Characterization of Putative Cation-selective Nicotinic Acetylcholine Receptors of the parasitic blood fluke *Schistosoma mansoni*

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ABSTRACT

Schistosomiasis is one of the most socioeconomically important parasitic diseases, affecting over 200 million people worldwide. Praziquantel is the only drug treatment available in most parts of the world and there is an urgent need to find a viable alternative to this drug. Acetylcholine-gated ion channels of the nicotinic acetylcholine receptor (nAChR) family have been shown to be effective drug targets for treatment of various helminth infections. Here, we describe a first investigation of putative nAChR subunits of the model parasite, *Schistosoma mansoni*. Four predicted subunits, smp_031680, smp_180570, smp_139330, and smp_012000, were cloned from *S. mansoni* by a combination of RT-PCR and RACE (rapid amplification of cDNA ends) procedures. All four full-length proteins contain prerequisite features of cation-selective nAChRs. Subsequent functional studies by RNA interference (RNAi) in parasite larvae (schistosomulae) and adult worms showed that the four nAChR subunits play an important role in the control of motor activity. RNAi targeting smp_031680 and smp_180570 caused hypoactivity, whereas knockdown of smp_139330 and smp_012000 caused hyperactivity, suggesting these subunits form channels that either stimulate or inhibit movement. Further confocal immunofluorescence studies of smp_031680 and smp_139330 showed that nAChRs are expressed extensively in both central and peripheral nervous systems of the parasite, musculature (smp_139330 only) and female reproductive system. Importantly, we observed distinct expression patterns of smp_031680 and smp_139330, which suggests that they are not part of the same channel. Our repeated efforts to heterologously express the four putative subunits in *Xenopus* oocytes were unsuccessful. We were unable to obtain functional channels from subunits expressed individually or in combination, in the presence and absence of *S. mansoni* orthologues of *Caenorhabditis elegans* ancillary proteins ric-3 and unc-50, which are known to improve heterologous expression of nAChRs. As an alternative strategy, we modified the subunits by inserting a fluorescent tag, sub-cloned the modified sequences into a mammalian expression vector, and tested for protein expression in transfected HEK293 cells by fluorescence microscopy. The results show that nAChRs can be expressed in the mammalian cells but additional experiments are needed to test for channel activity. All in all, this is a small yet significant first step in elucidating the parasite's cholinergic nervous system and identifying some of the receptors involved in cholinergic motor control in *S. mansoni*.

ABRÉGÉ

La schistosomiase est une des maladies parasitaires les plus importantes sur le plan socioéconomique, affectant plus de 200 millions de personnes à travers le monde. Le praziquantel est le seul médicament disponible pour le traitement de la schistosomiase dans la plupart des régions du monde et il est urgent de trouver un traitement de rechange viable pour complémenter ce médicament. Il a été démontré que les canaux ioniques de type pentamériques sensibles à de l'acétylcholine appartenant à la famille des récepteurs nicotiniques (nicotinic acetylcholine receptor, nAChR) sont la cibles de plusieurs médicaments vermifuges utilisés pour le traitement de diverses infections causées par des helminthes. Dans ce mémoire, nous décrivons pour la première fois l'existence de sous-unités nAChR putatives chez le parasite modèle *Schistosoma mansoni*. Les séquences codantes complètes de quatre sous-unités putatives, smp_031680, smp_180570, smp_139330, et smp_012000, ont été déterminées et clonées chez *S. mansoni* en utilisant une combinaison de RT-PCR (transcription inverse et réaction en chaîne par la polymérase) et de RACE (amplification rapide des extrémités d'ADNc). Les quatre séquences protéiques obtenues possèdent les caractéristiques typiques des nAChRs perméables aux cations. Nous démontrons également grâce à des études fonctionnelles d'interférence de l'ARN (RNAi) réalisées dans des larves de parasites (schistosomules) et des vers adultes que les quatre sous-unités nAChR jouent un rôle important dans le contrôle de l'activité motrice. En effet, nous avons observé que l'RNAi ciblant smp_031680 et smp_180570 causait l'hypoactivité motrice, tandis que la diminution de l'expression génique de smp_139330 et smp_012000 causait l'hyperactivité motrice, suggérant par conséquent que ces sous-unités forment des canaux ioniques capable de stimuler ou d'inhiber l'activité motrice. Par ailleurs, des études d'immunolocalisation et de microscopie confocale ont montré que les sous-unités nAChRs smp_031680 et smp_139330 sont abondamment exprimées dans les systèmes nerveux central et périphérique du parasite, ainsi qu'au niveau de la musculature (smp_139330 seulement) et du système reproducteur féminin. Il est important de noter nous avons observé des profils d'expression distincts entre smp_031680 et smp_139330, ce qui suggère que ces sous-unités ne font pas partie du même récepteurs. Nos tentatives répétées pour exprimer de manière hétérologue ces quatre sous-unités putatives dans des ovocytes de *Xenopus* ont été infructueuses.

Nous n'avons pas pu obtenir de récepteurs fonctionnels à partir de sous-unités exprimées individuellement ou en combinaison, en présence et en l'absence de protéines auxiliaires de *S. mansoni* orthologues aux protéines ric-3 et unc-50 de *Caenorhabditis elegans*, lesquelles sont connus pour améliorer l'expression hétérologue des nAChRs. Alternativement, nous avons modifié les sous-unités en y insérant un marqueur fluorescent et avons sous-cloné ces séquences dans un vecteur d'expression de mammifère. Nous avons ensuite visualisé par immunofluorescence l'expression des protéines d'intérêts transfectées dans des cellules HEK293. Les résultats montrent que les nAChRs sont exprimés dans les cellules de mammifères, mais d'autres expériences sont nécessaires afin de tester l'activité de ces récepteurs. Somme toute, bien que limités, nos résultats constituent un premier pas important dans la compréhension du système nerveux cholinergique du parasite et ont permis d'identifier quelques-uns des récepteurs impliqués dans le contrôle cholinergique des fonctions motrices chez *S. mansoni*.

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STATEMENT OF CONTRIBUTIONS

The scientific work described in this thesis was designed and performed by the author under the supervision of Dr. Paula Ribeiro who provided the necessary financial resources to realize the experiments and was involved in the design of the experiments, data analysis and editing this work.

STATEMENT OF ORIGINALITY

The following aspects of this thesis are considered original contributions to the knowledge:

Manuscript I:

In this study we report the cloning and sequence analysis of four putative nicotinic acetylcholine receptors (nAChRs) of the helminth parasite *Schistosoma mansoni*. To test for biological activity, we knocked down expression of these proteins in larval and adult stage animals using RNA interference (RNAi). We observed two distinct types of RNAi motor phenotypes: RNAi targeting subunits smp_031680 and smp_180570 caused hypomotility, whereas knockdown of smp_139330 and smp_012000 produced significant hypermotility. Double knockdown of smp_031680 and smp_180570 produced the same hypomotile phenotype as the individual subunit treatments. On the other hand, a double knockdown of smp_139330 and smp_012000 (hypermotile phenotypes) resulted in an opposite (hypomotile) phenotype. In other studies we examined the effects of an agonist (nicotine) and an antagonist (mecamylamine) in RNAi-treated larvae. Compared to control animals, the smp_031680 and smp_180570 RNAi-treated larvae were less responsive to nicotine, whereas, smp_139330 and smp_012000 RNAi animals showed resistance to mecamylamine. Moreover, by confocal immunofluorescence microscopy, using specific antibodies against smp_031680 and smp_139330, we characterized the expression patterns of these two proteins in both adult and larval (schistosomulae) stages of the parasite. We found expression of smp_031680 in the central (CNS) and peripheral nervous systems (PNS) of the adult worm and the PNS and the parenchyma of the larval stage, whereas smp_139330 was localized in the CNS, PNS and the musculature of the adult worm or the PNS and probable musculature of the schistosomulae. Importantly, we observed distinct expression patterns of smp_031680 and smp_139330, which suggests these subunits are not part of the same channel. The strong motor RNAi phenotypes, combined with the wide distribution of these receptors identify nAChRs as important proteins of the parasite's motor control system.

Manuscript II:

In this study, we sub-cloned the four putative nAChRs into suitable vectors for heterologous expression in *Xenopus* oocytes or mammalian cells. Moreover, we report identification and successful cloning of two S. mansoni orthologues of C. elegans ancillary ric-3 and unc-50 proteins, which have been implicated in proper folding and expression of nAChRs. Despite repeated attempts, we were unable to obtain functional S. mansoni nAChRs in *Xenopus* oocytes from various combinations of putative subunits in the presence and absence of ancillary proteins. However, we confirmed expression of smp_031680 and smp_139330 in the oocytes by confocal microscopy, using specific antibodies. In addition, we demonstrated subunit expression in transiently transfected mammalian cells using the same antibodies and several fluorescent-tagged constructs. These constructs are now available for future functional expression studies in the mammalian cell environment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Literature Review

Chapter 2: Manuscript I (Putative cation-selective nicotinic acetylcholine receptors of the human parasite *Schistosoma mansoni*)

Chapter 3: Manuscript II (Heterologous expression of putative cation-selective nicotinic acetylcholine receptors of *Schistosoma mansoni*)

LIST OF ABBREVIATIONS

AA: Amino Acid

AbD: Antibody Diluent

ACC: Acetylcholine-gated Chloride Channel subunit

Ach: Acetylcholine

ATP: Adenosine Triphosphate

BA: Biogenic Amine

ChAT: Choline Acetyltransferase

CNS: Central Nervous System

cDNA: Complementary Deoxyribonucleic Acid

cRNA: complementary Ribonucleic Acid

DNA: Deoxyribonucleic Acid

ELISA: Enzyme Linked Immunesorbent Assay

ER: Endoplasmic Reticulum

FITC: Fluorescein Isocyanate

GABA: Gamma-aminobutyric Acid

GAR: G Protein-linked Acetylcholine Receptor

GFP: Green Fluorescent Protein

GluCl: Glutamate-gated Chloride channel

GPCR: G Protein-Coupled Receptor

GSP: Gene Specific Primer

HEK293: Human Embryonic Kidney 293

HRP: Horseradish Peroxidase

IP: Immunoprecipitation

kDa: kilo Dalton

L-GIC: Ligand-Gated Ion Channel

M-MLV RT: Moloney Murine Leukemia Virus Reverse Transcriptase

mRNA: messenger Ribonucleic Acid

MW: Molecular Weight

nAChR: Nicotinic Acetylcholine Receptor

NADPH-d: Nicotinamide Adenine Dinucleotide Phosphate Diaphorase

NO: Nitric Oxide

PBS: Phosphate Buffered Saline

PNS: Peripheral Nervous System

RACE: Rapid Amplification of cDNA Ends

RNA: Ribonucleic Acid

RNAi: RNA interference

RT: Room Temperature

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrilamide Gel Electrophorasis

SiRNA: Small Interfering Ribonucleic Acid

TEVC: Two-Electrode Voltage-Clamp

TM: Transmembrane

VAChT: Vesicular Acetylcholine Transporter

INTRODUCTION

Schistosomiasis is a debilitating chronic parasitic infection, second only to malaria in terms of socioeconomic impact. Over 700 million people of 74 different countries are at risk of becoming infected worldwide [1,2]. The infected population exceeds 200 million [2]. The majority of the infected are located in developing countries of the world, where their daily activities in agriculture, domestic chores and recreation expose them to infested water.

There are two major types of schistosomiasis, intestinal, caused primarily by *Schistosoma mansoni* and *Schistosoma japonicum* and urogenital, brought on by infection with *Schistosoma haematobium*. Pathology occurs due to deposition of eggs in various tissues, particularly the liver and spleen. The mainstay of drug therapy for schistosomiasis is praziquantel. Praziquantel is effective against the adult stage of the parasite [3] and is much safer and cheaper than the alternative schistosomicides, metrifonate or oxamniquine [4]. However, there are reports of reduced efficacy in endemic areas of Egypt [5] and Senegal[6] and drug resistance can develop after only two generations of repeated exposure to sub-lethal doses of Praziquantel in mice [7]. With increasing usage of praziquantel due to mass treatment programs in many parts of the world, there is a real concern that resistance to the principal schistosomicidal agent may occur. Thus, there is an urgent need to search for alternative drug therapies and to find new drug targets.

The nervous systems of helminth parasites hold great potential for drug targeting, as evidenced by the success of nematocidal drugs such as ivermectin or levamisole, which target neuronal proteins. Schistosomes have a well-organized nervous system, comprising a central nervous system (CNS) and a peripheral nervous system (PNS). The CNS includes bilobed cerebral ganglia in place of a brain and two pairs of longitudinal ventral and dorsal nerve cords. Each pair is interconnected at regular intervals by transverse commissures. Neural branches develop from the CNS to form the PNS, which contains smaller nerve cords and nerve plexuses, reaching virtually every region of the body. This includes the somatic musculature, tegument, oral and ventral suckers, digestive tract and the reproductive system [8-10]. Due to the absence of a coelomic cavity and thus endocrine signalling through circulating body fluids, much of the regulatory activity in the parasite is performed by the nervous system. Critical functions such as

movement, host attachment, penetration and migration, feeding reproduction and excretion are all controlled through the parasite's nervous system [8].

An examination of currently available anthelmintics reveals that many of these drugs work by disrupting the activity of nicotinic acetylcholine receptors (nAChRs). Common nematocidal drugs such as pyrantel, levamisole, morantel, paraherquamide and monepantel all have nAChRs as their molecular targets [11-15]. nAChRs form homo or hetero pentameric ion channels, which open upon binding of the neurotransmitter ligand, acetylcholine (ACh), or competitive agonist nicotine[16]. nAChRs mediate many of the effects of ACh in vertebrates and invertebrates, notably neuromuscular effects associated with motor control, as well as effects on behavior, egg laying, feeding, grooming and modulation of cerebellar activity in various organisms [17-20]. Our knowledge of nAChRs is derived mainly from the classical vertebrate model, where the ion channels are located on post-synaptic membranes neuronal as well as neuromuscular. Activation of these ion channels by endogenous acetylcholine causes influx of cations and resulting depolarization of the downstream muscle fiber (and thus muscle excitation) or neuron (excitatory postsynaptic potential)[21].

It has long been known that acetylcholine, along with serotonin, dopamine and neuropeptides are major neurotransmitters that control muscle function and movement of the parasite [22-26]. In other species, acetylcholine is the quintessential excitatory neuromuscular transmitter that stimulates contraction of the major muscles of the body. This is caused by activation of cation-selective nAChRs at the neuromuscular junction, which leads to depolarization of the muscle fiber and ensuing muscle contraction [21] However schistosomes have a very different response to acetylcholine. Studies dating back to the 1970s and 80s have shown that treatment of schistosomes with acetylcholine causes muscle relaxation and flaccid paralysis [27,28], suggesting that acetylcholine has inhibitory effects in these parasites. Two types of acetylcholine receptors are found in the genome of *S. mansoni*: (i) muscarinic (GPCR) receptors and (ii) nicotinic acetylcholine receptors [29]. Two candidates of muscarinic receptors are found in the genome: smp_152540 and smp_145540 of which, the latter has been shown to be truncated (unpublished data).

Of the 22 putative nicotinic acetylcholine receptor candidates, 11 contain enough sequence information (cys loop with multiple transmembrane regions) to be considered actual

nAChRs. Among these 11, we see the presence of five putative cation – selective nAChRs and the rest are predicted anion-selective cholinergic channels (Ribeiro and Geary, 2010). Four of the 5 putative cation selective nAChRs were investigated in this study by means of RNA interference (RNAi) and confocal microscopy. These receptors are: smp_031680, smp_180570, smp_139330 and smp_012000. Included in this study are orthologues of two previously described *S. haematobium* nAChR subunits [30] one of which localized to the surface of the parasite and the other was found in the musculature and parenchyma [30]. Contrasting expression patterns and repeated unsuccessful efforts by the authors to form a functional ion channel in a heterologous expression system [30] indicated that these two subunits are not part of the same ion channel.

In this study, we seek to elucidate the biological role of cation-selective nAChR subunits in both larval and adult life stages of *S. mansoni*. We inquire what happens to the motility of the parasite upon abrogation of 4 nAChR subunits by RNAi in *S. mansoni*. We also seek to confirm and characterize further the expression patterns of these subunits by confocal immunofluorescence microscopy in the same life stages. Finally, we attempt to heterologously express the four putative subunits in two different expression systems: *Xenopus* oocytes and mammalian cell line for functional characterization of nAChR channel(s).

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Chapter 1

Literature Review

Schistosomiasis and *Schistosoma*: Schistosomiasis is a chronic parasitic disease caused by the platyhelminth blood fluke of the genus *Schistosoma*. The disease is a major world health concern with prevalence among 74 countries of the world [1,2]. More than 200 million people undergoes treatment every year, most of which (>90%) lives in Africa where schistosomiasis takes lives of approximately 200,000 people each year [1].

Humans obtain *Schistosoma* infection by coming in contact with fresh water infested with a larval stage of the parasite shed by snails. In many developing countries this is a common scenario since people use such fresh water sources as ponds and canals for day to day activities and irrigation. Two kinds of schistosomiasis are caused by three major species of the parasite: *S. mansoni* and *S. japonicum, which* cause intestinal schistosomiasis, and *S. haematobium,* which cases urogenital schistosomiasis (Table 1). Symptoms of schistosomiasis are caused by the eggs produced by paired adult worms, which often get lodged into blood vessels of the human body. Intestinal schistosomiasis is manifested by pain, diarrhoea and blood in the stool. In advanced cases, liver enlargement, accumulation of fluid in the peritoneal cavity, hypertension of the abdominal blood vessels and enlargement of the spleen is frequently identified [3]. Urogenital schistosomiasis patients classically exhibit haematuria (blood in urine). Late stage complications include fibrosis of bladder and ureter, bladder cancer, genital lesions along with bleeding and even infertility in both men and women [3]. In endemic areas, schistosomiasis is diagnosed by the presence of eggs in the stool or urine. Intestinal schistosomiasis is detected by staining stool samples by Kato-Katz technique [4]. In case of urogenital schistosomiasis, a filtration technique with nylon, polycarbonate or paper is employed [4]. Chemical reagent strips are a common way to detect blood in the urine of the patient [5]. In non-endemic areas, serological or immunological tests are commonly used to identify schistosomiasis [4].

Praziquantel remains the mainstay of treatment of both intestinal and urogenital schistosomiasis. A single dose of the drug at a rate of 40 mg/kg body weight is effective against the adult stage parasites of the body [6]. Oxamniquine can be used to treat intestinal schistosomiasis (15-60 mg/kg body weight). However it is less cost effective and has higher side effects [7]. Metrifonate is an alternative to praziquantel to treat urogenital schistosomiasis (10 mg/kg body weight). Metrifonate requires to be kept below 25^{o}C which is a major obstacle

towards its mass usage [8]. Both oxamniquine and metrifonate requires multiple dosages and as a result rate of compliance is low for these drugs among patients [8].

***Schistosoma mansoni*:** *Schistosoma mansoni* is one of the major causative agents of intestinal schistosomiasis. The parasite is found largely in Africa and South America. The major definitive host of the parasite is human. However, rodents and primates are also viable hosts for the parasite. Adult worms live in the mesenteric veins of the host and obtain nutrient in the form of blood and blood soluble nutrients from the host's intestine.

The lifecycle of the blood fluke *Schistosoma mansoni* (Figure 1) is both unique and complex. *S. mansoni* has an intermediate host (snails of the genus *Biomphalaria*) and a definitive host (human). Eggs of the parasite are shed by an infected human along with feces into the water in places where sanitation is inadequate. It takes about 10 hours for the egg to transform into miracidium, the larval stage infective to snails [9]. Upon infection of the snail, the parasite forms sporocysts (approximately 6 weeks), which reproduce asexually for multiple generations [10]. Maturation of a sporocyst to cercaria, the stage of the parasite infective to human host takes approximately 3 days [11]. These cercaria are motile due the presence of tails and when they come in contact with a human host are able to readily penetrate the skin through hair follicles. It takes a parasite 2-3 days to become schistosomula and find its way to the blood circulation via lymph vessels [11]. The parasite then migrates to the lungs, followed by hepatoportal circulation via the left ventricle of the heart. Ultimately, the parasite migrates to the liver and matures into an adult. *Schistosoma* is unique among Trematodes in that they are dioecious, as opposed to being hermaphroditic, which is the common norm of the phyla [12]. A female parasite pairs with its male counterpart by taking place in the gynecophoral cavity created by the male blood fluke and the duo travel to the mesenteric venules of the bowel against the blood flow [12]. In the mesenteries, female *Schistosoma* lays eggs, which circulate to the liver and ultimately are shed in the stool, infecting more snails upon maturation.

Morphology: *S. mansoni* are parasitic flatworms of the phylum Platyhelminthes. The organism is dioecious and dimorphic which means that male/female sexes exist in this organism [13]. Males can be up to 10 mm long, are stouter than their female counterparts and possess a body

cavity, termed as gynecophoral canal. Female adults can be up to 14 mm long but are more slender than the males. During sexual reproduction, a female worm takes place in a male's gynecophoric canal [14]. Both male and female body is surrounded by a double-layered tegument. However, the male tegument is dorsally ornate with tubercles, which females lack altogether. Both males and females have an oral sucker and a ventral sucker, which is located posterior to the bifurcation of the gut. It lacks a pharynx but possesses an esophagus with esophageal glands. A single caecum extends the whole body, which is located posterior to a pair of caeca which follows the esophagus. Males have 6-9 testes and the male genital pore is located below the ventral sucker on the ventral plane. On the female, a single ovary is located on the anterior portion of the body which is connected by a uterus to the female genital pore [14].

