cerebral ganglia with pairs of dorsal, ventral and lateral nerve cords extending

anteriorly and posteriorly from each lobe. The anterior cords extend to the anterior end of the animal, joining in a single neuropile or ganglion. The CNS longitudinal nerve cords are cross-linked with transverse commissures along the length of the worm, giving the appearance of an orthogonal (ladder-like) pattern, which is characteristic of the entire phylum (Hyman, 1951, Halton and Maule, 2004; Collins et al., 2011).

Flatworms, also have a peripheral nervous system (PNS) made up of finer nerve fibres and plexuses, which are outsourced to all the major bodily structures of the worm (Halton and Gustaffson, 1996; Halton, 2004; Halton and Maule, 2004). Such structures include the somatic musculature, the tegument, the oral and ventral suckers, the reproductive organs, and the alimentary tract (Halton and Gustaffson, 1996; Halton, 2004; Halton and Maule, 2004; Ribeiro and Geary, 2010). The PNS also innervates the worm tegument (Halton and Maule, 2004). In *S. mansoni*, numerous nerve endings and innervated papillae have been identified in the tegument, where they are presumed to play a role in sensation (Gordon et al., 1934; Vercammen-Granjean, 1951; Richard , 1968; Wagner , 1961; Smith et al., 1969; Morris and Threadgold, 1967; Robson and Erasmus, 1970; Matricon -Gondran, 1971; Richard, 1968, Dorsey and Stirewalt, 1971; Morris, 1971, Nuttman, 1971; Short and Cartrett, 1973; Short and Gagne, 1975; Short and Kuntz, 1976; Cousin and Dorsey, 1987; Cousin and Dorsey, 1991). There is also evidence in other flatworms of efferent neuronal fibers having a role in, sensation, as evidenced by surface innervation of both sensory organs and the surface membrane (Dixon and Mercer, 1965).

The schistosomal nervous system is under the control of both peptidergic and small classical transmitters. Among the classical transmitters are acetylcholine (ACh), glutamate and the biogenic amines (BAs). BAs are the largest subset of classical transmitters and are derived from metabolism of aromatic amino acids or histidine. In this study, we identify, for the first time, the presence of octopamine (OA) in the parasitic stages of *S. mansoni.* OA is an invertebrate-specific BA derived from tyrosine metabolism. In other invertebrates, OA and its precursor, tyramine, play an important role in motor control (Sombati & Hoyle, 1984; Hoyle,

1986; Mulloney et al., 1987; Bacon et al., 1995; Roeder et al., 1998; Verlinden et al., 2010). OA signaling in invertebrates is said to be the invertebrate counterpart to the adrenergic signaling system in vertebrates (Roeder, 1999; Blenau and Baumann, 2012). Although OA can be identified in "trace" amounts in mammals, and is thus referred to as a "trace amine", it is primarily an invertebrate signaling molecule. Prior to the findings of this manuscript, OA has never been identified or characterized in schistosomes. OA signaling is absent in mammals and OA signaling could prove to be an attractive drug target for anti-schistosomal drug design.

The aim of this study is to first, demonstrate the presence of OA in the *S. mansoni* nervous system and, second, to determine whether OA is involved in neuromuscular control of parasite motility. We determine that OA is widespread in the nervous system and localizes to neurons of the peripheral nerve net that typically innervate muscle, suggesting a neuromuscular role. Next, to determine a possible role in motility for OA, we performed *in vitro* motility studies to determine the effects of octopamine and related tyrosine derivatives. We also test synthetic compounds that affect tyrosine derivative signaling in other species. Our data suggests that not only is OA present in the parasite, but that it plays an important role in schistosome motility. Given the absence of OA signaling in the mammalian host, and the success of modulators of OA signaling in targeting invertebrate biology, the identification of this neurotransmitter in *S. mansoni* opens up new lines of inquiry for the development of anti-schistosomals.

2. Materials and methods

2.1 Parasites

Larval and adult parasitic stages used in this study were obtained from *S. mansoni*-infected *Biomphalaria glabrata* snails (kindly provided by the Schistosomiasis Resource Center of Biomedical Research Institute (BRI), Rockville, MD, USA). To obtain schistosomula, snails were made to shed cercariae by continuous light exposure for a period of 2 hours, 6-8 weeks following infection. Cercariae were collected and concentrated by incubation at 4 °C for 1 hour.

Cercariae were resuspended in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, MA, USA) with 500µg/ml streptomycin and 500U/ml penicillin (Gibco, Thermo Fisher Scientific, MA, USA) and transformed mechanically to schistosomula by vortexing (Lewis et al., 1986). Schistosomula were cultured in a humidified incubator at 37 °C, 5 % CO_2. To obtain adult stage parasites, 28-day old female CD1 mice were infected with ~200 cercariae each, via penetration through the tail (Smithers and Terry, 1965; Tucker et al., 2013). 7-8 weeks post infection, at the time of patency, mice were sacrificed and adult worms collected via perfusion of the mesenteric venules and the hepatic portal vein (Smithers and Terry, 1965; Carneiro and Lopes, 1986; Lewis, 2001). The described animal procedures were reviewed and approved under Protocol Number 3346 by the McGill University Facility Animal Care Committee, and in keeping with the guidelines of the Canadian Council on Animal Care.

2.2. Confocal microscopy

Parasites obtained using the above-described methods were washed, permeabilized and fixed for confocal immunolocalization, using a previously described protocol (Mair et al., 2000; Taman and Ribeiro, 2009; Patocka et al., 2014) with some modification. Freshly collected adult worms were washed in OPTI-MEM (Thermo Fisher Scientific, MA, USA) and incubated at room temperature for 25 minutes in a 6 well plate, to promote separation of coupled males and females. Adult worms were subsequently washed five times in 1x PBS and placed between two glass slides and submerged in 4 % paraformaldehyde (PFA) at 4 °C for four hours. Schistosomula were cultured for 7 days, washed twice in 1x PBS and incubated in 4 % PFA for four hours with end-over-end rotation. Following fixation, all worms were washed three times in 1x PBS, followed by a 5 min wash in 100mM glycine in 1x PBS. Worms were permeabilized in 2% SDS for 2 hours and then washed three times in antibody diluent (AbD) (0.5% Triton-X-100, 0.1% BSA (bovine serum albumin) in 1x PBS)) followed by an overnight incubation at 4 °C with end-over-end rotation. Worms were subsequently probed with primary antibody specific for the target protein or biogenic amine (BA) of interest in AbD, individually or in combination.

Negative controls include parallel experiments omitting primary antibody, and, for anti-octopamine antibody, preadsorbtion with 0.19 g/ml (1mM) OA. Primary antibodies, anti-OA (EMD Millipore, Merck Millipore), anti-serotonin (EMD Millipore, Merck Millipore) and a monoclonal anti-synapsin antibody (anti SYNORF1, submitted to the DSHB ((Developmental Studies Hybridoma Bank), http://dshb.biology.uiowa.edu/, created by the NIH NICHD and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242) by E. Buchner), were added at dilutions of 1:80, 1:100 and 1:35, respectively and incubated for 3 days at 4 °C, with end-over-end rotation. In schistosomula, anti-synapsin was added at a titer of 1:25, rather than 1:35. Adult worms were washed three times, and schistosomula once, and incubated overnight in AbD. Secondary antibody was added at a dilution of 1:800 with an overnight incubation. In some experiments, 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) at a dilution of 1:500 (20ng/ml) was also added, to stain nuclei, and secondary antibody, with or without DAPI, was subsequently incubated for two additional days. Secondary antibodies used include a FITC-conjugated goat anti-rabbit antibody and an HRP-conjugated goat anti-mouse antibody. Adult worms without anti-synapsin were washed 4 times, and schistosomula twice, in AbD and mounted for visualization with confocal miscroscopy. Worms probed with anti-synapsin were prepared as follows: Following incubation with secondary antibody, the Alexa-594-conjugated tyramide signal amplification (TSA) reagent from a kit (TSA™ Kit #15, Thermo Fisher Scientific, MA, USA) was added to the kit-provided AbD, supplemented with 0.0015% H_2O_2, at a dilution of 1:80, and incubated at 4 °C for 1 hour with end-over-end rotation. Adult worms were washed 5 times, and schistosomula 3 times, before mounting. Mounted specimens were visualized with a Zeiss LSM710 confocal microscope (Carl Zeiss Inc., Oberkochen, Germany) with ZEN 2010 software (Carl Zeiss Inc., Oberkochen, Germany). Argon (488 nm) and HeNe (594 nm) lasers were used to excite dyes and fluorophores and obtain images. Filter sets were adjusted to minimize overlap of emission wavelengths and non-specific bleed-through of the acquired signal due to spectral overlap.

2.3 Testing BAs and related compounds on schistosomula

In vitro transformed schistosomula were cultured in 24 well plates at ~150 schistosomula /well for 7-days in 850 µl Opti-MEM supplemented with 5% heat inactivated FBS, 100µg/ml streptomycin and 100U/ml penicillin. Animals were acclimated to room temperature for 15 minutes and subsequently, motility was analyzed to give baseline readings using a previously described protocol (El-Shehabi et al., 2012). The test compound was administered at concentrations ranging from 0 (vehicle alone control) to 500 µM. Recordings were taken prior to the addition of drug compound (baseline reading) and 20 minutes following addition of drug compound or vehicle (H_2O or 0.015% DMSO). Schistosomula were filmed for 1 min with a Nikon SMZ1500 microscope equipped with a QICAM Fast 1394 (mono 12 bit, QImaging, BC, CA) digital video camera and video acquired with SimplePCI software (version 5.2, Compix Inc., AB, CA), at ~2.5 frames per second (fps). A minimum of 12 schistosomula were recorded per well, in a total minimum of three distinct fields of vision, from 2 to 3 wells per treatment. All schistosomula in the field of the video were analyzed, unless in contact with other schistosomula, to avoid bias in selection. ImageJ software (version 1.41, NIH, USA) was used to quantify worm motility as a measure of length changes over the period of the 1 min recording, using the ImageJ Fit Ellipse algorithm, as has been previously described (Patocka and Ribeiro, 2013). The length of the ellipse, per frame, was measured using the Major Axis measurement of the ellipse, in ImageJ, as an indicator of schistosomulum length.

2.4 Phenotypic screening of schistosomula using putative agonists and antagonists of DA- and OA-signaling

Agonists and antagonists of DA-signaling were selected from a Food and Drug Administration (FDA) library (Pharmakon Pharmaceuticals, Inc., IN, USA) with a preference for compounds that also affect OA signalling in invertebrates (Table 1). Adrenergic signaling is considered the vertebrate counterpart to OA signaling and adrenergic compounds may affect OA signalling (Evans, 1981; Roeder, 1999; Blenau and Baumann, 2012). Agonists and antagonists of

adrenergic signalling were thus selected in addition to compounds which modulate OA-signaling in other invertebrates (Table 1) from the FDA library. Screens were performed on schistosomula at the University of California, San Francisco's Center for Discovery and Innovation in Parasitic Diseases (CDIPD) and Small Molecule Discovery Center (SMDC). Cercariae were obtained from *Biomphalaria glabrata* snails, suspended in Basch medium (Basch, 1981) and transformed via passage through a 22 G double-headed luer lock needle to remove cercarial tails (Abdulla et al., 2009; Stefanic et al., 2010). Schistosomula were washed three times in medium supplemented with 80 μg/ml streptomycin and 80 U/ml penicillin on ice. Schistosomula were resuspended in Basch medium supplemented with 5% FBS and added to 96 well round-bottomed clear plastic plates. Images of schistosomula were acquired in time-lapse an IN Cell Analyzer 2000 (GE Healthcare, IL, USA) over 20 sec. Parasite motility and length in the presence and absence of compound were compared and assessed manually by playing videos that had been generated using ImageMagick (http://www.imagemagick.org/) side by side. Videos were also compared to wells to which vehicle was added and assessed manually, to ensure that similar effects did not occur with vehicle in the corresponding experiment. All experiments were performed in triplicate and a minimum of 15 animals were analyzed per well.

3. Results

3.1. Labelling with neuronal marker synapsin reveals the organization of the nervous system in adult *S. mansoni*

Neuronal marker synapsin was labelled, using a monoclonal antibody against Drosophila synapsin, which was previously shown to recognize *S. mansoni* synapsin (Collins et al., 2011). Samples were treated with a commercial signal amplification reagent (Tyramide Signal Amplification, Thermo Fisher Scientific, MA, USA), as described previously (Collins et al, 2011), so as to increase the sensitivity of the assay. Immunolabelling without the TSA amplification reagent did not yield a detectable signal. The results show widespread labelling of synapsin throughout the parasite CNS (**Figure 1**). The characteristic bi-lobed brain and the the

commissural ring linking the cerebral ganglia (CG) are clearly visible (**Figure 1A**). All pairs of major longitudinal nerve cords are labeled and can be seen originating from the cerebral ganglia, including ventral (VNC) (**Figure 1A, B**), lateral (LNC) (**Figure 1C, D**) and dorsal (DNC) (**Figure 1C, E**) nerve chords. The main cords are periodically cross-linked by synapsin-positive transverse commissures, which are varicose in appearance, consistent with the classic orthogonal organization of the flatworm nervous system. A lateral nerve cord, with an associated finer longitudinal nerve fiber is visible running along the length of the worm body, with a network of finer neurons of the peripheral nervous system (PNS), which extend outwards, towards the surface of the worm, demonstrating innervations of the CNS and PNS all throughout the worm body (**Figure 1F**). Co-staining with DAPI reveals that synapsin labels what appears to be neuronal cell bodies at the junctions between a longitudinal nerve chord and connecting perpendicular fibers, likely of the PNS, suggesting synaptic junctions along longitudinal nerve fibers (**Figure 1G**). In the midbody, a ventral nerve chord is visible along the flap of the gynecophoral canal (**Figure 1H**). The gynecophoral canal flaps are highly innervated and sensation and control of the flaps are likely important in the coupling of male-female worms. To compare the distribution of synapsin with that of a known neurotransmitter, we co-labeled adult worms using antibodies against synapsin (red) and 5HT (green) (**Figure 1I**). Labelling of 5HT is proximal to that of synapsin, with some overlap, indicated by regions of yellow, though perfect colocalization is not observed. This labelling likely indicates that 5HT and synapsin are enriched in separate regions of the neuron, or that the level of synapsin in serotonergic neurons is below the detectable threshold under the conditions of this experiment. Negative controls labeled with secondary antibody only did not produce significant labelling (**Figure 1J**).

3.2 Synapsin labelling in schistosomula

Synapsin was used to label the nervous system of fixed 7-day old schistosomula. As in adult parasites, labelling synapsin primarily demonstrates the organization of the CNS. A single pair

of CG, as described in cercariae (Collins et al., 2011) are visible in the center of the worm body (**Figure 2A**). VNCs extend longitudinally from the CG (**Figure 2A**) and DNCs (**Figure 2B**) and LNCs (**Figure 2C, D**) are also visible. The DNCs, with LNCs and VNCs, produce a cage-like structure with transverse commissures (**Figure 2C**) joining the sets of longitudinal nerve chords along the length of the worm. The DAPI counterstain reveals a densely nucleated schistosomula with a concentration of nuclei within the cage formed by the CNS network (**Figure 2D**). CG are present in the body segment, as has been documented in the literature (Cousin and Dorsey, 1991; Collins et al., 2011) (**Figure 2D**). Labelling schistosomula with the HRP-conjugated secondary antibody alone and the TSA reagent does not result in significant labelling, demonstrating specificity of the antibody and TSA reagent (**Figure 2E**).

3.3. Labelling with anoctopamine (OA)-specific antibody reveals widespread labelling in neurons of the brain, and in the central and peripheral nervous system of adult schistosomes

Adult male and female worms were fixed, permeabilized, and probed with a commercial anti-octopamine (anti-OA) antibody, followed by a secondary antibody conjugated to Alexa-488. DAPI was used as a counterstain in some experiments, and the synapsin neuronal marker was used to verify localization to nervous tissue. OA was determined to be present in the CG and commissural ring, as well as the anterior ganglia and associated nerve chords that supply the oral sucker (**Figure 3A**). OA labelling was confirmed to be neuronal by generating a composite of OA and synapsin labelling, which showed regions of colocalization of OA and synapsin, indicated in yellow (**Figure 3B**). Two pairs of ganglia, labeled by synapsin and OA, are observed. Two pairs of ganglia have previously been described in other platyhelminthes, specifically, in tapeworms (Kotikova and Raikova, 2008). At higher magnification, we observed what appear to be cell bodies containing OA along the surface of a ganglion neuropile (**Figure 3C**). Besides the brain and oral sucker, OA is present in the longitudinal nerve chords and transverse commissures of the CNS throughout the length of the body, as well as in fine nerve fibres of the PNS that extend to the worm surface, both in males (**Figure 3D, E**) and females (**Figure 3F, G,**

H). The pattern of OA labelling is similar to what is observed with synapsin, once again indicating that OA localizes to the nervous system. OA was detected in the innervation of the gut, with a large nerve fibre running along the outside of the caecum, which could suggest a role in feeding and digestion (**Figure 3I**). In the male, at high magnification (63x magnification), we see strong OA labelling in a network of fibres resembling the peripheral submuscular nerve net of the PNS (**Figure 1J**), with a criss-crossing of fibers innervating body wall muscle (**Figure 1J**). This suggests a possible role for OA in neuromuscular control (motility). As negative controls, worms were probed with anti-OA antibody preadsorbed with OA (**Figure 1K**), or secondary antibody alone (**Figure 1L**). These negative controls did not yield significant fluorescence, demonstrating specificity of the antibody. Probing with preadsorbed OA does yield some non-specific fluorescence on the worm surfacebut there is no apparent labelling of neuronal structures or a pattern matching any of the worms probed with anti-OA alone.

3.4. Labelling of octopamine (OA) and synapsin reveals the presence of OA in the female ovary in the reproductive system and in the nerve net of the developing embryo

In females, OA labelling was prominent all along the reproductive tract (**Figure 4A**), including the ovary (**Figure 4B, C**), where we also see a concentration of DAPI-stained nuclei, as described (Neves et al., 2005). The ovary contains the precursors to schistosome eggs, oocytes, each with a single nucleus, prior to their passage through the oviduct. A schistosome egg co-labeled with OA and synapsin antibodies is shown adjacent to a female worm. The embryo within the schistosome egg, shows apparent colocalization of OA and synapsin (**Figure 4D, E, F**) as indicated by regions of intense yellow fluorescence. Individually, synapsin (**Figure 4E**) and OA (**Figure 4F**) both produce a web or mesh-like pattern throughout the length of the embryo. Apart from descriptions of the formation of the brain (Jurberg et al., 2009), not much is known about the organization of the nervous system in the embryo or the transmitters involved in signaling. The size of the embryo (over 100 μM in length) and the fact that it is elongated in shape and occupies a majority of the internal area of the egg (Jurberg et al., 2009) suggests that

it is in a later stage of development, possibly stage 7, of the 8 stages of embryonic development of schistosome eggs (Jurberg et al., 2009). The stage cannot be determined conclusively without further staining and histological assessment. These results suggest, for the first time, that OA could play a role in the developing nervous system of schistosome embryos.

3.5. Co-Labelling of octopamine (OA) with the neuronal marker synapsin reveals surface innervation of OA in adult parasites

OA-containing neurons were detected near the suface of adult male worms, including the tubercles (**Figure 5A**), which are enriched in sensory nerve fibers and nerve endings. OA and synapsin labelling in the tubercles is pronounced and the overlay shows intense yellow fluorescence, suggesting co-localization or, at least, close proximity of the two signals. From the tubercles, inwards, OA-positive nerve fibers are varicose in appearance, showing a typical "beads-on-a-string pattern", which closely matches that of synapsin labelling, to form a web-like organization beneath the worm surface. The innervation of tubercles connecting to the subtegumental nerve net suggests a role in sensation for OA. The progression of tubercle-innervating octopaminergic neurons downwards into the body, and intersection with synapsin labeled longitudinal nerve chords of the CNS, indicates a possible efferent innervation of octopaminergic sensory neurons onto the CNS. This labelling of synapsin from deeper in the worm body to the tubercles is evident with the synapsin-transmitted light image overlay (**Figure 5B**). A merging of PNS octopaminergic neurons with CNS synapsin-labeled neurons is also evident in larger nerve fibers in the midbody (**Figure 5C**). OA labelling of the surface progresses deeper into the body, merging with synapsin-labeled large nerve chords. Proximal to the synapsin-labeled longitudinal nerve fiber, perpendicular octopaminergic neurons closely match the labelling of finer synapsin-labeled nerve fibers (**Figure 5D**), indicating a progression of synapsin-rich CNS nerve fibers to OA-rich PNS nerve fibers in the midbody. Octopamine labelling also closely matches neurons on the outer flaps of the gynecophoral canal, indicating extensive innervation of the gynecophoral canal (**Figure 5E**). Short nerve fibers with OA and

synapsin labelling, positioned along the flap of the canal is apparent (**Figure 5E**). Labelling with secondary antibodies and the amplification reagent alone did not produce a non-specific signal (**Figure 5F**).

3.6. Immunolabelling of octopamine reveals localization to fine nerve fibers of the PNS in schistosomula

Octopamine (OA) labelling was also tested in fixed 7-day old schistosomula, using the same anti-OA antibody and FITC-conjugated secondary antibody described in the labelling of the adult parasite, and DAPI, sometimes used as a counterstain. Unlike synapsin labelling, which is primarily detectable in the CNS of the larvae, OA is largely restricted to the peripheral nervous system (PNS) in 7-day old schistosomula. Diffuse labelling is present in concentric circles near the surface of the worm (**Figure 6A**), a pattern similar to that previously described for other neuronal proteins in the larvae (Patocka et al., 2014), suggesting labelling of the peripheral nervous system (PNS). OA is also observed in fine nerve fibers, and what appear to be cell bodies, which are connected in a web-like organization along the length of the body, directly beneath the surface (**Figure 6B**). This labelling of OA demonstrates a more disorganized PNS than what is observed in adult schistosomes, consistent with a nervous system still under development. Labelling of a pair of punctate sites in the mid body is also apparent in some schistosomula, which may indicate OA in the CNS and the bi-lobed early developing cerebral ganglia (**Figure 6C**). Taken together, these images demonstrate the presence of OA-positive neurons in schistosomula, in PNS-like nerve fibers, and what appear to be the developing cerebral ganglia of the CNS. Labelling with secondary antibody alone did not produce non-specific labelling (**Figure 6D**).

3.7 Exogenous administration of tyrosine derivatives and related compounds to schistosomula causes concentration-dependent changes in length and frequency of body movements

Day 7 (D7) old schistosomula were incubated with 500 µM OA or related biogenic amine (BA) tyrosine derivatives, including other phenolamines and catecholamines. The goal of these experiments was to determine whether these compounds would cause changes in length of the larvae (**Figure 7**) and/or changes in the frequency of body movements (**Figure 8**), which might indicate a role in muscle control. This schistosomula parasitic stage is a primary focus of this study, as chemotherapy targeting the early larval migrating stage of schistosomiasis is not currently available. D7 old schistosomula correspond to the late lung stage of infection, which typically modulate the host immune response and are adept at evading the host immune system, more so than the early larval stages, including cercariae and 1 to 4-day old, pre-lung stage schistosomula (Gobert et al., 2007). Phenolamines were not known to affect schistosome motility. Here, however, we see that 3 phenolamines tested consistently produced a significant increase in the frequency of body movement. The strongest stimulation was observed with synephrine (SE), an OA derivative (~22-fold), followed by OA precursor tyramine (TA) (~12-fold), and OA itself (~5-fold). These effects were comparable to, or several fold higher than the stimulation caused by serotonin, which is a well-known myoactive substance in this parasite (Boyle and Yoshino, 2005; Patocka et al., 2014). Among the catecholamines, the strongest effect was observed with metanephrine (ME) (~14-fold), the methylated derivative of adrenaline. Other substances tested produced either very weak stimulation or had no measurable effect. HA, which has previously been determined to cause an increase in motility of *S. mansoni* cercariae (Ercoli et al., 1985), had no significant effect in schistosomula, compared to the vehicle control.

Besides measuring frequency of body movements, we also recorded the effects of amine transmitters on the average length of schistosomula, following a 20 minute incubation with 500 µM test amine. Dopamine (DA) was previously shown to cause a lengthening of the

parasite body (Pax et al., 1984) and we observed a similar effect of OA when added directly to schistosomula (**Figure 7**). It should be emphasized that the lengthening occurred quickly, within minutes of treatment, indicating that the change in morphology is due to an effect on the body wall muscles rather than growth of the parasite. A quantitative analysis revealed that catecholamines (DA, NE) and phenolamines (OA, TA, SE) all produced significant elongation of the body, representing an increase in length of ~20% compared to the untreated controls. The adrenaline derivative, ME, also produced an increase of about 20% in body length, whereas the remaining amines tested had minimal or no effect.

To further investigate the effects of phenolamines and dopamine on larval length and movement, assays were repeated over a concentration range of 1-500 µM (**Figure 9**). Effects were observed following a 20 minute incubation, and expressed as a fold change, compared to the vehicle-treated control (0 µM). Length and motility values were expressed as a fold change of baseline motility, or length, for each well, prior to normalization to the vehicle-treated control. As the changes in length observed, correspond to effects following a short incubation period, these effects are attributed to changes in muscle control, and not growth. The vehicle-treated negative control rules out the possibility that these effects are due to physical perturbation or a change in worm stasis due to addition of liquid to the worms in culture.

The phenolamines, OA, TA and SE all produced a concentration-dependent stimulation of movement, though OA was generally less potent than its precursor, TA, or metabolite, SE. TA and SE both caused significant stimulation at concentrations as low as 1 µM, whereas OA had no significant effect below 100 µM (**Figure 9**). Unlike the phenolamines, dopamine caused significant, concentration-dependent inhibition of movement at 10 to 100 µM. At 500 µM, however, the effect was reversed and motility was increased relative to the control, suggesting that dopamine can either inhibit or stimulate movement, depending of the concentration used.

Apart from producing changes in motility, phenolamines and dopamine also cause a concentration-dependent increase in schistosome length (**Figure 9**). OA produced a biphasic curve showing a modest (but statistically significant) shortening of the body at lower

concentrations (1-50 µM), whereas higher concentrations (100, 500 µM) caused a concentration-dependent increase in length up to ~20%. The other phenolamines and dopamine.all produced a concentration-dependent increase in length. Dopamine had the strongest effect, causing as much as 40- 60% increase in body length relative to the baseline.

3.8 A phenotypic compound screen to test putative agonists and antagonists of DA and OA signaling, identifies synthetic compounds producing pronounced changes on length and motility in schistosomula

Having thus confirmed concentration-dependent effects of tyrosine derivatives on schistosomula length and motility, we wanted to identify antagonists and agonists *in vitro*, by first screening synthetic compounds that may modulate tyrosine-derivative signaling, and secondly, performing concentration-response assays of select compounds. We screened 28 compounds that are agonists or antagonists of catecholamine and adrenergic signaling, as well as compounds identified in the literature as modulating OA signaling in an invertebrate species (**Table 1**). Modulators of adrenergic activity were selected based on the similarity between OA and adrenergic receptors (Roeder, 1999; Blenau and Baumann, 2001) and the possibility that these compounds may also modulate OA signaling. Compounds indicated in **Table 1** were tested at concentrations of 10 µM and 65 µM, to determine if a phenotype resulted, as compared to the well at baseline. Schistosomula were seeded at ~25 worms per well, in a 96 well, round bottom plate plate, and cultured for 7 days, prior to addition of the compounds. Worms were recorded at baseline, as well as 20 minutes following addition of the compound at each concentration, in triplicate. The experiment was repeated twice, not including subsequent dose response assays. Videos were generated using an IN Cell Analyzer 2000 (GE Healthcare, IL, USA), recorded for 20 seconds, to obtain 30 time-lapse images at a rate of 1.66 Hz equivalent to 1.5 fps. Videos to compare motility between vehicle-treated controls and wells treated with synthetic compounds were generated using ImageMagick (http://www.imagemagick.org/) software. Compounds producing the most robust motor responses at 65 µM are listed in **Table**

2. The most pronounced changes observed include hypermotility (highlighted in red) and hypomotility (blue). Among those drugs causing hyperactivity are amitraz and epinastine, two putative ligands of OA receptors in other species (Evans and Gee, 1980 , Hollingworth and Murdoch, 1980; Goldman et al., 1980; Dudai et al., 1987; Roeder et al., 1998). 6 compounds (acepromazine, carvedilol, chlorpromazine, clozapine, cyproheptadine and prazosin) produced a hypomotile effect. Prazosin was also previously determined to cause a slowing of parasite motility when tested over a longer, 24 hour, incubation period and in adult parasites (Abdullah et al., 2009), rather than short-term (20 minute) incubation period in schistosomula. Chlorpromazine (CPZ) and its related analogue, acepromazine, and carvedilol (CAR) produced paralysis of schistosomula, and chlorpromazine, producing the more pronounced effect, was selected for a dose response assay (**Figure 10**). CPZ was previously shown to inhibit DA signaling in schistosomula dopamine receptor SmD2 (Taman and Ribeiro, 2009). We also decided to pursue CAR, a mixed alpha- and beta-adrenergic antagonist, to identify a possible OA antagonist. 7 compounds produced a lengthening effect, all of which, excluding prazosin, an alpha-adrenergic antagonist, also produced a hypermotile effect, and include clonidine, levobunolol, mianserin, naphazoline, propranolol (PR) and tolazoline. All compounds causing a pronounced increase in relative motility, accompanied by an increase in length, are classified in the literature as having some adrenergic activity and are potential agonists of OA signaling, apart from mianserin, which has been identified as a 5HT antagonist in adult schistosomes (Willcockson and Hillman, 1984). We selected PR to perform a concentration-response assay, to identify a potential OA agonist, as this compound is associated with modulating adrenergic signaling (beta-adrenergic antagonist), and in some invertebrate species, OA signaling, and given the pronounced effect on length and motility that we observed in the screen. We wanted to identify both an agonist and antagonist of OA signaling, as agonists of OA signaling are proven drug compounds, as with the formamidine and imidazolidine classes of pesticides (Vans and Gee, 1980, Hollingworth and Murdoch, 1980; Goldman et al., 1980; Dudai et al., 1987). Given the stimulatory effect of OA on schistosomes, an antagonist of its activity would likely

cause paralysis, allowing for clearance of the parasite from the host, and would also be an ideal anti-schistosomicide.

CPZ was previously reported to cause moto-stimulation at a concentration of 1 µM in schistosomula (Abdullah et al., 2009). This was confirmed in the present study but the effects are concentration-dependent, both with respect to movement and body length (**Figure 10**). CPZ is stimulatory at concentrations of up to 10 µM, whereas at higher concentrations there is a significant decrease in movement and a shortening of the body relative to the control (at 500 µM). These effects are opposite to the effects of adding DA to parasites in culture and are consistent with a DA signaling antagonist. Carvedilol, an adrenergic drug (Beta-blocker) caused almost complete paralysis at most of the concentrations tested, as well as significant shortening of the body at higher concentrations (50 to 500 µM) (**Figure 10**). The paralytic effect of the drug mirrors the expected result of an OA antagonist, where a concentration-dependent inhibition of motility is expected. Finally, propranolol, also an adrenergic drug caused concentration-dependent increases both in movement and body length. These effects are consistent with possible agonist activity towards OA or TA receptors, though we cannot rule out interactions with other receptors.

4. Discussion

In this study, we discovered OA and characterized the effect of OA and related tyrosine derivatives in the schistosome parasite, specifically *S. mansoni*. We used an antibody against OA to determine that it is widespread and abundant in the NS. To verify that OA is found in neuronal tissue, and to gain a better understanding of the organization of the nervous system in the parasite, we used an antibody against the neuronal marker, synapsin.

The schistosome nervous system has previously been described as being comprised of a brain (CG), (Halton and Maule, 2004) and three pairs of longitudinal nerve chords, the VNCs, LNCs and DNCs. The longitudinal nerve chords are cross-linked by transverse commissures along the length of the worm, in an orthogonal pattern (Hyman, 1951, Halton and Maule, 2004;

Collins et al., 2011). These nerve chords and the CG make up the CNS. The PNS is comprised of finer nerve fibres and plexuses (Halton and Gustaffson, 1996; Halton, 2004; Halton and Maule, 2004) and innervate the main body structures including the body-wall muscle and the tegument. By labelling neuronal marker synapsin, we confirmed this organization of the CNS in the parasitic stages: cercariae and adult parasites. Labeling synapsin in these life stages is a valuable tool to visualize the nervous system and determine whether a specific neurotransmitter localizes to nervous tissue. In the adult parasite, we co-labelled synapsin with both OA and 5HT, demonstrating that this marker can be used to co-label neurotransmitters in the parasitic stages of *S. mansoni*. We also determined that there are 2 pairs of CG in the adult brain, which was not previously known. Based on co-labelling of OA and synapsin, OA seems indeed to be a schistosome neurotransmitter.

OA was observed in the four lobes of the CG in the adult parasite and throughout the CNS and PNS. Localization of OA to the PNS is also observed in schistosomula, where OA is largely restricted to the PNS, though OA also appears to be located in the CG of schistosomula.

The localization of OA to neurons lining the female reproductive tract, including the ovary, and in neurons of developing embryos, suggests a role for OA in egg-laying and egg development. A contribution by OA in egg-laying has been reported in the nematode *C. elegans* (Horvitz et al., 1982; Alkema et al., 2005; Chase and Koelle, 2007*)*.

The colocalization or close juxtaposition of OA and synapsin in tubercles and in neurons linking tubercles to the deeper regions in the body in adults indicates a role in sensation for OA and points to efferent signaling in schistosomes.

