# Exploring the Role of Cholinergic Receptors in *Schistosoma mansoni* Motor Function

**Kevin MacDonald**

Institute of Parasitology

McGill University, Macdonald Campus

Ste. Anne-de-Bellevue, Québec, Canada

January 2014

This thesis is submitted to the Faculty of Graduate Studies and Research

in partial fulfilment of the requirements of the degree of

Doctor of Philosophy

## Abstract

Schistosomiasis is a debilitating neglected tropical disease that affects hundreds of millions of people in developing tropical and subtropical areas of the world. As a long-term chronic infection, schistosomiasis causes significant annual mortality and morbidity, as well as economic losses. The causative agents of the disease are flatworms of the genus *Schistosoma*, which are vectored by several snail intermediate hosts into humans. Once inside the human host, schistosomes undergo a complex migratory and developmental period before maturing into adult worms. A key regulator of this process is the parasite nervous system, which controls a variety of essential biological functions in both larval and adult schistosomes. Signal transduction in the nervous system results from the interaction between neurotransmitters and their cognate receptors. In vertebrates, acetylcholine (ACh) is the quintessential excitatory neurotransmitter of the neuromuscular system. In schistosomes, on the other hand, there is evidence that it plays an inhibitory role in parasite motility. This divergence from the vertebrate mode of action suggests that the receptors responsible for mediating this activity may serve as good chemotherapeutic targets. To date, no schistosome cholinergic receptor has been characterized at the molecular level or linked to neuromuscular inhibition. In the present work, we demonstrate the existence of a novel family of schistosome cholinergic receptors that modulate inhibitory neuromuscular responses. Using bioinformatics, we identified five putative anion-selective acetylcholine receptor subunits (SmACCs) in the genome of *S. mansoni*. Silencing of these subunits in larval schistosomula using RNAi led to a hyperactive phenotype consistent with the removal of an inhibitory neuromuscular modulator. Immunolocalization studies indicated that two of these subunits are located in the peripheral nervous system of the parasite, close to sites of ACh-release, and may participate in the indirect regulation of motor function. Heterologous expression studies showed that one subunit, SmACC-1, forms a functional nicotinic chloride channel. Characterization of SmACC-1 using a mammalian cell-based assay also represents a new tool for high-throughput drug screens of parasite ion channels. Following on the characterization of cholinergic signaling through nicotinic receptors, we then examined the lone G protein-coupled acetylcholine receptor predicted in the schistosome genome. SmGAR was cloned from larval *S. mansoni* and

functionally characterized in a yeast expression system. SmGAR is selectively activated by cholinergic agonists and displays high levels of constitutive activity. Sequence analysis and homology modeling indicate the presence of several amino acid substitutions that are linked to agonist-independent signaling, suggesting that SmGAR may display wild-type constitutive activity *in vivo*. Furthermore, an RNAi behavioral screen confirmed that SmGAR does modulate early larval motor function in schistosomes, although its mechanism of action remains unclear. In sum, we have identified two cholinergic signaling systems in schistosomes that are involved in neuromuscular signal transduction. The unique characteristics of the receptors involved in this pathway and their essential role in controlling parasite larval motility suggest that schistosome cholinergic receptors merit further investigation as novel drug targets.

## Abrégé

La schistosomiase est une maladie tropicale négligée débilitante affectant des centaines de millions de personnes vivant dans les pays en développement situés dans les zones tropicales et subtropicales du monde. Cette infection chronique est associée à un taux de mortalité significatif et est une cause importante de morbidité, en plus d'entraîner des pertes économiques considérables dans les communautés affligées. Les agents étiologiques de cette maladie sont des vers plats du genre *Schistosoma*, lesquels sont transmis aux humains via plusieurs espèces d'escargots agissant comme hôtes intermédiaires. Une fois à l'intérieur de l'hôte humain, les schistosomes entament un processus complexe de migration et de développement les menant à leur maturation en vers adultes. Le système nerveux du parasite joue un rôle régulateur clé dans ce processus, puisqu'il contrôle une panoplie de fonctions biologiques essentielles tant chez le stade larvaire que chez le schistosome adulte. La transduction de signal dans le système nerveux est le résultat de l'interaction spécifique entre les neurotransmetteurs et leurs récepteurs. Chez les vertébrés, l'acétylcholine (ACh) est le neurotransmitteur le plus important pour la modulation de la transmission synaptique excitatrice du système

neuromusculaire. Chez les schistosomes, en revanche, certains indices suggèrent que l'ACh joue plutôt un rôle inhibiteur dans la motilité du parasite. Cette divergence de mode d'action comparativement aux vertébrés suggère que les récepteurs modulant l'effet inhibiteur de l'ACh pourraient constituer des cibles thérapeutiques intéressantes. À ce jour, aucun des récepteurs cholinergiques identifiés chez les schistosomes n'a été caractérisé au niveau moléculaire. En outre, aucun lien n'a été établi entre ces récepteurs et l'effet inhibiteur de l'ACh sur l'activité neuromusculaire. Les résultats présentés dans le cadre de cette thèse démontrent l'existence d'une nouvelle famille de récepteurs cholinergiques impliqués dans la modulation inhibitrice des réponses neuromusculaires chez les schistosomes. Une analyse bio-informatiques du génome de *S. mansoni* nous a permis d'identifier cinq gènes encodant des sous-unités putatives de récepteurs ACh perméables aux anions (SmACCs). Des expériences d'interférence de l'ARN (RNAi) ciblant ces sous-unités, réalisées dans le stade larvaire des schistosomes (schistosomule), induisent un phénotype hyperactif cohérent avec la suppression de la modulation inhibitrice des réponses neuromusculaires par l'ACh. Des études d'immunolocalisation et de microscopie confocale montrent également que deux de ces sous-unités sont exprimées dans le système nerveux périphérique du parasite, à proximité des sites de sécrétion de l'ACh. Ces sous-unités pourraient participer indirectement à la régulation des fonctions motrices. Nos expériences d'expression hétérologues montrent par ailleurs que l'une de ces sous-unités, SmACC-1, forme un récepteur nicotinique fonctionnel perméable aux ions $Cl^-$. L'utilisation d'un essai basé sur un système d'expression en culture cellulaire afin de caractériser SmACC-1 constitue en outre un nouvel outil pour le criblage à haut débit de composés d'intérêt pharmaceutique ciblant les canaux ioniques de parasites. Suite à la caractérisation de la signalisation cholinergique médiée par les récepteurs nicotiniques, nous avons examiné l'unique récepteur cholinergique couplé aux protéines G (G protein-coupled acetylcholine receptor, SmGAR) prédit dans le génome des schistosomes. Nous avons cloné SmGAR à partir du stade larvaire de *S. mansoni*, puis nous avons procédé à la caractérisation fonctionnelle de ce récepteur dans un système d'expression en levure. SmGAR est activé de manière spécifique par divers agonistes cholinergiques et possède une activité constitutive élevée. L'analyse de la séquence protéique et la

modélisation par homologie de SmGAR révèlent la présence de plusieurs substitutions d'acides aminés associés aux voies de signalisations indépendantes des agonistes, ce qui pourrait expliquer le que niveau basal d'activité de SmGAR observé *in vivo*. En outre, une analyse phénotypique par interférence de l'ARN nous a permis de confirmer que SmGAR module la fonction motrice des schistosomes et ce, dès le stade précoce du développement larvaire, bien que le mécanisme d'action ne soit pas encore défini. En somme, nous avons identifié deux systèmes distincts de signalisation cholinergique chez les schistosomes, tous deux impliqués dans la transduction de signal au niveau du système neuromusculaire. Les caractéristiques uniques des récepteurs impliqués dans cette voie de signalisation, combiné au rôle primordial qu'ils jouent dans le contrôle de la motilité chez les larves du parasite, suggèrent que l'étude des récepteurs cholinergiques des schistosomes en tant que nouvelles cibles thérapeutiques potentielles mérite d'être approfondie.

# Acknowledgements

I would first like to thank my supervisor Dr. Paula Ribeiro for giving me the opportunity to pursue my Ph.D. at the Institute of Parasitology. She has been a great mentor and resource for scientific knowledge, as well as a source of encouragement and guidance.

I would also like to thank my advisory committee, Dr. Tim Geary and Dr. Roger Pritchard for their continued support and advice concerning my academic and professional goals.

I owe a tremendous debt of gratitude to all of the academic and support staff at the Institute of Parasitology, as well as my labmates and fellow graduate students. The organizational and technical support provided by Shirley, Christiane and Gordon is unparalleled. My labmates, Nelly, Anastasia, Vitalie and Moe were a genuine pleasure to work with and I could not have been successful without their help and support. I am also especially grateful to Dr. Petra Rohrbach for allowing me to commandeer her confocal microscope and to Vanessa Dufour for her endless patience in teaching me electrophysiology methods.

Finally, I would like to thank my parents and my wife, Nicola. Without their love and support, I would be lost.

## Thesis Office Statement

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

If this option is chosen, **connecting texts, providing logical bridges between the different papers are mandatory.**

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

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

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

## Statement of Contributions

All experimental work presented in this thesis was performed by the author, under the supervision of Dr. Paula Ribeiro. Dr. Ribeiro also participated in the experimental design, presentation of data and pre-editing of manuscripts and the thesis. S. Buxton provided assistance with functional expression of cholinergic channels in *Xenopus* oocytes and electrophysiology. Financial support was provided by Dr. Ribeiro through a collaborative grant with Dr. M. Kimber and Dr. T. Day.

# Statement of Originality

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

*Manuscript I:*

Here, we describe a novel family of acetylcholine-gated chloride channels in *Schistosoma mansoni*. Bioinformatics analysis identified five schistosome nicotinic acetylcholine receptor subunits with a motif consistent with anion-selectivity, which we termed *S. mansoni* acetylcholine-gated chloride channels (SmACCs). These receptor subunits are unrelated to the *C. elegans* ACC genes and have a highly conserved ligand-binding pocket, suggesting a true nicotinic pharmacological profile. Silencing of SmACC genes resulted in hyperactive motor phenotypes in larval schistosomula, suggesting they play an inhibitory role in neuromuscular function. Characterization of one subunit, SmACC-1 was performed using a fluorescence-based functional assay in mammalian cells and demonstrates that SmACC-1 forms a functional chloride channel with nicotinic pharmacology. This represents the first characterization of a flatworm ion channel using a mammalian cell-based assay and opens the door for the development of high-throughput drug screening against parasite receptors. Finally, localization studies determined that two SmACC subunits are expressed in the nervous system in close proximity to sites of ACh-release and do not appear to be directly associated with body-wall muscle. This indicates an inhibitory modulatory role for these receptors. Combined, these results confirm the expression of anion-selective nicotinic channels that indirectly exert inhibitory modulation over schistosome neuromuscular function and strengthen their candidacy as novel drug targets.

*Manuscript II*

In this study, we describe the cloning and functional characterization of a novel G protein-coupled acetylcholine receptor in *Schistosoma mansoni* (SmGAR). This is the first receptor of this type ever to be cloned and characterized from any flatworm species.

In a heterologous expression system, SmGAR was selectively activated by cholinergic agonists but displayed high levels of agonist-independent signaling. The constitutive activity of SmGAR was inhibited by cholinergic drugs, atropine and promethazine, in a concentration-dependent manner, suggesting these drugs have inverse agonist activity towards the parasite receptor. Sequence analysis and homology modeling show several structure-altering amino acid substitutions in SmGAR, suggesting the possibility that the wild-type receptor may be constitutively active *in vivo*. Behavioral analysis and RNAi experiments also demonstrated that SmGAR modulates motor function in very early stage larval schistosomula. Together, these data provide the first evidence for the involvement of muscarinic signaling in modulating the motor function of parasitic flatworms.

## Table of Contents

# List of Figures

## List of Abbreviations

5-HT: 5-hydroxytryptophan

α-BTX: alpha bungarotoxin

AC: adenylate cyclase

ACC: acetylcholine-gated chloride channel

ACh: acetylcholine

AChE: acetylcholinesterase

cAMP: cyclic adenosine monophosphate

ChAT: choline acetyltransferase

CNS: central nervous system

ECD: extracellular domain

EST: expressed sequence tag

GABA: γ-aminobutyric acid

GAR: G-protein-linked acetylcholine receptor

GDP: guanosine diphosphate

GPCR: G-protein-coupled receptor

GTP: guanosine triphosphate

ICD: intracellular domain

LGIC: ligand-gated ion channel

mAChR: muscarinic acetylcholine receptor

MDA: mass drug administration

nAChR: nicotinic acetylcholine receptor

PCR: polymerase chain reaction

PLC: phospholipase C

PNS: peripheral nervous system

PTX: Pertussis toxin

PZQ: praziquantel

RNAi: RNA-interference

SmACC: *Schistosoma mansoni* acetylcholine-gated chloride channel

SmGAR: *Schistosoma mansoni* G-protein-linked acetylcholine receptor

TMD: transmembrane domain

YFP: yellow fluorescent protein

# Introduction

Second to the soil-transmitted helminths, schistosomiasis is the most prevalent parasitic worm infection in the developing world (Hotez *et al*., 2010). Affecting some 200 million people, the disease is most common in highly impoverished areas of sub-Saharan Africa, although intense foci of infection also exist in Asia and Brazil (Hotez and Kamath, 2009, Amaral *et al*., 2006, Bergquist and Tanner, 2010). The causative agents of the disease are blood-dwelling trematodes from the genus *Schistosoma*. There are five medically important species of schistosomes, *S. mansoni*, *S. japonicum*, *S. haematobium*, *S. intercalatum* and *S. mekongi*. All species except *S. haematobium* reside in the mesenteric venules and cause intestinal schistosomiasis. The urinary disease-causing *S. haematobium* prefer to live in the perivesical venules of the urinary bladder. The main pathologies related to the disease are chronic in nature and are caused by the accumulation of eggs released by adult female worms. Intestinal schistosomiasis is caused fibrotic and granulomatous immune reactions to the eggs in the affected tissues leading to portal hypertension and hepatosplenic disease (Gryseels, 2012). Urinary schistosomiasis has been associated with squamous cell carcinoma and increased susceptibility to HIV infection (Secor, 2012).

Treatment options for schistosomiasis are limited. There is no vaccine currently available and the WHO recommends the use of a single drug, praziquantel (PZQ). Mass drug administration programs utilizing PZQ have had some measure of success in reducing infection among endemic populations (Mo *et al*., 2014). However, this strategy has two major drawbacks. First, the dissemination of a single therapeutic compound in widespread treatment programs raises the possibility of selecting for drug resistant parasites. Several areas have reported reduced cure rates when using PZQ to treat schistosomiasis (Greenberg, 2013). In addition to concerns over emerging resistance, the effectiveness of PZQ against susceptible parasites is also limited. Sabah and colleagues demonstrated that while PZQ is effective in killing adult schistosomes, it has much lower efficacy against the migratory, larval stages (Sabah *et al*., 1986). The limitations of PZQ outlined above and the lack of a robust drug discovery pipeline

(Greenberg, 2013) highlight the dire need to develop novel therapeutics for the treatment of schistosomiasis.

Despite its shortcomings, PZQ does offer some clues that aid in the search for effective new drug targets. The exact molecular target of PZQ is still unknown. However its physiological effects, such as spastic muscle contractions (Xiao *et al*., 1985) and its interaction with voltage-gated $Ca^{2+}$ channels (Redman *et al*., 1996, Greenberg, 2005, Doenhoff *et al*., 2008) point to the schistosome nervous system. Evidence for the richness of targets in the nervous system is further strengthened by the success of several antinematodal drugs, such as ivermectin, pyrantel, levamisole and metrifonate (Maule *et al*., 2005). A major reason that the nervous system presents such a good drug target lies in the basic biology of flatworms. As acoelomates (lacking a body cavity), flatworms rely on neuronal signal transduction and paracrine signaling to control all essential biological functions, such as feeding, reproduction and host attachment. Furthermore, motor function is vital for the developmental cycle of schistosomes, as larval migration through the host is linked to parasite maturation (Maule *et al*., 2005). Interruption of this process by disrupting motor function presents an attractive chemotherapeutic target because it eliminates the worms before they reach the pathology-inducing (i.e. egg-producing) adult stage.

Identification and characterization of schistosome nervous system targets focuses mainly on the receptors that mediate neurotransmission. Neuroactive substances in schistosomes can be broadly classified into two groups, the small-molecule neurotransmitters (biogenic amines, glutamate, gamma-aminobutyric acid (GABA) and acetylcholine) and the neuropeptides (Ribeiro *et al*., 2005, McVeigh *et al*., 2012). Both signal via neuroreceptors that fall into one of two classes, the G protein-coupled receptors (GPCRs) and the ligand-gated ion channels (LGICs). Several biogenic amine GPCRs have been cloned and characterized in schistosomes (Hamdan *et al*., 2002, Taman and Ribeiro, 2009, El-Shehabi and Ribeiro, 2010, El-Shehabi *et al.*, 2012, Patocka et al, 2014). Several of these receptors play important roles in schistosome motor function (Ribeiro *et al*., 2012). More recently, a glutamate-gated chloride channel was also identified in S. mansoni (Dufour *et al*., 2013). These studies

represent a growing understanding of parasite neuroreceptors as they pertain to motor function. However, there is very limited knowledge about one of the most important neuromuscular modulators across phylogeny, the cholinergic system.

Signaling via cation-selective ion channels, acetylcholine (ACh) is the classical excitatory neurotransmitter at the neuromuscular junction of vertebrates and some invertebrate organisms. In schistosomes, however, ACh seems to have the opposite effect on muscular contraction. Early pharmacological studies determined that treatment of adult schistosomes with ACh causes muscular relaxation and flaccid paralysis that is consistent with neuromuscular inhibition (Barker *et al*., 1966). Activation of nicotinic ACh receptors (nAChRs) on schistosome muscles was found to be the cause of this flaccid paralysis (Day *et al*., 1996) but subsequent attempts to characterize cloned schistosome nAChRs were unsuccessful (Bentley *et al*., 2004). Later research in the model nematode *Caenorhabditis elegans* identified a possible mechanism for the apparent inhibitory action of some invertebrate nAChRs, the ACh-gated chloride channels (ACCs) (Putrenko *et al*., 2005). Although, the functional mechanism and full pharmacological profile of ACCs are still unclear, their high divergence from vertebrate cation-selective nAChRs makes them an attractive potential anthelminthic target. This is especially true, given the evidence of their presence in a wide variety of helminth parasites (Beech *et al*., 2013).

Similar to vertebrates, a second type of ACh receptor also plays a role in invertebrate neurotransmission. Muscarinic receptors (mAChRs) are GPCRs that may signal through excitatory or inhibitory pathways and indirectly modulate motor activity. In nematodes, three mAChRs (GAR-1, GAR-2 and GAR-3) have been cloned and characterized (Lee *et al*., 1999, Lee *et al*., 2000, Hwang *et al*., 1999). Suppression of *C. elegans* GARs by RNA-interference (RNAi) leads to behavioral phenotypes that suggest involvement in both sensory and motor function (Dittman and Kaplan, 2008). Evidence for the function of mAChRs in schistosomes, however, is conflicting. Behavioral studies indicate that classical muscarinic agonists and antagonists are ineffective on adult schistosomes (Barker *et al*., 1966, Day *et al*., 1996). However, annotation of the *S.*

*mansoni* genome suggests the presence of at least one mAChR (Berriman *et al*., 2009, Protasio *et al*., 2012).

The action of nAChRs and mAChRs as important inhibitory neuromodulators of *S. mansoni* motor function forms the central hypothesis of this thesis. Basic evidence supporting nAChR-mediated inhibition of schistosome muscular contraction exists at the behavioral level in adult worms. Genome analysis suggests the expression of anion-selective receptors capable of controlling this process. However, the schistosome cholinergic receptors responsible for this behavior have never been cloned or functionally characterized. Furthermore, the role of cholinergic neurotransmission in larval motility has never been confirmed. Therefore, the main goal of this study was the identification and functional characterization of schistosome cholinergic receptors, focusing on putative inhibitory nAChRs and the lone annotated mAChR. In addition to the pharmacological characterization of cholinergic receptors, we sought to define their inhibitory role in larval schistosomes by using RNA interference to directly link receptor activity to motor function. Finally, we performed immunolocalization studies to identify expression patterns of cholinergic receptors in schistosome tissue.

The results presented here provide the first molecular evidence of functional inhibitory nAChRs in *S. mansoni* and confirm their role as modulators of larval motor function (Manuscript I). Bioinformatics analysis led to the identification of five putative anion-selective nAChR subunits in schistosomes, which we termed *Schistosoma mansoni* acetylcholine-gated chloride channels (SmACCs). Abrogation of SmACC function both pharmacologically and by RNAi led to hyperactive motor phenotypes in *S. mansoni* schistosomula that were consistent with the removal of an inhibitory neuromodulator. Two subunit genes, SmACC-1 and SmACC-2 were selected for immunolocalization. Both subunits localized to the peripheral nervous system of adult and larval schistosomula, indicating that the receptors formed by these subunits mediate their motor effects in an indirect manner, through modulation of neuromuscular signalling rather than direct effects on muscle. A novel, fluorescence-based functional assay was used to characterize SmACC-1 in a heterologous expression system. SmACC-1 subunits form a homomeric chloride channel that is selectively activated by

cholinergic agonists, including nicotine, in a concentration-dependent manner. To our knowledge, this represents the first characterization of a parasite ion channel using a mammalian cell-based expression system and is a significant step toward the development of high-throughput drug screening assays for parasite receptors.

In the second manuscript, we turned our attention to the lone muscarinic receptor encoded in the *S. mansoni* genome, SmGAR (Manuscript II). We cloned SmGAR from early stage schistosomula and expressed the protein in a heterologous system, showing that it forms a functional GPCR with a cholinergic pharmacological profile. SmGAR displayed high levels of wild-type constitutive activity that were modulated by the muscarinic antagonist atropine and the partial cholinergic antagonist promethazine. We then used RNAi to assess the effects SmGAR on larval motility. Surprisingly, suppression of SmGAR expression caused a reduction in the motility of early stage schistosomula, although the mechanism of this behavior is still unclear. In sum, these studies contribute new knowledge about the cholinergic signalling of *S. mansoni* by confirming the inhibitory role nAChRs in schistosome motility and providing the first molecular evidence of mAChR function in any flatworm, free-living or parasitic.

## References


1. Amaral RS, Tauil PL, Lima DD, Engels D (2006). An analysis of the impact of the Schistosomiasis Control Programme in Brazil. Mem Inst Oswaldo Cruz **101** (Suppl 1): 79-85.

2. Barker, LR, Bueding E, Timms AR (1966). The possible role of acetylcholine in *Schistosoma mansoni*. Brit J Pharmacol **26**: 656-665.

3. Beech RN, Callanan MK, Rao VT, Dawe GB, Forrester SG (2013). Characterization of cys-loop receptor genes involved in inhibitory amine neurotransmission in parasitic and free living nematodes. Parasitol Int **62**(6): 599-605.

4. Bentley GN, Jones AK, Oliveros Parra WG, Agnew A (2004). ShAR1alpha and ShAR1beta: novel putative nicotinic acetylcholine receptor subunits from the platyhelminth blood fluke *Schistosoma*. Gene **329**: 27-38.

5. Bergquist R, Tanner M (2010). Controlling schistosomiasis in Southeast Asia: a tale of two countries. Adv Parasitol **72**: 109-44.

6. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, et al. (2009). The genome of the blood fluke *Schistosoma mansoni*. Nature **460**: 352–358.

7. Day TA, Chen GZ, Miller C, Tian M, Bennett JL, Pax RA (1996). Cholinergic inhibition of muscle fibers isolated from *Schistosoma mansoni* (Trematoda: Digenea). Parasitology **113**(Pt. 1): 55-61.

8. Dittman JS, Kaplan JM (2008). Behavioral impact of neurotransmitter-activated G-protein-coupled receptors: muscarinic and GABAB receptors regulate Caenorhabditis elegans locomotion. J Neurosci **28**(28): 7104-12.

9. Doenhoff MJ, Cioli D, Utzinger J (2008). Praziquantel: mechanisms of action, resistance and new derivatives for schistosomiasis. Curr Opin Infect Dis **21**(6): 659-67.

10. Dufour V, Beech RN, Wever C, Dent JA, Geary TG (2013). Molecular Cloning and Characterization of Novel Glutamate-Gated Chloride Channel Subunits from *Schistosoma mansoni*. PLoS Pathog **9**(8): e1003586.

11. El-Shehabi F, Taman A, Moali LS, El-Sakkary N, Ribeiro P (2012). A novel G protein-coupled receptor of *Schistosoma mansoni* (SmGPR-3) is activated by dopamine and is widely expressed in the nervous system. PLoS Negl Trop Dis **6**(2): e1523.

12. Greenberg RM (2005). Are Ca2+ channels targets of praziquantel action? Int J Parasitol **35**(1): 1-9.

13. Greenberg RM (2013). New approaches for understanding mechanisms of drug resistance in schistosomes. Parasitology **140**(12): 1534-46.

14. Gryseels B (2012). Schistosomiasis. Infect Dis Clin North Am **26**(2): 383-97.

15. Hamdan FF, Abramovitz M, Mousa A, Xie J, Durocher Y, Ribeiro P (2002). A novel Schistosoma mansoni G protein-coupled receptor is responsive to histamine. Mol Biochem Parasitol **119**(1): 75-86.

16. Hotez PJ, Bethony JM, Diemert DJ, Pearson M, Loukas A (2010). Developing vaccines to combat hookworm infection and intestinal schistosomiasis. Nat Rev Microbiol **8**(11): 814-26.

17. Hotez PJ, Kamath A (2009). Neglected tropical diseases in sub-saharan Africa: review of their prevalence, distribution, and disease burden. PLoS Negl Trop Dis **3**(8): e412.

18. Hwang JM, Chang DJ, Kim US, Lee YS, Park YS, *et al*. (1999). Cloning and functional characterization of a *Caenorhabditis elegans* muscarinic acetylcholine receptor. Receptors Channels **6**: 415–424.

19. Lee YS, Park YS, Chang DJ, Hwang JM, Min CK, *et al*. (1999). Cloning and expression of a G protein-linked acetylcholine receptor from *Caenorhabditis elegans*. J Neurochem **72**: 58–65.

20. Lee YS, Park YS, Nam S, Suh SJ, Lee J, *et al*. (2000). Characterization of GAR-2, a novel G protein-linked acetylcholine receptor from *Caenorhabditis elegans*. J Neurochem **75**: 1800–1809.

21. Maule AG, Day TA, Chappell CH (2005). Parasite neuromusculature and its utility as a drug target. Parasitology **131**: S1-S2.

22. McVeigh P, Kimber MJ, Novozhilova E, Day TA (2005). Neuropeptide signalling systems in flatworms. Parasitology **131**: S41-55.

23. Mo AX, Agosti JM, Walson JL, Hall BF, Gordon L (2014). Schistosomiasis elimination strategies and potential role of a vaccine in achieving global health goals. Am J Trop Med Hyg **90**(1): 54-60.

24. Protasio AV, Tsai IJ, Babbage A, Nichol S, Hunt M (2012). A systematically improved high quality genome and transcriptome of the human blood fluke *Schistosoma mansoni*. PLoS Negl Trop Dis **6**(1): e1455.

25. Putrenko I, Zakikhani M, Dent JA (2005). A Family of Acetylcholine-gated Chloride Channel Subunits in *Caenorhabditis elegans*. J Biol Chem **280**: 6392-6398.

26. Redman CA, Robertson A, Fallon PG, Modha J, Kusel JR, Doenhoff MJ, Martin RJ (1996). Praziquantel: an urgent and exciting challenge. Parasitol Today **12**(1): 14-20.

27. Ribeiro P, El-Shehabi F, Patocka N (2005). Classical transmitters and their receptors in flatworms. Parasitology **131**: S19-S40.

28. Ribeiro P, Gupta V, El-Sakkary N (2012). Biogenic amines and the control of neuromuscular signaling in schistosomes. Invert Neurosci **12**(1): 13-28.

29. Secor WE (2012). The effects of schistosomiasis on HIV/AIDS infection, progression and transmission. Curr Opin HIV AIDS **7**(3): 254-9.

30. Taman A, Ribeiro P (2009). Investigation of a dopamine receptor in *Schistosoma mansoni*: Functional studies and immunolocalization. Mol Biochem Parasitol **168**: 24–33.

31. Xiao SH, Catto BA, Webster LT Jr. (1985). Effects of praziquantel on different developmental stages of Schistosoma mansoni in vitro and in vivo. J Infect Dis **151**(6): 1130-7.

# Chapter 1: Literature Review

# 1. Literature Review

## A. Schistosomes

### *A.1 Schistosomes- General Biology*

Schistosomes are metazoan parasitic flatworms belonging to the phylum Platyhelminthes and the class Trematoda. There are over 18 species of *Schistosoma*, which infect mammalian and avian hosts. Five of these species are of medical importance: *S. mansoni*, *S. japonicum*, *S. haematobium*, *S. intercalatum*, and *S. mekongi* (Rollinson and Southgate, 1987, WHO, 2007). Four of these species parasitize the mesenteric portal veins of their human hosts, while *S. haematobium* prefers to live in the venous plexuses of the urinary bladder.

Schistosomes are the only dioecious members of the class Trematoda. Males typically range from 10-20 mm long and females from 15-25 mm (Webbe 1982). Adult female schistosomes reside in the gynaecophoric canal of the male during copulation and oviposition. Males may have between 4-13 testes, which lie posterior to the ventral sucker. Females have a single ovary, found in the posterior half of the worm. Females may produce between 20-3500 non-operculated eggs per day, dependent upon species (Webbe, 1982).

All basic features of the subclass Digenea, including bilateral body symmetry and oral and ventral suckers are present in schistosomes. They lack a muscular pharynx and have a blind digestive system consisting of an oesophagus that extends into bifurcated caecae, which reunite posterior to the ventral sucker. A protective syncytial tegument covers the outer layer of the worm, which is unique to schistosomes (McLaren and Hockley, 1977). The tegument protects the parasite from immune attack and also plays an important role in the transport of nutrients, such as glucose and amino acids (Skelly *et al*, 1999).

### *A.2 Life Cycle*

All schistosomes of medical importance share a similar invertebrate-vertebrate host life cycle. The intermediate hosts of *Schistosoma sp.* are snails and the definitive hosts may be either mammalian or avian. The genus of the intermediate host varies and is species-specific, although experimental infections are possible under laboratory conditions. The most important intermediate snail hosts are members of the genera *Biomphalaria* (the host of *S. mansoni*) and *Bulinus* (the host of *S. haematobium*). Snails of the genus *Oncomelania* are the intermediate host of *S. japonicum*.

The life cycle of schistosomes begins with the release of eggs from the adult female (Figure 1). Different egg morphology exists, depending upon the species of schistosome. The eggs of *S. mansoni* have a lateral spine and measure 114-175 μm in length by 45-68 μm in diameter. *S. haematobium* eggs are of similar size, however they have a terminal spine. The eggs of *S. japonicum* are smaller than both and spineless (Beaver *et al*, 1984).

Eggs are oviposited into the small venules of the submucosa and contain a maturing miracidium. As the eggs grow, they travel through the wall of the venules and into the lumen of the gut. The eggs are then passed out of the definitive host with the faeces, often into a water source. Upon contact with fresh water, the eggs absorb water and swell, causing them to hatch and release the miracidium. The miracidium is the free-swimming stage of the parasite and may survive for up to 16 hours without a host (Beaver *et al*, 1984). The miracidium then locates an appropriate snail host and penetrates its shell.

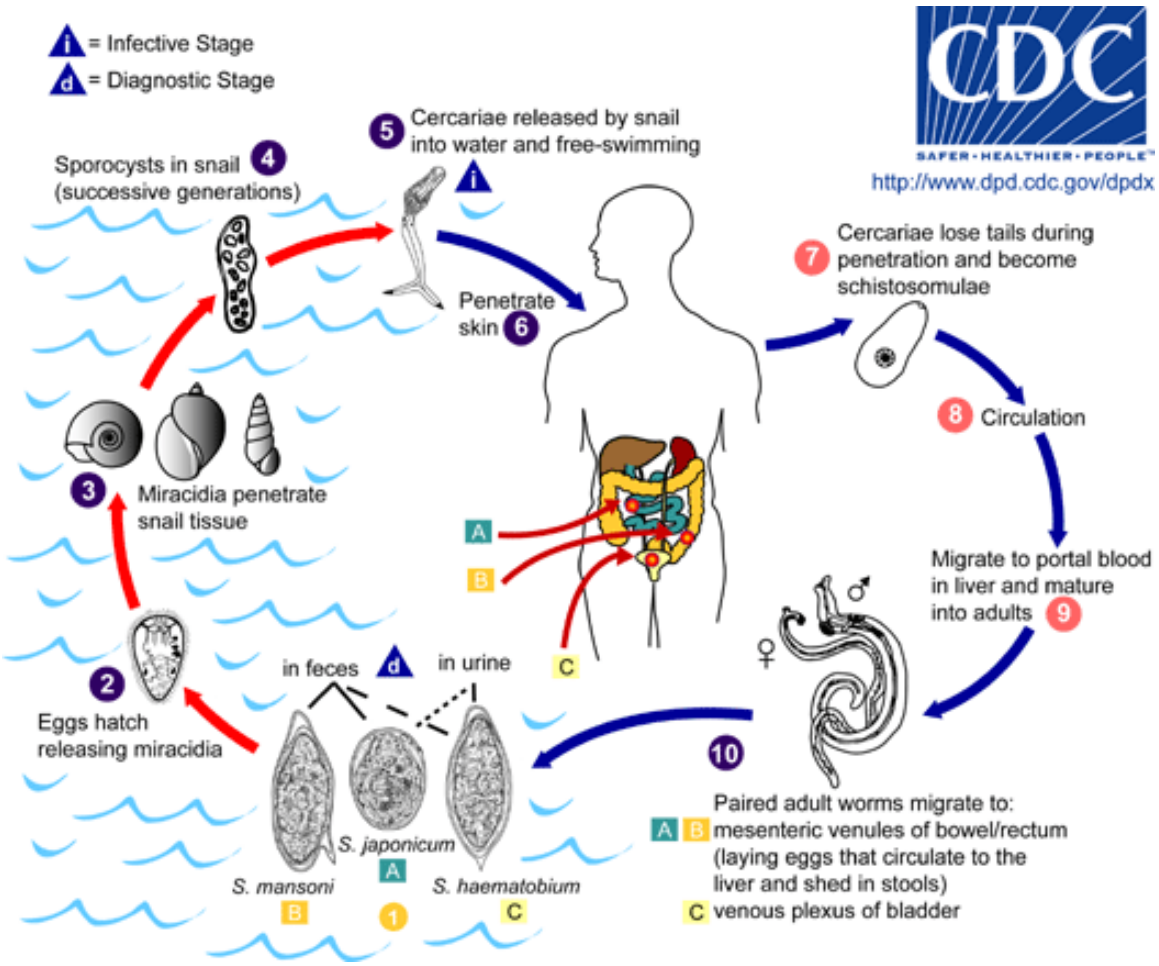


Figure 1: The life cycle of human schistosomes (*S. mansoni, S. haematobium, S. japonicum*).
Credit: CDC/DPDx

Once the miracidium has penetrated the snail, it sheds its epithelium and differentiates into a mother sporocyst. The mother sporocyst then differentiates into daughter sporocysts, inside which cercariae begin to develop. After a period of 4-6 weeks, the cercariae begin to emerge from the intermediate host and are released into the surrounding water. The bodies of cercariae are approximately 175-250 μm in length with bifurcated tails ranging from 235-350 μm. Cercariae are able to survive without a host for 1-3 days. Once the cercaria has found a mammalian host, it penetrates the skin and drops its tail, transforming into a schistosomulum. The schistosomulum then migrates to the circulatory system, where it is carried to the lungs and then the heart (Wilson *et al*, 1978). From the heart, the schistosomulum is carried to the liver. The mature worm then

migrates to the mesenteric portal vessels, or the venous plexuses of the bladder (Wilson *et al*, 1978). Once at their final destination, adult worms form couples and begin the cycle anew.

### *A.3 Pathogenesis*

There are three phases of pathogenesis during infection with schistosomes: the prepatent phase, the acute phase and the chronic phase. The majority of pathology caused by schistosomiasis occurs in the latter two phases, as a result of the host immune reaction to the production of eggs by the adult worms. The prepatent phase occurs between 5 days and 4 weeks after the penetration of the skin by cercariae. During this stage, the infected host may exhibit an urticarial rash, fever and occasionally diarrhea. The acute phase of the infection occurs approximately 4-10 weeks after infection and is seldom seen in patients from endemic areas (Caldas *et al*, 2008). Acute schistosomiasis (also known as Katayama Fever) is characterized by daily fever, dysentery, epigastric pain and an enlarged liver and spleen (Beaver *et al*, 1987). It is rarely seen in chronically exposed populations and more often affects immune-naïve travellers to endemic areas (Bottieau *et al*, 2006). Most cases of Katayama fever are self-limited, resolving within 2-10 weeks (Gryseels *et al* 2006); in some cases, however, disease may persist for up to a year (Doherty *et al.*, 1996).

Chronic schistosomiasis is the more prevalent form of the disease affecting endemic populations and is the result of the long-term host immune response to schistosome egg antigens (Dunne and Pearce, 1999). The most common pathology associated with this disease stage is inflammatory and obstructive disease in urinary, intestinal or hepatic tissues. During chronic infection, large numbers of eggs released by female schistosomes migrate through host tissues, often becoming trapped in the bowel walls and the liver. A combination of $T_H1$ and $T_H2$-derived host immune effector molecules cause the formation of granulomas around these trapped ova. As time progresses, these granulomas become fibrotic deposits and impede blood flow to vital organs, causing portal hypertension, hepatosplenomegaly and the formation of gastric varices (Chen *et al*, 1993). Damage to the bladder caused by *S. haematobium* infection has also been cited as a cause of squamous cell carcinoma of the bladder (WHO, 1994).