Cercariae are the snail-derived, infective larval stage of the parasite. They can be up to 1 mm in length, which includes a 200um long head and a bifurcated tail. The plasma membrane is trilaminate in nature at this stage [15]. The animal is covered by an 8.5 nm glycocalyx which is thought to protect the larva from hypo osmotic environment. It has five pairs of glands, two preacetabular and three postacetabular, all of which empties into the oral sucker via separate ducts [15]. Cercariae swim around in the water in a figure 8 pattern and once come in contact with human host, adhere to the skin by its suckers aided by mucoid secretions. A skin penetration is mostly facilitated by secretions of the preacetabular glands, which occurs after postacetabular gland secretions and contains highly enzymatic in contents. Following skin penetration, cercariae loses its tail, glycocalyx layer and contents of the penetration glands to become schistosomula [15].

Schistosomula undergo a number of changes before they mature into adults. The tegument of schistosomula is similar to that of the adult worms, more than cercaria both biochemically and structurally. Like the adults, the schistosomula tegument has a heptalaminate organization formed by two lipid bilayers [16]. A rudimentary gut starts to develop following skin penetration. In the lung stage schistosomula (6-8 days post penetration), an increase of the surface area of these animals occur although, the body mass remains largely unchanged [17,18]. This results in slight elongation of the parasite. Once schistosomula reach the liver, the true growth of these animals begins. The development of gut and reproductive system become pronounced and change into juvenile stage begins.

Nervous system of Schistosomes: Schistosome nervous system is a primitive form of organized nervous structure. However it has tremendous importance in the parasite's feeding, egg production and motility. Since, *S. mansoni* is an acoelomate and has no circulatory system or endocrine glands, it relies heavily on the nervous system for basic biological activities [19]. In this way, the nervous system can be thought of a multifaceted organization, which is capable of secreting modulatory substances in close proximity of the target cells in a synaptic or non-synaptic fashion.

The whole nervous system can be divided into two components: central nervous system (CNS) and peripheral nervous system (PNS). CNS is a bilateral assembly, which consists of a bilobed anterior pair of cerebral ganglia or the 'brain' and pairs of longitudinal nerve cords, which can be grouped into major or minor longitudinal nerve cords. Flatworms are the first instance of organisms with a brain-like structure [20]. The cords are connected to each other by commissures in a ladder-like manner. This kind of assembly is known as an 'orthogon' [19]. Projections from nerve cords branch out and create a meshwork of neurons, which innervate various tissues such as muscle, digestive and reproductive systems. Such specialized meshwork organization is known as a nerve plexus. A concentration of neurons is usually observed near the surface and in the muscular suckers used by the worm to attach to the host.

The nerve cords of *Schistosoma* can be categorized into two groups: main and minor nerve cords [21]. Main nerve cords have the following two characteristics: (i) they are well connected to the cerebral ganglia and (ii) they show presence of serotonin and acetylcholinesterase upon staining [22,23]. In *S. mansoni*, main nerve cords include a pair of ventral nerve cords and a pair of slender dorsal nerve cords. Minor nerve cords include a pair of lateral nerve cords, which run along the body on both sides.

The PNS encompasses fine neuronal projections protruding from nerve cords and its branches. It often creates intricate network of neuronal structure, which innervate various organs and tissues. Various specialized nerve nets have been described in *S. mansoni*. Subtegumental, submuscular, genital and intestinal nerve plexuses are of note[19].

Our knowledge regarding cellular structure of *S. mansoni* neurons comes from various forms of immunofluorescence studies performed not only on the organism itself but also from

similar studies done in other organisms. The size and shape of the nerve cells vary depending on their localization. Neurons of ganglia are predominantly unipolar, whereas bipolar and multipolar neurons are found in other regions of the CNS. PNS neurons are mostly of bipolar morphology. Size of neurons varies from 4.5x3 µm to a size of a magnitude larger [24]. It is important to note that, even in the same cord or peripheral site, neurons of different sizes exist. Serotonergic neurons are considerably thicker than cholinergic and peptidergic ones. NADPH-d (nicotinamide adenine dinucleotide phosphate diaphorase) positive neurons also occur in the main nerve cords, which tend to be large in size [19].

A noteworthy feature of flatworm nerve cells is the presence of a large number of vesicles associated with export of various transmitters. Substances are synthesized in the cell body and carried along by axonal transport for secretion. The common features of a non-ganglionic neuron is non-myelinated axon, a centrally placed euchromatic nucleus, numerous ribosomes, peripherally located mitochondria and neurotubules arranged in a parallel array like fashion [19]. Vesicles occur from Golgi bodies and are often seen to migrate and fuse with the plasma membrane. Various kinds of vesicles have been observed in flatworms. Usually a single type is found in one neuron. As a result, neurons can be described in terms of its vesicle profile [19]. The different types of vesicles encountered in flatworm neurons are:

1. Small, clear vesicles (20-40 nm in diameter), found in the proximity of synapse and are thought to be cholinergic or are associated with membrane and/or Ca^{2+} retrieval.
2. Dense cored vesicles (50-140 nm in diameter) of diverse types, regarded to be aminergic or peptidergic.
3. Large, dense vesicles (50-200 nm in diameter), usually viewed as peptidergic.
4. Large, lucent vesicles (60-300 nm in diameter), seen in sensory neurons and along the large, dense vesicles, contents unknown.

However, this vesicle type- content association has been found to vary significantly as observed in different immunoreactivity experiments [19]. Most likely, the variance is due to the species, life stage and the processing stage of the neuroactive substance of interest.

Flatworm neurotransmitters: Neurotransmitters are substances which are synthesized in neurons and exert an effect upon release in the synaptic cleft and binding to receptors usually located on the post-synaptic membrane. These substances are rapidly inactivated once a response

has been produced, either by enzymatic degradation or by carrier-mediated re-uptake into presynaptic terminals. Our knowledge regarding synthesis and functions of these transmitters in flatworms is still at its infancy. However, over the last four decades, a substantial amount of studies have shown the presence of an array of neurotransmitters in various flatworms. Moreover, recent advancements in sequencing flatworm genomes and transcriptomes have identified a number of candidate receptors for these molecules [25].

The vast array of neuroactive substances can be divided into two major groups: (i) classical neurotransmitters and (ii) neuropeptides. The classical neurotransmitters can be subcategorized into four distinct groups: (i) choline esters, represented by acetylcholine (ii) biogenic amines (iii) excitatory amino acids, mainly glutamate and (iv) inhibitory amino acids, predominantly gamma aminobutyric acid (GABA) and glycine. Additionally, two more types of neuroactive substances have been identified which are gases such as nitric oxide (NO) and purines, adenosine and adenosine triphosphate (ATP). The effects of various transmitters on flatworms are diverse depending on the species. For the same molecule, a different response is often observed in different species. These effects are summarized in Table 2 [26].

Acetylcholine (ACh): ACh is a major excitatory neuromuscular transmitter in vertebrates. However, it can be both excitatory and inhibitory among invertebrates, as shown in the nematode model organism, *Caenorhabditis elegans* [27]. Acetylcholine is produced in the cytoplasm of the axon terminal of a neuron by choline acetyltransferase (ChAT), which catalyzes the transfer of an acetyl group from acetyl-CoA to a choline molecule [28]. Synthesized acetylcholine molecules are stored into synaptic vesicles by the vesicular acetylcholine transporter (VAChT). The interior of the vesicle is enriched with protons by an ATP-dependent proton pump located on the vesicular membrane and the pH gradient provides the necessary energy required for the transport of an acetylcholine molecule into the vesicle in exchange for a proton [29]. When an action potential reaches the axon terminal, vesicle-stored ACh is released into the synaptic cleft and binds nicotinic acetylcholine receptors located on the muscle fiber adjacent to the axon terminal. Although nAChRs are mainly characterized in the context of vertebrate neuromuscular junctions, they are found in a variety of locations which include a wide range of epithelial surfaces and blood cells [30]. An important distinction between vertebrate and invertebrate nAChRs is that vertebrate nAChRs are invariably cation (Na^+, K^+, Ca^+) selective,

whereas invertebrate nAChRs may be either cation or anion (Cl^-) selective [31]. Examples of these receptors include anion-selective nAChRs of the mollusk *Lymnaea stagnalis* [32] and acetylcholine-gated chloride channel subunits (ACCs) of *C. elegans*. ACCs are however more closely related to GABA/Glycine channels rather than nAChRs [33]. It is noteworthy that, beside acetylcholine receptors, which form ion channels, G-protein coupled receptors (muscarinic receptors or GARs) exist in both vertebrates and invertebrates. Muscarinic receptors modulate the frequency of action potentials by either up or down regulating cellular potassium or down regulating calcium conductance in the cell [34].

Nicotinic acetylcholine-gated ion channels (nAChRs): The genome of *S. mansoni* reveals the presence of three major ligand-gated ion channel super families: Cys-loop family, glutamate gated cation channels and ATP-gated ion channels [25]. Cys-loop family subunits are so called due to the presence of a highly conserved disulfide linkage between two cysteine residues separated by 13 amino acids at the protein's N-terminal end [35]. nAChRs are characterized by their selectivity for acetylcholine as the natural ligand and high affinity for nicotine as a cholinergic agonist [36]. Presence of 22 nicotinic acetylcholine receptors has been predicted in the genome of *S. mansoni* [25]. However, only 11 of these predicted subunits contain the predicted number of transmembrane domains and the canonical cys-loop motif. Members of the nAChR family are divided into two principal groups: α- subunits, which contain an YxCC motif in the C-loop (complementary loop) of the acetylcholine binding region and non- α-subunits (β, δ, ε, and γ), which are classified based on their relative sequence similarities [36]. Both α and non- α subunits share similar physical characteristics in that all of them have an extracellular N-terminal domain containing the conserved Cys-loop and four transmembrane domains followed by an extracellular C- terminal short tail [37].

A typical pentameric organization is well conserved in all channels of the Cys-loop superfamily. This is achieved either by assembly of five α-subunits (homomeric channel) or two α and a combination of β, δ, ε, and γ subunits (heteromeric channel), which fill in the requirement for the other three subunits [37]. The acetylcholine binding site of a channel consists of a principal and a complementary binding site (Figure 2). The latter aids proper binding of an acetylcholine molecule to the principal binding site [38]. Both sites are necessary for successful ligand binding. Only α-subunits contain both a principal and a complementary acetylcholine

binding sites, whereas non- α-subunits only contain complementary binding sites. As a result, homomeric channels (example: α7 channel in humans) can bind 5 acetylcholine molecules, whereas heteromeric channels (example: α4β2 channel in humans) can bind only 2 of these molecules [39].

The 5 subunits of a channel form a central pore through which selective ions can pass upon ligand binding (Figure 3). The specificity of the ion is dictated by the second transmembrane region (TM2) of each subunit, which forms the inner wall of the pore [40]. In particular, the residues towards the boundaries of TM2 play important roles in conferring ion selectivity. Presence of a negatively charged amino acid such as aspartate or glutamate adjacent to the cytosolic TM2 boundary has been implicated in cation selectivity by the channel, whereas a neutral or positively charged residue has been shown to confer anion selectivity [38,41]. Also, absence of a proline within the three amino acids of the aforementioned ion-selective amino acid residue and presence of an amino acid with an aromatic side chain at a specific position within second transmembrane region has been implicated in cation selectivity [42,43].

Importance of characterizing schistosome nAChRs: Acetylcholine causes flaccid paralysis when added exogenously to *S. mansoni* schistosomula or adult worms in culture [44]. Studies dating back to the 1970s and 80s have shown that treatment of schistosomes with acetylcholine and carbachol (cholinomimetic drug that binds and activates acetylcholine receptors) causes relaxation of isolated muscle fibers [45-47], suggesting the flaccid paralysis is due to inhibitory effects on muscle or neuromuscular structures. Bentley et al. showed that a homologue of one of the channel subunits under investigation in this study (smp_031680 (shAR1α)) localizes to the surface (tegument) of the related schistosome species, *Schistosoma haematobium* [48]. Another schistosome nAChR subunit (orthologue of smp_139330 (shAR1β)) was identified in the body wall muscles and discrete cell bodies of the connecting parenchyma of *S. haematobium* [48]. Previous studies demonstrated that upon addition of cholinergic agonists to the media, adult *Schistosoma* increased glucose uptake [49]. These observations indicate that nAChRs have varied and important roles in schistosomes since they may be associated with such basic biological functions as locomotion and nutrient uptake. As a result, nAChRs are potentially important targets for chemotherapy against schistosomiasis. The great evolutionary distance

between the parasite channels and those of the human host could be exploited to screen for drugs that specifically target *Schistosoma* nAChRs.

Objectives of the project: The central hypothesis of this study is that cation-specific nAChRs are present in *S. mansoni*, they play a role in essential biological functions in the parasite, and therefore are exploitable targets for drug discovery. To test this hypothesis, we propose to undertake an investigation of four putative cation-specific nAChR subunits of *S. mansoni*: smp_031680, smp_180570, smp_139330 and smp_012000. The specific experimental objectives are as follows:

1) Cloning full -length cDNA sequences of all four putative subunits of interest.

2) Investigation of the effect of abrogating putative nAChRs by RNAi in cultured S. *mansoni* and examining their effects by measuring parasite motility.

3) Determination of smp_031680 and smp_139330 expression profiles using confocal immunofluorescence microscopy with specific antibodies in adult and schistosomulae life stages of the parasite.

4) Functional expression studies of smp_031680, smp_180570, smp_139330 and smp_012000 in *Xenopus* oocytes followed by electrophysiological experiments (two-electrode voltage clamp) to test for channel activity.

Tables and figures:

	Species	Geographical distribution
Intestinal schistosomiasis	*Schistosoma mansoni*	Africa, the Middle East, the Caribbean, Brazil, Venezuela, Suriname
	Schistosoma japonicum	China, Indonesia, the Philippines
	Schistosoma mekongi	Several districts of Cambodia and the Lao People's Democratic Republic
	Schistosoma guineensis and related *S. intercalatum*	Rain forest areas of central Africa
Urogenital schistosomiasis	*Schistosoma haematobium*	Africa, the Middle East

Table 1: Two major types of schistosomiasis (intestinal & urogenital), their respective causative agents and geographical distribution [50].

Transmitter	Activity	Species
Serotonin (5HT)	↑muscle contraction (↑motility)	Sm, Fh, Hd, Dm, Pl
	↑carbohydrate metabolism	Fh, Hd, Sm
	↑tissue regeneration	Dg
Catecholamines	↓muscle contraction	Sm
	↑muscle contraction	Dm
	hyperkinesia	Dg
Histamine (HA)	↑motility	Sm, Hd
Acetylcholine (ACh)	↓muscle contraction (flaccid paralysis)	Sm, Fh, Hd
	↑muscle contraction	Bc, Pl
	hypokinesia (tonic contraction)	Dg
	↑glucose transport	Sh, Sm
Glutamate	↑muscle contraction	Hd, Sm
	↑spike activity (CNS)	Gf
GABA	↓spike activity (CNS)	Na
Neuropeptides	↑muscle contraction	Sm, Fh, Bd, Pl

Bc, *Bdelloura candida*; Dm, *Diclidophora merlangi*; Dg, *Dugesia* spp; Fh, *Fasciola hepatica*; Gf, *Gyrocotyle fimbriata*; Hd, *Hymenolepis diminuta*; Na, *Notoplana acticola*; Pl, *Procerodes littoralis*; Sh, *Schistosoma haematobium*; Sm, *Schistosoma mansoni*.

Table 2: Effects of major known neurotransmitters in flatworms [26]. Species specific effects of these agents are particular visible.

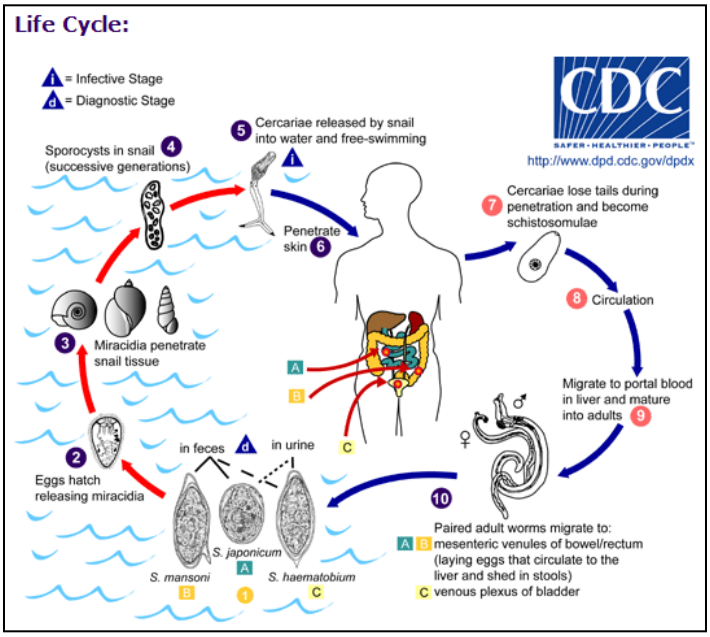

Figure 1: Life cycle of the blood fluke *Schistosoma* [51].

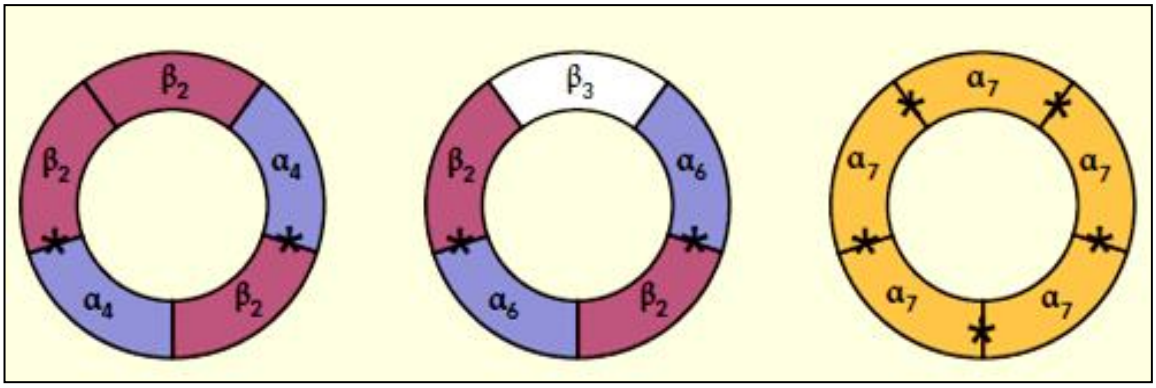

Figure 2: acetylcholine binding site (marked by *) in various mammalian heteromeric ($\alpha_4\beta_2$, $\alpha_6\beta_2\beta_3$) and homomeric (α_7) nicotinic acetylcholine gated ion channels [52].

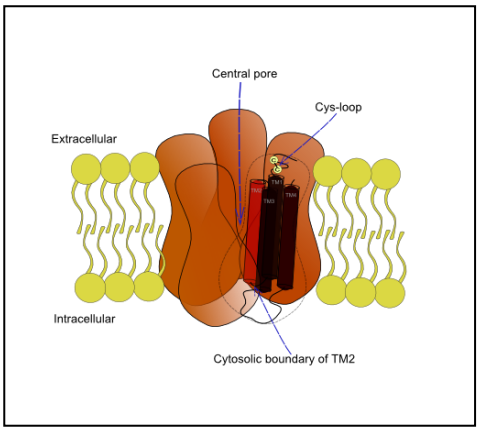

Figure 3: A pentameric organization of nAChRs to form a functional ion channel on the cytoplasm. Each subunit of the channel is constituted of (from N to C terminal) an extracellular ligand binding domain containing a cys loop, four transmembrane (TM) regions and a short extracellular tail. TM2 is located towards the central part of the channel and forms the pore through which ions pass upon channel opening and thus confers ion selectivity. A large intracellular loop (usually highly variable) is also present between the TM3 and TM4 regions.

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Chapter 2

Manuscript I

Putative Cation-Selective Nicotinic Acetylcholine Receptors of the Human Parasite *Schistosoma mansoni*

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Abstract:

Acetylcholine is an important neuroactive substance in all the parasitic helminths, including *Schistosoma mansoni*. Neuromuscular signalling and motor control are particularly dependent on cholinergic signalling. Nicotinic acetylcholine receptors (nAChRs), which are pentameric acetylcholine/ nicotine gated ion channels are an important part of this cholinergic system. nAChRs are molecular targets for a variety of anthelmintic drugs and they are also regarded as promising drug targets in *S. mansoni*. Here, we describe the cloning and first characterization of four putative nAChR subunits of S. mansoni. These are: smp_031680, smp_180570, smp_139330, and smp_012000. The four proteins were shown to be full-length and contain all the characteristic features of cation-selective nAChRs. RNA interference (RNAi) studies in cultured *S. mansoni* larvae (schistosomulae) and adults showed that nAChRs can affect parasite motility, either negatively (smp_031680 and smp_180570) or positively (smp_139330 and smp_012000), depending on the subunits targeted. Moreover, confocal immunofluorescence studies, using specific polyclonal antibodies against smp_031680 and smp_139330 showed that nAChRs are expressed extensively in neuromuscular structures, including both central and peripheral nervous systems of the parasite and, in the case of smp_139330, the musculature itself. Importantly, we observed distinct expression patterns of smp_031680 and smp_139330, which suggests these subunits are not part of the same channel. The strong motor RNAi phenotypes, combined with the wide distribution of these receptors identify nAChRs as important proteins of the parasite's motor control system.