Localization of OA to the PNS in both life stages and, in adult parasites, the nerve net which typically innervates body-wall muscle, suggests a neuromuscular role for OA.

Our discovery of OA, an invertebrate-specific neurotransmitter, in *S. mansoni* and its widespread localization and abundance in the parasite highlights its potential for drug targeting. Our use of the neuronal marker synapsin to label the schistosome nervous system

sheds new insight into the neuronal organization of schistosomes and identifies a neuronal marker which can be used to verify neuronal localization in the parasitic stages of *S. mansoni*.

Given the localization of OA to the peripheral nerves, typically innervating body-wall muscle and OA's contribution to motor control in other invertebrates (Sombati & Hoyle, 1984, 1986; Bacon et al., 1995; Roeder et al., 1998) it seems likely that OA plays a role in schistosome motility, a notion supported by the increased motility of the parasite in the presence of exogenous OA. To verify that OA is involved in motor control of schistosomes we incubated schistosomula with OA and related tyrosine derivatives and measured changes in motility and length using an established quantitative assay employing ImageJ (El-Shehabi et al., 2012; Patocka et al., 2013; Patocka et al., 2014; Macdonald et al., 2015). We determined that phenolamines OA, SE and TA and the catecholamine DA modulate schistosome length and motility in a concentration-dependent manner. Using a phenotypic screen, we identified a putative agonist and antagonist of OA signalling which we confirmed to be concentration-dependent using the established quantitative method. We also determined that the inhibitory effects of a DA antagonist are concentration-dependent.

Prior to the findings of this manuscript, it was not known that OA and related phenolamines affect schistosome motility. By quantifying the concentration-dependent effects of these phenolamines, we demonstrate that these BAs stimulate motility and cause a lengthening effect in schistosomes. By identifying a putative antagonist and agonist of phenolamine signalling and characterizing the effect of an antagonist of catecholamine signalling, we identify compounds which can be pursued to develop anti-schistosomals.

OA elicits a ~3-5 fold increase in schistosomular motility and compounds related to OA also induced concentration-dependent hypermotility, namely TA (~12 fold increase) and SE (~22-35 fold increase). The more pronounced effect of SE on schistosome motility is consistent with a trend observed for 5HT and DA in schistosomes, whereby their methylated derivatives more significantly altered motility than the precursor BA (Patocka et al., 2014; El-Shehabi et al., 2009). We also observed this effect for the EPN metabolite ME. This finding suggests that SE

may have a higher affinity for OA receptors, by virtue of the methyl group on the amine moiety, the only difference between the OA and SE structures. The concentration-dependent effects of DA on motility in D7 schistosomula differ somewhat from those observed in D4 schistosomula, which are characterized by a consistent decrease in relative motility up to 100 µM (El-Shehabi et al., 2012). In D7 schistosomula, an initial decrease in motility up to 50 µM is observed, with a moderate increase in motility at high concentration. These results suggest that the effect of BAs on the parasite may vary throughout schistosome development. Phenolamines produced a concentration-dependent increase in length up to 30%, while DA stimulation of length was most pronounced (60% increase).

OA signaling is considered the invertebrate equivalent of adrenergic signaling in vertebrates (Roeder, 1999; Blenau and Baumann, 2001), as both neurotransmitters are involved in glycogenolysis, in fat metabolism, act as stress hormones and modulate muscle contraction (Roeder, 1999). Moreover, OA receptors have signaling properties similar to beta- and alpha-adrenergic receptors (Evans, 1981; 1985; Evans and Robb, 1993; Roeder, 1995; Roeder, 1999). Thus, alpha- and beta-blockers are of interest as potential antagonists of OA signaling. Drug targets in invertebrates are typically OA receptors (Hollingworth and Murdock, 1980; Ahmed et al., 2015), and antagonists of OA might paralyze schistosomula.

Our phenotypic screen identified potential agonists and antagonists of OA and DA signaling. We selected three compounds to characterize further, including the DA antagonist, CPZ, a putative antagonist, CAR, and an agonist of OA signaling, PR. Concentration-specific effects were observed for these three compounds.

OA-signaling produces an increase in motility, as well as an increase in length, likely mediated by stimulation of circular muscle contraction and possibly relaxation of longitudinal muscle, as suggested by the effects of muscle contraction/relaxation on motor control (Pax et al., 1984; Halton and Maule, 2004). Hypermotility likely arises from stimulation of either circular or longitudinal muscle. Hypermotility resulting from an increase in longitudinal muscle tone and a relaxation of circular muscle produces an increase in motility without an

accompanying increase in length (Pax et al., 1984). Relaxation of longitudinal muscle has been associated with an increase in length (Pax et al., 1984). The increase in length and motility which we observe is therefore likely caused by a stimulation of circular muscle rather than longitudinal muscle, which may be accompanied by a relaxation of longitudinal muscle. Given the dynamic interplay between longitudinal and circular muscle in schistosomula, as well as the activity of BAs and related compounds, further study of these signaling systems is warranted. Heterologous expression of an OA receptor would allow more complete testing of putative antagonists. RNAi, or heterologous expression and characterization of BA signaling proteins, would give insight into the role of these pathways. The whole organism screen has the benefit of identifying compounds that modulate muscle control and movement, demonstrating the potential for future drug development.

BAs were also tested on adult parasites (not shown). An increase in motility of ~3-fold baseline motility was observed for 5HT, as previously reported, though the other BAs tested, including OA, TA and DA, did not produce a significant change in motility. The absence of an effect observed following addition of OA to the adult parasite is consistent with previous reports (Hillman and Senft, 1973). The abundance of OA in the nervous system and localization to the peripheral neurons that innervate muscle suggests that OA is an important neurotransmitter in adult parasites which may be involved in motor control. The absence of a significant effect following incubation with OA in adults may mean that these BAs are not taken up from the environment. Localization to the tegument is associated with uptake from the environment (Catto and Otteson, 1979; Patocka and Ribeiro, 2007; Faghiri and Skelly, 2009). While a putative OA transporter (OAT) has been identified in *S. mansoni* (Ribeiro and Patocka, 2013), it may not be localized to the worm surface. OA could not be obtained from the vertebrate host, negating the requirement for an OAT on the tegument.

Our discovery that OA is present in *S. mansoni*, that OA and related tyrosine derivatives play an important role in motor control and that antagonists and agonists of tyrosine derivative signaling also modulate schistosome motor control provides strong evidence for these

neurotransmitters as important signaling molecules in *S. mansoni*. Putative phenolamine signaling genes have been identified in *S. mansoni* (Ribeiro et al., 2012; Protasio et al., 2012), though these genes have not yet been characterized. While several DA signaling genes have been identified and characterized in *S. mansoni* (Taman and Ribeiro, 2009; El-Shehabi et al., 2012), their roles in *S. mansoni* motor control have not been fully elucidated. Further characterization of these tyrosine derivative related signaling genes and their roles in *S. mansoni* motor control using reverse genetics can shed further insight into the importance of tyrosine derivative signaling in schistosomes compared to other neurotransmitters.

OA and its precursor TA are the only non-peptide neurotransmitters that are specific to invertebrates (Degen et al., 2000). Given the role of phenolamine signaling in a wide range of invertebrate functions, its absence from the human host and the success of OA-based insecticides (Hamshezadeh et al., 1985), our discovery and description of OA signaling in *S. mansoni* and its modulation by small molecule agonsists and antagonists opens new lines of inquiry for the development of potent and specific schistosomicides.

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Figures

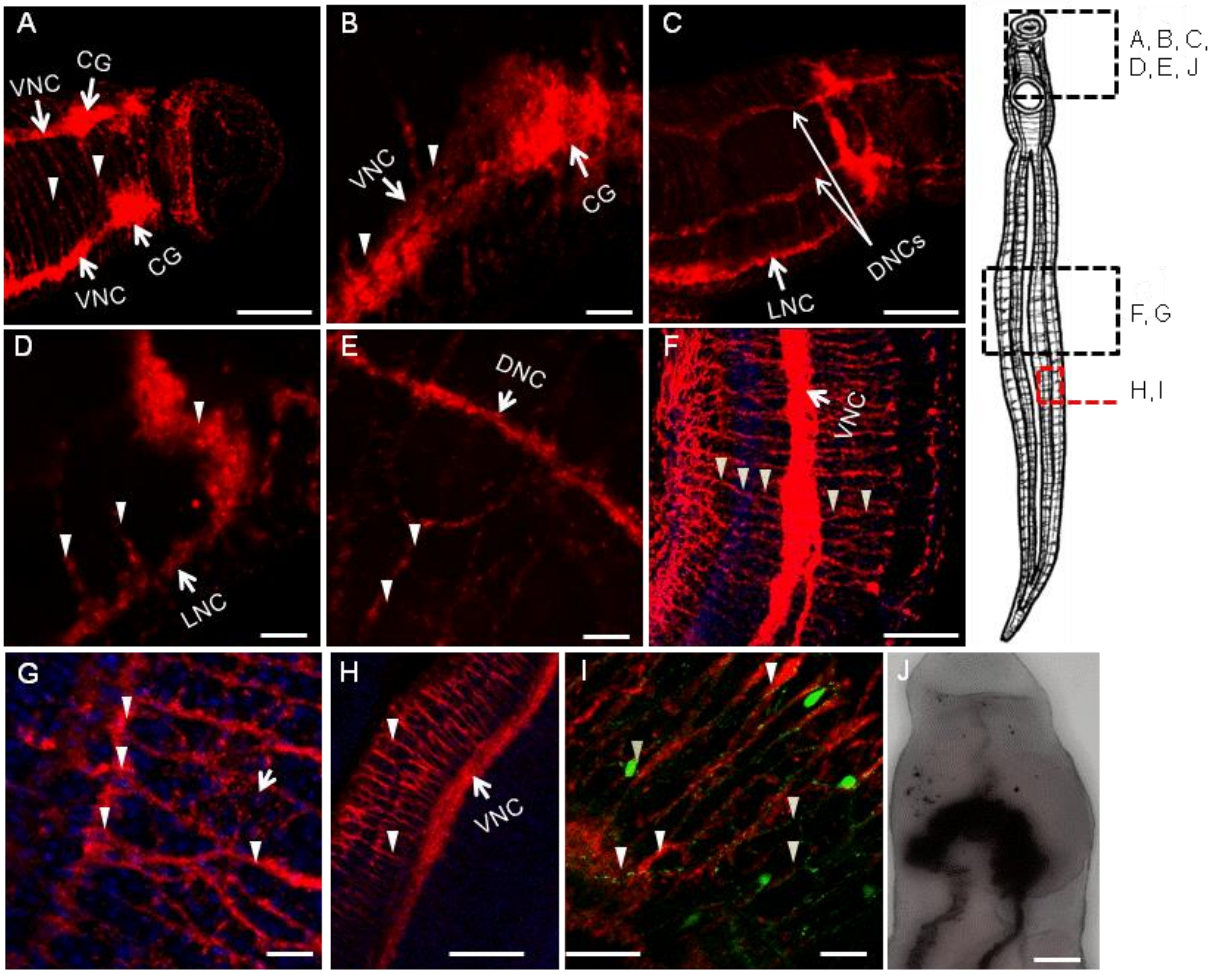

Figure 1. Visualizing the schistosome nervous system by labelling with an antibody against conserved neuronal marker synapsin. The neuronal marker synapsin was immunolabeled in adult worms to visualize the nervous system, using a signal amplification reagent (Tyramide Signal Amplification, Thermo Fisher Scientific, MA, USA) as previously performed in cercariae (Collins et al., 2011) (A). Labelling with synapsin reveals the organization of the schistosome CNS. The location of individual panels in the worm body is indicated in the diagram on the right. The pair of ventral nerve chords (VNCs), joined by transverse commissures, which form a

“rungs-on-a-ladder”-like organization, are visible, as are the cerebral ganglia (CG) (A-B). (C-D) Lateral nerve chords (LNCs) are visible on either side of the worm (C-D). The dorsal nerve chords (DNCs) are also observed (C, E). The fine nerve fibers of the PNS are visible throughout the body, extend to the surface (F), and intersect with the main longitudinal nerve chords (F, G). Short nerve fibers of the PNS extend from VNCs to the gynecophoral canal flap (H). Known schistosome neurotransmitter 5HT was co-labeled with with synapsin (I), and the labelling pattern shows a close juxtaposition of nerve fibers. Labelling worms with secondary antibody and the amplification reagent alone did not produce significant non-specific labelling (J). Scale bars represent 100 µm at 20x magnification, in panels A, C, F, H, J and 20 µm at 63x magnification, in panels B, D, E, G, I.

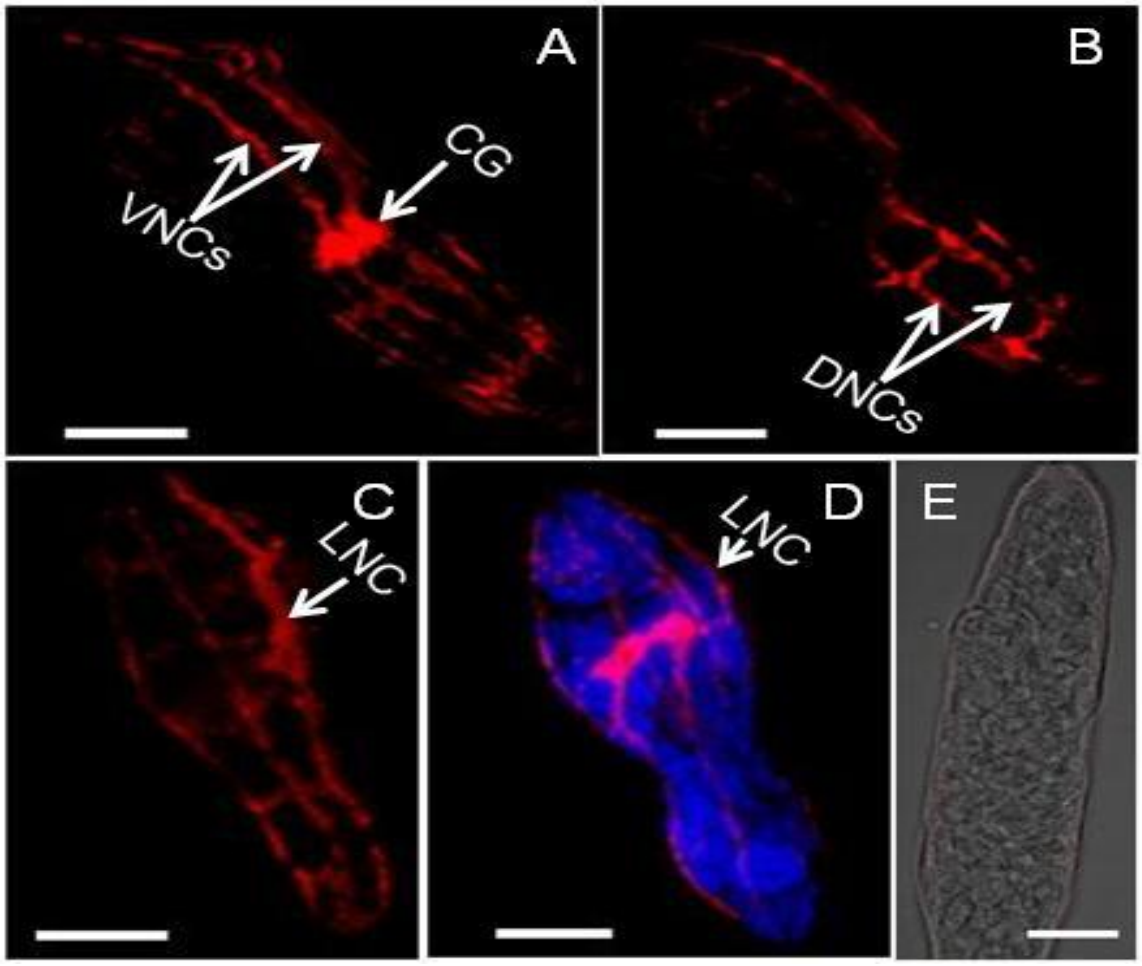

Figure 2. Labelling with neuronal marker synapsin reveals the organization of the central nervous system in post-infective larval stage schistosomula. Synapsin labelling reveals the CNS of 7- day old schistosomula that were fixed and permeabilized. CG are evident in the center of the worm body and VNCs project lonigitudinally from the CG (A). DNCs and LNCs are also labeled (B-C). Transverse commissures join longitudinal nerve chords, indicated by the arrowheads. The DAPI counterstain reveals the positioning of nerve chords in the worm body. Cerebral ganglia are also visible in the posterior of the worm (D). The labelling with the amplification reagent and secondary antibody alone, did not produce non-specific labelling, as demonstrated with the transmitted image overlay (E). Images were obtained at a 63x magnification and scale bars indicate 20 µm.

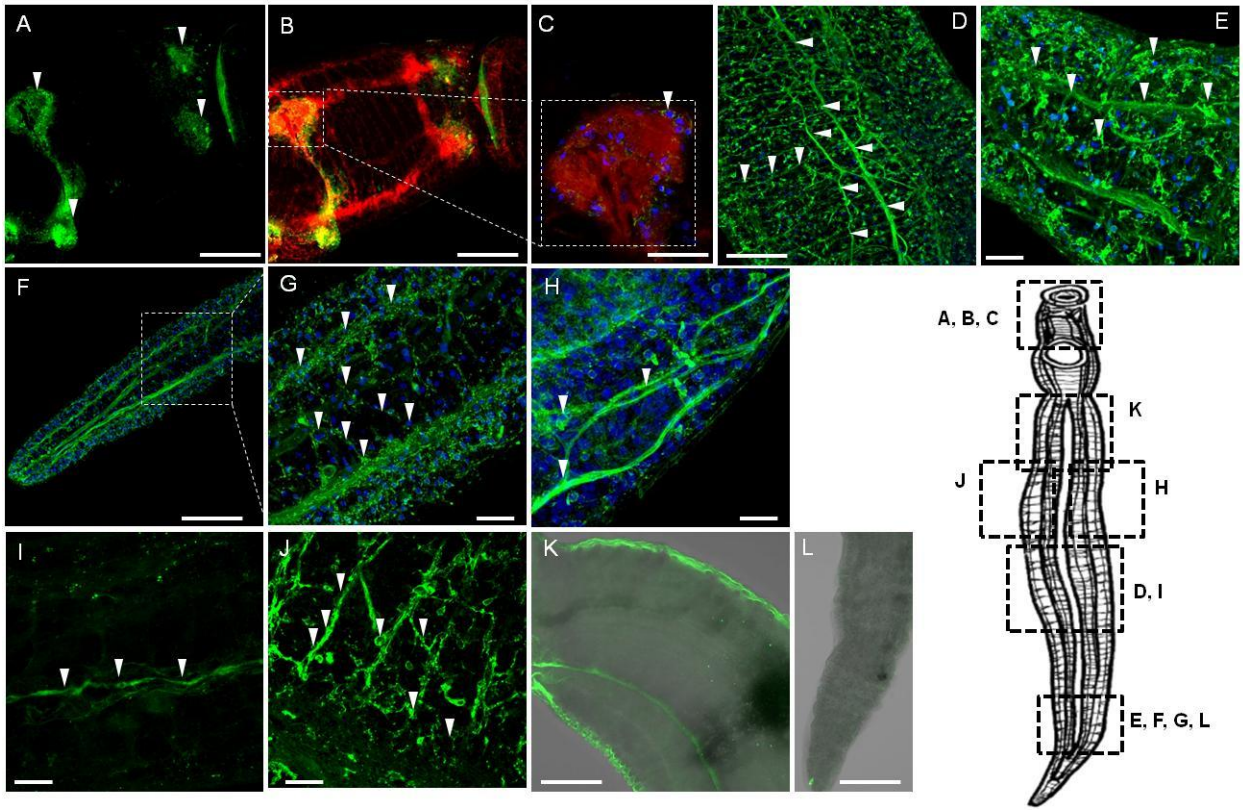

Figure 3. Labelling with an octopamine (OA)-specific antibody reveals widespread labelling in neurons of the brain, central and peripheral nervous system. OA is present in adult *S. mansoni*, indicated by labelling with Alexa-488 (green), with a DAPI counterstain. OA is present in four lobes of the CG, in the head (A-C), evidenced by the synapsin labelling (red). Labelling of OA in longitudinal nerve chords of the CNS and fine nerve fibers of the PNS (D-H), in both males (D, E) and females (F, G, H) is observed. At higher magnification, OA is identified in cell bodies (E, G, H). An octopaminergic neuron lines the caecum, indicated by arrowheads (I). Intersecting octopaminergic nerve fibres beneath the surface ressemble the submuscular peripheral nerve net of the PNS (J). Worms were also probed with anti-OA antibody preadsorbed with OA (K), or secondary antibody alone (L). These negative controls did not yield significant fluorescence. Some non-specific fluorescence is observed with the preadsorbed

control, on the worm surface, though this labelling does not resemble any neuronal structure observed when probing with anti-OA alone. Scale bars represent 100 µm at low magnification (20x) in panels A, B, D, F, K, L and 20 µm at high magnification (63x) in panels C, E, G, H, I, J.

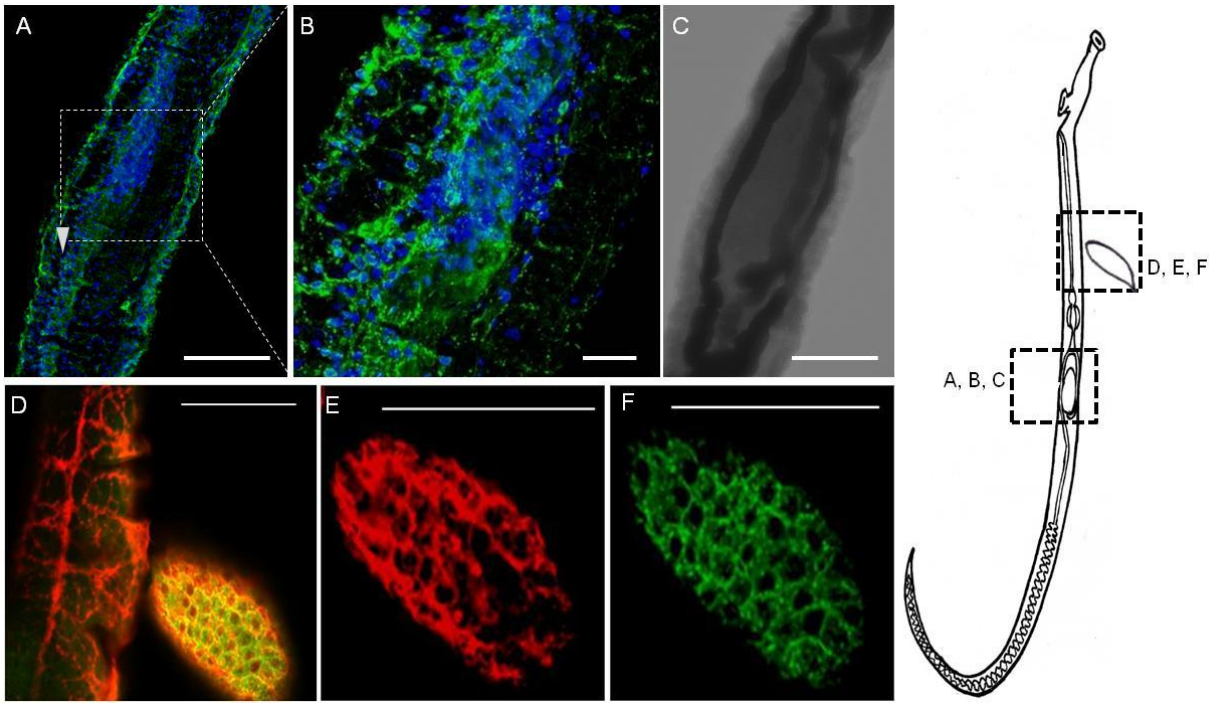

Figure 4. Labelling of octopamine (OA) and synapsin reveals the presence of OA in the ovary of the female reproductive system and in the developing embryo. The region in the female worm or adjacent egg is indicated in the diagram to the right of the figure. OA labelling reveals octopaminergic neurons concentrated along the length of the ovary (A, B, C), that's lining has a concentration of nucleii, as previously described (Neves et al., 2005) (A, B). Co-labelling of OA with synapsin reveals the organization of the nerve fibers of the developing embryo (D, E, F). Co-localization is indicated by regions in yellow. Individually, synapsin (E) and OA (F) labelling of the embryo show a similar web-like pattern. The scale bar represents 100 µm with a 20x magnification in panels A, C, D, E and F, and 20 µm at high magnification (63x) in panel B.

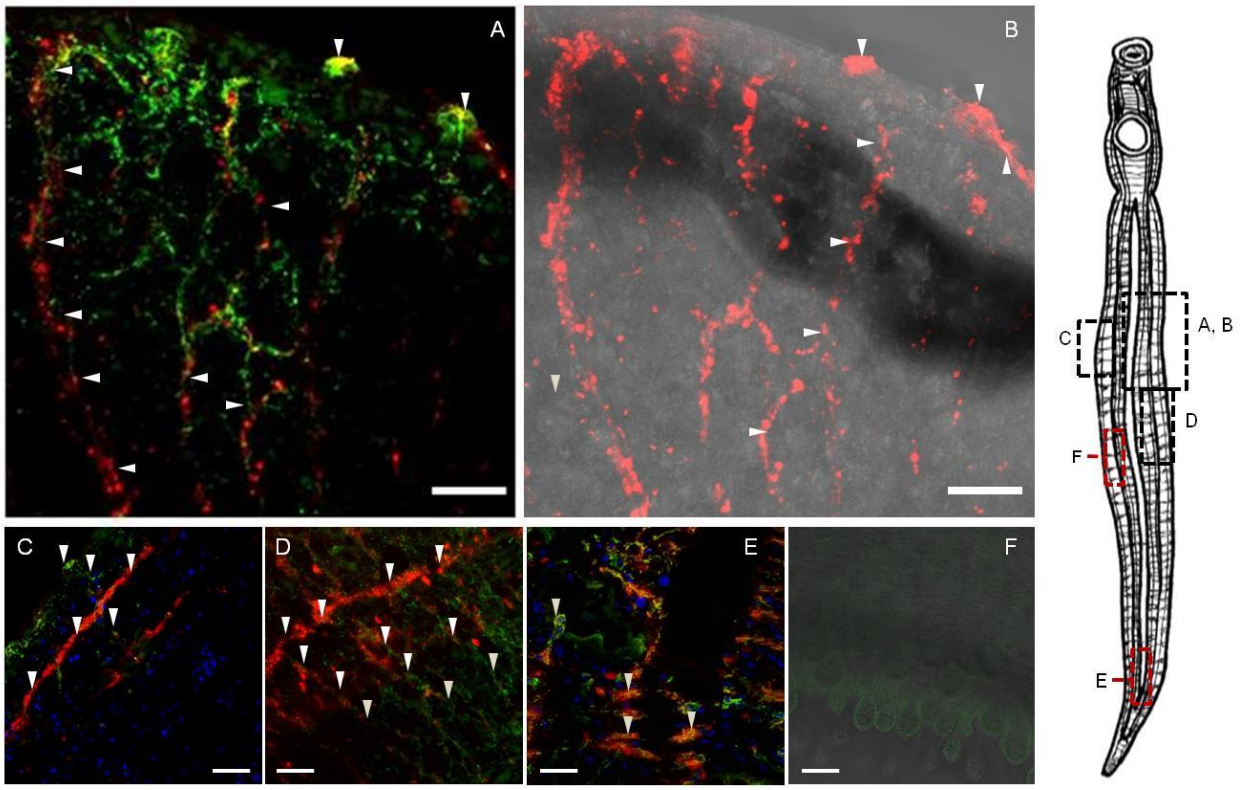

Figure 5. Co-labelling of octopamine (OA) with the neuronal marker synapsin reveals that OA is present in nervous tissue and demonstrates innervation of the worm surface. Regions corresponding to the panels are indicated in the diagram to the right. Labelling of OA (green), synapsin (red), and sites of colocalization (yellow), demonstrates overlap or a close juxtaposition of the signals (A). OA and synapsin labelling in the tubercles is punctate, and connects to nerve fibres in a web-like organization, which are varicose in appearance, as demonstrated by the synapsin-transmitted light image (B). The connection of octopaminergic neurons in tubercles to the subtegumental nerve net suggests that these neurons may be involved in efferent signaling. While there is a close juxtaposition of synapsin and OA, synapsin appears to be enriched in CNS nerve fibers, and OA, in PNS nerve fibers (C-D). The surface of the gynecophoral canal is innervated by a cluster of short nerve fibers containing OA and synapsin, indicated in yellow, indicating that this region is rich in octopaminergic sensory nerve

fibers (E). Scale bars represent 20 µm at high magnification (63x) in panels A, B, C, E and F and 100 µm at low magnification (20x), in panel D.

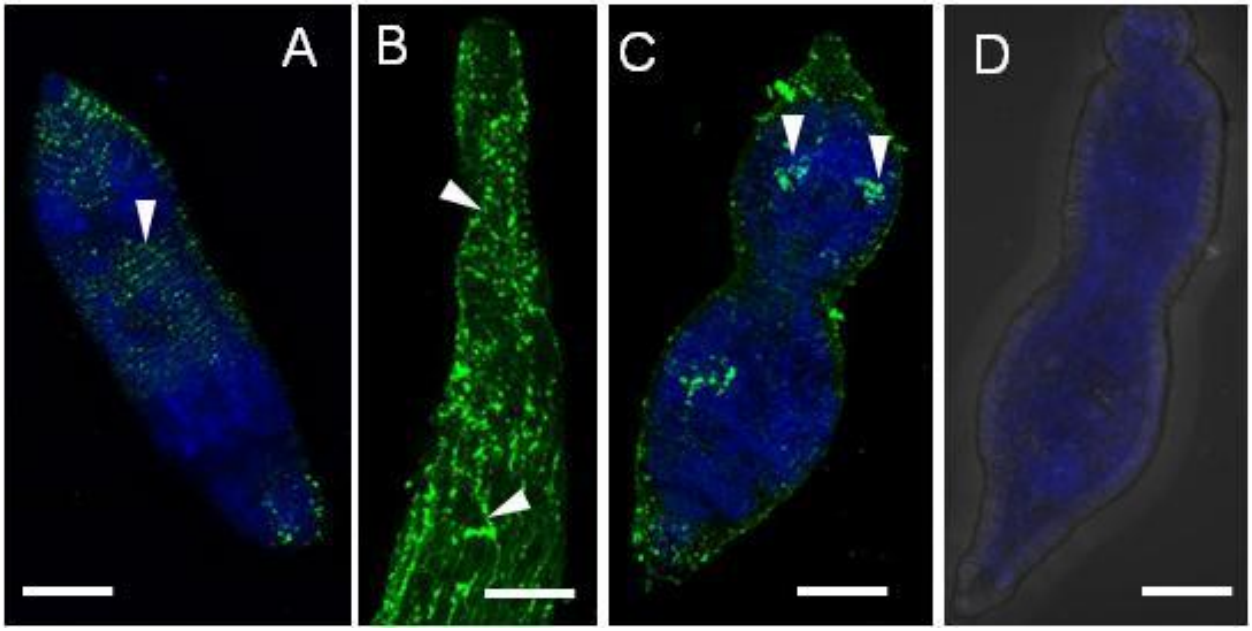

Figure 6. Octopamine labelling reveals localization to fine nerve fibers of the PNS in schistosomula. Octopamine (OA) is present in 7-day old schistosomula, indicated by labelling with FITC (green), with a DAPI counterstain. Diffuse staining is present in a beads-on-a-string pattern in concentric circles near the worm surface, suggesting innervation of the PNS (A). OA is also detected in neurons that are varicose in appearance, and arranged in a web-like organization (B). Arrowheads indicate two sites of punctate labelling in the worm posterior, possibly of the CG of the CNS (C). Labelling with secondary antibody alone did not produce non-specific labelling (D). Scale bars represent 20 µm at a 63x magnification.

Figure 7. Incubation with catecholamines and phenolamines produces a transient lengthening effect in schistosomula. 7-day old schistosomula pictured above were incubated with water vehicle (A) or octopamine (OA), at 65 µM (B), which produced a transient lengthening effect on schistosomula. Worms transiently lengthen and contract in. This lengthening effect is also observed with the tested catecholamines and phenolamines.