Infection with *S. haematobium* may also present as genital disease in women, leading to tubular infertility (Poggensee and Feldmeier, 2001) and increasing the risk of infection with HIV (Feldmeier *et al*, 1995). More rarely, *S. mansoni* or *S. japonicum* localize to the central nervous system, causing transverse myelitis and epilepsy (Kane and Most, 1948, Chen and Mott, 1989).

### *A.4 Diagnosis and Treatment of Human Schistosomiasis*

The gold standard diagnostic test for schistosomiasis is the identification of schistosome ova in a patient's stool or urine (*S. haematobium* only) by microscopic examination (Ross *et al,* 2002). The distinctive characteristics of schistosome eggs, such as their lateral or terminal spine, make positive identification relatively easy. Diagnosis of intestinal schistosomiasis may be made from observing a single egg in 2-10 mg of stool. In lightly infected patients, such as returned travellers, sedimentation and concentration of eggs by formalin may improve diagnostic sensitivity (Garcia *et al*, 1999). In endemic areas where higher parasite burden is more common, the WHO recommends the use of the Kato-Katz thick stool smear examination, which requires a larger stool sample (Katz *et al*, 1972). Examination for *S. haematobium* infection is performed by microscopic analysis of concentrated urine samples, combined with reagent stick tests for haematuria (Lengeler *et al*, 1991, Gryseels *et al* 2006).

Although direct parasitological examination is the gold standard for diagnosis of schistosomiasis, this approach does have limitations. The developmental cycle of schistosomes in the human host requires around two months before the production of eggs. Moreover, microscopy of stool and urine samples requires skilled manual labor. Finally, the predictive value of these examinations may vary between areas of high and low intensity of infection (de Jong *et al*, 1988) and egg counts may vary within a single patient on a daily basis (Savioli *et al*, 1990 and Barreto *et al*, 1990). In order to mitigate these limitations, several other methods of diagnosis have been developed.

In returned travellers and patients with low intensity of infection, PCR and antibody-based diagnostics may be utilized (Bottieau *et al*, 2006). Detection of worm DNA by PCR corroborates microscopic examination (Olivera *et al*, 2010) and is useful in diagnosing patients with Katayama fever and with low worm burden. Serological

analysis, which relies on host antibody response to circulating worm and egg antigens, is of less clinical relevance (van Lieshout *et al*, 1997). The inability of serological tests to distinguish between active and resolved infections (Rabello *et al*, 1997) and antigen cross-reactivity with other helminths (Chand *et al*, 2010) are major drawbacks of this approach.

Upon diagnosis, therapeutic options for schistosomiasis are severely limited. There is no vaccine available and a single dose of the drug praziquantel (PZQ) is the only treatment currently recommended by the WHO. In the past, the drugs oxamniquine and metrifonate were used to successfully treat schistosomiasis. However, their high cost and species-specific activity limit their effectiveness in large-scale mass drug administration programs (Feldmeier and Chitsulo, 1999).

Although PZQ is effective as an antischistosomal treatment, its target and mechanism of action remain relatively unclear. There is evidence to suggest that PZQ diminishes parasite viability through at least two mechanisms. Schistosome intracellular $Ca^{2+}$ homeostasis (Pax *et al*, 1978) may be disrupted by the drug's interaction with voltage-gated $Ca^{2+}$ channels ((Jeziorski and Greenberg, 2006) and cause spastic paralysis of the worm. PZQ also disturbs the structural integrity of the schistosome tegument, causing surface membrane blebbing (Becker *et al*, 1980) and the release of hidden or inaccessible antigens. Both of these potential mechanisms increase worm susceptibility to host immune attack. Although PZQ is effective against adult schistosomes, it is not active at biologically relevant doses against larval schistosomula (Sabah *et al*, 1986, Botros *et al*, 2005). The lack of PZQ efficacy against larval schistosomula and its inability to prevent reinfection limit its use in active, mature infections (Dupré *et al*, 1999).

Despite this shortcoming, PZQ has been used in several large mass drug administration (MDA) programs with some measure of success (King, 2009). However, most of this success has been in areas of low to moderate transmission (Geary, 2013). In areas of higher prevalence, MDA programs have failed to impact disease transmission (Black *et al*, 2010). There are several factors contributing to this failure including limited drug distribution infrastructure, poor patient compliance and the inability

of PZQ to prevent reinfection. Moreover, the intensive use of a single compound against this widely distributed parasite has led to fears concerning the emergence of praziquantel resistant schistosomes (Doenhoff *et al*, 2009, Greenberg, 2013). Although evidence of widespread drug resistance has yet to appear, parasites with reduced sensitivity to PZQ have already been reported in the field (Fallon and Doenhoff, 1994, Melman *et al*, 2009) and engineered in the laboratory (Couto *et al*, 2011). The looming prospect of widespread PZQ-resistance combined with the limited treatment options currently available highlight the need to discover novel therapeutic targets for the treatment of schistosomiasis. One area of promise in the search for new drug targets is the worm's neuromuscular system, which we discuss in the next section.

### *A.5 Schistosome Neurobiology*

As mentioned above, schistosomes are members of the phylum Platyhelminthes, or flatworms. Flatworms are the most primitive creatures on earth to have developed a bilateral nervous system. Similar to vertebrates, the schistosome nervous system controls neuromuscular function and a variety of other essential biological processes. Additionally, the nervous system may also provide an alternative mechanism for long distance signal transduction in schistosomes, which lack the circulatory system necessary for classical endocrine signalling (Halton and Gustaffson, 1996 and Ribeiro and Geary, 2010).

Morphologically, the schistosome nervous system is comprised of two main interconnected components. The central nervous system (CNS) consists of a bi-lobed cephalic ganglion and several pairs of longitudinal nerve cords that run along the entire length of the worm (Reuter and Gustafsson, 1996). The longitudinal nerve cords are connected to each other via a network of circular regularly spaced commisures. This structure resembles a ladder and is termed the "orthogon" (Reuter and Gustafsson, 1996). The schistosome peripheral nervous system (PNS) includes minor nerve cords and the nerve plexuses. Nerve plexuses are large meshed networks of neurons that innervate a wide variety of tissues including muscles and sensory organs. There are two distinct nerve plexuses in schistosomes- the submuscular plexus and the subtegumental plexus. The submuscular plexus innervates both body wall musculature and the oral and

ventral suckers (Mair *et al*, 2000). The subtegumental plexus is more closely associated with the innervation of sensory structures, such as papillae (Gobert *et al*, 2003), in the male gynocophoric canal and on the tegument of the worm (Mair *et al*, 2004). Many of these sensory organs are located at the anterior end of the worm (Short and Cartrett, 1973). In addition to motor and sensory functions, the nervous system has been shown to heavily innervate the flatworm reproductive system (Baguna and Ballester, 1978).

Signalling in the nervous system is mediated through the interaction of neurotransmitters with their cognate receptors and transporters. In the broadest sense, neurotransmitters are chemical messengers that are present in the neurons and are biologically active. Various types of neurotransmitter systems have been found to exist in flatworms, including choline esters, biogenic amines, neuroactive amino acids and neuroactive gasses (reviewed in Ribeiro *et al*, 2005). These neurotransmitters play important roles in several processes essential to the survival of the worm, such as locomotory function, metabolism, reproduction and nutrient transport.

### *A.6 Experimental Approaches to Study Schistosome Neurobiology*

Historically, studies of the schistosome nervous system were limited to crude behavioral and biochemical assays. Techniques included behavioral (Barker and Bueding, 1966), enzymatic (Bueding, 1952) and later, electrophysiological (Day *et al*, 1996) assays. This limited technical repertoire was due to the lack of genomic data and consequent inability to utilize molecular methods. The development of a schistosome expressed sequence tag (EST) database (Olivera and Johnston, 2001) and later the sequencing and annotation of the entire genome (Berriman *et al*, 2009 and Protasio *et al*., 2011) have removed this obstacle and allow for the identification and molecular characterization of schistosome neurotransmitter receptors and transporters. The goal of this section is to briefly outline the various techniques that are now available to study proteins involved in schistosome nervous system function, with a focus on pharmacological and RNAi-based behavioral assays.

The earliest studies on the neuromuscular function of schistosomes relied on qualitative behavioral assays that utilized the addition of neuroactive compounds onto cultured adult worms (examples include Mansour, 1957 and Barker and Bueding, 1966).

In these assays, gross behavioral and morphological changes, such as hypermotility or paralysis, may be observed. However, this method provides limited insight into the molecular characterization of neuronal receptors. Several confounding factors, such as drug permeability, non-selective binding and toxicity may complicate analysis. Moreover, the subjective, qualitative data generated by this method lacks standardization and diminishes technical reproducibility. Despite these drawbacks, drug-based behavioral assays offer the benefit of quickly identifying compounds that exert control over worm motor function and aid in the dissection of signalling pathways. Therefore, when modified to include more quantitative phenotype scoring, these studies may prove useful for identifying candidates from large drug screens that merit further mechanistic studies (Paveley *et al*, 2012).

The development of reverse genetics approaches such as RNA interference (RNAi) have greatly improved the ability to link a specific receptor to biological function at the behavioral level. First discovered in the nematode *Caenorhabditis elegans* (Fire *et al*, 1998), RNAi offers a gene-specific, *in vivo* tool for the suppression of endogenous gene function via the degradation of target mRNA. The RNAi pathway is conserved and functional in schistosomes (Boyle *et al*, 2003; Krautz-Peterson *et al*., 2007) and has been utilized to study various families of proteins (Correnti *et al*. 2005; Dinguirard and Yoshino 2006; Krautz-Peterson *et al*. 2007; Nabhan *et al*. 2007; Morales *et al*. 2008; Rinaldi *et al*, 2009). Similar to drug-based behavioral assays, RNAi is amenable for use in large-scale phenotypic screens (Stefanic *et al*, 2010). The use of RNAi has also been combined with quantitative behavioral assays in order to study the effects of target gene suppression on parasite morphology and motor function (Patocka and Ribeiro, 2013). This approach offers the ability to assess phenotypic changes in response to suppression of endogenous protein levels, thus eliminating the complications associated with the addition of exogenous drugs. Data generated from RNAi-based behavioral assays comprise a significant portion of the work presented in this thesis.

In addition to improving behavioral assays, the publication of the *S. mansoni* genome has allowed for the cloning, deorphanization and characterization of several neuroreceptors at the molecular level (Hamdan *et al*, 2002; Taman and Ribeiro, 2009;

El-Shehabi and Ribeiro, 2010; El-Shehabi *et al*, 2012; Dufour *et al.* 2013; Patocka et al, 2014). The most common tools for pharmacological characterization in these studies were yeast, mammalian cell or *Xenopus* oocyte heterologous expression systems.

## B. Acetylcholine

Acetylcholine (ACh) is a ubiquitous and essential neurotransmitter in both vertebrate and invertebrate nervous systems. ACh is the quintessential excitatory neurotransmitter of vertebrates and is most often associated with neuromuscular function. In invertebrate systems, however, there is mounting evidence for a higher diversity of biological processes modulated by ACh, including inhibitory neuromodulation. The following section provides a summary of cholinergic signalling, which is the main focus of this thesis. Particular emphasis will be placed upon invertebrate cholinergic systems.

### *B.1 Acetylcholine Biosynthesis and Degradation*

Acetylcholine is a small choline ester synthesized in neurons by the enzyme choline acetyltransferase (ChAT). ChAT transfers the acetyl group from the molecule acetyl-coenzyme A (acetyl-CoA) to choline, a quaternary ammonium alcohol. There are two sources from which choline is derived- synthesis inside the neuron by the metabolism of serine (Elwyn *et al*, 1955) or transport into the neuron from outside the cell by a high-affinity transporter (Birks and MacIntosh, 1961). This recycled choline, derived from the degradation of unbound acetylcholine in the synapse, provides the majority of synthetic reactant. As such, transport of choline into the neuron is considered the rate-limiting step in the biosynthesis of ACh.

After synthesis, ACh is stored in cytosolic vesicles until needed for release (Parsons *et al*, 1987). Initiation of an action potential in the neuron causes an influx of $Ca^{2+}$ ions via the opening of voltage-gated ion channels. This influx causes the ACh storage vesicles to fuse with the presynaptic cellular membrane and release the neurotransmitter into the synaptic cleft. Once released, ACh binds to receptors (discussed in Sections C and D) on the membranes of both the presynaptic and postsynaptic neurons, eliciting a cellular response. Unbound ACh is hydrolyzed by the enzyme acetylcholinesterase (AChE) into choline and acetate (Leuzinger *et al*, 1967).

The choline is then transported back into the neurons by high-affinity choline transporters and the cycle begins anew.

### *B.2 Biological Functions of Acetylcholine in Vertebrates*

Acetylcholine and its related receptors are widely distributed throughout the vertebrate nervous system. Both subtypes of ACh receptors, muscarinic and nicotinic, are present in vertebrates and participate in a wide variety of biological processes. Cholinergic neurons are localized to both the central and peripheral nervous systems and may be found on presynaptic and postsynaptic neurons, as well as non-neuronally. In the CNS, acetylcholine modulates neurotransmitter release (Pidoplichko *et al*, 1997) and controls attention, learning, memory and motor control (reviewed in Albuquerque *et al*, 2007). Deficiencies in CNS cholinergic signalling have been linked to many disease states, including Alzheimer's disease, schizophrenia and epilepsy (Wess *et al*, 2007 and Lindstrom, 1997). In the PNS, cholinergic signalling controls several different functions of both the autonomic and somatic nervous systems. Muscarinic receptors in the sympathetic and parasympathetic autonomic nervous system regulate cardiovascular function, glandular secretion and contraction of smooth muscle (Brown, 2010).

The best-known function of ACh in vertebrates is the transmission of fast excitatory action potentials at the neuromuscular junction. It is the only neurotransmitter used to directly control somatic motor function. ACh released by presynaptic neurons is bound by nicotinic receptors on the muscle membrane, eliciting the activation of cation channels. This influx of cations depolarizes the muscle cell, causing a contraction (Cohen-Corey, 2002). Although beyond the scope of this thesis, there are a plethora of extensive review articles on the topic of vertebrate neuromuscular signalling (see Fagerlund and Eriksson, 2009) available.

### *B.3 Biological Functions of ACh in Invertebrates*

Acetylcholine plays an important role in invertebrate neurotransmission. It is present across almost all invertebrate phyla and has been extensively studied in insects and mollusks. Similar to vertebrates, both muscarinic and nicotinic receptors function as part of the invertebrate cholinergic system. However unlike vertebrates, the cholinergic

system of invertebrate organisms is often absent from the neuromuscular junction, with expression limited to the CNS (Breer and Sattelle, 1987).

As a central modulator of neurotransmission, ACh regulates an array of important biological processes in insects. These include sensory and olfactory functions (Breer, 1987), vision (Kolodziejczyk *et al*, 2008) and learning and formation of memory (Dupuis *et al*, 2012). Insect cholinergic systems are comprised primarily of nicotinic acetylcholine receptors (Hannan and Hall, 1993) that act as mediators of fast excitatory neurotransmission (Oleskevich, 1999 and Lee and O'Dowd, 1999). Due to its central role in insect viability, the cholinergic system is the target of several classes of pesticides, including carbamates and organophosphates and a newer class of insecticide, the neonicotinoids (reviewed in Matsuda *et al*, 2001).

In mollusks, ACh was first discovered as an excitatory modulator of smooth muscle contraction in the mussel *Mytilus edulis* (Twarog, 1954). The cholinergic system of mollusks is now also known to control egg-laying (Vulfius *et al*, 1967) and feeding (Yeoman *et* al, 1993) behaviors. Work in the sea slug *Aplysia* established that cholinergic neurotransmission is localized to the CNS (Tauc and Gerschenfeld, 1961) and signals via nicotinic receptors (Kehoe, 1972). Interestingly, this study also provided evidence for an inhibitory role of ACh in mollusks, a marked departure from its canonical excitatory function in vertebrate systems. Inhibitory cholinergic neurotransmission in mollusks is mediated by anion-selective nicotinic receptors (van Nierop *et al*., 2006). In the snail *Lymnaea*, they are speculated to modulate neuropeptide release (van Nierop *et al*, 2006).

Perhaps the best-characterized invertebrate cholinergic system is that of the nematodes. The excitatory neuromuscular role of ACh in nematodes was discovered in the parasitic worm *Ascaris lumbricoides* (del Castillo *et al*, 1963 and del Castillo *et al*, 1967). However, much of what we know about cholinergic signaling in nematodes is derived from experiments in the free-living *C. elegans* model system. A large proportion of *C. elegans* neurons release ACh and its genome contains more than 35 acetylcholine receptors and receptor subunits (Lee *et al.* 1999, Lee *et al.* 2000, Park *et al.* 2003,

Jones and Sattelle, 2004, Putrenko *et al*, 2005). Several nematode ACh receptors will be discussed in further detail in later sections of this review.

The most prominent functional role of ACh in *C. elegans* is the control of locomotory behavior. The modulation of muscular contraction takes place at both the neuromuscular junction (Brenner, 1974) and also within the central nervous system (Winnier *et al*, 1999). Cholinergic signaling has also been shown to control feeding behavior (McKay *et al*, 2004), waste elimination (Thomas, 1999) and male reproductive function (Garcia *et al*., 2001).

### *B.4 Acetylcholine in Platyhelminths*

ACh was first discovered in the free-living flatworm cousins of schistosomes, the planarians, in the 1970s (Tiras, 1978). Control of planarian muscular function is controlled by a widespread cholinergic system, comprised of both nicotinic and muscarinic acetylcholine receptors (Nishimura *et al*, 2010). Treatment of planarian muscle fibres and whole animals with ACh causes muscle contraction and rigid paralysis in a dose-dependent manner (Blair and Anderson, 1994, Butarelli *et al*, 2000). There is some evidence that this excitatory response interacts with the dopaminergic system in order to prevent hyperkinesia, although the underlying mechanisms remain unclear (Butarelli *et al*, 2000). Although evidence does point to the existence of cholinergic receptors in planarians, none have been cloned or functionally characterized (Cebria *et al*, 2002, Mineta *et al*, 2003).

The effects of ACh in parasitic flatworms are the opposite of those observed in planarians. It was first postulated that ACh played an important role in parasitic flatworm nervous systems as early as the 1950s (Bueding, 1952, Chance and Mansour, 1953). Later experiments demonstrated that whereas ACh has an excitatory effect on muscle contraction and motility in nematodes and planarians, it mediates an inhibitory effect in parasitic flatworms (Barker *et al*, 1966, Holmes and Fairweather, 1984). Histochemical analysis using acetylcholinesterase (AChE) as a marker of ACh established that cholinergic neurons are present throughout flatworms (Halton, 1967). This was confirmed by a study that visualized ACh molecules in the longitudinal nerve cords of the CNS and somatic and sub-tegumental muscle fibers of cestodes (Samii and Webb,

1990). AChE has also been localized to the outer tegument of *S. mansoni* (Jones *et al*, 2002) and cholinergic receptors have been identified in both the body-wall muscles and surface of *S. haematobium* (Bentley *et al*, 2004). The distribution of the cholinergic system in schistosomes closely resembles that of the peptidergic neuronal system (Halton and Gustaffson, 1996) and interaction between the two systems is a possibility.

ACh has a profound effect on the motor activity of parasitic flatworms. Early studies demonstrated that treatment of cut worms or isolated muscle fibers with exogenous ACh inhibits muscle contraction and induces flaccid paralysis (Sukhdeo, 1984 and 1986, McKay, 1989). The mechanism by which ACh inhibits muscle contraction is unknown, however nicotinic-type ACh receptors are thought to mediate this process (Day *et al*, 1996). In addition to affecting the motility of parasitic flatworms, ACh also affects nutrient transport. There is evidence to suggest that cholinergic receptors localized to the tegument of *S. mansoni* regulate the transport of exogenous glucose into the parasite (Camacho *et al*, 1995, Camacho and Agnew, 1995, Jones *et al*, 2002). Treatment of worms with ACh stimulates glucose transport in a dose-dependent manner. A nicotinic receptor appears to control this response, perhaps by modulation of the GLUT1 glucose transporter, which has also been localized to the parasite tegument (Jones *et al*, 2002). Finally, the presence of ACh in muscularized reproductive organs (Samii and Webb, 1990) suggests a possible reproductive function for the flatworm cholinergic system.

### C. Cys-Loop Receptors and Ionotropic Acetylcholine Signalling

Given the unique effects of ACh on schistosomes and its important role in a number of biological processes, ACh receptors are an attractive target for the development of novel therapeutics. The largest class of ACh receptors in vertebrates are ligand-gated ion channels (LGICs). LGICs are members of the Cys-loop ligand-gated ion channel (LGIC) superfamily (Nys *et al*, 2013). The name Cys-loop is derived from a characteristic disulphide bond formed between two cysteine residues in the N-terminal domain of each receptor subunit. Functionally, Cys-loop receptors act as neurotransmitter receptors and play an important role in fast signal transduction via the conductance of ions across neuronal and muscular cell membranes. This mechanism of

signalling classifies Cys-loop receptors as ionotropic, as opposed to metabotropic receptors, which signal through a second messenger system. In vertebrates, Cys-loop receptors mediate either excitatory or inhibitory neurotransmission for a number of native ligands, such as acetylcholine, serotonin, glutamate, glycine and γ-amino butyric acid (GABA) (Thompson *et al*., 2010). Invertebrates possess a slightly larger repertoire of Cys-loop receptors that includes homologs of the classical vertebrate proteins as well as several invertebrate-specific receptors. A large portion of these unique invertebrate receptors fall into the larger family of ionotropic ACh receptors.

### *C.1 Structure of Nicotinic Acetylcholine Receptors*

The majority of Cys-loop cholinergic channels identified to date are nicotinic acetylcholine receptors (nAChRs). Much of what is known about the structure of these channels originates from analysis of the *Torpedo* ray nicotinic acetylcholine receptor (nAChR) (Unwin, 2005). Structurally, nAChRs are composed of five membrane-spanning protein subunits arranged into a barrel shape around a central ion-selective pore (Kistler *et al*, 1982). Subunits are split into two types, alpha (α-subunit) and non-α (β, γ, δ or ε-subunit). Receptor pharmacology and localization are determined by subunit composition, with only α-subunits capable of forming functional homomeric ion channels (Millar and Gotti, 2009). There are 10 α-subunits that have been described in vertebrate organisms, which are classified according to the presence of a double cysteine motif in the N-terminus (Noda *et al*., 1982). All nAChRs must contain at least one type of α-subunit (Millar and Gotti, 2009). The non-α subunits are further classified as neuronal (β-subunits) and muscle-type (γ, δ or ε-subunits).

There are three main functional domains of Cys-loop receptor subunits: an N-terminal extracellular domain (ECD), the pore-forming transmembrane domains (TMD) and the intracellular domains (ICDs). Each nAChR contains two ACh binding sites (Green and Wanamaker, 1998), located at the interface of the ECDs of neighboring subunits. Six loops (A-F), three from each subunit, form the binding pocket for ACh (Brejc *et al*, 2001). The main molecular interaction involved in binding ACh is a cation-π bond, in which the quaternary ammonium of ACh binds with a highly conserved tryptophan in Loop B (Trp143) of the α-subunit (Zhong *et al.*, 1998 and Brejc *et al.*,

2001). In addition to the orthosteric binding sites for ACh, the activity of nAChRs may also be modulated via multiple allosteric binding sites (reviewed in Faghih *et al.*, 2008). A secondary role of the ECD, particularly the M2-M3 extracellular loop, is to transfer the energy of ligand binding into channel opening (reviewed in Thompson *et al.*, 2010).

The TMD of nAChRs is composed of four membrane-spanning α-helical domains (Noda *et al.*, 1982, Miyazowa *et al.*, 2003). These helices are termed M1-M4 and form the pore through which the channel conducts ions. Together, the M domains form two concentric charged rings connected by a hydrophobic constriction that acts as the ion gate (Panicker *et al.*, 2002). The M1, M3 and M4 domains form the outer and cytoplasmic charged rings that protect the inner charged ring formed by the pore lining M2 domains (de Planque *et al.* 2004). The funnel-shaped structure of the M2 pore creates a hydrophobic constriction. The charge-dependent size of this constriction confers the ion selectivity of the pore (Beckstein *et al.*, 2004). Interestingly, mutagenesis studies have demonstrated that the ion-selectivity of Cys-loop receptors may be reversed by altering the charge of the intermediate M2 ring with amino acid substitutions (Galzi *et* al., 1992, Keramidas *et al.*, 2002). In addition to their function in ion gating, portions of the TMDs have also been implicated in receptor assembly and trafficking to the cell surface (Wang *et al*., 2002).

The intracellular loops joining the TMDs form the ICD of nAChRs. The M3-M4 loop is a large, structurally undefined region that is hypothesized to form a “hanging basket” conformation in the cytoplasm (Unwin, 2005 and Hales *et al*., 2006). The sequence of the M3-M4 loop is highly divergent and the least conserved portion of the protein (McKinnon *et al.*, 2012). Functionally, this loop contributes to receptor assembly in the endoplasmic reticulum, trafficking to the cell surface and channel conductance (Ren *et al.*, 2005, Hales *et al.*, 2006, Kracun *et al.*, 2008). Portions of the ICD also interact with intracellular proteins, such as kinases, in order to modulate receptor activity (reviewed in Swope *et al.*, 1999).

### *C.2 Function of nAChRs in Vertebrates*

All vertebrate nAChRs are cation-selective and thus mediate excitatory neurotransmission. In the vertebrates, nAChRs are classified into neuronal nAChRs and

muscle-type nAChRs, according to their classical location of expression (Fagerlund and Eriksson, 2009). They may also be classified pharmacologically by their affinity for the alpha-type antagonist α-bungarotoxin (α-Btx), which strongly binds muscle-type and some neuronal nAChRs (Stroud *et al.* 1990 and Chini *et al.*, 1992). Neuronal nAChRs are comprised of the α2-α10 and β2- β4 subunits. They are present in both the CNS and PNS, as well as in some non-neuronal tissues (Tracey, 2007). The homomeric α7 and heteromeric α2β4 receptors are the most highly abundant nAChRs in the vertebrate CNS (Francis and Papke, 2000), where they control synaptic plasticity and cognition (Dajas-Bailador and Wonnacott, 2004). Most likely this is achieved through the modulation of neurotransmitter release rather than direct transmission of action potentials (De Filippi *et al.*, 2005). Due to their importance in maintaining normal brain function, CNS nAChRs have been extensively studied and linked to many diseases, including Parkinson's and Alzheimer's and schizophrenia (reviewed in Posadas *et al*., 2013).

In the vertebrate PNS, the complete role of neuronal nAChRs is less clear. However, a wide variety of neuronal nAChR subunits provide widespread regulation of synaptic transmission (Conroy and Berg, 1995). Expressed on presynaptic neurons at the neuromuscular junction, neuronal nAChRs act as autoreceptors and stimulate the continued release of ACh into the synaptic cleft during periods of high frequency stimularion (Bowman *et al*., 1990). Most neuronal nAChRs expressed in the PNS localize to the parasympathetic nervous system, such as the ciliary ganglia (Williams *et al*., 1998) and the intracardiac ganglia (Cuevas and Berg, 1998). They may also control breathing and glandular secretion (Lecci *et al*., 2010) Neuronal α9α10 nAChRs have been localized to the cochlear hair cells of the PNS, where they are involved in auditory function (Elgoyhen *et al*, 2001). Despite their name, expression of neuronal nAChRs outside the CNS is not limited to neuronal cells. Other cell types expressing non-muscle type nAChRs include glia, endothelial cells and epithelial cells (Gahring and Rogers, 2005). Nicotinic receptors are also vital regulators of the inflammatory immune response (Wang *et al.*, 2003).

Although neuronal-type receptors are involved in a wide variety of essential biological pathways, the main focus of PNS nAChR research is the muscle-type nAChR. Muscle-type nAChRs control all excitatory neuromuscular signalling, have a high affinity for α-Btx and are divided into two groups. Fetal (α1β1γδ) nAChRs are short-lived and quickly replaced by adult muscle-type receptors (Mishina *et al.*, 1989). The more predominant adult receptors (α1β1εδ) (Fagerlund and Eriksson, 2009) are expressed on the postsynaptic membrane of somatic muscle cells in large clusters (Wheeler *et al.*, 1993). In both cases, activation of muscle-type nAChRs causes an influx of cations (mostly $Ca^{2+}$ and $Na^{+}$), which is amplified by a dense region of $Na^{+}$ channels in the perijunctional zone and leads to muscular contraction (Cohen-Corey, 2002).

### *C.3 nAChRs in Invertebrates*

The physiological importance of nAChRs is not limited to vertebrate organisms. Cholinergic neurotransmission is a vital regulator of a number of essential processes in invertebrates including insects, mollusks and worms. ACh acts as a neuromodulator through neuronal-type nAChRs in the CNS of both insects and mollusks (Thany, 2010). In nematodes and flatworms, nAChRs are found in both the CNS and at the neuromuscular junction (Jones and Sattelle, 2004 and Ribeiro *et al*., 2005).

Although they are a major target of insecticides, relatively little information exists about the pharmacology and function of insect nAChRs. Immunolocalization studies demonstrated that insect nAChRs are widely distributed in the brains of several insects such as bees (Scheidler *et al.*, 1990), flies (Dudai and Amsterdam, 1977) and locusts (Breer *et al*., 1985). Evolutionarily, these insect receptors diverged from their vertebrate and nematode homologs families very early (Le Novére and Changeux, 1995) and are one of the smallest gene clusters of nAChRs known, ranging from 10-12 subunits (Dupuis *et al*., 2012). Nevertheless, insect nAChRs may possess a greater functional diversity that this number suggests due to alternative splicing of transcripts (Jones *et al.*, 2005).

Similar to vertebrate nAChRs, insect receptors are split into two families- α-subunits and non-α subunits (Jones and Sattelle, 2010). Pharmacological characterization of insect nAChRs remains a challenge due to the difficulty of expressing

functional receptors in a heterologous system. To date there have been few successful attempts, notably the characterization of the *Drosophila* Dα2 receptor, which confirmed the presence of nicotinic cholinergic receptors in insects (Sawruk *et al.* 1990). To ameliorate the difficulty of expressing insect nAChRs in heterologous systems, attempts have been made to form functional hybrid nAchRs using insect α-subunits and vertebrate β-subunits (Bertrand *et al.*, 1994 and Dederer *et al.*, 2011). The heteromeric, hybrid nAChRs formed in these experiments exhibited both α-BTX sensitive and insensitive pharmacology. This agrees with α-BTX co-precipitation experiments that suggest heteromeric α-BTX sensitive insect nAChRs exist *in vivo* (Chamaon *et al.*, 2000 and Chamaon *et al.*, 2002). There is also limited *in vivo* evidence that α-BTX insensitive nAChRs are also expressed (Courjaret and Lapied, 2001). In sum, further research is needed to determine the subunit composition and complete pharmacological profiles of insect nAChRs.

Molluscan nAChRs are present in the CNS and exhibit control over feeding behavior (Benjamin and Elliott, 1989 and Kehoe and McIntosh, 1998) and cardiac function (Buckett *et al.*, 1990). Unlike insect and vertebrate receptors, mollusk nAChRs are capable of both excitatory and inhibitory neurotransmission (Kehoe, 1972). Later work in the snail *Lymnaea* determined that mutations in the M2 TMD of the inhibitory receptors are the cause for the shift in ion-selectivity towards anions (van Nierop *et al.*, 2005). The same study demonstrated that cation-selective, excitatory *Lymnaea* nAChRs share a common pharmacological profile with the vertebrate α9 receptor and other true nAChRs- namely they are activated by nicotinic agonists and are α-BTX-sensitive. The inhibitory snail nAChRs also share this vertebrate pharmacology due to conservation of the ligand-binding domain but a non-overlapping expression pattern with the excitatory nAChRs points to a different functional role. Yet, other immunolocalization experiments show the expression of both excitatory and inhibitory snail nAChRs in the same neurons (Wachtel and Kandel., 1971, Syed *et al.*, 1990, Wooden *et al.*, 2002). This apparent discrepancy may result from the peculiar feature of molluscan interneurons to mediate multiple types of fast synaptic neurotransmission (van Nierop *et al*., 2005).

The cholinergic receptors of nematodes, specifically *C. elegans*, are perhaps the most extensively studied invertebrate nAChRs. ACh was first discovered in the parasitic nematode *Ascaris lumbricoides* (Mellanby, 1955) and was later determined to be the major excitatory neurotransmitter at the nematode neuromuscular junction (del Castillo *et al.*, 1963). Work continued in the free-living nematode, *C. elegans*, where mutants resistant to the drug levamisole identified several genes linked to nematode cholinergic neurotransmission (Brenner *et al.*, 1974 and Lewis *et al*., 1980). Following the sequencing of the *C. elegans* genome in 1998, several more nematode nAChR subunits were identified (Mongan *et al*., 1998, Mongan *et al.*, 2002, Jones and Sattelle, 2004). More recently, homologs of *C. elegans* nAChRs have been identified in parasitic nematodes, such as *Ascaris* (Williamson *et al*., 2009) and *Haemonchus contortus* (Rufener *et al*, 2009).

In total, the *C. elegans* genome contains 32 nAChR-like subunits that are divided into 5 groups (ACR-16, UNC-38, UNC-29, DEG3 and ACR-8) (Holden-Dye *et al*., 2013). Receptors are clustered by sequence homology and contain both α- and non-α subunit types (Jones and Sattelle, 2004). The similarity of each *C. elegans* receptor group to other phyla varies widely. The UNC-38 group is most similar to insect nAChRs, while the ACR-16 group is most closely related to vertebrate receptors (Rand, 2007). There also exist nematode-specific receptor groups, such as ACR-8 and DEG-3 (Brown *et al*., 2006). Although 5 groups of *C. elegans* nAChRs have been classified by homology, only two pharmacologically distinct groups exist. Thus, subunits from different groups may form functional receptors together. Electrophysiological and heterologous expression studies have led to the characterization of several *C. elegans* neuromuscular receptors, each sensitive to either levamisole (L-type) or nicotine (N-type) but not both (Richmond and Jorgensen, 1999). L-type receptors are heteropentamers that always contain the UNC-29, UNC-38 and UNC-63 subunits (Fleming *et al*., 1997, Richmond and Jorgensen, 1999, Culetto *et al.*, 2004). N-type *C. elegans* receptors, on the other hand, are composed solely of ACR-16 α-subunits (Francis *et al.*, 2005, Touroutine *et al*., 2005). A third type of divergent receptor has also been shown to exist. Receptors of the DEG-3 family are preferentially activated by choline and modulated by the amino-acetonitrile derivative drug monopantel (Yassin *et al*, 2001, Rufener *et al*., 2010).

Despite the thorough characterization of *C. elegans* nAChRs at the pharmacological level, understanding of their full function at the behavioral level is still lacking. It is known that proper function of both LEV and nicotine-sensitive receptors is not essential for normal *C. elegans* motor function. However, loss of both receptor types causes severe uncoordinated phenotypes (Francis *et al.*, 2005, Touroutine *et al*., 2005). In addition to motor behavior, there is also evidence for non-neuronal, sensory function of nAChRs in nematodes (Yassin *et al*. (2001). This functional diversity in *C. elegans* is supported by its widespread expression pattern in nerve cords, sensory neurons, reproductive organs and body wall muscle (reviewed in Rand, 2007).

### *C.4 Invertebrate "non-nicotinic" cholinergic channels*

Aside from classical excitatory LEV and nicotine-sensitive nAChRs, nematodes possess a divergent and unique type of cholinergic receptor. First discovered in *C. elegans*, the ACC genes ((ACC-1, ACC-2, ACC-3, ACC-4) form ACh-gated chloride channels that do not exhibit nicotinic pharmacology (Putrenko *et al*., 2005). Subunits ACC-1 and ACC-2 are capable of forming functional homopentamers, while ACC-3 and ACC-4 are obligate heteromers. Phylogenetically, the ACC receptors are more closely related to the inhibitory GABA/glycine anion-selective receptors than to nicotinic receptors, indicating a different evolutionary origin (Putrenko *et al.*, 2005). This evolutionary path and unique pharmacology separates the ACCs distantly from the previously discussed *Lymnaea* nicotinic chloride channels (van Nierop *et al.*, 2005). Homologs of ACC receptors have been found in several parasitic nematode species (Beech *et al*., 2013). Recent genome annotations suggest that acetylcholine-gated chloride channels may also exist in parasitic flatworms (Berriman *et al.*, 2009, Protasio *et al.*, 2012, Huang *et al*., 2013). Due to the invertebrate-specificity and divergent pharmacology of these receptors, they present an attractive target for the development of novel antiparasitics. A major focus of this thesis is the identification and characterization of putative ACh-gated chloride channels in the trematode *S. mansoni*.

## D. G-protein-coupled Receptors and Muscarinic Acetylcholine Signalling

Besides Cys-loop gated channels, ACh exerts its effects by interacting with G-protein-coupled receptors (GPCRs). GPCRs are the largest, most diverse superfamily of cell surface receptors and represent a substantial portion (<30%) of currently utilized drug

targets (Salon *et al.*, 2011). This size and functional diversity is reflected in invertebrates, with the *C. elegans* genome predicted to encode over 1300 GPCRs (Hobert, 2013) and the *S. mansoni* genome predicted to have 117 (Zamanian *et al.*, 2011). Moreover, the discovery of parasite-specific families of GPCRs in schistosomes (El-Shehabi *et al.*, 2009) and other parasites (Kimber *et al.*, 2009) indicates an opportunity for the development of novel antiparasitics. In the following section, we will first introduce the general structure and signalling mechanisms of GPCRs. We will then discuss the functions of cholinergic GPCRs in vertebrates and invertebrates with a focus on nematodes and flarworms.