Introduction:

Schistosomiasis is a debilitating chronic parasitic infection, second only to malaria in terms of socioeconomic impact. Over 700 million people of 74 different countries are at risk of obtaining the disease worldwide [1,2]. The infected population exceeds 200 million [2]. The majority of infected people are located in developing countries of the world, where their daily activities in agriculture, domestic chores and recreation expose them to infested water. There are two major types of schistosomiasis, intestinal, caused primarily by *Schistosoma mansoni* and *Schistosoma japonicum* and urogenital, brought on by infection with *Schistosoma haematobium*. Pathology occurs due to deposition of eggs in various tissues, particularly the liver and spleen. The mainstay of drug therapy for schistosomiasis is praziquantel. Praziquantel is effective against the adult stage of the parasite [3] and is much safer and cheaper than the alternative schistosomicides, metrifonate or oxamniquine [4]. However, there are reports of reduced efficacy in endemic areas of Egypt [5] and Senegal[6] and drug resistance can develop after only two generations of repeated exposure to sub-lethal doses of Praziquantel in mice [7]. With increasing usage of praziquantel due to mass treatment programs in many parts of the world, there is a real concern that resistance to the principal schistosomicidal agent may occur. Thus, there is an urgent need to search for alternative drug therapies and to find new drug targets.

The nervous systems of helminth parasites hold great potential for drug targeting, as evidenced by the success of nematocidal drugs such as ivermectin or levamisole, which target neuronal proteins. Schistosomes have a well-organized nervous system, comprising of a central nervous system (CNS) and a peripheral nervous system (PNS). The CNS includes bilobed cerebral ganglia in place of a brain and two pairs of longitudinal ventral and dorsal nerve cords. Each pair is interconnected at regular intervals by transverse commissures. Neural branches develop from the CNS to form the PNS, which contains smaller nerve cords and nerve plexuses, reaching virtually every region of the body. This includes the somatic musculature, tegument, oral and ventral suckers, digestive tract and the reproductive system [8-10]. Due to the absence of a coelomic cavity and thus endocrine signalling through circulating body fluids, much of the regulatory activity in the parasite is performed by the nervous system. Critical functions such as movement, host attachment, penetration and migration, feeding, reproduction and excretion are all controlled through the parasite's nervous system [8].

An examination of currently available anthelmintics reveals that many of these drugs work by disrupting the activity of nicotinic acetylcholine receptors (nAChRs). Common nematocidal drugs such as pyrantel, levamisole, morantel, paraherquamide and monepantel all have nAChRs as their molecular targets [11-15]. nAChRs form homo or hetero pentameric ion channels, which open upon binding of the neurotransmitter ligand, acetylcholine (ACh), or competitive agonist nicotine[16]. nAChRs mediate many of the effects of ACh in vertebrates and invertebrates, notably neuromuscular effects associated with motor control, as well as effects on behavior, egg laying, feeding, grooming and modulation of cerebellar activity in various organisms [17-20]. Our knowledge of nAChRs is derived mainly from the classical vertebrate model, where the ion channels are located on post-synaptic membranes, neuronal as well as neuromuscular. Activation of these ion channels by endogenous acetylcholine causes influx of cations and resulting depolarization of the downstream muscle fiber (and thus muscle excitation) or neuron (excitatory postsynaptic potential) [21].

Structurally, nAChR subunits belong to the superfamily of Cys-loop ligand-gated ion channels [16] and are divided into two principal groups: α- subunits, which contain an YxCC motif in the N-terminal region and non- α-subunits , which are further classified (β, δ, ε, and γ) based on their relative sequence similarities [16]. α and non- α subunits share similar physical characteristics in that they all have an extracellular N-terminal domain containing the canonical Cys-loop motif, followed by four transmembrane (TM) domains and an extracellular C-terminal domain, which is often much shorter than its N-terminal counterpart [21]. The typical pentameric organization is well conserved in all channels of the Cys-loop superfamily. In the case of nAChRs, this is achieved either by an assembly of five α-subunits (homomeric channel) or two α and three non-α type subunits (heteromeric channel) [21]. The 5 subunits of a channel form a central pore through which selective ions can pass upon ligand binding (Fig. 1A). Ion specificity is dictated by the second transmembrane region (TM2) of each subunit, which forms the inner wall of the pore [22]. In particular, the residues towards the boundaries of TM2 play important roles in conferring ion selectivity [23-26]. For example, presence of a negatively charged amino acid such as aspartate or glutamate adjacent to the cytosolic TM2 boundary has been implicated in cation selectivity, whereas a neutral or positively charged residue has been shown to confer anion selectivity [23,24].

It has long been known that acetylcholine, along with serotonin, dopamine and neuropeptides are major neurotransmitters that control muscle function and movement of the parasite [25-29]. In other species, acetylcholine is the quintessential excitatory neuromuscular transmitter that stimulates contraction of the major muscles of the body. This is caused by activation of cation-selective nAChRs at the neuromuscular junction, which leads to depolarization of the muscle fiber and ensuing muscle contraction [21] However schistosomes have a very different response to acetylcholine. Studies dating back to the 1970s and 80s have shown that treatment of schistosomes with acetylcholine and carbachol (cholinomimetic drug that binds and activates acetylcholine receptors) cause muscle relaxation and flaccid paralysis [30-32], suggesting that acetylcholine has inhibitory effects in these parasites.

Two types of acetylcholine receptors are found in the genome of *S. mansoni*, G protein-coupled acetylcholine receptors (GARs, also known as muscarinic receptors) and nAChRs [33]. Of the 22 putative nAChR candidates encoded in the genome, 11 carry the canonical features of nAChR channel subunits, including the Cys loop motif and the characteristic four transmembrane topology. Some of these sequences are predicted acetylcholine – gated chloride channels (Ribeiro et al, 2005), others are distant orthologues of vertebrate cation-selective nAChRs. How these receptors contribute to the inhibitory effects of acetylcholine in the worm remains unclear. Three putative nAChR subunits were previously cloned from a related schistosome species, *S. haematobium*, but they have not been characterized in vitro and their functions are unknown [34,35].

In this study we report the first characterization of 4 predicted subunits of cation-selective ion channels in *S. mansoni*. We used RNAi to investigate the role of these subunits in the control of schistosome motility, both in larvae and adult worms. Additionally, we examined the expression patterns of two of the subunits (smp_031680 and smp_139330) by confocal immunofluorescence microscopy.

Experimental procedures:

Parasites: The NMRI strain (Puerto Rican) of *S. mansoni* was used throughout the study. Parasites were collected from infected snails *Biomphalaria glabrata*, kindly donated by the

Biomedical Research Institute, Rockville, Maryland, USA. Snails were induced to shed cercaria upon light exposure and were mechanically transformed into schistosomula [36,37]. 28 days old CD1 mice were infected via the tail [38,39] and adult S. mansoni worms were obtained by portal perfusion [40] after approximately 7 weeks.

Cloning: cDNA sequences of smp_031680, smp_139330, smp_180570 and smp_012000 were identified in the *S. mansoni* database (http://www.genedb.org/Homepage/Smansoni). Among these, smp_031680 and smp_139330 were found to be of full-length and carried a signal peptide. These two sequences were cloned directly from adult worm cDNA using forward and reverse primers smp_031680F, smp_031680R & smp_139330F, smp_139330R (see supplemental Table S1 for primer sequences). To obtain the cDNA, total RNA was extracted from adult worms using Trizol (Life Technologies) and oligodT reverse-transcribed with M-MLV reverse transcriptase (Life technologies) according to standard procedures. The other two nAChR sequences of interest, smp_180570 and smp_012000, were found to be incomplete based on sequence analysis. The annotation for Smp_01200 lacked a signal peptide at the 5' end and Smp_180570 lacked both a signal peptide and the fourth TM region. The missing ends were obtained by RACE (Rapid Amplification of cDNA Ends) procedures, using commercially available kits (Life technologies). For the 3' end, total adult RNA was reverse-transcribed using the oligo-dT anchor primer supplied with the kit. The resulting cDNA was used to amplify the target region using a gene-specific sense primer (smp_180570GSP1, Table S1) and the anti-sense anchor primer provided with the kit. To ensure specificity of the amplicon, further nested PCR was done using another downstream gene specific primer (smp_180570GSP2, Table S1) and the same anchor primer. To amplify the 5' ends, gene-specific primers (smp_180570GSP3, smp_012000GSP1, Table S1) were used to reverse-transcribe total adult RNA. An anchor was added to the 5' end of the resulting cDNA according to the kit protocol and PCR was subsequently performed with anchor forward primer and gene-specific reverse primers (smp_180570GSP4, smp_012000GSP2, Table S1). Amplified products were purified, sequenced and were aligned with the genomic sequence to verify their position in the gene. Full-length coding sequences were then amplified by RT-PCR using forward and reverse primers that targeted the beginning and end of the sequence. Each of the four full-length cDNAs was cloned into vector pJET (CloneJET PCR Cloning Kit, Thermo Scientific) and confirmed by DNA sequencing of three different clones.

Phylogenetic analysis: A phylogenetic tree was constructed using predicted nAChRs along with a range of various different cys-loop ligand gated ion channel (LGIC) subunits. Included in this alignment were the four nAChR subunits of interest, smp_031680, smp_139330, smp_180570 and smp_012000, known cation-selective nAChRs from other species and various other LGIC subunits, including serotonin, GABA, glycine or glutamate gated cys-loop LGIC subunits. The protein sequences were aligned using the multiple sequence alignment tool ClustalW2 [41,42], which was then edited manually using JalView [43]. A bootstrap data set (1000 replicates) was generated by the Neighbour Joining method [44] using the PHYLIP package [45] and the tree was rendered and rooted using human Ca^{2+} activated K^{+} channel subunit as the outgroup with the aid of TreeDyn [46]. Accession numbers for the protein sequences used in the alignment are listed in supplemental Table S2.

RNA interference (RNAi) in schistosomula: We used the protocol described by Nabhan et al. (2007) [47] to transfect schistosomula with small interfering RNA (siRNA). Unique regions of approximately 200 bp were identified for each target gene by BLASTn analyses. These regions corresponded to (nucleotide positions): 695-890 bp of smp_031680, 690-895 bp of smp_139330, 1022-1226 bp of smp_180570 and 1227-1465 bp of smp_012000. These regions were amplified by PCR, using primers that introduced flanking T7 promoters at both ends of the PCR product and verified by DNA sequencing. The resulting products were purified and used to generate double-stranded RNA by *in vitro* transcription of both cDNA strands, using MEGAscript® T7 Kit according to manufacturer's protocol. The dsRNAs were subsequently digested with *E. coli* RNase III (Life technologies) to produce siRNA pools, which were purified using Microcon®Ultracel YM-30 filter units (Millipore). Transfections were performed as described [42] in a 24-well plate with approximately 300 freshly-transformed schistosomulae in 300µl Opti-MEM (Life technologies) per well. The transfection mix was prepared with siPORT™ *NeoFX*™ Transfection Agent and siRNA in Opti-MEM, as per the manufacturer's recommendations and a 50 µl aliquot was added to each well so that the final concentration of siRNA was 50 nM. As negative controls, we used (i) an equal amount of non-specific scrambled siRNA (Silencer FAM Labeled Negative Control siRNA#1, Life technologies), (ii) transfection reagent alone (mock-transfected) and (iii) no transfection reagent (untransfected). Samples were supplemented with 10% heat inactivated fetal calf serum and antibiotics (0.25µg/ml fungizone, 100 µ/ml streptomycin and 100 units/ml penicillin, Life technologies) approximately 10 hr post-

transfection and the animals were cultured for up to 7 days in complete medium at 37^{o}C and 5% CO_2.

Measurement of motor activity in schistosomula: We used the method described by El-Shehabi et al. (2012) [48] to quantify motility of the siRNA-treated schistosomulae. Motility was recorded at 7 days post-transfection. Videos were obtained for periods of 1 min at a rate of approximately 2.3 frames/ second with a compound microscope (Nikon SMZ1500), equipped with a digital camera (QICAM Fast 1394, mono 12 bit, Qimaging) and the software SimplePCI version 5.2 (Compix Inc.). Data analysis was done using ImageJ software v1.46r (NIH, USA). As described previously [43], we used the 'Fit Ellipse' function of the software to draw an elipse of best fit around each schistosomulum at every frame of the recorded clip. This way, the length of the animal was represented by the major axis of the fitted ellipse. We calculated the change of major axis from frame to frame. A 3% (or greater) frame to frame change was considered to be a body movement. 30 animals per treatment were examined and each experiment was done three times (total of 90 animals per treatment). At least one of the experiments was performed blind. Statistical comparisons were done with Student's T-tests (2-tailed, equal variance) at $P < 0.05$.

RNAi in adult worms: Freshly collected adult worms were electroporated with 5 µg of pooled siRNAs, prepared as described above and administered as outlined by Krautz-Peterson et al.[49]. As our negative control, we used a pool of siRNAs against the common reporter gene, mCherry (147-350 bps). Unpaired worms (5 males and 5 females) were electroporated per treatment in a 4mm cuvette in 100 µl RPMI media using a single square wave 20 ms impulse at 125 V at room temperature. Post-electroporation, the worms were transferred to a 12-well plate and cultured in 1 ml of complete RPMI media (RPMI medium supplemented with 10 mM Hepes, 2 mM glutamate, 5% FBS and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) at 37C, 5% CO_2. Video recordings were obtained at 24 or 40 hours post electroporation. A short 2 min video clip of each treatment was recorded at a 2.3 frames/ second rate using a stereo microscope (Nikon SMZ1500), equipped with a digital camera (QICAM Fast 1394, mono 12 bit, Qimaging) and the software SimplePCI version 5.2 (Compix Inc.). All data collections and analyses were done by ImageJ and Microsoft Excel 2007. Single knockdown experiments were carried out three times, whereas double knockdown experiments were performed twice. Each experiment contained 5 males and 5 females per treatment.

In order to measure motility of adult worms, we used a simple imaging method based on pixel displacement, as described previously by Patocka et al (2013)[50]. To do so, each video clip was first corrected for variable illumination intensity by the light source using the 'stack deflicker' function of the 'wrMTrck' plugin of ImageJ. The images were then converted into binary format (white or black pixels) by a thresholding algorithm available in Image J. We selected individual binary objects representing single worms and first calculated the average number of pixels/ frame by performing a 'Z-axis Profile' of the selected area. Then, we used the 'Image Subtraction' process of ImageJ to subtract frame n from frame (n+1) for the entire stack of frames to obtain a frame by frame displacement of pixels for each worm. Worm movement was measured as the average pixel displacement per frame over the course of the 2 min video. Average pixel displacement was normalized relative to the total number of pixels in the binary object to account for variations in worm size. A schematic representation of the method is provided in supplemental Fig. S1 and further details can be found in Patocka et al. (2013) [50].

Real-Time Quantitative PCR (qPCR): Total RNA was purified, using the RNeasy Micro kit (QIagen) and reverse-transcribed with oligo$(dT)_{12-18}$ primers and M-MLV reverse transcriptase (Life technologies) according to standard procedures. qPCRs were performed using Platinum® SYBR® Green qPCR SuperMix-UDG kit (Life technologies) in a RotorGene RG-3000 (Corbett Research) thermal cycler. Primers for qPCRs were designed to amplify regions spanning two exons and avoiding RNAi target regions. A list of primer sequences is included (Fig. S1). As a housekeeping gene, a 206 bp fragment of *S. mansoni* glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Accession# M92359) was used for normalization as described by Taman and Ribeiro (2011) [51]. Non template and non reverse-transcribed controls were included with every experiment and specificity of reaction was examined by melting curve analysis. Relative expression quantifications were calculated using the method described by Pfaffl (2001)[52].

Antibody production and western blot analyses: Polyclonal antibodies against smp_031680 and smp_139330 were purchased from 21st Century Biochemicals (Marlboro, MA). Antibody against smp_031680 was produced in rabbits against two synthetic peptides (YSRNGEFHLSGSSVRRYAQRYEC & PYRLYNSVGNFNSKHIQDL) corresponding to positions 205-227 and 566-584 of the protein sequence. The smp_139330 antibody was raised in goats against two synthetic peptides (IRQINLPPERIWKPDI & IKAFTSRGAPSQPM)

(positions 103-118 and 719-132 of the full length protein, respectively). Each peptide was conjugated to ovalbumin as a carrier protein. Peptides were found to be highly specific upon BLASTp analysis against the *S. mansoni* genome [33]. Antisera were tested first by ELISA against the peptide antigens and were found to be of high titer. The sera were affinity-purified, using the peptide antigens with the aid of Sulfolink Immobilization kit for peptides (Thermo Scientific). Subsequent western blot analyses with purified antibodies were performed on a preparation of solubilized membrane proteins collected from adult worms.

In order to perform western blots, solubilized membrane proteins were obtained from adult worms using the Proteoextract® Native Membrane Protein Extraction kit, according to the manufacturer's protocol. Western blot analyses were performed according to standard procedures using 30 µg of adult worm membrane protein per lane. Protein assays were performed according to the Bradford method, using the Bio-Rad Protein Assay kit to determine protein concentrations. The samples were resolved in a 4-12% tris-glycine SDS-PAGE gel (Life technologies), transferred onto PVDF membrane and probed with peptide-purified primary antibody (1: 333) followed by incubation with secondary antibody (1:20,000). For antibody against smp_031680, which was generated in rabbits, a horseradish peroxidase (HRP) conjugated goat anti-rabbit polyclonal antibody was used. For smp_139330 generated in goat, a HRP conjugated rabbit anti-goat antibody was used. For preadsorbed antibody controls, a 10-fold molar excess of pooled peptide antigens was added to primary antibody in blocking buffer, incubated overnight with end-over-end rotation at 4^{o}C and centrifuged at 4^{o}C for 15 minutes at 16,000g. The resulting supernatant was used instead of primary antibody. In western blots, single immunoreactive bands of ≈90 kDa and ≈95 kDa sizes were observed for smp_031680 and smp_139330 respectively using these affinity-purified antibodies (supplemental. Figure S2). Upon preadsorption, no band was observed, which suggests that the antibodies are highly specific.

Immunolocalization: Confocal immunolocalization was performed according to the method of Mair et al. (2000) [53] with some modifications. Adult worms were flat-fixed between two glass slides with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS 0.1M, pH 7.4) for 4 hours and washed three times with PBS at 4^{o}C. An additional wash with PBS supplemented with 0.1 M glycine was performed to reduce non-specific background due to unquenched fixation.

Samples were then permeabilized with 1% SDS for 20 minutes at room temperature (RT) with end-over-end rotation and washed five times with antibody diluent (AbD: 0.1 M PBS, pH 7.4, containing 0.1% Triton X-100, 1% bovine serum albumin and 0.1% sodium azide) at RT. Overnight blocking at 4^{o}C in AbD supplemented was performed before washing followed by addition of primary antibody in AbD (dilutions smp_031680 1:100, smp_139330 1:75 and human choline acetyl transferase 1:50) and incubated for two days with end-over-end rotation at 4^{o}C. Subsequently, the worms were washed and incubated overnight in AbD containing secondary antibody (dilution 1: 1000) and washed again seven times at RT with AbD before examination by scanning confocal microscopy (Nikon LSM 710 with ZEN 2011 software package). Schistosomula samples were prepared as above except they were fixed overnight with 4% PFA with-end-over end rotation at 4^{o}C. Negative controls used in this study include (1) omission of primary antibody and (2) preadsorption of primary antibody with 10 fold molar excess of each peptide antigen.

Results:

Cloning of subunits and sequence analysis: Orthologues of smp_031680 and smp_139330 were previously cloned from *S. haematobium and* named shAR1alpha (GenBank: AAR84357.1) and shAR1beta (GenBank: AAR84358.1) respectively [34]. A comparison between *S. mansoni* putative sequences found on the database (http://www.genedb.org/Homepage/Smansoni) with those of *S. haematobium* revealed a strong resemblance between these sequences. Smp_031680 is 80.97% identical to shAR1alpha whereas, smp_139330 is 82.32% identical to shAR1beta. Both smp_031680 and smp_139330 contained four transmembrane domains (analysis with TMHMM server v. 2.0 [54,55]) and N-terminal signal peptides (analysis with SignalP 4.1 [56]). Our cloned smp_031680 sequence was identical to that of the prediction in GeneDB. However, we detected a 39 bp insertion after nucleotide 1316 for smp_139330.

Smp_180570 and smp_012000 were not previously cloned from any schistosome species. TMHMM and SignalP analyses of the putative sequences available in GeneDB database revealed that smp_180570 lacked the signal peptide at the 5`end and the fourth TM region towards the 3'

end. Smp_012000 was found to be missing the signal peptide only. We performed 5'and 3'RACE to identify the missing sequences and the full-length cDNAs were subsequently amplified by RT-PCR. The sizes of smp_031680, smp_139330, smp_180570 and smp_012000 were found to be 2061, 2199, 2028 and 2673 bp (encoding 686, 732, 675 and 890 AAs), respectively. The cDNAs were deposited in GenBank with the following accession numbers : KF738247 (smp_031680), KF738248 (smp_139330), KF738249 (smp_180570) and KF738250 (smp_012000).