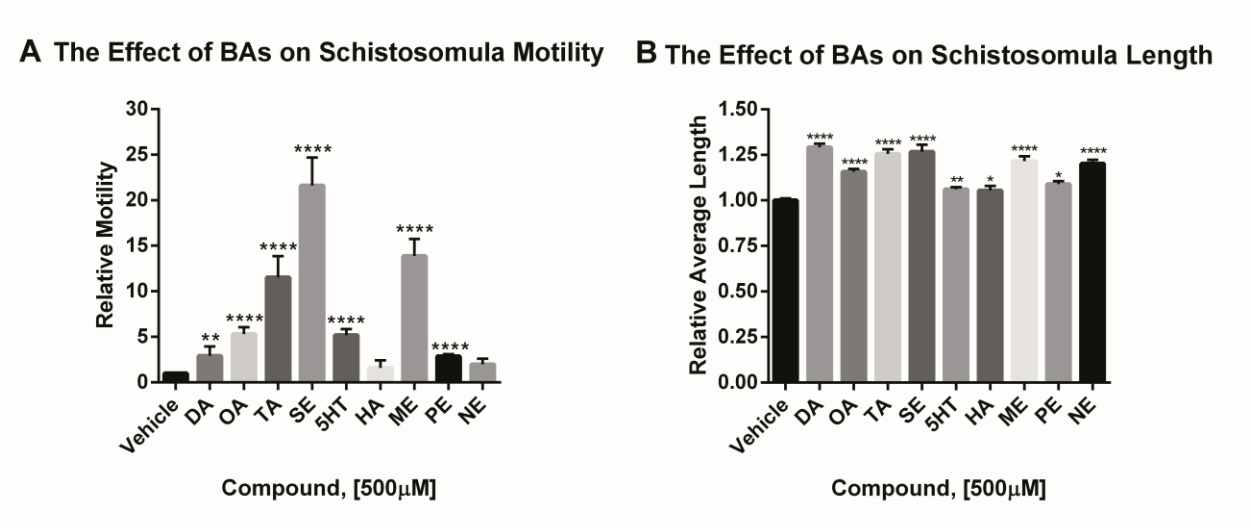

Figure 8. Incubating schistosomula with tyrosine derivatives and other biogenic amines (BAs) produces short term changes in length and motility. Schistosomula were cultured for 7 days following transformation and were incubated with BAs and related compounds for a 20 minute incubation period. Relative motility was quantified using ImageJ with the described method for

determining length changes as a measure of frequency of body movements (A). Motility is expressed as a fold change compared to the same well, preceding addition of the BA at 500 µM and normalized to the vehicle-treated control. 25 to 430 schistosomula were analyzed per treatment. The standard error of the mean value (SEM) is represented by error bars. Significance of the mean values, compared to the mean of the associated vehicle control, was determined using the unpaired student t-test, and p-values < 0.05 (*) were considered to be significant. The vehicle control corresponds to schistosomula incubated with 0.015% DMSO for synephrine (SE), and H2O for all other compounds. BAs that cause a significant increase in relative frequency of body movements at 500 µM include catecholaimes dopamine (DA) and metanephrine (ME), and phenolamines octopamine (OA), tyramine (TA), SE and phenylethylamine (PE), which is structurally related to tyramine. OA motor stimulation is comparable to that of 5HT, at ~5 fold that of the vehicle treated control. Tyrosine derivatives that cause the most pronounced stimulation of motility are OA, TA, SE and ME at ~5, 12, 22 and 14-fold stimulation, compared to the vehicle-treated control. The average length of schistosomula following a 20 minute incubation with the BA at 500µM was also determined with the described method using ImageJ (B). Bars in graphs in which the y-axis indicates a relative average length, represents the mean relative length, expressed as a fold change over the baseline, normalized to the vehicle-treated control. Compounds showing a pronounced significant increase in length (P-Values: <0.0001), of ~15-30% compared to treatment with vehicle alone are as follows: DA> SE> TA> ME > NE > OA.

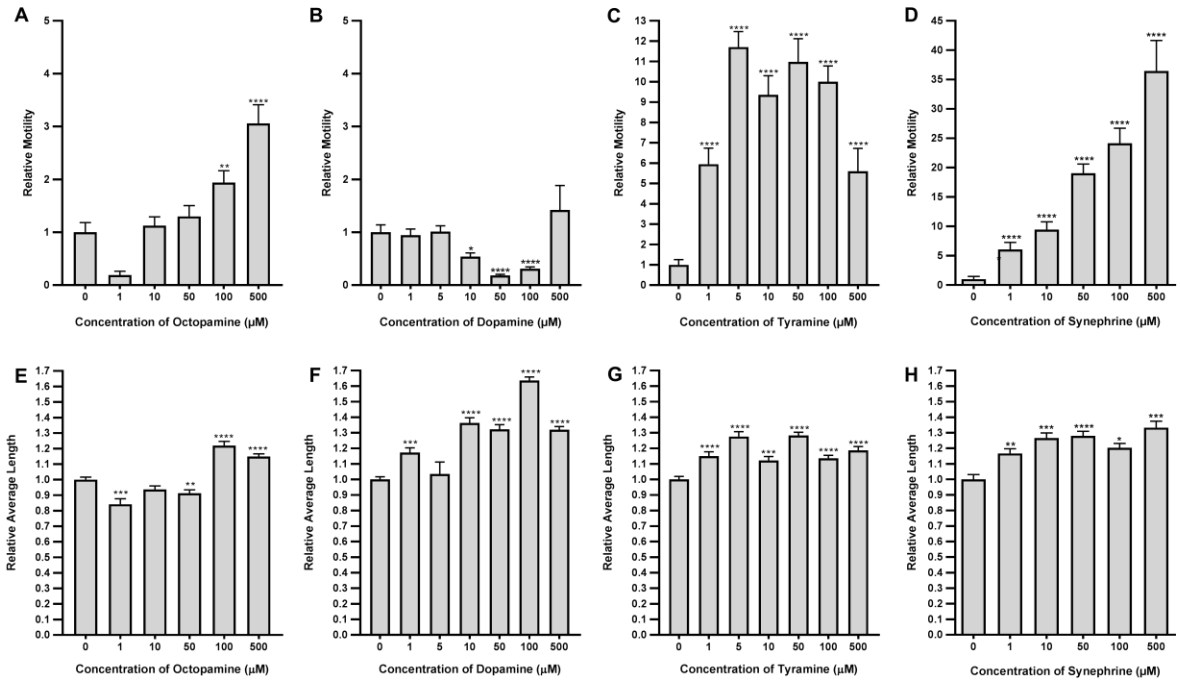

Figure 9. Octopamine and related amines produce short-term changes in relative length and motility in schistosomula. Dose response assays were performed for four tyrosine derivatives that we determined to cause significant changes in motility (A-D) and length (E-H). Mean relative motility values were determined by normalization to the corresponding wells, at baseline, followed by normalization to the vehicle-treated control. Changes in motility were determined for OA (A), DA (B), OA precursor tyramine (TA) (C) and OA metabolite synephrine (SE). Compounds were tested at a concentration range from 1 to 500 µM. OA produces an initial decrease in relative motility at 1 µM, followed by a progressive concentration-dependent increase up to 3-fold baseline motility at 500 µM (A) indicating concentration-dependent stimulation of motility. DA causes a dose dependent decrease in motility, though a reversal of this trend is observed at 500 µM. TA produces a pronounced increase in motility, up to ~12 fold at 5 µM (C). SE produces the most significant increase in motility, at ~37 fold at 500 µM (D). OA also produced concentration-dependent effects on length (E), which are most pronounced at higher concentrations of 100-500 µM, producing length changes of up to ~25%. There is an

initial slight decrease for average relative length following OA incubation. Concentration-dependent effects on length, up to ~60%, are most pronounced for DA (F). TA, similarly to OA, produces a maximum increase in length of ~25% (G). SE produces the most pronounced increase in length after DA, at ~30% (H). In all panels (A-H) the standard error of the mean value (SEM) is represented by error bars. Significance of the mean values, compared to the mean of the associated vehicle control, was determined using the unpaired student t-test, and p-values < 0.05 (*) were considered to be significant.

	Compound	Activity Reported in Invertebrate or Vertebrate Species	References
1	**Acepromazine**	DA (+), OA (+)	Collard and Maggs, 1958; Monteiro et al., 2007
2	**Albuterol**	ADRB (+)	Colice, 2008
3	**Amitraz**	ADRA (+), OA (+)	Roeder, 1999
4	**Carvedilol**	ADRB (-), ADRA (-)	Frishman, 1998, Yue et al., 1992
5	**Chlorpromazine**	DA (-), ADRB (-)	Harold et al., 1987; McNaughton et al., 2001; Besser et al., 1980; Huerta-Bahena et al., 1983; Roeder and Gewecke, 1990; Roeder and Gewecke, 1999
6	**Clonidine**	ADRA (+), OA (+)	Seedat et al., 1969, Bender and Abdel-Rahman, 2008, Roeder and Gewecke, 1990, Brooker et al., 2011
7	**Clozapine**	DA (-), ADRA (-), OA (-)	Nathanson and Hunnicutt, 1981; Dougan and Wade, 2007; Persinger et al., 2001
8	**Corgard**	ADRB (-)	Frishman, 1981
9	**Cyproheptadine**	5HT (-), ADR (-), OA (-)	Osborne, 1996, Gillman, 1999; Nathanson and Hunnicutt, 1981
10	**Epinastine**	HA (-), OA (-)	Tasaka et al., 1990; Tadashi et al., 1997; Packham et al., 2010; Roeder et al., 1998
11	**Hytrin**	ADRA (-)	Achari and Laddu, 1992
12	**Labetalol**	ADRA (-), ADRB (-)	Richards, 1976
13	**Levalbuterol**	ADRB (+)	Berger, 2003
14	**Levobunolol**	ADRB (-)	Kaplan et al, 1971
15	**Lopressor**	ADRB (-)	Gattis, 2001
16	**Loxapine**	DA (-)	Lescot et al., 2007
17	**Metoclopramide**	DA (-), OA (-)	Osborne, 1996; Kluge et al., 2007; Besser et al., 1980
18	**Mianserin**	5HT (-), ADR (-), OA (-)	Doggrell, 1980; Wakeling, 1983; Roeder and Gewecke, 1990, Roeder, 1990; Osborne, 1996; Evans, 1980; Kasper et al., 1994; Valentini et al., 2004
19	**Naphazoline**	ADRA (+), OA (+)	Roeder and Gewecke, 1990; Sanders et al., 1975
20	**Phentolamine**	ADRA (-), OA (-)	Sanders et al., 1975; Wilson and Yates, 1977; Nathanson and Hunnicutt, 1981; Roeder and Gewecke, 1990; Osborne, 1996
21	**Pindolol**	ADRB (-)	Gugler et al., 1974; Bastick, 1995
22	**Prazosin**	ADRA (-)	Greengrass and Bremmer, 1979; Simpson et al., 2009
23	**Propranolol**	ADRB (-), OA (-)	Nathanson and Hunnicutt, 1981; Nathanson, 1985; Oost et al., 2007; Sun et al., 2011
24	**Sulpiride**	DA (-), OA (-)	Dougan and Wade, 2007
25	**Tenormin**	ADRB (-)	Elliot et al., 1975
26	**Terbutaline**	ADRB (+)	Lam et al., 1998; Lam and Gill, 2005
27	**Tolazoline**	ADRA (-), OA (+)	Roeder and Gewecke, 1990 ; Sanders et al., 1975; Nuntnarumit et al., 2002; Casbeer and Knych, 2013
28	**Yohimbine**	ADRA (-), OA (-)	Roeder and Gewecke, 1990, Roeder and Gewecke, 1999, Robb et al., 1994; Verwaerde et al., 1997

* Agonistic (+), antagonistic (-), Dopamine (DA), Octopamine (OA), adrenergic (ADR), beta adrenergic (ADRB), alpha adrenergic (ADRA), histamine (HA), serotonin (5HT) signaling. Signaling activity indicated represents what has been reported in vertebrate or invertebrate species in the literature and does not represent the classification of the compound.

Table 1. Synthetic compounds that are modulators of biogenic amine signaling were tested on schistosomula to determine effects on motor control. 28 compounds that have agonistic (+) or antagonistic (-) effects on octopamine (OA) receptors in invertebrates, and agonists or antagonists of dopamine (DA) and adrenergic (ADR) signaling were identified. Compunds modulating both alpha (ADRA) and beta (ADRB) adrenergic signaling were selected. Compounds that modulate histamine (HA) and serotonin (5HT) signaling are also indicated. The type of signaling indicated represents what has been reported in vertebrate or invertebrate species in the literature. Modulators of ADR activity were selected based on the similarity between OA and ADR receptors, and the possibility that these compounds may also modulate OA signaling.

Compounds in BA Antagonist Screen Displaying Phenotypic Response			
Compound	**Hypermotility**	**Hypomotility**	**Lengthening Effect**
Acepromazine		x	
Amitraz	x		
Carvedilol		x	
Chlorpromazine		x	
Clonidine	x		x
Clozapine		x	
Cyproheptadine		x	
Epinastine	x		
Levobunolol	x		x
Metoclopramide	x		
Mianserin	x		x
Naphazoline	x		x
Phentolamine	x		
Pindolol	x		
Prazosin		x	x
Propranolol	x		x
Tolazoline	x		x
**x: Indicated phenotype was observed*			

Table 2. Synthetic compounds that modulate catecholamine and phenolamine signaling in metazoans produce short-term changes in length and motility phenotypes in schistosomula. Worms were seeded at ~25 worms per well, in a 96 well plate, and cultured for 7 days, prior to addition of the compounds indicated in **Table 1**. Worms were recorded at baseline, and 20 minutes following addition of the compound at 10 µM and 65 µM, in triplicate. The experiment was repeated twice. Videos were generated using an IN Cell Analyzer 2000 (GE Healthcare), where each well was recorded for 20 seconds, to obtain 30 time-lapse images at a rate of 1.5 fps. Videos to compare motility between vehicle-treated controls and wells treated with synthetic compounds were generated using ImageMagick (http://www.imagemagick.org/)

software. Pronounced changes observed at 65 µM include hypermotility (highlighted in red), hypomotility (blue), and a lengthening effect (marked by an “x”).

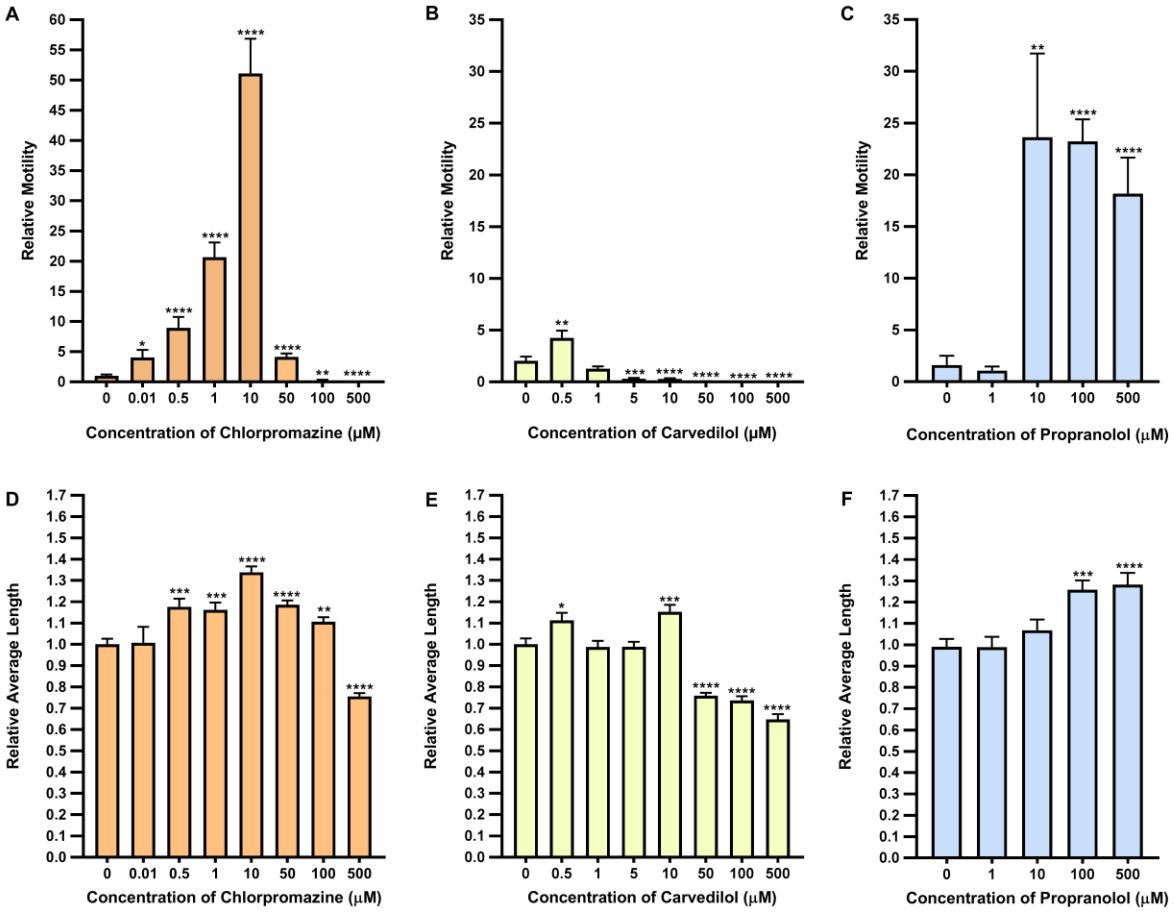

Figure 10. Synthetic modulators of tyrosine derivative signaling chlorpromazine, carvedilol and propranolol produce concentration-dependent effects on motility and length in schistosomula. Three compounds that caused changes in length and motility in the initial screen of synthetic compounds were tested further, to determine concentration-dependent effects. The described method to quantify relative body movements and changes in relative average length was used. Chlorpromazine (CPZ), a DA signaling antagonist in schistosomula (Taman and Ribeiro, 2009), caused an increase in motility, up to ~50-fold, at 10 µM, followed by concentration-dependent hypomotility and paralysis between 50 to 500 µM. Carvedilol (CAR), a mixed alpha- and beta-adrenergic antagonist, causes an increase in motility of ~4 fold at 0.5 µM, and a subsequent concentration-dependent decrease in motility and paralysis between 5

and 50 µM, suggesting concentration-dependent antagonistic activity (B). Propranolol (PR), a beta-adrenergic antagonist, caused hypermotility of ~23-fold baseline, between 10 and 500 µM, suggesting concentration-dependent agonistic activity (C). CPZ causes a concentration-dependent increase in relative average length up to ~35% at 10 µM, followed by a concentration-dependent decrease of up to ~25%, at 500 µM (D). CAR causes a modest concentration-dependent increase in length, of ~15%, with a decrease in motility at concentrations higher than 10 µM (E). PR causes a concentration-dependent increase in length, that is most significant at higher concentrations of 100 to 500 µM (F).

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Connecting Statement I

In the previous chapter, we identified octopamine (OA) and labelled the nervous system of *Schistosoma mansoni* with a neuronal marker, synapsin, gaining new insight into both the structure and organization of the nervous system, and identifying a previously unknown schistosome signaling system with much potential for drug targeting. Confocal immunolocalization studies and colocalization of OA and synapsin confirm that OA is found in neuronal tissue, particularly in the peripheral nervous system (PNS), suggesting a role in muscle control and sensation. Next, we identified a neuromuscular function for OA and related tyrosine derivatives, and identified putative agonists and antagonists of their signaling. These findings demonstrate the importance of tyrosine derivative signaling in schistosome neurobiology and motor control. The success of the insect OA system as a pesticide target highlights the potential of neuroactive tyrosine derivatives for anthelminthic (antischistosomal) drug discovery. In the next chapter, we investigate the role of tyrosine derivative signaling further. We determine the role of tyrosine derivative-related signaling genes in motor control of *S. mansoni* using reverse genetics. We identify three putative OA GPCRs, Smp_150180, Smp_180140 and Smp_120620, which we target via RNAi, as well as putative *S. mansoni* OA synthesis enzyme SmTBH (tyramine beta-hydroxylase). We also target four receptors previously shown to be involved in dopamine (DA) signaling, including known DA GPCRs SmD2, SmGPR3 and DA biosynthesis enzymes, tyrosine hydroxylase (SmTH) and SmAADC (aromatic amino acid decarboxylase). We perform RNAi on both larval and adult parasitic stages of *S. mansoni*, to determine whether these TA signaling genes confer the motor phenotypes we observed in the previous chapter, and are associated with the expected effects on motor control and whether these genes may serve as drug targets for development of anti-schistosomicides.

CHAPTER III (Manuscript II)

"An RNAi screen of *S. mansoni* to determine the role of catecholamine and phenolamine-related signaling genes in schistosome motility"

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ABSTRACT

Schistosomiasis is a debilitating disease infecting more than 250 million people worldwide, and putting more than 800 million people at risk of contracting infection. A single drug compound, praziquantel (PZQ) is the mainstay of schistosomiasis control, despite reports of reduced efficacy in curing infection. One area that holds potential for drug development is the schistosome nervous system, which plays an important role in motor control and infection. Our recent identification of the neurotransmitter octopamine (OA), in *Schistosoma mansoni* (El-Sakkary and Ribeiro, in preparation), suggests that phenolamine signaling, which plays an important role in motor function in other invertebrates, is involved in control of schistosome motility. Here, we describe the identification of putative OA signaling genes, including *Schistosoma mansoni* tyramine beta-hydroxylase (SmTBH) (Smp_243830) and three putative OA GPCRs, Smp_150180, Smp_120620 and Smp_180140. We target these genes, as well as other previously characterized tyrosine derivative signaling genes involved in dopamine (DA) signaling, including GPCRs SmD2 (Taman and Ribeiro, 2009), SmGPR-3 (El-Shehabi et al., 2012) and the two enzymes responsible for DA synthesis, *S. mansoni* tyrosine hydroxylase (SmTH) (AAC62256) and *S. mansoni* amino acid decarboxylase (SmAADC). We downregulate expression of these genes in both schistosomula and adult stages of the parasite and use robust imaging assays to determine changes in motility, length, posture and coordination. Together, these findings provide further evidence that tyrosine-derived neurotransmitters play an important role in schistosome motor control and identifies 8 genes that can potentially be targeted in developing novel, urgently needed, anti-schistosomicides.

1. Introduction

Schistosoma mansoni is a major cause of schistosomiasis, a neglected parasitic disease that infects > 250 million people worldwide, with > 280,000 deaths per annum (Fenwick et al., 2003; Knudsen et al., 2005; Steinmann et al., 2006; Doenhoff et al., 2008), and >800 million people at risk of contracting infection (King and Dangerfield-Cha, 2008). A single chemotherapeutic drug, praziquantel (PZQ), has been used for the past 35 years, despite being ineffective against the juvenile stages of the parasite (Sabah et al., 1986). Morever, increased usage of PZQ, particularly through mass administration programs aroud the world, raises serious concerns over the prospect of drug resistance. While resistance to PZQ at the population level has not been reported to date, (Cioli et al., 2012), it is possible to induce PZQ resistance in laboratory mouse strains (Xiao et al., 1985) and there have been incidences of human schistosomiasis that did not respond to treatment (Cioli , 2000), suggesting resistance can also occur in the field.

The schistosome nervous system offers great potential for drug targeting, given its role in controlling virtually all aspects of parasite biology. Among the many activities of the nervous system is the regulation of muscle function, which enables the control of movement, host-penetration, larval migration through the host (Crabtree and Wilson, 1980) to complete successful infection, as well as feeding, male-female coupling and egg-laying (Maule et al., 2005), all of which are required for schistosome survival and infectivity. Signaling in the schistosome nervous system involves a rich diversity of neurotransmitters, including two different types of tyrosine-derived BAs, catecholamines and, as shown in the previous chapter, invertebrate-specific phenolamines. Catecholamine signalling is mediated primarily by dopamine (DA), which is present in the parasite nervous system, and causes a relaxation of longitudinal muscle (Pax et al., 1984), resulting in elongation of the body. (Day et al., 1994). DA biosynthesis requires two enzymes, both of which are present in schistosomes. The first, rate-limiting enzyme is tyrosine hydroxylase (SmTH), which was previously cloned from *S. mansoni* and fully characterized *in vitro* (Hamdan and Ribeiro, 1998). The second enzyme, *Schistosoma*

mansoni amino acid decarboxylase (SmAADC, locus tag: Smp_171580), was identified via bioinformatics analyses (Ribeiro et al., 2012), based on homology with AADCs from other species. In addition to endogenous biosynthesis, recent evidence demonstrated the presence of a specific DA transporter (Larsen et al., 2011) and at least two DA-activated GPCRs in *S. mansoni*, SmD2 (Taman and Ribeiro, 2009) and SmGPR3 (El-Shehabi et al., 2012), all of which have been cloned and characterized *in vitro*, though their biological functions are poorly understood.

The other major group of neuroactive, tyrosine derivatives are the phenolamines, including octopamine (OA) and its precursor tyramine (TA). These play an important role in motor control in many invertebrate phyla (Sombati and Hoyle, 1984; Hoyle, 1985; Bacon et al., 1995; Mulloney et al., 1987; Roeder et al., 1998; Roeder et al., 2003; Verlinden et al., 2010) but were only recently discovered in schistosomes. We have shown that OA, at least, is widely distributed in the nervous system of *S. mansoni*, particularly the PNS, and that exogenously applied OA and TA both produce motor effects in cultured schistosomula, resulting in a lengthening of the body and strong hyperacivity (El-Sakkary et al., *in preparation*). A search of the most current annotation of the *S. mansoni* genome (Protasio et al., 2012) identified two putative enzymes involved in phenolamine biosynthesis, including *Schistosoma mansoni* homologues of tyrosine decarboxylase (SmTDC) (Smp_135230 and Smp_130860), the enzyme that catalyzes tyrosine to TA (Zhu et al., 2016), followed by tyramine beta-hydroxylase (SmTBH) (Smp_243830), which converts TA to OA (Wallace, 1976; Nishimura et al., 2008; Ribeiro et al., 2012). TDC catalyzes the removal of the carboxyl group from tyrosine (Cole et al., 2005). TBH synthesizes OA via hydroxylation of the beta carbon of TA (Stuart et al., 1974; Wallace, 1975; Walker and Kurkut, 1978; Monastirioti et al., 1996; Herman et al., 2006; Nishimura et al., 2008) and shares homology with mammalian dopamine beta-hydroxylase (DBH) (Klinman, 2006).

Besides biosynthetic enzymes, our bioinformatics studies identified several putative amine-like GPCRs that share some homology with OA/TA receptors from other invertebrate species (Smp_150180, Smp_180140 and Smp_120620). OA/TA receptors are classified based

on their sequence homology with adrenergic receptors and include β-adrenergic-like OctβR receptors, α-adrenergic-like OctαR receptors (Evans, 1981; Evans and Maqueira, 2005), as well as the TyrR receptors which signal through tyramine (Nagaya et al., 2002; Roeder et al., 2003). Octopamine GPCRs are believed to be good drug targets as they are invertebrate-specific (Roeder, 1999), and their targeting would not likely adversely affect host biology.

To determine if any of these genes are involved in neuromuscular control, we performed RNAi phenotypic screens in cultured schistosomula and adult parasites. Animals were transfected with short-interfering RNA (siRNAs) targeting individual genes of interest, and effects on motor activity were subsequently measured, using well-established, robust imaging assays (Patocka et al, 2014; Rashid et al, 2015).

Ribonucleic Acid interference (RNAi) is a useful tool by which treatment with a sequence-specific dsRNA (or siRNA), causes downregulation of protein expression, primiarily via targeted mRNA degradation (Fire et al., 1998; Tabara et al., 1998; ; Kennerdell and Carthew, 1998; Hunter, 1999; Da'dara and Skelly, 2015). RNAi is possible in organisms that have the required machinery to perform this gene-specific targeting of protein expression and has been demonstrated in such diverse organisms as plants, fungi, arthropods, protozoans and vertebrates (de Lang et al., 1995; Wianny and Zernicka-Goetz, 2000, Schoppmeier and Damen, 2001; Morris et al., 2002; Bushman, 2003; Shi et al., 2004; Gordon and Waterhouse, 2007; Younis et al., 2014). Schistosomes have the ability to perform RNAi (Krautz-Peterson and Skelly, 2007) and there are RNAi protocols available for all major life stages of schistosomes (Da'dara and Skelly, 2015). RNAi has been used to understand aspects of schistosome biology (Correnti et al., 2005; Ndegwa et al., 2007; Krautz-Peterson et al., 2007; Mourao et al., 2009; Faghiri and Skelly, 2009; Rinaldi et al., 2009; Krautz-Peterson et al., 2010; Stefanic et al., 2010; Krautz-Peterson et al., 2010; Bhardwaj et al., 2011; Patocka and Ribeiro, 2013; Macdonald et al., 2014; ,2015; Patocka et al, 2015) and to validate potential protein targets for chemotherapy in schistosomes. The success of RNAi-mediated gene silencing in schistosomes, including genes involved in neuronal signaling (Patocka and Ribeiro, 2013; Macdonald et al., 2014, 2015;

Patocka et al, 2015), highlight the potential of RNAi as a tool to identify proteins of the schistosome motor control system, which are potential chemotherapeutic targets in *S. mansoni*. The RNAi screen described here represents a thorough investigation of schistosomula and adult phenotypes associated with neuromuscular control and identifies 8 genes that, when downregulated, produce changes in motility, length, posture and/or coordination. Our results are consistent with previous findings in schistosomula that both phenolamine and catecholamine signaling are associated with an elongation of the body, indicating disruption of body wall muscle function. The RNAi-associated effects on motility and coordination that we observe suggest that SmTBH contributes to the hypermotile effect observed in schistosomula and that both OA and DA receptors, particularly Smp_150180, produce significant changes in posture and coordination. Together, these findings provide further evidence that tyrosine derivative signaling plays an important role in schistosome motor control and identifies 8 genes that can potentially be targeted in developing novel and badly needed, anti-schistosomicides.

2. Materials and methods

2.1 Bioinformatics studies

Genes of interest, including predicted BA GPCRs and biosynthetic enzymes, were obtained from the *Schistosoma mansoni* Genome Database (SchistoDB, http://www.schistodb.org) as well as NCBI (the National Center for Biotechnology Information) (https://blast.ncbi.nlm.nih.gov). In NCBI, BA GPCR sequences were identified by performing searches using keywords including the selected species' name, gene name, ligand specificity ("octopamine" or "dopamine" or "adrenaline", etc.) or abbreviations of ligand specificities ("OA" or "DA" or "ADR", etc.) together with "receptor", "GPCR" or enzyme. Enzymes involved in tyrosine-derivative synthesis were previously identified (Hamdan and Ribeiro, 1998; Ribeiro et al., 2012) and are indicated in **Figure 1**. DA synthesis enzyme SmTH (AF030336) was previously cloned from *S. mansoni* and characterized (Hamdan and Ribeiro, 1998). DA synthesizing enzyme SmAADC (Smp_171580) and OA synthesizing enzyme SmTBH

(Smp_243830) are annotated in the *Schistosoma mansoni* Genome Database (SchistoDB, http://www.schistodb.org) and can be identified using the search terms "amino acid decarboxylase" or "dopamine beta hydroxylase", respectively. The NCBI BLASTp tool was used to identify sequences sharing homology with schistosome BA GPCRs and biosynthetic enzymes. In SchistoDB, keywords "amine" and "GPCR" were used to identify sequences of interest. 120 GPCR sequences from both NCBI and the *Schistosoma mansoni* Genome Database (http://www.schistodb.org) were used to generate a multisequence alignment (MSA). The EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute) Clustal Omega tool (ClustalO, Version 1.1.2) (Söding, 2005; Sievers et al., 2011) was used to to generate the MSA. A neighbor-joining (nj) output was used to generate a tree, in Newick format, which was subsequently formatted using FigTree software (Version 3.0) (Morariu et al., 2008). The phylogenetic tree includes 22 amine-like ("orphan") and 6 known schistosome BA GPCR sequences. The remaining are BA GPCR sequences from vertebrate species, as well as invertebrate species including Drosophila, cestodes, roundworms and flatworms. All types of BA GPCR ligand specificities were included, with a larger proportion of vertebrate adrenergic receptors, which are most closely related to invertebrate OA receptors. Adrenergic signaling has not been identified in invertebrates, where it is believed to be replaced by octopaminergic signaling. Orphan amine-like GPCRs are classified based on homology with BA GPCRs of different subtypes and conservation of key residues specific to BA GPCR types. The aligned sequences were inspected manually to insure they were full-length and carried the expected 7 transmembrane regions and canonical residues required to form a functional GPCR. The predicted boundaries of TM regions were determind with TMHMM (Tied Mixture-Hidden Markov Model) (Version 2.0) software (Center for Biological Sequence Analysis – Technical University of Denmark (CBS-DTU), http://www.cbs.dtu.dk), using crystal structures of known vertebrate BA GPCRs as templates (Cherezov et al., 2007; Chien et al., 2010; Warne et al., 2008; Wacker et al., 2013; Wang et al., 2013). TMHMM posterior probability plots quantify the probability that each residue is located in a TM region (Krogh et al., 2001) and were studied to

predict the number of TMs. BA GPCR sequences having 4-8 predicted TM regions as predicted by the TMHMM algorithm or by manual inspection of the posterior probability plot were included in the tree, taking into account possible errors in TM region prediction and possible over- or under- estimation of TM number. A portion of the orphan *S. mansoni* GPCRs were inspected manually to identify BA GPCR-conserved residues (Ballesteros and Weinstein, 1995) and TM regions.