### *D.1 Overview of G-protein-coupled Receptor Structure and Function*

GPCRs are classified into several structural families (Fredriksson *et al.*, 2003, Peng *et al.*, 2010). Class A, or rhodopsin-like receptors, is the largest of these families and includes all known types of cholinergic GPCRs. The rhodopsin-like Class A GPCRs share common structural features (Lefkowitz, 2000), which are characterized by a relatively short extracellular N-terminus, seven transmembrane (TM) domains linked by intra- and extracellular loops of variable length (ICL, ECL) and an intracellular C-terminal end. The TM domains arrange into an anticlockwise helical bundle (Baldwin *et al.*, 1997, Palczewski *et al.*, 2000) that plays several important roles. In the case of receptors that bind small molecule ligands, such as ACh, the binding pocket is formed from portions of the TM helices, in particular TM3, TM5, TM6 and TM7 (Kristiansen, 2004). Other highly conserved residues in the TM domains are essential for the structural integrity of the receptor and play a role in G-protein interaction (discussed below and reviewed in Katritch *et al.*, 2013). The C-terminal end of GPCRs contains an eighth, non-transmembrane helix and is maintains several functional roles, including proper protein folding and localization, G-protein coupling and receptor desensitization (Chen *et al.*, 2004, Piserchio *et al*, 2005). Within the TM regions, an invariant amino acid residue has been identified for each domain and arbitrarily assigned the number 50 (Ballasteros and Weinstein, 1995). The positions of all other residues within a TM domain are based upon this residue. Therefore, the highly conserved arginine residue of the third TM region would be referred to as 3.50 and the amino acid 5 positions upstream would be

3.45. This system of nomenclature will be used throughout this section when describing residues important for GPCR structure and function.

From a mechanistic standpoint, GPCRs signal via a ligand-induced change in the structural orientation of the receptor. The interactions formed by bound ligands with both ECL and TM domain residues cause the repositioning and rotation of several TM helices. This rearrangement causes the cytoplasmic portion of the helical bundle to open, revealing the G protein binding site. Mutational and structural analyses have shown that the outward rotation of TM6 and a repositioning of TM5 are essential for the activation of all Class A GPCRs (Nygaard *et al.*, 2009, Reiter *et al.*, 2012). Inter- and intrahelical salt-bridges formed by amino acids in TM3 have also been shown to affect receptor activation. A highly conserved Arg at position 3.50 interacts with negatively charged residues in TM6 to form an “ionic lock” that stabilizes some GPCRs in an inactive conformation (Vogel *et al*., 2008). However, the magnitude and functional importance of TM3 movement is variable among GCPR subtypes (Vanni *et al.*, 2007, Xu *et al.*, 2011, Lebon *et al*., 2011). There is also more recent evidence to suggest that movements in TM3 and TM7 may affect G-protein independent signalling pathways (Liu *et al.*, 2012).

Heterotrimeric G-proteins act as the primary intermediaries between GPCRs and downstream effector proteins. They are comprised of three different subunits, designated α, β and γ. Each subunit protein family has multiple members, with the Gα as the largest with 25 unique proteins identified (Cabrera-Vera *et al.*, 2003). At the functional level, G-proteins are divided into four main groups ($G\alpha_s$, $G\alpha_q$, $G\alpha_i$, and $G_{12/13}$) according to the homology of their Gα-subunits. Two families, $G\alpha_s$ and $G\alpha_q$ are responsible for stimulatory cellular responses, mainly by activating adenylate cyclase (AC) and phospholipase C (PLC). The $G\alpha_i$ proteins serve an inhibitory role through various pathways, such as the down-regulation of adenylate cyclase, opening of $K^+$ channels and closing of $Ca^{2+}$ channels. $G_{12/13}$ regulates cell growth and differentiation by the activation of Rho GTPases (Stalhman *et al*., 1991, Dhanaseraren *et al.*, 1996).

Regardless of subunit composition and their downstream effects, the cycle of heterotrimeric G-protein activation and inactivation is invariant. The process begins with the ligand-induced rearrangement of the GPCR helical bundle and the exposure of the

receptor G-protein binding pocket. The inactive, cytosolic G protein/guanosine diphosphate (GDP) complex occupies this space and is bound to the GPCR by its Gα-subunit. Several domains of the GPCR play important roles in the binding of G-protein α-subunits, including TM3, TM5 and TM6 and the second and third intracellular loops (i2 and i3) (Scheerer *et al.*, 2008). The interaction between the receptor and G protein catalyzes the replacement of GDP with guanosine triphosphate (GTP). This reaction causes the dissociation of the G protein into its constituent subunits, which interact with a variety of effector molecules to mediate cellular responses (reviewed in Johnston and Siderovsky, 2007). The activity of G-protein subunits continues until hydrolytic inactivation by GTPase converts the GTP back to GDP and the three subunits oligomerize, forming the inactive complex. Other mechanisms leading to the inactivation of G protein signalling include ligand reuptake or degradation and receptor desensitization (Bohm *et al*. 1997).

In addition to the classical G protein signalling cascades, GPCRs may also signal via G-protein-independent pathways. The observation that cholinergic GPCRs could evoke $K^+$ and $Na^+$ currents in G protein-depleted cells first led to this discovery (Olsen *et al*., 1988, Shirayama *et al*., 1993). Since then, this capability has been noted in several other GPCRs, such as the metabotropic glutamate and adrenergic receptors (Anwyl *et al*., 1999, Tang *et al*., 1999). The mechanism of G-protein independent signalling is not completely clear but interacting partners such as MAP and tyrosine kinases and small (monomeric) G proteins have been identified (Heuss and Gerber, 2000).

*D.2 Ligand-independent (constitutive) activity of GPCRs*

As discussed later, the cholinergic GPCR of S. mansoni has a propensity towards spontaneous activation (see Chapter 3) and therefore this aspect of GPCR activity is of particular relevance to this thesis. At one time, all GPCR-mediated signalling was thought to be ligand-dependent. However, the discovery of spontaneously activated opioid and adrenergic receptors (Koski *et al.*, 1982, Cerione *et al.*, 1984) refuted this dogma and led to the development of the “Two-State Model” of GPCR activation. Briefly, this model states that native GPCRs exist in equilibrium between inactive (R) and active (R*) structural conformations (Lefkowitz *et al*., 1993). Most GPCRs remain in the R-state

until the binding of a ligand shifts the equilibrium to favor the R* conformation and thus initiate signalling. Constitutively active GPCRs, on the other hand, have structural anomalies that cause them to adopt the R* conformation even in the absence of ligand. It is now known that over 40% of wild-type GPCRs exhibit at least a minimal level of agonist-independent activation (Seifert and Wenzel-Seifert, 2002). There is also mounting evidence that dysfunction of constitutively active GPCRs (both wild-type and mutant) may be associated with several disease conditions, such as dwarfism (Schipani *et al*., 1999), congenital night blindness (Dryja *et al*., 1993) and atherosclerosis (Casarosa *et al*., 2001). A rapidly growing area of GPCR research therefore, focuses on compounds known as inverse agonists, which reduce the high level of basal activity exhibited by spontaneously activated GPCRs and shift the equilibrium of back toward the inactive R pose.

### *D.3 Muscarinic Acetylcholine Receptors*

H.H. Dale first described the actions of ACh on two different receptor types, muscarinic and nicotinic, in 1914 (Dale, 1914). Muscarinic receptors (mAChRs) are distinguished by their preferential activation by the fungal toxin muscarine and their blockade by the antagonist atropine. Further pharmacological analysis provided evidence for several pharmacologically and functionally distinct subtypes of mAChRs (Riker and Wescoe, 1951, Roszcowski, 1961, Birdsall and Hulme, 1983). These early studies were validated with the cloning of five separate receptor genes from humans (M1-M5) and resulted in the mAChR subtype classification that is currently utilized (reviewed in Caulfield and Birdsall, 1998).

mAChRs share sequence homology with other members of the Class A GPCR superfamily and they have the same general topology. Sequence identity across the mAChR subtypes is also highly conserved, including the amino residues that form the binding pocket for ACh (Hulme *et al*., 2003). In addition to the conserved orthosteric ACh-binding site, mAChRs are known to have at least two allosteric binding sites (Lanzafame *et al.*, 2006), which may also contribute to differing subtype pharmacology. The availability of a number of cloned and heterologously expressed receptor genes has allowed for extensive mutagenesis studies to elucidate the structure-function

relationships mAChRs (Leach *et al*., 2012). Key residues involved in ligand binding (Jones *et al*., 1995), signalling efficiency (Wess *et al*., 1992, Spalding *et al*., 1998) and constitutive activity (Lu and Hulme, 1999, Schmidt *et al*., 2003) were identified in this manner. However, the extensive nature of this topic is beyond the scope of this review. More recently, crystal structures of the $M_2$ and $M_3$ receptors have been solved (Haga *et al*. 2012, Kruse *et al*., 2012).

The majority of what is known about mAChR structure, pharmacology and function is derived from studies of vertebrate organisms, especially the cloning and characterization of the human mAChRs. Located in both the CNS and PNS of vertebrates, they have a variety of sensory, cognitive, and motor roles. They are also key modulators of smooth muscle contraction and glandular secretion and more recently discovered, immune function (Noramura *et al*., 2003). Receptors M1, M3 and M5 usually couple to $G_{q/s}$ subunits, are pertussis toxin (PTX) insensitive and generate an excitatory postsynaptic potential (Langmead *et al*., 2008). The majority of these receptors activate phospholipase C (PLC) and adenylyl cyclase (Nathanson, 2008), resulting in an increase of intracellular $Ca^{2+}$, and the activation of various kinases and the cAMP-dependent pathway. Receptor types M2 and M4, on the other hand, mediate inhibitory postsynaptic potentials. They couple to PTX-sensitive $G_{i/o}$ subunits, inhibit adenylyl cyclase and do not activate PLC (Nathanson, 2008). M2/M4 receptors are also capable of activating ion channels, especially $K^+$ channels (Soejima and Noma, 1985). Both of these mechanisms serve to decrease the amount of intracellular $Ca^{2+}$ and cAMP. M2/M4 are presynaptic inhibitory receptors used to regulate neurotransmitter release. M2 receptors, in particular, serve as autoreceptors controlling ACh-release in the brain (Douglas *et al*., 2001) and from presynaptic neurons at neuromuscular junctions (Slutsky *et al*, 1999, Minic *et al*, 2002). . The M2 receptor also plays an important role in the regulation of cardiac muscle (Caulfield, 1993) and smooth muscle contraction (Elhert *et al*., 1997).

Dysfunction of mAChRs has been linked to a number of clinical diseases, which is not surprising given the variety of essential functions they mediate. M1 and M5 receptor abnormalities are known to be involved in neurodegenerative diseases, such as

Parkinson's and Alzheimer's, as well as addictive behaviors. Also, the parasite *Trypanosoma cruzi* induces production of host antibodies against M2 receptors, causing cardiomyopathy in the late stages of Chagas disease (Hernandez *et al.*, 2003). Due to the receptor-specific nature of these pathologies, there is much interest in the development of subtype-selective agonists and antagonists to treat disease (Conn *et al.*, 2009).

### *D.4 Cholinergic GPCRs in Invertebrates*

Knowledge about the structure and pharmacology of invertebrate mAChRs is limited, although there is evidence that they exist in a number of phyla (Trimmer, 1995). For the most part, our knowledge of invertebrate mAChRs comes from studies of insects and nematodes, primarily C. elegans. Unlike vertebrates, insect and nematode cholinergic signalling is biased toward nicotinic receptors, which heavily outnumber mAChRs (Breer and Sattelle, 1987). The gene structure of invertebrate mAChRs (and other GPCRs) also differs from vertebrates as well. The majority of genes encoding human GPCRs are intronless. Invertebrate receptor genes, on the other hand, contain many introns are well known to undergo extensive alternative splicing (Fridmanis *et al*., 2007). Differences such as these point to fundamental differences in the function and pharmacology of invertebrate and vertebrate receptors.

Insect mAChRs are pharmacologically divided into two receptor subtypes (Knipper and Breer, 1988). This initial classification was performed on isolated tissues and was confirmed with the cloning of the first insect mAChR in *Drosophila*, Dm1 (Shapiro *et al*., 1989). Subsequent investigation of *Drosophila* and beetle mAChRs revealed that the two subtypes of insect mAChR are evolutionarily divergent (Collin *et al*., 2013), leading to a re-classification of invertebrate mAChRs as "A-type" and "B-type" receptors. The A-type receptors are evolutionarily related to vertebrate mAChRs and display classical muscarinic pharmacology. B-type receptors diverged during the Protostome/Deuterostome split some 700 million years ago (Collin *et al*., 2013). Although activated by ACh, these receptors show a very low affinity for muscarine and do not respond to the classical antagonists, atropine, scopolamine and QNB. Although several insect cholinergic GPCRs have been cloned and characterized *in vitro*, little is

known about their biological function. However, there is evidence that insect mAChRs act as autoreceptors to modulate ACh release (Breer and Knipper, 1984, Knipper and Breer, 1989) and also may modulate motor behavior (Trimmer, 1995).

As insects and nematodes are both protostomes, A-type and B-type cholinergic GPCRs are also present in *C. elegans*. However in nematodes, muscarinic receptors are referred to as GAR (G-protein-linked Acetylcholine Receptor) or GAR-like receptors (Lee *et al*., 1999). Three GAR genes (GAR-1, GAR-2, GAR-3) have been identified and cloned from *C. elegans* (Lee *et al*., 1999, Lee *et al*., 2000, Hwang *et al*., 1999). The *in vitro* characterization of GAR-1 and GAR-2 revealed that both receptors exhibit pharmacological profiles that are consistent with B-type invertebrate mAChRs. The endogenous agonist ACh activated both receptors but neither responded to the classical vertebrate mAChR agonist, oxotremorine (Lee *et al*., 1999, Lee *et al*., 2000). Treatment of GAR-1 with the antagonist atropine caused limited effects and GAR-2 showed no response to atropine. As both GAR-1 and GAR-2 were able to elicit a current via GIRK1 *in vitro*, they are hypothesized to couple to $G\alpha_{i/o}$ subunits and act in an inhibitory manner. More recently, a homolog of GAR-1 from the parasitic nematode *Ascaris* was cloned and characterized (Kimber *et al*., 2009). This receptor, AsGAR-1 displays similar pharmacology to its *C. elegans* relative, with the exception that it is activated by oxotremorine. Localization experiments using the GAR-1 promoter in *C. elegans* and tissue-specific PCR in *Ascaris* demonstrated that these receptors are expressed in sensory neurons and not the body wall musculature, indicating a neuromodulatory role. The GAR-3 gene is the closest homolog of human mAChRs. It is the only *C. elegans* cholinergic receptor to fall in the A-type. Similar to the human M1/M3/M5 receptors, it has a classical muscarinic pharmacological profile and stimulates PLC and cAMP production (Hwang *et al*., 1999, Park *et al*., 2006).

Although the effects of cholinergic agonists in flatworms are known (Ribeiro *et al*., 2005), there is very little information available about the receptors involved in this signaling. Some muscarinic agonists are known to affect the motor behavior of free-living planarians (Butarelli *et* al., 2000). In parasites, sequencing and annotation of the *S. mansoni* genome (Berriman *et al*. 2009, Protasio *et al*., 2012) indicates the presence of

one full-length mAChR-like sequence. Similarly, the genomes of other parasitic flatworms, such *Clonorchis*, *Taenia* and *Echinococcus* suggest small complements of mAChRs. However, no functional characterization of flatworm cholinergic GPCRs currently exists. Thus, the second major focus of this thesis is to characterize the lone putative mAChR encoded by *S. mansoni* and to investigate its function in the worm.

### *References*


1. Albuquerque EX, Pereira EF, Alkondon M, Rogers SW (2009). Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev **89**(1): 73-120.
2. Anwyl R (1999). Metabotropic glutamate receptors: electrophysiological properties and role in plasticity. Brain Res Brain Res Rev **29**(1): 83-120.
3. Baguñà J, Ballester R (1978). The nervous system in planarians: Peripheral and gastrodermal plexuses, pharynx innervation, and the relationship between central nervous system structure and the acoelomate organization. J. Morphol **155**: 237–252.
4. Baldwin JM, Schertler GF, Unger VM (1997). An alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein-coupled receptors. J Mol Biol **272**(1): 144-64.
5. Ballasteros J, Weinstein H (1995). Integrated methods for the construction of three dimensional models and computational probing of structure-function relations in G-protein coupled receptors. Methods in Neurosciences **25**: 366-428.
6. Barker, LR, Bueding E, Timms AR (1966). The possible role of acetylcholine in *Schistosoma mansoni*. Brit J Pharmacol **26**: 656-665.
7. Barreto ML, Smith DH, Sleigh AC (1990). Implications of faecal egg count variation when using the Kato - Katz method to assess *Schistosoma mansoni* infections. Trans R Soc Trop Med Hyg **84**: 554-555.
8. Beaver PC, Jung RC, Cupp EW (1984). Clinical parasitology. 9th ed, pp.733-746. Lea & Febiger, Philadelphia.
9. Becker B, Mehlhorn H, Andrews P, Thomas H, Eckert J (1980). Light and electron microscopic studies on the effect of praziquantel on *Schistosoma mansoni*, *Dicrocoelium dendriticum*, and *Fasciola hepatica* (Trematoda) in vitro. Z Parasitenkd **63**: 113-128.
10. Beckstein O, Tai K, Sansom MS (2004). Not ions alone: barriers to ion permeation in nanopores and channels. J Am Chem Soc. **126**(45): 14694-5.
11. Beech RN, Callanan MK, Rao VT, Dawe GB, Forrester SG (2013). Characterization of cys-loop receptor genes involved in inhibitory amine neurotransmission in parasitic and free living nematodes. Parasitol Int **62**(6): 599-605.
12. Benjamin PR, Elliott CJH (1989). Snail feeding oscillator: the central pattern generator and its control by modulatory interneurons. In: Neuronal and cellular oscillators, Jacklet JW, (ed), pp.173-214, Dekker, New York.

13. Bennett JL, Gianutsos G (1977). Distribution of catecholamines in immature *Fasciola hepatica*: a histochemical and biochemical study. Int J Parasitol **7**(3): 221-5.
14. Bentley GN, Jones AK, Oliveros Parra WG, Agnew A (2004). ShAR1alpha and ShAR1beta: novel putative nicotinic acetylcholine receptor subunits from the platyhelminth blood fluke *Schistosoma*. Gene **329**: 27-38.
15. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, et al. (2009). The genome of the blood fluke *Schistosoma mansoni*. Nature **460**: 352–358.
16. Bertrand D, Ballivet M, Gomez M, Bertrand S, Phannavong B, Gundelfinger ED (1994). Physiological properties of neuronal nicotinic receptors reconstituted from the vertebrate beta 2 subunit and Drosophila alpha subunits. Eur J Neurosci **6**(5): 869-75.
17. Birdsall NJM, Hulme EC (1983). Muscarinic receptor subclasses. Trends Pharmacol Sci **4**: 459–463.
18. Birks RI, McIntosh FC (1961). Acetylcholine metabolism of a sympathetic ganglion. Can J Biochem Physiol **39**: 787-827.
19. Birnbaumer L, Abramowitz J, Yatani A, Okabe K, Mattera R, Graf R, Sanford J, Codina J, Brown AM (1990). Roles of G proteins in coupling of receptors to ionic channels and other effector systems. Crit Rev Biochem Mol Biol **25**(4): 225-44.
20. Black CL, Muok EM, Mwinzi PN, Carter JM, Karanja DM, Secor WE, Colley DG (2010). Increases in levels of schistosome-specific immunoglobulin E and CD23(+) B cells in a cohort of Kenyan children undergoing repeated treatment and reinfection with *Schistosoma mansoni*. J Infect Dis **202**(3): 399-405.
21. Blair KL, Anderson PAV (1994). Physiological and pharmacological properties of muscle cells isolated from the flatworm *Bdelloura candida* (Tricladia). Parasitology **9**(3): 325-335.
22. Bohm A, Gaudet R, Sigler PB (1997). Structural aspects of heterotrimeric G-protein signaling. Curr Opin Biotechnol **8**(4): 480-7.
23. Botros S, Sayed H, El-Dusoki H, Sabry H, Rabie I, El-Ghannam M, Hassanein M, El-Wahab YA, Engels D (2005). Efficacy of mirazid in comparison with praziquantel in Egyptian Schistosoma mansoni-infected school children and households. Am J Trop Med Hyg **72**(2): 119-23.
24. Bottieau E, Clerinx J, de Vega MR, Van den Enden E, Colebunders R, Van Esbroeck M, Vervoort T, Van Gompel A, Van den Ende J (2006). Imported Katayama fever: clinical and biological features at presentation and during treatment. J. Infect **52**(5): 339–345.
25. Bowman WC, Prior C, Marshall IG (1990). Presynaptic receptors in the neuromuscular junction. Ann N Y Acad Sci **604**: 69-81.
26. Boyle JP, Wu XJ, Shoemaker CB, Yoshino TP (2003) Using RNA interference to manipulate endogenous gene expression in *Schistosoma mansoni* sporocysts. Mol Biochem Parasitol **128**(2): 205-15.
27. Breer H, Knipper M (1984). Characterization of acetylcholine release from insect synaptosomes. Insect Biochem **14**: 337-344.
28. Breer H, Kleene R, Hinz G (1985). Molecular forms and subunit structure of the acetylcholine receptor in the central nervous system of insects. J Neurosci **5**(12): 3386-92.

29. Breer H, Sattelle DB (1987). Molecular properties and functions of insect acetylcholine receptors. J Insect Physiol **33**: 771-790.
30. Breer H, Knipper M (1988). Subtypes of muscarinic receptors in insect nervous system. Comp Biochem Physiol **90**C: 275-280.
31. Brejc K, van Dijk WJ, Klaassen RV, Schuurmans M, van Der Oost J, Smit AB, Sixma TK (2001). Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature **411**(6835): 269-76.
32. Brenner S (1974). The genetics of *Caenorhabditis elegans*. Genetics **77**(1): 71-94.
33. Brown LA, Jones AK, Buckingham SD, Mee CJ, Sattelle DB (2006). Contributions from *Caenorhabditis elegans* functional genetics to antiparasitic drug target identification and validation: nicotinic acetylcholine receptors, a case study. Int J Parasitol **36**(6): 617-24.
34. Brown DA (2010). Muscarinic acetylcholine receptors (mAChRs) in the nervous system: some functions and mechanisms. J Mol Neurosci **41**(3): 340-6.
35. Brownlee DJ, Fairweather I (1996). Immunocytochemical localization of glutamate-like immunoreactivity within the nervous system of the cestode *Mesocestoides corti* and the trematode *Fasciola hepatica*. Parasitol Res **82**(5): 423-7.
36. Buckett KJ, Dockray GJ, Osborne NN, Benjamin PR (1990). Pharmacology of the myogenic heart of the pond snail *Lymnaea stagnalis*. J Neurophysiol **63**(6): 1413-25.
37. Bueding, E (1952). Acetylcholinesterase activity of *Schistosoma mansoni*. British Journal of Pharmacology and Chemotherapy, 7: 563–566.
38. Butarelli FR, Pontieri FE, Margotta V, Palladini G (2000). Acetylcholine/dopamine interaction in planaria. Comp Biochem Physiol C Toxicol Pharmacol **125**(2): 225-31.
39. Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, Hamm HE (2003). Insights into G protein structure, function, and regulation. Endocr Rev **24**(6): 765-81.
40. Caldas IR, Campi-Azevedo AC, Oliveira LF, Silveira AM, Oliveira RC, Gazzinelli G (2008). Human schistosomiasis mansoni: immune responses during acute and chronic phases of the infection. Acta Trop **108**(2-3): 109-17.
41. Camacho M, Agnew A (1995). Schistosoma: rate of glucose import is altered by acetylcholine interaction with tegumental acetylcholine receptors and acetylcholinesterase. Exp Parasitol **81**(4): 584-91.
42. Camacho M, Alsford S, Jones A, Agnew A (1995). Nicotinic acetylcholine receptors on the surface of the blood fluke Schistosoma. Mol Biochem Parasitol **71**(1): 127-34.
43. Casarosa P, Bakker RA, Verzijl D, Navis M, Timmerman H, Leurs R, Smit MJ (2001). Constitutive signaling of the human cytomegalovirus-encoded chemokine receptor US28. J Biol Chem **276**(2): 1133-7.
44. Caulfield MP (1993). Muscarinic receptors--characterization, coupling and function. Pharmacol Ther **58**(3): 319-79.
45. Caulfield MP, Birdsall NJ (1998). International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev **50**(2): 279-90.

46. Cebrià F, Kudome T, Nakazawa M, Mineta K, Ikeo K, Gojobori T, Agata K (2002). The expression of neural-specific genes reveals the structural and molecular complexity of the planarian central nervous system. Mech Dev **116**(1-2): 199-204.
47. Cerione RA, Codina J, Benovic JL, Lefkowitz RJ, Birnbaumer L, Caron MG (1984). The mammalian beta 2-adrenergic receptor: reconstitution of functional interactions between pure receptor and pure stimulatory nucleotide binding protein of the adenylate cyclase system. Biochemistry **23**(20): 4519-25.
48. Chamaon K, Schulz R, Smalla KH, Seidel B, Gundelfinger ED (2000). Neuronal nicotinic acetylcholine receptors of *Drosophila melanogaster*: the alpha-subunit dalpha3 and the beta-type subunit ARD co-assemble within the same receptor complex. FEBS Lett **482**(3): 189-92.
49. Chamaon K, Smalla KH, Thomas U, Gundelfinger ED (2002). Nicotinic acetylcholine receptors of Drosophila: three subunits encoded by genomically linked genes can co-assemble into the same receptor complex. J Neurochem **80**(1): 149-57.
50. Chance MR, Mansour TE (1953). A contribution to the pharmacology of movement in the liver fluke. Br J Pharmacol Chemother **8**(2): 134-8.
51. Chand MA, Chiodini PL, Doenhoff MJ (2010). Development of a new assay for the diagnosis of schistosomiasis, using cercarial antigens. Trans R Soc Trop Med Hyg **104**: 255-8.
52. Changeux JP, Kasai M, Lee CY (1970). Use of a snake venom toxin to characterize the cholinergic receptor protein. Proc Natl Acad Sci U S A **67**(3): 1241-7.
53. Chen M, Mott K (1989). Progress in the assessment of morbidity due to *Schistosoma japonicum* infection: a review of recent literature. Trop Dis Bull **85**: R1-R56.
54. Chen LS, Lin HC, Lee FY, Hou MC, Lee SD 1996. Portal hypertensive colopathy in patients with cirrhosis. Scand J Gastroenterol **31**: 490-494.
55. Chen C, Li J, Bot G, Szabo I, Rogers TJ, Liu-Chen LY (2004). Heterodimerization and cross-desensitization between the mu-opioid receptor and the chemokine CCR5 receptor. Eur J Pharmacol **483**(2-3): 175-86.
56. Chini B, Clementi F, Hukovic N, Sher E (1992). Neuronal-type alpha-bungarotoxin receptors and the alpha 5-nicotinic receptor subunit gene are expressed in neuronal and nonneuronal human cell lines. Proc Natl Acad Sci U S A **89**(5): 1572-6.
57. Cohen GB, Oprian DD, Robinson PR (1992). Mechanism of activation and inactivation of opsin: role of Glu113 and Lys296. Biochemistry **31**(50): 12592-601.
58. Cohen-Cory S (2002). The Developing Synapse: Construction and Modulation of Synaptic Structures and Circuits. Science **298**(5594): 770-776.
59. Collin C, Hauser F, de Valdivia EG, Li S, Reisenberger J, Carlsen EM, Khan Z, Hansen NO, Puhm F, Søndergaard L, Niemiec J, Heninger M, Ren GR, Grimmelikhuijzen CJ (2013). Two types of muscarinic acetylcholine receptors in Drosophila and other arthropods. Cell Mol Life Sci **70**(17): 3231-42.
60. Conn PJ, Jones CK, Lindsley CW (2009). Subtype-selective allosteric modulators of muscarinic receptors for the treatment of CNS disorders. Trends Pharmacol Sci **30**(3): 148-55.

61. Conroy WG, Berg DK (1995). Neurons can maintain multiple classes of nicotinic acetylcholine receptors distinguished by different subunit compositions. J Biol Chem **270**(9): 4424-31.
62. Correnti JM, Brindley PJ, Pearce EJ (2005). Long-term suppression of cathepsin B levels by RNA interference retards schistosome growth. Mol Biochem Parasitol **143**:209–215
63. Courjaret R, Lapied B (2001). Complex intracellular messenger pathways regulate one type of neuronal alpha-bungarotoxin-resistant nicotinic acetylcholine receptors expressed in insect neurosecretory cells (dorsal unpaired median neurons). Mol Pharmacol **60**(1): 80-91.
64. Couto FF, Coelho PM, Araújo N, Kusel JR, Katz N, Jannotti-Passos LK, Mattos AC (2011). *Schistosoma mansoni*: a method for inducing resistance to praziquantel using infected *Biomphalaria glabrata* snails. Mem Inst Oswaldo Cruz **106**: 153-157.
65. Cuevas J, Berg DK (1998). Mammalian nicotinic receptors with alpha7 subunits that slowly desensitize and rapidly recover from alpha-bungarotoxin blockade. J Neurosci **18**(24): 10335-44.
66. Culetto E, Baylis HA, Richmond JE, Jones AK, Fleming JT, Squire MD, Lewis JA, Sattelle DB (2004). The *Caenorhabditis elegans* unc-63 gene encodes a levamisole-sensitive nicotinic acetylcholine receptor alpha subunit. J Biol Chem **279**(41): 42476-83.
67. Cully DF, Paress PS, Liu KK, Schaeffer JM, Arena JP (1996). Identification of a *Drosophila melanogaster* glutamate-gated chloride channel sensitive to the antiparasitic agent avermectin. J Biol Chem **277**(33): 20187-20191.
68. Curtis CA, Wheatley M, Bansal S, Birdsall NJ, Eveleigh P, Pedder EK, Poyner D, Hulme EC (1989). Propylbenzilylcholine mustard labels an acidic residue in transmembrane helix 3 of the muscarinic receptor. J Biol Chem **264**(1): 489-95.
69. Dale HH (1914). The action of certain esters and ethers of choline, and their relation to muscarine. J Pharmacol Exp Ther **6**: 147–190.
70. Dajas-Bailador F, Wonnacott S (2004). Nicotinic acetylcholine receptors and the regulation of neuronal signalling. Trends Pharmacol Sci **25**(6): 317-24.
71. Davis RE, Stretton AOW (1995). Neurotransmitters in helminths, pp.257-87. In Marr JJ, Muller M (eds), Biochemistry and Molecular Biology of Parasites. Academic Press, New York.
72. Day TA, Maule AG, Shaw C, Halton DW, Moore S, Bennett JL, Pax RA (1994). Platyhelminth FMRFamide-related peptides (FaRPs) contract *Schistosoma mansoni* (Trematoda: Digenea) muscle fibres in vitro. Parasitology **109**(Pt 4): 455-9.
73. Day TA, Chen GZ, Miller C, Tian M, Bennett JL, Pax RA (1996). Cholinergic inhibition of muscle fibers isolated from *Schistosoma mansoni* (Trematoda: Digenea). Parasitology **113**(Pt. 1): 55-61.
74. Dederer H, Werr M, Ilg T (2011). Differential sensitivity of Ctenocephalides felis and *Drosophila melanogaster* nicotinic acetylcholine receptor α1 and α2 subunits in recombinant hybrid receptors to nicotinoids and neonicotinoid insecticides. Insect Biochem Mol Biol **41**(1): 51-61.

75. Del Castillo J, De Mello WC, Morales T (1967). The initiation of action potentials in the somatic musculature of *Ascaris lumbricoides*. J Exp Biol **46**(2): 263-79.
76. De Filippi G, Baldwinson T, Sher E (2005). Nicotinic receptor modulation of neurotransmitter release in the cerebellum. Prog Brain Res **148**: 307-20.
77. De Jonge N, Gryseels B, Hilberath GW, Poederman AM, Deelder AM (1988). Detection of circulating anodic antigen by ELISA for seroepidemiology of Schistosomiasis mansoni. Trans R Soc Trop Med Hyg **82**: 591–594.
78. De Planque MR, Rijkers DT, Liskamp RM, Separovic F (2004). The alphaM1 transmembrane segment of the nicotinic acetylcholine receptor interacts strongly with model membranes. Magn Reson Chem **42**(2): 148-54.
79. Dhanasekaran N, Dermott JM (1996). Signaling by the G12 class of G proteins. Cell Signal **8**(4): 235-45.
80. Dinguirard N, Yoshino TP (2006). Potential role of a CD36-like class B scavenger receptor in the binding of modified low-density lipoprotein (acLDL) to the tegumental surface of *Schistosoma mansoni* sporocysts. Mol Biochem Parasitol **146**:219–230.
81. Doherty JF, Moody AH, Wright SG (1996). Katayama fever: an acute manifestation of schistosomiasis. BMJ **313**(7064): 1071–1072.
82. Doenhoff MJ, Hagan P, Cioli D, Southgate V, Pica-Mattoccia L, *et al*. (2009). Praziquantel: its use in control of schistosomiasis in sub-Saharan Africa and current research needs. Parasitology **136**(13): 1825-35.
83. Douglas CL, Baghdoyan HA, Lydic R (2001). M2 muscarinic autoreceptors modulate acetylcholine release in prefrontal cortex of C57BL/6J mouse. J Pharmacol Exp Ther **299**(3): 960-6.
84. Dryja TP, Berson EL, Rao VR, Oprian DD (1993). Heterozygous missense mutation in the rhodopsin gene as a cause of congenital stationary night blindness. Nat Genet **4**(3): 280-3.
85. Dudai Y, Amsterdam A (1977). Nicotinic receptors in the brain of Drosophila melanogaster demonstrated by autoradiography with [125I]alpha-bungarotoxin. Brain Res **130**(3): 551-5.
86. Dufour V, Beech RN, Wever C, Dent JA, Geary TG (2013). Molecular Cloning and Characterization of Novel Glutamate-Gated Chloride Channel Subunits from *Schistosoma mansoni*. PLoS Pathog **9**(8): e1003586.
87. Dunne DW, Pearce EJ (1999) Immunology of hepatosplenic schistosomiasis mansoni: a human perspective. Microbes Infect **1**: 553–560.
88. Dupre L, Herve M, Schacht AM, Capron A, Riveau G (1999). Control of schistosomiasis pathology by combination of Sm28GST DNA immunization and praziquantel treatment. J Infect Dis **180**: 454-463.
89. Dupuis J, Louis T, Gauthier M, Raymond V (2012). Insights from honeybee (Apis mellifera) and fly (Drosophila melanogaster) nicotinic acetylcholine receptors: from genes to behavioral functions. Neurosci Biobehav Rev **36**(6): 1553-64.
90. Eccles JC, O'connor WJ (1941). Abortive impulses at the neuro-muscular junction. J Physiol **100**(3): 318-28.
91. Elgoyhen AB, Vetter DE, Katz E, Rothlin CV, Heinemann SF, Boulter J (2001). alpha10: a determinant of nicotinic cholinergic receptor function in mammalian

vestibular and cochlear mechanosensory hair cells. Proc Natl Acad Sci U S A **98**(6): 3501-6.
92. Ehlert FJ, Ostrom RS, Sawyer GW (1997). Subtypes of the muscarinic receptor in smooth muscle. Life Sci **61**(18): 1729-40.
93. El-Shehabi F, Ribeiro P (2010). Histamine signalling in Schistosoma mansoni: immunolocalisation and characterisation of a new histamine-responsive receptor (SmGPR-2). Int J Parasitol **40**(12): 1395-406.
94. El-Shehabi F, Taman A, Moali LS, El-Sakkary N, Ribeiro P (2012). A novel G protein-coupled receptor of *Schistosoma mansoni* (SmGPR-3) is activated by dopamine and is widely expressed in the nervous system. PLoS Negl Trop Dis **6**(2): e1523.
95. Elwyn D, Weissbach A, Henry SS, Sprinson DB (1955). The biosynthesis of choline from serine and related compounds. J Biol Chem **213**(1): 281-95.
96. Ercoli N, Payares G, Nunez D (1985). *Schistosoma mansoni*: neurotransmitters and the mobility of cercariae and schistosomules. Exp Parasitol **59**: 204–216.
97. Fagerlund MJ and Eriksson LI (2009). Current concepts in neuromuscular transmission. Br J Anaesth **103**(1): 108-114.
98. Faghih R, Gopalakrishnan M, Briggs CA (2008). Allosteric modulators of the alpha7 nicotinic acetylcholine receptor. J Med Chem **51**(4): 701-12.
99. Fallon PG, Doenhoff MJ (1994) Drug-resistant schistosomiasis: resistance to praziquantel and oxamniquine induced in *Schistosoma* mansoni in mice is drug specific. Am J Trop Med Hyg **51**: 83–88.
100. Feldmeier H, Krantz I, Poggensee G (1995). Female genital schistosomiasis: a neglected risk factor for the transmission of HIV? Trans R Soc Trop Med Hyg **89**: 237.
101. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391**(6669): 806-11.
102. Fleming JT, Squire MD, Barnes TM, Tornoe C, Matsuda K, Ahnn J, Fire A, Sulston JE, Barnard EA, Sattelle DB, Lewis JA (1997). *Caenorhabditis elegans* levamisole resistance genes lev-1, unc-29, and unc-38 encode functional nicotinic acetylcholine receptor subunits. J Neurosci **17**(15): 5843-57.
103. Francis MM, Papke RL (1996). Muscle-type nicotinic acetylcholine receptor delta subunit determines sensitivity to noncompetitive inhibitors, while gamma subunit regulates divalent permeability. Neuropharmacology **35**(11): 1547-56.
104. Francis MM, Evans SP, Jensen M, Madsen DM, Mancuso J, Norman KR, Maricq AV (2005). The Ror receptor tyrosine kinase CAM-1 is required for ACR-16-mediated synaptic transmission at the C. elegans neuromuscular junction. Neuron **46**(4): 581-94.
105. Fredriksson R, Lagerström MC, Lundin LG, Schiöth HB (2003). The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol **63**(6): 1256-72.
106. Gahring LC, Persiyanov K, Rogers SW (2005). Mouse strain-specific changes in nicotinic receptor expression with age. Neurobiol Aging **26**(6): 973-80.