Analysis of conserved protein motifs using InterProScan [57,58] reveals that the four proteins are related to nAChRs with E values of 1.7E-15, 1.6E-14, 1.9E-7 and 1.5E-42 for smp_031680, smp_139330, smp_180570 and smp_012000, respectively. Phylogenetic analyses show that the four schistosome subunits clade together with cation-specific nAChRs from other species (Fig. 1 C). Smp_031680 and smp_139330 and their *S. haematobium* orthologues are most closely related to *C. elegans* ACR-16 and human alpha-7, whereas the other two subunits (smp_180570 and smp_012000) are more divergent and form a separate sub-clade of their own.

The four schistosome subunits all contain a N-terminal signal peptide and common features of Cys-loop ligand-gated ion-channels, including a conserved N-terminal ligand binding domain containing two cysteine residues 13 amino acids apart (cys-loop), followed by four transmembrane domains and a short extracellular tail. Smp_031680 was found to be of alpha type nAChR, which contains an YXCC motif (YECC: 221-224AA) important for ligand binding [59]. The other three, however, lack this motif and thus have been identified as non-alpha.

A structural alignment with known cation-selective nAChRs using PROMALS3D [60] identified three major structural features of cation-selective Cys-loop channels [61] [62] (Fig. 1 B). These include the presence of a negatively charged amino acid (glutamate or aspartate) residue at the cytosolic side of the TM2 region (Fig. 1 B, red box), absence of a neighbouring proline within three upstream residues of this negatively charged amino acid (Fig. 1 B, dotted green box) and a hydrophobic amino acid at the hydrophobic ring within the TM2 region (Fig. 1 B, blue box), which is a methionine in all four subunits of our interest.

Confocal analysis of the cholinergic system using anti ChAT antibody: In order to visualize the cholinergic system of the parasite we performed confocal immunofluorescence microscopy

with an antibody against choline acetyl transferase (ChAT), the enzyme responsible for acetylcholine biosynthesis and a common marker of cholinergic neurons. We used a commercially available polyclonal antibody against human ChAT, which shares high sequence homology with the parasite predicted orthologue. ChAT staining in adult worms (Fig. 2) clearly shows the enzyme's expression in the nervous system of the parasite, both the CNS, including the cerebral ganglia, ventral and dorsal nerve cords (Fig. 2A) and PNS comprising lateral nerve cords and peripheral innervation of the musculature (Fig. 2 B, C). In 7 day-old schistosomulae, expression of ChAT was observed mainly in nerve fibers of the PNS just beneath the outer layer of the larval body. ChAT was not detected in the CNS of the larvae.

Immunolocalization of smp_139330: Having mapped the cholinergic nervous system of *S. mansoni*, we proceeded to investigate the expression patterns of two of the nAChR subunits in these two developmental stages. We obtained polyclonal antibodies against smp_031680 and smp_139330 and performed similar confocal immunofluorescence microscopy.Smp_031680 was found to be expressed abundantly in both the CNS and PNS (Fig 3), near regions that also express the ChAT cholinergic marker. In the head region of the adult worm, expression was observed in the cerebral ganglia and ventral nerve cords and anterior longitudinal nerve cords and its branches (Fig. 3 A). Expression in ventral and dorsal nerve cords is present throughout the rest of the body (Fig. 3 B, C and E). Transverse commissures, which join these two sets of cords, are also rich in the protein (figure 3 B). Expression in swollen bodies known as varicosities is of particular interest (Fig. 3 B). Varicosities are sites of neurotransmitter release [63] and the presence of nAChRs at these sites suggests a potential role in the modulation of neuronal signalling. Numerous branches from both dorsal and ventral nerve cords towards the surface of the parasite can be seen throughout the body (figure 3 C). Most of these branches divide further and form a nerve plexus that supplies the muscle layers of the parasite. An interesting expression trait observed for smp_031680 is that the protein is present not only in nerve fibers but also in neuronal cell bodies (Fig. 3 A to C and E). This trait is especially visible in the tail region of the parasite (Fig. 3 E). Similar expression patterns were observed in the nervous systems of male and female worms. However in females, the protein was also found to be expressed in reproductive structures (Fig. 3 D). We detected significant Smp_031680 expression in the ootype, where egg maturation and fertilization occurs [64].

In 7 days old schistosomulae (figure 3 G), expression of smp_031680 was observed in fine varicose nerve fibers in the outer layer of the body, a region that also contain cholinergic neurons (Fig. 2). In addition, we observed strong expression in the ventral sucker and throughout the parenchyma of the larvae (Fig. 3) The immunoreactive parenchymal cells could be undifferentiated neuronal, muscle (or other) cells of the developing parasite where the receptor is expressed.

Immunolocalization of smp_139330: The other subunit tested in this study, Smp_139330, also localized to the nervous system of the parasite. As in smp_031680, expression of Smp_139330 was observed in the cerebral ganglia (faint expression), the dorsal nerve cords and their transverse comissures of the CNS (Fig. 4 A). However, the level of expression was less pronounced compared to that of smp_031680. In the head region, Smp_139330 was present in the anterior longitudinal nerve cords, lateral nerve cords and the extensive nerve net around the oral sucker (fig. 4 A, B). In addition we observed diffuse expression associated with the submuscular nerve net throughout the body of the worm (Fig. 4 B, E) and possibly the musculature itself (Fig. 4 D). In males, a number of Smp_139330 –expressing nerve fibers were seen extending all the way to the tegument, ending in swollen bodies within the tubercles of the worm (fig. 4 C). Neuronal expression of this protein was associated mainly with axonal processes and nerve fibers, not cell bodies, unlike smp_031680 which was present in both. Expression of smp_139330 in the CNS seemed to be highest in the head region of the parasite but diminished as we examined further down of the body. In contrast there was significant expression in the submuscular nerve net and possibly, the muscle itself throughout the length body with same intensity (fig. 4 B and E). Besides the nervous system, Smp_139330 is expressed in the female reproductive system of the parasite. The Mehlis' gland responsible for aiding in egg shell production [65,66] was found to be rich in this protein (Fig. 4 F). In 7 days-old schistosomulae, smp_139330 expression was observed in the peripheral nerve fibers of the larvae's body wall body. Expression in parenchymal cells was not observed as in smp_031680.

Dual labelling experiments: To compare the spatial distribution of the two subunits, we performed dual labelling experiments with antibodies against smp_031680 and smp_139330, followed by secondary antibodies conjugated to alexa fluor 488 (green) and alexa fluor 594 (red). The results show that the expression patterns of the two proteins are similar but they do not

overlap, as demonstrated by the lack of yellow fluorescence in these dual labelling experiments (Fig. 5). We detected both smp_031680 and smp_139330 in the CNS and PNS of the parasite, as described above. In the head region (fig. 5 A) of the adult worm, smp_031680 is strongly expressed in the cerebral ganglia and the ventral nerve cords, whereas smp_139330 is expressed faintly in the cerebral ganglia, the dorsal and lateral nerve cords. As we moved further down the body (fig. 5 B and C), we saw expression of smp_031680 in the ventral and dorsal nerve cords and smp_139330 in the submuscular nerve net and the musculature of the parasite. This separation of signals was maintained throughout the rest of the body. Moreover, in the female reproductive tract, non-overlapping expression patterns of the two proteins continued (fig. 5 D). Smp_031680's expression in the ootype is clearly distinct from smp_139330's expression in the Mehlis' gland. Dual labelling of smp_031680 and smp_139330 in 7 day-old schistosomulae confirmed that these subunits have different expression patterns (fig. 5 F). Although, the proteins are both expressed in the peripheral innervation of the body wall, their labeling patterns do not overlap. Moreover, parenchymal expression was observed for only one of the subunits (smp_031680).

RNAi in schistosomula: Given that acetylcholine has strong effects on worm movement, we hypothesized that the four nAChR subunits might play a role in motor control. With the aid of RNAi and established protocols [47], we tested whether silencing expression of nAChRs subunits had any effect on parasite motility in culture. Four subunits were tested in schistosomulae and all four produced an RNAi motor phenotype compared to the controls transfected with irrelevant (scrambled) siRNA (Fig. 6). The data were from 90 animals per RNAi treatment and from three separate experiments (30 animals per treatment per experiment), each statistically significant at $P < 0.05$. Two types of phenotypes were observed. Animals, transfected with Smp_031680 or smp_180570 siRNAs were significantly less motile than the controls, both treatments causing about a 50% decrease in movement (fig. 6 B). On the other hand, transfection with smp_139330 or smp_012000 siRNAs caused significant hyperactivity ranging from 45-65% greater motility compared to the controls (fig. 6 B). In subsequent double-knockdown experiments, we treated schistosomula with a combination of siRNAs against smp_031680 and smp_180570 or siRNAs against smp_139330 and smp_012000. Our goal was to see whether there was any further decrease (smp_031680+smp_180570) or increase (smp_139330+smp_012000) of motility in the double-knockdowns compared to the individual

siRNA treatments. The smp_031680 and smp_180570 combination did not result in a further decrease in motility (fig. 6 C). The double-knockdown decreased movement by about 50%, the same as the individual subunit treatments. On the other hand, the smp_139330 and smp_012000 combination showed a markedly different phenotype from that seen in the individual treatments. We observed a significant decrease (≈ 50%) in motility in the double knockdowns, in contrast to the hyperactivity seen in the single subunit knockdowns (fig. 6 C). There was no apparent effect of any of the treatments on larval viability, as determined by the dye (methylene blue) exclusion method of Gold (1994) [67]. To verify the knockdown, we performed quantitative real time PCR (qPCR) at the same time as the motility assays (7 days post-transfection) and compared the transcript level of each nAChR subunit in schistosomulae treated with specific siRNA relative to irrelevant 'scrambled' siRNA. We saw a marked decrease of all four nAChRs at the transcript level, ranging from 60-95% (fig. 6 A).

siRNA-treated schistosomula are resistant to cholinergic drugs: siRNA-treated schistosomulae were exposed to either an agonist, nicotine (10μM) or an antagonist, mecamylamine (10 μM) to test if the RNAi decreased the sensitivity to cholinergic substances. Larval motility was recorded 5 min after addition of test drug and was normalized relative to basal motility measured before drug treatment. Upon addition of nicotine, the control (scrambled) group exhibited a strong (≈60%) reduction of motility compared to that of pre-treatment (fig. 6 D), a response consistent with the known inhibitory effects of cholinergic agonists on worm movement [30,31]. A similar response was observed in animals treated with three of the subunit siRNAs, smp_031680, smp_139330 and smp_012000, but not 180570 (fig. 6 D). Those animals transfected with 180 siRNAs were essentially unresponsive to nicotine, suggesting this subunit is required for the effect of the drug on motility (fig. 6 D). Animals treated with smp_031680 siRNAs also appeared less responsive to nicotine than the controls but the difference was not statistically significant at $P< 0.05$. Experiments were repeated with mecamylamine, a classical antagonist of nAChRs which is known to be a potent stimulator of schistosome motility in vitro [32]. Treatment with mecamylamine increased motility in the control group (scrambled) as expected, and also stimulated movement in two of our test groups (smp_031680 and smp_180570) roughly to the same extent as the control (fig. 6 E). However, the groups with hypermotile RNAi phenotypes (smp_139330 and smp_012000) showed no further increase in movement when treated with this drug (fig. 6 E).

RNAi in adult worms: Having shown that nAChR subunits are required for motor control in the larvae, we questioned whether the same was true in adult worms. To test for function in the adults, we used a previously described transfection protocol, where male and female worms were electroporated with siRNA against target nAChRs. Worm movement was quantified by means of an imaging assay to identify RNAi motor phenotypes. Target groups were compared to the control which was electroporated with equal amount (5µg) siRNA against a non-specific target (mCherry). We observed pronounced effects of the siRNA treatments. However, the adult worms seemed to recover quickly and at variable rates depending on the subunit targeted. For smp_180570-siRNA treated worms, the maximum RNAi phenotype was observed at 24 hr post-transfection and recovery occurred sooner (by 30 hours) than the other groups. In contrast, smp_139330 and smp_012000 siRNA treatments showed maximum RNAi phenotypes at around 40 hours post-transfection and the smp_031680's phenotype was consistent both at 24hr and 40 hr time points. As a result, we recorded worms at two different time points, 24 hr for smp_180570, smp031680 and, the double knockdown (smp_031680 and smp_180570), or 40 hr, for the remaining test groups. An irrelevant control (mCherry) was included at each time point and motility data were normalized relative to the irrelevant control. The results (Fig. 6 F) show that the various siRNA treatments produced similar effects in the adult worms as in the larvae. As in larvae, we saw a significant decrease in motility upon treatment with siRNA against smp_031680 or smp_180570 and an increase of motility due to treatments with siRNA against smp_139330 or smp_012000. The similarity between the schistosomula and the adult stages was evident in the double knockdowns as well. Combination of smp_031680 and smp_180570 showed the same reduced motility as the individual knockdowns, whereas the double smp_139330/ smp_012000 knockdown had the opposite phenotype of the individual treatments. Female worms were generally more active than the males but the relative effects of the siRNA treatments were similar in the two sexes.

The RNAi knockdown of the various subunits was verified at the same time as the motility assays, either by western blot (smp_031680 and smp_139330) or qPCR (smp_180570 and smp_012000). Western blots were performed with the same anti-smp_031680 and anti-smp_139330 antibodies described above. Densitometry analyses of the blots by ImageJ showed approximately 47% and 41 % knockdowns for smp_031680 and smp_139330 siRNA treated adult worms, respectively, compared to the mCherry control (fig. 6 G). For smp_180570 and

smp_012000, the RNAi silencing was verified only at the RNA level by qPCR since we did not have antibodies against these subunits. In both cases, we observed virtually complete loss of mRNA transcript (≈100%) compared to mCherry control group, confirmed by three separate qPCR experiments (fig. 6 H).

Discussion:

The chronic parasitic disease, schistosomiasis, is one of the most socioeconomically important parasitic diseases of the world. The mainstay of treatment, praziquantel, is effective at present but there are growing concerns over the prospect of drug resistance. Since this is the only drug available, there is an urgent need to find a viable alternative to praziquantel. Nicotinic nAChRs, so named due to their affinity towards nicotine, are effective drug targets in other helminth parasites. Thus we conducted a first investigation of these channels at the molecular level in *S. mansoni*, with the goal of identifying possible new targets for anti-schistosomal drug discovery. In this project, we describe the cloning, immunolocalization and RNAi phenotypic analysis of four cation-selective nAChR subunits in two life stages of *S. mansoni*.

Sequence analyses of the four putative nAChRs revealed many of the characteristic features of the Cys-loop ligand-gated ion channel superfamily. These include a signal peptide for membrane translocation, a large extracellular domain containing a cys-loop (two cysteine residues, 13 amino acids apart), four TM regions, a large intracellular loop between TM3 and TM4 and a short extracellular tail. Analysis of these proteins with InterProScan for conserved motifs confirmed their identity as nAChRs. A close look at the TM2 region and its vicinity by structural alignment against well-characterized nAChRs identified residues typically associated with selectivity for cations. Moreover, a phylogenetic analysis revealed that the four subunits clade together with other well characterized nAChRs. Subunits smp_031680 and smp_139330 are evolutionarily closer to nAChRs from other species, whereas smp_180570 and smp_012000 are more divergent and constitute a separate subclade in the nAChR tree.

Using RNAi and motility assays, we demonstrated that the putative nAChRs can affect parasite motility, either positively or negatively, depending on the subunit. We observed two kinds of phenotypes upon RNAi treatment. Reduction of motility for smp_031680 and smp_180570 in the RNAi experiments would suggest that these two subunits are positively

associated with motor control. Moreover, the double-knockdown did not cause a further decrease in motility, indicating that the individual RNAi effects are not additive. It is tempting to speculate that these two nAChRs may be part of the same ion channel, such that the loss of either subunit would be sufficient to cause a decrease in movement. Upon addition of nicotine, we observed that the smp_031680-RNAi animals were less responsive to treatment than the scrambled controls, and the smp_180570-RNAi animals were virtually resistant to the paralytic effect of nicotine. This is consistent with the notion that the channels formed by these two subunits are sensitive to nicotine. On the other hand, addition of the classical cholinergic antagonist, mecamylamine, produced the same effect in the RNAi-suppressed animals as in the scrambled control and therefore these channels do not appear to be sensitive to mecamylamine.

RNAi targeting the other two subunits, smp_139330 and smp_012000, produced strongly hyperactive phenotypes, which indicates that they normally restrict parasite movement. Interestingly, however, the double knock down produced the opposite phenotype, a strong decrease in motility compared to the controls. This suggests that these subunits are most probably part of different channels and the presence of at least one of these channels is important for maintaining parasite motility. Unlike the two subunits described above, the smp_139330- and smp_012000-RNAi animals were as sensitive to nicotine treatment as the scrambled controls but they did not respond to the antagonist mecamylamine. This suggests that these ion channels are insensitive to nicotine but responsive to mecamylamine. Nicotine-insensitive nAChRs are not unprecedented among invertebrates. Noteworthy among these channels are the levamisole-sensitive, nicotine-insensitive nAChR subunits of *C. elegans* [12,68].

Ultimately the agonist/antagonist specificity of the schistosome nAChRs will have to be confirmed by testing the recombinant proteins in a heterologous expression system, such as *Xenopus* oocytes. It should be mentioned here that we have attempted heterologous expression of the four putative subunits in *Xenopus* oocytes (supplementary table S3). Attempts were made to express subunits individually and in various combinations, according to established protocols [69] with or without addition of *Haemonchus contortus* ancillary proteins (ric-3.1, unc-50 and unc-74), which have been shown to facilitate nAChR expression [70]. However, no functional channels were detected in response to cholinergic agonists or any of the other ligands tested, including biogenic amines and neuroactive amino acids. Subunits smp_031680 and smp_139330

were found to be expressed on the *Xenopus* oocyte surface by confocal microscopy [71] (supplementary figure S3) but the expression levels were low. This suggests that either we did not have the necessary subunit composition, or the expression levels of the *S. mansoni* subunits were too low to form functional ion channel(s).

With the aid of confocal immunofluorescence microscopy, we examined spatial expression patterns of smp_031680 and smp_139330 in 7 day old schistosomula and adult life stages, using specific polyclonal antibodies. Widespread expression of these two proteins was observed. Smp_031680 expression was observed in the peripheral nerve net and in parenchymal cells of the larval stage, whereas in adult stage, it was found in both central and peripheral nervous systems of the parasite. Subunit smp_139330 was found to be expressed in the peripheral nerve net of the larvae and the CNS, PNS and muscle fibers of the adult stage. Moreover, both smp_031680 and smp_139330 were localized in the female reproductive system of the parasite. Smp_031680 was localized in the ootype where eggs undergo maturation and fertilization [64] whereas, smp_139330 was localized in Mehlis' gland which contributes to the egg shell production[65,66]. However, upon RNAi treatment of adult worms, we did not see a significant difference of egg count in the culture dish compared to the control group. This suggests that the depletion of these putative nAChRs have no significant effect on egg production.

To identify the cholinergic system in the two life stages, we performed similar confocal microscopy analysis of schistosomulae and adult worms using a polyclonal antibody against human ChAT, which is 43.1% similar to the putative orthologue in S. mansoni. ChAT is the enzyme response for acetylcholine biosynthesis and is often used as a marker of cholinergic neurons [72]. The results showed a pattern of immunoreactivity similar to that of the channel subunits. ChAT was detected in the peripheral nerve net of the body wall in the larvae, whereas the adults expressed ChAT both in the central and peripheral nervous systems.

The neuronal localization of the two putative nAChRs suggests these channels work by depolarizing neurons and probably affect motility indirectly through modulation of neuromuscular circuits. Depending on which nAChR receptor is activated, this kind of signalling could be mediated by excitatory molecules, such as serotonin, glutamate and neuropeptides, or inhibitory molecules such as GABA and glycine. This would explain the

contrasting hyper and hypoactive RNAi phenotypes observed for the different sets of subunits. An interesting feature of smp_031680 expression was its prevalence in the cell bodies of neurons as well as varicosities of neuronal fibers, which are known sites of neurotransmitter release [8,9]. This expression pattern is consistent with a presynaptic receptor that acts by modulating neuronal signaling.

Whereas smp_031680 is predominantly neuronal, smp_139330 was detected both in the nervous system and muscle fibers. Direct expression of the protein on muscle fibers would be consistent with the role of a classical (vertebrate-like) nAChR, which mediates Ca^{2+} influx into the muscle and thus directly stimulates muscle contraction. If we decrease concentration of a muscle-based nAChR, we would expect decreased muscle depolarization and the resulting phenotype should be a relaxed, less motile phenotype. However, our RNAi screen showed the opposite result (hypermotility), which argues against a direct effect and suggests that smp_139330 also works via neuromodulation. A possible explanation is that the smp_139330 channel is expressed in nerve endings associated with the musculature, rather than the muscle itself. The function of these channels may be to stimulate release of inhibitory molecules, such as GABA and glycine, which would act upon the muscle fibers to decrease muscle contraction and movement.