2.2 SiRNA preparation for RNAi targeting

RNAi was used to target expression of the following GPCRs: Smp_127310 (SmD2), Smp_043290 (SmGPR3), Smp_150180, Smp_180140, Smp_120620, Smp_126730 (Sm5HTR, positive control (Patocka et al., 2014) and synthesis enzymes: AAC62256 (SmTH), Smp_243830 (SmTBH), Smp_171580 (SmAADC) in *S. mansoni*. SiRNAs were synthesized commercially (Sigma-Aldirch, Millipore Sigma, MA, USA or Qiagen Inc., MD, USA) or were obtained by RNA transcription from a cloned cDNA template, using dsRNA and siRNA synthesis kits. In the latter case, the cDNA of interest was amplified via reverse transcription from total adult (mixed male/female) *S. mansoni* RNA, using the First-Strand cDNA Synthesis RT-PCR (reverse transcriptase polymerase chain reaction) kit with Oligo (dt)12-18 primers and an M-MLV RT (Moloney Murine Leukemia Virus reverse transcriptase). A 100-400 base pair target sequence was amplified from cDNA with a proofreading Phusion High Fidelity Polymerase (New England Biolabs) using primers indicated in **Table 1**. Primers were designed using Oligo software (version 6.2, Molecular Biology Insights, Inc) (Rychlik, 2007) to amplify a unique region in the target gene sequence. To diminish the chance of off-target effects, dsRNA target regions were chosen based on a BLAST (Basic Local Alignment Sequencing Tool) analysis (http://www.ncbi.nlm.nih.gov/) against the schistosome genome *in sillico*, to identify unique target regions. For GPCRs, the variable loop regions, rather than the relatively conserved transmembrane regions, were chosen, particularly the highly variable third intracellular loop, and for biosynthetic enzymes, variable domains, as determined by BLAST analysis, were selected. Primers were also compared to sequences in the

genome via BLAST analysis, to confirm low homology with other sequences in the genome. The amplified cDNA was resolved in a 1% agarose gel and visualized under UV light to confirm the appropriate size of the amplicon, gel purified and ligated to pJET cloning vector with the CloneJET Cloning kit (Thermo Fisher Scientific, MA, USA). The Megascript RNAi kit (Ambion, Thermo Fisher Scientific, MA, USA) was used to generate dsRNA from the template. Primers matching those described in **Table 1** were designed with an additional 5'-end T7 promoter sequence (5'-TAATACGACTCACTATAGGGAGA-3'), to act as a promoter site for T7 viral polymerase transcription of dsRNA. The amplified sequence, with attached T7, was generated in a standard PCR reaction using Phusion High Fidelity Polymerase. dsRNA was generated by *in vitro* transcription of the resulting amplicon, using 300-2000ng of the template, T7 RNA polymerase, and nucleotides ATP, CTP, GTP and UTP. The mixture was heated to 37 °C for 18 hours to generate dsRNA, incubated with DNAse I/RNAse A at 37 °C for two hours, to remove genomic contaminants, followed by a column-based purification step, as per the kit protocol. siRNA was generated by digestion with *E. coli* Ribonuclease III (RNAse III), with subsequent purification, using the Silencer siRNA kit (Ambion, Thermo Fisher Scientific, MA, USA).

2.3 Parasites

Larval and adult parasitic stages used in this study were obtained from *S. mansoni*-infected *Biomphalaria glabrata* snails, provided courtesy of the Biomedical Research Institute (BRI), and biodefense and emerging infections (BEI) resources (Rockville, Maryland, USA) (Lewis et al., 1986). To obtain larval-stage parasites, infected snails were made to shed cercariae by continuous light exposure for a period of 2 hours, according to standard procedures (Lewis et al, 1986). Cercariae were collected and concentrated by incubation at 4 °C for 1 hour. Cercariae were resuspended in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, MA, USA) with 500µg/ml streptomycin and 500U/ml penicillin (Gibco, Thermo Fisher Scientific, MA, USA) and transformed mechanically to schistosomula by vortexing. Schistosomula were washed three times in Opti-MEM (Thermo Fisher Scientific, MA, USA)

supplemented with 1000µg/ml streptomycin and 1000U/ml penicillin (Thermo Fisher Scientific, MA, USA). Schistosomula were resuspended in Opti-MEM, without streptomycin and penicillin, and cultured in 24 well plates in Opti-MEM and transfected with siRNA. To obtain adult stage parasites, 28-day old female CD1 mice were infected with ~200 cercariae each, via penetration through the tail (Smithers and Terry, 1965; Tucker et al., 2013). 7-8 weeks post infection, at the time of patency, mice were sacrificed and adult worms collected via perfusion of the mesenteric venules and the hepatic portal vein (Smithers and Terry, 1965; Carneiro and Lopes, 1986; Lewis, 2001). Worms were washed 5-6 times in reduced RPMI (Thermo Fisher Scientific, MA, USA) supplemented with 1000µg/ml streptomycin and 1000U/ml penicillin and cultured in 12 well plates, prior to electroporation and transfection with siRNA. The described animal procedures were reviewed and approved under Protocol Number 3346 by the McGill University Facility Animal Care Committee, and in keeping with the guidelines of the Canadian Council on Animal Care.

2.4 Transfection of schistosomula with siRNA

Schistosomula were cultured in 24 well plates, as described above. Prior to plating, the siRNA-lipid transfection reagent complex was prepared as follows. 2µl of siPORT Neo FX was incubated with 25 µl of Opti-MEM, mixed, and incubated for 10 minutes. Gene-specific siRNA or scrambled siRNA control (Thermo Fisher Scientific, MA, USA) were each subsequently mixed with 25 µl of Opti-MEM, mixed with the lipid transfection reagent mixture, and incubated for 10 minutes at room temperature to produce the transfection reagent complex. Schistosomula were pipetted into the 24 well plate with the transfection reagent complex in triplicate. siRNA was added to a final concentration of 50nM when generated by the transcription-based method, and to 75nM when synthesized commercially. OPTI-MEM was added to each well and schistosomula were incubated at 37 °C, as described, for 4 hours, to allow transfection to occur in the newly-transformed schistosomula. FBS was added to a final concentration of 4%, and after another incubation of 14 hours at 37 °C, to allow for transfection to continue further,

streptomycin and penicillin were added to final concentrations of 100µg/ml 100U/ml respectively. Schistosomula were monitored daily to determine if there were any effects on motility compared to untransfected or scrambled siRNA-treated controls. A change in motility was typically observed at 5-7days days following transfection, at which point motility was quantified. Transfection was verified using FAM-labeled siRNA with examination by confocal microscopy as described previously, to verify successful transfection (Nabhan et al., 2007) and downregulation of siRNA was confirmed by qRT-PCR.

2.5 Quantifying motility and length changes in schistosomula

RNAi-transfected schistosomula were cultured in 24 well plates, as described in the above protocol, at ~150 schistosomula /well, for 5-7 days in 850 µl Opti-MEM supplemented with 5% heat inactivated FBS, 100µg/ml streptomycin and 100U/ml penicillin. Worms were monitored daily to determine visible phenotypes, and motility was recorded when a change was observed, typically following incubation for 5 days. To record schistosomula, animals were acclimated to room temperature for 15 minutes prior to taking the recording. Smp_150180 and SmTBH siRNA-transfected animals were also incubated with 10 µM OA, following the initial baseline recording, using a previously described protocol (El-Shehabi et al., 2012). This was done to determine if the RNAi had any effect on OA-stimulated movement, as well as basal motility of the worm. Schistosomula were filmed for 1 min with a Nikon SMZ1500 microscope equipped with a QICAM Fast 1394 (mono 12 bit, QImaging) digital video camera and video acquired with SimplePCI software (version 5.2, Compix Inc.), at ~2.5 frames per second (fps). Between 2 to 3 wells were recorded per treatment, with a minimum of 12 schistosomula per well and each treatment was repeated over a minimum of three separate transfections using ImageJ software. All schistosomula in the field of the video were analyzed, unless in contact with other schistosomula, to avoid bias in selection. ImageJ software (version 1.41, NIH, USA) was used to quantify worm motility as a measure of length changes over the period of the 1 min recording, using the ImageJ Fit Ellipse algorithm, as has been previously described (Patocka and Ribeiro,

2013). The length of the ellipse, per frame, was measured using the Major Axis measurement of the ellipse, in ImageJ, as an indicator of schistosomula length.

2.6 Transfection of adult schistosomes with siRNA

Adult worms were obtained and washed as described above, and transfected with siRNA as follows. 4-5 adult male and female pairs (8-10 parasites in total) were electroporated in RPMI (Thermo Fisher Scientific, MA, USA) with 5 µg sequence-specific or control siRNA in a 4mm electroporation cuvette and electroporated, using a single 20 ms square wave pulse at 125 V, as previously described (Patocka et al., 2014). Worms were subsequently cultured in a 12 well plate in RPMI supplemented with 2 mM glutamate, 10 mM Hepes, 5% FBS and 100 g/ml streptomycin and 100 U/ml penicillin.

2.7 Quantitative and qualitative methods for determining motility and phenotype changes in adult *S. mansoni*

Phenotypes in siRNA-transfected adult worms in culture were determined as follows: Motility was recorded daily and analyzed when a change in phenotype was observed. A pixel displacement method, described previously (Patocka et al., 2014; Rashid et al, 2015), was used to quantify motility of adult worms. Briefly, using ImageJ, video recordings of two minutes of all worms in culture were converted to binary format, such that worms in the videos are represented by black pixels against a white background. Single worms were isolated for analysis, the video duplicated (V1 and V2) and first and last frames of the original (V1) and duplicated (V2) videos were deleted (V1-f0 and V2-fn, respectively) such that the videos were staggered, with a single-frame time lapse when played simultaneously. The videos were subtracted from one another to quantify the number of displaced pixels from frame-to-frame, using the Image Calculator algorithm in ImageJ. A worm motility index was subsequently calculated as the proportion of displaced pixels relative to total pixels in the original frame. Average motility was calculated from 3-8 transfections per treatment. Besides effects on

motility, the videos were also examined for changes in locomotory behaviours that might indicate discuption of muscle function. For RNAi-targeted adult worms, videos were assessed to identify at least 6 distinct motor phenotypes. These phenotypes are: (1) adhesion of the sucker to the bottom of the dish (supplemental video S1 and S2) and (2) a c-shaped, or bent posture, involving a limited range of motion, where the ends of the worm remain bent to the same side, in what resembles a "C" shape throughout the duration of the video (supplemental video S3). Also observed was (3) rigidity of the body, in, but not limited to the worm midbody (supplemental video S4), (4) a tight curl in the body, where the worm curls tightly into itself, in a ball, such that head, midbody and tail are not easily distinguishable (supplemental video S5), (5) thickness in the body, typically in the midbody (supplemental video S6) and (6) a lengthening effect, where worms extended to 2-6 times its shortest length between contractions. Proportions of worms displaying the phenotype were averaged for each transfection. Statistics were generated for 20-180 worms, per treatment, for each of the 6 phenotypes The unpaired student t-test was used to compare the means of control and test populations and p-values < 0.05 were considered to be significant. Downregulation of gene expression was confirmed by qRT-PCR.

2.8 RNA extraction from schistosomes

RNA from schistosomula was extracted using the RNeasy Micro RNA extraction Kit (Qiagen Inc., MD, USA). Briefly, schistosomula were harvested, washed, resuspended in 5 ul of 4ng/ul carrier RNA in RLT lysis buffer and sonicated for 2 minutes with alternate 10 second pulse and rest periods, to extract RNA. RNA was purified using the kit-provided column. RNA from adults was obtained from ~8-10 worms at a time, using a TRIZOL (Thermo Fisher Scientific, MA, USA) - chloroform separation and subsequent purification with the Total RNA Purification kit (Norgen Biotek Corp.). Adult worms were suspended in TRIZOL, homogenized with a mortar and pestle, aspirated through a 25 G, 2" needle, (Becton, Dickinson and Company (BD)) 20 times and sonicated as above, to extract RNA. The mixture was then centrifuged for 10 minutes at 12,000

xg, at 4 °C, and the supernatant, mixed with chloroform at 20% of the total volume. Following a subsequent centrifugation, the separated aqueous phase was mixed with an equal volume of the kit-provided RL lysis buffer, followed by 70% ethanol, to eliminate salts. RNA was purified using the kit-provided column, as per the kit protocol. All RNA was quantified using a Nanodrop spectrophotometer (ND1000, Thermo Fisher Scientific, MA, USA). Reverse-transcription, to generate cDNA, was performed as described in section *2.3* above, using equal amounts of RNA template for each gene target extract, per experiment and controls omitting reverse transcriptase as well as omitting an RNA template template, to verify specificity of the RT reactions.

2.9 Verifying RNAi knockdown by qRT-PCR

qRT-PCR was performed on RNA extractions from siRNA-targeted samples, using EvaGreen qPCR MasterMix-Rox (Diamed, ON, Canada). qPCR primers were designed to amplify a ~200bp region of the target gene. Primers indicated in **Table 1** were designed, using the same requirements ascribed to designing the RNAi primers. A distinct region was selected as the target sequence, to avoid overlap with the RNAi target sequence and to ensure detection of gene knockdown, rather than a possible off-target hit. Another set of primers targeting *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase (GAPDH, accession number: M92359) was used as an internal control for each gene target. The following primers targeting sense and anti-sense strands of GAPDH, respectively, were used: 5'-GTTGATCTGACATGTAGGTTAG-3' and 5'-ACTAATTTCACGAAGTTGTTG-3', as previously described (Macdonald et al., 2014). Primer efficiency was determined for each primer set, using the standard curve method to verify optimal efficiencies (100 +/- 15%) and similar efficiencies between primer sets (Rutlege and Cote, 2003). To determine primer efficiency, the targeted gene sequence, conjugated to PJet cloning vector, using the same protocol described above, was amplified in serial 2-fold dilutions of template. Amplification of GAPDH between SYBR Green and EvaGreen (Diamed, ON, Canada) was compared side-by-side in a qPCR experiment, and lower Ct values were observed

for both GAPDH and the tested gene for EvaGreen, indicating higher sensitivity with EvaGreen, under the selected experimental conditions. Accordingly, EvaGreen was used for the described qPCR experiments. A final concentration of 35 nM of forward and reverse primers, respectively, 2 µl of cDNA template and 10 µl of the EvaGreen mastermix was used in final reaction volumes of 20 µl. The qPCR reaction was performed in a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, MA, USA) and threshold cycle (Ct) and melting curve temperatures determined using StepOnePlus Software (Version 2.3), with the following thermal cycling conditions: 95 °C/10 min, followed by 50 cycles of 95 °C/ 15 s, 56 °C/ 30s, 72 °C/30 s, modified from (El-Shehabi and Ribeiro, 2010). Following amplification of cDNA via qRT-PCR, relative gene expression levels were determined using the comparative ΔΔCT method (Livak and Schmittgen, 2001) to determine fold downregulation of the targeted gene, compared to scrambled siRNA-targeted samples reference genes, using GAPDH as an internal control. A minimum of 4 separate transfections were analyzed per gene target and 2-3 transfections were pooled per RNA extraction.

3. Results

3.1. Identification of putative tyrosine derivative signaling genes via *in silico* analysis

We previously identified and characterized SmTH (AF030336), involved in the rate-limiting step of DA synthesis (Hamdan and Ribeiro, 1998), as well as putative DA synthesis enzymes SmAADC (Smp_171580) and putative OA synthesis enzyme SmTBH (Smp_243830), via an *in silico* analysis of the *S. mansoni* genome (**Figure 1**). To identify GPCRs putatively involved in OA signaling, a phylogenetic tree of biogenic amine (BA) GPCRs was generated from the Clustal Omega (ClustalO, Version 1.1.2) (Söding, 2005; Sievers et al., 2011) Phylogeny tool in EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute), using FigTree (Version 3.0) (Morariu et al., 2008), for visualization (**Figure 2**). 60 vertebrate sequences and 60 invertebrate sequences were obtained from NCBI (http://ncbi.nlm.nih.gov) and SchistoDB (http://www.schistodb.org), based on their annotation and included in the alignment. 28

schistosome BA GPCR sequences are included in the phylogenetic tree, including 6 known and 22 putative sequences. The phylogenetic tree includes BA GPCRs from the following vertebrate species: *Homo sapiens*, *Bos taurus*, *Mus musculus* and the boar species *Sus scrofa* and such invertebrate species as *Drosophila melanogaster*, *D. pseudoobscura*, tick species *Rhipicephalus microplus*, the cockroach species *Periplaneta americana*, and *Apis mellifera*, *Clonorchis sinensis* and *Schistosoma japonicum*. BA GPCRs of different ligand specificities include beta-adrenergic receptors (ADRB), alpha-adrenergic receptors (ADRA), octopaminergic receptors (OAR), D1-D4 type dopamine (DA) receptors, H1-H4 histaminergic receptors, serotonergic receptors and M1-M4 type muscarinic acetylcholinergic receptors. The tree contains known and orphan receptors. Known *Schistosoma mansoni* dopamine (DA) receptors, SmD2 (Smp_127310) (Taman and Ribeiro, 2009) and SmGPR3 (Smp_043290) (El-Shehabi et al., 2012), as well as a known serotonin receptor, Sm5HTR (Smp_126730) (Patocka et al., 2014) and known acetylcholinergic receptor SmGAR (Smp_145540) are indicated. Other known *S. mansoni* BA GPCRs included in the phylogenetic analysis include histaminergic (HA) SmGPCRs, SmGPR1 (Smp_ 043260) (El-Shehabi et al., 2009) and SmGPR2 (Smp_ 043340) (El-Shehabi and Ribeiro, 2010). While the schistosome 5HTR and SmD2 and SmGAR BA GPCRs share homology with other 5HT, dopamine (DA) and acetylcholinergic receptors of other species, respectively, SmGPR1-3, as previously described, group with the SmGPCR clade of *S. mansoni* receptors that share low levels of homology with other BA GPCRs. The tree highlights BA GPCRs that are of interest for de-orphanization. One such BA GPCR includes Smp_150180, which shares homology with beta-adrenergic receptors and may be an OAR, given the role of OA as the invertebrate equivalent to mammalian adrenergic signaling. Other BA GPCRs of interest, indicated by the arrows, include Smp_120620 and Smp_180140, which are closely related to the SmGPCRs, indicating that they diverge from vertebrate GPCRs, which may indicate that they would be specific drug targets. TMHMM2 software (Center for Biological Sequence Analysis – Technical University of Denmark (CBS-DTU), http://www.cbs.dtu.dk) reveals that both Smp_150180 and Smp_120620 have the 7 predicted transmembrane regions required to form a

functional GPCR. While TMHMM software predicts 6 TM regions for Smp_180140, closer inspection of the posterior probability plot reveals two separate regions of hydrophopic residues predicted to be a single TM region, likely indicating that there are indeed 7 TM regions in this receptor. Moreover, manual inspection of an alignment of Smp_180140 with vertebrate BA GPCRs indicates that the 7 TM regions are likely present, as the predicted TM regions align with those of vertebrate receptors. In an BLASTp (NCBI) analysis, Smp_180140 shares ~24-26% identity with two predicted beta adrenergic receptors and ~21-27% identity with putative 5HT GPCRs. Smp_120620 shares 21-31% identity with alpha-adrenergic receptors and 31-34% identity with putative DA GPCRs. This data indicates a possible OA specificity for these BA GPCRs, given their similarity to adrenergic receptors, though these analyses cannot conclusively determine the associated ligand for these receptors, and *in vitro* studies are required to deorphanize these BA GPCRs.

3.2 An RNAi screen of proteins putatively involved in catecholamine and phenolamine signaling in schistosomula

Genes involved (or putatively involved) in OA- and DA-signaling were targeted by RNAi to identify the associated phenotype.and to determine if they play a role in muscle control, as predicted from the prior BA screen. Specifically, we questioned whether downregulating these genes caused a change in motility, a decrease in relative body length and/or aberrant motor phenotypes, opposite to the effects observed following OA or DA administration. Such a result would further validate the role of these neuroractive amines in schistosome muscle control.

In schistosomula, DA-related genes SmTH (accession number: AAC62256), SmD2 (Smp_127310) and SmGPR-3 (Smp_043290), which have previously been cloned and characterized (Hamdan and Ribeiro, 1998; Taman and RIbeiro, 2009; El-Shehabi et al., 2012), as well as DA-related gene SmAADC (Smp_171580) and OA-related genes SmTBH (Smp_243830), Smp_150180 and Smp_180140 were targetted by RNAi. When cercariae lose their tail and transform to schistosomula, their membrane undergoes remodeling from a single unit membrane

phospholipid bilayer to a double-bilayer tegument. This remodeling means that the tegument is more permeable to siRNA at the time of transformation (Skelly et al., 2003; Nabhan et al., 2007). Thus, transfection is performed within 2 hours of transformation to maximize uptake of the siRNA with the lipid transfection reagent. Successful labelling of schistosomula by successfully transfected FITC-conjugated siRNA with the described conditions was confirmed with confocal microscopy before proceeding with the described experiment (Nabhan et al., 2007).

Relative motility and length of schistosomula was quantified with the described methods for length changes as a measure of frequency of body movements (El-Shehabi et al., 2012; Patocka and Ribeiro, 2013). Worms were cultured for up to seven days, and motility was recorded when a change was observed, typically following 5 days in culture, following transfection with 50 nM siRNA with a lipid transfection reagent (SiPORT Neo FX, Thermoscientific), or 75 nM of synthesized siRNA (Sigma-Aldirch, MilliporeSigma, MA, USA). Pooled, in vitro digested siRNA was generating using an RT (reverse-transcriptase) PCR–based method using the Megascript RNAi kit (Thermo FIsher Scientific, MA, USA). Worms were recorded using a microscope equipped with a video camera to generate videos of a 1 minute duration at ~2.5 frames per second (fps) (~150 frames per video). A total of 45 to 300 schistosomula were analyzed per treatment from a minimum of three separate transfections.
When downregulated via RNAi, dopamine BA GPCRs SmD2 and SmGPR3 and synthesis enzyme SmTH, which performs the rate limiting step in DA synthesis, synthesizing DA precursor L-DOPA (Hamdan and Ribeiro, 1998), as well as putative DA synthesis enzyme, SmAADC, show similar trends with respect to relative motility (Figure 3A). That is, downregulating these genes causes significant hypomotility of ~50% or lower, consistent with the effects of incubating schistosomula with DA at high concentration (500 µM). Mean motility values were calculated for each treatment, indicated in the graphs, normalized to the scrambled-treated control. The standard error of the mean (SEM) is indicated by the error bar and significant values are indicated by asterisks, as determined by student t-tests at p values < 0.05 (*). The shortening

effect is most pronounced for SmGPR3, which shows an associated decrease in motility compared to the scrambled treated control of ~ 75%. The order of hypomotility, from most to least pronounced for DA-related RNAi-targeted genes is as follows: SmGPR3 > SmTH > SmAADC > SmD2. While DA causes a concentration-dependent decrease in motility in schistosomula, this effect on motility was determined to be biphasic, and at high concentration (500 µM), an increase in motility is observed. This data is consistent with inhibition of movement at high concentrations of DA.

OA-related targeted genes include putative OA synthesis enzyme, SmTBH and putative receptors Smp_150180 and Smp_180140. Downregulation of SmTBH in seven-day old schistosomula significantly decreased motility (p value < 0.005) (**Figure 3B**). RNAi targetting Smp_150180 also appears to produce a decrease in motility but the effect is not statistically significant at p <0.05, while RNAi targeting of Smp_180140 had no measurable effect on motility. The reponse to SmTBH RNAi is expected, as SmTBH is a putative OA synthesis enzyme and its downregulation would presumably decrease the availability of endogenous OA for all downstream GPCR signaling. In contrast, the weak (or no) response to Smp_150180 and Smp_180140 suggests these may not be the receptors associated with OA-stimulated motility in schistosomula or, alternatively, redundancy may exist in OA signaling, such that targeting of a single OA-related receptor does not produce an observable effect on motility.

SmTBH and Smp_150180 were incubated with 10 µM OA, following the baseline recording of siRNA-targeted animals (**Figure 3C**). As expected, addition of OA to SmTBH-targetted animals reversed the effects of the RNAi, where a decrease in motility was no longer observed. This data is consistent with providing the schistosomula with OA which has been depleted by the downregulation of SmTBH.

In addition to changes in motility, we observed a lengthening effect associated with DA and OA signaling in schistosomula. To determine whether the genes of interest are involved in the elongation response, we quantified the relative average length of schistosomula following siRNA treatment relative to the scrambled control (**Figure 3D, E, F**). Targetting DA-related

signaling genes caused a significant decrease in length of up to 20%, with the exception of SmAADC, which was not associated with a measurable change in length compared to the scrambled-treated control (**Figure 3D**). AADC is a predicted DA – biosynthetic enzyme but it has not yet been characterized in vitro. Moreover, SmAADC may be involved in 5HTR signaling (Ribeiro et al., 2012), as a homologous decarboxylase enzyme is involved in serotonin biosynthesis from its immediate precursor, 5-hydroxytryptophan (5HTP). Serotonin is associated with an increase in schistosomula motility. If SmAADC is involved in 5HT synthesis, this could explain why only motility is affected by the RNAi. If, alternatively, SmAADC is involved in DA biosynthesis from its immediate precursor, L-3,4-dihydroxyphenylalanine (L-DOPA), L-DOPA may counteract effects of the RNAi on length. L-DOPA has previously been determined to affect DA signaling and motor control (Opacka-Juffry et al., 1998; Fisone et al., 2011). A significant shortening effect is observed following targeting of these OA-signaling genes (**Figure 3E**), as is expected, given the concentration-dependent increase in parasite length observed following incubation of schistosomula with OA (see Chapter 2). The order of OA-related gene siRNA targeted genes producing the most significant decrease in length is as follows: Smp_150180 > SmTBH > Smp_180140. The decrease in length compared to the scrambled control ranges from ~10% for Smp_180140 to ~15% for Smp_150180. These effects are consistent with the expected effect of RNAi downregulation of OA signaling-related genes.

Adding 10 µM OA following SmTBH downregulation reverses effects on motility. Adding 10 µM OA to schistosomula does not reverse the significant effects on parasite length for SmTBH and Smp_150180. These effects range between a ~10 to 15% decrease for SmTBH and Smp_150180, respectively (Figure 3F). The data are consistent with the expected result for schistosome length, where exogenously applied OA and DA cause a significant lengthening effect and RNAi silencing of genes associated with OA and DA signaling causes the opposite effect (shortening). These effects may indicate that, compared to other BAs, OA plays a more significant role in determining length changes than motility. While the in vitro studies in Chapter 2 suggest that OA has a significant effect on schistosomula motility, other receptors

involved in motility may counteract the effects observed by RNAi on motility. Furthermore, the absence of an effect on motility following Smp_150180 downregulation suggests that other receptors are involved in the effects of OA on motility.

3.3 An RNAi Screen of genes involved in tyrosine-derivative signaling in adult schistosomes

As in schistosomula, DA- and OA-related genes were downregulated in adult parasites to shed insight into the role of DA and OA signaling in adults. In adult stage parasites, as with schistosomula, changes in frequency of body movements were quantified. The method of quantification of adult motility following RNAi downregulation, described here, uses pixel displacement to measure relative motility, using ImageJ software (Patocka et al., 2014) (**Figure 4**). Worms in culture were recorded using a microscope equipped with a video camera (Nikon) and SimplePCI software to generate videos. Videos were converted to binary format to detect pixel units and each worm was selected for analysis with a drawing tool in ImageJ. The videos were subtracted from one another to quantify the displaced pixels, which were expressed as a portion of total pixels in the original video. Mixed populations were composed of 8-10 worms, containing an equal number of males and females per well, in 24 well plates. siRNA treatments were performed in triplicate on a total of 20-130 worms per treatment, in 3-8 separate transfections, per RNAi treatment, for the 10 siRNA samples, including the scrambled siRNA and Sm5HTR (Smp_126730) control. Sm5HTR was previously validated using the same method to quantify motility in adult schistosomes (Patocka et al., 2014). Untransfected and un-electroporated samples were also used as negative controls. Worms were transfected using electroporation, as described (Patocka et al., 2014), and motility, quantified following 3-4 days of maintenance in culture. Worms in culture were recorded daily, using a microscope equipped with a video camera and SimplePCI software to generate videos of a 2 min duration at ~2.5 frames per second (fps) (~300 frames per video). Bars in the graph represent mean values of motility, expressed as fold change in motility, normalized to the scrambled siRNA-treated control (**Figure 5**). Motility was quantified using a mixed, male and female population (**Figure**

5A) and, analyzing males and females separately. For OA-related genes where significant changes in motility were observed, relative motility compared to the scrambled-treated control, in male and female populations, is shown (**Figure 5B**). Mixed population (**Figure 5A**) and single-sex population analysis of DA-related genes, SmTH, SmAADC, SmD2 and SmGPR3, did not display a significant change in motility (not shown for single-sex analysis).

In the mixed sex population of OA-related genes, Smp_150180 downregulation is associated with a decrease of ~40% in motility. Analyzing males and females separately, reveals that this downregulation is primarily due to the effects of Smp_150180 downregulation in the female population of ~60%, comparable to that of the Sm5HTR control, indicated by an arrow **Figure 5B**). Downregulation of OA-related Smp_120620 yielded a significant ~30% increase in motility in males, indicated by an arrow (**Figure 5B**). In the mixed population, Smp_150180 shows a modest decrease of ~40%, though this decrease is due to a significant ~60% decrease in the female population, indicated by arrows (**Figure 5B**).

3.4 RNAi downregulation of OA- and DA-related genes in adult *S. mansoni* causes changes in posture and motor control phenotypes

Apart from the effects of RNAi on frequency of body movements, effects on posture, coordination and shape of the adult worms, indicative of changes in muscle tone, were also observed. The most frequent and identifiable phenotypes are indicated in **Figure 6**. Proportions of worms showing the indicated phenotypes were quantified, using the videos from the same population of 20-130 worms, from 3-8 separate transfections, on which the siRNA experiments were performed. In this way, worms could be assessed further, identifying phenotypes that did not neccessarily change the relative motility of the parasites and would not be identified in the first analysis. Accordingly, the most frequently occurring phenotypes were determined, named according to their salient feature(s), and, for each experiment (transfection), the number of worms displaying the phenotype were counted and then the proportion of worms showing that phenotype was determined for each transfection (**Figure 7**).

Compared to the scrambled control worms, the analyzed phenotypes include: adhesion of the worm to the bottom of the dish via the oral sucker; a C-shape/bending phenotype; rigidity, which typically occurs in the midbody and is indicative of paralysis in the region in which it is observed; a lengthening of the body; and thickening of the body, also typically most pronounced in the midbody. The described phenotypes can be indicative of worm health, as is the case of sucker adhesion, or variations in muscle tone, which can contribute to differences in posture and types of movement.

A single worm was analyzed at a time and the mean percentage of worms demonstrating the phenotype was determined to verify if there were any trends associated with DA and OA-related signaling genes in adult worms (**Figure 7)**. Error bars represent the standard error of the means, and asterisks represent p values < 0.05, using student t tests.

Downregulating DA-related genes causes an increase in the percentage of worms showing the thickness phenotype, particularly for SmD2, where 20% of the animals display this phenotype, compared to none in the scrambled-treated control group.

Putative DA synthesizing enzymes SmTH and SmAADC and DA receptor SmGPR3 downregulation both result in a significant percentage of the population demonstrating the rigidity phenotype, at 52%, 31% and 27%, respectively, compared to in 4% of the control group.

There is also an increase in the percentage of worms retaining a C-shaped posture compared to the control, following downregulation of DA-related genes. Approximately 40% of worms in populations where SmTH or SmGPR-3 are downregulated demonstrate the C-bend phenotype, compared to 26% of the animals in the scrambled siRNA-treated control group.

In 14%, ~20% and 23% of the population, the tight-curl phenotype is observed following SmTH, SmAADC and SmGPR-3 downregulation, compared to 7% in the scrambled-treated control.

Downregulating OA-related genes also results in changes in the percentage of worms displaying different phenotypes associated with posture and coordination. A significant increase in the percentage of worms showing the thickness phenotype is observed following

downregulation of Smp_180140 (33%) and Smp_120620 (6%) compared to 0% in the scrambled-treated control

The rigidity phenotype occurs in an average of 65%, 55%, 32% and 21% of populations where Smp_150180, Smp_180140, Smp_120620 and SmTBH are downregulated, respectively. This phenotype demonstrates a paralysis in the midbody and suggests that OA-related genes, particularly Smp_150180, are important for motility in the midbody. This rigidity points to an inability for the parasite to bend or straighten, relative to its resting posture.

The C-shaped phenotype is observed in 56%, 54% and 44% of the population following Smp_150180, Smp_180140 and Smp_120620 downregulation, respectively, compared to in 34% in the scrambled-treated control population. SmTBH downregulation, conversely, causes a decrease in the percentage of worms displaying the C-shape phenotype, which occurs in 9% of the population.

An increase in the percentage of worms with the tight curl phenotype is observed in populations following downregulation OA-related genes SmTBH (11%), Smp_120620 (13%), Smp_150180 (21%) and Smp_180140 (26%), compared to the scrambled-treated control population (1%).

In populations where OA-related genes are downregulated, there is a significant decrease in the proportion of worms which adhere to the dish. This sucker adhesion phenotype occurs in 7%, 1% and 0.2% of the population following Smp_150180, Smp_180140 and Smp_120620 downregulation, respectively, compared to 15% in the scrambled-treated control population.

3.5 qRT-PCR confirms RNAi knockdown in schistosomula and adult parasites

qRT-PCR was used to determine RNA expression levels of the indicated genes (**Figure 8**). cDNA from 2-3 pooled transfections per qPCR experiment from a minimum of 4 independent transfections, was amplified and the ΔΔCt method was used to calculate fold downregulation of RNA expression in both schistosomula and adult parasites (**Figure 8**), normalized to the

schistosome GAPDH housekeeping gene, using scrambled siRNA samples as a reference gene. All targeted genes show a significant downregulation at the RNA levels of ~50% or less compared to the scrambled-treated control, indicating that RNAi silencing of these DA- and OA-related genes was successful.

4. Discussion

Our RNAi studies in schistosomula and adult parasites reveal neuromuscular effects of both DA- and OA-related signaling genes, highlighting the importance of tyrosine derivative signaling in *S. mansoni* motor control. RNAi silencing of DA-related genes in schistosomula caused a shortening of the body and a decrease in motility. These effects are consistent with the expected effects of inhibiting DA-signaling at high concentration which we reported as being associated with an increase in motility in the previous chapter. We performed a concentration response assay to determine the effect of DA on schistosomula. While DA was associated with a concentration-dependent decrease in motility, at high concentration (500 μM), an increase in motility was observed. DA signaling is associated with a relaxation of longitudinal muscles (Pax et al., 1984). The decrease in worm length that we observed is therefore consistent with the expected result, following RNAi downregulation of DA-signaling genes. RNAi-induced stimulation of longitudinal muscle contration may therefore be contributing to the decrease in motility and shortening effect.