107. Galzi JL, Devillers-Thiéry A, Hussy N, Bertrand S, Changeux JP, Bertrand D (1992). Mutations in the channel domain of a neuronal nicotinic receptor convert ion selectivity from cationic to anionic. Nature. **359**(6395): 500-5.
108. Garcia LR, Mehta P, Sternberg PW (2001). Regulation of distinct muscle behaviors controls the *C. elegans* male's copulatory spicules during mating. Cell **107**(6): 777-88.
109. Geary TG (2013). Are new anthelmintics needed to eliminate human helminthiases? Curr Opin Infect Dis **25**(6): 709-17.
110. Gentles AJ, Karlin S (1999). Why are human G-protein-coupled receptors predominantly intronless? Trends Genet **15**(2): 47-9.
111. Gether U (2000). Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev **21**(1): 90-113.
112. Gobert GN, Stenzel DJ, McManus DP, Jones MK (2003) The ultrastructural architecture of the adult *Schistosoma japonicum* tegument. Int J Parasitol **33**(14): 1561-75.
113. Green WN, Wanamaker CP (1988). Formation of the nicotinic acetylcholine receptor binding sites. J Neurosci **18**(15): 5555-64.
114. Greenberg RM (2013). New approaches for understanding mechanisms of drug resistance in schistosomes. Parasitology **140**(12): 1534-46.
115. Gryseels B, Polman K, Clerinx J, Kestens L (2006). Human schistosomiasis. Lancet **368**: 1106–18.
116. Haga K, Kruse AC, Asada H, Yurugi-Kobayashi T, Shiroishi M, Zhang C, Weis WI, Okada T, Kobilka BK, Haga T, Kobayashi T (2012). Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. Nature **482**(7386): 547-51.
117. Hales TG, Deeb TZ, Tang H, Bollan KA, King DP, Johnson SJ, Connolly CN (2006). An asymmetric contribution to gamma-aminobutyric type A receptor function of a conserved lysine within TM2-3 of alpha1, beta2, and gamma2 subunits. J Biol Chem **281**(25): 17034-43.
118. Halton DW (1967). Histochemical studies of carboxylic esterase activity in *Fasciola hepatica*. J Parasitol **53**(6): 1210-6.
119. Halton DW and Gustaffson MKS (1996). Functional morphology of the platyhelminth nervous system. Parasitology **113**: S47–S72.
120. Halton DW, Maule AG (2004). Flatworm nerve–muscle: structural and functional analysis. Can J Zool **82**(2): 316-333.
121. Hamdan FF and Ribeiro P (1998). Cloning and characterization of a novel form of tyrosine hydroxylase from the human parasite, *Schistosoma mansoni*. J Neurochem **71**(4): 1369-80.
122. Hamdan FF, Abramovitz M, Mousa A, Xie J, Durocher Y, Ribeiro P (2002). A novel Schistosoma mansoni G protein-coupled receptor is responsive to histamine. Mol Biochem Parasitol **119**(1): 75-86.
123. Hannan F, Hall LM (1993). Muscarinic acetylcholine receptors in invertebrates: comparisons with homologous receptors from vertebrates. EXS **63**: 98-145.
124. Hernández CC, Barcellos LC, Giménez LE, Cabarcas RA, Garcia S, Pedrosa RC, Nascimento JH, Kurtenbach E, Masuda MO, Campos de Carvalho

AC (2003). Human chagasic IgGs bind to cardiac muscarinic receptors and impair L-type Ca2+ currents. Cardiovasc Res **58**(1): 55-65.
125. Heuss C, Gerber U (2000). G-protein-independent signaling by G-protein-coupled receptors. Trends Neurosci **23**(10): 469-75.
126. Hillman GR, Senft AW (1973). Schistosome motility measurements: response to drugs. J Pharmacol Exp Ther **185**(2): 177-84.
127. Hobert O (2013). The neuronal genome of Caenorhabditis elegans. WormBook, pp. 1-106.
128. Holden-Dye L, Joyner M, O'Connor V, Walker RJ (2013). Nicotinic acetylcholine receptors: a comparison of the nAChRs of *Caenorhabditis elegans* and parasitic nematodes. Parasitol Int **62**(6): 606-15.
129. Holmes SD, Fairweather I (1984). Fasciola hepatica: the effects of neuropharmacological agents upon in vitro motility. Exp Parasitol **58**(2): 194-208.
130. Hulme EC, Lu ZL, Saldanha JW, Bee MS (2003). Structure and activation of muscarinic acetylcholine receptors. Biochem Soc Trans **31**(Pt 1): 29-34.
131. Humphries JE, Kimber MJ, Barton YW, Hsu W, Marks NJ, Greer B, Harriott P, Maule AG, Day TA (2004). Structure and bioactivity of neuropeptide F from the human parasites *Schistosoma mansoni* and *Schistosoma japonicum*. J Biol Chem **279**: 39880–39885.
132. Hwang JM, Chang DJ, Kim US, Lee YS, Park YS, *et al*. (1999). Cloning and functional characterization of a *Caenorhabditis elegans* muscarinic acetylcholine receptor. Receptors Channels **6**: 415–424.
133. Jeziorski MC, Greenberg RM (2006). Voltage-gated Ca2+ channel subunits from schistosomes and other platyhelminths: potential role in praziquantel action. Int J Parasitol **36**: 625-632.
134. Joffe B**,** Kotikova EA (1991). Distribution of catecholamines in turbellarians (with discussion of neuronal homologies in the Platyhelminthes). Studies in Neuroscience **13**: 77-113.
135. Johnston CA, Siderovski DP (2007). Structural basis for nucleotide exchange on G alpha i subunits and receptor coupling specificity. Proc Natl Acad Sci U S A **104**(6): 2001-6.
136. Jones PG, Curtis CA, Hulme EC (1995). The function of a highly-conserved arginine residue in activation of the muscarinic M1 receptor. Eur J Pharmacol **288**(3): 251-7.
137. Jones AK, Bentley GN, Oliveros Parra WG, Agnew A (2004). Molecular characterization of an acetylcholinesterase implicated in the regulation of glucose scavenging by the parasite *Schistosoma*. FASEB J **16**(3): 441-3.
138. Jones AK, Marshall J, Blake AD, Buckingham SD, Darlison MG, Sattelle DB (2005). Sgbeta1, a novel locust (Schistocerca gregaria) non-alpha nicotinic acetylcholine receptor-like subunit with homology to the Drosophila melanogaster Dbeta1 subunit. Invert Neurosci **5**(3-4): 147-55.
139. Jones AK, Sattelle DB (2004). Functional genomics of the nicotinic acetylcholine receptor gene family of the nematode, *Caenorhabditis elegans*. Bioessays **26**(1): 39-49.
140. Jones AK, Sattelle DB (2010). Diversity of insect nicotinic acetylcholine receptor subunits. Adv Exp Med Biol **683**: 25-43.

141. Kane CA, Most H (1948). Schistosomiasis of the central nervous system: experiences in World War II and a review of the literature. Arch Neurol Psychiatry **59**:141-83.
142. Katritch V, Cherezov V, Stevens RC (2013). Structure-function of the G protein-coupled receptor superfamily. Annu Rev Pharmacol Toxicol **53**: 531-56.
143. Katz N, Chaves A & Pellegrino J (1972) A simple device for quantitative stool thick smear technique in *Schistosomiasis mansoni*. Revista Do Instituto de Medicina Tropical de Sao Paulo **14**: 397–400.
144. Katz B, Miledi R (1961). The localized action of 'end plate drugs' in the twitch fibres of the frog. J Physiol **156**: 24-25.
145. Kehoe J (1972). Three acetylcholine receptors in Aplysia neurons. J Physiol **225**(1): 115-46.
146. Kehoe J, McIntosh JM (1998). Two distinct nicotinic receptors, one pharmacologically similar to the vertebrate alpha7-containing receptor, mediate Cl currents in aplysia neurons. J Neurosci **18**(20): 8198-213.
147. Keramidas A, Moorhouse AJ, Pierce KD, Schofield PR, Barry PH (2002). Cation-selective mutations in the M2 domain of the inhibitory glycine receptor channel reveal determinants of ion-charge selectivity. J Gen Physiol **119**(5): 393-410.
148. Kim JM, Altenbach C, Thurmond RL, Khorana HG, Hubbell WL (1997). Structure and function in rhodopsin: rhodopsin mutants with a neutral amino acid at E134 have a partially activated conformation in the dark state. Proc Natl Acad Sci U S A **94**(26): 14273-8.
149. Kimber MJ, Sayegh L, El-Shehabi S, Song C, Zamanian M *et al*. (2009). Identification of an *Ascaris* G protein-coupled acetylcholine receptor with atypical muscarinic pharmacology. Int J Parasitol **39**(11): 1215-22.
150. Kistler J, Stroud R, Klymkowsky M, Lalancette R, Fairclough R (1982). Structure and function of an acetylcholine receptor. Biophys J **37**: 371–383.
151. Knipper M, Breer H (1989). Muscarinic receptors modulating acetylcholine release from insect synaptosomes. Comp Biochem Physiol C **93**(2): 287-92.
152. Kolodziejczyk, A, Sun, X, Meinertzhagen IA, Nässel DR (2008). Glutamate, GABA and acetylcholine signaling components in the lamina of the *Drosophila* visual system. PLoS ONE **3**(5): e2110.
153. Koski G, Streaty RA, Klee WA (1982). Modulation of sodium-sensitive GTPase by partial opiate agonists. An explanation for the dual requirement for Na+ and GTP in inhibitory regulation of adenylate cyclase. J Biol Chem **257**(23): 14035-40.
154. Kracun S, Harkness PC, Gibb AJ, Millar NS (2008). Influence of the M3-M4 intracellular domain upon nicotinic acetylcholine receptor assembly, targeting and function. Br J Pharmacol **153**(7): 1474-84.
155. Kreutz-Peterson G, Radwanska M, Ndegua D, Shoemaker CK, Skelly PJ (2007). Optimizing gene suppression in schistosomes using RNA interference. Mol Biochem Parasitol **153**(2): 194-202.
156. Kristiansen K (2004). Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular

modeling and mutagenesis approaches to receptor structure and function. Pharmacol Ther **103**(1): 21-80.
157. Kruse AC, Hu J, Pan AC, Arlow DH, Rosenbaum DM, Rosemond E, Green HF, Liu T, Chae PS, Dror RO, Shaw DE, Weis WI, Wess J, Kobilka BK (2012). Structure and dynamics of the M3 muscarinic acetylcholine receptor. Nature **482**(7386): 552-6.
158. Kues WA, Sakmann B, Witzemann V (1995). Differential expression patterns of five acetylcholine receptor subunit genes in rat muscle during development. Eur J Neurosci **7**(6): 1376-85.
159. Langley JN (1905). On the reaction of cells and of nerve-endings to certain poisons, chiefly as regards the reaction of striated muscle to nicotine and to curari. J Physiol **33**(4-5): 374-413.
160. Langmead CJ, Watson J, Reavill C (2008). Muscarinic acetylcholine receptors as CNS drug targets. Pharmacol Ther **117**(2): 232-43.
161. Lanzafame AA, Sexton PM, Christopoulos A (2006). Interaction studies of multiple binding sites on m4 muscarinic acetylcholine receptors. Mol Pharmacol **70**(2): 736-46.
162. Larsen MB, Fontana AC, Magalhães LG, Rodrigues V, Mortensen OV (2011). A catecholamine transporter from the human parasite *Schistosoma mansoni* with low affinity for psychostimulants. Mol Biochem Parasitol **177**(1): 35-41.
163. Leach K, Simms J, Sexton PM, Christopoulos A (2006). Structure-function studies of muscarinic acetylcholine receptors. Handb Exp Pharmacol **208**: 29-48.
164. Lebon G, Warne T, Edwards PC, Bennett K, Langmead CJ, Leslie AG, Tate CG (2011). Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation. Nature **474**(7352): 521-5.
165. Lecci A, Santicioli P, Maggi CA (2010). Pharmacology of transmission to gastrointestinal muscle. Curr Opin Pharmacol **2**(6): 630-41.
166. Lee D, O'Dowd DK (1999). Fast excitatory synaptic transmission mediated by nicotinic acetylcholine receptors in Drosophila neurons. J Neurosci **19**(13): 5311-21.
167. Lee YS, Park YS, Chang DJ, Hwang JM, Min CK, *et al*. (1999). Cloning and expression of a G protein-linked acetylcholine receptor from *Caenorhabditis elegans*. J Neurochem **72**: 58–65.
168. Lee YS, Park YS, Nam S, Suh SJ, Lee J, *et al*. (2000). Characterization of GAR-2, a novel G protein-linked acetylcholine receptor from *Caenorhabditis elegans*. J Neurochem **75**: 1800–1809.
169. Lefkowitz RJ, Cotecchia S, Samama P, Costa T (1993). Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. Trends Pharmacol Sci **14**(8): 303-7.
170. Lefkowitz RJ (2000). The superfamily of heptahelical receptors. Nat Cell Biol **2**(7): E133-6.
171. Lengeler C, Kilima P, Mshinda H, Morona D, Hatz C, Tanner M (1991). Rapid, low-cost, two-step method to screen for urinary schistosomiasis at the district level: the Kilosa experience. Bull World Health Organ **69**(2): 179-89.

172. Le Novère N, Changeux JP (1995). Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells. J Mol Evol **40**(2): 155-72.
173. Lewis JA, Wu CH, Levine JH, Berg H (1980). Levamisole-resistant mutants of the nematode *Caenorhabditis elegans* appear to lack pharmacological acetylcholine receptors. Neuroscience **5**(6): 967-89.
174. Lindstrom J (1997). Nicotinic acetylcholine receptors in health and disease. Mol Neurobiol **15**(2): 193-222.
175. Liu JJ, Horst R, Katritch V, Stevens RC, Wüthrich K (2012). Biased signaling pathways in β2-adrenergic receptor characterized by 19F-NMR. Science **335**(6072): 1106-10.
176. Lu ZL, Hulme EC (1999). The functional topography of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor, revealed by scanning mutagenesis. J Biol Chem **274**(11): 7309-15.
177. Mair GR, Maule AG, Day TA, Halton DW (2000). A confocal microscopical study of the musculature of adult Schistosoma mansoni. Parasitology **121** (Pt 2): 163-70.
178. Mair GR, Niciu MJ, Stewart MT, Brennan G, Omar H, Halton DW, Mains R, Eipper BA, Maule AG, Day TA (2004). A functionally atypical amidating enzyme from the human parasite *Schistosoma mansoni*. FASEB **18**(1): 114-21.
179. Marks NJ, Maule AG (2010). Neuropeptides in helminths: occurrence and distribution. Adv Exp Med Biol **692**: 49-77.
180. Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M, Sattelle DB (2001). Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. Trends Pharmacol Sci **22**(11): 573-80.
181. Maule AG, Halton DW, Johnston CF, Shaw C, Fairweather I. (1990). A cytochemical study of the serotoninergic, cholinergic and peptidergic components of the reproductive system in the monogenean parasite, *Diclidophora merlangi*. Parasitol Res **76**(5): 409-19.
182. McKay DM, Halton DW, Allen JM, Fairweather I (1989). The effects of cholinergic and serotoninergic drugs on motility in vitro of *Haplometra cylindracea* (Trematoda: Digenea). Parasitology **99**(Pt 2): 241-52.
183. McKay JP, Raizen DM, Gottschalk A, Schafer WR, Avery L (2004). eat-2 and eat-18 are required for nicotinic neurotransmission in the *Caenorhabditis elegans* pharynx. Genetics **166**(1): 161-9.
184. McKinnon NK, Bali M, Akabas MH (2012). Length and amino acid sequence of peptides substituted for the 5-HT3A receptor M3M4 loop may affect channel expression and desensitization. PLoS One **7**(4): e35563.
185. McLaren DJ, Hockley DJ (1977). Blood flukes have a double outer membrane. Nature **269**: 147-149.
186. Miyazawa A, Fujiyoshi Y, Unwin N (2003). Structure and gating mechanism of the acetylcholine receptor pore. Nature **423**(6943): 949-55.
187. McVeigh P, Mair GR, Atkinson L, Ladurner P, Zamanian M, Novozhilova E, Marks NJ, Day TA, Maule AG (2009). Discovery of multiple neuropeptide families in the phylum Platyhelminthes. Int J Parasitol **39**(11): 1243-52.

188. McVeigh P, Mair GR, Novozhilova E, Day A, Zamanian M, Marks NJ, Kimber MJ, Day TA, Maule AG (2011). Schistosome I/Lamides--a new family of bioactive helminth neuropeptides. Int J Parasitol **41**(8): 905-13.
189. Mellanby H (1955). The identification and estimation of acetylcholine in three parasitic nematodes (Ascaris lumbricoides, Litomosoides carinii, and the microfilariae of Dirofilaria repens). Parasitology **45**(3-4): 287-94.
190. Mellin TN, Busch RD, Wang CC, Kath G (1983). Neuropharmacology of the parasitic trematode, *Schistosoma mansoni*. Am J Trop Med Hyg **32**(1): 83-93.
191. Melman SD, Steinauer ML, Cunningham C, Kubatko LS, Mwangi IN, *et al* (2009). Reduced Susceptibility to Praziquantel among Naturally Occurring Kenyan Isolates of *Schistosoma mansoni*. PLoS Negl Trop Dis **3**(8): e504.
192. Millar NS, Gotti C (2009). Diversity of vertebrate nicotinic acetylcholine receptors. Neuropharmacology **56**(1): 237-46.
193. Mineta K, Nakazawa M, Cebria F, Ikeo K, Agata K, Gojobori T (2003). Origin and evolutionary process of the CNS elucidated by comparative genomics analysis of planarian ESTs. Proc Natl Acad Sci U S A **100**(13): 7666-71.
194. Moneypenny CG, Kreshchenko N, Moffett CL, Halton DW, Day TA, Maule AG (2001). Physiological effects of FMRFamide-related peptides and classical transmitters on dispersed muscle fibres of the turbellarian. *Procerodes littoralis*. Parasitology **122**: 447–455.
195. Mongan NP, Baylis HA, Adcock C, Smith GR, Sansom MS, Sattelle DB (1998). An extensive and diverse gene family of nicotinic acetylcholine receptor alpha subunits in Caenorhabditis elegans. Receptors Channels **6**(3): 213-28.
196. Mongan NP, Jones AK, Smith GR, Sansom MS, Sattelle DB (2002). Novel alpha7-like nicotinic acetylcholine receptor subunits in the nematode *Caenorhabditis elegans*. Protein Sci **11**(5): 1162-71.
197. Morales ME, Rinaldi G, Gobert GN, Kines KJ, Tort JF, Brindley PJ (2008). RNA interference of *Schistosoma mansoni* cathepsin D, the apical enzyme of the hemoglobin proteolysis cascade. Mol Biochem Parasitol **157**:160–168.
198. Nabhan JF, El-Shehabi F, Patocka N, Ribeiro P (2007). The 26S proteasome in *Schistosoma mansoni*: bioinformatics analysis, developmental expression, and RNA interference (RNAi) studies. Exp Parasitol **117**(3): 337-47.
199. Nathanson NM (2008). Synthesis, trafficking, and localization of muscarinic acetylcholine receptors. Pharmacol Ther **119**(1): 33-43.
200. Nishimura K, Kitamura Y, Taniguchi T, Agata K. (2010). Analysis of motor function modulated by cholinergic neurons in planarian *Dugesia japonica*. Neurosci **168**(1): 18-30.
201. Noda M, Takahashi H, Tanabe T, Toyosato M, Furutani Y, Hirose T, Asai M, Inayama S, Miyata T, Numa S (1982). Primary structure of alpha-subunit precursor of *Torpedo californica* acetylcholine receptor deduced from cDNA sequence. Nature **299**(5886): 793-7.
202. Nomura J, Hosoi T, Okuma Y, Nomura Y (2003). The presence and functions of muscarinic receptors in human T cells: the involvement in IL-2 and IL-2 receptor system. Life Sci **72**(18-19): 2121-6.

203. Nygaard R, Frimurer TM, Holst B, Rosenkilde MM, Schwartz TW (2009). Ligand binding and micro-switches in 7TM receptor structures. Trends Pharmacol Sci **30**(5): 249-59.
204. Nys M, Kesters D, Ulens C (2013). Structural insights into Cys-loop receptor function and ligand recognition. Biochem Pharmacol **86**(8): 1042-53.
205. Oleskevich S (1999). Cholinergic synaptic transmission in insect mushroom bodies in vitro. J Neurophysiol **82**(2): 1091-6.
206. Oliveira G, Johnston DA (2001). Mining the schistosome DNA sequence database. Trends Parasitol **17**: 501-503.
207. Oliveira LM, Santos HL, Gonçalves MM, Barreto MG, Peralta JM (2010). Evaluation of polymerase chain reaction as an additional tool for the diagnosis of low-intensity Schistosoma mansoni infection. Diagn Microbiol Infec Dis **68**(4): 416-21.
208. Olsen KL, Edwards E, Schechter N, Whalen RE (1988). Muscarinic receptors in preoptic area and hypothalamus: effects of cyclicity, sex and estrogen treatment. Brain Res **448**(2): 223-9.
209. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000). Crystal structure of rhodopsin: A G protein-coupled receptor. Science **289**(5480): 739-45.
210. Panicker S, Cruz H, Arrabit C, Slesinger PA (2002). Evidence for a centrally located gate in the pore of a serotonin-gated ion channel. J Neurosci **22**(5): 1629-39.
211. Park YS, Cho TJ, Cho NJ (2013). Stimulation of cyclic AMP production by the Caenorhabditis elegans muscarinic acetylcholine receptor GAR-3 in Chinese hamster ovary cells. Arch Biochem Biophys **450**(2): 203-7.
212. Parsons SM, Bahr BA, Gracz LM, Kaufman R, Kornreich WD, Nilsson L, Rogers GA (1987). Acetylcholine transport: fundamental properties and effects of pharmacologic agents. Ann N Y Acad Sci **493**: 220-33.
213. Patocka N and Ribeiro P (2013). The functional role of a serotonin transporter in *Schistosoma mansoni* elucidated through immunolocalization and RNA interference (RNAi). Mol Biochem Parasitol **187**: 32-42.
214. Patocka N, Sharma N, Rashid M, Ribeiro P (2014). Serotonin Signaling in *Schistosoma mansoni*: A Serotonin–Activated G Protein-Coupled Receptor Controls Parasite Movement. PLoS Pathog 10(1): e1003878.
215. Paveley RA, Mansour NR, Hallyburton I, Bleicher LS, Benn AE, et al. (2012). Whole Organism High-Content Screening by Label-Free, Image-Based Bayesian Classification for Parasitic Diseases. PLoS Negl Trop Dis **6**(7): e1762.
216. Pax RA, Bennett JL, Fetterer JL (1978). A benzodiazepine derivative and praziquantel: effects on musculature of *Schistosoma mansoni* and *Schistosoma haematobium*. Naunym-Schmiedberg's Arch Pharmacol **304**: 309-315.
217. Peng ZL, Yang JY, Chen X (2010). An improved classification of G-protein-coupled receptors using sequence-derived features. BMC Bioinformatics **11**: 420.
218. Pidoplichko VI, DeBiasi M, Williams JT, Dani JA (1997). Nicotine activates and desensitizes midbrain dopamine neurons. Nature **390**:401-404.

219. Piserchio A, Zelesky V, Yu J, Taylor L, Polgar P, Mierke DF (2005). Bradykinin B2 receptor signaling: structural and functional characterization of the C-terminus. Biopolymers **80**(2-3): 367-73.
220. Poggensee G, Feldmeier H (2001). Female genital schistosomiasis: facts and hypotheses. Acta Trop **79**: 193-210.
221. Posadas I, López-Hernández B, Ceña V (2013). Nicotinic receptors in neurodegeneration. Curr Neuropharmacol **11**(3): 298-314.
222. Protasio AV, Tsai IJ, Babbage A, Nichol S, Hunt M (2012). A systematically improved high quality genome and transcriptome of the human blood fluke *Schistosoma mansoni*. PLoS Negl Trop Dis **6**(1): e1455.
223. Putrenko I, Zakikhani M, Dent JA (2005). A Family of Acetylcholine-gated Chloride Channel Subunits in *Caenorhabditis elegans*. J Biol Chem **280**: 6392-6398.
224. Rabello A (1997). Diagnosing Schistosomiasis. Mem Inst Oswaldo Cruz **92**(5): 669-76.
225. Rand JB (2007). Acetylcholine. WormBook, pp.1-21.
226. Reiter E, Ahn S, Shukla AK, Lefkowitz RJ (2012). Molecular mechanism of β-arrestin-biased agonism at seven-transmembrane receptors. Annu Rev Pharmacol Toxicol **52**: 179-97.
227. Ren XQ, Cheng SB, Treuil MW, Mukherjee J, Rao J, Braunewell KH, Lindstrom JM, Anand R (2005). Structural determinants of alpha4beta2 nicotinic acetylcholine receptor trafficking. J Neurosci **25**(28): 6676-86.
228. Reuter M, Gustaffson M (1996). Neuronal signal substances in asexual multiplication and development in flatworms. Cell Mol Neurobiol **16**(5): 591-616.
229. Ribeiro P, Webb RA (1983). The synthesis of 5-hydroxytryptamine from tryptophan and 5-hydroxytryptophan in the cestode *Hymenolepis diminuta*. Int J Parasitol **13**(1): 101-6.
230. Ribeiro P, El-Shehabi F, Patocka N (2005). Classical transmitters and their receptors in flatworms. Parasitology **131 Suppl**: S19-40.
231. Ribeiro P and Geary TG (2010). Neuronal signaling in schistosomes: Current status and prospects for post-genomics. Can J of Zool **88** 1-22.
232. Richmond JE, Jorgensen EM (1999). One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction. Nat Neurosci **2**(9): 791-7.
233. Riker WF, Wescoe WC (1951). Pharmacology of flaxedil. Int Rec Med Gen Pract Clin **164**(12): 716.
234. Rollinson D, Southgate VR (1987). The genus *Schistosoma*: a taxonomic appraisal, p. 1-49. In D Rollinson, AJ Simpson (eds), The Biology of Schistosomes: from Genes to Latrines. Academic Press, London.
235. Roszkowski AP (1961). An unusual type of sympathetic ganglionic stimulant. J Pharmacol Exp Ther **132**: 156-70.
236. Rosenbaum DM, Rasmussen SG, Kobilka BK (2009). The structure and function of G-protein-coupled receptors. Nature **459**(7245): 356-63.
237. Ross AGP, Bartley PB, Sleigh AC, Olds GR, Li Y, Williams GM, McManus DP (2002). Schistosomiasis. N Eng J Med **346**: 1212-9.

238. Rufener L, Baur R, Kaminsky R, Mäser P, Sigel E (2010). Monepantel allosterically activates DEG-3/DES-2 channels of the gastrointestinal nematode *Haemonchus contortus*. Mol Pharmacol **78**(5): 895-902.
239. Sabah AA, Fletcher C, Webbe G, Doenhoff J (1986). *Schistosoma mansoni*: chemotherapy of infections of different ages. Exp Parasitol **61**: 294–303.
240. Salon JA, Lodowski DT, Palczewski K (2011). The significance of G protein-coupled receptor crystallography for drug discovery. Pharmacol Rev. **63**(4): 901-37.
241. Samii SI, Webb RA (1996). The stimulatory effect of L-glutamate and related agents on inositol 1,4,5-trisphosphate production in the cestode *Hymenolepis diminuta*. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol **113**(3): 409-20.
242. Savioli L, Hatz C, Dixon H, Kisumku UM, Mott KE (1990). Control of morbidity due to *Schistosoma haematobium* on Pemba Island: Egg excretion and hematuria as indicators of infection. Am J Trop Med Hyg **43**: 289–295.
243. Sawruk E, Schloss P, Betz H, Schmitt B (1990). Heterogeneity of Drosophila nicotinic acetylcholine receptors: SAD, a novel developmentally regulated alpha-subunit. EMBO J **9**(9): 2671-7.
244. Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, Hofmann KP, Ernst OP (2008). Crystal structure of opsin in its G-protein-interacting conformation. Nature **455**(7212): 497-502.
245. Scheidler A, Kaulen P, Brüning G, Erber J (1990). Quantitative autoradiographic localization of [125I]alpha-bungarotoxin binding sites in the honeybee brain. Brain Res **534**(1-2): 332-5.
246. Schipani E, Langman C, Hunzelman J, Le Merrer M, Loke KY, Dillon MJ, Silve C, Jüppner H (1999). A novel parathyroid hormone (PTH)/PTH-related peptide receptor mutation in Jansen's metaphyseal chondrodysplasia. J Clin Endocrinol Metab **84**(9): 3052-7.
247. Schmidt C, Li B, Bloodworth L, Erlenbach I, Zeng FY, Wess J (2003). Random mutagenesis of the M3 muscarinic acetylcholine receptor expressed in yeast. Identification of point mutations that "silence" a constitutively active mutant M3 receptor and greatly impair receptor/G protein coupling. J Biol Chem **278**(32): 30248-60.
248. Seifert R, Wenzel-Seifert K (2002). Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. Naunyn Schmiedebergs Arch Pharmacol **366**(5): 381-416.
249. Shapiro RA, Wakimoto BT, Subers EM, Nathanson NM (1989). Characterization and functional expression in mammalian cells of genomic and cDNA clones encoding a Drosophila muscarinic acetylcholine receptor. Proc Natl Acad Sci U S A **86**(22): 9039-43.
250. Shirayama T, Matsumoto K, Pappano AJ (1993). Carbachol-induced sodium current in guinea pig ventricular myocytes is not regulated by guanine nucleotides. J Pharmacol Exp Ther **265**(2): 641-8.
251. Short R, Cartrett ML (1973). Argentophilic "papillae" of *Schistosoma mansoni* cercariae. J Parasitol **59**: 1041-1059.

252. Skelly PJ, Tielens AG, Shoemaker CB (1998). Glucose Transport and Metabolism in Mammalian-stage Schistosomes. Parasitol Today **14**(10): 402-6.
253. Solis-Soto JM, De Jong Brink M (1994). Immunocytochemical study on biologically active neurosubstances in daughter sporocysts and cercariae of *Trichobilharzia ocellata* and *Schistosoma mansoni*. Parasitology **108**(Pt 3): 301-11.
254. Soejima M, Noma A (1985). Mode of regulation of the ACh-sensitive K-channel by the muscarinic receptor in rabbit atrial cells. Pflugers Arch **400**(4): 424-31.
255. Spalding TA, Burstein ES, Henderson SC, Ducote KR, Brann MR (1998). Identification of a ligand-dependent switch within a muscarinic receptor. J Biol Chem **273**(34): 21563-8.
256. Stefanić S, Dvořák J, Horn M, Braschi S, Sojka D, Ruelas DS, Suzuki B, Lim KC, Hopkins SD, McKerrow JH, Caffrey CR (2010). RNA interference in *Schistosoma mansoni* schistosomula: selectivity, sensitivity and operation for larger-scale screening. PLoS Negl Trop Dis. **4**(10): e850.
257. Stroud RM, McCarthy MP, Shuster M (1990). Nicotinic acetylcholine receptor superfamily of ligand-gated ion channels. Biochemistry **29**(50): 11009-23.
258. Sukhdeo MV, Hsu SC, Thompson CS, Mettrick DF (1984). *Hymenolepis diminuta*: behavioral effects of 5-hydroxytryptamine, acetylcholine, histamine and somatostatin. J Parasitol **70**(5): 682-8.
259. Sukhdeo SC, Sangster NC, Mettrick DF (1986). Effects of cholinergic drugs on longitudinal muscle contractions of *Fasciola hepatica*. J Parasitol **72**(6): 858-64.
260. Swope SL, Moss SJ, Raymond LA, Huganir RL (1999). Regulation of ligand-gated ion channels by protein phosphorylation. Adv Second Messenger Phosphoprotein Res **33**: 49-78.
261. Syed NI, Bulloch AG, Lukowiak K (1990). In vitro reconstruction of the respiratory central pattern generator of the mollusk Lymnaea. Science **250**(4978): 282-5.
262. Taman A, Ribeiro P (2009). Investigation of a dopamine receptor in *Schistosoma mansoni*: Functional studies and immunolocalization. Mol Biochem Parasitol **168**: 24–33.
263. Tauc L, Gerschenfeld HM (1961). Cholinergic transmission mechanisms for both excitation and inhibition in molluscan central synapses. Nature **192**: 366-7.
264. Thany SH (2010). Electrophysiological studies and pharmacological properties of insect native nicotinic acetylcholine receptors. Adv Exp Med Biol **683**: 53-63.
265. Thompson CS, Mettrick DF (1989). The effects of 5-hydroxytryptamine and glutamate on muscle contraction in *Hymenolepis diminuta* (Cestoda). Can J Zool **67**(5): 1257-1262.
266. Thompson AJ, Lester HA, Lummis SC (2010). The structural basis of function in Cys-loop receptors. Q Rev Biophys **43**(4): 449-99.
267. Tierney, AJ (2001). Structure and function of invertebrate 5-HT receptors: A review. Comp Biochem Physiol **128A**: 791-804.

268. Tiras KhP (1978). Acetylcholinesterase activity in the nervous system of normal planaria and during regeneration. Ontogenez **9**(3): 262-8.
269. Tomosky TK, Bennett JL, Bueding E (1974). Tryptaminergic and dopaminergic responses of *Schistosoma mansoni*. J Pharmacol Exp Ther **190**(2): 260-71.
270. Touroutine D, Fox RM, Von Stetina SE, Burdina A, Miller DM 3rd, Richmond JE (2005). acr-16 encodes an essential subunit of the levamisole-resistant nicotinic receptor at the *Caenorhabditis elegans* neuromuscular junction. J Biol Chem **280**(29): 27013-21.
271. Tracey KJ (2007). Physiology and immunology of the cholinergic antiinflammatory pathway. J Clin Invest **117**(2): 289-96.
272. Trimmer BA (1995). Current excitement from insect muscarinic receptors. Trends Neurosci **18**(2): 104-11.
273. Tsai IJ, Zarowiecki M, Holroyd N, Garciarrubio A, Sanchez-Flores A *et al*. (2013). The genomes of four tapeworm species reveal adaptations to parasitism. Nature **496**(7443): 57-63.
274. Twarog BM (1954). Responses of a molluscan smooth muscle to acetylcholine and 5-hydroxytryptamine. J Cell Physiol **44**(1): 141-63.
275. van Lieshout L, Polderman AM, Visser LG, Verwey JJ, Deelder AM (1997). Detection of the circulating antigens CAA and CCA in a group of Dutch travellers with acute schistosomiasis. Trop Med Int Health **2**: 551-557.
276. Unwin N (2005). Refined structure of the nicotinic acetylcholine receptor at 4A resolution. J Mol Biol **346**(4): 967-89.
277. Vanni S, Neri M, Tavernelli I, Rothlisberger U (2009). Observation of "ionic lock" formation in molecular dynamics simulations of wild-type beta 1 and beta 2 adrenergic receptors. Biochemistry **48**(22): 4789-97.
278. van Nierop P, Keramidas A, Bertrand S, van Minnen J, Gouwenberg Y, *et al.* (2005). Identification of Molluscan Nicotinic Acetylcholine Receptor (nAChR) Subunits Involved in Formation of Cation- and Anion-Selective nAChRs. J Neurosci **25**(46): 10617-10626.
279. Vogel R, Mahalingam M, Lüdeke S, Huber T, Siebert F, Sakmar TP (2008). Functional role of the "ionic lock"--an interhelical hydrogen-bond network in family A heptahelical receptors. J Mol Biol **380**(4): 648-55.
280. Vulfius EA, Veprintzev BN, Zeimal EV, Michelson MJ (1967). Arrangement of cholinoreceptors on the neuronal membrane of two pulmonate gastropods. Nature **216**(5113): 400-1.
281. Wang JM, Zhang L, Yao Y, Viroonchatapan N, Rothe E, Wang ZZ (2002). A transmembrane motif governs the surface trafficking of nicotinic acetylcholine receptors. Nat Neurosci **5**(10): 963-70.
282. Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Wang H, Yang H, Ulloa L, Al-Abed Y, Czura CJ, Tracey KJ (2003). Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature **421**(6921): 384-8.
283. Webbe G (1982). The intermediate hosts and host-parasite relatioships, pp.16-49. In Jordan P, Webbe G (eds), Schistosomiasis: Epidemiology, Treatment and Control. Heinemann, London.