In conclusion, we demonstrate that putative cation-selective nAChRs of the blood fluke *S. mansoni* are important players in maintaining the normal motile state of the parasite. The receptors localize to important physiological sites, such as central and peripheral nervous systems, musculature and reproductive system of the parasite and therefore are potential targets for chemotherapeutic drugs to treat schistosomiasis. The four nAChRs studied in this project allows us a glance into the complex nature of the parasite's nervous system. Acetylcholine, which has been partially explored in this project, constitutes only a component of this system and we have demonstrated that interference with the cholinergic system yields significant phenotypic outcomes. This is a small, yet significant first step in elucidating the parasite's cholinergic nervous system and identifying the molecular players involved in this signaling.

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Figures:

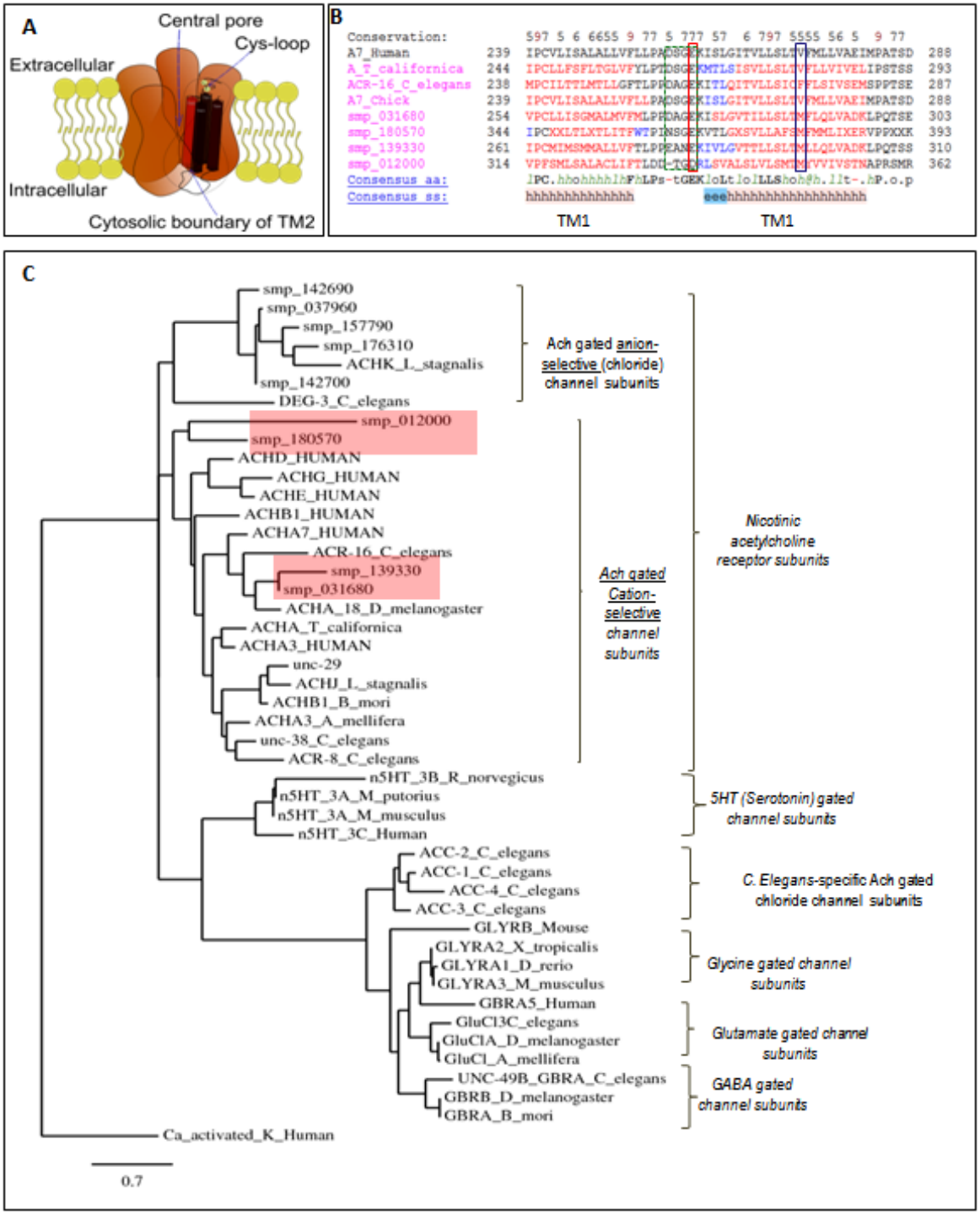

Figure 1: Arrangement of a ligand-gated ion channel (A), structural alignment of the second TM region of the four subunits of interest against similar well characterized nAChRs (B) and phylogenetic analysis of these putative subunits (C). A: Pentameric organization of an ion

channel formed by nAChRs. Five nAChRs create the channel where the central pore is lined with TM2 of each subunit. Each subunit possesses an extracellular ligand binding domain containing a cys-loop, four TM regions and an extracellular tail (N to C terminal). TM2 and its intracellular boundary dictates ion-specificity of the channel. B: A structural alignment of TM2 region constructed using PROMALS3D tool. Each of the four subunits of interest include a negatively charged amino acid (glutamate or aspartate) residue at the cytosolic side of the TM2 region (red box), absence of a proline within three upstream amino acid residues (dotted green box) of this negatively charged amino acid and a hydrophobic amino acid within the TM2 region (blue box). C: Phylogenetic analysis of the four subunits and cation-specific nAChRs from other species. A bootstrap data set (1000 replicates) for the tree was generated using Neighbour Joining method. The tree was rooted using a human Ca^{2+} activated K^{+} specific channel subunit as the outgroup. All the accession numbers are listed in the supplementary table 2 (Table S2).

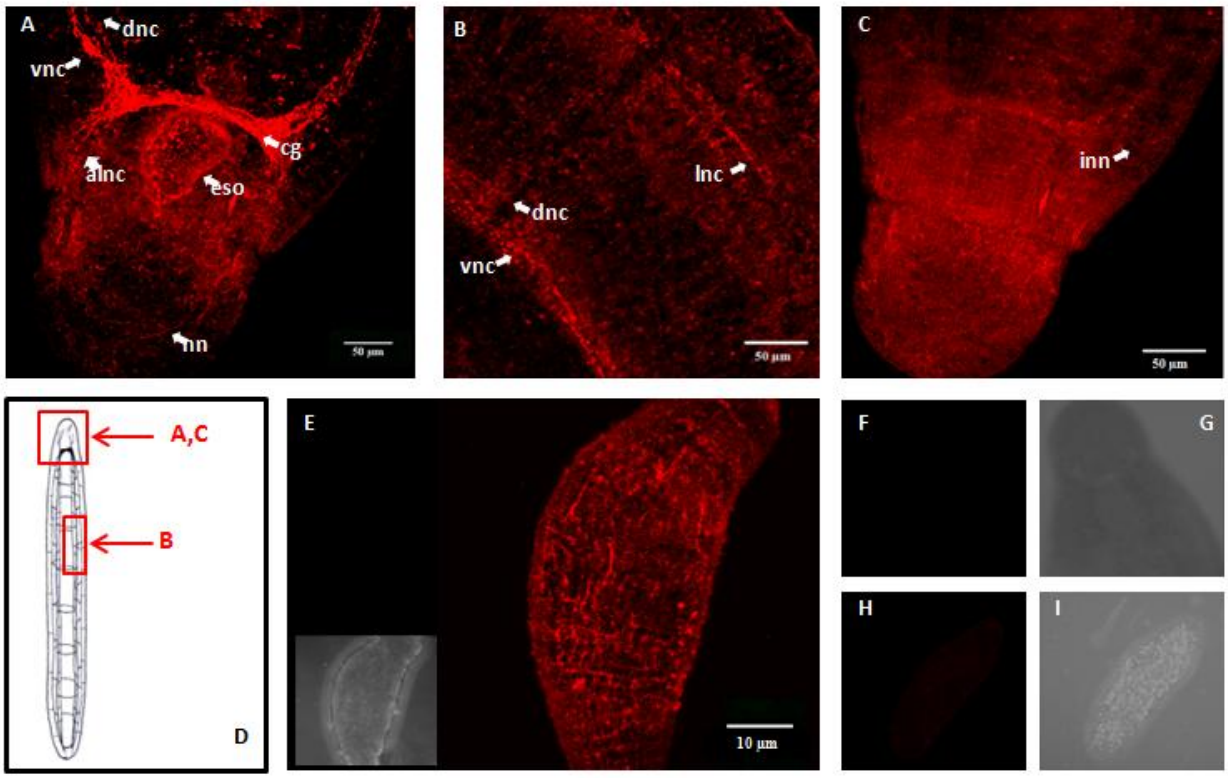

Figure 2: Expression patterns of choline acetyltransferase (ChAT) in the nervous system of adult (A-C) and schistosomulae (E) life stages of the parasite.(A) In adult male worms we observe expression of ChAT in the cerebral ganglia (cg), ventral nerve cords (vnc), dorsal nerve cords (dnc) and anterior longitudinal nerve cords (alnc) of the CNS. Expression in the peripheral nervous system includes expression in the nerve net (nn) around oral sucker (A), lateral nerve cords (lnc) (B) and peripheral innervations of musculature (inn) (C). (D) A schematic diagram of an adult schistosome shows the location of the confocal images in panels A-C. A 7 day-old schistosomulum labeled with anti-ChAT antibody (E) and corresponding transmission linght image (inset). Expression of ChAT in schistosomulae was observed along the periphery of the body in an irregular mesh-like pattern. No significant labeling was observed in the negative controls (omission of primary antibody) for the adult worms (F, G) or 7 day- old schistosomulae (H, I).

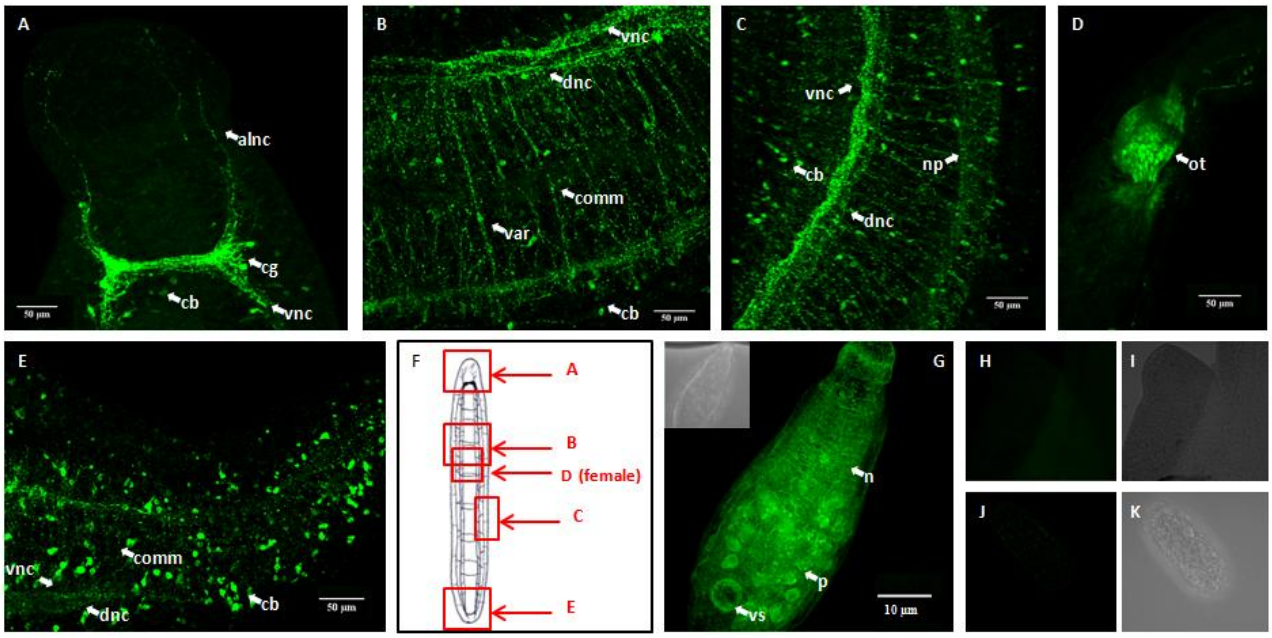

Figure 3: Expression patterns of smp_031680 in adult males (A-C), adult females (D, E)) and schistosomulae (G) stages of the parasite. (A) Expression in adult worm includes cerebral ganglia (cg), ventral (vnc) and anterior longitudinal nerve cords (alnc) in the head region. (B) In the midbody, the protein is expressed in both the ventral nerve cords (vnc) and dorsal nerve cords (dnc) as well as the connectors known as transverse commissures (comm). The transverse commissures show numerous swollen bodies or varicosities (var), which are rich in the protein. (C) In the midbody, immunoreactive nerve fibers can be seen branching from the main nerve cords to form the peripheral nerve plexus (np) of the worm's body wall. (D) In female worms, strong expression was observed in the ootype (ot). (E) Tail end of a male showing immunoreactivity in neuronal cell bodies (cb). (F) A schematic diagram shows the location of the confocal images in panels A-E. (G) In a 7 day-old schistosomulum, smp_031680 localizes to fine nerve fibers of the peripheral nerve net (n) just beneath the surface of the body. In addition, expression was observed in probable parenchymal cells of the growing larva. Pronounced expression around ventral sucker (vs) is visible as well. Fig. H and I: preadsorbed control of adult and its corresponding transmission image; Fig. J and K, preadsorbed control of 7 days old schistosomulum and its transmission image.

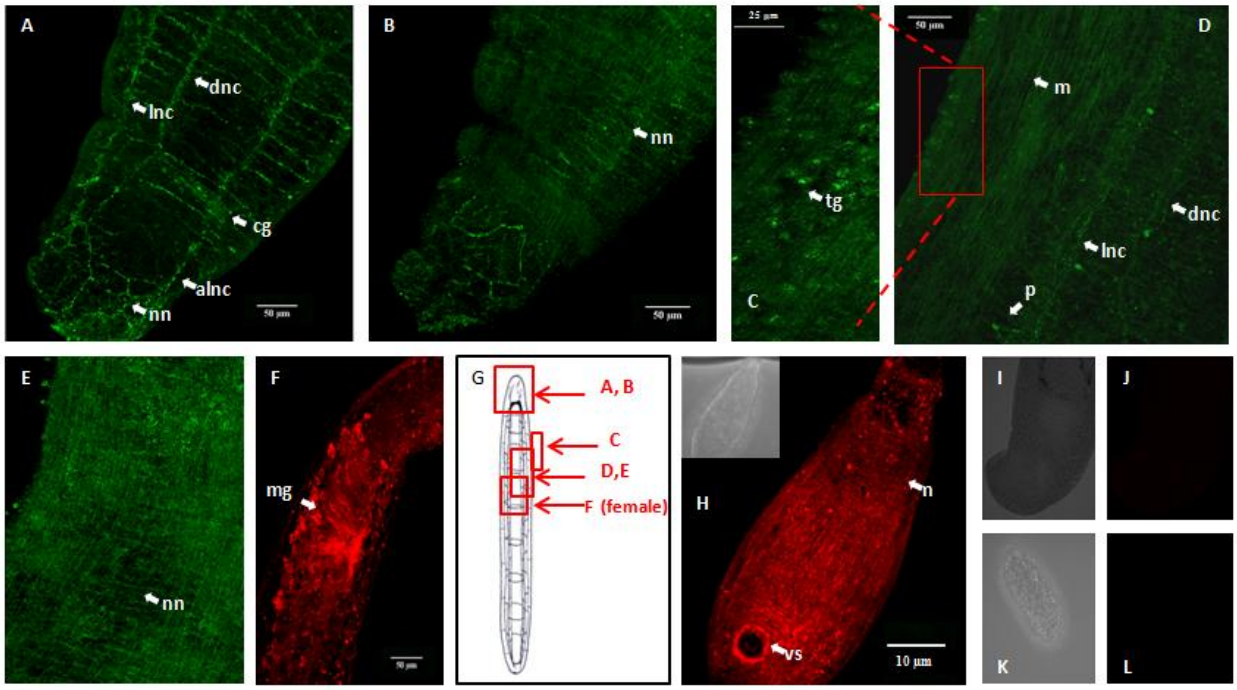

Figure 4: Smp_139330 expression in the nervous system and musculature of the parasite. In the head region (panel A), the protein is expressed faintly in the cerebral ganglia (cg) but strongly in dorsal (dnc), lateral (lnc) and anterior longitudinal nerve cords and nerve net (nn)around oral sucker. Throughout the body (panels B, E), expression is observed in the submuscular nerve net (nn) and muscle fibers (m, panel D). Dorsal and lateral nerve cords are interconnected by neurons expressing smp_139330 (panel D). Fine outward projections from lateral nerve cords reach the bulb-like tubercles of the teguments (tg) (panel C: body surface). Panel G: A schematic diagram shows the location of the confocal images. In a 7 day- old schistosomulum, the protein was observed in the peripheral nerve net (n) on the outer layer of the body. Strong localization around ventral sucker is also evident. Panels I and J: preadsorbed control of adult and its corresponding transmission image. Panels K and L preadsorbed control of 7 day- old schistosomulum and its transmission image. In panels A to E, polyclonal donkey anti-goat Alexa Fluor 488 was used as 2^{o} antibody. In panels F and H the secondary antibody was donkey anti-goat Alexa Fluor 594.

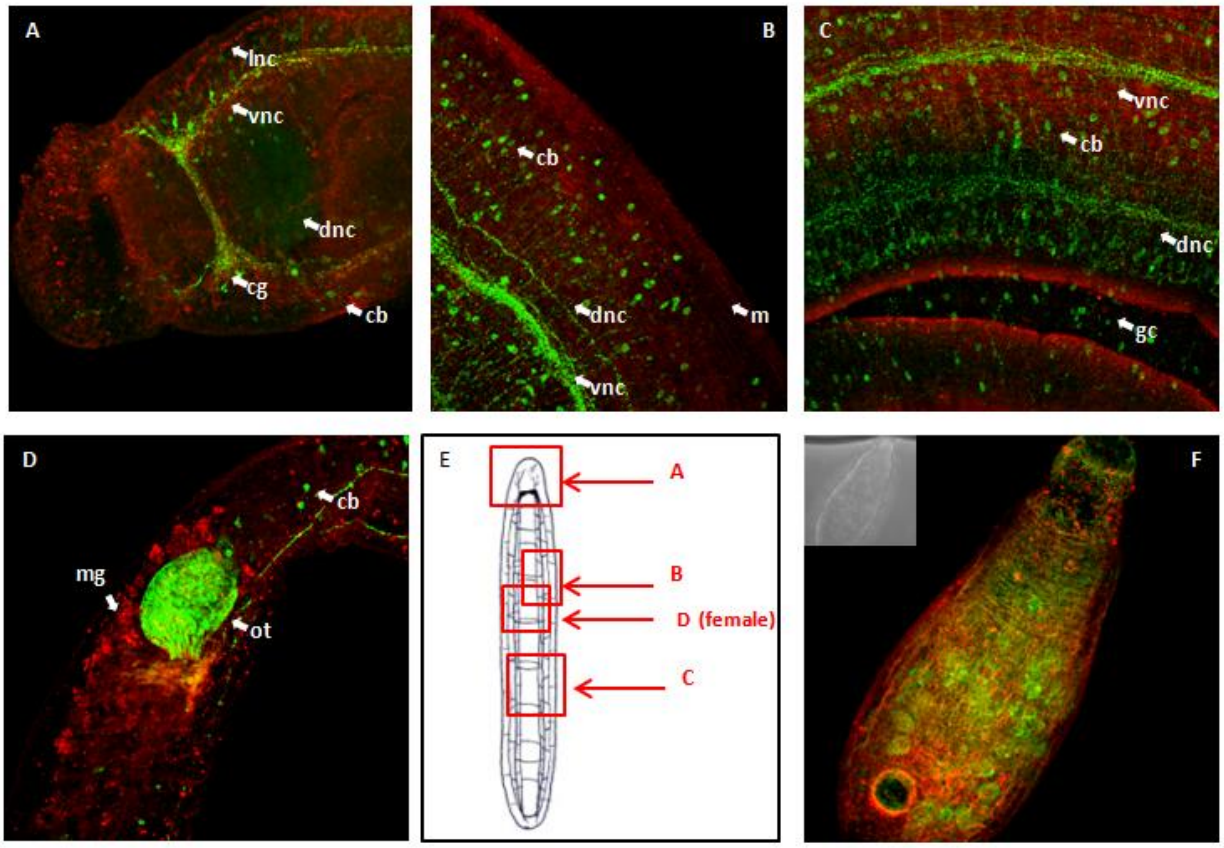

Figure 5: Dual labelling of smp_031680 (green) and smp_139330 (red) shows non-overlapping expression patterns of the two proteins in adult worms (A-D) and 7 day- old schistosomulae (F). (A) head region of a typical male worm showing expression of smp_031680 in cerebral ganglia (cg) and ventral nerve cords (vnc) ; smp_139330 expression is visible in the dorsal (dnc) and lateral (lnc) nerve cords. In the midbody (B) smp_031680 is enriched in neuronal cell bodies and nerve cords of the CNS whereas smp_139330 localizes to the peripheral nerve fibers that innervate the body wall muscles (m) or possibly the musculature itself. Panel C shows expression of the two proteins around the male gynecophoric canal (gc) region. Expression of smp_031680 in cell bodies was observed throughout the body (cb) (panels A to D). In female reproductive system (panel D), smp_031680 is localized in the ootype (ot) whereas smp_139330 is found in the Mehlis' gland (mg). In 7 day- old schistosomulum, discrete mesh-like pattern on the outer layer of the body is visible for the two proteins. Moreover, expression of smp_031680 in parenchymal cells is visible in this image as well.