Downregulation of all tested DA-related genes in schistosomulawas associated with the expected effect, a decrease in motility and length, consistant with inhibition of DA signaling at high concentration, when the observed effect was statistically significant. Only SmAADC downregulation was not associated with a decrease in length. SmAADC shares homology with the AADC that catalyzes both DA and 5HT synthesis. If SmAADC is involved in 5HT synthesis, its downregulation would likely cause a decrease in motility due to disruption of 5HT signaling, consistent with the effect of RNAi. Alternatively, if SmAADC is downregulated, this could result in an increase in the DA precursor, L-DOPA, the putative substrate of SmAADC. L-DOPA could

itself modulate DA signaling and motor control (Opacka-Juffry et al., 1998; Fisone and Bezard, 2011), which may counteract effects on length.

RNAi silencing of OA-related genes, when statistically significant, was associated with the expected effects of inhibiting OA signaling. SmTBH downregulation causes a decrease in motility and length, which is opposite to the concentration-dependent effects of OA reported in the previous chapter. The absence of an effect on motility following Smp_150180 and Smp_180140 downregulation indicates that these receptors may only be involved in causing the changes in length which we observed. Effects of RNAi on SmTBH motility which were not observed with the targeted receptors may be attributable to the fact that SmTBH is involved in OA synthesis and its downregulation would decrease the availability of OA in the parasite, affecting all OA signaling pathways rather than a single pathway. SmTBH downregulation could therefore inhibit OA receptors which may be involved in the lengthening effect stimulated by OA.

The effect of adding 10 µM OA following RNAi silencing also suggests that OA contributes to changes in length, more than it does motility. Addition of OA to schistosomula causes a concentration-dependent increase in length and motility. This effect on motility may not be as pronounced in the RNAi studies, as was observed in the motility studies in Chapter 2, because other neurotransmitter-related genes may have redundant functions and compensate for the decrease in endogenous OA. Incubation with 10 µM OA may therefore be adequate to reverse effects of SmTBH downregulation on motility, though not on length. As indicated in Chapter 2, effects of OA on length and motility are consistent with the relaxation of longitudinal muscle and/or a stimulation of circular muscle contraction. This role in muscle contraction is suggested by the combination of the concentration-dependent increase in length and motility. An increase in motility likely requires a stimulation of a muscle type, as suggested by the effects of muscle contraction/relaxation on motor control (Pax et al., 1984; Halton and Maule, 2004). A stimulation of circular muscle, not longitudinal muscle, is consistent with an increase in length and motility (Halton and Maule, 2004). This effect does not rule out the possible effect of OA

causing relaxation of longitudinal muscle as well (Pax et al., 1984). Relaxation of longitudinal muscle, combined with contraction of circular muscle, could still result in a parasite with an increase in relative length and motility. Therefore, if OA signaling stimulates circular muscle contraction, RNAi of OA-related genes would result in relaxation of circular muscle, which could lead to shortening of the body, without necessarily affecting frequency of body movements. Whereas inhibition of longitudinal muscle contraction and/or stimulation of circular muscle may cause an increase in motility, the relaxation of circular muscle and possible stimulation of longitudinal muscle resulting from RNAi may only be sufficient to cause a shortening of the body, without necessarily causing a pronounced decrease in motility. This effect may be due to the distribution of OA receptors on, or in neurons innervating, the different muscle types. These receptors can be distributed differently, have different EC50 values (Hawley et al., 1995) and therefore, cause different effects on motor control. Targeting a single receptor by RNAi can therefore produce a different effect than stimulating all of the receptors via incubation with OA or inhibiting all of the receptors via SmTBH downregulation.

To determine if tyrosine derivative-related signaling genes played an important role in neuromuscular control in adult parasites, as observed in schistosomula, RNAi was also performed in adult schistosomes. While DA-related signaling genes did not produce a significant change in motility compared to the scrambled-treated control, downregulating two OA-related signaling genes, Smp_150180 and Smp_120620, caused changes in motility. These effects on motility were gender-specific and occurred in only the male or female population. These gender-specific effects may point to different roles of both Smp_150180 and Smp_120620 between the sexes. The different RNAi phenotypes may be due to differences in protein function and differences in protein expression between males and females (Protasio et al., 2012).

OA effects on posture, coordination and shape of the adult worms, indicative of changes in muscle tone, were also observed following downregulation of tyrosine derivative-related signaling genes by RNAi. RNAi targeting DA-related genes, excluding SmD2, produces a tight

curl phenotype. This tight curling may be due to reduced longitudinal muscle relaxation, along with loss of coordination between circular and longitudinal body wall muscles.

While SmGPR3 is widespread in the nervous system (El-Shehabi et al., 2012), SmD2, has been identified in body wall muscle (Taman and Ribeiro, 2009). This localization suggests different effects on muscle control for these receptors. Effects of SmGPR3 downregulation differ from that of SmD2 and resemble that of the other DA-targeted genes, except for SmAADC. The thickness phenotype observed following RNAi of all DA-related genes can be attributed to a stimulation of longitudinal muscle. These effects on muscle are opposite to, and therefore consistent with the effects of DA on schistosomes, which includes a relaxation of longitudinal muscle (Pax et al., 1984). If longitudinal muscle is stimulated following RNAi and is in a state of constant tension, circular muscle may lose tone, giving the appearance of a thicker worm.

The fact that SmAADC downregulation does not contribute to an increase in frequency of the C-bend phenotype, only the tight-curl and rigidity phenotype, may be due to the disruption of DA biosynthesis increasing endogenous L-DOPA concentration, which may counteract the effect of downregulation of DA-signaling, preventing worms from remaining in a hunched C-shape posture. Some inhibition of longitudinal muscle may still allow for circular muscle contraction, producing straight, rigid worms, with periodic tight curling. This gene may also be involved in production of 5HT from 5-HTP and effects of SmAADC downregulation may alternatively be associated with 5-HTP production (Anden et al., 1968). 5-HTP may cause the relaxation of circular muscle and increase longitudinal muscle tone, effects which are associated with 5HT signaling in schistosomes (Pax et al., 1984). This combination of effects on muscle may decrease the percentage of worms with the C-bend phenotype.

OA-targetted RNAi resulted in a decrease in sucker adhesion, particularly for Smp_120620 and Smp_180140, where the worms cease to adhere to the bottom of the dish. Given the pronounced increase in motility in male worms associated with Smp_120620 downregulation, the decrease in sucker adhesion may be due to worms that are moving too

quickly to adhere and remain attached to the culture dish. Alternatively, the reduced adhesion may be caused by a decrease in worm health, resulting in uncoordinated movement and a loss of motor control.

SmTBH displays a phenotype that differs slightly from the tested OA-related receptors. SmTBH is the only gene for which a significant decrease in sucker adhesion and increase in the thickness phenotype are not observed, and which is associated with a reduced percentage of worms showing the C-bend phenotype. As with schistosomula, differences in the effects observed following RNAi may be due to differences in where receptors are located and the fact that SmTBH would inhibit all OA receptors rather than a single OA receptor. SmTBH downregulation may inhibit circular muscle and stimulate longitudinal muscle to a different extent than the individual OA-related receptors. The most pronounced effect of siRNA-targeting for these OA-related genes, as compared to the scrambled-treated control, is in worm rigidity, particularly for Smp_150180. This phenotype demonstrates a paralysis in the midbody and suggests that OA-related genes are important for motility in the midbody.

If OA signaling is involved in longitudinal muscle relaxation and/or circular muscle contraction, based on what we observe in schistosomula, the effects we observe in the adult parasites would support this idea. The phenotype of a hunched worm, with a thick body, restricted in its ability to extend, with the tight-curl phenotype occurring periodically is consistent with a stimulation of longitudinal muscle and relaxation of circular muscle types. In summary, there are similarities in the trends in motility changes between OA- and DA-signaling genes following addition of the neurotransmitter to schistosomula in culture, as well as downregulation of signaling genes. These trends include a concentration-dependent lengthening effect following addition of these BAs, as well as uncoordinated movement and changes in motor control, following RNAi. Differences in these trends, include a consistent decrease in motility following RNAi of DA-signaling genes in schistosomula, which is observed following downregulation of OA-related genes when the effects are significant. These differences indicate that these tyrosine derivatives may act on different muscle types and/or

regulate muscle contraction differently. In adult parasites, while these phenotypes that result following RNAi downregulation may point to inhibition of longitudinal muscle contraction, or relaxation of circular muscle, to yield variations in predominant muscle types contributing to motility, a common observation is a resulting uncoordinated movement. This uncoordinated movement highlights the importance of these signaling genes in control of motility, and their potential for drug targeting. This data should not be taken alone, as a direct confirmation of the role of DA- and OA-related signaling genes on types of muscle control, given the complexity of the system in the whole organism, but to demonstrate that drastic changes in control of motility are observed, and that these genes, particularly Smp_150180, Smp_120620, Smp_180140 and SmTH which produce the most pronounced effects, are important for motor function.

Our previous study of the effects of tyrosine derivatives on schistosomula motility and length, combined with the RNAi studies performed here in adult and schistosomula parasitic stages of *S. mansoni* provide strong evidence for the importance of tyrosine derivative signaling in schistosomes and identify 8 genes that produce significant changes in length, motility, posture and coordination, which may be targeted to develop anti-schistosomals for this widespread and debilitating disease. Genes associated with the most pronounced changes in motor control in the RNAi studies can be tested to determine if the RNAi effects can predict future drug efficacy. Functional studies of tyrosine derivative-related signaling genes, with unknown ligands/substrates, can be used to verify ligand- or substrate-specificity. Following the functional studies, the same assays can be used and scaled up to provide a readout for drug screens, to identify compounds which can specifically inhibit tyrosine derivative signaling proteins and interfere with schistosome neurobiology.

Figures

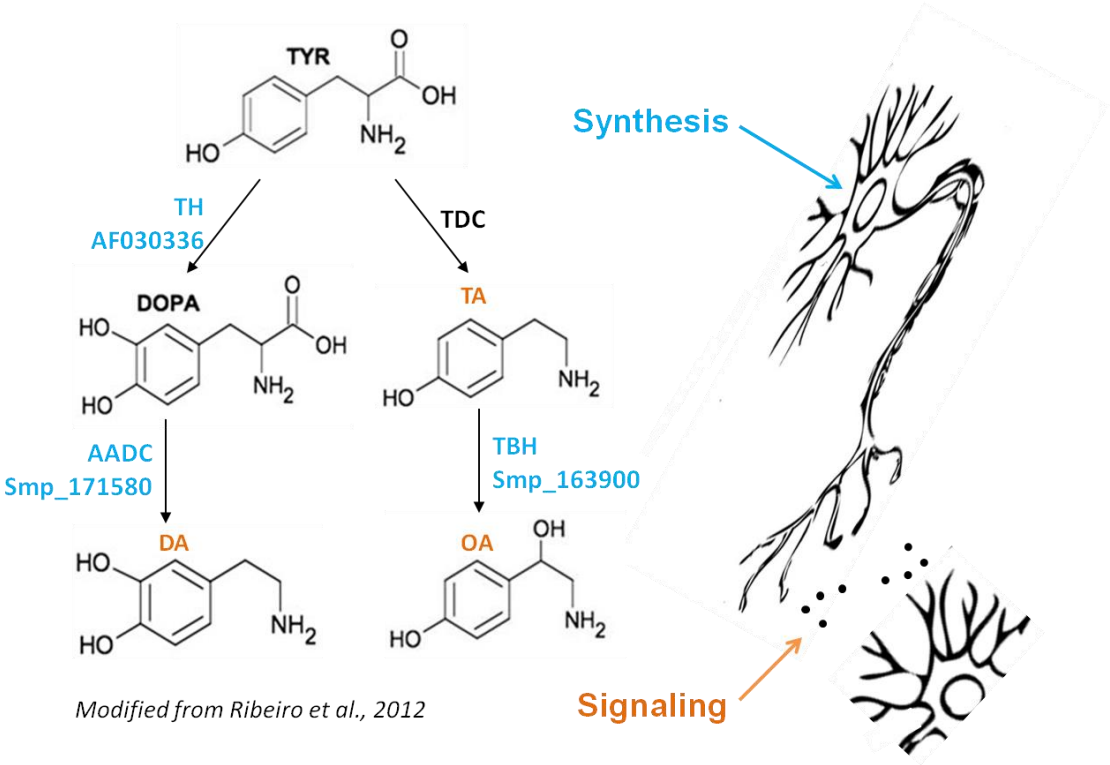


Figure 1. Homologues of genes involved in tyrosine derivative synthesis are present in *Schistosoma mansoni*. We previously identified genes involved in, and putatively involved in, tyrosine derivative synthesis in *S. mansoni* (Ribeiro et al., 2012). The synthesis of these biogenic amines (BAs) typically occurs in the neuronal cell bodies, as indicated. Synthesis pathways for catecholamine and phenolamine synthesis homologues in *S. mansoni* are indicated in blue. Catecholamines and phenolamines involved in signaling between neurons, which we previously tested on schistosomula, in *in vitro* motility studies, are indicated in orange. Tyrosine derivative signaling genes in *S. mansoni* include *S. mansoni* tyrosine hydroxylase (SmTH) (accession number.: AAC62256), the rate-limiting enzyme involved in catecholamine synthesis and that synthesizes L-DOPA (3, 4-dihydroxy phenylalanine) (Hamdan and Ribeiro, 1998) from dopamine (DA). SmTH was previously cloned and characterized in *S. mansoni*, indicating that the parasite

can perform endogenous synthesis of DA (Hamdan and Ribeiro, 1998). *S. mansoni* amino acid decarboxylase (SmAADC, locus tag: Smp_171580) and tyramine beta-hydroxylase (SmTBH) (Smp_243830), were identified, based on homology with enzymes in other species, via bioinformatics analyses (Ribeiro et al., 2012). SmAADC putatively synthesizes DA from L-DOPA via a decarboxylation reaction and SmTBH putatively synthesizes octopamine (OA) from hydroxylation of the tyramine (TA) beta carbon.

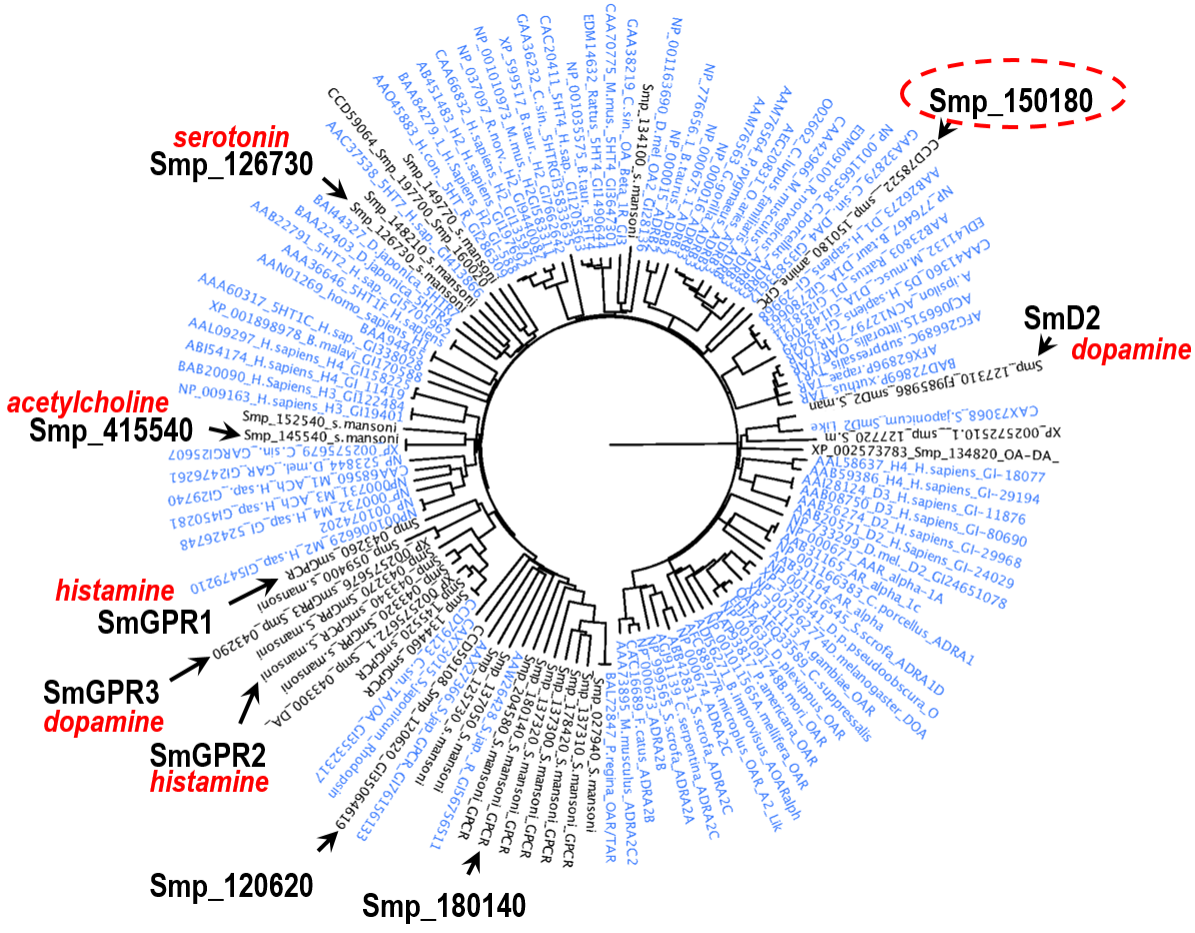


Figure 2. A phylogenetic tree analysis of biogenic amine (BA) receptors identifies putative octopamine (OA) GPCRs Smp_150180, Smp_120620 and Smp_180140. A phylogenetic tree of biogenic amine G protein-coupled receptors (BA GPCRs) was generated from an EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute) Clustal Omega (ClustalO, Version 1.1.2) (Söding, 2005 ; Sievers et al., 2011) multiple sequence alignment. The EMBL-EBI tree, generated with a neighbor-joining (nj) output, was visualized and reformatted using FigTree (Version 3.0) (Morariu et al., 2008). 120 sequences were obtained from NCBI (http://ncbi.nlm.nih.gov), including vertebrate and invertebrate BA GPCRs. 28 schistosome BA GPCR sequences, including known and putative sequences, are indicated in black. Locus_tags are indicated for *S. mansoni* BA GPCRs and GenBank accession numbers are indicated for other sequences. Descriptions of the indicated genes include abbreviated species names, including H. sap (*Homo sapiens*), B. taur (*Bos taurus*), M. Musc (*mus musculus*), D. mel (*Drosophila melanogaster*), C. sin (*C. sinensis*) and *S. jap* (*Schistosoma japonicum*). BA GPCR abbreviations include ADRB (beta-adrenergic receptor), ADRA (alph-adrenergic receptor), OAR (octopaminergic receptor) D1-D4 (type 1-4 dopaminergic receptors), H1-H4 (histaminergic 1-4 type receptors), 5HT (serotonergic receptor) and M1-M4 Ach (muscarinic acetylcholinergic type 1 to 4 receptors). The tree contains known and orphan receptors. Known *Schistosoma mansoni* dopamine (DA) receptors, SmD2 (Smp_127310) (Taman and Ribeiro, 2009) and SmGPR3 (Smp_043290) (El-Shehabi et al., 2012), as well as a known serotonin receptor, Sm5HTR (Smp_126730) (Patocka et al., 2014) and known acetylcholinergic receptor SmGAR (Smp_145540) are indicated by arrows. Other known *S. mansoni* BA GPCRs include histamine (HA) SmGPCRs SmGPR1 (Smp_ 043260) (El-Shehabi et al., 2009) and SmGPR2 (Smp_ 043340) (El-Shehabi and Ribeiro, 2010), also indicated by arrows. BA GPCRs of interest for RNAi studies are also indicated by arrows. One such BA GPCR, Smp_150180, shares homology with beta-adrenergic receptors, which may indicate that it is an OAR, given the homology between OARs and ADRs. Other BA GPCRs of interest, indicated by the arrows, include Smp_120620 and Smp_180140, which are closely related to the SmGPCRs.

Gene Name	GenBank Accession Number or Locus Tag	Forward (F) or Reverse (R)	T7-RNAi Target Primer Sequence	qPCR Target Primer Sequence
SmGPR3	Smp_043290	F	5'-GCGTAATACGACTCACTATAGGGAGA-AAGTTTATTGAATAAAC-3'	5'-TATTGATATAACAGATGCGTTATC-3'
SmGPR3	Smp_043290	R	5'-GCGTAATACGACTCACTATAGGGAGA-CTTTATTAAATATAAC-3'	5'-CGTCTAAATACGATTCATCAATTAC-3'
SmD2	FJ985986	F	5'-GCGTAATACGACTCACTATAGGGAGA-ACGCAGTTACCCAGAGTC-3'	5' GTC AAT CAA GTG GTA TTG GAA CC 3'
SmD2	FJ985986	R	5'-GCGTAATACGACTCACTATAGGGAGA-GTACCAAGACGCCATTTC-3'	GCG TCG GTT GAA GTT TAT TGG
SmAADC	Smp_171580	F	5'-GCGTAATACGACTCACTATAGGGAGA-CTACCGAATGAAGCACC-3'	5' CCA TTG GCA ACA TCC TC 3'
SmAADC	Smp_171580	R	5'-GCGTAATACGACTCACTATAGGGAGA-CCATCCAATCAATCATAAC-3'	5' CCT TGT ATG ACT CCA CCA CTG 3'
SmTH	AAC62256	F	5'-GCGTAATACGACTCACTATAGGGAGA-CTACAGAGAGCGACG-3'	5'-CGTCGCTCTCTGTAGAC-3'
SmTH	AAC62256	R	5'-GCGTAATACGACTCACTATAGGGAGA-CCTGAAGTACGATGC-3'	5'-GAGTTTATTGTACGAAGAAT-3'
SmTBH	Smp_243830	F	5'-GCGTAATACGACTCACTATAGGGAGA-TCTCCCTATAGTGAGTCGTATTACGC-3'	5' GTG TGG TGG AGT GGA AAT C 3'
SmTBH	Smp_243830	R	5'-GCGTAATACGACTCACTATAGGGAGA-CCTGTTGAACCCATAGC-3'	5' GAT AGA TGA CAT CAT GTC TAC TG 3'
Putative OAR	Smp_150180	F	5'-TAATACGACTCACTATAGGGAGA-TGGCGATATGAACAAAATCACT-3'	5' AAAATCCTATCACGCATACATC 3'
Putative OAR	Smp_150180	R	5'-TAATACGACTCACTATAGGGA-GACCAATGTTTTAGCTGCTTTCTG-3'	5' CGGAGAGCTATTATCATAATCC 3'
Putative OAR	Smp_180140	F	5'-TAATACGACTCACTATAGGGAGA-TAGCCTATGATTCACAGTTTGC-3'	5' TGTAGTACGCAGATTGGGA 3'
Putative OAR	Smp_180140	R	5'-TAATACGACTCACTATAGGGAGA-TGTGAAAATGTTGAAGTATTCC-3'	5' CTTAGTAGAACGGATTGAACC 3'
Putative OAR	Smp_120620	F	5'-TAATACGACTCACTATAGGGAGA-TCAACAACATCGACAGAGTTGC-3'	5' ATTGGAACTTCAGAATGGTCAA 3'
Putative OAR	Smp_120620	R	5'-TAATACGACTCACTATAGGGAGA-ATCATGGAACTCTTGATCTTTAGG-3'	5' TGTTAGTGTTGGATGAATTCCC 3'
Sm5HTR	Smp_126730	F	N/A (Synthesized siRNA)	5' GAACACCACAACGTATGGC 3'
Sm5HTR	Smp_126730	R	N/A (Synthesized siRNA)	5' CCTGCTGTCATTTTTGACT 3'

Table 1. List of primers to amplify RNAi and qPCR target sequences. Forward and reverse primer sequences used to amplify the dsRNA template for siRNA synthesis, primer sets to amplify the sequence for qPCR reactions, as well as gene accession numbers or locus tags (http://ncbi.nlm.nih.gov) are indicated. Primers were designed to amplify 100-400 bp regions and 100-300 bp regions for RNAi and qPCR primer sets, respectively. Primers were designed to target unique regions of the indicated genes and BLAST analysis was used to ensure a low probability of off target effects or non-specific amplification.

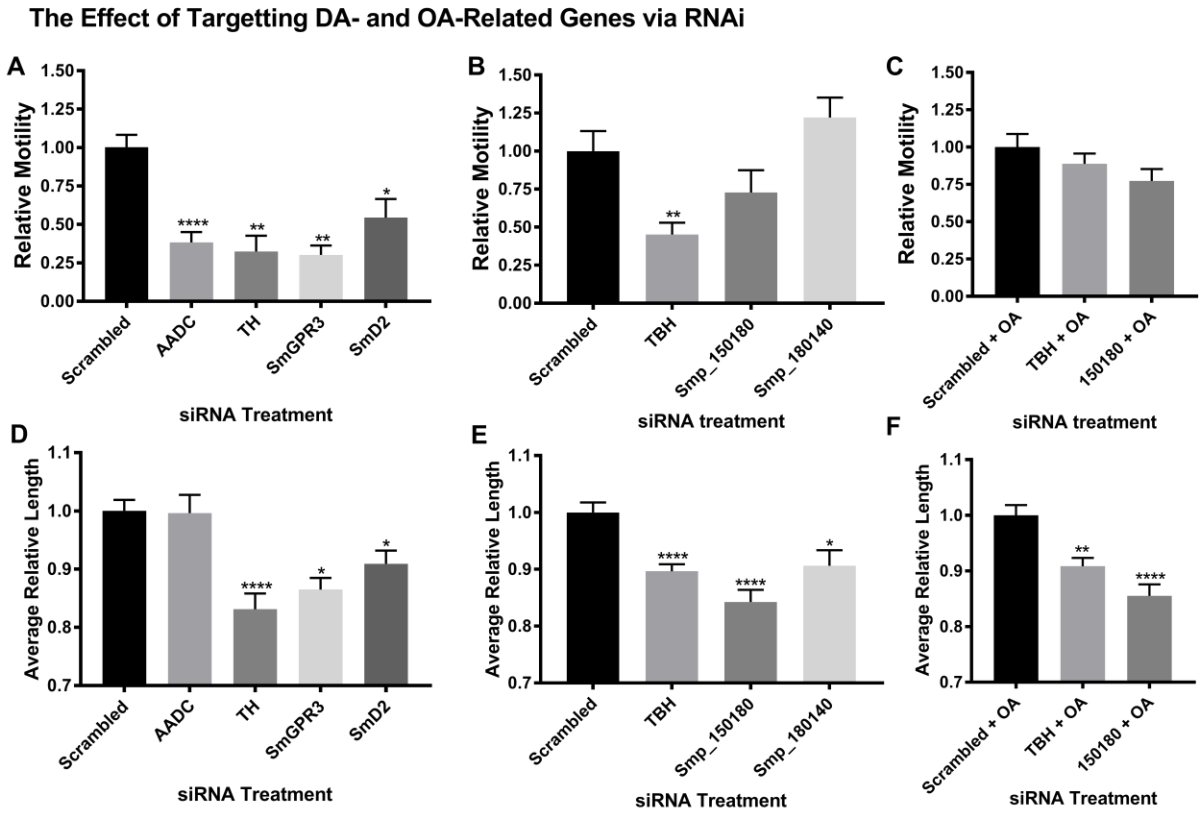
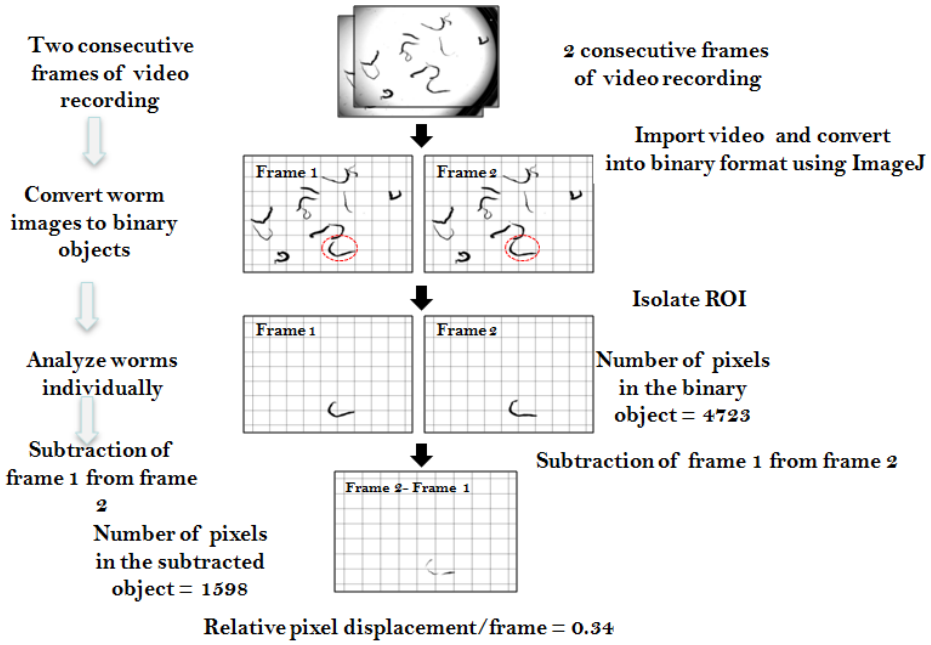


Figure 3. Quantification of schistosomula motility of OA-related genes following RNAi downregulation. Motility of schistosomula was quantified with the described method for determining length changes as a measure of frequency of body movements. Bars in the graphs represent mean values of average motility per treatment, normalized to that of the scrambled-treated siRNA control. Standard errors of the mean (SEM) are represented by error bars. Significance was determined using the unpaired student t-test, and p-values < 0.05 (*), < 0.005 (**), < 0.0005 (***) and < 0.00005 (****) were considered to be significant. Worms were cultured for up to seven days, and motility was recorded when a change was observed, typically following 5 days in culture, following transfection with 75 nM synthesized siRNA (Sigma-Aldrich, MilliporeSigma, MA, USA) or 50 nM pooled siRNA, with a lipid transfection reagent (SiPORT Neo FX, Thermoscientific). Worms were recorded using a microscope equipped with a video camera and SimplePCI software to generate videos of a 1 minute duration at ~2.5 frames per second

(fps) (~150 frames per video). A total of 45 to 300 schistosomula were analyzed per treatment from a minimum of three separate transfections using ImageJ software. Motility (A-C) and length changes (D-F) were quantified with the described method. Downregulation of all RNAi-targetted DA-related genes, SmAADC, SmTH, SmGPR3 produced a significant decrease in motility between ~25 to 50 % (A). Of RNAi-targetted OA-related signaling genes, only SmTBH produced a significant decrease in motility of ~60%, while Smp_150180 and Smp_180140 did not produce significant changes in motility. Following incubation of RNAi-targetted schistosomula with 10 µM OA no significant changes were observed on motility (C). Downregulation of DA-related signaling genes produced significant decreases in length between ~10-15%, except for SmAADC (D). Targeting OA-related signaling genes produces a significant decrease in schistosomula length between 10 to 15% (E). These effects are not reversed by incubation of schistosomula with 10 µM OA, indicating that they are pronounced and specific.



Adapted from *Patocka at el., 2014*

Figure 4. A pixel displacement method is used to quantify motility of RNAi-targetted adult *S. mansoni* motility. Motility of adult worms was quantified with the described pixel displacement method, pictured in the above schemata (adapted from Patocka et al., 2014). Worms in culture were recorded using a microscope equipped with a video camera (Nikon) and SimplePCI software to generate videos of a 2 minute duration at ~2.5 frames per second (fps) (~300 frames per video). Video was analyzed using ImageJ software. Each worm was analyzed independently. Videos were converted to binary format and the region of interest (ROI), containing a single worm, was selected with a drawing tool in ImageJ. The video was duplicated and the first and last frame of the first and second video, respectively, were subtracted to generate two videos containing an equal number of frames (total original frame number (ftotal) -1), with a single-frame lapse. The videos were subtracted from one another to quantify the displaced pixels. The portion of displaced pixels is represented as a fraction of total pixels in the original video to indicate motility.