284. Wachtel H, Kandel ER (1971). Conversion of synaptic excitation to inhibition at a dual chemical synapse. J Neurophysiol **34**(1): 56-68.
285. Wess J, Maggio R, Palmer JR, Vogel Z (1992). Role of conserved threonine and tyrosine residues in acetylcholine binding and muscarinic receptor activation. A study with m3 muscarinic receptor point mutants. J Biol Chem **267**(27): 19313-9.
286. Wess J, Eglen RM, Gautam D (2007). Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nat Rev Drug Discov **6**(9): 721-33.
287. Wheeler SV, Jane SD, Cross KM, Chad JE, Foreman RC (1994). Membrane clustering and bungarotoxin binding by the nicotinic acetylcholine receptor: role of the beta subunit. J Neurochem **63**(5): 1891-9.
288. WHO (1994). Schistosomes, Liver flukes and *Helicobacter pylori*. IARC Monographs on the evaluation of carcinogenic risks to humans **61**: 45-119.
289. Williams BM, Temburni MK, Levey MS, Bertrand S, Bertrand D, Jacob MH (1998). The long internal loop of the alpha 3 subunit targets nAChRs to subdomains within individual synapses on neurons in vivo. Nat Neurosci **1**(7): 557-62.
290. Williamson SM, Robertson AP, Brown L, Williams T, Woods DJ, Martin RJ, Sattelle DB, Wolstenholme AJ (2009). The nicotinic acetylcholine receptors of the parasitic nematode *Ascaris suum*: formation of two distinct drug targets by varying the relative expression levels of two subunits. PLoS Pathog **5**(7): e1000517.
291. Wilson RA, Draskau T, Miller P, Lawson JR (1978). *Schistosoma mansoni*: the activity and development of the schistosomulum during migration from the skin to the hepatic portal system. Parasitology **77**(1): 57-73.
292. Winnier AR, Meir JY, Ross JM, Tavernarakis N, Driscoll M, Ishihara T, Katsura I, Miller DM 3rd (1999). UNC-4/UNC-37-dependent repression of motor neuron-specific genes controls synaptic choice in *Caenorhabditis elegans*. Genes Dev **13**(21): 2774-86.
293. Xu F, Wu H, Katritch V, Han GW, Jacobson KA, Gao ZG, Cherezov V, Stevens RC (2011). Structure of an agonist-bound human A2A adenosine receptor. Science **332**(6027): 322-7.
294. Yassin L, Gillo B, Kahan T, Halevi S, Eshel M, Treinin M (2001). Characterization of the deg-3/des-2 receptor: a nicotinic acetylcholine receptor that mutates to cause neuronal degeneration. Mol Cell Neurosci **17**(3): 589-99.
295. Yeoman MS, Parish DC, Benjamin PR (1993). A cholinergic modulatory interneuron in the feeding system of the snail, *Lymnaea*. J Neurophysiol **70**(1): 37-50.
296. Zamanian M, Kimber MJ, McVeigh P, Carlson SA, Maule AG, Day TA (2011). The repertoire of G protein-coupled receptors in the human parasite *Schistosoma mansoni* and the model organism *Schmidtea mediterranea*. BMC Genomics **12**: 596.
297. Zhong W, Gallivan JP, Zhang Y, Li L, Lester HA, Dougherty DA (1998). From ab initio quantum mechanics to molecular neurobiology: a cation-pi binding site in the nicotinic receptor. Proc Natl Acad Sci U S A **95**(21): 12088-93.

# Chapter II (Manuscript I)

"Functional Characterization of a Novel Family of Acetylcholine-gated Chloride Channels in *Schistosoma mansoni*"

Kevin MacDonald[1], Samuel Buxton[2], Michael J Kimber[2], Tim A Day[2] and Paula Ribeiro[1]*

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

[2]Department of Biomedical Sciences, Iowa State University, Ames, IA 50011, USA

Tel: (514) 398-7607

Fax: (514) 398-7857

E-mail: paula.ribeiro@mcgill.ca

*To whom correspondence should be addressed

**(Submitted to PLoS Pathogens)**

## Abstract

Acetylcholine is the canonical excitatory neurotransmitter of the mammalian neuromuscular system. However, in the trematode parasite *Schistosoma mansoni*, cholinergic stimulation leads to muscle relaxation and a flaccid paralysis, suggesting an inhibitory mode of action. Information about the pharmacological mechanism of this inhibition is lacking. Here, we used a combination of techniques to assess the role of cholinergic receptors in schistosome motor function. The neuromuscular effects of acetylcholine are typically mediated by gated cation channels of the nicotinic receptor (nAChR) family. Bioinformatics analyses identified numerous nAChR subunits in the *S. mansoni* genome but, interestingly, nearly half of these subunits carried a motif normally associated with chloride-selectivity. These putative schistosome acetylcholine-gated chloride channels (SmACCs) are evolutionarily divergent from those of nematodes and form a unique clade within the larger family of nAChRs. Pharmacological and RNA interference (RNAi) behavioral screens were used to assess the role of the SmACCs in larval motor function. Treatment with antagonists produced the same effect as RNAi suppression of SmACCs; both led to a hypermotile phenotype consistent with abrogation of an inhibitory neuromuscular mediator. Antibodies were then generated against two of the SmACCs for use in immunolocalization studies. SmACC-1 and SmACC-2 localize to regions of the peripheral nervous system that innervate the body wall muscles, yet neither appears to be expressed directly on the musculature. One gene, SmACC-1, was expressed in HEK-293 cells and characterized using an iodide flux assay. The results indicate that SmACC-1 formed a functional homomeric chloride channel and was activated selectively by a panel of cholinergic agonists. The results described in this study identify a novel clade of parasite nicotinic chloride channels that act as inhibitory modulators of schistosome neuromuscular function. Additionally, the iodide flux assay used to characterize SmACC-1 represents a new high-throughput tool for drug screening against these unique parasite ion channels.

## Author Summary

Schistosomiasis is a widespread, chronic disease affecting over 200 million people in developing countries. Currently, there is no vaccine available and treatment depends on the use of a single drug, praziquantel. Reports of reduced praziquantel efficacy, as well as its ineffectiveness against larval schistosomula highlight the need to develop new therapeutics. Interference with schistosome motor function provides a promising therapeutic target due to its importance in a variety of essential biological processes. The cholinergic system has been shown previously to be a major modulator of parasite motility. In our research, we have described a novel clade of schistosome acetylcholine-gated chloride channels (SmACCs) that act as inhibitory modulators of this pathway. Our results suggest that these receptors are invertebrate-specific and indirectly modulate inhibitory neuromuscular responses, making them an attractive drug-target. We have also validated a new functional assay to characterize these receptors, which may be modified for future use as a high-throughput drug screening method for parasite chloride channels.

## Introduction

Flatworms of the genus *Schistosoma* are the causative agents of the debilitating parasitic infection schistosomiasis, afflicting over 230 million people in 74 endemic countries [Gryseels *et al*., 2006]. The majority of human schistosomiasis can be attributed to three species- *S. mansoni*, *S. japonicum* and *S. haematobium*- which cause a wide spectrum of chronic pathology, including hepatosplenomegaly, portal hypertension and squamous cell carcinoma [Gryseels *et al*., 2006]. Currently, praziquantel (PZQ) is the only drug used to treat schistosomiasis and there is no vaccine available. Widespread and exclusive use of PZQ has led to concerns of emerging drug resistance. Laboratory strains of PZQ-resistant *S. mansoni* have been successfully generated and there are now several reports of reduced PZQ cure rates in the field [Doenhoff *et al*., 2009; Melman *et al*., 2009]. Moreover, PZQ is ineffective in

killing larval schistosomulae [Sabah *et al*., 1986]. The stage-limited efficacy of PZQ and looming prospect of drug resistance signal the importance of exploring novel therapeutic targets for the treatment of schistosomiasis.

An area of interest for the treatment of helminth parasites is the neuromuscular system, which is targeted by the majority of currently approved and marketed anthelminthics [Robertson and Martin, 2007]. Inhibition of neuromuscular activity provides two modes of treatment. First, motor inhibition may interfere with parasite maturation, which is closely tied with migration during the larval stage [Crabtree and Wilson, 1980]. Second, a loss of muscle function would disrupt essential activities, including attachment to the host, feeding, mating and others [Maule *et al*., 2005], ultimately causing the parasite to be eliminated from the host. The cholinergic system has proved especially successful as a neuromuscular anthelminthic target. Common antinematodal drugs such as levamisole, pyrantel and monepantel [Robertson and Martin, 2007; Kaminsky *et al*, 2008], and the antischistosomal drug, metrifonate [Bueding *et al*., 1972], all disrupt neuromuscular signaling by interacting with proteins of the worm's cholinergic system.

Acetylcholine (ACh) is an important neurotransmitter in both vertebrate and invertebrate species. The neuromuscular effects of ACh are typically mediated by postsynaptic nicotinic acetylcholine receptors (nAChRs), so named because of their high-affinity for nicotine. Structurally, nAChRs are members of the Cys-loop ligand-gated ion channel (LGIC) superfamily. They form homo- and heteropentameric structures, which are organized in a barrel shape around a central ion-selective pore [Albuequerque *et al.*, 2009]. Vertebrate nAChRs are invariably cation-selective ($Na^+$, $K^+$) and mediate excitatory responses. Invertebrates, on the other hand, have both cation and anion-selective ($Cl^-$) nAChRs. The latter mediate $Cl^-$ - driven membrane hyperpolarization and therefore are believed to play a role in inhibitory responses to ACh. One example of these unique invertebrate receptors is the acetylcholine-gated chloride channel (ACC) of the snail, *Lymnaea*, which is structurally related to nAChRs, yet is selective for chloride ions [van Nierop *et al*., 2005]. In addition, nematodes have an unusual type of ACC, which is a functional acetylcholine-gated chloride channel but is more closely related to

other chloride channels (GABA and glycine receptors) than nAChRs [Putrenko *et al*., 2005; Beech *et al*., 2013]. A defining feature of the ACCs is the presence of a Pro-Ala motif in the pore-lining M2 domains of the constituent subunits. This motif, which has been shown to confer anion-selectivity to other LGICs, replaces a Glu residue normally found in the cation-selective channels [Keramidas *et al*., 2002].

ACCs have not been identified in any of the flatworms, free-living or parasitic. However, there is experimental evidence supporting an inhibitory role for ACh in the parasites, which could be mediated by this type of receptor. Early studies in the 1960s observed that addition of exogenous cholinergic agonists to parasite cultures caused flaccid paralysis of adult trematodes and cestodes [Barker *et al*., 1966; Wilson and Schiller, 1969]. Flaccid paralysis indicates muscular relaxation and is in direct contradiction to the excitatory response of tonic contraction expected from cholinergic stimulation. Later research established a causal relationship between activation of a nicotinic-like receptor in *S. mansoni* muscle fibers and the flaccid paralysis caused by ACh in whole worms [Day *et al*., 1996]. However, this work was performed in the pre-genomic era and no attempt was made to clone or characterize the receptors involved. More recently, the publication of the *S. mansoni* genome [Berriman *et al*., 2009] has provided cause to revisit the unusual inhibitory activity of ACh in schistosomes. Several candidate genes have been annotated as nAChR subunits [Berriman *et al*., 2009; Protasio *et al*., 2012] and the present work aims to confirm the presence of and functionally characterize cholinergic chloride channels in *S. mansoni*.

One strategy that has been used for assessing the therapeutic value of candidate genes in parasites, particularly helminths, is RNA interference (RNAi) [Behm *et al*., 2005; Boyle *et al*., 2003; Kreutz-Peterson *et al*., 2007]. A strength of this reverse genetics strategy is the ability to screen living animals for phenotypic and behavioral changes as a result of abrogation of a particular gene's function, as demonstrated by the large-scale screens in the free-living platyhelminth cousins of schistosomes, the planarians [Reddien *et al*., 2005]. The RNAi pathway is conserved in *S. mansoni* [Behm *et al*., 2005; Boyle *et al*., 2003] and has previously been used to probe the neuropeptidergic system of the parasite [McVeigh *et al*., 2011] and, more recently, the serotonergic system as well

[Patocka and Ribeiro, 2013]. However, the effects of silencing other important neuroactive pathways, such as the cholinergic system, are not known.

Here we describe a novel clade of anion-selective nAChR subunits (SmACCs) that appear to be invertebrate-specific. The ion channels formed by these subunits play an inhibitory role in the neuromuscular activity of the parasite, as suggested by the results of RNAi and pharmacological behavioral assays, their tissue distribution and pharmacological properties.

## Materials and Methods

### *Parasites*

A Puerto Rican strain of *S. mansoni*-infected *Biomphalaria glabrata* snails were kindly provided by Dr. Fred Lewis (Biomedical Research Institute and BEI Resources, MD, USA) and used for all experiments. To obtain larval schistosomula, 6-8 week-old snails were exposed to bright light for 2 hours at room temperature. The resulting cercarial suspension was mechanically transformed *in vitro* by vortexing, washed twice with Opti-MEM (Gibco) containing 0.25µg/ml fungizone, 100µg/ml streptomycin and 100units/ml penicillin and cultured in Opti-MEM/antibiotics supplemented with 6%FBS (Gibco) [Lewis, 2001]. To obtain adult worms, 40-day old female CD1 mice were injected intraperitoneally with 250 mechanically transformed schistosomula [Lewis, 2001]. After 8 weeks, adult worms were collected by perfusion of the mouse hepatic portal vein and mesenteric venules, as previously described [Lewis, 2001]. Animal procedures were reviewed and approved by the Facility Animal Care Committee of McGill University (Protocol No. 3346) and were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

### *Bioinformatics*

To generate a target list of putative nicotinic acetylcholine receptor (nAChR) subunits, the *S. mansoni* Genome Database was searched using the keywords “nicotinic”

and “acetylcholine receptor” [Berriman *et al.*, 2009; Protasio *et al*., 2012]. A BLASTp homology search was also performed using the *Torpedo* nAChR (AAA96704.1) as a query. The resulting list of nAChR subunit sequences was used as a query against the general NCBI protein database and aligned with other Cys-loop receptor superfamily proteins by CLUSTALX [Larkin *et al*., 2007]. The alignments were analyzed manually to identify the presence of the vicinal C motif, indicative of nAChR α-subunits, and key amino acids involved in ion-selectivity. Phylogenetic trees were built in PHYLIP using the neighbor-joining method and bootstrapped with 1,000 replicates [Felsenstein, 1989]. Trees were visualized and annotated using FigTree3.0 [Morariu *et al*., 2008] and manually inspected to ensure that bootstrap values for each node were above a 70% threshold.

### *siRNA Design and Synthesis*

Five putative nAChR subunits were targeted by RNA interference (RNAi): Smp_157790, Smp_037960, Smp_132070, Smp_176310 (SmACC-1) and Smp_142690 (SmACC-2). For each target sequence, we amplified a unique 200-300 bp PCR fragment by RT-PCR. Total RNA was extracted from pooled adult male and female *S. mansoni,* using the RNeasy Micro Kit (Qiagen) and reverse-transcribed with MML-V (Invitrogen) and Oligo-dT (Invitrogen). PCR amplification was performed with a proofreading Phusion High Fidelity Polymerase (New England Biolabs), according to standard protocols. PCR primers (Table S2) were designed using Oligo6.2 [Rychlik, 2007] and the unique fragment sequences were identified by BLAST analysis. Amplicons were ligated to the pJET1.2 Blunt Vector (Fermentas) and verified by sequencing of multiple clones. For synthesis of double-stranded RNAs (dsRNA), the T7 promoter sequence (5’-TAATACGACTCACTATAGGGAGA-3’) was added to both ends of each target fragment by PCR. Long dsRNAs were generated from the resulting T7-flanked PCR products by in vitro transcription of both DNA strands, using the MegaScript T7 Transcription Kit (Ambion), according to the kit protocol. The dsRNAs were subsequently digested with RNAseIII, using the Silencer siRNA Kit (Ambion), to generate a mixture of siRNAs for each target. The siRNA was quantitated and assessed for purity using a Nanodrop ND1000 spectrophotometer.

### *Transfection of Schistosomula and Motility Assays*

Larval schistosomula were obtained by the standard protocol (see above) with some modification. After the final wash, freshly transformed schistosomula were re-suspended in Opti-MEM without antibiotics or FBS and plated at a concentration of 100 animals/well in a 24-well plate. Animals were transfected using siPORT NEO FX Transfection Agent (Ambion) and either an irrelevant scrambled siRNA (Ambion) or nAChR subunit-specific siRNA at a final concentration of 50nM. Transfections were performed blind to rule out selection bias during analysis. Opti-MEM containing antibiotics and supplemented with 6%FBS was added to transfected schistosomula 24 hours post-treatment. A previously described larval motility assay was performed 6 days post-transfection [El-Shehabi *et al*., 2012]. Briefly, schistosomula were filmed for 45s using a Nikon SMZ1500 microscope equipped with a digital video camera (QICAM Fast 1394, mono 12 bit, QImaging) and SimplePCI version 5.2 (Compix Inc.) software. Three distinct fields were recorded for each well. ImageJ (version 1.41, NIH, USA) software was then used to quantitate worm motility using the Fit Ellipse algorithm in ImageJ, as described [Patocka and Ribeiro, 2013]. The data shown here are derived from three independent experiments in which a minimum of 12 animals was measured per experiment. Pharmacological motility assays were carried out with 6-day old schistosomulae in the same manner, but without the transfection with siRNA. Baseline measurements of schistosomula motility were recorded prior to drug addition. Compounds of interest (arecoline, nicotine, mecamylamine, d-tubocurarine) were subsequently added at a final concentration of 100 μM and larval motility was measured again after 5 minutes exposure. Viability of drug-treated and siRNA-treated schistosomula was routinely monitored by a dye exclusion assay, according to the method of Gold [Gold, 1997].

### *Real-Time Quantitative PCR*

Six-day old siRNA-treated schistosomula were washed twice with 1X PBS, re-suspended in the lysis buffer provided with the RNEasy Micro RNA Extraction Kit (Qiagen) and sonicated with 6 pulses of 10s each. Total RNA was then extracted from the lysate following the manufacturer's instructions. RNA was quantified and assessed

for purity using a Nanodrop ND1000 spectrophotometer. 100ng total RNA was used for each 20µl MML-V (Invitrogen) reverse transcription (RT) reaction, which was performed according to standard protocols. A negative control lacking reverse transcriptase was also prepared in order to rule out contamination with genomic DNA. Quantitative real-time PCR (qPCR) was performed using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) in a 25µl reaction volume. Primers located in a unique region of each gene and separate from those regions used to generate siRNA were designed using Oligo6.2 and may be found in Table S2. Primers targeting the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Accession #M92359) were used as an internal control and are as follows: forward 5'-GTTGATCTGACATGTAGGTTAG- 3' and reverse 5'-ACTAATTTCACGAAGTTGTTG-3'. Primer validation curves were generated to ensure similar efficiency of target and housekeeping gene amplification. Cycling conditions were as follows: 50°C/2 min, 95°C/2 min, followed by 50 cycles of 94°C/15 s, 57°C/30 s, 72°C/30 s. Cycle threshold (Ct) values were normalized to GAPDH and then compared to the scrambled siRNA control, as well as an off-target gene (another nAChR subunit) to ensure transcript-specific silencing. All expression data was analyzed using the comparative ΔΔCt method [Livak and Schmittgen, 2001] and was generated from three separate experiments done in triplicate.

### *Cloning of Full Length SmACC-1 and SmACC-2*

Two putative anion-selective subunit sequences, Smp_176310 (SmACC-1) and Smp_142690 (SmACC-2) were chosen for further study and cloned by conventional RT-PCR (see above) using primers targeting the beginning and end of each cDNA. For SmACC-1 we used primers: forward 5'-ATGGATCTAATATACTTG-3' and reverse: 5'-TTAGGTAGTTTCTGAATC-3'. PCR conditions were as follows: 98°C/30s, 30 cycles of 98°C/10s, 55°C/60s, 72°C/90s and final extension of 72°C/5min. In the case of SmACC-2, the full-length cDNA was amplified with primers 5'-ATGGAAAAATCACTTATTCG-3'(forward) and 5'-TTATTGTAGATCAACTACG-3'(reverse), using the following cycling conditions: 98°C/30s, 30 cycles of 98°C/10s, 54°C/60s, 72°C/60s and a final extension of 72°C/5min. The 5' end of SmACC-2 was further verified by 5' RACE (rapid amplification of cDNA ends), using a commercial kit (Invitrogen) and a gene-specific

primer for the reverse transcription [5'-GCAGGTACATAATCTGAG-3'], according to manufacturer's instructions. All PCR products were ligated to the pJet1.2 Blunt cloning vector (Thermo Scientific) and verified by DNA sequencing of at least two independent clones.

### *Antibody Production*

Peptide-derived polyclonal antibodies were generated in rabbits against subunits SmACC-1 and SmACC-2 (21$^{st}$ Century Biochemicals – Marlborough, MA). Two peptides were used for each target. For SmACC-1, both peptides 1(NAKVNRFGKPHGNKFC) and 2(CSKKALSAANAKWNSPLQY) are located in the third intracellular loop of the protein. For SmACC-2, peptide 1 (TDGEAERHIRHEDRVHQLRSVC) and peptide 2 (LQNINMKQIKLEYKNSLGC) are located at the N- and C-terminal ends, respectively. All peptides were conjugated to the carrier protein ovalbumin and were BLASTed against the *S. mansoni* genome database and the NCBI general database to ensure specificity. Whole antisera were tested for specificity and titer against the immunogenic peptides by ELISA. The Pierce Sulfolink Kit for Peptides (Thermo Scientific) was used to purify anti-nAChR-specific IgG fractions, according to manufacturer's instructions. ELISA was performed to determine the titer of affinity-purified antibody fractions. Protein was quantified by the Bradford assay, using a commercial kit (BioRad, USA). Goat anti-human choline acetyltransferase (anti-ChAT) was obtained from a commercial source (Millipore, USA) and used as a marker for cholinergic neurons.

### *Confocal Microscopy*

Parasites were prepared for confocal microscopy according to previously described protocols [Mair *et al*., 2000; Taman and Ribeiro, 2009]. Briefly, 6-day old *in-vitro*-transformed schistosomula or freshly collected adult worms were washed two times in 1X PBS and fixed in 4%PFA for 4 hours at 4°C. Parasites were washed twice, each for 5 minutes in 1X PBS containing 100µM glycine and then permeabilized with 1%SDS in 1X PBS for 25 minutes [Collins *et al.*, 2011]. After permeabilization, animals were incubated overnight at 4°C in antibody diluent (AbD) containing 0.1%Tween-20, 1% BSA in PBS to block non-specific binding. After 3 washes of 10 minutes each in the AbD, animals were then incubated with either affinity-purified anti-SmACC alone (1:100) or in

combination with anti-ChAT (1:100 each) for three days at 4°C. Samples were then washed 3 times in AbD and incubated in secondary antibody (1:1000) conjugated to Alexa Fluor 488 or 594 (Invitrogen, USA). In some experiments, tetramethylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin (200 µg/ml) was added with secondary antibody and used to visualize the musculature. Secondary antibody incubation lasted for 2 days and animals were again washed three times before mounting for microscopy. Slides were examined using a Zeiss LSM710 confocal microscope (Carl Zeiss Inc., Canada) equipped with the Zeiss Zen 2010 software package. The lasers used for image acquisition were an Argon 488 nm and a HeNe 594 nm, with the filter sets adjusted to minimize bleed-through due to spectral overlap. Negative control slides were prepared by incubating samples in either pre-immune serum or primary antibody preadsorbed with 0.5mg/mL of mixed peptide antigen. At least 5 independent samples were examined for each peptide-derived antibody.

### *Western Blot Analysis*

Membrane-enriched protein fractions were extracted from adult *S. mansoni* using the ProteoExtract Native Membrane Protein Extraction Kit (Calbiochem, USA) and following the manufacturer's instructions. Protein was quantified by the Bradford Assay (BioRad, USA) and used for SDS-PAGE and Western blot analysis. Approximately 20 µg of membrane extract was loaded on a 4-12% Tris-Glycine gel (Invitrogen, USA) and resolved by SDS-PAGE, then transferred to a PVDF membrane (Millipore, USA). A standard Western blot protocol was followed to visualize proteins. Primary antibodies used were peptide-purified anti-SmACC-1 or anti-SmACC-2 (both 1:1000). Secondary antibody (1:5000) was goat-anti-rabbit conjugated to horseradish peroxidase (Invitrogen, USA). Membranes were also probed with peptide antigen-preadsorbed primary antibody (1:1000) as a negative control.

### *Heterologous Expression and Functional Characterization of SmACC-1 in HEK-293 Cells*

For mammalian expression studies, a human codon-optimized construct of SmACC-1 was synthesized (Genescript, USA) and inserted into the pCi-Neo (Promega) expression vector, using *NheI* and *SmaI* restriction sites. A C-terminal FLAG tag was

also included in the SmACC-Neo construct to aid in the monitoring of expression. HEK-293 cells were grown to 50% confluence Dulbecco's Modified Essential Media (DMEM) supplemented with 20 mM HEPES and 10% heat inactivated fetal calf serum. Cells were transiently transfected with either the SmACC-1 construct or empty vector, using XtremeGENE 9 transfection reagent (Roche), as recommended by the manufacturer. 24 hours post-transfection, cells were transduced with Premo Halide Sensor (Invitrogen), a halide-sensitive fluorescent indicator used to assess ligand-gated chloride channel function [De La Fuente *et al*., 2008; Galietta *et al*., 2001]. Following transduction, cells were incubated at 37°C, 5% $CO_2$ overnight and seeded onto a 96-well plate at a density of 50,000 cells per well. After an 8 hour incubation at 37°C, 5% $CO_2$, growth media was removed and cells were equilibrated with iodide assay buffer provided with the Premo Halide Sensor assay kit for at least 30 minutes at 37°C in the reading chamber of a FlexStation II scanning fluorometer (Molecular Devices). YFP fluorescence was measured for 10s before and up to 2 minutes after the addition of test compounds. Compounds were added at a final concentration of 100 µM, or as indicated, in a total sample volume of 200 µl. Water was used as a vehicle-only negative control. Receptor activity was calculated by measuring the reduction in YFP fluorescence (ΔRF) due to iodide influx over the time of measurement. Briefly, a fluorescence measurement was taken 10s after the addition of drug ($RF_{initial}$) and again after a period of 120s ($RF_{final}$). The $RF_{final}$ was subtracted from the $RF_{initial}$ to generate ΔRF. ΔRF was then divided by the $RF_{initial}$ and multiplied by 100, resulting in a measurement of %YFP quench, as described [Galietta *et al*., 2001]. Readings were normalized to water-treated controls and reported as Fold-Change in YFP Quench [Johansson *et al*., 2013]. Receptor activation was also calculated by the linear-regression slope method [Verkman and Galietta, 2009] with similar results. The minimum quench threshold for all experiments was set at zero [Kruger *et al*., 2005]. Dose response curves were fit using the non-linear regression function of Prism 6 software (Graphpad Software, USA). Student's t-tests were performed to determine statistically significant differences at $P < 0.05$.

### *Other Methods*

The Calcium 4 FLIPR Assay Kit (Molecular Devices, USA) was used according to a previously established protocol [Xie *et al*., 2005] in order to rule out the possibility of

YFP Quench resulting from activation of endogenous calcium activated $Cl^-$ channels rather than SmACC-1-mediated $Cl^-$ conductance. SmACC-1 antagonist assays were carried out using the protocol outlined in the section above, with the following modification. Cells were pre-incubated with cholinergic antagonists (mecamylamine, tubocurarine, atropine) at a concentration of 100 µM during the iodide assay buffer equilibration step. Cells were then treated with either 100 µM nicotine or H2O and YFP Quench was measured.

## Results

### *Identification of Acetylcholine-gated Chloride Channel Subunits in S. mansoni (SmACCs)*

A combination of BLAST and keyword searches were used to generate a list of potential nAChR subunits in the genome database of *S. mansoni* [Berriman *et al*., 2009]. In total, nine putative receptor subunits were identified. All sequences were predicted to have the defining features of a nAChR subunit, including a Cys-loop motif and four transmembrane domains [Thompson *et al*., 2010] and all subunit genes identified are predicted to contain full-length coding sequences. A structural alignment of the putative schistosome nAChR subunits with two previously characterized human nAChR alpha subunits, the *Lymnaea* nicotinic chloride channels and the crystal structure of the *Torpedo* nAChR suggests the presence of both cation and anion-selective schistosome nAChR subunits. Figure 1 shows the M2 domain of the structural alignment in which several of the schistosome nAChR subunits, including SmACC-1 (Smp_176310) and SmACC-2 (Smp_142690) display the canonical Pro-Ala motif of anion-selective Cys-loop receptors, including those of *Lymnaea*. In contrast, the *Torpedo*, human and remaining schistosome receptor subunits contain a Glu residue, characteristic of a cation-selective channel, at the corresponding location.

The predicted schistosome nAChRs were then aligned with cation and anion-selective Cys-loop receptor subunits from other representative vertebrate and invertebrate species, including the acetylcholine-gated chloride channel (ACC) subunits from *C. elegans* [Putrenko *et al*., 2005] and the molluscan (snail) anion-selective

nAChRs. A phylogenetic tree of the alignment (Figure 2) shows the unique clade formed by the Pro-Ala motif-containing schistosome nAChR subunits is located firmly in the larger group of cation-selective nAChR subunits. Also present in this clade are the previously characterized nicotinic chloride channel of the snail *Lymnaea* [van Nierop *et al*., 2005] and putative homologs from fellow flatworms *Clonorchis* and *Dugesia*. This is in contrast to the *C. elegans* ACC subunits, which group more closely to the anion-selective GABA/glycine receptors and have low affinity for nicotine [Putrenko *et al*., 2005]. Thus, the nAChR subunits in schistosomes are all structurally related to cation-selective nicotinic receptors but those carrying the Pro-Ala motif appear to have diverged and may have acquired selectivity for anions. The structural relationship of the schistosome sequences to known chloride-selective nAChRs of *Lymnaea* points to their potential function as nicotinic anion channels. Moreover, the presence of putative homologs in closely related flatworms and their apparent absence in host species indicate that these receptors may be good targets for broad-spectrum antiparasitics.

Two of the predicted anion-selective subunits, SmACC-1 and SmACC-2 were selected for full-length cloning. SmACC-1 contains a predicted ORF of 2415 bp distributed over 9 exons, encoding a protein of 92kDa. SmACC-1 contains an N-terminal signal peptide and an N-terminal double cysteine motif (YxCC) that is the defining characteristic of nAChR alpha-type subunits [Kao and Karlin, 1996]. Full-length SmACC-1 was successfully amplified by PCR and sequencing of multiple SmACC-1 clones verified the predicted ORF (GenBank accession # KF694748). The coding sequence of SmACC-2 was predicted to be 2745 bp. However, further sequence analysis by BLAST predicted a large (~1kb) N-terminal nucleotide-binding domain (NBD), a feature not normally present in Cys-loop receptors. This excess sequence may have been a result of the concatenation of two distinct proteins during annotation. To identify the correct start codon of SmACC-2, 5'RACE experiments were performed and an alternative start site downstream of the predicted start codon was identified, removing the NBD sequence. New PCR primers were designed and full-length SmACC-2 was amplified, resulting in a product of 1528 bp and a corresponding protein of 60kDa (GenBank accession # KF694749). The new SmACC-2 coding sequence was in frame with the predicted ORF and retained both its Cys-loop and transmembrane domains but does not

contain a signal peptide. SmACC-2 also lacks the vicinal cysteine motif, suggesting that it is a non-alpha-type nAChR subunit.

### *Schistosome nAChRs Act as Inhibitory Modulators of Motor Function*

A previously described behavioral assay [Patocka and Ribeiro, 2013; El-Shehabi *et al*., 2012] was used to evaluate the effect of cholinergic compounds on *S. mansoni* larval motility. Animals were treated with either cholinergic agonists (arecoline, nicotine) or antagonists (mecamylamine, d-tubocurarine) alone at a concentration of 100 μM and the frequency of body movements (shortening and elongation) was calculated as a measure of motility [Patocka and Ribeiro, 2013; El-Shehabi *et al*., 2012]. Treatment of 6-day old schistosomula with cholinergic agonists caused rapid, near complete paralysis when compared to the water-treated controls (Figure 3A). Conversely, the nicotinic antagonists caused a 2-3.5-fold increase in larval motility. These results are consistent with previous studies [reviewed in Ribeiro *et al*., 2005] and support the hypothesis that cholinergic receptors inhibit neuromuscular function in *S. mansoni*.

To examine the role of the predicted anion-selective nAChR subunits in larval motor behavior, we targeted individual nAChR subunits by RNA interference (RNAi), using pooled sequence–specific siRNAs. A mock–transfected sample (lipid transfection reagent only) and a nonsense scrambled siRNA control were included as negative controls; there was no significant decrease in motor behavior in either control compared to untransfected larvae. In contrast, animals treated with nAChR siRNAs all showed a significant ($P < 0.05$) hyperactive motor phenotype (Figure 3B). Depending on the subunit, the increase in larval motility ranged from 2-4-fold when compared to the negative scrambled control. The two subunits generating very strong hyperactive phenotypes were SmACC-2 (~6-fold) and SmACC-1 (~4.5-fold). The hyperactivity in the nAChR RNAi-treated animals is consistent with the phenotype seen in animals where nAChR activity has been pharmacologically abrogated by receptor antagonists (Fig. 3A).

Knockdown at the mRNA level was confirmed by qPCR for SmACC-1 and SmACC-2 (Figure 4A). SmACC-2 expression was reduced 60% at the transcript level and SmACC-1 expression was reduced by 90%. In both cases the knockdown was

observed only in RNAi-suppressed larvae, indicating the effect was specific. Transfection with SmACC-1 siRNAs had no effect on the expression level of the other subunit, SmACC-2, or vice-versa (Fig. 4A). Knockdown at the protein level was confirmed by western blot analysis of SmACC-1, using a specific antibody (Figure 4B). The siRNA-treated animals show a drastic reduction in protein expression, as evidenced by the absence of the expected 92kDa band in the treated sample lane, whereas no difference was seen in the loading control.

### *Immunolocalization of SmACC-1 and SmACC-2*

In order to determine the tissue localization of SmACC-1 and SmACC-2, we obtained custom commercial antibodies against each target. Polyclonal antibodies were generated using two unique peptide antigens for each gene of interest, each peptide being conjugated to ovalbumin. The antibodies were peptide affinity-purified and tested by ELISA and western blotting. Adult worm membrane fractions probed with anti-SmACC-1 showed a band corresponding to the expected size of SmACC-1 (Figure S1). Probing with antibodies specific for SmACC-2 again resulting in a band corresponding to the predicted protein size (Figure S1). A peptide-preadsorbed negative control for each antibody was also used to probe the adult membrane fractions; no immunoreactivity was present in either negative control sample, indicating specificity of binding for the intended protein.

For the immunolocalization study, adult and larval schistosomes were stained with either anti-SmACC-1 or anti-SmACC-2 and an Alexa-488 conjugated secondary antibody. Some animals were also counterstained with either TRITC-conjugated phalloidin or a commercial antibody against whole recombinant human choline acetyltransferase (ChAT). The phalloidin was used to label muscle and cytoskeletal features, while ChAT served as a marker of cholinergic neurons. The human enzyme shares high sequence homology with the predicted *S. mansoni* ChAT (Smp_146910) and therefore the antibody was expected to recognize the parasite protein.

The results of our *in situ* immunofluorescence studies suggest that SmACC-1 is localized to the peripheral nervous system (PNS) of the worm (Figure 5). Negative

control worms labeled with peptide-preadsorbed anti-SmACC-1 showed no appreciable fluorescence (Figure 5A). Parasites labeled with SmACC-1 show a diffuse network of punctate, neuronal expression in apparent cell bodies and fine varicose nerve fibers. Labeling was observed in the peripheral nerve net of the suckers (Fig. 5B), the subtegumental nerve plexus just under the surface of the parasite (Fig. 5G) and the innervation of the body wall muscles along the length of the body (Fig. 5F). Co-labeling studies using anti-SmACC-1 and anti-ChAT antibodies demonstrate that SmACC-1 is present in close proximity to cholinergic neurons, where it can be activated by endogenously released ACh. The pattern of anti-ChAT-labeled cholinergic neurons in the PNS is strikingly similar to that of SmACC-1, heavily innervating the acetabulum (Fig. 5C, D), as well as the peripheral nerve plexuses of the worm's body wall (Fig. 5F). Consistent with previous localization studies in other flatworms [45], anti-ChAT immunoreactivity was also present in the central nervous system (CNS) of *S. mansoni*, including main nerve cords and cerebral ganglia (not shown). In contrast, expression of SmACC-1 was limited to the peripheral nervous system. We did not observe significant SmACC-1 immunoreactivity in the CNS of *S. mansoni*.

Similar to SmACC-1, SmACC-2 localizes primarily to the parasite PNS (Figure 6). Beneath the surface, anti-SmACC-2 staining revealed numerous varicose nerve fibers in the peripheral innervation of the body wall (Figure 6B). Some of these fine, immunoreactive nerve fibers can be seen criss-crossing the length of the body, where they come into close contact with the body wall musculature (Figure 6C). However there was no visible overlay between the antibody labeling (green) and the phalloidin-stained muscles (red), suggesting that SmACC-2 is expressed in nerve fibers rather than the body wall muscle itself. Another region where we observed significant SmACC-2 immunoreactivity was the surface of the worm. This occurred in both sexes but it was particularly enriched in the tubercles of male worms (Figure 6D). It is unknown if this labeling is associated with the tegument itself or possibly sensory nerve endings that are present on the surface of the worm. No comparable fluorescence could be seen in any of the negative controls tested, including a peptide-preadsorbed antibody control (Figure 6A) and therefore the labeling is considered to be specific.