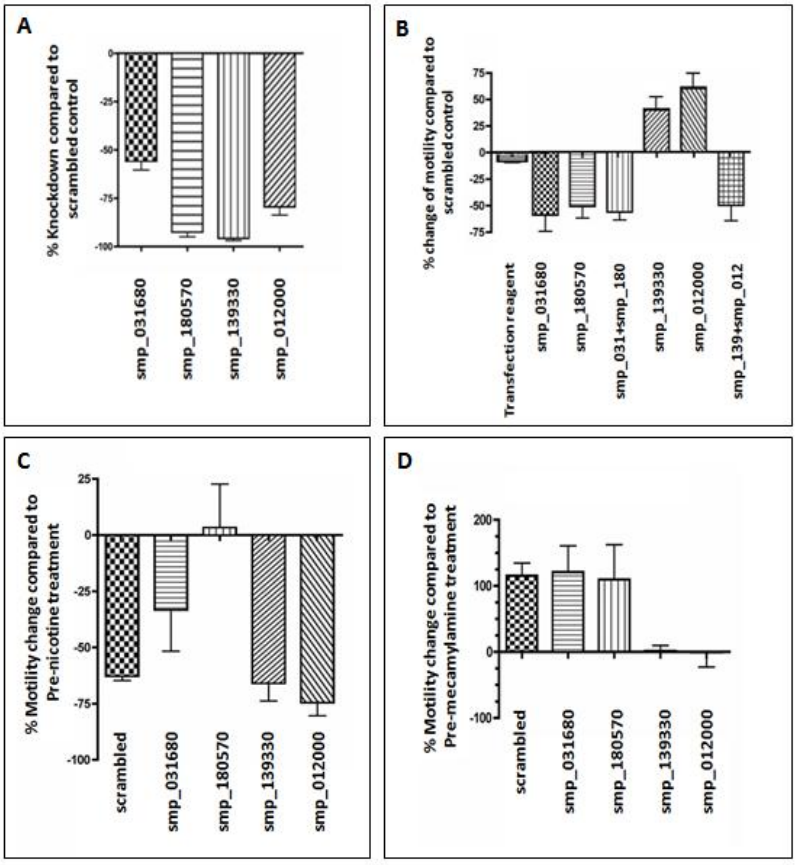

Figure 6: RNA interference (RNAi) targeting the four putative nAChRs in cultured schistosomula. Freshly transformed S. mansoni schistosomulae were treated with 50 nM nAChR-specific siRNAs, 50 nM irrelevant (scrambled) siRNAs or transfection reagent alone (mock-transfected) for 7 days and then examined for RNAi silencing and motor phenotypes. (A) Confirmation of knockdown in siRNA-treated schistosomula by quantitative RT-qPCR. % knockdown was calculated by the Pfaffll method and was normalized to the control treated with scrambled siRNA. (B) Effects of single and combination siRNA treatments on larval motility compared to non-specific 'scrambled' control. For dual siRNA treatments, each siRNA was added at a final concentration of 50 nM, for a total concentration of 100 nM, and the corresponding scrambled siRNA control was 100 nM. Larval motility was measured using an imaging assay, as described [47] and then normalized relative to the corresponding scrambled siRNA control. The normalized motility data are the means and SEM of 90 animals from 3 independent transfections. C: Effects of 10 μM nicotine on siRNA- treated larvae. Schistosomulae were treated with subunit-specific or scrambled siRNAs, as above, and tested for

RNAi motor phenotypes at 7 days post-transfection. For each treatment, larval motility was measured prior to drug addition and again 5 min after addition of nicotine (10 µM final concentration). The data were normalized relative to the pre- nicotine baseline. Each data point is the mean and SEM of 90 animals from 3 transfections. D: Effects of 10 µM mecamylamine on siRNA- treated larvae. Experiments were performed as in panel C except that larvae were treated with the cholinergic antagonist, mecamylamine instead of nicotine. The data are the means and SEM of 90 animals from 3 independent experiments.

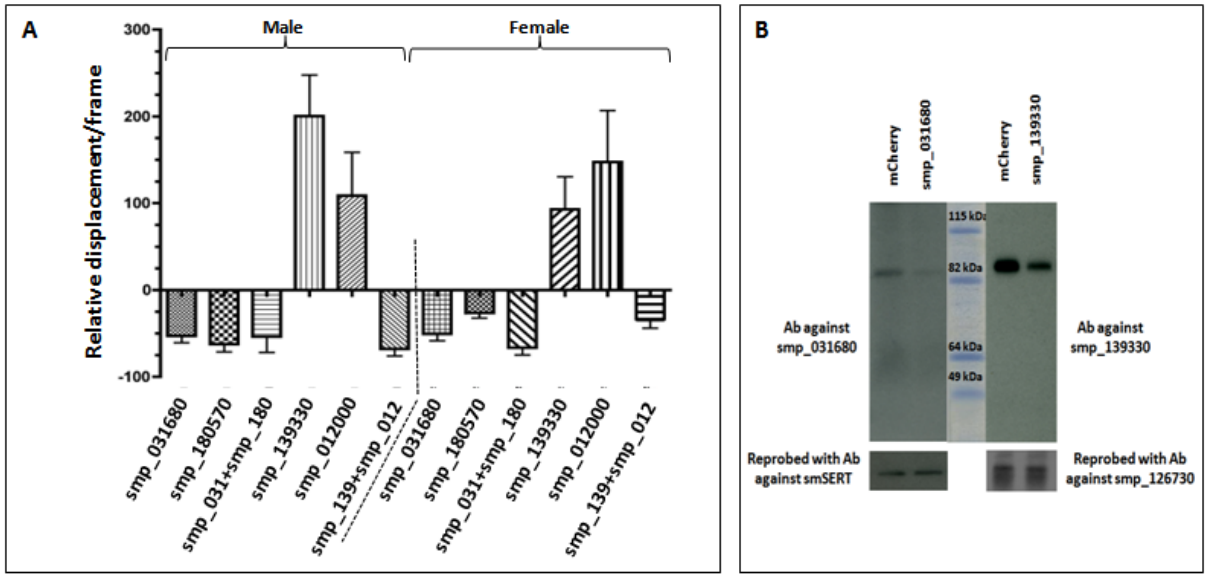

Figure 7: RNAi in adult worms. Adult males and females were electroporated with subunit-specific siRNA or irrelevant (mCherry) control siRNA and motility was recorded approximately 24 hr (smp_031680, smp_180570 and their combination) or 40 hr (smp_139330, smp_012000 and combination of smp_139330 and smp_012000) post-transfection, using an imaging assay. Worm movement was recorded for periods of 2 min and motility data were normalized relative to the irrelevant siRNA control. Each data point is the mean and SEM of 15 worms obtained from 3 separate RNAi experiments. (B) RNAi knockdown of smp_031680 and smp_139330 was verified at the protein level by western blot analysis. Worms were harvested immediately after motility recording and membrane proteins were extracted. Equal amounts of membrane proteins from test and control worms (mixed males and females) were probed with anti-smp_031680 and smp_139330 antibody or an antibody against a different membrane protein (SmSERT: smp_126730 [73]) as a loading control. Worms, electroporated with subunit-specific siRNAs had significantly less western positive nAChR protein than the control worms electroporated with irrelevant mCherry siRNA. Densitometric analysis of the blots using ImageJ revealed ≈47% and ≈ 42% knockdowns for smp_031680 and smp_139330 respectively. Knockdown of smp_180570 and smp_012000 in adults was verified at the RNA level by qPCR to be almost 100% compared to mCherry control group (data not shown).

Supplementary Tables and Figures:

	Primer name	Primer Sequence
Cloning primers	smp_031680F	ATG AAT TCA ATC ATT AAA ATA TTT GG
	smp_031680R	TTA TGG CAT AAA TGA AGC AAT TAA AT
	smp_139330F	ATG TAT AAA TTA TAT AAC ATT TTG TAT CAT
	smp_139330R	TTA CAT TGG TTG TGA TGG TG
	smp_180570GSP1	CGT CGT ATT ACT CAA TTT GGT TTA TGT CAA TTC
	smp_180570GSP2	CTT ATT TAT CAA TCA ACG CTT ATT GTT ACA AG
	smp_180570GSP3	ATT ACA CAT ACA ACA ACT G
	smp_180570GSP4	AGA ACA GCT TCA ATA CTA AAT CAG TTT GTC
	smp_012000GSP1	ACC ACA TAC ATA GTC ATA C
	smp_012000GSP2	AAC CGA TCT CCT GTA TCA TCT AAT GTG
qPCR primers	q031680F	TTA ACG CTT CAC AAC CTG ATT GTA C
	q031680R	GTT GTG CAT AAC GTC GAA CTG AAG
	q139330F	TCG TCA GGA AAT CCT GTA CTT GTC T
	q139330R	GAC AAT TTA GTA AAA CCA CAG CGC A
	q180570F	CGT GTT CCA CCA ACA GCT AA
	q180570R	CCA TTT TGG TGC ACG TGA TA
	q012000F	GAT CGG TTA TCA GTT GCA CTT TC
	q012000R	ATT ACA GCA AGT ATC GTC GCA AT
	qGapdhF	GTT GAT CTG ACA TGT AGG TTA G
	qGapdhR	ACT AAT TTC ACG AAG TTG TTG

Table S1: A list of various primers used in this project. Cloning and qPCR primers are shown separately.

Protein name	Accession/Ref. number	Protein name	Accession/Ref. number
smp_031680	KF738247	ACC-3_C_elegans	NP_508810.2
smp_139330	KF738248	ACC-4_C_elegans	NP_499789.1
smp_180570	KF738249	ACHA_18_D_melanogaster	ABO26063.1
smp_012000	KF738250	ACHA3_A_mellifera	NP_001073029.1
smp_037960	CCD82627.1	ACHB1_B_mori	NP_001103398.1
smp_157790	XP_002577842.1	ACHA_T_californica	0811252A
smp_142700	CCD82626.1	ACHJ_L_stagnalis	ABA60390
smp_176310	XP_002580708.1	ACHK_L_stagnalis	ABA60389
smp_142690	CCD82628.1	UNC-49B_GBRA_C_elegans	NP_001022785
ACHA7_HUMAN	NP_000737.1	GBRB_D_melanogaster	AAA28556
ACHA3_HUMAN	NP_000734.2	GBRA5_Human	EAW57656
ACHB1_HUMAN	NP_000738.2	GBRA_B_mori	NP_001093294
ACHG_HUMAN	NP_005190.4	GLYRB_Mouse	CAM06613
ACHD_HUMAN	NP_000742.1	GLYRA1_D_rerio	NP_571477
ACHE_HUMAN	NP_000071.1	GLYRA2_X_tropicalis	XP_002939916
Ca_activated_K_Human	NP_001258447.1	GLYRA3_M_musculus	Q91XP5
ACR-16_C_elegans	NP_505207.1	5HT_3C_Human	NP_570126
unc-38_C_elegans	NP_491472.1	5HT_3A_M_musculus	CAA80453
unc-29_C_elegans	NP_492399.1	5HT_3B_R_norvegicus	EDL95431
ACR-8_C_elegans	NP_509745.2	5HT_3A_M_putorius	XP_004749922
DEG-3_C_elegans	NP_505897.1	GluCl3C_elegans	NP_504441.1
ACC-1_C_elegans	NP_501715.1	GluClA_D_melanogaster	NP_001163655
ACC-2_C_elegans	NP_501567.1	GluCl_A_mellifera	NP_001071277.1
A7_chick	NP_989512		

Table S2: Accession/Ref. numbers of various protein sequences used to construct structural alignment (fig. 1 B) and phylogenetic tree (fig. 1 C) in this paper.

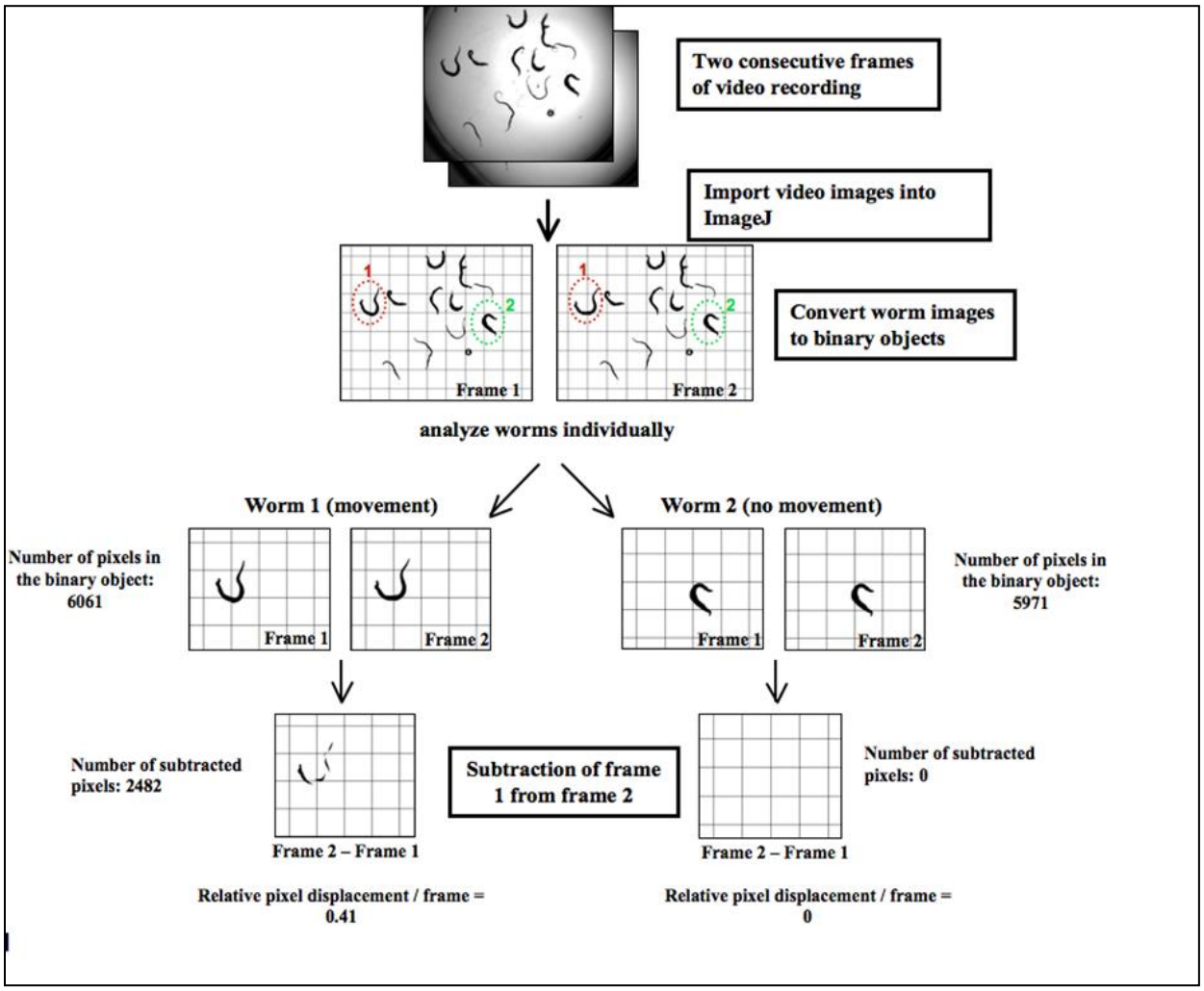

Figure S1: A schematic diagram of the motility assay for adult schistosomes. Videos are converted into a binary format by using a thresholding algorithm available in ImageJ. A binary object representing a single worm is selected and each frame is subtracted from the next using the software to generate a frame to frame displacement profile. An average of the displacement in pixels relative to the average size of the worm (in pixels) is then calculated. This value described as 'Relative Displacement/Frame', is used as a measure of worm motility. Further details of the imaging assay are described in Patocka et al., 2013 [50].

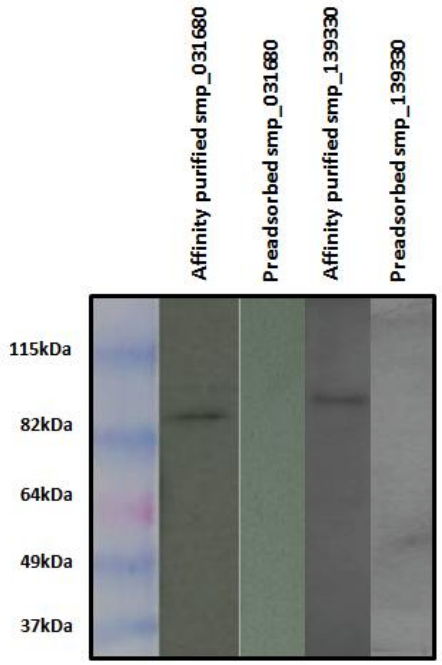

Figure S2: Western blot analyses of smp_031680 and smp_139330. Aliquots of adult worm membrane proteins (30 µg per lane) were resolved on a 4-12% gradient SDS-PAGE and immunoblotted with peptide affinity-purified antibodies or antigen-preadsorbed antibody controls, as indicated. A single immunoreactive band of the expected size was observed for each antibody but not the corresponding preadsorbed negative control.

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Connecting statement:

In the previous chapter we described the first cloning and characterization of four putative nAChR subunits of *S. mansoni*. We showed that nAChR subunits are required for proper motor control and they are widely expressed in neuronal and neuromuscular structures of the parasite. The next step in this project is to test whether the putative subunits form functional ion channels *in vitro*. In the following chapter, we describe our attempts to heterologously express the four nAChR subunits in two expression systems, *Xenopus* oocytes and mammalian cell line (HEK 293 cells), for future functional assays of ion channel activity.

Chapter 3

Manuscript II

Heterologous Expression of Putative Cation-Selective Nicotinic Acetylcholine Receptors of *Schistosoma mansoni*

Sections of this chapter appear in the following manuscript:

M. Rashid and P. Ribeiro. Characterization of Putative Nicotinic Acetylcholine Receptors of the Human Parasite *Schistosoma mansoni (in preparation for submission to PLoS NTD)*

Abstract: Schistosomiasis, caused by helminth parasites of the genus *Schistosoma* is a major parasitic infection of third-world countries. The mainstay of disease control is praziquantel, which is effective at present but there are growing concerns over the prospect of drug resistance in endemic areas. Nicotinic acetylcholine receptors (nAChRs) are proven drug targets for treatment of parasitic diseases and four subunits of nAChRs have been cloned as part of this project. In order to screen for drugs against these receptors, it is necessary to express the proteins and form functional channel(s) in vitro, using a suitable heterologous system. Here, we attempted to express putative *S. mansoni* nAChR subunits in *Xenopus* oocytes by microinjection of the cRNAs, both individually and in combination, into the oocyte's cytoplasm. However, despite repeated efforts, we could not detect a functional ion channel by two-electrode voltage clamp (TEVC) electrophysiology. Injections were repeated with *S. mansoni* orthologues of ancillary proteins ric-3, unc-50 and unc-74, which are known to aid proper folding and expression of nAChRs in nematodes but no ion channel activity was detected. Using confocal microscopy and specific antibodies, we demonstrated that the schistosome nAChRs were expressed on the oocytes' surface. It is possible the expressed protein levels were too low to form a functional channel, or we lacked the correct subunit composition. As an alternative strategy, the expression constructs were modified by addition of Green fluorescent protein (GFP) or mCherry and the fluorescently tagged subunits were expressed in transfected HEK 293 cells. Preliminary results suggest that the schistosome subunits can be expressed in the mammalian cell environment. Further experiments are needed to improve transfection efficiency and to test for channel activity.

Introduction: Schistosomiasis (also known as Bilharziasis or snail fever) is a chronic parasitic infection caused by blood flukes of the genus *Schistosoma*. Over 240 million people are treated for the disease worldwide every year [1]. An estimated 700 million people of 74 countries are thought to be at risk of being infected [2]. The mainstay of chemotherapy against schistosomiasis consists of a single dose of 40 mg/kg body weight of praziquantel (Biltricide), which is effective against adult stages of all three major species of *Schistosoma* (*S. mansoni, S. haematobium and S. japonicum*) [3]. Although, praziquantel is an effective schistosomicide at present, there are growing concerns about the prospect of drug resistance. Artificial selection in the laboratory has produced resistant strains of *S. mansoni* in only 2 generations of repeated exposure to sub-lethal doses of praziquantel in a mouse model [4]. Moreover, reduced cure rates have already been reported in endemic foci of Senegal [5] and Egypt [6]. As a result, it is safe to speculate that we need an alternative to praziquantel to ensure treatment of schistosomiasis in the future. In an effort to find alternatives to praziquantel, one might look into the mode of action of the existing anthelmintics. A close examination of the mode of action reveals that most of these drugs exert their effects by interfering with ion channels (appendix, Table 1, in *italic*), in particular nicotinic acetylcholine-gated ion channels (appendix, table 1, ***bold and italic***), four of which have been cloned in this project.