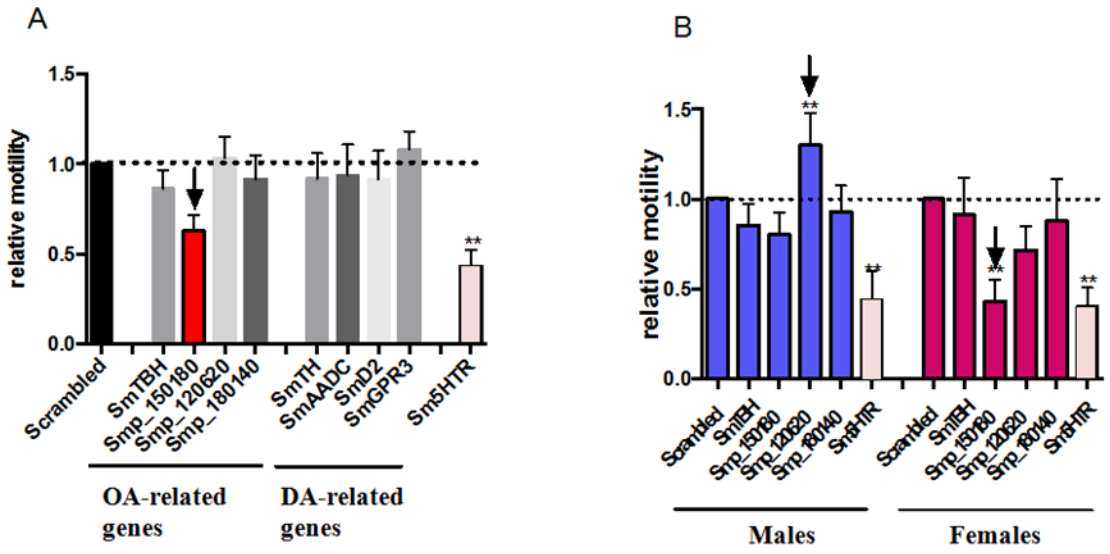


Figure 5. Targeting tyrosine derivative-related signaling genes in adult schistosomes produces gender-specific effects on motility. Motility of adult worms was quantified following targeting of DA and OA-related signaling genes via RNAi, using the pixel displacement method, described previously (Patocka et al., 2014). Worms were recorded daily, to generate videos of a 2 minute duration and motility was analyzed when a phenotype was observed, typically 3-4 days following transfection. Analysis was performed for 3-8 transfections per each treatment for a total of 20-130 worms in 3 to 8 transfections per RNAi treatment. Significance was determined using unpaired student t-tests and p-values < 0.05 (*) and < 0.005 (**) were considered to be significant. Sm5HTR, which was previously validated in adult schistosomes (Patocka et al., 2014), was used as a positive control, and scrambled siRNA as a negative control. Analyzing the pooled population of males and females did not result in pronounced changes in relative motility. Smp_150180 downregulation resulted in a modest decrease in motility of ~40% in adult schistosomes, which, upon analyzing males and females separately (B), was attributed to a significant decrease in motility in females. A significant increase in motility of Smp_120620

was observed in males, suggesting gender-specific roles of these phenolamine-related signaling genes.

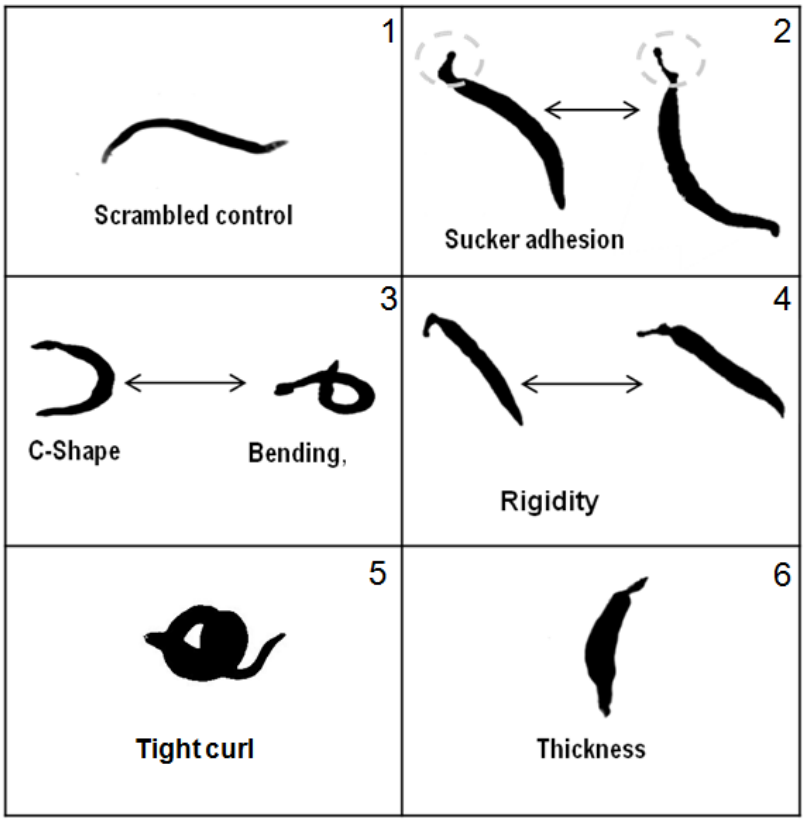


Figure 6. Adult schistosomes targeted via RNAi are associated with changes in posture and coordination. The most frequently ocurring and readily identifiable phenotypes observed in adult schistosomes targeted via RNAi are indicated. Worms were recorded using a microscope equipped with a video camera, and SimplePCI software to generate videos of a 2 minute duration at ~2.5 frames per second (fps) (~300 frames per video). The above examples are images of cultured male worms, showing a (1) control worm and the indicated phenotypes. These phenotypes include (2) sucker adhesion, (3) a C-shape/bending phenotype, (4) rigidity, which typically occurs in the midbody, and is indicative of paralysis, (5) a tight curl phenotype and (6) a thickening of the body, also typically most pronounced in the midbody. The

described phenotypes can be indicative of worm health, as in the case of (2) sucker adhesion, or variations in muscle tone that can contribute to differences in posture and types of movement.

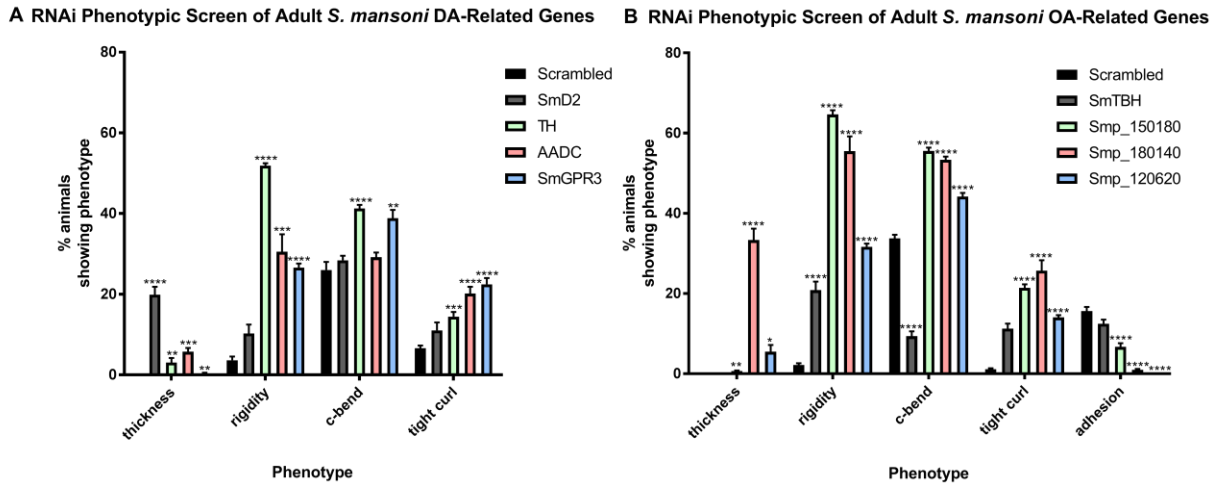


Figure 7. RNAi-targeted adult worms display phenotypes associated with changes in posture and coordination, indicating a role in neuromuscular control. Worms showing the above phenotypes were quantified in the population of 20-130 worms that were targeted by siRNA in 3-8 separate transfections. The percentage of animals showing a phenotype was determined for each experiment and average percent data were further calculated as the means ± SEM from 3-8 seprate experiments. Significance was determined using unpaired student t-tests and p-values < 0.05 (*), < 0.005 (**), < 0.0005 (***) and < 0.00005 (****) were considered to be significant. A mixed, male and female population was used throughout these studies. Phenotypes included in the graphs correspond to those for which significant changes were observed for DA- (A) and OA-related genes (B), respectively. Targeting DA-related SmD2, SmTH, SmAADC and SmGPR-3 results in pronounced changes in phenotypes in body-thickness, rigidity, the C-bend posture and tight curl phenotypes (A). Targeting OA-related signaling genes SmTBH,

Smp_150180, Smp_180140 and Smp_120620 produced pronounced changes in the thickness, rigidity, C-bend, tight curl as well as the adhesion phenotype (B).

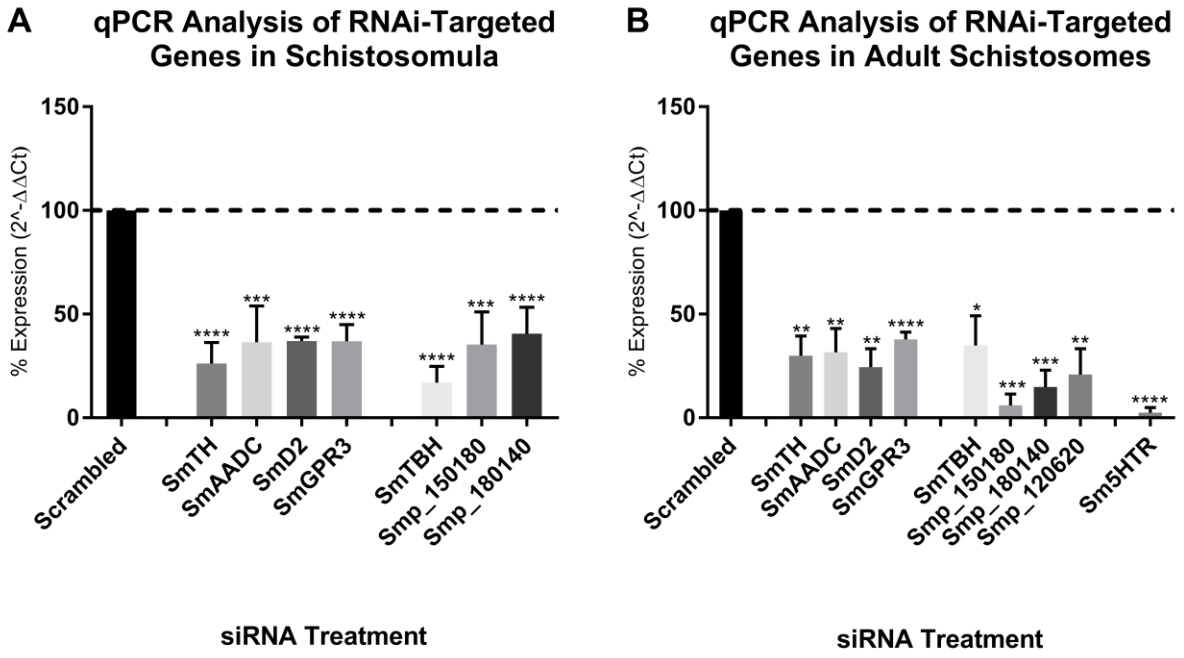


Figure 8. qRT-PCR of RNAi-targeted genes indicates downregulation via qRT-PCR. The ΔΔCT method (Livak and Schmittgen, 2001) was used to quantify RNA expression of the targeted tyrosine derivative-related signaling genes following downregulation. Following amplification of cDNA via qRT-PCR, relative mean gene expression levels ± SEM were determined, compared to the scrambled siRNA-targeted samples, serving as reference genes, using GAPDH as an internal control. Significance was determined using unpaired student t-tests and p-values < 0.05 (*), < 0.005 (**), < 0.0005 (***) and < 0.00005 (****) were considered to be significant. A minimum of 4 separate transfections were analyzed per gene target and 2-3 transfections were

pooled per RNA extraction. All targeted genes in both schistosomula (A) and adult parasites (B) produced a decrease in RNA expression greater than 50%.

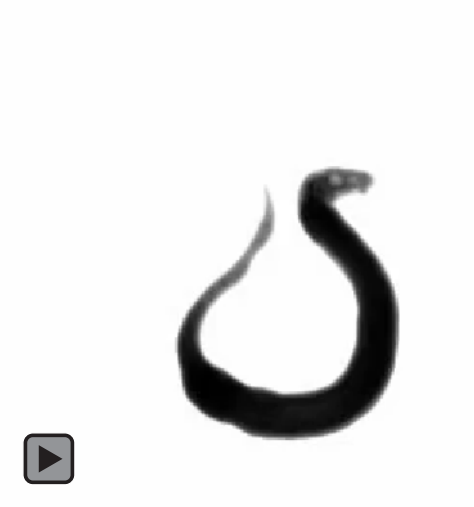
Supporting Information

Video S1

Scrambled siRNA-transfected control adult worm. Video of representative worm in culture displays the typical range of body movements where the worm extends and contracts in without restriction.

Video S2

Adhesion of adult worms to the bottom of the culture dish via the sucker. Adhesion of the worms to the culture dish via sucker attachment is a phenotype which is a sign of health. Following RNAi, this phenotype may become less frequent as parasite motility becomes less coordinated and the worm can no longer remain attached to the dish.



Video S3

The c-shaped posture or bending phenotype in adult worms. A C-shaped posture may be observed following RNAi where the worm retains a hunched posture and does not fully extend. This phenotype can be accompanied by paralysis in the midbody and uncoordinated movement, as is observed in the representative video.

Video S4

The rigidity phenotype is observed in adult schistosomes following RNAi. The rigidity phenotype is observed when a section of the worm body remains rigid and cannot bend. This phenotype, as in the corresponding video, typically occurs in the midbody and is associated with uncoordinated movement and paralysis.

Video S5

The tight curl phenotype occurs in adult schistosomes following RNAi. The tight curl phenotype is characterized by an adult worm that's body curls to form one or more complete loops and is restricted in its range of movement.

Video S6

The thickness phenotype results from RNAi of adult schistosomes. The thickness phenotype is observed when the adult worm's body is thick and enlarged, typically in the midbody. This phenotype is typically accompanied by paralysis in the enlarged region, as in the corresponding video.

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Connecting Statement II

Having determined that inhibiting tyrosine derivative signaling via downregulation of associated signaling genes causes pronounced changes in motor control, in the previous chapter, we characterized these signaling proteins to verify if they are involved in tyrosine-derivative signaling. In the next chapter, we cloned, expressed and characterized two genes which we determined via RNAi studies in the previous chapter, to play an important role in neuromuscular control: the putative octopamine (OA) synthesis enzyme tyramine beta-hydroxylase (SmTBH, Smp_243830) and the putative OA GPCR Smp_150180. Apart from these genes, we also cloned cDNAs encoding 5 additional BA GPCRs, which can be deorphanized in the future to potentially identify additional OA receptors. Next, we cloned 3 cDNAs encoding putative enzymes which can be downregulated to determine similarities in the effects of RNAi downregulation compared to in *S. mansoni* and potentially validate *D. tigrina* as a model organism for the study of schistosomes. The discovery that tyrosine derivative-related signaling genes play an important role in schistosome motor control provides incentive to further characterize these genes. Identifying and characterizing these genes through functional studies can shed insight into the extent of the involvement of tyrosine derivative signaling in *S. mansoni* neurobiology compared to what has been determined for other neurotransmitters in schistosomes. The success of anthelmintics and pesticides that target invertebrate nervous system function by acting as agonists of OA GPCRs demonstrate the potential of targeting OA signaling genes, and our preliminary characterization of two putative OA signaling genes provides strong evidence for their potential as targets for schistosomicide discovery.

CHAPTER IV

Characterization of octopamine signaling proteins SmTBH (Smp_243830) and Smp_150180

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ABSTRACT

Schistosomiasis is a greatly debilitating disease that afflicts more than 250 million people worldwide, in more than 75 tropical and subtropical countries. More than 800 million people are at risk of infection. The disease is associated with chronic morbidity and mortality, with severe pathology of the liver a common outcome. A single drug, praziquantel (PZQ), has been the primary treatment for schistosomiasis, despite reports of reduced efficacy and inefficacy against the larval parasitic stage, signaling the need to identify new drugs and drug targets for schistosomiasis. Recently, we identified octopamine (OA) in *S. mansoni* as an invertebrate-specific neurotransmitter that can provide the basis for discovery of new drugs that specifically target the parasite without affecting host biology. OA is a phenolamine biogenic amine (BA) of particular interest, as it is an important neuromodulator and neurotransmitter, involved in modulating a vast spectrum of behaviours and physiological processes in other invertebrates. OA is involved in locomotion, motivation and behaviour, feeding, the 'fight or flight' response, female reproduction, learning and memory. We previously showed that OA causes hypermotility in schistosomula and we identified a putative OA receptor (Smp_150180) and a putative OA-synthesizing enzyme, SmTBH (Smp_243830), both of which, when downregulated, produce changes in posture and motor control. OA receptors typically belong to the G protein-coupled recepor (GPCR) superfamily. Here, we describe the cloning of cDNAs encoding 6 orphan schistosome BA GPCRs, including Smp_150180. We performed heterologous expression studies to characterize Smp_150180 and found that it is specifically stimulated by OA. We also cloned cDNAs encoding 3 BA synthesis enzymes in *Dugesia tigrina*, which are homologues of schistosome tyrosine derivative synthesis enzymes that we previously targeted via RNAi downregulation. These genes can be downregulated in *D. tigrina* to test whether this organism is a model for the study of *S. mansoni*. Lastly, we cloned, recombinantly expressed and characterized SmTBH and showed that the enzyme can synthesize OA.

1. Introduction

Schistosoma mansoni is one of the most prevalent etiological agents that cause schistosomiasis. Schistosomiasis is the leading cause of hepatic fibrosis globally (Doenhoff et al., 2008) and infects more than 250 million people worldwide, resulting in more than 280,000 deaths per annum (Fenwick et al., 2003; Knudsen et al., 2005; Steinmann et al., 2006; Doenhoff et al., 2008). Schistosomiasis is widespread in many tropical and subtropical regions, putting more than 800 million people at risk of contracting infection. *S. mansoni* causes severe pathology of the liver, including hepatosplenomegaly, as well as ascites, colon obstruction and mega-colon. Despite the chronic morbidity and mortality associated with schistosomiasis and the pervasiveness of the disease, a single drug, praziquantel (PZQ), has been used for its treatment for the past 35 years. PZQ is ineffective against juvenile stages of the parasite (Sabah et al., 1986; Cioli and Pica-Mattoccia, 2003) and incidences of reduced sensitivity (Cioli, 2000) and inefficacy (Ismail et al., 1996; Ismail et al., 1999) have been reported, highlighting the need for novel schistosomicides. In other helminths, the nervous system is a proven drug target. The anthelmintics levamisole and ivermectin (IVM) (Robertson and Martin, 2007; Kaminsky et al, 2008; Gutman et al., 2010; Wolstenhome, 2011), for example, interfere with signaling of the nematode nervous system, resulting in paralysis and leading to clearance of the parasite from the host (Geerts et al., 1989; Gill et al., 1991; Geary et al., 1993). The schistosome nervous system is central to worm survival and infectivity and is involved in control of such functions as maturation, migration, feeding, egg-laying, and host penetration.

Octopamine (OA) signaling holds great potential for drug targeting, given its important role in schistosome neurobiology. The presence of an OA signaling system in *S. mansoni* and its importance as a schistosome neurotransmitter is evidenced by our findings in confocal immunolocalization and motility studies, as well as our RNAi screen of OA-related signaling genes, described in the first and second manuscripts in this thesis. OA is widespread in the schistosome nervous system and plays a role in neuromuscular control, producing a

hypermotile effect and an increase in length of schistosomula and uncoordinated movement and changes in posture in adults following RNAi downregulation.

In other invertebrates, OA GPCRs are proven drug targets. The imidazolidine and formamidine classes of pesticides target OA GPCRs in the arthropod nervous system, causing spasticity and uncoordinated movement. The discovery of OA signaling in *S. mansoni* is novel and OA GPCRs have not yet been identified in *S. mansoni*. The sequencing of the *S. mansoni* genome (Berriman et al., 2009) and its subsequent reannotation (Protasio et al., 2012) has revealed a variety of BA GPCRs, many of which diverge from classical BA GPCR homologues (Zamanian et al., 2011; El-Shehabi and Ribeiro, 2010; El-Shehabi et al., 2012; Macdonald et al., 2015). In the second manuscript of this thesis, we generated a phylogenetic tree and identified 28 candidate BA GPCRs, only 6 of which have known ligands, based on their annotation and homology with other BA GPCRs. A recent classification of known and orphan BA GPCRs in schistosomes estimates 15 *S. mansoni* BA GPCRs (Campos et al., 2014). Given the importance of OA signaling in schistosome biology, these 15-22 predicted orphan GPCRs, may include OA-selective GPCRs. Here we describe the cloning of cDNAs encoding 11 predicted orphan GPCRs. We cloned 6 putative orphan BA GPCRs and characterized one of them, Smp_150180, via heterologous expression in mammalian cells. Heterologous expression in mammalian cells, which has been used to de-orphanize schistosome BA GPCRs (Hamdan et al., 2002; Taman and Ribeiro, 2009), suggests that Smp_150180 is an OA GPCR.

We also cloned four *Dugesia tigrina* cDNAs, 3 of which encode homologues of putative *S. mansoni* BA synthesis enzymes that we previously targeted via RNAi. The cloned sequences include tyrosine hydroxylase (TH)-like, amino acid decarboxylase (AADC)-like and tyramine beta hydroxylase (TBH)-like enzymes. The cloning of these cDNAs is part of an RNAi screen to validate *D. tigrina* as a flatworm model. The annotation and assembly of *D. tigrina* transcriptomic data has been performed (Wheeler et al., 2015). While the free-living nematode *C. elegans* is as a model organism for the study of parasitic species, a flatworm model organism is lacking. *D. tigrina* is more amenable to *in vitro* studies than schistosomes, which are prone to

issues with contamination and in maintaining viability in culture (Kaletta and Hengartner, 2006).

Of the cloned *S. mansoni* BA synthesis enzymes, SmTBH (Smp_243830) is of particular interest. Given the presence of OA signaling in the parasite, SmTBH would likely be the sole source of OA because OA is an invertebrate-specific BA, and it could not be obtained from the vertebrate host. TBH could be a suitable drug target in *S. mansoni* because it diverges from the host dopamine beta-hydroxylase (DBH) homologue. Previous RNAi studies targeted SmTBH to produce a decrease in schistosomula motility and length, and caused uncoordinated movement in adult parasites, consistent with inhibiting OA-mediated stimulation of motility and supporting the hypothesis that OA is an important signaling molecule. These data highlight SmTBH as a target for novel schistosomicide discovery.

These studies demonstrate the power of tools in bioinformatics in facilitating the cloning of flatworm genes and characterizion of schistosome proteins, driving schistosomiasis research forward. These studies also highlight new targets for schistosomicide discovery, including Smp_150180 and SmTBH.

2. Materials and methods

2.1 Bioinformatics analysis and cloning of *S. mansoni* and *D. tigrina* biogenic amine signaling genes

Sense and antisense primers were designed using Oligo software (version 6.2, Molecular Biology Insights, Inc) (Rychlik, 2007) against the 5' and 3' ends of the predicted coding DNA sequence (CDS) of 11 putative *S. mansoni* BA GPCRs: Smp_134100, Smp_027940, Smp_178420, Smp_180140, Smp_137300, Smp_043300, Smp_137050, Smp_204580, Smp_134820, Smp_120620, and Smp_150180. Primers were also designed against 4 predicted *D. tigrina* cDNAs, including a putative Rho-GTPase-activating-protein (Locus_30706) and three putative tyrosine derivative synthesis enzymes, including an amino acid decarboxylase (AADC)-like (Locus_30706), a tyrosine hydroxylase (TH)-like (Locus_21544), and a tyramine beta

hydroxylase (TBH)-like (Locus_32684) sequence. For schistosomes, predicted full length CDSs were obtained from the GeneDB *Schistosoma mansoni* homepage (http://www.genedb.org/Homepage/Smansoni; Protasio et al., 2012) using the search words "amine" and "GPCR". Orphan BA GPCRs having the highest number of predicted TM regions as determined by TMHMM (Tied Mixture-Hidden Markov Model) (Version 2.0) topology software (Center for Biological Sequence Analysis – Technical University of Denmark (CBS-DTU), http://www.cbs.dtu.dk) closest to the 7 TM regions required for a functional GPCR, were selected. *Dugesia tigrina* sequences having the highest level of homology with *S. mansoni* homologues SmTBH (Smp_243830), SmAADC (Smp_171580) and SmTH (accession no.: AAC62256), and BA GPCR SmD2 (Smp_127310), via the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/), Magic-Basic Local Alignment Sequencing Tool (BLAST version 2.2.26+), were selected. *Dugesia tigrina* sequences were obtained from transcriptomic data (Wheeler et al., 2015) assembled from a collection of 15,802,710 nucleotides from 38,056 sequences using a BLOSUM62 substitution matrix with a neighboring words threshold of 11 and existence and extension gap penalties (Schaffer et al., 2001) of 11 and 1, respectively (Alejandro et al., 2001). cDNA was synthesized from RNA extracted from mixed sex populations of adult *S. mansoni* (2 µg/ul) provided by the Biomedical Research Institute (BRI, Rockville, MD, USA). *D. tigrina* cDNA was extracted from five worms that were starved for almost one week using a TRIZOL (Thermo Fisher Scientific, MA, USA) - chloroform separation and subsequent purification with an RNAeasy Mini kit (Qiagen Inc., MD, USA). Worms were suspended in TRIZOL and homogenized on ice with a mortar and pestle to extract RNA. The mixture was centrifuged at 12,000 x g for 10 minutes, at 4 °C and the supernatant was mixed with chloroform at a ratio of 1:4. Following a subsequent centrifugation, the aqueous phase containing RNA was mixed with an equal volume of ethanol and added to the kit-provided column for purification following the kit specifications. The purified RNA was quantified using a Nanodrop spectrophotometer (ND1000, Thermo Fisher Scientific, MA, USA). cDNA was synthesized from RNA using a First Strand cDNA Synthesis reverse transcriptase Kit

(Thermo Fisher Scientific MA, USA). Briefly, 1 µg RNA was used as a template and the reaction mixture containing OligodT primers, 0.5 mM dNTP (deoxynucleotide triphosphates) (10mM), first strand buffer, RNAseOUT and 200 U MMLV-RT (Murine Leukemia Virus Reverse Transcriptase) (Thermo Fisher Scientific, MA, USA) was incubated for 1.5 hours to synthesize cDNA. The reaction was terminated by 15 min incubation at 70 °C. A negative control lacking MMLV-RT was used to rule out genomic DNA contamination in subsequent amplification reactions. 2 µg *D. tigrina* or *S. mansoni* cDNA was used in each PCR reaction. CDNA was amplified in a PCR reaction using proof reading Phusion High Fidelity Polymerase (New England Biolabs, MA, USA) with the following cycling conditions: 98°C/30s; (98°C/8s, 54.7°C/25s, 72°C/28s) x 35 cycles; 72°C/8min or 98°C/30s; (98°C/8s, 55.4°C/25s, 72°C/28s) x 35 cycles; 72°C/8min, testing annealing temperatures of both 54.7°C and 55.4°C. cDNA was quantified using a Nanodrop spectrophotometer (ND1000, Thermo Fisher Scientific, MA, USA). PCR products were visualized under UV light after electrophoresis through a 1% agarose gel with ethidium bromide. Amplified bands measuring more than 650 bp were subcloned into pJET 1.2 cloning vector using the CloneJET Cloning kit (Thermo Fisher Scientific, MA, USA), according to the kit protocol. In *S. mansoni*, this method was used as a shotgun approach, without preference for any of the targeted putative BA GPCRs, with the exception of Smp_150180, which was the primary candidate for heterologous cell expression studies. *D. tigrina* sequences were subsequently ligated into the L4440 (Grishok et al., 2005) RNAi silencing vector between NotI (5'-GCGGCCGC-3') and NheI (5'-GCTAGC-3') digestion sites. Primers were designed against 5' and 3' ends of amplified *D. tigrina* sequences, with restriction sites preceded by four nucleotides (5'-GATC-3') to promote efficient cleavage. Primers used to introduce digestion sites to the 5' and 3' ends of the sequences via PCR are indicated in Table 2. All gene sequences were confirmed by sequencing a minimum of two individual clones (Génome Québec Innovation Centre – Nanuq, QC, CA). The sequence was compared against the predicted sequence targeted by the cloning primers. A global sequence alignment between the predicted and cloned CDS was generated using the EMBL-EBI European Molecular Biology

Open Software Suite (EMBOSS) (http://www.emboss.org/) stretcher tool, which was also used to determine the percent identity and percent gaps between the sequences. For BA GPCRs, the number of predicted TM regions in amplicons and predicted sequences were determined using the TMHMM protein topology tool. TMHMM-generated posterior probability plots were also observed, counting peaks of hydrophobic stretches of residues, to determine the possible underestimation of predicted TM regions, though the software-predicted TM number was used to represent TM number prediction for each receptor. For other putative BA signaling genes, the percent identity of amplified sequences was compared against genes in the genome of *Dugesia japonica*, a related planarian, and in the absence of a match, against another flatworm gene sequence having the highest level of identity, as determined using the protein BLAST (BLASTp) tool from NCBI.

2.2 Heterologous expression of Smp_150180 in HEK293F mammalian cells

Mammalian Human Embryonic Kidney (HEK) 293 F cells (American Type Culture Collection, VA, USA) were transfected with codon-optimized Smp_150180 encoding an N-terminal FLAG tag, C-terminal hemaglutinin (HA) tag, and 5' NcoI and 3' BamH1 digestion sites. The sequence with tags and digestion sites was submitted to Genscript (Genscript USA Inc., NJ, USA), specifying codon optimization for the Smp_150180 coding sequence for expression in a mammalian cell. We compared the translated codon-optimized sequence against the submitted sequence to confirm conservation of the amino acid (AA) sequence. 2 µg of the pCI-neo-Smp_150180 construct was transfected into HEK293F cells using the Lipofectamine3000 transfection reagent (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. Negative control cells were transfected with empty pCI-neo vector or with vehicle only. Briefly, a pre-incubated mixture of the transfection reagent with or without the Smp_150180 construct or the empty pCI-neo plasmid was added to cells at 50-60% confluency in OPTI-MEM (Thermo Fisher Scientific, MA, USA) in a 6 well plate. Transfection proceeded for 3-5 hours before the media was supplemented with 5% FBS (Thermo Fisher Scientific, MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. In parallel, cells were seeded and transfected into a 6 well plate with

a sterilized coverslip in each well for immunofluorescence assay (IFA) monitoring of construct expression. Cells were maintained at 37°C with 5% CO_2 in a humidified incubator. Preceding and following transfection, cells were cultured in DMEM (Thermo Fisher Scientific, MA, USA) supplemented with 5% FBS (Thermo Fisher Scientific, MA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. 24-48 hours following transfection, at ~80% confluency, expression of the Smp_150180 construct was confirmed via IFA as described (Nabhan and Ribeiro, 2007; El-Shehabi et al., 2009) using a fluorescent microscope. Briefly, cells were fixed with 4 % PFA at room temperature for 15 minutes, permeabilized for 15 minutes with 0.1% ice cold Triton-X in PBS, and probed with a mouse anti-FLAG antibody (Sigma-Aldrich, Millipore Sigma, MA, USA) and a Fluorescein Isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich, Millipore Sigma, MA, USA) in blocking buffer (0.5% BSA in PBS). To visualize nucleii, a SlowFade Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, MA, USA) was added up to 2 hours before visualization. When expression of the pCI-neo-Smp_150180 construct was confirmed in HEK293F transfectants, cells were subjected to selective drug pressure with 500-800 µg/ml gentamcin sulfate (G418) (Wisent Bioproducts, QC, Canada) for four passages, and the highest concentration permitting cell growth selected for further culturing. IFA was used intermittently to confirm continued expression of Smp_150180.

2.3 Functional characterization of Smp_150180 in HEK293F transfectants

cAMP levels were quantified using a CAMP-Glo cAMP detection kit (Promega Corporation, WI, USA) as per the manufacturer's instructions. Briefly, HEK293F stable pCI-neo-Smp_150180 transfectants or untransfected cells, as a negative control, were seeded at 50 000 cells in 100 µl of complete media per well in a 96 well white-walled clear bottom plate, coated with Poly-D-Lysine. Cells were incubated overnight at 37 °C with 5% CO_2. The following day, the cells were incubated in 100 µl induction buffer (1x PBS with 500 µM IBMX and 100 µM Ro20-1724 (4-(3-butoxy-4-methoxy-benzyl) imidazolidone), phosphodiesterase inhibitors) with the BA of interest at 0 - 100 µM, and cells were incubated for 1 hour to induce Smp_150180 GPCR activation.

Following incubation, cAMP Lysis buffer was added to lyse the cells, followed by the cAMP-Glo assay buffer, containing protein kinase A (PKA). Kinase-Glo (Promega Corporation, WI, USA) was added to each well, and luminescence determined using a Synergy-H4 plate reader (BioTek Instruments, Thermo Fisher Scientific, MA, USA). Relative luminescence values (RLUs), as a measure of cAMP production and relative adenylate cyclase inhibition or stimulation, were determined by subtracting the RLU of the tested BA from that of a vehicle-treated control.