Immunolocalization studies were repeated in larval schistosomula and the labeling patterns of SmACC-1 and 2 were found to be similar. In both cases, immunoreactivity occurred in a network of fine varicose nerve fibers that run just below the surface and along the entire length of the body (Figure 6E). This resembles the expression pattern seen in the adults and suggests the receptor is expressed in the developing PNS of the larvae [Nishimura *et al*., 2010]. As with the adults, we were unable to detect specific labeling in the CNS of the larvae with either antibody.

### *SmACC-1 Forms a Functional, Nicotinic Chloride Channel*

HEK293 cells were transfected with codon-optimized (humanized) SmACC-1 and protein expression was monitored by *in situ* immunofluorescence. Transfected cells were immunoreactive for SmACC-1 when probed either with specific antibody or anti-FLAG antibody (Figure 7A). No immunofluorescence was noted in the negative control cells transfected with empty plasmid (Figure 7B). Transfected cells were transduced with a YFP –like fluorescence sensor (Premo Halide Sensor) and seeded on a 96-well plate for the iodide ($I^-$) flux assay. The principle of the assay has been described in detail [De La Fuente *et al*., 2008; Galietta *et al*., 2001; Johansson *et al*., 2013; Verkman and Galietta, 2009] and is shown schematically in Fig. 7C. Cells expressing a chloride channel of interest are bathed in an iodide buffer, which serves as a surrogate for chloride ($Cl^-$) anions. After a period of equilibration, test compounds are added and if the chloride channel of interest is activated, an influx of $I^-$ occurs, quenching the fluorescence of the YFP sensor. Channel activity was quantified by measuring either the slope of the curve or the decrease in fluorescence following drug addition, as described [Johansson *et al*., 2013]. Figure 7D shows representative tracings of cells expressing SmACC-1 and mock-transfected cells, each treated with 100 μM nicotine. Activation of SmACC-1 (red dots) by nicotine caused a significant decrease in YFP fluorescence (ΔRF) compared to nicotine-treated mock-transfected cells (black dots). No significant reduction in fluorescence was seen in SmACC-1 expressing cells treated with water, suggesting YFP quench was agonist-dependent. Indirect YFP quench resulting from SmACC-1 activation of the endogenous calcium activated chloride channels of HEK-293 cells was ruled out using a calcium flux assay. Treatment of cells expressing SmACC-1 with nicotinic agonists caused no increase in intracellular $Ca^{2+}$ when compared to mock-

transfected cells and indicates that SmACC-1 is an anion-selective channel. Experiments were repeated with different test substances and the results are shown in Figs. 8-9. None of the compounds used stimulated a significant influx of $I^-$ in the mock control. In contrast the cells expressing SmACC-1 were responsive to several cholinergic agonists, particularly nicotine. Treatment with nicotine (100μM) caused a significant ($p<0.05$) 6-fold increase in YFP quench in cells expressing SmACC-1. Smaller but statistically significant responses were also seen with other cholinergic agonists (ACh, choline chloride, carbachol and arecoline). Non-cholinergic substances, including biogenic amines (serotonin (5HT), dopamine) and glutamate, had no effect on the cells (Fig. 8). These data suggest that SmACC-1 is capable of forming a functional homomeric chloride channel that displays a preference for nicotine and related cholinergic substances. Furthermore, SmACC-1 was activated by nicotine in a dose-dependent manner with an $EC_{50}$ = 5.49μM (Figure 8, inset). At a concentration of 100 μM, the cholinergic antagonist d-tubocurarine was able to significantly block the activation of SmACC-1 by nicotine (Figure 9). However, mecamylamine and atropine were ineffective at this concentration.

## Discussion

Acetylcholine (ACh) has long been known as the quintessential excitatory neurotransmitter of the vertebrate neuromuscular system. Signaling through cation-selective nAChRs, ACh mediates muscular contraction via membrane depolarization due to an influx of $Na^+$ or $Ca^{2+}$. More recently, a distinct class of anion-selective nAChRs and other types of acetylcholine-gated chloride channels (ACCs) has been reported in several invertebrate organisms, including mollusks and nematodes [van Nierop *et al*., 2005; Putrenko *et al*., 2005]. These chloride-permeable channels initiate membrane hyperpolarization, causing an inhibition of action potentials. However, none of these invertebrate channels has been directly implicated in the control of motor function.

The effects of ACh on invertebrate neuromuscular activity vary depending upon the organism in question. As in vertebrates, ACh has excitatory neuromuscular effects in many invertebrate phyla, including some helminths such as nematodes and planarians [Walker *et al*., 2000; Butarelli *et al*., 2000]. In trematodes, however, ACh appears to act

in exactly the opposite manner. Exogenous application of cholinergic agonists onto trematodes in culture causes a rapid flaccid paralysis due to relaxation of the body wall muscles [Barker *et al*., 1966; Holmes and Fairweather, 1984]. A similar type of paralysis was observed in tapeworms (cestodes) treated with exogenous ACh [Wilson and Schiller, 1969]. This inhibitory response to cholinergic drugs appears unique to parasitic flatworms (trematodes and cestodes), and the receptors mediating this activity may therefore hold value as a therapeutic target. Earlier electrophysiology studies of *S. mansoni* tentatively identified these receptors as nAChR-like based on their pharmacological properties [Day *et al*., 1996] but the receptors themselves were not identified. The sequencing of the *S. mansoni* genome [Berriman *et al*., 2009; Protasio *et al*., 2012] led to the annotation of several candidate nAChR subunit genes, which are the focus of the present work.

Using a combination of BLAST and keyword searches, a total of nine nAChR subunit genes were found in the genome of *S. mansoni*. A structural alignment of the schistosome nAChR subunits with the *Torpedo* nAChR was then performed to identify peptide motifs associated with ion-selectivity. Cation-selective ion channel subunits have a negatively charged intermediate ring, formed by the presence of Glu residues in the M1-M2 linking region [Wilson *et al*., 2000]. Anion-selective Cys-loop receptor subunits replace the Glu in this region with a Pro-Ala motif, disrupting the electrostatic interactions in the intermediate ring and conferring anion-selectivity to the channel [Keramidas *et al*., 2002]. The results of our structural alignment indicate that 5 of the schistosome nAChR subunits (SmACC-1, SmACC-2, Smp_157790, Smp_037910 and Smp_132070) contain this anion-selectivity determinant and they were tentatively identified as *S. mansoni* SmACCs. Furthermore, a dendrogram analysis suggests that the SmACCs are evolutionarily distinct from the ACCs found in *C. elegans*. Unlike the *C. elegans* ACCs, the schistosome subunits are structurally related to vertebrate and invertebrate nAChRs [Putrenko *et al*., 2005], suggesting that the SmACCs are descended from ancient nicotinic channels but have evolved selectivity for chloride. This allies the SmACCs more closely with the anion-selective nAChRs of the snail *Lymnaea*, with which they share more them 40% identity at the protein level. Interestingly, certain species of *Lymnaea* are permissive intermediate hosts of schistosomes. However, it is

unclear if the presence of anion-selective nicotinic channels in both organisms is due to horizontal gene transfer, common ancestry or convergent evolution. There is also evidence of closely related, putative nAChR chloride channels present in the genome of the trematode *Clonorchis sinensis* [Huang *et al*., 2013], suggesting a unique clade of platyhelminth-specific nicotinic chloride channels.

The next step after identifying the SmACCs was to study their role in the motor function of the parasite. The flaccid paralysis of adult schistosomes caused by treatment with cholinergic compounds is well characterized. However, very little is known about the role of cholinergic receptors in the motor activity of larval schistosomula. Given that larval migration is vital to parasite development and survival [Crabtree and Wilson, 1980] and the cholinergic system is a major regulator of motor function in adult worms, we hypothesized that SmACCs play an important role as inhibitory modulators in larval neuromuscular function. To test this, two types of behavioral assay were employed- pharmacological and RNAi. The results of the pharmacological motility assay agree with previous studies implicating ACh as an inhibitor of schistosome movement [Barker *et al*., 1966; Day *et al*., 1996]. Treatment of 6-day old schistosomula with the cholinergic agonists arecoline and nicotine caused nearly complete paralysis whereas classical antagonists, mecamylamine and d-tubocurarine stimulated movement by 3-4 fold over water-treated control animals. These results suggest that the schistosome cholinergic system mediates inhibitory neuromuscular responses, possibly via an influx of chloride generated by SmACC activation.

Although the results of the pharmacological motility assay agree with previously published studies, motor phenotypes elicited by treatment of worms with exogenous compounds are not necessarily of biological or behavioral relevance. Drug permeability across the tegument, non-selective targeting and toxic effects may all induce motor behaviors that obscure the true role of the receptors in question. Silencing of receptor function by RNAi mitigates these issues by targeting receptors individually and by measuring effects on basal motor activity in the absence of added drugs. The results of our RNAi assay show that the ion channels formed by the SmACC subunits act as inhibitory mediators of motor activity in schistosomula. Knockdown of each of the 5

identified SmACC subunits resulted in a 3-6-fold hypermotile phenotype, mirroring the hyperactivity seen in antagonist-treated schistosomula. It is unclear why the individual subunits all produced similar hypermotile RNAi phenotypes. It is possible these are all components of the same inhibitory channel, such that the loss of any one subunit results in loss of channel function and hyperactivity. As discussed below, our immunolocalization studies show that two of these subunits, at least (SmACC-1 and SmACC-2) have similar distribution patterns, suggesting they could be components of the same channel in the worm. Alternatively these could assemble into different channels that have similar inhibitory effects on movement.

To identify the possible mechanisms by which the SmACCs mediate inhibitory motor responses, immunolocalization studies were performed by confocal microscopy. The tissue distribution of two SmACCs in which silencing elicited large hypermotile phenotypes, SmACC-1 and SmACC-2, was examined in both adult and larval stages. SmACC-1 is present in neuronal cell bodies and along the varicose nerve fibers of the parasite's PNS. In adult worms, the distribution of SmACC-1 closely resembles that of acetylcholine-producing neurons labeled with anti-ChAT. However, whereas ChAT can be found both in the CNS and PNS of the parasite (not shown), SmACC-1 is present only in the PNS. SmACC-2 is distributed more prominently than SmACC-1 but presents a similar pattern of PNS localization. Again, SmACC-2 is present in varicose nerve fibers, which cross the body of the worm in a chain-link pattern. Counterstaining of both SmACCs with the muscular marker phalloidin suggest that neither subunit is expressed directly on the body wall musculature. Rather, SmACC-1 and SmACC-2 appear to be expressed in the peripheral nerve net of the worm's body wall [Koopowitz and Chien, 1974], including neurons of the submuscular nerve plexus that control the somatic muscles responsible for movement. This suggests that SmACC-1 and SmACC-2 mediate their inhibitory effects in an indirect manner, perhaps by modulating the release of other neurotransmitters or by acting as autoreceptors. In flatworms, as well as vertebrate model systems, nicotinic receptors are well known to mediate the release of other neurotransmitters, including neuropeptides and dopamine [Barik and Wonnacott, 2006; Akasu *et al*., 1984; Di Angelantonio *et al*., 2003]. In schistosomes, the cholinergic and neuropeptidergic system (which is excitatory in flatworms), are in very close

proximity [Halton and Maule, 2004; Halton and Gustaffson, 1996]. The balance between these systems may, therefore, be an important factor in the regulation of motor behavior. It would be of interest to determine if ACh inhibits neuropeptide release through these receptors, and whether this inhibition might explain the flaccid paralysis and other motor effects of ACh in these parasites.

SmACC-2 immunoreactivity was also seen on the surface of the parasite. Discreet, punctate staining is present along and in between the tubercles of adult male worms and along the surface of adult females. This marks the second time a nAChR has been localized to the schistosome tegument [Camacho *et al*., 1995]. Surface nAChRs in schistosomes have previously been linked to modulation of glucose uptake and are postulated to act through tegumental GLUT-1 like transporters [Camacho and Agnew, 1996]. The possibility also exists that tegumental SmACC-2 may provide sensory cues affecting motor function. The tubercles are known to contain innervated sensory structures [Kruger *et al*., 1986], which interface with the peripheral nerve net below and ultimately the CNS. The presence of SmACC-2 at both of these locations points to a potential role for ACh and this receptor in mediating host-parasite interactions affecting worm motor behavior.

While behavioral assays and microscopy serve to elucidate the behavioral role of the SmACCs, they provide only limited insight into receptor function at the molecular level. Therefore, functional expression analysis of a SmACC receptor was carried out in a heterologous expression system. A previous study cloned and expressed two cation-selective nAChR subunits from *S. haematobium* in *Xenopus* oocytes [Bentley *et al*., 2004]. However, neither subunit was able to form a functional ion channel either alone or when co-expressed. Our initial attempts to express SmACC-1 and SmACC-2 failed to produce functional channels, either individually or in combination and in two different expression environments, HEK293 cells and *Xenopus* oocytes (data not shown). SmACC-2 lacks the YxCC motif of nAChR alpha-subunits and therefore is not capable of forming functional homomeric channels. Further examination with appropriate antibodies of cells transfected with the SmACC-1 subunit determined that the level of protein expression was low, which could explain the apparent lack of activity. It has been

shown that differences in codon-usage can significantly decrease the expression of recombinant schistosome proteins in heterologous systems [Hamdan *et al*., 2002]. Thus we obtained a codon-optimized (humanized) cDNA for SmACC-1 and repeated the analysis in HEK293 cells. The humanized construct produced higher levels of protein expression and some of this protein appeared to be correctly targeted to the cell surface, as determined by immunofluorescence analysis. Subsequent functional studies showed that human codon-optimized SmACC-1 produced a functional homomeric ion channel in HEK-293 cells. Several nAChR subunits are known to form functional homomeric channels *in vivo*. Examples of this include the vertebrate alpha-7 nAChR and the ACR-16 of *C. elegans* [García-Guzmán *et al*., 1994; Raymond *et al*., 2000]. However, the expression of functional homomeric nAChRs is limited to neuronally expressed channels [Le Novère *et al*., 2002]. Moreover, only alpha-type nAChR subunits are capable of forming homopentameric channels. Thus, the formation of a functional homomeric SmACC-1 channel, together with its neuronal expression pattern in the worm, both suggest that SmACC-1 is a neuronal-type alpha nAChR subunit.

Activity assays were performed using a relatively novel, fluorescence-based assay, the Premo Halide Sensor (Invitrogen). The results of the activity assay show that SmACC-1 is activated by cholinergic agonists but not other biogenic amines. Nicotine and ACh induced the largest response (6-fold and 2.5-fold, respectively) when compared to water-treated control cells. An $EC_{50}$ = 5.49 μM was calculated for nicotine, which falls within the reported range for vertebrate neuronal nAChR response to nicotine, as well as an nAChR characterized in the parasitic nematode *A. suum* [Papke *et al*., 2007; Williamson *et al*., 2009]. The response of SmACC-1 to nicotine was antagonized by d-tubocurarine but not mecamylamine or atropine. This suggests that the effects on larval motility resulting from mecamylamine treatment are caused by activation of nAChRs that do not include the SmACC-1 subunit.

Functional analysis of SmACC-1 in a mammalian expression system represents a departure from the more classical electrophysiological method in *Xenopus* oocytes. Although electrophysiological characterization is the gold standard for measurement of ion channel activity, this method is technically demanding, labor-intensive and generally

unsuitable for screening large numbers of compounds. In order to mitigate these issues, researchers have turned to mammalian cell-based ion channel functional assays. Expression of target ion channels in mammalian cells still allows direct measurement of ion flux and membrane potential, however it does so in a high-throughput format. Assays exist for a variety of ion channel types ($Ca^{2+}$, $Na^{+}$, Cl-) and many are commercially available (reviewed in [Trivedi *et al*., 2010]). Moreover, the data from these HTS assays generally correlate well with results generated by traditional electrophysiological methods [Trivedi *et al*., 2010]. The Premo Halide Assay employed in this study is based upon technology used to identify small molecule inhibitors of CFTR chloride channels [De La Fuente *et al*., 2008]. The high-throughput format of the assay allows for the possibility of screening large chemical libraries against parasite receptors that may have highly divergent pharmacology. Given the major effects the SmACCs exert over worm motor function, this is an option worth pursuing.

The work described here adds to the mounting evidence of acetylcholine's role as a major inhibitory transmitter in schistosomes. We have described a novel clade of nicotinic acetylcholine-gated chloride channel subunits (SmACCs) that are phylogenetically distant from the *C. elegans* ACCs and play a major role in inhibitory neuromuscular modulation as it pertains to larval motor behavior. The localization of the SmACCs to the peripheral nerve plexus points to their broad, indirect role in this modulation. Functional studies in mammalian cells indicate that the SmACC subunits are capable of forming functional nicotinic chloride channels *in vitro*. Finally, the use of a fluorescent, mammalian cell-based functional assay for a helminth ion channel represents a new tool in the search for new drugs targeting this new clade of parasite-specific chloride channels.

## Acknowledgements

The authors are grateful to the Biomedical Research Institute (Bethesda, MD, USA) and BEI Resources, (Manassas, VA, USA) for providing *Biomphalaria glabrata* snails infected with *S. mansoni*. We also thank Claudia Wever (McGill University) and Dr.

Joe Dent (McGill University) for their technical assistance with the functional expression studies.

## References

1. Albuequerque EX, Pereira EF, Alkondon M, Rogers SW (2009). Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Res **89**(1): 73-120.

2. Akasu T, Ohta Y, Koketsu K (1984). Neuropeptides facilitate the desensitization of nicotinic acetylcholine-receptor in frog skeletal muscle endplate. Brain Res **290**(2): 342‑347.

3. Barker, LR, Bueding E, Timms AR (1966). The possible role of acetylcholine in *Schistosoma mansoni*. Brit J Pharmacol **26**: 656-665.

4. Barik J, Wonnacott S (2006). Indirect Modulation by _7 Nicotinic Acetylcholine Receptors of Noradrenaline Release in Rat Hippocampal Slices: Interaction with Glutamate and GABA Systems and Effect of Nicotine Withdrawal. Molec Pharmacol **69**(2): 618-628.

5. Beech RN, Callanan MK, Rao VTS, Dawe GB, and Forrester SG (2013). Characterization of Cys-loop receptor genes involved in inhibitory amine neurotransmission in parasitic and free-living nematodes. Parasitology Int Apr 16 doi: 10.1016/j.parint.2013.03.010. [Epub ahead of print].

6. Behm CA, Bendig MM, McCarter JP, Sluder AE (2005). RNAi-based discovery and validation of new drug targets in filarial nematodes. Trends Parasitol **21**(3): 97-100.

7. Bentley GN, Jones AK, Oliveros Parra WG, Agnew A (2004). ShAR1alpha and ShAR1beta: novel putative nicotinic acetylcholine receptor subunits from the platyhelminth blood fluke *Schistosoma*. Gene **329**: 27-38.

8. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, et al. (2009). The genome of the blood fluke *Schistosoma mansoni*. Nature **460**: 352–358.

9. Boyle JP, Wu XJ, Shoemaker CB, Yoshino TP (2003). Using RNA interference to manipulate endogenous gene expression in *Schistosoma mansoni* sporocysts. Mol Biochem Parasitol **128**(2): 205-15.

10. Bruckner DA, Vogel M (1974). The nervous system of larval *Schistosoma mansoni* as revealed by acetylcholinesterase staining. J Parasitol **60**(3): 437-446.

11. Bueding E, Liu CL, Rogers SH (1972). Inhibition by metrifonate and dichlorvos of cholinesterases in schistosomes. Br J Pharmacol **46**(3): 480-7.

12. Butarelli FR, Pontieri FE, Margotta V, Palladini G (2000). Acetylcholine/dopamine interaction in planaria. Comp Biochem Physiol C Toxicol Pharmacol **125**(2): 225-31.

13. Camacho M and Agnew A. (1995). *Schistosoma*: rate of glucose transport is altered by acetylcholine interaction with tegumental acetylcholine receptors and acetylcholinesterase. Exp Parasitol **81**: 584–591.

14. Camacho M, Alsford S, Jones A, Agnew A (1995). Nicotinic acetylcholine receptors on the surface of the blood fluke Schistosoma. Mol Biochem Parasitol **71**: 127–1.

15. Collins JJ III, King RS, Cogswell A, Williams DL, Newmark PA (2011). An Atlas for *Schistosoma mansoni* Organs and Life-Cycle Stages Using Cell Type-Specific Markers and Confocal Microscopy. PLoS Negl Trop Dis **5**(3): e1009.

16. Crabtree JE, Wilson RA (1980). *Schistosoma mansoni*: a scanning electron microscope study of the developing schistosomulum. Parasitology **81**(Pt 3): 553-64.

17. Day TA, Chen GZ, Miller C, Tian M, Bennett JL, Pax RA (1996). Cholinergic inhibition of muscle fibers isolated from *Schistosoma mansoni* (Trematoda: Digenea). Parasitology **113** (Pt. 1): 55-61.

18. De La Fuente R, Namkung W, Mills A, Verkman AS (2008). Small-molecule screen identifies inhibitors of a human intestinal calcium-activated chloride channel**.** Mol Pharmacol **73**: 758-768.

19. Di Angelantonio S, Giniatullin R, Costa V, Sokolova E, Nistri A (2003). Modulation of neuronal nicotinic receptor function by the neuropeptides CGRP and substance P on autonomic nerve cells. Br J Pharmacol **139**(6): 1061-73.

20. Doenhoff MJ, Hagan P, Cioli D, Southgate V, Pica-Mattoccia L, *et al*. (2009). Praziquantel: its use in control of schistosomiasis in sub-Saharan Africa and current research needs. Parasitology **136**(13): 1825-35.

21. El-Shehabi F, Taman A, Moali LS, El-Sakkary N, Ribeiro P (2012). A novel G protein-coupled receptor of *Schistosoma mansoni* (SmGPR-3) is activated by dopamine and is widely expressed in the nervous system. PLoS Negl Trop Dis **6**(2): e1523.

22. Felsenstein, J (1989). PHYLIP - Phylogeny Inference Package (Version 3.2). Cladistics **5:** 164-166.

23. Galietta LJ, Haggie PM, Verkman AS (2001). Green fluorescent protein-based halide indicators with improved chloride and iodide affinities. FEBS Lett **499**: 220-224.

24. García-Guzmán M, Sala F, Sala S, Campos-Caro A, Criado M (1994). Role of two acetylcholine receptor subunit domains in homomer formation and intersubunit recognition, as revealed by alpha 3 and alpha 7 subunit chimeras. Biochem **33**(50): 15198-203.

25. Gold D (1997). Assessment of the viability of *Schistosoma mansoni* schistosomula by comparative uptake of various vital dyes. Parasitol Res **83**: 163-169.

26. Gryseels B, Polman K, Clerinx, J, Kestens L, (2006). Human Schistosomiasis. Lancet **368**(9541): 1106-1118.

27. Halton DW and Gustaffson MKS (1996). Functional morphology of the platyhelminth nervous system. Parasitology **113**: S47–S72.

28. Halton DW, Maule AG (2004). Flatworm nerve–muscle: structural and functional analysis. Can J Zool **82**(2): 316-333.

29. Hamdan FF, Mousa A, Ribeiro P (2002). Codon optimization improves heterologous expression of a *Schistosoma mansoni* cDNA in HEK293 cells. Parasitol Res **88**(6): 583-6.

30. Holmes FP, Fairweather I (1984). *Fasciola hepatica*: the effects of neuropharmacological agents in *in vitro* motility. Exp Parasitol **58**: 194-208.

31. Huang Y, Chen W, Wang X, Liu H, Chen Y, *et al* (2013). The carcinogenic liver fluke, *Clonorchis sinensis*: new assembly, reannotation and analysis of the genome and characterization of tissue transcriptomes**.** PLOS One **8**(1): e54732.

32. Johansson T, Norris T, Peilot-Sjögren H (2013). Yellow Fluorescent Protein-Based Assay to Measure $GABA_A$ Channel Activation and Allosteric Modulation in CHO-K1 Cells. PLoS ONE **8**(3): e59429.

33. Kaminsky R, Gauvry N, Schorderet Weber S, Skripsky T, Bouvier J, *et al.* (2008). Identification of the amino-acetonitrile derivative monepantel (AAD 1566) as a new anthelminthic drug development candidate. Parasitol Res **103**(4): 931-939.

34. Kao PN, Karlin A (1996). Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystinyl residues. J Biol Chem **261**(18): 8085-8088.

35. Keramidas A, Moorhouse AJ, Pierce KD, Schofield PR, Barry PH (2002). Cation-selective Mutations in the M2 Domain of the Inhibitory Glycine Receptor Channel Reveal Determinants of Ion-Charge Selectivity. J Gen Physiol **119**: 393-410.

36. Koopowitz H, Chien P (1974). Ultrastructure of the Nerve Plexus in Flatworms. Cell Tiss Res **155**: 337-351.

37. Kreutz-Peterson G, Radwanska M, Ndegua D, Shoemaker CK, Skelly PJ (2007). Optimizing gene suppression in schistosomes using RNA interference. Mol Biochem Parasitol **153**(2): 194-202.

38. Kruger FJ, Hamilton-Attwell VL, Tiedt L, Du Preez L (1986). Further observations on an intratubercular sensory receptor of *Schistosoma mattheei.* Onderstepoort J Vet Res **53**(4): 239-40.

39. Kruger W, Gilbert D, Hawthorne R, Hryciw DH, Frings S, Poronnik P Lynch JW (2005). A yellow fluorescent protein-based assay for high-throughput screening of glycine and GABAA receptor chloride channels. Neurosci Lett **380**(3): 340-5.

40. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, *et al* (2007). Clustal W and Clustal X version 2.0. Bioinformatics **23**(21): 2947-2948.

41. Le Novère N, Corringer PJ, Changeux JP (2002). The diversity of subunit composition in nAChRs: evolutionary origins, physiologic and pharmacologic consequences. J Neurobiol **53**(4): 447-56.

42. Lewis F. (2001) Schistosomiasis. Current Protocols in Immunology **28**: 19.1.1–19.1.28.

43. Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(t)) Method. Methods **25**(4): 402-8.

44. Mair GR, Maule AG, Day TA, Halton DW (2000). A confocal microscopical study of the musculature of adult *Schistosoma mansoni*. Parasitology **121**(Pt 2): 163–170.

45. Maule AG, Day TA, Chappell CH (2005). Parasite neuromusculature and its utility as a drug target. Parasitology **131**: S1-S2.

46. McVeigh P, Mair GR, Novozhilova E, Day A, Zamanian M, Marks NJ, Kimber MJ, Day TA, Maule AG (2011). Schistosome l/Lamides--a new family of bioactive helminth neuropeptides. Int J Parasitol **41**(8): 905-13.

47. Melman SD, Steinauer ML, Cunningham C, Kubatko LS, Mwangi IN, *et al* (2009). Reduced Susceptibility to Praziquantel among Naturally Occurring Kenyan Isolates of *Schistosoma mansoni*. PLoS Negl Trop Dis **3**(8): e504.

48. Morariu VI, Srinivasan BV, Raykar VC, Duraiswami R, Davis LS (2008). Automatic online tuning for fast Gaussian summation**.** Advances in Neural Information Processing Systems (NIPS) 2007. Available: http://books.nips.cc/papers/files/nips21/NIPS2008_0257.pdf. Accessed 13 October 2013.

49. Nishimura K, Kitamura Y, Taniguchi T, Agata K. (2010). Analysis of motor function modulated by cholinergic neurons in planarian Dugesia japonica. Neurosci **168**(1): 18-30.

50. Papke RL, Dwoskin LP, Crooks (2007). The pharmacological activity of nicotine and nornicotine on nAChRs subtypes: relevance to nicotine dependence and drug discovery. J Neurochem **101**(1): 160-7.

51. Patocka N and Ribeiro P (2013). The functional role of a serotonin transporter in *Schistosoma mansoni* elucidated through immunolocalization and RNA interference (RNAi). Mol Biochem Parasitol **187**: 32-42.

52. Protasio AV, Tsai IJ, Babbage A, Nichol S, Hunt M (2012). A systematically improved high quality genome and transcriptome of the human blood fluke *Schistosoma mansoni*. PLoS Negl Trop Dis **6**(1): e1455.

53. Putrenko I, Zakikhani M, Dent JA (2005) A Family of Acetylcholine-gated Chloride Channel Subunits in *Caenorhabditis elegans*. J Biol Chem 280: 6392-6398.

54. Raymond V, Mongan NP, Sattelle DB (2000). Anthelmintic actions on homomer-forming nicotinic acetylcholine receptor subunits: chicken alpha7 and ACR-16 from the nematode *Caenorhabditis elegans*. Neurosci **101**(3): 785-91.

55. Reddien PW, Bermange AL, Murfitt KJ, Jennings JR, Sánchez Alvarado A (2005). Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. Dev Cell **8**(5): 635-49.

56. Ribeiro P, El-Shehabi F, Patocka N (2005). Classical transmitters and their receptors in flatworms. Parasitology **131**: S19-S40.

57. Robertson AP and Martin RJ (2007). Ion-channels on parasite muscle: pharmacology and physiology. Invert Neurosci **7**(4): 209-17.

58. Rychlik W (2007). OLIGO 7 Primer Analysis Software. In: Yuryev A, editor. Methods in Molecular Biology Vol. 402: PCR Primer Design. Totowa: Humana Press. pp 35-59.

59. Sabah AA, Fletcher C, Webbe G, Doenhoff J (1986). *Schistosoma mansoni*: chemotherapy of infections of different ages. Exp Parasitol **61**: 294–303.

60. Taman A, Ribeiro P (2009). Investigation of a dopamine receptor in *Schistosoma mansoni*: Functional studies and immunolocalization. Mol Biochem Parasitol **168**: 24–33.

61. Thompson AJ, Lester HA, Lummis SCR (2010). The Structural Basis of Function in Cys-Loop Receptors. Q Rev Biophys **43**(4): 449-99.

62. Trivedi S, Liu J, Ruifeng L, Bostwick R (2010). Advances in functional assays for high-throughput screening of ion channels targets. Expert Opin Drug Discov **5**(10): 995-1006.

63. van Nierop P, Keramidas A, Bertrand S, van Minnen J, Gouwenberg Y, *et al.* (2005). Identification of Molluscan Nicotinic Acetylcholine Receptor (nAChR) Subunits Involved in Formation of Cation- and Anion-Selective nAChRs. J Neurosci **25**(46): 10617-10626.

64. Verkman AS, Galietta LJ (2009). Chloride channels as drug targets. Nat Rev Drug Discov **8**(2): 153-71.

65. Walker RJ, Franks CJ, Pemberton D, Rogers C, Holden-Dye L (2000). Physiological and pharmacological studies on nematodes. Acta Biol Hung **51**(2-4): 379-94.

66. Williamson SM, Robertson AP, Brown L, Williams T, Woods DJ, Martin RJ, Sattelle DB, Wolstenholme AJ (2009). The nicotinic acetylcholine receptors of the parasitic nematode *Ascaris suum*: formation of two distinct drug targets by varying the relative expression levels of two subunits. PLoS Pathog **5**(7): e1000517.

67. Wilson CVLC, Schiller EL (1969). The neuroanatomy of *Hymenolepis dimimuta* and *H. nana*. J Parasitol **55**(2): 261-70.

68. Wilson GG, Pascual JM, Brooijmans N, Murray D, Karlin A (2000). The Intrinsic Electrostatic Potential and the Intermediate Ring of Charge in the Acetylcholine Receptor Channel. J Gen Physiol **115**: 93-106

69. Xie J, Dernovici S, Ribeiro P (2005). Mutagenesis analysis of the serotonin 5-HT2C receptor and a *Caenorhabditis elegans* 5HT2 homologue: Conserved residues of helix 4 and 7 contribute to agonist-dependent activation of 5HT2 receptors. J Neurochem **92**: 375–387.

## Figures

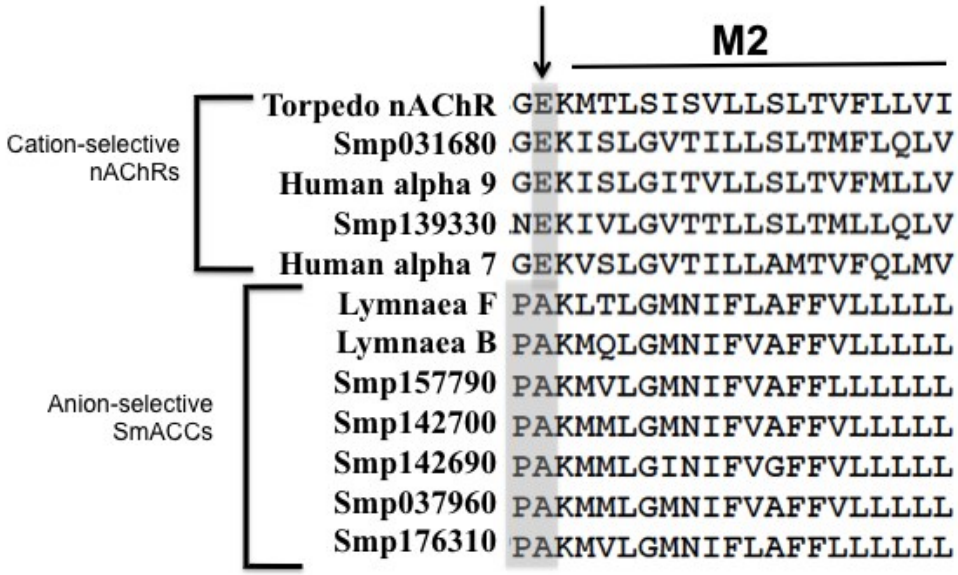


**Figure 1. Predicted Ion-selectivity of Putative *S. mansoni* nAChRs.** A structural alignment of human, *Lymnaea* and *S. mansoni* nAChR subunits was generated using the *Torpedo* nAChR structure (PDB Accession # 2BG9) as a template. The M1-M2 linker region, shown here, is a key determinant of ion-selectivity in Cys-loop ligand gated ion channels. A glutamate residue (arrow) confers cation-selectivity and is present in all vertebrate subunits, as well as two of the *S. mansoni* subunits. The remaining schistosome and snail subunits display a Pro-Ala motif in this position, suggesting anion-selectivity. We have termed these receptors *S. mansoni* acetylcholine-gated chloride channels (SmACCs).

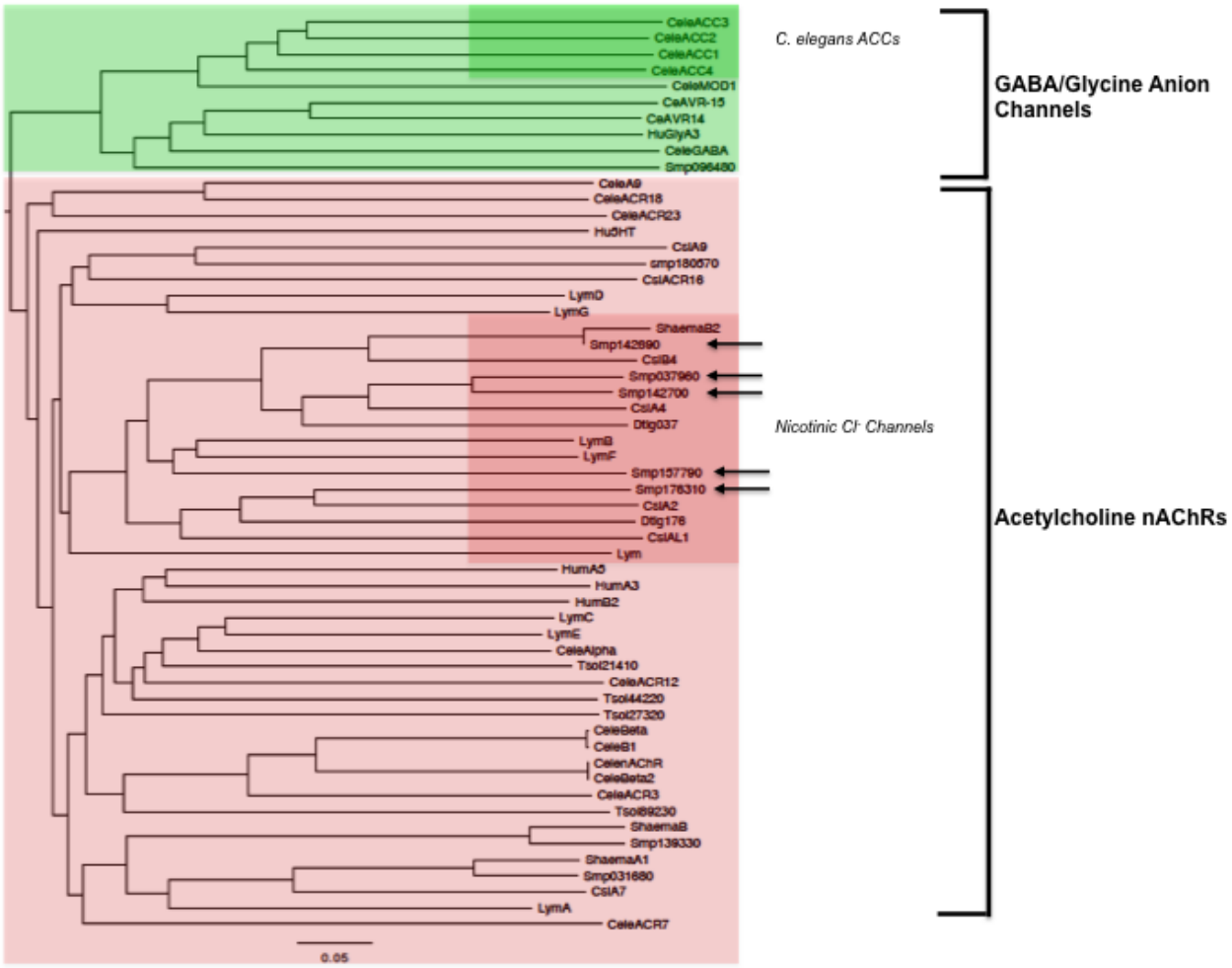


**Figure 2**. **Phylogenetic Analysis of Cys-Loop Ion Channel Subunits**. A bootstrapped, neighbor-joining tree was constructed in PHYLIP from a CLUSTALX alignment of vertebrate and invertebrate Cys-loop superfamily receptor subunits. The tree is midpoint-rooted and was visualized using FigTree 3.0. Two distinct groups of receptors can be seen, the γ-aminobutyric acid (GABA) /glycine-like anion channels and the nicotinic acetylcholine receptors (nAChRs). The *C. elegans* acetylcholine-gated chloride channels (ACC) form a distinct clade within the larger group of GABA/glycine anion channels (green inset). In contrast the predicted *Schistosoma* acetylcholine-gated chloride channels (SmACCs) align with cholinergic nicotinic nAChRs, suggesting divergent evolutionary paths. The SmACCs described here are indicated by arrows and they constitute a separate clade in the nAChR tree along with putative homologs from flatworms *Dugesia (Dtig)* , *Clonorchis (Cs) and S. haematobium*, as well as the snail

*Lymnaea (Lym)*. Accession numbers for sequences used in the alignment are listed in Table S1.