To test the potential of schistosome nAChRs for drug discovery, it is necessary first to demonstrate that the schistosome subunits form functional ion channels in vitro and then test the effects of candidate agonists and antagonists [7]. Oocytes of *Xenopus laevis* have been widely used for heterologous expression of recombinant ion channels, both vertebrate and invertebrate [8] and they remain the system of choice for electrophysiology studies. In this method, complementary RNA is microinjected into defolliculated oocytes. Upon successful expression of the subunit(s) and formation of ion channels on the plasma membrane, deviation of membrane potential from a preset value due to ligand binding can be recorded, using two-electrode voltage clamp system (Fig. 1). The process is well established and straightforward but very elaborate and not suitable for high-throughput drug screening. As an alternative expression system, mammalian cells are being increasingly explored for studies of recombinant channels. With the aid of a voltage sensitive fluorescent protein, it is now possible to detect change of membrane potential of cultured mammalian cells [9]. This allows us to identify formation of ion channel or impact of a test substance on an ion channel expressed by mammalian cells [9-12]. Various types

of cell lines have been used previously, including Drosophila S2 [13], CHO-K1 (Chinese hamster ovary K1) [14] and HEK 293 [15] (human embryonic kidney 293) cell lines. Among these, HEK293 cells have been extensively used to study nAChRs due to their permissive nature for heterologous expression, availability and ease of use.

Despite the availability of multiple assays and expression systems, functional expression studies of helminth ion channels remain an experimental challenge. A very relevant example is the previous attempt to express *S. haematobium* orthologues of smp_031680 and smp_139330 in Xenopus oocytes [16], which was unsuccessful after repeated attempts. The reason these experiments are so challenging is the wide variability of subunits among heteromeric ion channels and the difficulty in establishing the right subunit composition. While some channels form functional homomeric assemblies, others require the combination of multiple types of subunits, which must be cloned and co-expressed to generate active channels. Another complicating factor is that the heterologously expressed subunits may fail to fold properly and/or to be correctly targeted to the cell membrane in the foreign cell environment. It has been demonstrated in both nematodes and vertebrates that the proper folding and export of nAChRs requires various ancillary proteins. These include ric-3, unc-50, unc-74 in the nematodes *C. elegans* [17-19] and *H. contortus* [20] and ric-3, unc-50 and VILIP-1 in humans [21,22]. Ric-3 is a transmembrane resident of the endoplasmic reticulum, which is thought to aid nAChR folding [18,23,24]. Unc-50 is a multipass membrane protein of the Golgi apparatus, which specifically assists vesicular transport of levamisole-sensitive acetylcholine receptor (L-AChR) in *C. elegans* and *H. contortus* and prevents their lysosomal degradation [17,20]. Unc-74 is a member of the protein disulfide isomerase family and is the least characterized among the three ancillary proteins. It contains a thioredoxin domain closely related to human TMX3 protein and is thought to be a resident of the endoplasmic reticulum. Mutations in this gene results in lack of L-AChR expression in *C. elegans* and *H. contortus* [19,20,25]. Given the importance of these proteins, researchers often co-express nematode nAChR subunits of interest with *C. elegans* ric-3, unc-50 and/or unc-74 to improve the folding / targeting of the channels to the membrane [19].

In this section of the project, we describe our efforts to heterologously express four putative nicotinic acetylcholine receptors (nAChR) of *S. mansoni*: smp_031680, smp_139330, smp_180570 and smp_012000. *Xenopus* oocyte expression system was attempted first. To do so, the four putative nAChRs were sub-cloned into an appropriate transcription vector.

Complementary RNAs were generated and microinjected into oocytes with or without ancillary proteins' RNAs (*H. contortus* ric-3, unc-50 and unc-74 or putative *S. mansoni* ric-3 and unc-50 orthologues) and the oocytes were tested by TEVC. Besides *Xenopus* oocytes, a mammalian cell line was also explored as a possible expression system. As a first step of this process, we sub-cloned the four putative subunits into a mammalian expression vector and inserted fluorescent tags (GFP/mCherry) between the third and fourth transmembrane domains of each putative nAChR to produce tagged fusion proteins, according to established protocols [26]. Mammalian cells were then transfected with these tagged subunits and examined with a fluorescent microscope to assess expression efficiency.

Materials and methods:

Identification and Cloning of nAChR subunits and Ancillary proteins: Full-length cDNA sequences of all four nAChR subunits of interest were cloned as described in Chapter II and verified by DNA sequencing (Accession numbers smp_031680: KF738247, smp_139330: KF738248, smp_180570: KF738249 & smp_012000: KF738250). In order to identify orthologues of ancillary proteins in the *S. mansoni* genome, we took the mRNA sequence of well characterized *C. elegans* ancillary proteins from NCBI nucleotide database and searched in the *S. mansoni* genome using tBLASTx tool, which takes a translated nucleotide query and looks for similar sequence in all six possible frames. We identified possible ric-3 and unc-50 orthologues in *S. mansoni* genome to be smp_141650 and smp_029040 respectively. Identifying the orthologue of unc-74 turned out to be more difficult because the thioredoxin domain is present in many different *S. mansoni* proteins and pinpointing one probable candidate was not possible based on sequence analysis. Hence, we restricted the analysis to *S. mansoni* ric-3 and unc-50 orthologues only (smp_141650 and smp_029040 respectively). To clone these putative ancillary protein orthologues, we designed forward and reverse primers to amplify full-length cDNA sequences. All the primers for this purpose are listed in Table 2. In both cases we observed one clean sharp band upon agarose gel electrophoresis of the PCR product. We purified and cloned these fragments into vector pJET (CloneJET PCR Cloning Kit, Thermo Scientific) and confirmed sequences by DNA sequencing of three different clones in each case.

Subcloning of cDNAs and complementary RNA (cRNA) production: In order to produce cRNA, full length cDNA sequences of all four subunits of interest and ancillary proteins (smp_141650 and smp_029040) were subcloned into transcription vector pT7-TS for cRNA production. Plasmids containing full-length smp_031680 and smp_139330 were used as templates to amplify and append restriction sites at both ends (Bgl-II at 5' and Spe-I at 3' for smp_031680 and Bgl-II at both 5' and 3' for smp_139330 respectively) by PCR. These fragments were then ligated into the Bgl-II and Spe-I sites of transcription vector pT7-TS (Figure 2). pT7-TS contains a T7 promoter site and both 5' and 3' UTRs of the *Xenopus* beta globin gene flanking the multiple cloning site. For smp_180570, smp_012000 and the ancillary protein genes, the subcloning was done using the In-Fusion HD cloning kit (Clontech Laboratories Inc.). First the insert was amplified by PCR using forward and reverse primers, which had 15 base pairs overlap with the site of insertion (EcoRV) on the plasmid. The plasmid was linearized at the target site using EcoRV. With the aid of a proprietary enzyme supplied by the kit, the insert was then cloned into the target site. In all cases, full-length sequences were confirmed from three different bacterial clones. Identities of full-length protein sequences were verified using the on-line tool Interproscan. For *in vitro* transcription, the vector pT7 was linearized by digestion with EcoRV and the linearized plasmids were then used as a template for cRNA synthesis using mMESSAGE mMACHINE T7 kit (Ambion), according to the manufacturer's guidelines.

Oocyte acquisition and microinjection:

In order to obtain oocytes from adult female *Xenopus laevis,* the animals were anaesthetized by immersing into a 0.03% benzocaine solution in water for 20 minutes. A 1 cm incision was made in the lower abdominal at one side to remove oocytes surgically. Collected oocytes were incubated for 2 hours at room temperature in ND96 solution (93.5 mM NaCl, 2 mM KCl, 2mM $MgCl_2$ and 5 mM HEPES) without $CaCl_2$ and supplemented with 2.5 mg/ml collagenase II. Following incubation, oocytes were washed 4 times with ND96 with $CaCl_2$ (1.8 mM) and resuspended in the same media. Healthy, defolliculated oocytes were chosen for microinjection with the aid of a stereomicroscope.

A total of 46 nl cRNA at a concentration of ≈1 μg/μl (approximately 50 ng/injection) were microinjected into each oocyte, using Nanoject II (Drummond Scientific Co.) with a glass needle having an opening size of 10-30 microns. cRNAs were injected individually or in

combination into at least 20 oocytes over at least two experiments. In some experiments, subunits were co-injected with predicted *S. mansoni* ric-3 and unc-50, or with cRNAs encoding known ancillary proteins from *Haemonchus contortus*, Hco ric-3, unc-50 and unc-74 (kindly provided by Thomas Duguet and Dr. Robin Beech). Co-injections were performed with 46 ng of cRNA per oocyte. The ng amount was divided among the cRNA species when various combinations were tested. Oocytes were examined by two-electrode voltage clamp over the course of 5 days post-injection.

The microinjection technique involved insertion of two electrodes, both made of glass, filled with KCl solution into an oocyte. Both electrodes were inserted into the oocyte under examination, where one of the electrodes (voltage electrode) measured the voltage of the oocytes plasma membrane while the other electrode (current electrode) was used to supply current in order to maintain a set membrane potential across the plasma membrane and a ground electrode. Inherent membrane potential of a healthy oocyte was found to be approximately -40 mV and the system was set to a -80mV clamp by the two electrodes. All the electrodes were mounted on holders, which were connected to an amplifier by silver wire coated with $AgCl_2$. Any change of potentials was visualized onto a computer screen using AxoClamp 2B and Digidata 1322A 16-bit data acquisition system (Axon Instruments, Foster City, CA), and recordings were sampled at 1 kHz using Clampex 8.1 digital oscilloscope software (Axon Instruments, Foster City, CA). After accomplishing insertions of both the electrodes and holding the membrane potential to -80 mV, the oocyte could be exposed to various potential ligand solutions in the same standard solution.

Confocal microscopy on injected oocytes: To test the surface expression of our subunits of interest, we examined the oocytes microinjected with the four subunits of interest along with *H. contortus* ancillary proteins (Hco-ric-3.1, Hco-unc-50 and Hco-unc-74) by confocal microscopy (Nikon LSM-710) after immunolabeling with specific polyclonal antibodies against smp_031680 (produced in rabbits) and smp_139330 (produced in goat) purchased from 21st Century Biochemicals (Marlboro, MA), as described by Balduzzi et al (2001) [27]. Details of the antibodies are provided in Chapter II. The oocytes were fixed in 3.7% paraformaldehyde in PBS buffer (0.01M, pH 7.4) for 3 hours, washed three times for 10 minutes each in the PBS buffer supplemented with 3% bovine serum albumin (BSA) and incubated

overnight at 4^{o}C with the specific antibodies (1:100 dilutions for both smp_031680 and smp_139330) in the BSA supplemented PBS buffer. The oocytes were then washed three times for 10 minutes each at room temperature, again with the BSA supplemented PBS buffer and incubated overnight with secondary antibodies: Alexa Fluor 488 conjugated donkey anti rabbit IgG and Alexa Fluor 594 conjugated donkey anti goat IgG both at a dilution of 1:200 in BSA supplemented PBS buffer. The oocytes were then washed 10 times, each for 5 min with PBS at room temperature before examining them by confocal microscopy. As a negative control we used oocytes injected with *H. contortus* ancillary proteins (Hco-ric-3.1, Hco-unc-50 and Hco-unc-74) but no nAChR subunits.

Expression of *S. mansoni* nAChR subunits in mammalian cells: The four full-length subunits of interest were first cloned into a mammalian expression vector pCI-neo (Promega Corp.) and verified by DNA sequencing. The constructs were subsequently modified by insertion of a fluorescent reporter (GFP or mCherry) into the predicted intracellular loop region between the third and fourth transmembrane domains (TM3-TM4 loop) of each subunit. This region was chosen based on the guidelines described by Nashmi et al [26]. In introducing the fluorescent tag, we avoided motifs that have been reported to interfere with the natural processing or activity of the ion channels. These include putative phosphorylation sites [28] and motifs associated with endocytosis [29], endoplasmic retention [30] or protein export [31], as identified by MyHits Motif Scan tool (http://myhits.isb-sib.ch/cgi-bin/motif_scan). Protein secondary structures were identified with the PredictProtein tool (https://www.predictprotein.org). Once appropriate regions were identified, we looked for available restriction sites in these areas. We chose restriction sites (nucleotide positions relative to full-length cDNA): XcmI (1262), PciI (1693), SwaI (1790) and SwaI (2171) of smp_031680, smp_139330, smp_180570 and smp_012000, respectively, for GFP or mCherry tag insertion. After linearizing the pCI-neo plasmid containing a subunit of interest with the selected restriction enzyme, we used the In-Fusion HD cloning kit for cloning GFP or mCherry into the target site. Primers used for these experiments are listed in Table 2. For transfection, HEK 293 cells were cultured in sterile humidified environment at 37^{o}C and 5% CO_2 in DMEM supplemented with 10% heat inactivated foetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies). Cells were seeded onto 12-well cell culture plates the day before transfection so that they reached approximately 60% confluency.

X-tremeGENE 9 DNA Transfection Reagent (Roche Applied Science) was used according to the manufacturers' protocol to transfect the cells with constructs of GFP- or mCherry-tagged subunits. The ratio of µl of transfection reagent to µg of plasmid DNA was optimized (2:1 to 6:1) in preliminary experiments, as recommended by the manufacturer. The transfection plates were examined with a Nikon inverted fluorescent microscope capable of detecting GFP and mCherry signals in live cells. Examination was done at different time points between 24 and 72 hours post transfection.

Results and Discussion:

cRNAs were microinjected into *Xenopus* oocytes and tested by two-electrode voltage clamp electrophysiology. A summary of the various subunit combinations tested in this project is shown in Table 3. To test for activity, the oocytes were treated with cholinergic agonists (acetylcholine, nicotine and carbachol) as well as other neuroactive substances, which are known to interact with Cys-loop ion channels, for example serotonin, dopamine, histamine, glutamate, gamma-aminobutyric acid (GABA), tyramine, octopamine, tryptamine, epinephrine, norepinephrine and glycine, each at a concentration of 1 mM. The oocytes were tested at different times post-injection up to 8 days. Unfortunately, no activity was detected in any of the samples tested. As a positive control, we tested a *Haemonchus contortus* levamisole/acetylcholine-sensitive ion channel, which is composed of Hco-unc-29.1, Hco-unc-38, Hco-unc-63a and Hco-acr-8, together with the species-specific ancillary proteins, Hco-ric-3.1, Hco-unc-50 and Hco-unc-74, as described by Boulin et al (2011) [20]. The positive control showed a typical response to acetylcholine, as expected (data not shown), suggesting the assay was performed properly. Our negative controls included water-injected oocytes or the *H. contortus* levamisole-acetylcholine channel (Hco-unc-29.1, Hco-unc-38, Hco-unc-63a and Hco-acr-8) without the species-specific ancillary proteins (Hco-ric-3.1, Hco-unc-50 and Hco-unc-74). No activity was detected in these negative controls.

One reason for the lack of channel activity could be that the schistosome subunits were not expressed in the oocytes. In order to test for expression at the protein level, we performed confocal microscopy with injected oocytes using specific antibodies against smp_031680 and smp_139330. Details of these antibodies were provided in Chapter II. The results show faint expression of both smp_031680 and smp_139330 on the surface of the injected oocytes (Fig. 3).

No immunoreactivity was detected in the negative controls (oocytes injected with *H. contortus* ancillary proteins Hco-ric-3.1, Hco-unc-50 and Hco-unc-74 but no subunits) indicating that the signal was specific.

Due to lack of signal in the Xenopus system, we explored mammalian cells (HEK 293 cells) as an alternative expression system. In preparation for these experiments, subunits were subcloned into a mammalian expression vector (pCi-neo) and the resulting constructs were modified by insertion of a fluorescent reporter (GFP or mCherry) in order to facilitate detection of protein expression in the transfected cells. The fluorescent tag was inserted into the intracellular loop between TM3 and TM4, as recommended previously [26]. A schematic of the fluorescent-tagged constructs generated for this study is shown in Fig. 4. HEK 293 cells were transfected with individual subunits first before testing subunit combinations. Observation of the live cells at 48 hour post-transfection by fluorescence microscopy revealed some expression of fluorescent- tagged subunits on the cell surface (Fig. 5). However, the expression level was generally weak for all four subunits and transfection efficiency was less than 1-5%. Further optimization is needed to improve transfection efficiency and expression levels before testing for channel activity.

Despite repeated attempts at heterologous expression of our subunits of interest, we failed to see any detectable signal in the two electrode voltage clamp experiments. We microinjected the four subunits in various combinations, with or without ancillary proteins of *H. contortus* [20] or putative ancillary proteins of *S. mansoni* (smp_029040, smp_141650), which were cloned for this study. All our attempts were unsuccessful. The confocal microscopy analysis showed that two of the subunits were expressed on the oocytes' surface but the expression levels were low.

There are two possible explanations for the absence of signal in the TEVC experiments: Either the protein expression on the surface was too low to detect activity due to differences in codon usage, inadequate folding, targeting to membrane etc, or we did not have the subunit composition required for formation of a functional channel. In this project, we co-injected oocytes with *H. contortus* ancillary proteins such as ric-3.1, unc-50 and unc-74 or putative *S. mansoni* orthologues of ric-3 and unc-50 during microinjection of the four putative nAChRs to aid adequate folding and appropriate targeting. However, the possibility remains that the lack of signal was due to differences in codon usage between *S. mansoni* and *Xenopus*. In our lab we have recently shown that a chloride-selective nAChR subunit of *S. mansoni* (smp_037690) can

form functional homomeric ion channels in both mammalian cells (HEK 293) and *Xenopus* oocytes only after optimizing the codon for mammalian expression (MacDonald et al., 2013, submitted). This gives us hope that, upon similar codon optimization, the four putative cation selective nAChRs of *S. mansoni* will form functional ion channel(s).

Our efforts to express fluorescently tagged subunits in mammalian cells need to be optimized to increase transfection/expression efficiency. Since optimizing transfection by varying the ratio between transfection reagent and plasmid did not help, one might explore electroporation as an alternative method of transfection. This is a widely used technique and is recommended for plasmids with large inserts, such as the ones used in this study (approximate size of plasmids with inserts ≈7-8 k bp). Alternatively, we can also try co-transfecting the putative nAChRs with human ric-3, which has been shown to increase the expression of specific mammalian nAChRs [32].

In conclusion, we demonstrate that putative cation-selective nAChRs of the blood fluke *S. mansoni* can be expressed in both *Xenopus* oocytes and mammalian cell expression systems but the expression levels are low and no functional channels could be detected in either system. Formation of functional ion channel(s) will require cloning and expression of additional nAChR subunits, or codon-optimization of existing subunits to improve protein expression levels. Preliminary results from mammalian expression seem hopeful. However, better plasmid delivery is required to improve expression efficiency in cultured cells. Recent developments in fluorescent protein-based membrane potential sensor systems could potentially be explored to develop new functional and pharmacological assays for studies of schistosome nAChRs.

Tabled and figures:

Antiparasitic drug	Mode of action
Benzimidazole	Inhibits tubule polymerization
DEC	Inhibits arachidonic acid meta. in microfilaria
Niclosamide	Uncouples oxidative phosphorilation
Praziquantel	*Probably by activating Ca^{2+} ion channels*
Octadepsipeptides	*Activating GPCRs & Ca^{2+} gated K^{+} channels*
Macrocyclic lactones	*Activates glutamate gated chloride channels*
Pyrantel pamoate	***Acetylcholine agonist, nicotinic acetylcholine receptor agonists***
Levamisole	***Acetylcholine agonist, nicotinic acetylcholine receptor agonists***
Morantel tartate	***Acetylcholine agonist, nicotinic acetylcholine receptor agonists***
Paraherquamide	***Acetylcholine antagonist, nicotinic acetylcholine receptor antagonists***
Monepantel	***Acetylcholine agonist, nicotinic acetylcholine receptor agonists***

Table 1: A list of antiparasitic drugs and their modes of action.