2.4 Cloning and *in silico* analysis of the *Schistosoma mansoni* tyramine beta-hydroxylase (SmTBH) sequence

The predicted *S. mansoni* tyramine beta-hydroxylase (SmTBH) was cloned from adult cDNA by PCR. RNA was extracted from an adult *S. mansoni* (2 µg/ul) mixed sex population obtained from the BRI (Biomedical Research Institute, Rockville, MD, USA) and cDNA was synthesized from RNA in a reverse transcriptase reaction using a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA) as described above. cDNA was amplified in a PCR reaction using proofreading Phusion High Fidelity Polymerase (New England Biolabs, MA, USA). Sense (5'-ATGATCAACAATTTATTTGTTTTAATTTTG-3') and anti-sense (5'-TTACTTATCATCCAATGTATTCAAG-3') primers were designed using Oligo software (Version 6.2, Molecular Biology Insights, Inc) (Rychlik, 2007). The primers were used to amplify 2 µg cDNA with the following cycling conditions: 98°C/30s; 98°C/8s, 56.5°C/25s, 72°C/28s x 35 cycles; 72°C/8min. The sequence was ligated into the pJET 1.2 cloning vector using the CloneJET Cloning kit (Thermo Fisher Scientific, MA, USA) according to the kit protocol, and the identity of the sequence verified by sequencing of two independent clones. The sequence was analyzed in MacVector (Version 7.1.1, Accelrys, Inc) using the "open reading frame" analysis tool to identify a continuous open reading frame (ORF). A single ORF exceeding 200 nt was identified and the cloned sequence modified by PCR to exclude 35 out-of-frame nucleotides at the 5' end of the sequence using a different sense primer (5'-ATGGCAACTTCTTGTCGTGAAAATGG-3'). Restriction sites, as well as a C-terminal FLAG (DYKDDDDK)-encoding sequence at the 3' end, were introduced into the sequence via

PCR with the following sense and antisense primers, respectively: 5'-CCGCTCGAGATGGCAACTTCTTGTCGTG-3' (sense) and 5'-CCGGATCCTTATTTGTCATCGTCATCTTTGTAGTCCTTATCATCCAATGTATTCAAGAATATATTTAATG-3' (antisense).

2.5. *In silico* analysis of schistosome tyramine beta-hydroxylase (SmTBH)

A phylogenetic tree of the cloned *S. mansoni* SmTBH sequence was generated via multiple sequence alignment (MSA) using the Clustal Omega tool (ClustalO, Version 1.1.2) (Söding, 2005; Sievers et al., 2011) in EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute), with a neighbor-joining (nj) output, to generate a tree in Newick format. Copper type-II monooxygenase SmTBH and SmDBH sequences were obtained from NCBI. Sequences were identified based on homology with SmTBH using the Basic Local Alignment Sequencing Tool for amino acid sequences (BLASTp) or by using key search word "dopamine beta hydroxylase" in the "protein" database of the National Center for Biotechnology Information (NCBI) (http://ncbi.nlm.nih.gov). The tree was visualized with FigTree (Version 3.0) (Morariu et al., 2008). To analyze the cloned *S. mansoni* tyramine beta-hydroxylase (SmTBH) (Smp_243830) sequence and identify catalytic domains and conserved residues, the sequence was aligned in a ClustalW multiple sequence alignment (MSA) using MacVector (Version 7.1.1, Accelrys, Inc). Copper type-II monooxygenase, TBH- and DBH-encoding sequences were obtained from the NCBI database. Only cloned and characterized DBH/TBH sequences were selected. DjTBH was selected based on homology between schistosome and *Dugesia* flatworm species and TBH-1, based on the role of *C. elegans* as a model for the study of helminth species. The alignment was inspected visually to identify conserved cysteine residues and the two TBH/DBH catalytic domains with conserved histidines. NCBI BLASTp analysis of SmTBH was used to confirm homology with other TBH/DBH sequences and to identify conserved domain database (CCD, NCBI) conserved protein domain family

(pfam) domains consistent with TBH/DBH copper-type-II-monooxygenase domains in the N-terminal and C-terminal regions and determine a possible structure-function relationship.

2.6 Expression and western blot analysis of SmTBH

The cloned SmTBH sequence was subcloned into the pET-15b bacterial expression vector between an N-terminal histidine (6xHis) tag-encoding sequence and Xho1 and BamH1 sites. BL21(DE3) pLysE chemically competent *E. coli* (Sigma-Aldrich, Millipore Sigma, MA, USA) were transformed with 250 µg of the SmTBH construct using a heat shock method, adhering to the manufacturer's protocol. Expression of SmTBH was induced following transformation by inoculating a single colony in Luria broth (LB) with ampicillin at 37°C to an optical density of 0.6-1 at 600 nm (OD_{600}). Cells were induced by addition of Isopropyl beta-D-1-thiogalactopyranoside (IPTG) to a final concentration of 100 mM and incubated at 37°C for 2 hours. Cells were subsequently pelleted by centrifugation at 5,000 xg for 5 min at 4 ˚C, washed in 10 ml of ice-cold Tris-HCl (50 mM, pH 8) and centrifuged a second time. Untransformed cells were grown in parallel and subjected to the same conditions as a negative control. As an additional negative control, transformed cells not subjected to induction by IPTG were grown in parallel. Soluble protein was extracted under cold conditions. Briefly, protease inhibitor (Sigma-Aldrich, Millipore Sigma, MA, USA) added to the pellet according to the manufacturer's instructions, followed by sodium phosphate buffer (0.5 M NaCl, 20 mM phosphate buffer, pH 7.4). Cells were subjected to several freeze-thaw cycles to activate endogenous PLysE activity and sonicated for 1 minute ((6x(10s pulse; 30s rest period)), 35% amplitude). The homogenate was subsequently centrifuged at 12,000 xg for 15 min at 4 ˚C and protein concentration determined for both pellet and supernatant (membrane and soluble fractions) using a Bradford kit (Bio-Rad Laboratories, QC, Canada). For analysis of SmTBH, 10-15 µg soluble protein extract was loaded into a 4-10% Tris-Glycine resolving precast gel (Thermo Fisher Scientific, MA, USA) following protein denaturation in Laemmli buffer at a final concentration of 0.1 M DTT (dithiothreitol) with boiling for 10 minutes prior to loading. Protein was transferred to a

polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Merck Millipore, Darmstadt, Germany). The protein was subsequently probed with a commercially obtained mouse monoclonal antibody to probe the C-terminal FLAG tag (Sigma-Aldrich, MilliporeSigma, MA, USA) (1:500 dilution), followed by probing with an HRP-conjugated rabbit anti-mouse secondary antibody (Sigma-Aldrich, MilliporeSigma, MA, USA) (1:60000 dilution) for detection of protein with a chemiluminescent substrate (Pierce, Thermo Fisher Scientific, MA, USA). Negative controls include loading untransfected BL21(DE3) cell extract, uninduced SmTBH-transfected BL21(DE3) cell extract and the insoluble membrane fraction extract. As a positive control, 10 or 25 µg (1:500 or 1:200 dilution) anti-FLAG antibody was precleared with 50 µg or 125 µg untransfected BL21(DE3) cell extract, respectively. The predicted size of the protein was determined with SIB (Swiss Institute of Bioinformatics) ExPASy (Expert Protein Analysis System) Compute pI/Mw (isoelectric point/molecular weight) software tool, and putative phosphorylation sites, via Center for Biological Sequence Analysis – Technical University of Denmark (CBS-DTU, http://www.cbs.dtu.dk) Netphos software (Version 2.0).

2.7 Measurement of SmTBH activity

We measured SmTBH activity using a previously described method (Nagatsu and Udenfriend, 1972) to quantify the amount of OA produced from tyramine (TA) following incubation with the enzyme. OA was converted to p-hydroxybenzaldehyde and the reaction mixture excited at 330 nm in a spectrophotometer; absorbance is proportional to the concentration of OA. A standard curve was generated using pure OA (0-150 µM) converted to p-hydroxybenzaldehyde. Conversion to p-hydroxybenzaldehyde was performed via addition of sodium periodate ($NaIO_4$), which oxidizes OA to the aldehyde derivative. The reaction was stopped via addition of sodium periodate ($NaIO_4$), and resultant excess IO_4- anions were titrated via addition of sodium metabisulphite ($Na_2S_2O_5$). The linear range for detecting p-hydroxybenzaldehyde (0 – 20 µM) was selected for future standard curve assays. The enzyme activity assay used 0.7 mg crude protein extract, induced by IPTG to express SmTBH. Boiled protein extract and PBS were used

in parallel reactions as controls. A 1 ml total reaction volume in triplicate was prepared as described (Nagatsu and Udenfriend, 1972) containing 50 µl 0.02 M TA, 50 µl 20 mM pargyline, 150 µl f 0.2 M N-ethylmaleimide (NEM) as an enzyme cofactor, 200 µl 1 M sodium acetate buffer (pH 5), 50 µl 0.2 M ascorbate, 50 µl 0.2 M sodium fumarate and 50 µl 1,500 U bovine catalase in PBS (Sigma-Aldrich, MilliporeSigma, MA, USA) to prevent H_2O_2 formation. The reaction mixture was supplemented with 1 µM OA to raise baseline absorbance values and increase sensitivity. The control boiled enzyme was generated by heating to 100°C for 20 minutes. Reaction mixtures were incubated in 25 ml glass tubes, exposed to air, for 1 hour with shaking at 37°C. Samples were then centrifuged at 4,000 xg for 10 minutes to collect precipitate in the bottom of the tube. The supernatant was run through a Dowex-50 (H, 200-400 Mesh, Sigma-Aldrich, MilliporeSigma, MA, USA) packed volume column, washed with 5 ml of water, and the bound OA eluted by neutralizing the acidic column via addition of 3 M NH_4OH.

3. Results

3.1. Cloned *S. mansoni* and *D. tigrina* genes are putatively involved in BA neurotransmitter signaling

In *S. mansoni*, 11 orphan BA GPCRs annotated as amine GPCRs in GeneDB (http://www.genedb.org/Homepage/Smansoni), having closest to the required 7 TM regions to form a functional GPCR as determined by TMHMM software (CBS-DTU), http://www.cbs.dtu.dk), were selected (**Table 1**). In *D. tigrina*, 3 putative tyrosine derivative BA neurotransmitter synthesis enzymes and a Rho-GTPase-activating protein (Wheeler et al., 2015) having homology with *S. mansoni* predicted homologues were selected for cloning (**Table 2**). The Rho-GTPase-activating protein (Rho-GAP) is putatively involved in GPCR signaling and a potential positive control for siRNA targeting studies. All amplified *D. tigrina* sequences and 6 of the 11 *S. mansoni* sequences yielded PCR amplicons of the approximate expected size. Amplified *S. mansoni* cDNAs included Smp_204580, Smp_137300, Smp_134100, Smp_180140,

Smp_178420, Smp_027940, Smp_120620 and Smp_150180. Amplified *Dugesia tigrina* cDNAs included genes with locus tags: Locus_30706, Locus_32684, Locus_21544 and Locus_24292 (Wheeler et al., 2015). *D. tigrina* sequences were subsequently subcloned into the L4440 RNAi silencing vector (Grishok et al., 2005) between the NotI and NheI restriction sites. Smp_134100 and Smp_120620 had sequences that diverged significantly from the target sequence. In these two sequences, the number of TMHMM predicted TM regions varied significantly between the predicted and cloned sequences, whereas in the cloned sequences, no TM regions were detected, while the CDSs have 4-5 predicted TM regions. Smp_150180 shares 63.8% identity with the predicted CDS, with 9 nts in the amplicon differing from those in the predicted CDS. The homology of the predicted *D. tigrina* synthesis enzymes between *D. japonica* or *C. sinensis* sequences ranges from ~53-74%. The highest level of identity between predicted *D. tigrina* and *D. japonica* genes is with the AADC-like sequences that share 74% identity. The Rho-GTPase-activating protein, which may inactivate G-proteins involved in a BA GPCR signaling cascade, or terminate signaling (Moon and Zheng, 2003), was also cloned and, if RNAi targeting produces an observable phenotype, will serve as a positive control.

3.2. Smp_150180 is selectively activated by OA

To determine whether Smp_150180 is an OA GPCR, the receptor was codon-optimized for expression in mammalian cells. In GeneDB, Smp_150180 is annotated as an OA GPCR and the receptor contains 7 predicted TM regions. The receptor was therefore selected for heterologous expression. HEK293F cells were stably transfected with a pCI-neo-FLAG-Smp_150180-6xHis construct, or with pCI-neo alone, or left untransfected. Cells stably-transfected with the Smp_150180 construct showed pronounced labelling with the fluorescently-labelled antibody, while mock-transfected and untransfected cells did not produce non-specific labelling, indicating that the stable transfectants produce the Smp_150180 BA GPCR (**Figure 1A**). While labelling was observed in the cytoplasm of the cell, punctate sites of relatively more pronounced labelling at the edge of the cell indicate that the

receptor is expressed on the cell surface. cAMP production following incubation with tyrosine-derived BAs was assayed to determine ligand specificity. Smp_150180 was assayed using a luminescence-based cAMP detection kit (cAMP-Glo, Promega Corporation, WI, USA) (**Figure 1B**). Luminescence produced is inversely proportional to ligand-mediated GPCR stimulation and associated inhibition or stimulation of AC. BAs were tested at concentrations ranging from 0 to 100 µM, and 10 µM produced the most significant changes in AC stimulation. Other tyrosine derivatives tested included tyramine (TA), dopamine (DA) and the OA metabolite synephrine (SE). Incubation with vehicle alone served as a negative control. Only OA produced a significant decrease in baseline RLU (p-value, using the Student's t-test: < 0.0005), indicating a pronounced stimulation of AC (**Figure 1B**). The other tyrosine derivatives tested produced delta RLU values of 0 to -3000, indicating either no pronounced inhibition of AC or moderate inhibition of AC, though not significant. The data represent 3 technical replicates from up to two experiments. The experiment will be repeated with a broader range of BAs and a concentration-response assay of OA stimulation of cAMP production will be performed to verify the preliminary data. The kit used enables detection of ligand binding resulting in GPCR-mediated stimulation or inhibition of adenylate cyclase (AC), resulting in cAMP production, or an inhibition of production (**Figure 1C**). cAMP binds to and releases regulatory subunits of protein kinase A (PKA), resulting in consumption of ATP available for oxyluciferin (Promega Corporation, WI, USA) production, associated with a release of light, is effectively decreased in proportion to cAMP availability. Stimulation of AC, typically by Gαs-associated G-protein stimulation, or inhibition of AC, typically by G α i/o s-associated G-protein stimulation are therefore associated with a decrease or increase in luminescence, respectively.

3.3. *In silico* analysis of predicted schistosome tyramine beta-hydroxylase (SmTBH) teveals homology between other type-II copper monooxygenases

In invertebrates, phenolamine synthesis utilizes a tyramine beta-hydroxylase (TBH) that catalyzes the conversion of the tyrosine derivative tyramine (TA) to octopamine (OA) via

hydroxylation of the beta carbon of the TA side chain. Prior analysis of the schistosome genome (Berriman et al., 2009; Protasio et al., 2012; Ribeiro et al., 2012) revealed a putative *S. mansoni* homologue of tyramine beta-hydroxylase (TBH), SmTBH (Smp_243830) (Ribeiro et al., 2012). BLASTp analyses of the TBH were performed in NCBI. The putative SmTBH sequence (Smp_243830) annotated in NCBI shares 55% identity with both *Schmidtea mediterranea* (GenBank accession number: AFD34360) and *Lymnea stagnalis* (BAM35937) TBH, and 49% identity with *Dugesia japonica* TBH (DjTBH, BAG86630), indicating sequence conservation between these homologues. The cloned sequence matches different portions of the genomic and predicted CDS (http://www.genedb.org/; Protasio et al., 2012).

To determine homology of SmTBH with other copper type II monooxygenases, a phylogenetic tree of SmTBH was generated (**Figure 2**). Invertebrate TBHs clade together, with the exception of TBH from the pond snail *L. stagnalis*, which shares a common node with vertebrate DBHs. Within the invertebrate TBH clade, a flatworm Platyhelminthes phylum subclade is apparent and includes *D. japonica*, *S. mediterranea*, *Clonorchis sinensis* and *S. mansoni* TBH. *C. elegans* TBH (TBH-1), while more distantly related to SmTBH than the flatworm subclade of TBHs, shares homology with SmTBH. Similarly, vertebrate DBHs clade together in two subclades. DBHs from *Homo sapiens*, *Pan paniscus* and *Gorilla gorilla* clade together, as do DBHs from Rodentia, M*us musculus* and *Rattus norvegicus*). *Bos taurus* DBH, while not part of a subclade, clades with other vertebrate DBHs and is more closely related to primate than rodent DBHs.

To analyze SmTBH and identify catalytic domains and conserved residues, the sequence was aligned with a smaller subset of copper type-II monooxygenases (**Figure 3**). Included in the MSA were sequences from *D. japonica* and *C. elegans* as well as human, mouse and bovine DBH. The MSA revealed a high level of conservation in regions conferring catalytic activity. Nine conserved cysteines that typically confer intramolecular disulfide linkages were identified in each of the TBH/DBH sequences, including SmTBH. The TBH sequences diverge from the DBH sequences at the N-terminal regulatory domain, with little conservation until the first

conserved cysteine. In *S. mansoni*, the N-terminus is shorter than in other species and the first cysteine does not align with the other sequences. Two TBH/DBH catalytic domains were identified in all of the sequences. The catalytic domains contain the conserved histidine residues and the methionine residue that are involved in redox reactions that use molecular oxygen to hydroxylate the beta carbon of tyramine and dopamine in TBH/DBH, respectively. Two histidines and a methionine in the N-terminal catalytic domain and two histidines in the C-terminal catalytic domain are involved in ascorbate reduction and subsequent cupric ion reduction are also present in all sequences. N-terminal and C-terminal domains, pfam01082 (protein family # 01082) and pfam03712, respectively, were also identified in the SmTBH sequence using the NCBI Conserved Domain Database (CDD, http://ncbi.nlm.nih.gov), identifying SmTBH as a copper type-II monooxygenase and indicating a potential structure-function relationship for OA production.

3.4. Western blot verification of SmTBH expression.

Western blotting was used to verify expression of recombinant SmTBH by probing the C-terminal FLAG tag. 10 µg soluble protein extract from SmTBH-Pet15b-transfected cells and the BL21(DE3) untransfected cell control were loaded into lanes of a 4-10% Tris-Glycine SDS-PAGE gel. Protein was transferred to a PVDF membrane and probed with a monoclonal anti-FLAG antibody followed by an HRP-conjugated secondary antibody for detection of protein with a chemiluminescent substrate. Primary antibody precleared with 50 µg or 125 µg of BL21(DE3) cell extract at dilutions of 1:500 (**Figure 4A**) and 1:200 (**Figure 4B**), respectively, was used to determine specificity of primary antibody for 10 µg of the transfected protein. A non-specific band at ~94kDa was observed in the crude, untransfected and transfected cell extracts, which serves as an internal loading control. The expected SmTBH band size is ~49 kDa and bands resolve at ~50-55 kDa (**Figure 4A-C**). This shift in expected band size may be due to phosphorylation of the protein and altered SDS loading (Rath et al., 2009). Netphos software (Version 2.0, CBS-DTU) predicts 23 possible phosphorylation sites. A second lower band,

possibly a degradation product, resolved at ~45 kDa (**Figure 4A-C**). Both bands were absent in untransfected cells (**Figure 4A-C**). Probing with secondary antibody alone did not produce any non-specific banding, further demonstrating that the primary antibody produced specific bands (**Figure 4D**). Probing with a higher dilution of precleared antibody produced fainter bands (**Figure 4A**), while decreasing the dilution from 1:500 to 1:200 resulted in stronger banding (**Figure 4B**). Preclearing is expected to reduce signal, due to expected non-specific binding of antibody to the protein. Bands visualized with the precleared antibody at higher concentration (**Figure 4B**) and the band observed with the 1:500 dilution (**Figure 4C**), with a fainter band of inferior size, are therefore specific. Probing of the insoluble fraction did not produce any specific banding (not shown). The data suggest that the cloned SmTBH (Smp_243830) sequence is expressed as a soluble protein of ~50-55 kDa.

3.5 Production of OA from TA via SmTBH is quantifiable by spectrophotometric measurement

To determine whether SmTBH (Smp_243830) can synthesize OA from TA, we used an enzyme assay (Nagatsu and Udenfriend, 1972). SmTBH was cloned, expressed and SmTBH-containing total protein was extracted as described. OA (0 - 20 µM) was derivatized to p-hydroxybenzaldehyde and quantified spectrophotometrically at 330 nm (Nagatsu and Udenfriend, 1972), and a standard curve was generated (**Figure 5A**). 0.7 mg freshly obtained crude extract from *E. coli* in PBS induced to express SmTBH was incubated in a reaction mixture and OA converted to p-hydroxybenzaldehyde. The standard curve demonstrates OA concentration-dependent absorbance values, which at concentrations up to 20 µM are linearally proportional to the concentration of OA. Increasing TA concentration in the reaction mixture containing recombinant enzyme produced a corresponding increase in absorbance. Concentration of OA was calculated using the equation of the line of the standard curve (**Figure 5A**). Enzyme activity plateaued under the conditions of the assay, between 20 and 50 mM TA substrate indicating saturation of the enzyme (**Figure 5B**).

4. Discussion

The publication of the *S. mansoni* genome (Beriman et al., 2009) and its subsequent reannotation (Protasio et al., 2012) has facilitated the identification and characterization of schistosome proteins. An area of particular interest in schistosomes is BA signaling proteins of the nervous system, particularly those involved in OA signaling. The nervous system, largely under control of BAs (Pax et al., 1984; Boyle and Yoshino, 2005; El-Shehabi et al., 2012; Patocka et al., 2014), is central to the control of *S. mansoni* biology, including migration, feeding and egg laying (Crabtree and Wilson, 1980; Maule et al., 2005). BAs are the largest subset of classical neurotransmitters, and unlike the neuropeptide class of neurotransmitters, are readily available commercially, facilitating screens required to determine ligand and substrate specificity. BA signaling proteins include BA GPCRs and BA synthesis enzymes. GPCRs are proven drug targets, accounting for ~30-40 % of drug targets (Sautel and Milligan, 2000; Wise et al., 2002; Bockaert et al., 2004; Eglen, 2005; Eglen et al., 2007; Gilchrist, 2008). Targeting BA GPCRs in the schistosome nervous system may facilitate elimination of the parasite from the host. Indeed, in other helminth species, as well as with the anti-schistosomal metrifonate, targeting proteins involved in neuronal signaling has proven to be an effective strategy for eliminating the parasite from the host. Targeting a BA synthesis enzyme may serve to interrupt a signaling pathway, extending to all related BA GPCRs involved in downstream signaling. In higher invertebrates, BA GPCRs, particularly OA receptors, are known targets of pesticides, including the imidazolidine and formamidine classes. The annotation of the schistosome genome, supported by the cloning of several cDNAs encoding proteins involved in BA signaling, has revealed a high level of divergence between schistosome and human BA signaling pathways (Hamdan and Ribeiro, 1998; Hamdan et al., 2002; El-Shehabi et al., 2012), indicating their potential for selective targeting. The discovery of OA in the schistosome nervous system and the importance of OA and related tyrosine derivatives in motor control, described in the second and third chapter of this thesis, provide promising leads for future drug design. In addition to the potential applications for drug design, the study of proteins involved in tyrosine derivative

signaling is also an important step in characterizing and evaluating the importance of these neurotransmitters and their signaling pathways in schistosomes.

We showed that OA, an invertebrate-specific BA, is in the schistosome nervous system and plays an important role in muscle control. These findings are of particular interest, given the potential to specifically affect schistosome biology by targeting OA signaling. To identify an OA GPCR, we cloned 6 orphan BA GPCRs predicted in the *S. mansoni* genome. In a parallel study, we also cloned tyrosine-derivative-related signaling genes, homologous to SmTBH, SmAADC, and SmTH, and targeted them via RNAi to investigate *D. japonica* as a model species for the study of *S. mansoni*. While *C. elegans* serves as a model organism in the study of parasitic nematodes, a flatworm model organism is lacking in platyhelminth research. A free-living flatworm, which, like *C. elegans* (Raizen and Avery, 1994; Kaletta et al., 2006; Laing et al., 2015), is readily culturable, would circumvent issues with contamination and difficulties in maintaining viable cultures for *in vitro* studies with an organism out of its natural host environment (Stiernagle, 2006). The establishment of a planarian flatworm model organism would be facilitated by the availability of sequenced genomes, as are available for *D. tigrina* (Wheeler et al., 2015), *D. japonica* (Sakai and Sakaizumi, 2012; Nishimura et al., 2012) and *S. mediterranea* (Robb et al., 2008). Planarians have been proposed as model organisms (Sanchez Alvarado et al., 2002, Zamanian et al., 2011). In this study, we describe the cloning of cDNAs encoding 3 *D. tigrina* BA synthesis enzymes identified ny homology with schistosome genes that we previously targeted in RNAi studies. The *D. tigrina* sequences were obtained from an assembly of transcriptomic sequences from the sequencing project and annotation of this species' genome (Wheeler et al., 2015). As we have already established phenotypes associated with targeting the cloned *D. tigrina* enzymes in *S. mansoni*, we will compare the effects of targeting these homologues in *D. tigrina* and *S. mansoni*. Our cloning of these cDNAs into the L4440 RNAi vector, which can be transformed into *E. coli* and fed to the flatworm for dsRNA delivery, will help determine its suitability as a flatworm model organism. As a part of this study, other genes in *D. tigrina* were cloned and RNAi studies performed (Wheeler et al., 2015).

These studies provide a valuable tool in widening the scope of schistosomiasis research, potentially providing a tool for characterizing, screening, and validating the function of schistosome proteins.

In a prior phylogenetic tree analysis of predicted orphan BA GPCRs, described in the first manuscript of this thesis, Smp_150180 was the only BA GPCR to clade with adrenergic receptors, which typically share a high level of homology with OA GPCRs. Smp_150180 has 7 predicted transmembrane regions and is annotated as an OA receptor, and we pursued this receptor to determine if it is an OA GPCR. The remaining 5 cloned putative BA GPCRs can be de-orphanized to determine ligand specificity. Stable transfectants of Smp_150180 in HEK293F cells were generated and the cAMP-Glo assay (Promega Corporation, WI, USA) was used to quantify endogenous levels of second messenger cAMP production via luminescence. IFA was used to verify expression of the receptor, using an antibody against the encoded FLAG tag. Labelling was specific, as it was not observed in untransfected and mock-transfected cells. Of the BAs tested, only OA produced a decrease in baseline relative luminescence (RLU), having a mean delta RLU value of ~5000, indicative of a pronounced stimulation of AC.

Tyramine (TA), dopamine (DA) and synephrine (SE) caused no or moderate inhibition of AC, producing delta RLU values of 0 to -3000. While OA-mediated stimulation of AC by Smp_150180 was pronounced and significant, the experiment must be repeated with more BAs, and a concentration-response curve for OA constructed.

OA, being invertebrate-specific, would necessitate an endogenous synthesis pathway in schistosomes as it cannot be acquired OA from a vertebrate host. We cloned and expressed a predicted SmTBH (Smp_163900). Using bioinformatics analysis, the homology between the cloned SmTBH and other TBHs, as well as the orthologous SmAADC was determined. Conserved residues and regions in SmTBH characteristic of a copper type-II monooxygenase were identified. A phylogenetic tree generated with 6 invertebrate TBHs and 6 vertebrate DBHs revealed a separation of invertebrate TBHs from vertebrate DBHs, with DBHs forming a separate clade in the phylogenetic tree. These results suggest that this enzyme is likely a TBH

rather than a DBH. The separation of flatworm TBHs into a distinct clade containing *S. mansoni*, *D. japonica*, *S. mediterranea*, and *C. sinensis* sequences, suggests that flatworm TBHs are related.

The cloned SmTBH sequence was also aligned with a smaller subset of copper type-II monooxygenases to identify conserved residues and regions. Sequences and moieties conferring TBH and DBH catalytic activity were highly conserved. Each of the 6 TBH/DBH sequences, including the cloned *S. mansoni* sequence, contains 9 conserved cysteines, which typically form intramolecular disulfide linkages. The two conserved catalytic domains containing conserved histidines and a conserved methionine involved in reduction of ascorbate with subsequent reduction of copper, the enzyme cofactor (Cu++ → Cu+), are also present in all copper-type-II-monooxygenases. The presence of the conserved copper-type-II-monooxygenase N-terminal and C-terminal domains, pfam01082 and pfam03712, in the SmTBH-encoding sequence points to a structure-function relationship for SmTBH. While the *S. mansoni* N-terminal sequence is shorter than those of other species, the presence of all conserved residues, catalytic domains and protein family domains suggests that SmTBH encodes a functional protein, capable of synthesizing OA from TA in schistosomes.

Recombinant expression of SmTBH in BL21 (DE3) *E. coli* and Western blot analysis of the cell protein extract revealed that it is expressed as a soluble protein of the expected size of 50-55 kDa. A faint band of lower mass (~46 kDa) may be a degradation product. Protease inhibitors were added during the protein extraction and the sample was kept on ice throughout extraction. The 50-55 kDa band was observed in the transfected cell extract, but not in the untransfected cell extract, demonstrating specificity. The apparent shift to a higher molecular weight from the predicted 49 kDa may indicate post-translational modification of the protein, such as phosphorylation, and altered SDS loading (Rath et al., 2007).

We also assayed SmTBH activity to verify that this enzyme can synthesize OA from TA. We employed an activity assay where the concentration of OA produced was quantified by measuring the absorbance of OA derivative p-hydroxybenzaldehyde (Nagatsu and Udenfriend,

1972). Under the described assay conditions, we also determined that at concentrations between 0 and 1 µM, the assay is less sensitive. We observed this lower sensitivity when performing the activity assay using the reaction mixture with the TA substrate, and crude protein extract containing SmTBH, with subsequent derivitization of the product to p-hydroxybenzaldehyde. Sensitivity of the assay was improved when we supplemented the reaction mixture with 1 µM OA to raise absorbance baseline. Increasing TA substrate concentration produced a corresponding increase in absorbance of the product. Enzyme activity plateaued at an OA concentration of 8.616 µM OA, between 10 and 20 mM TA, indicating saturation of the enzyme. This plateau, combined with the initial increase in absorbance from substrate concentrations of 0 to 10 µM, produces a hyperbolic curve, typical of a type-II copper monooxygenase enzyme activity assay (Saxena et al., 1985). The data suggest that SmTBH can produce OA, which, along with the discovery of OA GPCR Smp_150180, localization of OA in the schistosome nervous system and the discovery that OA causes motor-stimulation of schistosomes, provides strong support for the presence of OA signaling in *S. mansoni*.

The research described here highlights the value of using bioinformatics to identify genes of interest in *S. mansoni*, and the advantage that the availability of the schistosome genome provides to further the study of schistosome proteins as potential drug targets and in gaining a better understanding of schistosome neurobiology. Similarly, the availability of planarian genomes (*D. japonica*, *S. mediterranea*, and *D. tigrina*) solidifies the possibility of using a planarian species as a flatworm model organism, much in the way that *C. elegans* hasbeen used to progress the study of parasitic nematodes (Kaletta et al., 2006; Laing et al., 2015). Bioinformatics studies of *S. mansoni* have led to deorphanization of several BA GPCRs (Taman and Ribeiro, 2009; El-Shehabi and Ribeiro, 2010; El-Shehabi et al., 2012; Macdonald et al., 2015) and facilitated the discovery of a putative OA GPCR, Smp_150180 and the SmTBH, though validation of these studies is required to confirm our findings. Following further characterization of these proteins to confirm a role in OA signaling, these functional expression

studies can be adapted to a high-throughput setup. Putative antagonists of OA signaling can then be screened, including the putative antagonists identified in the second chapter of this thesis, to identify compounds which may interefere with schistosome neurobiology. If compounds are identified which can robustly decrease SmTBH synthesis of OA or second messenger production by Smp_150180, analogues of these compounds can be further tested, using medicinal chemistry to improve antagonist specificity (Baraldi et al., 2008; Long et al., 2016). Apart from the potential of functional expression studies for adaptation to drug screens, this preliminary confirmation of a role in OA signaling highlights the importance of OA signaling in schistosome neurobiology.

Figures

Locus Tag	% Identity compared to GeneDB Predicted CDS	% Gaps, compared to GeneDB Predicted CDS	Length of CDS	TMHMM Predicted TM Region No.	Length of Cloned Sequence	TMHMM Predicted TM Region No.
Smp_204580	100	0	1230	7	1230	7
Smp_137300	100	0	987	6	987	6
Smp_134100	20.2	75.7	1149	4	834	0
Smp_180140	100	0	1065	6	1065	6
Smp_178420	100	0	987	3	880	3
Smp_027940	100	0	975	6	975	6
Smp_120620	32.9	57.9	1795	5	743	0
Smp_150180	63.8	33.9	2451	7	2211	7

Table 1. Cloned *S. mansoni* putative BA GPCRs.

Eight *S. mansoni* cDNAs (Smp_204580, Smp_137300, Smp_134100, Smp_180140, Smp_178420, Smp_027940, Smp_120620 and Smp_150180) were cloned from *S. mansoni*. Sense and antisense primers were designed using Oligo software against 5' and 3' ends of the predicted coding sequence (CDS). *S. mansoni* gene sequences were obtained from GeneDB

(http://www.genedb.org/Homepage/Smansoni) via selection using search words "amine" and "GPCR" in GeneDB. Sequences were confirmed by sequencing of a minimum of two independent clones. Percent identity and gaps between the cloned and target sequence were determined by generating a global sequence alignment using the EMBL-EBI EMBOSS (http://www.emboss.org/) stretcher tool and are indicated. The number of TMHMM-predicted TM regions in the predicted and cloned sequences is also indicated.