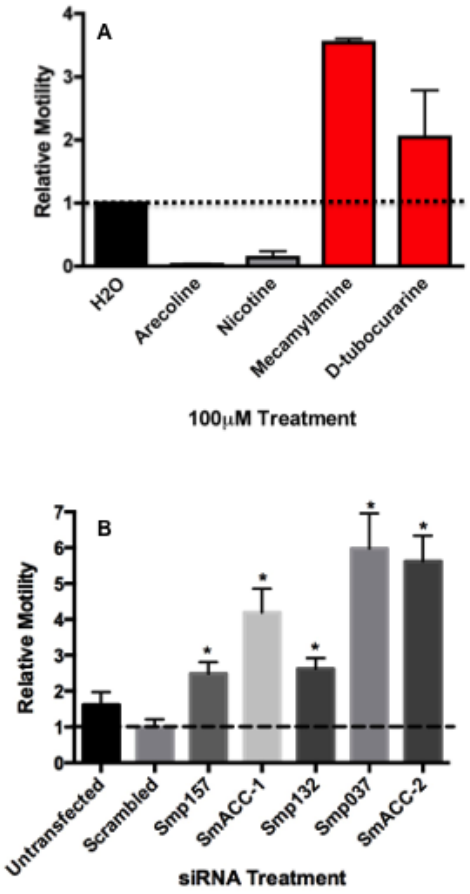


**Figure 3. Pharmacological and RNAi Behavioral Assays in Schistosomula.** (A) Relative motility of 6-day old larvae was measured before and 5 minutes after the addition of cholinergic compounds, each at 100μM. Data were normalized to baseline motility measured before drug addition. Treatment with the receptor agonists, nicotine and arecoline caused complete paralysis when compared to the water treated control. A 2-3.5-fold increase in motility was seen in parasites treated with the nicotinic receptor antagonists, mecamylamine and d-tubocurarine. The data are the means and SEM of three independent experiments, each containing at least 12 animals. (B) Freshly transformed schistosomula were transfected with 50 nM sequence-specific siRNA or 50 nM irrelevant (scrambled) siRNA and cultured for 6-days. Larval motility was measured and normalized relative to untransfected larvae cultured for the same period of time. The

results show that silencing of SmACC subunits causes a significant increase in relative motility ranging from 2-6-fold when compared to the negative scrambled siRNA control whereas no difference can be seen in the untransfected control. The two subunits generating strong hypermotility chosen for further study were SmACC-1 and SmACC-2. All data shown are the means of three independent experiments, each containing at least 12 animals. All changes in motility were statistically significant, as measured by a Student's t-test with a cutoff p-value of 0.05.

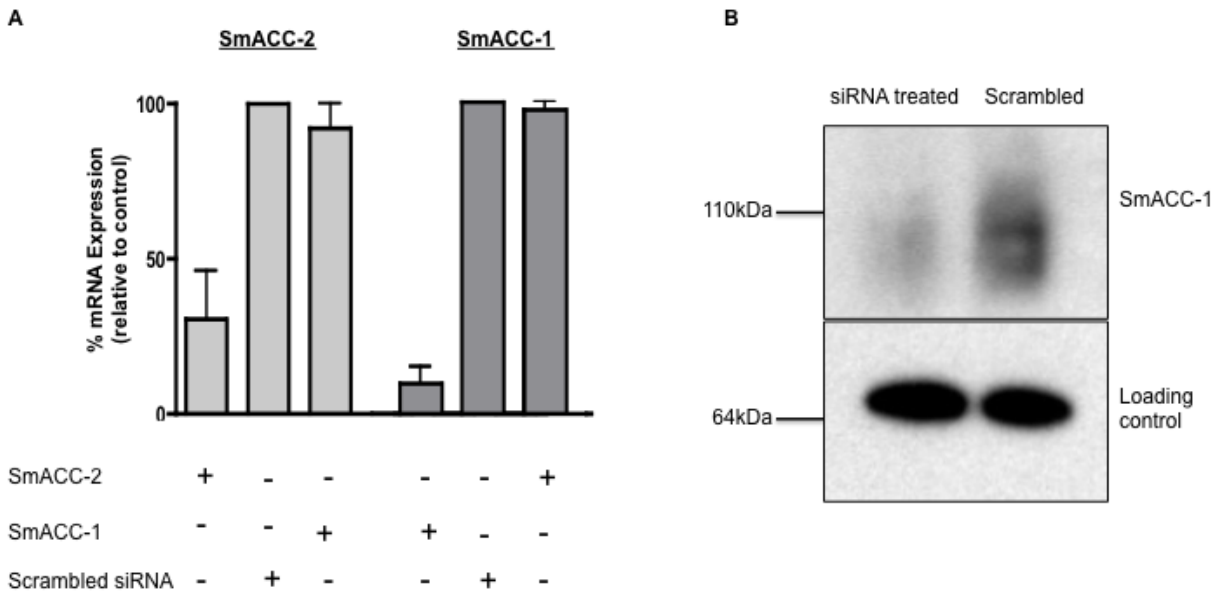


**Figure 4. Confirmation of SmACC Knockdown.** (A) Knockdown of SmACC-1 and SmACC-2 was confirmed at the mRNA level. RNA extracted from treated parasites was oligo-dT reverse-transcribed and qPCR was performed using primers targeting either a specific or a non-relevant SmACC subunit. Relative expression was calculated using the comparative ΔΔCt method after normalization to a housekeeping gene (GAPDH). Transfection with SmACC-1 siRNA (right panel) caused a 90% reduction in expression

of SmACC-1 mRNA compared to the negative scrambled siRNA control but had no effect on the expression level of SmACC-2, indicating the siRNA effect was specific. Similarly, transfection with SmACC-2 siRNA (left) produced significant (60%) knockdown of SmACC-2 mRNA compared to the scrambled negative control and no silencing of the SmACC-1 off-target control. Silencing of both subunits was statistically significant, as measured by Student's t-test in three independent experiments, each with 3 replicates. (B) Western blot analysis was performed to assay for silencing of SmACC-1 at the protein level. Crude membrane protein extracts from SmACC-1 siRNA-treated and negative control schistosomula were resolved on a SDS-PAGE gel, transferred to a PVDF membrane and probed with affinity-purified anti-SmACC-1 or a loading control (anti-Sm5-HTR). A band of the expected size is present in the negative control lane but not in the siRNA-treated lane, indicating silencing of SmACC-1 at the protein level. There is no difference in band intensity of the samples when probed with the anti-Sm5-HTR loading control.

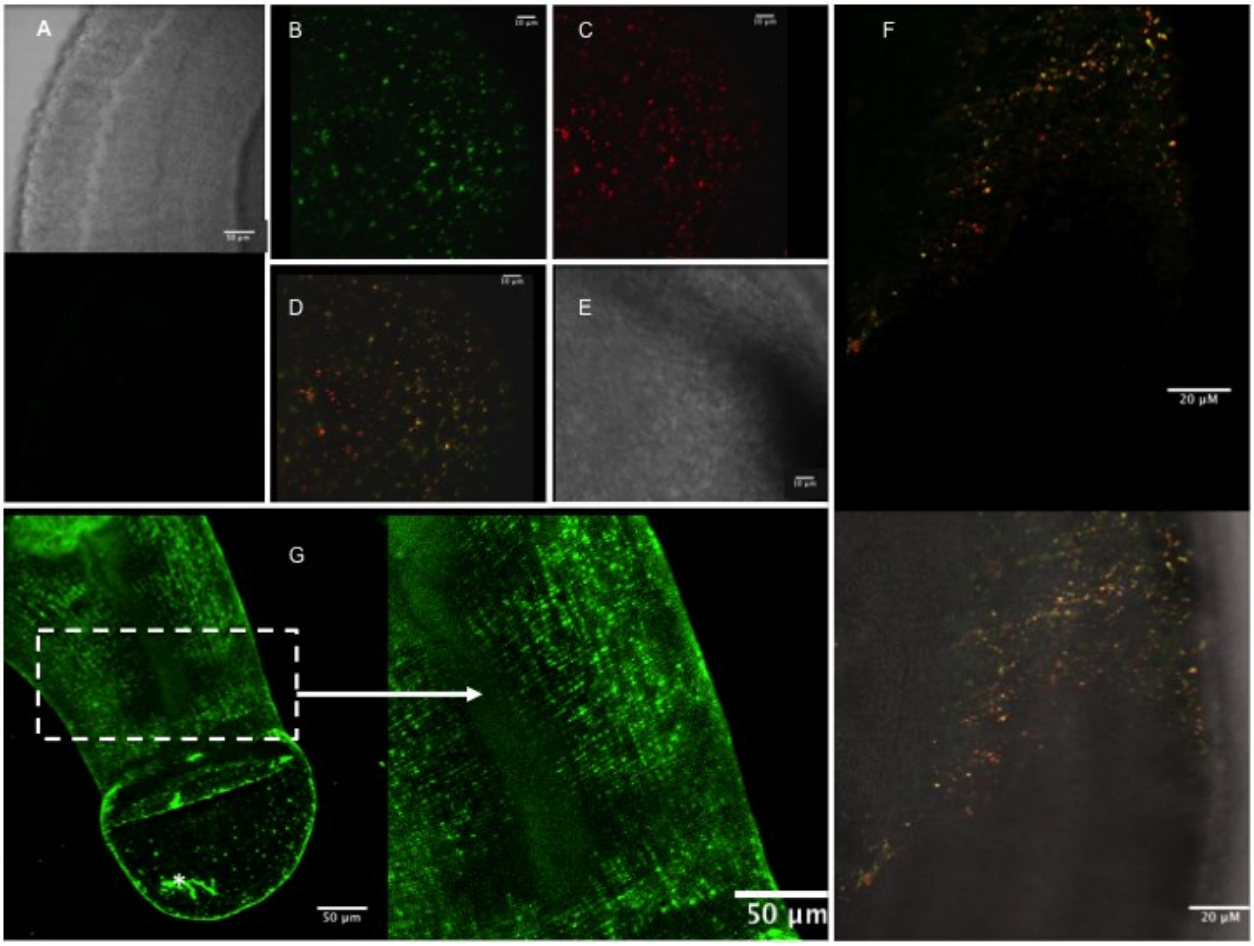


**Figure 5. Immunolocalization of SmACC-1 in Adult *Schistosoma mansoni*.** Fixed adult worms were treated with affinity-purified anti-SmACC-1, followed by Alexa 488-conjugated secondary antibody. Some animals were counterstained with goat anti-human choline acetyltransferase (ChAT), a marker of cholinergic neurons, and Alexa 594-conjugated secondary antibody. (A) Transmitted and fluorescence images of negative control worms labeled with peptide-preadsorbed anti-SmACC-1 showed no significant off-target immunoreactivity or autofluorescence. (B-E) Ventral sucker of an adult male schistosome labeled with anti-SmACC-1 (B, green) and anti-ChAT (C, red). Scale bars are 10 µm. Both proteins are present in a diffuse, punctate pattern that is characteristic of neuronal staining in the nerve plexuses of the peripheral nervous system. Co-labeling of SmACC-1 and ChAT (D) indicates that the two proteins are in close proximity (yellow) but do not share 100% co-localization. (E) Adult ventral sucker shown under transmitted light with no fluorescent overlay. (F) SmACC-1 and ChAT

immunoreactivity was also present in the fine nerve fibers of the subtegumental nerve plexus. (G) A Z-projection of SmACC-1 immunoreactivity in an adult male worm. SmACC is present in both the oral sucker and in the varicose nerve fibers that form the peripheral nerve plexus (dashed box). Non-specific fluorescence resulting from tissue damage is shown by a star (*).

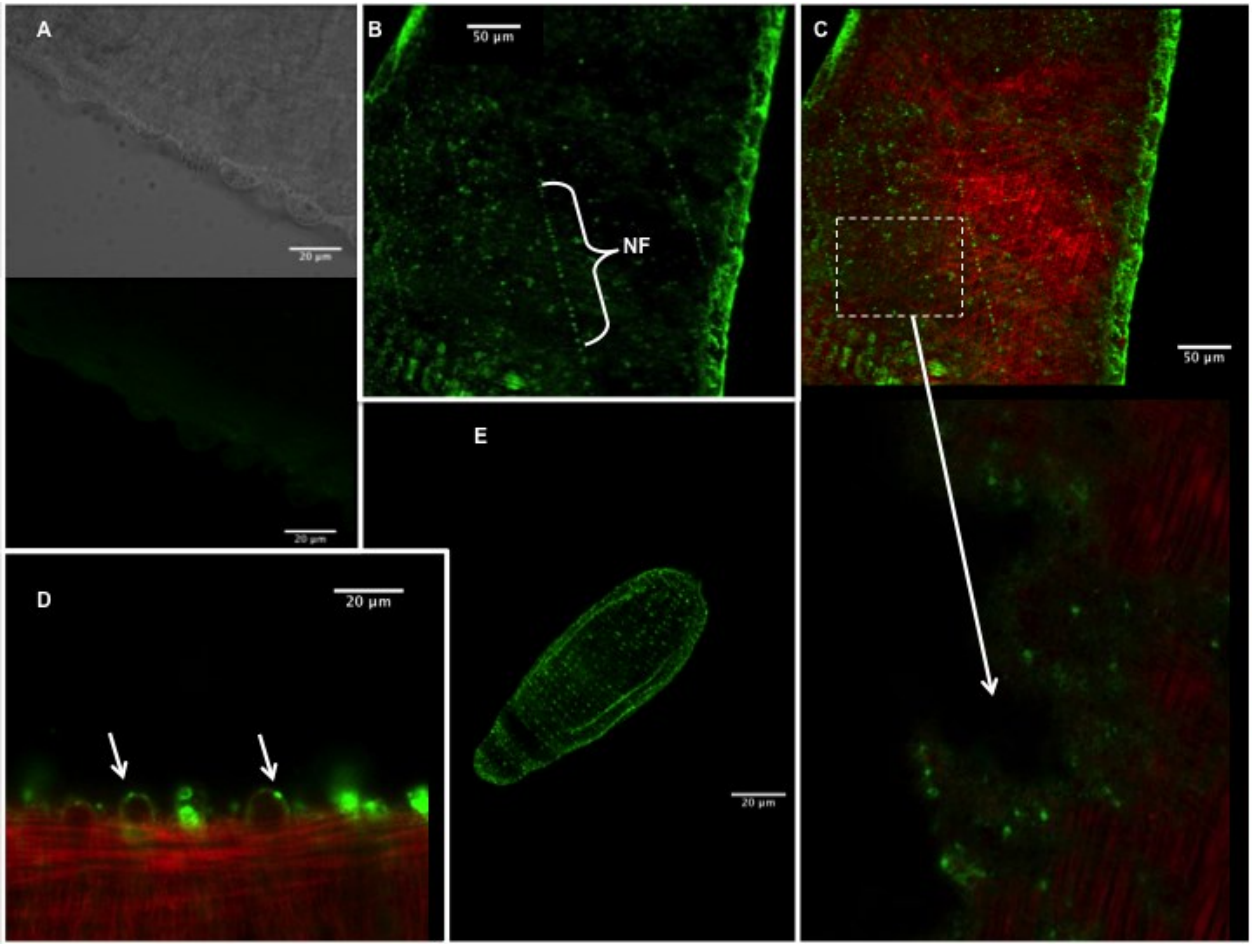


**Figure 6. Immunolocalization of Sm-ACC-2 in Adult and Larval *S. mansoni*.** Adult and 6-day old schistosomula were fixed and incubated with affinity-purified anti-SmACC-2, followed by Alexa 488-conjugated secondary antibody (green). Body wall musculature was counterstained with tetramethylrhodamine B isothiocyanate (TRITC)-labeled phalloidin (red). (A) No significant immunoreactivity was seen inside or on the surface of negative control worms treated with peptide-preadsorbed anti-SmACC-2. (B) SmACC-2 immunoreactivity (green) is present in varicose nerve fibers (NF) that cross the body in a mesh-like pattern indicative of PNS staining. A similar pattern was seen in adult females.

(C) Z-projection of an adult male worm stained with anti-SmACC-2 and phalloidin. SmACC-2 and the body wall musculature are present at different depths of the animal, indicating that SmACC-2 does not directly innervate muscle. SmACC-2 is localized to the submuscular or subtegumental nerve plexus surrounding the musculature. (D) Tubercles of an adult male worm labeled with anti-SmACC-2 and phalloidin. Specific, punctate SmACC02 immunoreactivity can be seen along the surface of the tubercles (arrows). A similar pattern of surface SmACC-2 staining was also seen in female worms (not shown). (E) SmACC-2 forms a pattern of concentric, varicose nerve fibers that run the entire length of 6-day old schistosomula. Labeling is present just under the surface, at the subtegumental or submuscular level, indicating the presence of SmACC-2 in the developing peripheral nervous system of larval *S. mansoni*. A similar expression pattern was observed in schistosomula labeled with anti-SmACC-1 antibody (not shown).

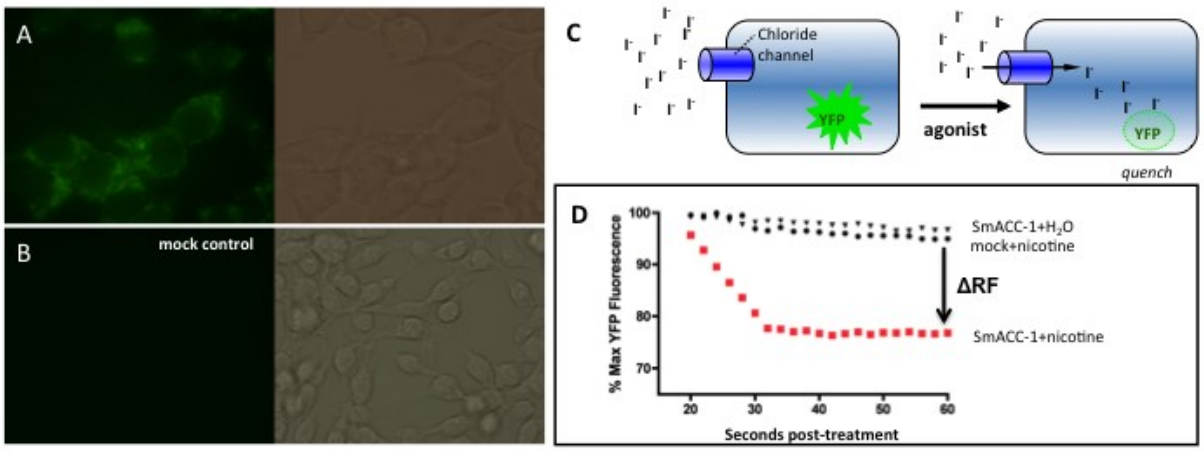


**Figure 7. Functional Characterization of SmACC-1 in HEK-293 cells.** HEK-293 cells were transfected with a human codon-optimized SmACC-1 construct and labeled with affinity-purified anti-SmACC-1 antibody, followed by FITC-conjugated secondary antibody (green). (A) The results show specific immunoreactivity along the surface of the cells, consistent with protein expression. (B) No immunofluorescence is present in cells

transfected with empty vector (mock control). (C) Schematic representation of the Premo Halide Sensor YFP quench assay. Cells expressing YFP and the chloride channel of interest are bathed in buffer containing iodide ($I^-$), which is used as a surrogate for chloride ions. Agonist-induced activation of the channel causes an influx of $I^-$ into the cell and quenches YFP fluorescence. (D) Representative data from individual wells containing cells transfected with either SmACC-1 or empty vector (mock). Treatment of SmACC-1 expressing cells with 100 μM nicotine (solid red squares) resulted in a significant reduction in YFP fluorescence (ΔRF) when compared to both a water-treated negative control (solid triangles) and mock-transfected cells treated with 100 μM nicotine (solid circles). Data were normalized relative to maximum YFP fluorescence for each sample.

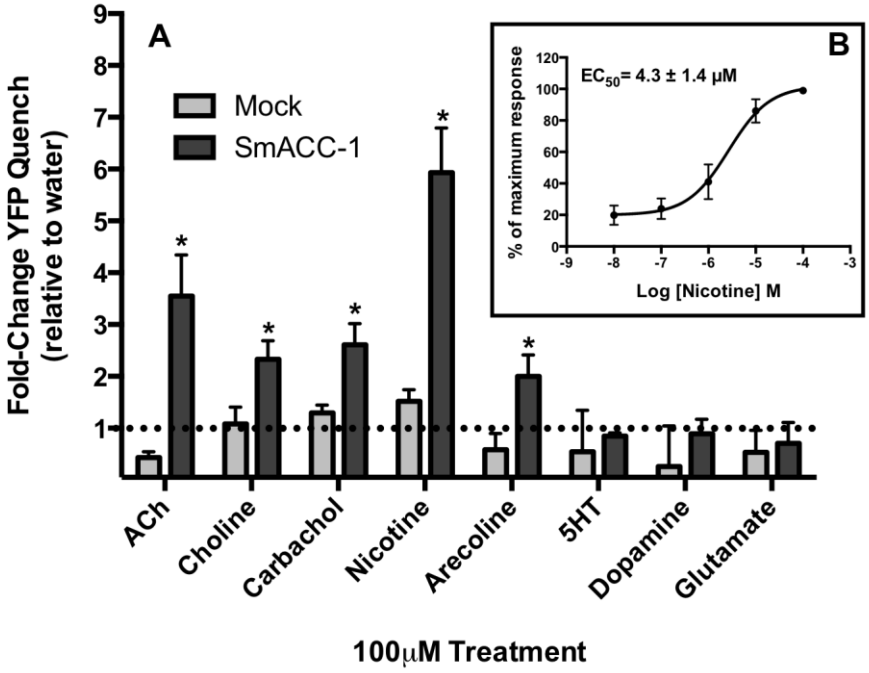


**Figure 8. SmACC-1 is selectively activated by cholinergic substances in transfected HEK-293 cells.** (A) A panel of cholinergic receptor agonists (acetylcholine (ACh), choline, carbachol, nicotine, arecoline) was tested against SmACC-1 expressing or mock-transfected cells. The YFP quench data were normalized relative to the water-treated control measured in the same experiment and on the same plate. Results are the means and SEM of 3-4 experiments, each containing 6 technical replicates per

treatment. All cholinergic agonists caused a significant reduction in YFP fluorescence at $P < 0.05$ (*) compared to the water control. Treatment of SmACC-1-expressing cells with serotonin (5HT), glutamate or dopamine did not result in significant YFP quench. (B) SmACC-1 expressing cells were treated with variable concentrations of nicotine and YFP quench was calculated. The YFP quench data were normalized relative to the maximum response for each experiment and an $EC_{50}$ value was calculated by nonlinear regression analysis of the normalized data. The results are the means ± SEM of 3 independent experiments, each with six replicates.

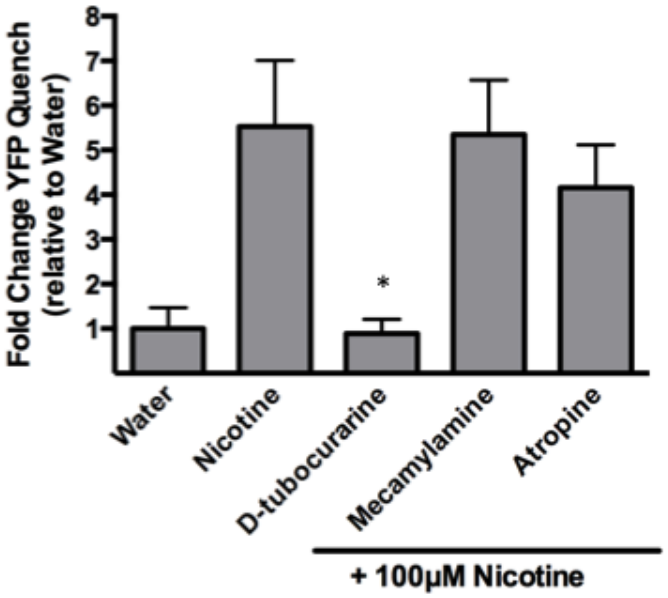


**Figure 9. SmACC-1 is selectively antagonized by d-tubocurarine in transfected HEK-293 cells.** Mock-transfected and SmACC-1-expressing cells were pre-incubated with cholinergic antagonists (mecamylamine, d-tubocurarine, atropine) at a concentration of 100 μM. Cells were then treated with 100 μM nicotine and YFP quench was measured. The YFP quench data were normalized relative to the water-treated control measured in the same experiment and on the same plate. Results are the means and SEM of two separate experiments, each containing 6 technical replicates. Control SmACC-1 cells were activated by nicotine in the absence of antagonist. Pre-incubation with d-tubocurarine caused a significant inhibition of nicotine activation. Mecamylamine and atropine did not antagonize nicotine activation of SmACC-1.

| Gene Name | *Species* | GenBank Accession Number/NCBI Reference Sequence | Comment |
|---|---|---|---|
| GABA-A subunit | *Caenorhabditis elegans* | AAN65376.1 | |
| AVR-15 | *Caenorhabditis elegans* | CAA04171.1 | |
| Nicotinic Beta 1 subunit | *Caenorhabditis elegans* | CAA58765.1 | |
| AVR-15 | *Caenorhabditis elegans* | NP001020963.1 | |
| ACR-17 | *Caenorhabditis elegans* | NP001023961.1 | |
| LEV-1 | *Caenorhabditis elegans* | NP001255705.1 | |
| UNC-63 | *Caenorhabditis elegans* | NP491533.1 | |
| UNC-29 | *Caenorhabditis elegans* | NP492399.1 | |
| ACR-7 | *Caenorhabditis elegans* | NP495647.1 | |
| ACC-4 | *Caenorhabditis elegans* | NP499782.1 | |
| ACC-2 | *Caenorhabditis elegans* | NP501567.1 | |
| ACC-1 | *Caenorhabditis elegans* | NP501715.1 | |
| ACR-23 | *Caenorhabditis elegans* | NP504024.2 | |
| ACR-18 | *Caenorhabditis elegans* | NP506868.2 | |
| ACC-3 | *Caenorhabditis elegans* | NP508810.2 | |

| | | | |
|---|---|---|---|
| ACR-3 | *Caenorhabditis elegans* | NP509129.1 | |
| ACR-12 | *Caenorhabditis elegans* | NP510262.1 | |
| MOD-1 | *Caenorhabditis elegans* | NP741580.1 | |
| Nicotinic Alpha 2 subunit | *Clonorchis sinensis* | GAA28402.2 | |
| Nicotinic Alpha 1 subunit | *Clonorchis sinensis* | GAA31334.2 | |
| Nicotinic Alpha 9 subunit | *Clonorchis sinensis* | GAA49744.1 | |
| ACR-16 | *Clonorchis sinensis* | GAA49755.1 | |
| Nicotinic Beta 4 subunit | *Clonorchis sinensis* | GAA51700.1 | |
| Nicotinic Alpha 4 subunit | *Clonorchis sinensis* | GAA51702.1 | |
| Nicotinic Alpha 7 subunit | *Clonorchis sinensis* | GAA53652.1 | |
| Putative Smp_176310 homolog | *Dugesia tigrina* | Locus 7348_Transcript 1 | |
| Putative Smp_037960 homolog | *Dugesia tigrina* | Locus 5943_Transcript 2 | |
| 5HT-gated ion channel subunit | *Homo sapiens* | 220340A | |
| Nicotinic Alpha 5 subunit | *Homo sapiens* | AAA58357.1 | |
| Nicotinic Alpha 3 subunit | *Homo sapiens* | EAW99159.1 | |
| Nicotinic Beta 2 subunit | *Homo sapiens* | NP_000739 | |
| Glycine Alpha 3 subunit | *Homo sapiens* | NP_006520.2 | |
| Nicotinic subunit A | *Lymnaea stagnalis* | ABA60380.1 | |
| Nicotinic subunit B | *Lymnaea stagnalis* | ABA60381.1 | |
| Nicotinic subunit C | *Lymnaea stagnalis* | ABA60382.1 | |
| Nicotinic subunit | *Lymnaea* | ABA60383.1 | |

| | | | |
|---|---|---|---|
| D | *stagnalis* | | |
| Nicotinic subunit E | *Lymnaea stagnalis* | ABA60384.1 | |
| Nicointic subunit F | *Lymnaea stagnalis* | ABA60385.1 | |
| Nicotinic subunit G | *Lymnaea stagnalis* | ABA60386.1 | |
| Nicotinic subunit F | *Lymnaea stagnalis* | ABA60387.1 | |
| Nicotinic Alpha 1 subunit | *Schistosoma haematobium* | AAR84357.1 | |
| Nicotinic Beta 1 subunit | *Schistosoma haematobium* | AAR84358.1 | |
| Nicotinic Beta 2 subunit | *Schistosoma haematobium* | AAX59989.1 | |
| SMP_031680 | *Schistosoma mansoni* | AAR84361.1 | |
| Smp_139330 | *Schistosoma mansoni* | AAR84362.1 | |
| Smp_012000 | *Schistosoma mansoni* | CCD81766.1 | |
| SmACC-2 | *Schistosoma mansoni* | KF694749 | |
| GABA-gated ion channel subunit | *Schistosoma mansoni* | XP_002580535.1 | |
| Smp_142700 | *Schistosoma mansoni* | XP00257155.1 | |
| Smp_037960 | *Schistosoma mansoni* | XP002575154.1 | |
| Smp_157790 | *Schistosoma mansoni* | XP002577842.1 | |
| Smp_096480 | *Schistosoma mansoni* | XP002580535.1 | |
| SmACC-1 | *Schistosoma mansoni* | KF694748 | |
| Smp_180570 | *Schistosoma mansoni* | XP002581250.1 | |
| Taenia putative nAChR subunit | *Taenia solium* | TsM_000273200 | No NCBI Accession Number; GeneDB reference used |
| Taenia putative nAChR subunit | *Taenia solium* | TsM_000214100 | No NCBI Accession Number; GeneDB reference used |
| Taenia putative nAChR subunit | *Taenia solium* | TsM_000442200 | No NCBI Accession Number; GeneDB reference used |

| | | | |
|---|---|---|---|
| Taenia putative nAChR subunit | *Taenia solium* | TsM_000892300 | No NCBI Accession Number; GeneDB reference used |

**Table S1. List of Cys-loop receptor sequences used for phylogenetic analysis of SmACCs.**

| Gene | Primer Pair (Forward, Reverse) | dsRNA/qPCR |
|---|---|---|
| SmACC-1 | 5'-GAGATTATGAAAAACGTGCG-3' | dsRNA |
| | 5'-ATACCAAATGATAATCCGTC-3' | dsRNA |
| | 5'-TGTGCACATGGTTACACGTGATG -3' | qPCR |
| | 5'-AACACCATACCGACTGCTCTCC-3' | qPCR |
| | | |
| SmACC-2 | 5'-GATAATCAAACTATTCATTCTG-3' | dsRNA |
| | 5'-CCAGCAATAATAATTAATG-3' | dsRNA |
| | 5'-GGACAAATTGTTGGGCTGT-3' | qPCR |
| | 5'-GTGTCCATATTTGATGTGG-3' | qPCR |
| | | |
| Smp_037960 | 5'-CAGCGAATTCCTCTACCAG-3' | dsRNA |
| | 5'-GAGCAAACCAATTACTAGC-3' | dsRNA |
| | | |
| Smp_132070 | 5'-CACCTGTAGTGGCGGATGC-3' | dsRNA |
| | 5'-CAGGGGATGAAATCTTATTG-3' | dsRNA |
| | | |
| Smp_157790 | 5'-CCGCAGTGCCTGAGAAGG-3' | dsRNA |
| | 5'-GTTCATTATTTGAATTAA-3' | dsRNA |

**Table S2. List of PCR Primers used for generation of siRNA and qPCR of SmACCs.**

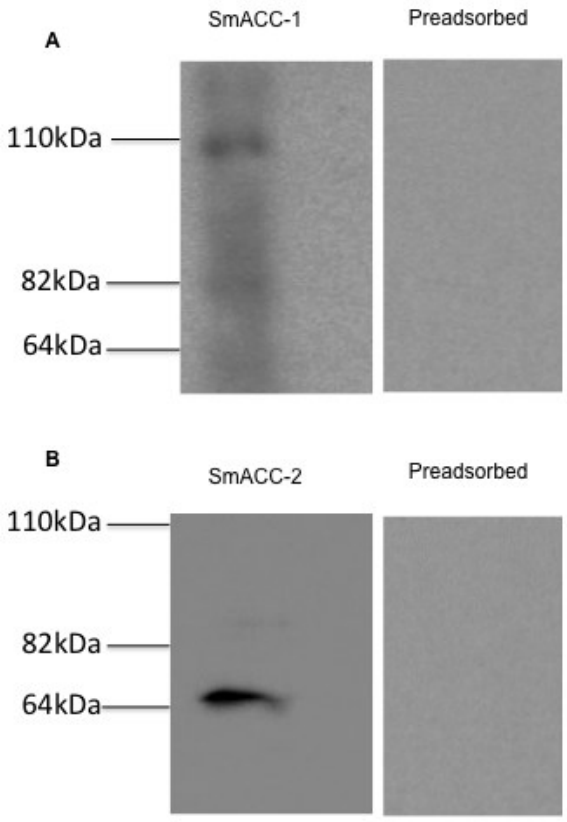


**Figure S1. Validation of anti-SmACC antibodies in Adult Schistosomes.** Crude membrane protein extract from adult *S. mansoni* was run on an SDS-PAGE gel, transferred to a PVDF membrane and probed with peptide-derived anti-SmACC antibodies, followed by horseradish peroxidase (HRP) conjugated secondary antibody. (A) Western blot of adult worm protein probed with anti-SmACC-1. A band corresponding to the predicted size of SmACC-1 is present when probed with anti-SmACC-1 but absent when probed with the peptide-preadsorbed SmACC-1. (B) Probing adult worm extract with anti-SmACC-2 results in a band corresponding to the expected size of the protein. There was no immunoreactivity in the peptide-preadsorbed negative control.

## Connecting Statement

In the previous chapter, we identified and characterized a novel family of acetylcholine-gated chloride channels in *S. mansoni* (SmACCs). Functional and RNAi assays confirmed that the SmACCs are nicotinic chloride channels that are located in the parasite PNS and indirectly modulate inhibitory neuromuscular behavior. Building upon this information, we decided to examine the second type of receptor mediating classical cholinergic signaling, muscarinic acetylcholine receptors (mAChRs). There exists no prior behavioral or biochemical investigation of mAChRs in parasitic flatworms. However, the modulatory function of mAChRs in other invertebrates, including nematodes is well known. Moreover, the annotation of the *S. mansoni* genome predicts the expression of a single full-length mAChR-like receptor. In the next chapter, we attempt to confirm the presence of a mAChR in *S. mansoni* and elucidate its behavioral function and pharmacological profile.

## Chapter III (Manuscript II)

# "Cloning and Characterization of a GAR-like Acetylcholine Receptor from *Schistosoma mansoni*"

Kevin MacDonald[1] and Paula Ribeiro[1*]

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

Tel: (514) 398-7607

Fax: (514) 398-7857

E-mail: paula.ribeiro@mcgill.ca

*To whom correspondence should be addressed

**(For Submission to Molecular and Biochemical Parasitology)**

## Abstract

The neuromuscular system of helminths controls a variety of essential biological processes and therefore represents a good source of novel drug targets. Acetylcholine has previously been shown to act as a major inhibitory modulator of muscular contraction in *Schistosoma mansoni* but the mode of action is poorly understood. Here, we present evidence of the first functional G protein-coupled acetylcholine receptor in *S. mansoni*, which we have termed SmGAR. A bioinformatics analysis indicated that SmGAR belongs to a unique clade of flatworm GAR-like receptors, which are distantly related to the GAR-2 receptor of *C. elegans* and vertebrate muscarinic acetylcholine receptors. Functional expression studies in yeast showed that SmGAR is constitutively active but can be further activated by acetylcholine and the cholinergic agonist, carbachol. Common cholinergic antagonists (atropine, promethazine) were found to have inverse agonist activity towards SmGAR, causing a significant decrease in the receptor's basal activity. Further sequence analysis identified several amino acid substitutions that may contribute to the constitutive activity of SmGAR. An RNAi phenotypic assay revealed that suppression of SmGAR activity in early-stage larval schistosomula leads to a drastic reduction in larval motility. In sum, our results provide the first molecular evidence that cholinergic GAR -like receptors are present in schistosomes and are required for proper motor control in the larvae. The results further identify SmGAR as a possible candidate for antiparasitic drug targeting.