Primer name	Primer Sequence
Smp_141650F	**ATG TCT CAG ATA TCA GTT CGT G**
Smp_141650F	**TCA GGT AGA TGT AAC CAA ACG A**
Smp_029040F	**ATG ACC CTC GGA CAT TCT AC**
Smp_029040R	**TTA AAA TAA ACG GAA CTG GTA AAA ATG G**
inf031GFPF	**AAA CTA AGC CAT TTA TTG GTT GGG GTG CAG GTA GTA AAG GAG AAG AAC TTT TCA**
inf031GFPR	**TCA TAA TTC CAA CCA ATA AAT GGA CCT GCA CCT TTG TAT AGT TCA TCC ATG**
inf031MCHF	**AAA CTA AGC CAT TTA TTG GTT GGG GTG CAG GTG TGA GCA AGG GCG AG**
inf031MCHR	**TCA TAA TTC CAA CCA ATA AAA TGG ACC TGC ACC CTT GTA CAG CTC GTC CAT G**
inf139MCHF	**ATT ACA ATA TAC ATG TGG TGC AGG TGT GAG CAA GGG CGA G**
inf 39MCHR	**TAT TTC GTT TAC ATG TAC CTG CAC CCT TGT ACA GCT CGT CCA TG**
inf180GFPF	**CAT ATT ACA TAA TTT AAA TGG TGC AGG TAG TAA AGG AGA AGA ACT TTT CA**
inf180GFPR	**AAA TTA AAT TGA TTT AAA TTA CCT GCA CCT TTG TAT AGT TCA TCC ATG**
inf180MCHF	**CAT ATT ACA TAA TTT AAA TGG TGC AGG TGT GAG CAAA GGG CGA G**
inf180MCHR	**AAA TTA AAT TGA TTT AAA TTA CCT GCA CCC TTG TAC AGC TCG TCC ATG**
inf012GFPF	**TGG TGT AAC AGA TTT AAA TGG TGC AGG TAG TAA AGG AGA AGA ACT TTT CA**
inf012GFPR	**AGT TGA GAT GGA TTT AAA CCT GCA CCT TTG TAT AGT TCA TCC ATG**

Table 2: A list of cloning primers used in this project.

cRNA tested	Role	Experiments 1	2	3	4	5	6	7	8	9	10	11
Smp_031680	Putative cation-selective nAChR	✓				✓		✓	✓	✓		
Smp_139330	Putative cation-selective nAChR		✓				✓	✓	✓	✓		
Smp_180570	Putative cation-selective nAChR			✓		✓		✓	✓	✓		
Smp_012000	Putative cation-selective nAChR				✓		✓	✓	✓	✓		
hc ric-3.1, unc-50 and unc-74	Functional ancillary proteins of *H. contortus*								✓		✓	
hc unc-29.1, unc-38, unc-63, acr-8	Functional Levamisole-sensitive-AChRs of *H. contortus*										✓	✓
Smp_141650; smp_029040	Putative ric-3; unc-50 orthologues in *S. mansoni*									✓		✓

Table 3: Various complementary RNA (cRNA) combinations were microinjected into *Xenopus* oocytes and tested by two electrode voltage clamp electrophysiology. The four putative nAChRs were tested individual or in different combinations as shown. Additionally, we co-injected the subunits with previously characterized *Haemonchus contortus* ancillary proteins (ric-3, unc-50, unc-74), which aid in expression of nAChRs. Co-injections were also performed with two predicted *S. mansoni* ric-3 and unc-50 orthologues (smp_141650 and smp_029040 respectively). No ion channel activity was observed in any of the injected oocytes after treatment with cholinergic agonists (acetylcholine, nicotine, carbachol) or a panel of other neuroactive substances (serotonin, dopamine, histamine, glutamate, gamma-aminobutyric acid, tyramine, octopamine, tryptamine, epinephrine, norepinephrine and glycine). A positive control (*H. contortus* levamisole- sensitive nAChR, lane 10) was also tested and produced the expected response upon treatment with acetylcholine (not shown).

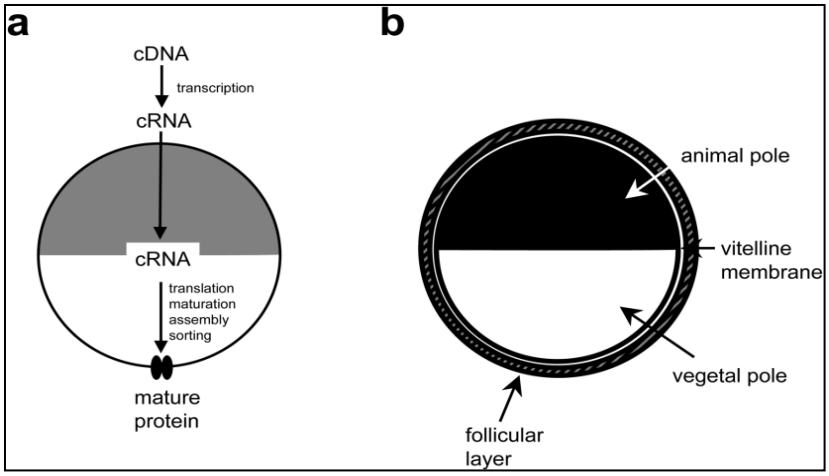


Figure 1: (a) Schematic representation of expression of ion channel subunits in *Xenopus* oocyte by microinjection of complementary RNA. (b) An intact oocyte and its various features [33].

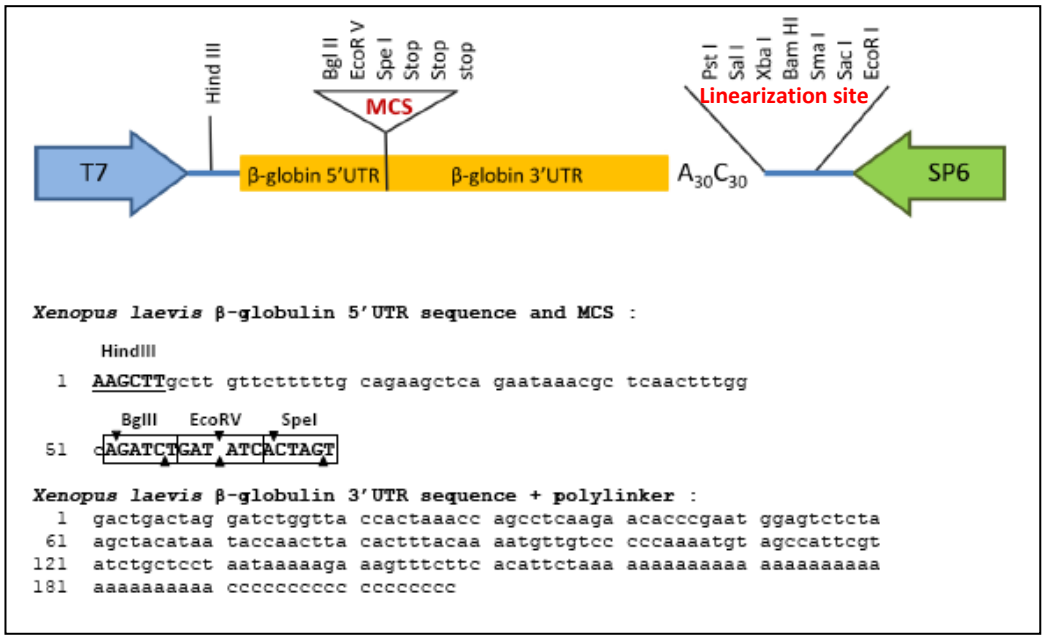


Figure 2: Vector map of *Xenopus* transcription vector pT7-TS.

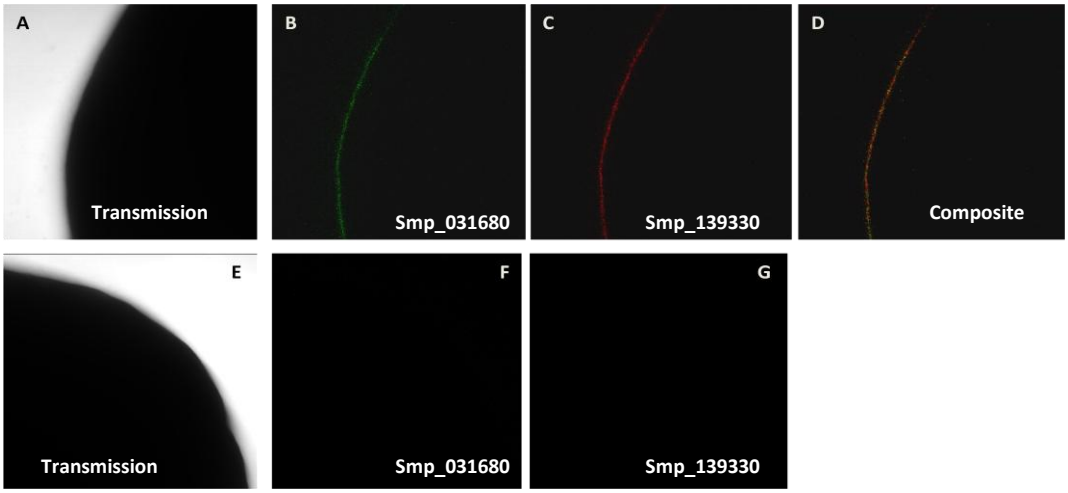
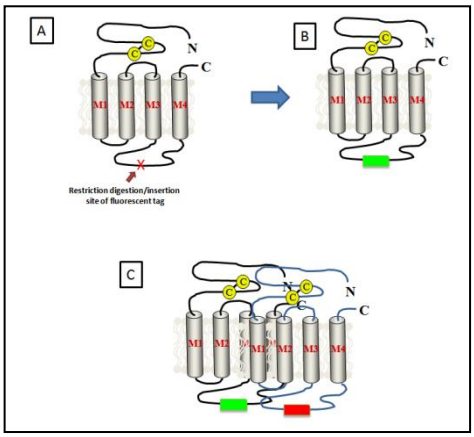


Figure 3: Confocal microscopy of injected oocytes, using antibodies against smp_031680 and smp_139330. Oocytes injected with all four subunits along with *H. contortus* ancillary proteins (A-D) were compared to oocytes injected with the *H. contortus* ancillary proteins only (E-G). Smp_031680 immunoreactivity was visualized using Alexa Fluor 488 (green), whereas smp_139330 was visualized using Alexa Fluor 594 (Red). The overlay of the signals (composite panel D) shows the two subunits expressed in close proximity on the oocyte surface. No immunofluorescence was detected in the controls lacking nAChR subunits (panels F and G).



Subunit	Tagged with
Smp_031680	GFP and mCherry separately
Smp_139330	mCherry
Smp_180570	GFP and mCherry separately
Smp_012000	GFP

Figure 4: Construction of fluorescently (GFP/mCherry) tagged nAChRs for expression in HEK293 cells. A: Determination of restriction site in the loop between third and fourth transmembrane regions. B: Insertion of fluorescent tag at this site. In total six tagged clones were generated for the four putative nAChRs so that combinations of two subunits could be co-expressed with different (GFP or mCherry) tags. C: co-expression of two differently tagged subunits to be identified by fluorescent microscopy.

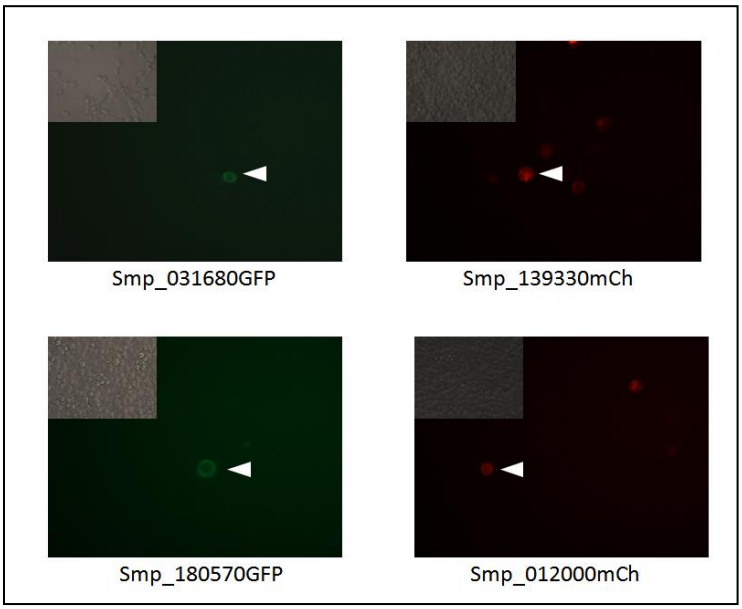


Figure 5: Expression of individual subunits in HEK 293 cells. The results show diffused but specific expression on the cell surface indicative of membrane expression.

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Final Discussion and Conclusions:

The chronic parasitic infection, schistosomiasis, is one of the most socioeconomically important parasitic diseases. The mainstay of treatment, Praziquantel, is effective at present. However, since it is the only drug available, there is an urgent need to find a viable alternative to this drug [1]. nAChRs have been demonstrated to be effective drug targets in various types of helminth parasites [2,3]. Thus they are considered promising targets for discovery of anti-schistosomal drugs. In this project we have characterized four cation-selective nAChRs (smp_031680, smp_139330, smp_180570 and smp_012000) by cloning the full-length sequences, knocking down gene expression in both larval and adult stages of the parasite and identifying the expression patterns of two subunits (smp_031680 and smp_139330) in these life stages. Attempts were made to heterologously express the four putative nAChRs in *Xenopus* oocytes, along with *H. contortus* ancillary proteins ric-3, unc-50 and unc-74, or *S. mansoni* orthologues of ric-3 and unc-50, which were cloned for this study. Moreover, the four subunits were also fluorescently tagged and sub-cloned into a mammalian expression vector in an effort to explore mammalian cells as an alternative to the conventional *Xenopus* oocyte heterologous expression system.

With the aid of bioinformatics, we demonstrated that each of the four subunits possesses a large extracellular domain containing a cys-loop, four transmembrane (TM) regions and a short extracellular tail. Structural alignment analyses of the second TM region, which is important for ion selectivity in nAChRs [4,5], showed that the four receptor subunits contain features found in cation-selective nAChRs. Furthermore, phylogenetic analyses cladded the four subunits with cation-selective nAChRs from other species. These results identify the four proteins as cys-loop nicotinic acetylcholine receptors.

Acetylcholine is a known neuromuscular transmitter in schistosomes [6] and therefore we hypothesized that the nAChRs might play a role in the control of parasite movement. To test this hypothesis, we employed a reverse-genetics approach based on RNA interference (RNAi) both in larvae and adult worms. We transfected freshly cultivated schistosomulae with either subunit-specific or non-specific (control) small interfering RNA (siRNA) and compared their motility, using an established method [7]. For the adult stage, we used electroporation to introduce siRNAs instead of liposome-based transfection since, in the literature, a similar protocol was

already found to be effective [8]. However, measuring the complex and irregular movement of adult worms was challenging. To overcome this problem, we designed a simple, yet powerful adult motility assay based on measurements of pixel displacement, using freely available software ImageJ.

In both schistosomulae and adult life stages, we demonstrated that the four subunits are required for normal motility of the parasite. In both stages, we identified a hypomotile phenotype upon knockdown of smp_031680 and smp_180570, whereas a hypermotile phenotype was observed upon knockdown of smp_139330 and smp_012000. A combination RNAi treatment of smp_031680 and smp_180570 revealed that knockdown of both subunits produced a similar hypoactive phenotype of the same magnitude, thus increasing the probability of these two subunits being part of the same ion channel. The RNAi-abrogated animals were resistant to nicotine and sensitive to mecamylamine, suggesting that this channel is normally responsive to nicotine but not mecamylamine. On the other hand, we observed an opposite phenotype upon combining smp_139330 and smp_012000 RNAi treatments (hypomotility instead of hypermotility as seen in individual treatments). This suggested that these two subunits are probably part of different ion channels and at least one of these ion channels is required to maintain motility of the parasite. Moreover, the effect of the cholinergic drugs on these RNAi treated animals suggested that the channels are sensitive to mecamylamine but not nicotine.

It should be mentioned here that although we saw significant effects on motility of the parasite, none of the RNAi treatments generated a lethal phenotype as evaluated by methylene blue dye exclusion assay. Thus the abrogation of these receptors does not have a direct lethal effect on the parasite in vitro. *In vivo*, however, the loss of receptor activity could impact on the parasite's ability to undergo its normal migration, to mature and ultimately survive within the host. The biological relevance of these receptors *in vivo* is not known at present but could be explored in future research by infecting mice with RNAi-treated schistosomulae [9].

We examined spatial expression patterns of smp_031680 and smp_139330 in 7 days old schistosomula and adult life stages by confocal microscopy using specific polyclonal antibodies and observed widespread expression of these two proteins. Smp_031680's expression was observed in the peripheral nerve net of the body in a mesh-like organization and in parenchymal cells of the larval stage, whereas in adults it was found in both central and peripheral nervous

systems of the parasite. Smp_139330 was also expressed in the peripheral nerve net of the larvae and both central and peripheral nervous systems as well as muscle fibers of the adult stage. However, despite the similarity in the distribution patterns, there was no apparent co-localization of the two subunits, consistent with the notion that these are members of different channels. The smp_031680 and smp_139330 antibodies were found to label different regions of the CNS and PNS in both larval and adult stages of the parasite. We performed similar confocal microscopic analysis with a polyclonal antibody against human ChAT, a common marker of cholinergic neurons [10], which is 33.2% similar to the putative ChAT orthologue in *S. mansoni*. ChAT immunoreactivity was observed in close proximity to the expression sites of smp_031680 and smp_139330, suggesting that the two subunits are parts of the cholinergic system of *S. mansoni.*

The RNAi analysis provided the first molecular evidence that nAChRs contribute to the control of parasite motility in *S. mansoni*. We also demonstrated for the first time that the schistosome cholinergic system can both increase and decrease parasite motility. The reason behind this dual effect can be attributed to the complexity of the nervous system and the possibility of acetylcholine having both direct and indirect effects on motor control. In the direct mechanism, acetylcholine may exert its effect via muscle-bound nAChRs, whereas the indirect effects are likely mediated by neuronal nAChRs and depolarization of neurons, resulting in neuroactive substance release to modulate motility. We observed that RNAi treatments of smp_031680 and smp_180570 yielded a hypoactive phenotype, suggesting these nAChRs are positively associated with motor activity (since their depletion reduced motility). Activation of ion channel(s) containing smp_031680 and smp_180570 subunits could cause membrane depolarization of neurons that release neuroactive substances with excitatory effects, such as serotonin and neuropeptides, which in turn stimulate motility. On the other hand, depletion of smp_139330 and smp_012000 by RNAi resulted in hypermotility and therefore these receptors appear to be negatively associated with motility. A possible explanation for this effect is that the channels containing these subunits stimulate release of inhibitory neuroactive substances, such as gamma-aminobutyric acid (GABA) and glycine, which could cause muscle relaxation in an indirect manner.

Besides expression in nervous system and muscle, smp_031680 and smp_139330 were found to be localized in the female reproductive system in the adult stage of the parasite.

Smp_031680 was found to be expressed in the ootype which is associated with egg maturation and fertilization [11]and smp_139330 was detected in the Mehlis' gland, which contributes to formation of the egg shell [12]. As a result, we expected a difference in egg production among RNAi-treated worms compared to the control group. However, we found no difference in terms of egg count or egg morphology between various specific RNAi-treated groups and the control RNAi treatment group (data not shown). It is possible the RNAi did not effectively reduce nAChR expression in these particular tissues, or the effect of the RNAi could not be detected under the conditions of our assay. It is also possible the putative nAChRs have no significant role on egg production despite their presence in female reproductive tissues.

An important objective of our study, and the reason to clone the full length subunits, was to pharmacologically characterize the ion channel(s) resulting from the four subunits of interest. However, our repeated efforts to heterologously express functional channels were unsuccessful.. In an effort to optimize conditions, we tested subunits individually and in all possible combinations. We co-injected nematode (*H.* contortus) ancillary proteins, which are known to improve nAChR folding and targeting to the membrane [13]. Moreover, we identified the orthologues of these ancillary proteins in *S. mansoni*, cloned the cDNAs and tried co-injection with these putative candidates as well. However, none of these efforts yielded a detectable signal based on two-electrode voltage clamp (TEVC) electrophysiology of injected *Xenopus* oocytes. Confocal immunofluorescence microscopy using specific antibodies against smp_031680 and smp_139330 revealed that the proteins are present on the oocytes' surface but at low level. At this point, a possible explanation for the lack of signal in the TEVC experiments is, either we do not have all the subunits to reconstitute functional ion channels, or the expression levels are too low to result in a detectable signal. Although, in many species, alpha type nAChRs result in homopentameric ion channels [14,15], this was not the case for our alpha type nAChR, smp_031680. In order to obtain functional ion channel(s), the schistosome cDNAs could be codon- optimized. We know that codon usage in flatworms is very different from vertebrate species and these differences can significantly decrease translational efficiency in the heterologous expression environment [16].In our laboratory, an alpha type anion selective *S. mansoni* nAChR was recently shown to form functional homopentameric ion channels only after optimizing the codon for mammalian expression (MacDonald et al. 2013, submitted). It is worth

optimizing the codon usage of the four subunits of interest of this project and trying microinjection of their cRNAs into *Xenopus* oocytes.

Besides heterologous expression in *Xenopus* oocytes, we also explored mammalian cells as an expression system. We fluorescently tagged and sub-cloned the four subunits into a mammalian expression vector. However, upon transfection of mammalian cells with these plasmids, we saw low expression with very poor (<5%) transfection efficiency. In order to improve the efficiency, one needs to either explore alternative methods of introducing plasmids into cells such as electroporation or by co-transfecting with mammalian ancillary protein, such as human Ric-3, which has been demonstrated to increase the expression rate [17].

In conclusion, we demonstrate that putative cation-selective nAChRs of the blood fluke *S. mansoni* are important players in controlling parasite motility. The receptors are expressed in important physiological sites such as central and peripheral nervous systems, musculature and reproductive system of the parasite, and as a result hold potential as targets for chemotherapeutic drugs. The four nAChRs studied in this project allow us a glance into the complex nature of the parasite's nervous system. The cholinergic pathway, which has been partially explored in this project, constitutes only a part of this system and we have demonstrated that interference with cholinergic signaling has significant phenotypic outcomes. This is a small, yet significant first step in elucidating the parasite's cholinergic system and identifying the receptors involved in this pathway.

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