Locus Tag	Protein Class	Not1-Sense Primer	Nhe1-Antisense Primer	% Identity of CDS Compared to *D. japonica*	% Identity of CDS Compared to *C. Sinensis*
Locus_30706	Rho-GTPase-Like	**5'-GATCGCGGCCGC-CCTGGCTCATTTCC-3'**	**5'-GATCGCTAGC-CGTTTGGCGTCTTTG-3'**		59
Locus_32684	TBH-Like	**5'-GATCGCGGCCGC-CCAAAGTGGATGG-3'**	**5'-GATCGCTAGC-GACTCACCAATAACG-3'**	53	
Locus_21544	TH-Like	**5'-GATCGCGGCCGC-GCTATGAGCACCAATCG-3'**	**5'-GATCGCTAGC--GTGTGAAGAGGCTTAGAATG-3'**	69	
Locus_24292	AADC-Like	**5'-GATCGCGGCCGC-GGCATCGGTTGCATTG-3'**	**5'-GATCGCTAGC-GGAAGTCAGGGCACTTGC-3'**	74	

Table 2. Primers used to clone *D. tigrinia* cDNAs for ligatation into the vector L4440.

Primers used to ligate the indicated *D. tigrina* cDNAs into the L4440 vector for dsRNA synthesis are indicated. The 5' bolded portion of the primer sequences are absent in the cloning primers used. The complete primer sequences were used to ligate the cloned gene sequence into L4440 and include the Not1 and Nhe1 restriction sites, GCGGCCGC and GCGGCCGC, respectively. *Dugesia tigrina* sequences were obtained from an assembly of transcriptomic data (Wheeler et al., 2015) derived from a collection of 38, 056 sequences, using a BLOSUM62 substitution matrix, with existence and extension gap penalties (Schaffer et al., 2001) of 11 and 1, respectively. *D. tigrina* predicted BA synthesis enzymes were selected based on homology with known and predicted schistosome proteins (SmDBH (Smp_243830), SmAADC (Smp_171580) and SmTH (accession number: AAC62256) via NCBI BLASTp analysis. Rho-

GTPase (Locus_30706) was selected as a positive control for future RNAi studies. All enzyme sequences share significant homology with *D. japonica* or *C. sinensis* homologues.

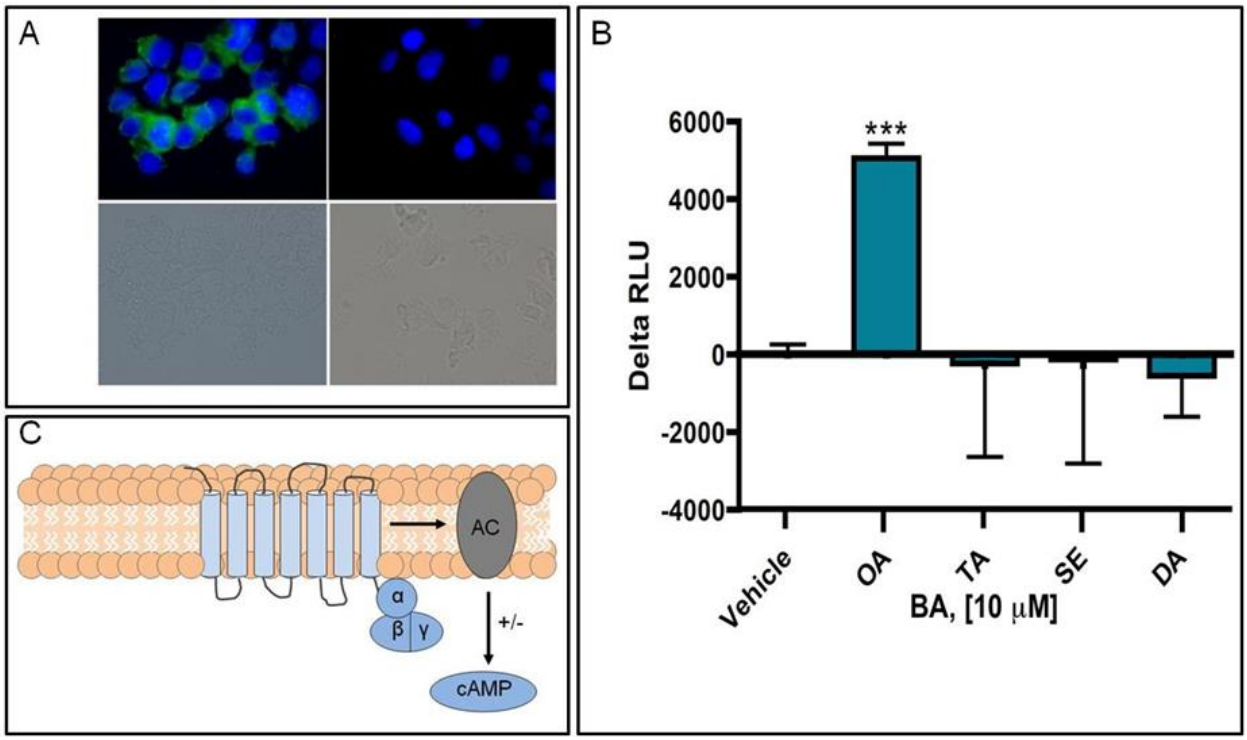

Figure 1. Functional expression studies of putative octopamine receptor Smp_150180. HEK 293 cells stably transfected with a plasmid construct expressing FLAG-Smp_150180-6xHis, or untransfected cells were fixed and permeabilized with ice-cold 4% paraformaldehyde (PFA). Cells were probed with an anti-FLAG primary antibody followed by a FITC-conjugated secondary antibody and visualized with an inverted fluorescent microscope. While stably-transfected cells showed pronounced labelling with the fluorescently-labelled antibody (green), untransfected cells and mock-transfected cells transfected with empty pCI-neo plasmid did not show non-specific labelling. DAPI was used as a counterstain to visualize nuclei. cAMP was assayed using a luminescence-based cAMP production detection kit (B). OA at 10 µM concentration produced the most significant change in receptor stimulation. cAMP production

or inhibition of production, resulting from GPCR stimulation by ligand interaction and associated inhibition or stimulation of adenylate cyclase (AC), is inversely proportional to luminescence produced, as quantified with a luminometer (C). The data are preliminary and are derived from 3 technical replicates from 1-2 experiments, though the effect of OA on Smp_150180-mediated AC stimulation is significant.

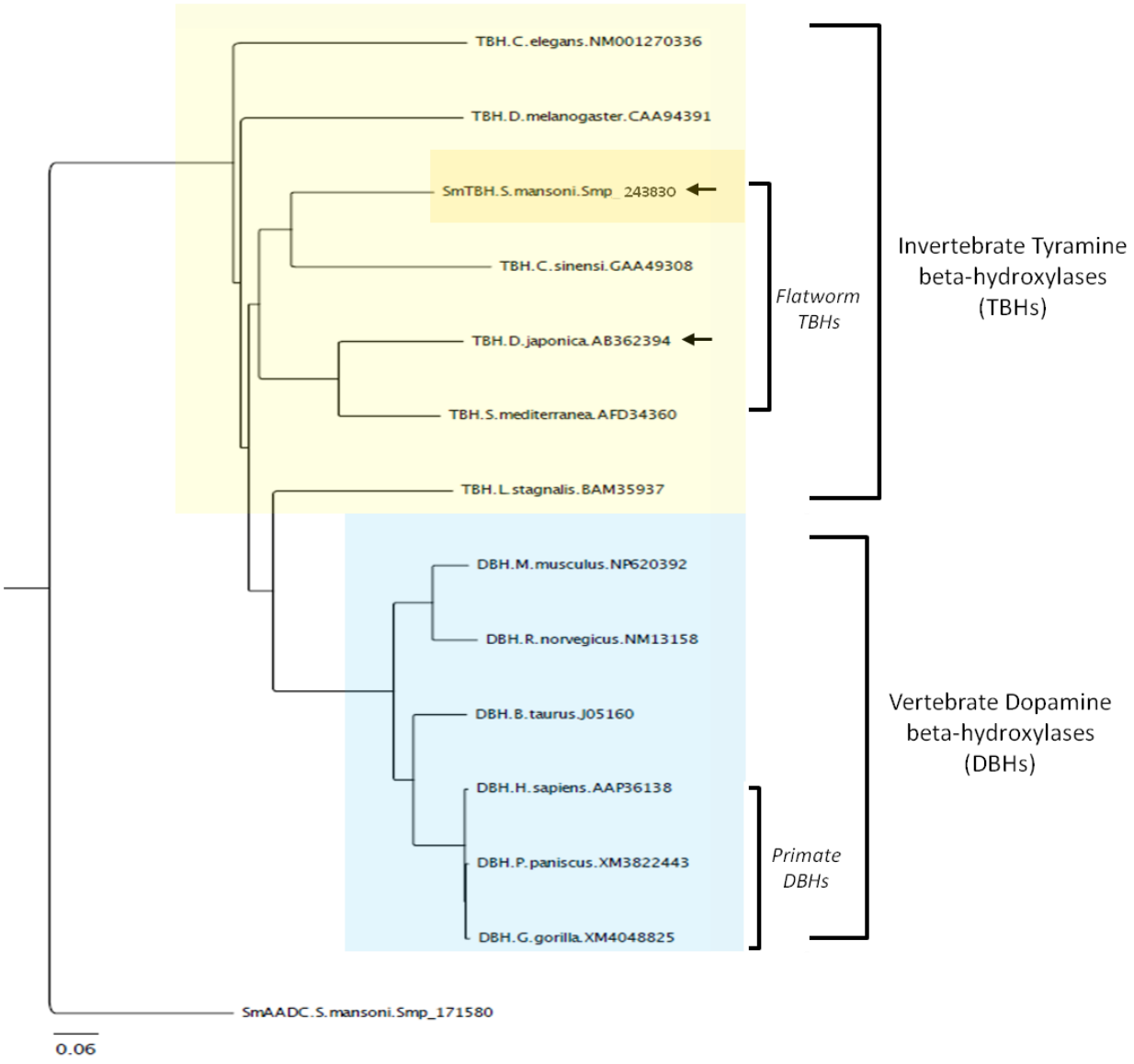

Figure 2. Phylogenetic tree analysis of copper type-II monooxygenases reveals homology with *Schistosoma mansoni* tyramine beta-hydroxylase. A phylogenetic tree of copper type-II monooxygenase homologues tyramine beta-hydroxylases (TBHs) and dopamine beta-hydroxylases (DBHs) was generated from an EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute) Clustal Omega (ClustalO, Version 1.1.2) (Söding, 2005; Sievers et al., 2011) multiple sequence alignment. Sequences were obtained from NCBI (http://ncbi.nlm.nih.gov). The *S. mansoni* SmTBH sequence corresponds to the cloned

sequence. The phylogenetic tree is midpoint-rooted and a putative schistosome amino acid decarboxylase (SmAADC) (Ribeiro et al., 2012) was included as an outgroup. GenBank locus_tags are indicated for *S. mansoni* genes, and accession numbers indicated for other genes.

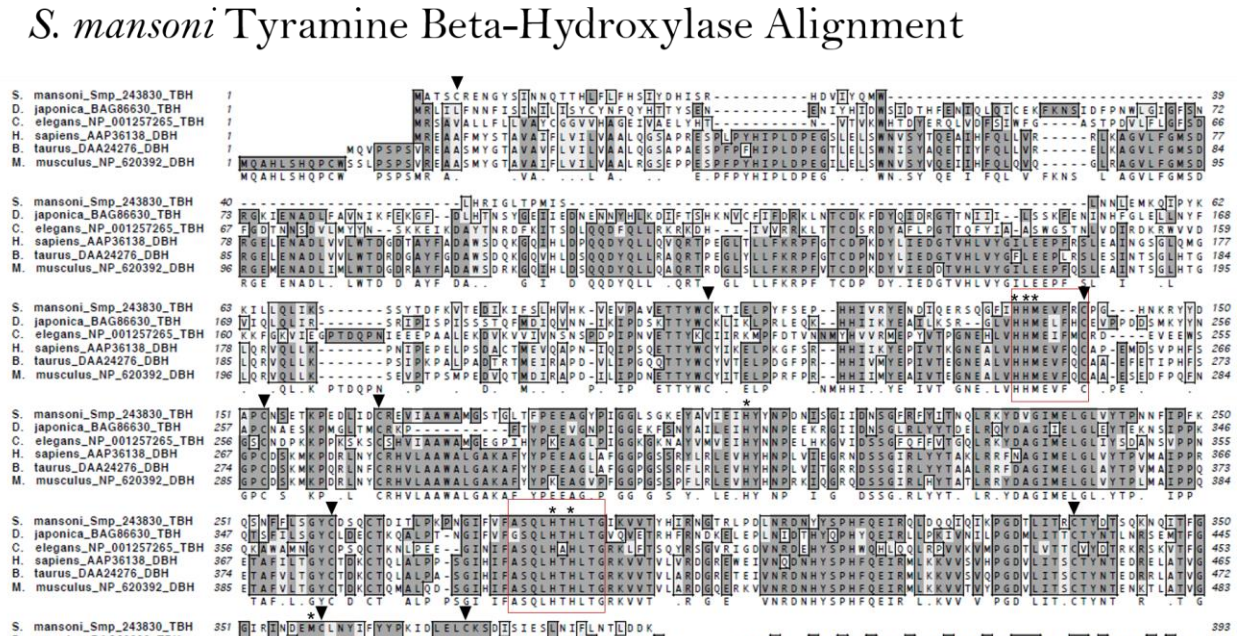

Figure 3. ClustalW alignment of SmTBH with copper type-II monooxygenases reveals conservation of catalytic domains. The predicted *S. mansoni* tyramine beta-hydroxylase (SmTBH, GenBank Locus_tag: Smp_243830) (Ribeiro et al., 2012; Protasio et al., 2012), cloned from adult cDNA, was included in a ClustalW alignment. The alignment also includes *D.*

japonica and *C. elegans* TBH. Coding sequences of dopamine beta-hydroxylase (DBH) were also included from *H. sapiens*, *B. taurus* and *M. musculus*. Black arrowheads indicate nine conserved cysteine residues, which form intramolecular disulfide linkages in the enzyme. Catalytic domains are indicated in the red boxes and contain conserved histidine residues and a methionine residue that are involved in the oxidation/reduction reaction that uses molecular oxygen to hydroxylate the beta carbon of tyramine and dopamine, for TBH and DBH, respectively. Histidines (*) and methionines (*) reduce ascorbate, enabling ascorbate-mediated reduction of the enzyme's cofactor, copper (Cu++ → Cu+).

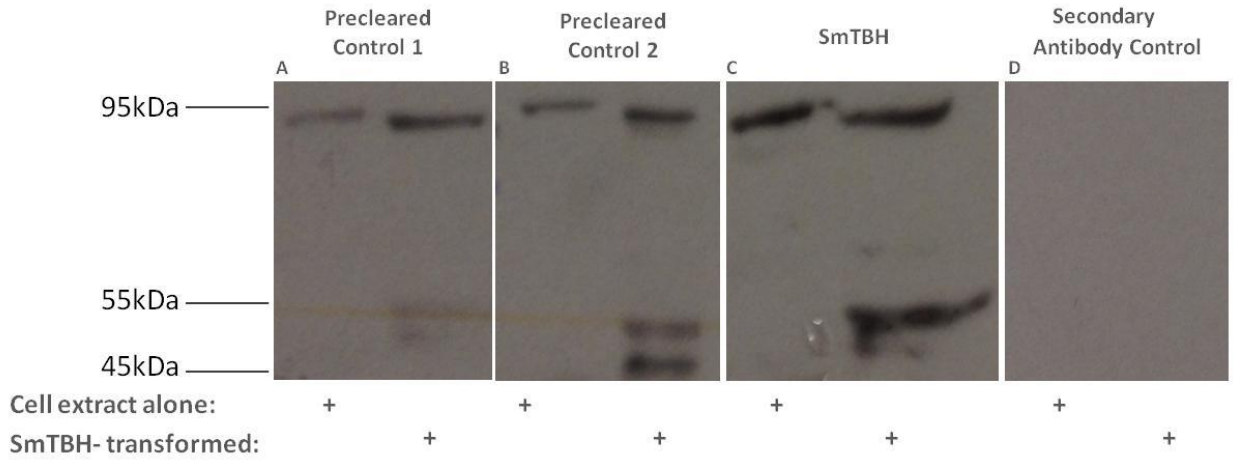

Figure 4. Western blot analysis verifies expression of soluble *Schistosoma mansoni* predicted tyramine beta-hydroxylase protein expression of the predicted molecular weight. Western blotting was used to verify expression of the cloned SmTBH protein by probing the attached C-terminal FLAG tag. 10ug of soluble protein extract from BL21(DE3) untransfected cells and SmTBH-Pet15b-transfected cells were loaded into each lane, as indicated, of a 4-10% Tris-Glycine gel. Primary antibody precleared with BL21(DE3) cell extract was used to determine specificity of primary antibody for the transfected protein, over native *E. coli* proteins, at dilutions of 1:500 (A) and 1:200 (B). Probing with higher dilutions of preadsorbed antibody produce faint bands (A), while decreasing the dilution from 1:500 to 1:200 produces stronger banding (B). Probing with a 1:500 dilution of primary antibody without preclearing produces the most pronounced band (C). A non-specific band at ~94kDa serves as a loading control. The expected band size is 49 kDa and bands resolve at ~50-55 kDa (A-C). A second band of inferior size resolves at ~45kDa. (A-C) Both bands are absent in untransfected cells. (D) Probing with secondary antibody alone did not produce any non-specific banding.

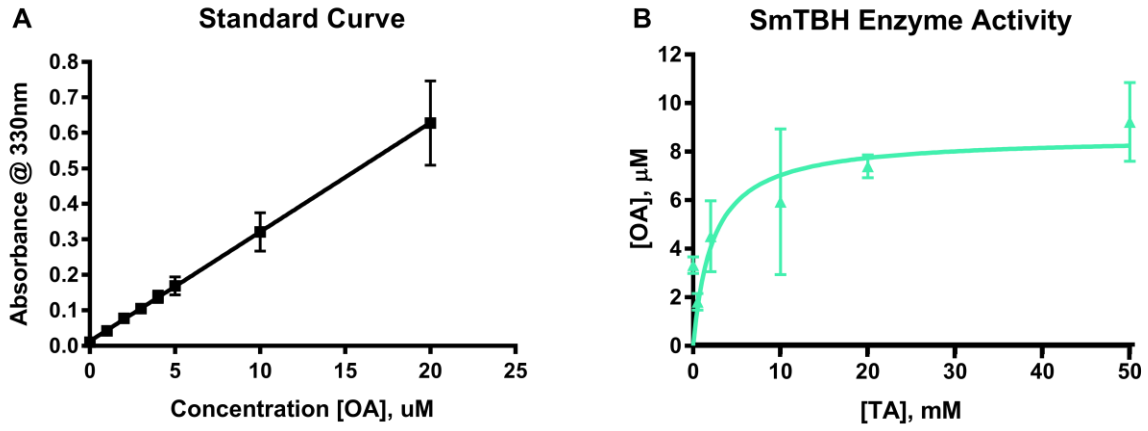

Figure 5. Quantification of OA metabolite p-hydroxybenzaldehyde to measure production of OA from TA by SmTBH. SmTBH was cloned and expressed to determine its activity. P-hydroxybenzaldehyde produced from concentrations of OA ranging from 0 to 20 µM, was quantified spectrophotometrically at 330 nm, as previously described (Nagatsu and Udenfriend, 1989), to generate a standard curve (A). 0.7 mg of crude extract obtained from *E. coli* containing SmTBH was incubated in a reaction mixture to allow for synthesis of OA from TA. OA was subsequently derivatized via an oxidation reaction, to p-hydroxybenzaldehyde (Nagatsu and Udenfriend, 1972; Aunis et al., 1980). The standard curve demonstrates OA concentration-dependent absorbance values, which, at low concentrations (0 to 20 µM), is directly proportional to the concentration of OA (A). Increasing the concentration of TA substrate produces a corresponding increase in OA. Enzyme activity plateaus between 20 to 50 mM TA substrate, as indicated in the hyperbolic curve in green. The assay was repeated twice, in triplicate and error bars indicate SEMs.

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Chapter V- Final Discussion and Conclusions

Schistosomiasis is a neglected tropical disease (NTD) that is most common in rural tropical and sub-tropical regions of poverty that lack access to clean water (Engels and Savioli, 2006; Hotez and Ferris, 2006; King and Dangerfield-Cha, 2008). Efforts to control and minimize the spread of the disease include educating communities in infected regions to avoid infection and reinfection via contact with cercaria-contaminated water (Chitsulo et al., et al., 2000) and implementing measures to improve sanitation. Despite successful efforts to control the spread of infection in countries like Egypt, Morocco, Brazil and China (Katz, 1998; Chen and Zheng, 1999; Laamrani et al., 2000; Hagan et al., 2003) and continued efforts on the part of such groups as the Bill and Melinda Gates Foundation Schistosomiasis Control Initiative (SCI) and Merck-KGaA (Darmstadt, Germany), schistosomiasis remains the second most prevalent NTD after malaria (http://www.cdc.gov/globalhealth/ntd/diseases/schisto_burden.html).

Schistosomiasis has been reported in 78 countries, with more than 800 million people at risk of contracting infection (WHO Fact Sheet No. 115).

Schistosomiasis is associated with severe pathology of the liver, causing hepatospleenomegaly, and with associated pathologies, such as ascites, colon obstruction and malaise. Schistosomiasis is the leading cause of hepatic fibrosis in the world (Doenhoff et al., 2008) and infects more than 250 million people worldwide, resulting in more than 280,000 deaths per annum (Fenwick et al., 2003; Knudsen et al., 2005; Steinmann et al., 2006; Doenhoff et al., 2008). The World Health Organization (WHO) reports that this NTD is endemic in 52 countries with moderate to high transmission rates, making schistosomiasis a major global health concern.

Schistosomiasis was discovered more than 150 years ago (Siebold, 1853) and despite the availability of the drug praziquantel (PZQ) for schistosomiasis treatment, there are concerns about the the possible development of resistance relating to the use of this single drug. Firstly, given the scope and spread of the disease, dependence on a single drug compound to treat continents of people is troubling. Historically, since the introduction of the first antibiotic in the 1930s, extensive use of a drug against pathogenic organisms has been associated with resistance (D'Costa et al., 2006; Davies and Davies, 2010). Furthermore, while mass drug

administration (MDA) has undeniably aided in controlling the spread of schistosomiasis (Secor, 2015), the US Center for Disease Control (CDC) reports that MDA is a factor that can contribute to resistance. The schistosomiasis MDA program is expanding, providing more tablets to affected areas, including a pledge of 200 million tablets per year from MerckKGaA (Darmstadt, Germany) over the next ten years, highlighting an increased possibility for drug resistance (Hotez and Fenwick, 2009). Secondly, clinical reports of inefficacy of PZQ are available (Ismail et al., 1996; Ismail et al., 1999); it is also possible to select a schistosome strain in the laboratory and *in vitro* with decreased PZQ sensitivity (Cioli et al., 2012; Cioli, 2000; Doenhoff and Mattoccia, 2006; Melman et al., 2009; Doenhoff et al., 2008; Greenberg, 2013). Thirdly, PZQ is ineffective against juvenile stages of the parasite (Sabah et al., 1986; Picquet et al., 1998 ; Cioli and Pica-Mattoccia, 2003), the stage that precludes schistosomiasis-associated morbidity and mortality and is present in the host 4-6 weeks preceding the development of egg-laying adults. This inefficacy means that treatment would not be protective against the development of morbidity and associated pathology in a patient treated once a year in an MDA program who who were infected up to 5-6 weeks earlier. Added to this is the constant risk of reinfection in affected areas, so that schistosomiasis treatment to reduce morbidity in people infected requires repeated and long-term PZQ administration.

The success in targeting the helminth nervous system (NS) with anthelmintics such as ivermectin (IVM) and levamisole (Robertson and Martin, 2007; Kaminsky et al, 2008; Gutman et al., 2010; Wolstenholme , 2011), and the role of the nervous system in schistosome migration through the host (Crabtree and Wilson, 1980), feeding and egg laying (Maule et al., 2005), processes that are central to infectivity, highlights the potential for the NS and its associated proteins for drug targeting.

Furthermore, the NS is of interest in the study of the evolution of the central nervous system (CNS). Early bilateria are purported to have the first nervous systems displaying the brain-nerve chord organization observed in metazoans. Flatworms, the earliest form of bilateria (Ruiz-Trillo et al., 1999), are postulated to have one of the earliest evolutionarily

derived NSs with a CNS (Holland et al., 2013; Strausfield and Hirth, 2013). The deviation from a "nerve net", typical of cnidarians, to the development of a CNS having grouped neurons, represents a pivotal point in the evolution of the NS. Flatworms, being acoelomates, lack an endocrine system, meaning their biology is even more heavily under the control of the NS, further highlighting the potential of the NS and its associated proteins for drug targeting. The schistosome NS is, in large part, under the control of the largest subset of classical transmitters, the biogenic amines (BA), which signal through G protein-coupled receptors (GPCRs). GPCRs are proven drug targets, making up between 30-40 % of all pharmaceuticals (Sautel and Milligan, 2000; Wise et al., 2002; Bockaert et al., 2004; Eglen, 2005; Eglen et al., 2007; Gilchrist, 2008). Our discovery of the invertebrate-specific BA octopamine (OA) in *S. mansoni*, described in this thesis, can potentially be specifically targeted to treat schistosomiasis. BA synthesis enzymes are also of interest in the study of the NS, as they represent a bottleneck in the process of BA signaling, at which production of a BA can be inhibited. Targeting *S. mansoni* OA synthesis enzymes provides the opportunity to inhibit all OA-specific GPCR signaling.

The study of the schistosome NS, aided by the publication of the *S. mansoni* genome (Berriman et al., 2009) and its subsequent reannotation (Protasio et al., 2012), and tools for *in sillico* analysis of genomic sequences, greatly accelerates the cloning and characterization of schistosome proteins.

In the first manuscript, we used confocal microscopy-based immunolocalization to identify OA in the parasite for the first time. The identification of OA in the NS is a landmark discovery, as it opens new lines of inquiry in the search for novel schistosomicides and for gaining a better understanding of NS function. Invertebrate-specific OA is an important neuromodulator and neurotransmitter and is involved in controlling and modulating a wide spectrum of behaviours and biological processes, including motility. We determined that OA is present in developing embryos, the schistosomula and the adult stages of *S. mansoni*, and is widespread in both the CNS and peripheral nervous system (PNS). Localization to the PNS includes the neuronal plexus that typically innervates musculature, suggesting a role in

neuromuscular control. We confirm localization of OA, using the neuronal marker synapsin, in the brain and in neurons throughout the body. The appearance of a 4-lobed, rather than the previously documented flatworm bi-lobed brain, was observed in adult parasites. In other flatworms, the brain has been described as being round (Quiroga et al., 2015) or bi-lobed (Bullock and Horridge, 1965; Sukhdeo et al., 1988; Cousin and Dorsey, 1991; Agata et al., 1998; Kotikova et al., 2002; Cebrià, 2007; Quiroga et al., 2015). Two pairs of ganglia have been described in tapeworms, and this organization is proposed to be more developed than a bi-lobed brain (Kotikova and Raikova, 2007).

To verify whether tyrosine derivatives of the phenolamine and catecholamine classes, are involved in NS signaling and to determine a motor-phenotype in schistosomes, we performed BA and small molecule screens of larval and adult stage parasites, as described in the first manuscript of this thesis. We tested OA- and DA-related metabolites, precursors and known and putative agonists and antagonists of these BA receptors. We included DA in parallel studies with OA to compare and contrast the two signaling systems in schistosomes.

In schistosomula, we observed an OA concentration-dependent increase in motility and an increase in average body length, also observed with the OA metabolite synephrine (SE) and the OA precursor tyramine (TA), using a previously described method (El-Shehabi et al., 2012) that determines the frequency of body-length changes. The effect on stimulation of motility was most pronounced with SE. These effects suggest a role for phenolamines in stimulation of circular muscle and/or relaxation of longitudinal muscle. We confirmed the previously described DA concentration-dependent decrease in motility and increase in length, and also observed an unusual biphasic response in frequency of body movements, which was also observed for TA, the adrenergic antagonist and putative OA agonist propranolol (PR), the putative OA antagonist carvedilol (CAR), and the DA antagonist chlorpromazine (CPZ). This biphasic response is a testament to the complexity of neuromuscular control, which could indicate concentration-dependent effects on different receptors and muscle types in the same signaling system. Testing different putative agonists and antagonists of OA signaling identified

5 candidate OA agonists that produce a hypermotile effect in schistosomula. Concentration-dependent effects on length and motility were observed for CPZ, CAR and PR, consistent with CPZ being an antagonist of DA signaling, CAR an antagonist of phenolamine signaling, and PR an agonist of phenolamine signaling. Agonists of phenolamine signaling are pesticides, causing uncoordinated movement and interrupting NS function in arthropods, including the formamidine and imidazolidine classes (Vans and Gee, 1980, Hollingworth and Murdoch, 1980; Goldman et al., 1980; Dudai et al., 1987). A putative antagonist of OA signaling, such as CAR, may paralyze the parasite, considering the stimulatory effect of OA on schistosomes. Our identification of putative agonists and antagonists of *S. mansoni* OA signaling provides a starting point for a line of inquiry to identify schistosomicides that may act by perturbing OA signaling.

Following the novel discovery of OA in the NS and characterization of the role of OA, TA, DA and SE on motility and length, we sought to identify related signaling proteins in schistosomes and to determine their role in tyrosine derivative signaling. We identified the putative orphan GPCRs Smp_150180, Smp_120620 and Smp_180140, which may use OA as a ligand. A phylogenetic analysis of 22 putative BA GPCRs reveals that Smp_150180 clades with adrenergic receptors, the mammalian equivalent of octopaminergic receptors.

We used RNAi to explore the role of OA and DA-associated NS proteins by downregulating gene expression and determining changes in motility and posture in schistosomula and adult parasites. Downregulation of DA and OA-related signaling mRNAs in schistosomula cause a shortening of the body, consistent with inhibition of longitudinal muscle relaxation, and/or an inhibition of circular muscle contraction. While SmTBH downregulation decreased length and motility, as expected, the absence of this effect when down-regulating the expression of putative OA GPCRs may reflect a disparity in the effect of downregulating BA signaling functions and BA stimulation. That is, while stimulation presumably raises baseline activity, downregulation minimizes the effect of a BA, which is more likely subject to masking by proteins with redundant or similar functions. OA and DA-related proteins may constitute a

greater portion of, or more significantly contribute to the effect of, BA receptors involved in lengthening.

Determining the effect of RNAi on adult parasites is more complex, given the larger size of this life stage (7-20mm) and the greater number of degrees of freedom associated with bending and extending. Following RNAi exposure, we observed changes in frequency of body movements, posture and coordination. Downregulating the putative OA receptor Smp_150180 decreased motility of females, while downregulating Smp_120620 increased motility of males, suggesting variations in the level of expression of these receptors between males and females. While changes in posture and coordination varied, the rigidity in the body and C-shaped posture phenotypes were clearly affected, particularly when the OA-related GPCRs were downregulated (Smp_150180 > Smp_180140 > Smp_120620), suggesting relaxation of circular muscle contraction and/or inhibition of longitudinal muscle relaxation.

Following our validation of the role of tyrosine derivative-related signaling genes in neuromuscular control, we cloned and performed a preliminary characterization of putative OA signaling proteins as described in the fourth chapter of the thesis. We cloned a cDNA encoding the putative OA receptor Smp_150180, as well as cDNAs encoding other putative OA receptors, and expressed Smp_150180 and SmTBH in heterologous systems to verify their ligand/substrate. We expressed Smp_150180 in mammalian cells and measured adenylate cyclase (AC) stimulation and cAMP levels using a luminescence-based kit. We incubated Smp_150180-expressing cells with OA, TA, SE and DA. While other tyrosine derivatives had no significant effect on AC, OA produced a pronounced and significant stimulation of AC, identifying Smp_150180 as an OA GPCR.

Recombinant expression of SmTBH (Smp_243830) demonstrated specific catalysis of the conversion of TA to OA in an absorbance-based activity assay. We incubated varying concentrations of TA in a reaction mixture with SmTBH, which produced a concentration-dependent increase in OA production.

As we established that OA signaling is present in schistosomes and validated that catecholamines and phenolamines play an important role in motility, we wanted to validate *Dugesia tigrina* as a model for the study of schistosome neurobiology. A flatworm model would serve as a useful tool in validating the findings of schistosome research, as well as providing an efficient system in which to perform RNAi screens to determine the phenotype of schistosome proteins, without the difficulties associated with RNAi-targeting of schistosomula. Such difficulties include variation in the viability of cultures, the complex and resource-intensive maintenance of the schistosome life cycle, and the high risk of contamination. In the second manuscript of this thesis, we report the cloning of cDNAs encoding 3 BA synthesis enzymes, the homologues of which were previously characterized in RNAi studies in schistosomes. The study is ongoing, but if successful, the data can provide a new tool in the study and deorphanization of schistosome NS proteins.

In this thesis, we performed an extensive study of OA-related tyrosine derivatives and signaling proteins, which highlights OA-related signaling proteins as potential drug targets. The absence of OA signaling in the mammalian host presents the opportunity for selective drug targetting. The abundance of OA in the NS, the concentration-dependent effects of OA and related amines on schistosome motility and length, and effects on length, motility, posture and coordination associated with downregulating tyrosine derivative-related genes, further highlight the importance of signaling of tyrosine derivatives in schistosome neuromuscular function. The discovery of phenolamine signaling in schistosomes and the characterization of catecholamine and phenolamine signaling in *S. mansoni* sheds new insight into schistosome neurobiology and its importance in motor control. Our identification of putative agonists and antagonists of tyrosine derivative signaling identifies lead compounds, which can be modified using medicinal chemistry (Baraldi et al., 2008; Long et al., 2016) to generate selective schistosomicides. Targeting tyrosine derivative signaling is promising and presents new lines of inquiry for the discovery of an alternative to PZQ for this widespread and debilitating disease.

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