**Keywords:**

Schistosome, G-protein-coupled receptor, motility, GAR, muscarinic, RNAi

## Introduction

Schistosomiasis is a debilitating, chronic infection that affects over 200 million people in 74 endemic countries [Gryseels *et al*., 2006]. Trematodes of the genus *Schistosoma* are the causative agents of the disease, with *S. mansoni* responsible for more than 90% of infections [Gryseels *et al*., 2006]. Currently, there is a single therapeutic option, praziquantel, and no vaccine is available. Reports of emerging resistance to praziquantel [reviewed in Wang *et al*., 2012], as well as its lack of efficacy against the migratory larval stages of the parasite [Sabah *et al*., 1986] underpin the need to develop new therapeutic targets. One area that has been especially productive in the search for new drug targets is the parasite nervous system, exemplified by the success of ivermectin, pyrantel and the more recently discovered octadepsipeptides [Wilson *et al*., 2003].

The schistosome nervous system is involved in a variety of processes that are essential to parasite survival including migration, attachment, feeding and reproduction [Maule *et al*., 2005]. It is also hypothesized to play a role in long-distance signal transduction via synaptic and paracrine mechanisms, as schistosomes lack a circulatory system and thus the capability for classical endocrine signaling. The key interaction controlling neuronal signaling in schistosomes involves neuroactive compounds binding to their cognate receptors and eliciting effects directly or via second messenger cascades [reviewed in Ribeiro *et al*., 2005]. These receptors fall into two broad classes: the Cys-loop ligand-gated ion channels and the metabotropic, heptahelical G-protein coupled receptors. Sequencing of the *S. mansoni* genome [Berriman *et al*., 2009; Protasio *et al*., 2012] has provided a large complement of putative neuroreceptors from both classes. Several have been cloned and characterized, including receptors for dopamine, histamine, glutamate and serotonin [El-Shehabi *et al*., 2012; El-Shehabi and Ribeiro, 2010; Taman and Ribeiro, 2011; Patocka *et al*., 2014]. Relatively less, however, is known about the cholinergic system of schistosomes.

Acetylcholine (ACh) is a quaternary amine neurotransmitter that elicits a variety of biological effects. In vertebrates, ACh acts as a primarily excitatory neurotransmitter and controls processes such as muscular contraction, glandular secretion and memory

formation [Wess *et al*., 2007]. ACh plays a similar excitatory role among invertebrates and its role in nematode motor function is very well characterized [Ribeiro *et al*., 2005]. A notable exception to the excitatory role of ACh occurs in schistosomes, where there is evidence of ACh acting as a major inhibitory neurotransmitter or modulator [Barker *et al*., 1966; Day *et al*., 1996]. Activation of ACh receptors (AChRs) in schistosomes manifests as muscular relaxation resulting in flaccid paralysis. The genome of *S. mansoni* contains several putative AChRs that may be responsible for this phenomenon [Berriman *et al*., 2009; Protasio *et al*., 2012]. The majority of these receptors are predicted to be nicotinic ion channels. However, a single full-length muscarinic acetylcholine receptor gene (SmGAR) is also predicted.

Muscarinic acetylcholine receptors (mAChRs) are members of the heptahelical G protein -coupled receptor superfamily and are structurally related to rhodopsin (Family A GPCRs). They mediate their effects by interaction with heterotrimeric G-proteins, causing changes in intracellular $Ca^{2+}$ or cyclic adenosine monophosphate (cAMP). The term “muscarinic” is derived from these receptors’ preferential binding and activation by the fungal toxin muscarine [Dale, 1914]. There are 5 subtypes of mAChRs in vertebrate organisms [reviewed in Eglen, 2012]. Vertebrate mAChRs are located in both the central and peripheral nervous systems and are involved in a vast array of physiological processes such as memory, smooth muscle contraction and regulation of neurotransmitter release. Invertebrate mAChRs, termed G-protein linked acetylcholine receptors (GARs), share this functional diversity with their vertebrate homologs. Three GAR subtypes have been identified in both parasitic and free-living nematodes [Lee *et al*., 1999; Hwang *et al*., 1999; Lee *et al*., 2000; Kimber *et al*., 2009]. Similar to vertebrate receptors, they may act in either an excitatory or inhibitory manner and are located on neurons contributing to several important nematode activities, such as muscular contraction, sensory perception and reproduction. Although structural similarity and broad expression patterns define the GARs and mAChRs as homologs, there are significant differences in the pharmacological profiles of vertebrate and invertebrate metabotropic acetylcholine receptors [Hannan and Hall, 1993]. This unique pharmacology, combined with their functional importance, marks the GARs as good targets for antiparasitics.

There are several tools available to examine the pharmacology and functional relevance of helminth mAChRs. As mentioned above, several schistosome GPCRs have been cloned and characterized, using a yeast-based pharmacological assay [El-Shehabi *et al*., 2012; Kimber *et al*., 2009]. Besides being amenable to high-throughput screening, another benefit of the yeast system is the ability to characterize constitutively active receptors by the screening of inverse agonists. Although once thought of as an artifact of heterologous protein expression, there is mounting evidence for the biological relevance of constitutively active muscarinic receptors in both normal and disease-states [Spalding and Burstein, 2006]. As no flatworm mAChR has ever been functionally expressed, it is important to allow for the possibility of a wild type, constitutively active schistosome GARs.

In addition to pharmacological characterization, it is important to interrogate receptor function at the behavioral level. In schistosomes, RNA interference (RNAi) screens have been especially successful in elucidating the role of neuroreceptors in motor function [Patocka *et al*., 2014; Zamanian *et al*., 2012]. Silencing of specific receptors with siRNA allows motor phenotypes to be observed without the variability introduced by the addition of exogenous drugs. It also allows for the high-throughput screening of entire neurotransmitter pathways. Used in combination with cell-based pharmacological assays, RNAi behavioral screens are powerful tools for assessing the function of novel parasite receptors.

In the present work, we describe the cloning and functional characterization of SmGAR, the only predicted G-protein coupled acetylcholine receptor in *S. mansoni*. SmGAR is structurally similar to *C. elegans* GAR-2 and its expression is predicted to be highly up-regulated during the early larval stages of the parasite [Berriman *et al*., 2009]. Functional analysis in the yeast expression system determined that SmGAR forms a constitutively active receptor with a muscarinic pharmacological profile. Furthermore, RNAi screens revealed that silencing of SmGAR causes a substantial reduction in larval motility, suggesting a probable role in early parasite migration.

## Materials and Methods

### *Parasites*

*Biomphalaria glabrata* snails infected with a Puerto Rican strain of *S. mansoni* were generously provided by Dr. Fred Lewis (Biomedical Research Institute and BEI Resources, MD, USA). Cercariae were obtained by exposing 6-8 week-old snails to bright light [Lewis, 2001] for 2 hours. Cercariae were then transformed into larval schistosomula *in vitro* by mechanical shearing [Lewis, 2001]. Schistosomula were washed with Opti-MEM containing antibiotics (100 $\mu$ g/ml streptomycin, 100 units/ml penicillin and Fungizone 0.25 $\mu$ g/ml) and cultured for 1-3 days in Opti-MEM (no antibiotics) supplemented with 6% fetal bovine serum at 37°C/5% $CO_2$ [Lewis, 2001]. Adult worms were recovered by portal perfusion [Lewis, 2001] from adult female CD1 mice 7 weeks post-infection with 250 freshly shed cercariae/mouse.

### *Full Length Cloning of SmGAR*

Total RNA was extracted from either pooled adult worms or 24-hour-old schistosomula using Trizol (Invitrogen) or the RNeasy Micro Kit (Qiagen), according to manufacturers' instructions. RNA was reverse-transcribed (RT) using MML-V and Oligo-dT (Invitrogen). A negative control RT reaction, lacking RNA template, was used to rule out the possibility of contamination of cDNA with genomic DNA. Primers to amplify the full length, predicted coding sequence of Smp_145540 (SmGAR) were designed using Oligo 6.2 [Rychlik, 2007]. Primer sequences were as follows: Forward 5'-ATGAATCTATTATTTTGTTTTC-3' and Reverse 5'-TTATAATCTTCTAAAATCACC-3'. A proofreading Phusion High Fidelity Polymerase (New England Biolabs) was used for PCR amplification according to standard protocols. Cycling conditions were as follows: 98°C/30s, 30 cycles of 98°C/10s, 54°C/60s, 72°C/60s and a final extension of 72°C/5min. All PCR products were ligated to the pJet1.2 Blunt cloning vector (Thermo Scientific) and verified by DNA sequencing of at least two independent clones.

### *Bioinformatics*

The predicted protein sequence of SmGAR (Smp_145540) was used as a query for a BLASTp search of the NCBI non-redundant protein dataset. Homologs were aligned with SmGAR using PROMALS3D [Pei *et al.*, 2008] and the resulting multiple sequence alignment was then inspected manually to ensure the correct alignment of

highly conserved Family A GPCR transmembrane (TM) motifs. Reference residues in these motifs are described by the Ballesteros and Weinstein numbering system [Ballasteros and Weinstein 1995]. Identification of TM regions was performed by TMHMMv2.0 [29] and comparison of SmGAR with the crystal structure of the human $\beta$ 2-adrenergic receptor (PDB: 2rh1). A neighbor-joining phylogenetic tree with 1000 bootstrap replicates was built from the multiple sequence alignment and visualized with FigTree 3.0 [Morariu *et al*., 2008]. Accession numbers of the sequences used in the alignment can be found in Table S1.

### *Yeast Expression*

Full-length SmGAR was ligated into a previously described yeast expression vector, Cp4258 [Kimber *et al*., 2009; Wang *et al*., 2006], using NcoI and BbsI restriction sites. The resulting construct (Cp4258-SmGAR) was confirmed by DNA sequencing and used to transform *Saccharomyces cervesiae* strain Cy13393 (*MAT* $\alpha$ $P_{FUS1}$*-HIS3 GPA1-G* $\alpha$*i2(5) can1 far1* $\Delta$*1442 his3 leu2 lys2 sst2* $\Delta$*2 ste14::trp1::LYS2 ste18* $\gamma$*6-3841 ste3* $\Delta$*1156 tbt1-1 trp1 ura3*); kindly provided by J. Broach, Penn State University). This strain expresses the *HIS3* gene under the control of the *FUS1* promoter [Wang *et al*., 2006] and also includes an integrated copy of a chimeric G$\alpha$ gene in which the first 31 and last 5 codons of the native yeast G$\alpha$ (GPA1) were replaced with those of human G$\alpha_{i2}$ subunit. Strains containing G$\alpha_q$ and G$\alpha_s$ were also tested but found to yield no receptor activity when compared to Cy13393. Yeast was cultured according to a previously established protocol [Kimber *et al*., 2009] until mid-log phase. Yeast (200 $\mu$l) were then transformed by the lithium acetate method using 200 $\mu$g of carrier DNA and 1 $\mu$g of Cp4258-SmGAR or empty plasmid as a negative control. Positive transformants were then selected on synthetic complete (SC) media containing 2% glucose media lacking leucine (SC/leu$^-$) and verified by diagnostic restriction enzyme digestion.

### *Yeast Receptor Activity Assays*

The principle of the receptor activity assay is based upon the protocols of Wang *et al.* [Wang *et al*., 2006] and has been previously described in helminths [El-Shehabi and Ribeiro, 2010; El-Shehabi *et al*., 2012; Kimber *et al*., 2009]. Briefly, single colonies

carrying the CP4258-SmGAR construct or empty plasmid (mock control) were grown in SC/leu$^-$ overnight at 30°C, 250 rpm. Cells were then washed 3 times in SC medium lacking leucine and histidine (SC/leu$^-$/his$^-$) and finally resuspended in SC/leu$^-$/his$^-$ supplemented with 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 6.8 and 1.5 mM 3-Amino-1, 2, 4-Triazole (3-AT). The addition of 3-AT reduces background signaling-induced basal yeast growth by inhibiting the gene product of *HIS3* [Stevenson *et al*., 1992]. Yeast cells were plated at a density of 3000 cells/well to a flat-bottom 96-well plate with either test agonist at the specified concentration, vehicle alone, or SC/leu$^-$/his$^+$ media at a final volume of 200 $\mu$l and incubated at 30$^\circ$ C for a period of 24-30 hours, after which 20 $\mu$l of Alamar Blue dye (Invitrogen) was added to each well. Plates were returned to 30$^\circ$ C incubator until Alamar Blue began to turn pink (2-4 hours) and fluorescence (560nm excitation/590 emission) was measured every 30 minutes for a total of 4 hours using a Synergy H4 microplate fluorometer (BioTek, USA). Baseline fluorescence values from cell-free wells were subtracted from test wells and fluorescence for each test group was normalized to water-treated control cells. All results are derived from at least three separate experiments each in 6 replicates. Statistical analysis and curve fitting was done using Prism v5.0 (GraphPad Software).

### *Synthesis of siRNA*

A unique 219 bp fragment of SmGAR sequence was identified using BLAST analysis and amplified using Phusion High Fidelity Polymerase (New England Biolabs). Amplification primers were designed using Oligo 6.2 [Rychlik, 2007] and are as follows: Forward 5-CGAAAACAACCAAACTTGGGG-3' and Reverse 5'-CGGTTTCTGGAACTTCATTTAAACG-3'. Products were ligated to pJET 1.2Blunt vector (Fermentas, USA) and verified by DNA sequencing. For synthesis of long double stranded RNA (dsRNA), a T7 promoter site (5'-TAATACGACTCACTATAGGGAGA-3') was added to each end of the target fragment by PCR. The T7-flanked target sequence was used as a template for *in vivo* transcription of both DNA strands by the MegaScript T7 Transcription Kit (Ambion) according to the manufacturer's instructions. The resulting dsRNA was digested by RNAseIII (Invitrogen) and purified using a Centricon YM-30 filter unit (Millipore) in order to generate a heterogeneous pool of specific siRNA. The

purity and concentration of pooled siRNA was assessed using a Nanodrop ND1000 spectrophotometer.

### *RNAi and Motility Assay*

Cercariae were shed from snails and transformed *in vitro* by the standard protocol (see above, [Lewis, 2001]) with a slight modification. Following the final wash step, parasites were resuspended in Opti-MEM containing no antibiotics or FBS and plated at a density of 100 animals/well in a 24-well culture plate. Transfection of schistosomula with SmGAR or non-relevant negative control siRNA was performed as previously described [Nabhan *et al*., 2007] in the presence of siPORT Neo FX Transfection Agent (Ambion, USA) at a final concentration of 50 nM. Animals were cultured for 24 hours and then assayed for motor phenotypes or harvested for quantitative PCR. The principle of the motility assay is based upon a previously established protocol described in [Patocka and Ribeiro, 2013]. Schistosomula were filmed for a period of 60 seconds using a Nikon SMZ1500 microscope equipped with a digital video camera (QICAM Fast 1394, mono 12 bit, QImaging) and SimplePCI version 5.2 (Compix Inc.) software. Parasite motility was then calculated using the Fit Ellipse function in the ImageJ software package (version 1.41, NIH, USA), as previously described [Patocka and Ribeiro, 2013]. Three distinct fields were recorded for each well and a minimum of 12 animals per treatment group were measured in each experiment. The data shown are the result of three independent experiments.

### *Quantitative PCR (qPCR)*

Total RNA was extracted from siRNA-treated schistosomula using the RNeasy Micro RNA Extraction Kit (Qiagen) as per manufacturer's instructions with the following modification. Animals were washed in 1X phosphate-buffered saline (PBS) and resuspended in the provided lysis buffer prior to sonication for 1 minute (6 pulses of 10s/each), as described in [Patocka and Ribeiro, 2013]. Total RNA was then extracted from the resulting lysate and assessed for quantity and purity using a Nanodrop ND 1000 spectrophotometer. RT reactions were performed as above, using 100 ng of RNA template per reaction. Primers to amplify a unique 150 bp fragment separate from the region used to generate the siRNA of SmGAR were designed using Oligo [Rychlik, 2007] and are as follows: Forward 5'-CAGCCTGTTTAACCTCCC-3' and Reverse 5'-

TTGAAGATAGGGTCCGTT-3'. Quantitative real-time PCR (qPCR) was performed using Platinum SYBR Green UDG SuperMix (Invitrogen) in a 25 $\mu$l reaction volume on a RotorGene RG3000 (Corbett Life Sciences, Australia). Cycling conditions were 50°C/2 min, 95°C/2 min, followed by 50 cycles of 94°C/15 s, 57°C/30 s, 72°C/15 s. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Accession # M92359) was used as an internal control and qPCR primers were: Forward 5'-GTTGATCTGACATGTAGGTTAG- 3' and Reverse 5'-ACTAATTTCACGAAGTTGTTG-3'. Relative gene expression was calculated using the Pfaffl's method [Pfaffl, 2001]. Results shown are derived from three separate experiments, each done in triplicate.

### *Homology Modeling*

Homology modeling of SmGAR was carried out using the UCSF Chimera Package (Computer Graphics Laboratory, University of California, San Francisco) [Pettersen *et al*., 2004] and Modeller v9.12 [38]. SmGAR was aligned with several GPCR crystal structures available in the general Protein Database (PDB) (Accession numbers 2rh1, 4daj, 1u19, 3eml) and the rat (*R. norvegicus*) M3 muscarinic receptor (4daj) was selected as the best template according to similarity scores. The alignment between SmGAR and M3 receptor was edited to remove areas of low structural resolution, including portions of the N-terminal and the third intracellular (i3) loop. Deletion of the i3 loop also removed the portion of the M3 structure containing the T4 lysozyme structure. The default automodel feature of Modeller v9.12 was used for subsequent modeling steps. The rat M3 structure (4daj) and resulting model of SmGAR were superimposed using the Matchmaker tool. Model accuracy was assessed by GA341 model score and RMSD [Melo *et al*., 2002].

## Results

### *SmGAR is a C. elegans GAR-2 homolog*

Smp_145540 (SmGAR) is the single putative full-length muscarinic GAR-like receptor annotated in the genome of *S. mansoni* [Berriman *et al*., 2009; Protasio *et al*., 2012]. The predicted coding sequence of SmGAR spans 3 exons and 1938 bp, encoding a 74 kDa protein. However, amplification of full-length SmGAR by PCR and sequencing of multiple clones resulted in a 2802 bp coding sequence, with a predicted

protein size of 106 kDa. This longer coding sequence matches the predicted genomic sequence of SmGAR and the extra sequence is located in the highly variable third intracellular loop (i3). The absence of amplification in the negative control PCR reactions lacking reverse transcriptase suggests that the SmGAR coding sequence does not contain any introns, similar to mammalian Family A GPCRs [Friedmans *et al*., 2007]. Furthermore, sequence analysis of SmGAR indicates that it lacks a 5' splice leader (SL) sequence and therefore the sequence does not appear to be trans-spliced. Analysis of the SmGAR transcript with TMHMM and SignalP indicate that it retains all features of a full-length, functional Class A GPCR, including the canonical 7TM regions.

In order to identify putative homologs, the sequence of SmGAR was used as a query for a BLASTp search of the NCBI non-redundant protein dataset and aligned with the resulting hits. SmGAR shared the highest homology with a putative GAR-2-like receptor from the trematode *Clonorchis sinensis* (42%) and previously characterized GAR-2 receptors from the nematodes *Caenorhabditis elegans* (44%) and the *Ascaris suum* GAR-1 (35%,). Although the *C. elegans* GAR shares a higher percent similarity with SmGAR, the *Clonorchis* receptor had higher coverage (96%), even across the highly divergent i3 loop. Putative homologs of SmGAR also appear in the genomes of the cestodes *Taenia solium* and *Echinococcus granulosus* [Huang *et al*., 2013; Tsai *et al*., 2013]. Phylogenetic analysis (Figure 1) shows that SmGAR is more closely related to the *C. elegans* GAR receptors than the human muscarinic receptors but despite the high similarity of SmGAR to its nematode homologs, the flatworm GARs form their own distinct clade. All members of this clade, including SmGAR, have longer amino acid sequences than their human and nematode counterparts.

### *SmGAR is a constitutively active acetylcholine receptor*

The activity of SmGAR was assessed using a previously described yeast functional assay [Wang *et al*., 2006; Kimber *et al*., 2009]. Briefly, a plasmid containing the coding sequence for SmGAR was transformed into *S. cervesiae* yeast that are auxotrophic for histidine. Activation of the heterologously expressed GPCR with the appropriate ligand allows for expression of the *HIS3* reporter gene, which is coupled to the yeast endogenous pheromone pathway and allows the yeast to grow in histidine-

deficient media. Receptor activity is then measured by yeast growth in selective media using the fluorometric redox indicator Alamar Blue (Invitrogen). Coupling to the correct guanosine nucleotide-binding protein (G protein) alpha subunit is important for receptor function. Therefore, yeast strains expressing different G$\alpha$ subunits were tested and a strain expressing inhibitory G$\alpha_i$ (CY13393) produced the strongest response when compared to mock-transfected controls. This suggests that SmGAR couples to G$\alpha_i$ and agrees with our prediction of SmGAR as a GAR-2 homolog. GAR-2 was previously described as a Gi/o-coupled receptor [Lee *et al*., 2000].

Further analysis of SmGAR revealed that the receptor has high basal activity when expressed in yeast, suggesting a propensity towards spontaneous activation. Once thought of as an artifact of heterologous expression, there is mounting evidence for the biological relevance of constitutively active GPCRs [Spalding and Burstein, 2006]. Moreover, constitutively active GPCRs often retain their ability to respond to agonists and signal above their elevated baseline [Burstein *et al*., 1998]. In order to test this, SmGAR expressing CY13393 were treated with a panel of cholinergic (acetylcholine, carbachol) and other neuroactive substances (tyramine, histamine, glutamate) at a concentration of 100 $\mu$M. The results show that SmGAR is activated by the cholinergic agonists but not the biogenic amines (tyramine and histamine) or glutamate (Figure 2A). Both acetylcholine and the classical muscarinic receptor agonist, carbachol, caused a moderate (1.5-2-fold) but statistically significant ($P<0.05$) activation of SmGAR when compared to the water-treated control cells. This activation was above the elevated baseline fluorescence caused by the constitutive activity of the receptor. When treated with varying concentrations of acetylcholine (Figure 2B), the SmGAR response was found to be dose-dependent with an $EC_{50}$ of 61.5 ±1.8 nM. The effect of carbachol was also dose-dependent but less potent ($EC_{50}$ = 23.6 $\pm$3.25 $\mu$M).

One way to characterize the pharmacology of constitutively active receptors is through the use of inverse agonists. Inverse agonists are compounds that inhibit the ligand-independent signaling of constitutively active GPCRs [de Ligt *et al*., 2000]. Often inverse agonists act as neutral antagonists on non-spontaneously activated receptors. We therefore decided to test several known muscarinic antagonists for inverse agonism

on SmGAR activity. Yeast cells expressing SmGAR were treated with varying concentrations of atropine, promethazine or pirenzipine in the absence of acetylcholine. Atropine and pirenzipine are classical muscarinic receptor antagonists. Promethazine has mixed antagonist activity towards muscarinic and H1 histamine receptors [Yanai, 2012] and is the most effective antagonist of the *Ascaris* GAR-1 receptor [Kimber *et al*., 2009]. The results of the functional assay show that both atropine (Figure 3A) and promethazine (Figure 3B) are able to decrease the high basal activity of SmGAR in a dose-dependent manner, with $IC_{50}$ of 14 $\pm$1.3 $\mu$M and 242 ±6.5 $\mu$M, respectively. In contrast, pirenzipine had no effect on receptor activity up to a concentration of 1 mM (Figure 3C). In order to confirm that inhibition was receptor-mediated and to control for drug-induced cytotoxicity, cells were plated in non-selective media (SC supplemented with histidine) containing the highest concentration of antagonist tested (1 mM). There was no inhibition of growth in these control wells or in the mock-transfected, antagonist-treated control cells.

### *SmGAR sequence analysis and homology modeling*

In order to examine whether SmGAR contained any unique sequence features that may explain its high basal activity, a structural alignment of SmGAR with mAChRs and GAR-like receptors from humans, nematodes and other Platyhelminthes was generated. A homology model of SmGAR was also generated, using the M3 muscarinic receptor (PDB# 4DAJ) as a template. The results of the homology modeling agree with the bioinformatics analysis of SmGAR. The hypothesized model of SmGAR contains 7TM domains that align very closely with the template receptor (Figure 4).

Although SmGAR contains all of the structural elements of a functional muscarinic receptor, our structural alignment reveals several amino acid substitutions that could impact on the activity of the receptor (Figure 5). The overall architecture of the SmGAR TM regions closely aligns with those of other mAChRs, including the highly conserved TM seed residues, which are identified by Ballesteros and Weinstein nomenclature [Ballasteros and Weinstein, 1995]. SmGAR also contains the highly conserved $Asp^{3.32}$ residue that is essential for binding the positively charged headgroup of acetylcholine [46] and other key residues of the orthosteric ACh-binding pocket,

including Tyr$^{3.33}$, Tyr$^{6.51}$ and Tyr$^{7.39}$ [Leach *et al*., 2012]. However, two substitutions are present in the predicted orthosteric binding site of SmGAR. In human mAChRs, Tyr$^{5.39}$ forms a direct bond with ACh and related compounds; mutation of this site leads to changes in agonist binding [Leach *et al*., 2012]. All invertebrate GARs in the structural alignment, including SmGAR, contain a substitution to Asn$^{5.39}$ at this position. The second difference in the orthosteric binding site of SmGAR occurs in TM6. Mutagenesis studies have shown that the residue in position 6.52 exerts some control over the binding affinity of mAChRs to atropine [Blüml *et al*., 1994]. In human mAChRs and the closely related *C. elegans* GAR-3, there is a highly conserved Asn at this position (Asn$^{6.52}$). However, the remaining *C. elegans* GARs contain a Tyr at position 6.52 and SmGAR and its flatworm homologs all substitute a His residue (His$^{6.52}$), as seen in Figure 5B. These differences may explain the varying sensitivities of human, nematode and flatworm receptors to atropine. The key residues of the allosteric binding sites of SmGAR appear to be highly conserved, including Tyr$^{3.28}$, Try$^{7.35}$ and aromatic residues in the second extracellular loop (e2) [Leach *et al*., 2012].

The substitutions present in the binding pockets of SmGAR may give some understanding of its pharmacology but these changes do not directly affect the signaling capabilities of the receptor. Given the high level of agonist-independent SmGAR signaling in the yeast assay, we hypothesized that SmGAR also contained amino acid substitutions that favored constitutive activity. We inspected the TM3/i2 loop interface and the cytoplasmic end of TM6, two regions known to influence receptor activation in GPCRs [Leach *et al.,* 2012]. Several substitutions are present at positions that have been shown to affect constitutive activity in vertebrate mAChRs [Burstein *et al*., 1998; Hogger *et al*., 1995]. Two of these substitutions are located in the proximity of residues forming the cytoplasmic ionic lock, at positions 3.52 (Figure 5A) and 6.30 (Figure 5B). At SmGAR 3.52, a Cys residue is substituted for a highly conserved Phe. In vertebrate mAChRs, the mutation of Phe$^{3.52}$ to Cys$^{3.52}$ causes constitutive activity, most likely due to destabilization of bonding interactions that favor the off-state of the receptor [Burstein *et al*., 1998]. In TM6, Ala$^{6.30}$ replaces a highly conserved Glu$^{6.30}$ residue of the vertebrate receptors [Leach *et al*., 2012]. The removal of this negative charge abolishes the

interhelical bonding necessary to form the cytoplasmic ionic lock, destabilizing the inactive conformation of the receptor.

### *SmGAR is plays a role in larval parasite motility*

The GAR-2 receptor from *C. elegans* has been localized to motor neurons and shown to play a role in worm locomotion [Dittman and Kaplan, 2008]. Here, we utilized an RNAi behavioral assay to determine whether SmGAR functions in a similar manner. The expression of SmGAR is predicted to be highly up-regulated during the first 24 hours after cercarial transformation into schistosomula [Berriman *et al*., 2009; Protasio *et al*., 2012]. Therefore, freshly transformed schistosomula were treated with a pool of heterogeneous SmGAR-specific siRNA and parasite motility was measured 24 hours post-transfection. Animals treated with nonsense scrambled siRNA were also included as a negative control. Suppression of SmGAR expression resulted in a significant ($p < 0.01$) 70% reduction in the motility of siRNA-treated parasites when compared to the negative control (Figure 5A). This hypoactive phenotype agrees with the RNAi data from *C. elegans* [Dittman and Kaplan, 2008] and suggests that SmGAR may act as a stimulator of larval motor function. Silencing of SmGAR at the mRNA level was confirmed by qPCR and the results are shown in Figure 5B. Treatment with gene-specific siRNA completely silenced the expression of SmGAR at the transcript level. However, there was no change in the expression of an unrelated control gene, SmACC-1 (Accession KF694748), indicating that suppression of SmGAR was specific.

## Discussion

Cholinergic neurotransmission is a key modulatory pathway in both nematode and flatworm motor behavior [Maule *et al*., 2005]. In nematodes, acetylcholine (ACh) acts in a predominantly excitatory manner, whereas in schistosomes ACh is a major inhibitory neuromodulator [Ribeiro *et al*., 2005]. Despite these opposing reactions, the effects of ACh in both types of worm are mediated through two types of receptors-ionotropic nicotinic acetylcholine receptors (nAChRs) and the metabotropic muscarinic receptors (mAChRs). Fast cholinergic neurotransmission is mediated by nAChRs, which are members of the Cys-loop superfamily of ligand-gated ion channels. They may be either cation or anion-selective and are expressed both neuronally and directly on muscle. Due to their importance as antiparasitic drug targets, several nematode

nAChRs have been cloned and pharmacologically characterized (reviewed in [Holden-Dye *et al*., 2013]). More recently, a nicotinic chloride channel in *S. mansoni* was also cloned and characterized [MacDonald *et al*., 2014].

In comparison to the nAChRs, relatively little is known about the structure and function of metabotropic acetylcholine receptors, termed GAR receptors, in worms. GAR receptors belong to the Class A G-protein-coupled receptor family (GPCR) and are homologs of the muscarinic acetylcholine receptors (mAChRs) in humans. GARs control a variety of processes in nematodes including the modulation of sensory perception, locomotion and reproductive behaviors. Three GAR receptor subtypes have been identified in both free-living and parasitic nematodes [Lee *et al*., 1999; Hwang *et al*., 1999; Lee *et al*., 2000; Kimber *et al*., 2009] and similar to vertebrate mAChRs, GAR receptors may behave in either an excitatory or inhibitory manner. It is important to note that while the GARs are activated by acetylcholine, they display a divergent pharmacological profile to the human mAChRs. This unique pharmacology and the control exerted over motor function by cholinergic signaling make GAR homologs found in parasitic worms potential therapeutic targets.

Previously, a GAR-1 homolog was identified and characterized in the parasitic nematode *A. suum* [Kimber *et al*., 2009]. Yet, there is little information available about the function of muscarinic receptors in parasitic trematodes. Pharmacological studies carried out on the free-living flatworms *Dugesia* do suggest the involvement of muscarinic receptors in the control of motor function [Butarelli *et al*., 2000]. Furthermore, the annotation of the *S. mansoni* genome [Berriman *et al*., 2009; Protasio *et al*., 2012] predicts one full-length putative muscarinic receptor. Here, report the cloning and functional characterization of this receptor, a *C. elegans* GAR-2 homolog, which we have termed SmGAR.

The results of our bioinformatics analysis indicate that SmGAR shares a high level of homology (44% similarity) with the *C. elegans* GAR-2 receptor. As far as gene structure, however, SmGAR appears to diverge from nematode GARs. Whereas all nematode GARs contain intronic regions, the coding sequence of SmGAR is intronless and consequently, SmGAR is unable to form multiple receptor isoforms. This feature

allies the gene structure of SmGAR more closely to vertebrate mAChRs [Friedmans *et al*., 2007]. The divergence of SmGAR from the nematode GAR receptors is further highlighted by the identification of several close homologs in the recently published genomes of fellow Platyhelminthes [Huang *et al*., 2013; Tsai *et al*., 2013]. A phylogenetic tree generated from the alignment of SmGAR and its homologs with vertebrate and nematode receptors shows the formation of a separate clade of flatworm GAR-like receptors. The coding sequence length of these flatworm GARs is significantly longer than those of nematodes or vertebrates. However, the functional relevance of this increased protein length remains unclear.

In order to probe whether SmGAR possesses similar or divergent pharmacology to those of nematodes, a yeast functional assay was utilized. Briefly, SmGAR cDNA was transformed into a modified yeast strain and treated with several neurotransmitters. Activation of the receptor (SmGAR) results in an increase in yeast growth, which then may be measured by a fluorometric assay. This strategy has been utilized to deorphanize several GPCRs in both nematodes [Kimber *et al*., 2009] and schistosomes [El-Shehabi *et al*., 2012; El-Shehabi and Ribeiro, 2010; Patocka *et al*., 2014]. The results of the functional assay demonstrate that SmGAR is selectively activated by cholinergic agonists but not other biogenic amines or glutamate. Treatment with varying concentrations of both acetylcholine and the non-selective muscarinic agonist carbachol resulted in a dose-dependent growth of SmGAR-expressing cells. The $EC_{50}$ calculated for acetylcholine (61 nM) and carbachol (23 $\mu$M) agree with the trend of acetylcholine acting as a more potent agonist than carbachol in both *C. elegans* GAR-2 [Lee *et al*., 2000] and the *Ascaris* AsGAR [Kimber *et al*., 2009].

In addition to being activated by the addition of exogenous cholinergic agonists, SmGAR-expressing yeast cells exhibited a high level of background growth when compared to cells transformed with empty vector. This growth in the absence of ligand indicates that SmGAR is acting in a constitutively active manner. There are several causes for the constitutive activation of GPCRs, including receptor or effector protein overexpression and conformational changes affecting G-protein coupling [Spalding and Burstein, 2006]. Although ligand-independent activity was once thought of as an artifact

of heterologous expression, there is now mounting evidence for the importance of *in vivo* constitutively active GPCRs in many biological functions [de Ligt *et al*., 2000]. Therefore, we decided to interrogate the constitutive activity of SmGAR more closely by studying its response to known inverse agonists.

Inverse agonists are compounds that display a negative intrinsic activity- they are capable of reducing the basal activity of a receptor in the absence of activating ligand [Bond and Ijzerman, 2006]. Due to their ability to modulate the ligand-independent activity of receptors, they are increasingly recognized as an important tool in drug discovery. Often compounds previously characterized as receptor antagonists will display inverse agonism when tested on constitutively active receptors [Kenakin, 2004]. Therefore, we tested well-known muscarinic antagonists atropine and pirenzipine, as well as the partial muscarinic antagonist promethazine on the basal activity of SmGAR. The results show that atropine and promethazine are able to reduce constitutive activity and SmGAR does not respond to pirenzepine. The response of SmGAR to both atropine and promethazine but not pirenzipine is similar to results of antagonist studies performed on AsGAR [Kimber *et al*., 2009] and suggests the possibility of similar pharmacological profiles for parasite GAR receptors. If true, this would make parasite GAR receptors attractive targets for broad-spectrum antiparasitics. Given the high-throughput nature of the yeast assay system, the screening of large compound libraries to more fully characterize the pharmacology of SmGAR merits further investigation.

Conformational changes affecting G-protein coupling are one of the known causes of GPCR constitutive activity [Leach *et al*., 2012]. Many times, these changes are the result of amino acid substitutions that affect inter- and intra-helical bonding interactions. A particularly important conformational change inducing constitutive activity occurs with the disruption of the ionic lock, a salt-bridge formed between residues in TM3 and TM6 [Vogel *et al*., 2008]. The results of aligning SmGAR with several worm and vertebrate muscarinic receptors indicate that SmGAR contains several substitutions that may prevent the formation of the ionic lock and cause constitutive activity. In particular, SmGAR contains a Cys at position 3.52 instead of a highly conserved Phe and an Ala at position 6.30 in place of a key Glu residue. Mutation at these positions has

been shown to cause constitutive activation in human mAChRs [Burstein *et al*., 1998; Hogger *et al*., 1995], most likely due to the destabilization of the inactive form of the receptor by the prevention of ionic lock formation. Interestingly, the GAR-1 receptor of *Ascaris* contains a substitution at one of these positions (3.52) and displays a significant level of constitutive activity in the yeast expression system [Kimber *et al*., 2009]. Also, all invertebrate sequences analyzed contain the $Glu^{6.30}$ to $Ala^{6.30}$ substitution. This conserved substitution may suggest a family of constitutively active muscarinic receptors in invertebrates or point to a fundamental difference in G-protein coupling between invertebrates and vertebrates.

Although yeast functional assays generated information about the pharmacological profile of SmGAR, they provide limited insight as to whether the receptor is involved in parasite motor function. Behavioral assays utilizing the addition of neuroactive drugs onto parasites may yield some information about receptor function but have several drawbacks, including toxicity and off-target activity. A better alternative is suppression of target receptors using RNA interference (RNAi), which allows for the silencing of a specific receptor and the measurement of baseline motor activity without the addition of exogenous compounds. This strategy has been particularly successful in elucidating the functional roles of several receptors in both free-living and parasitic flatworms [Patocka *et al*., 2014; Zamanian *et al*., 2012; Reddien *et al*., 2005]. Here, we modified a previously developed RNAi behavioral assay [Patocka and Ribeiro, 2013] to assess the role of SmGAR in larval schistosomula. Transcriptional profiling of SmGAR indicates that it is most highly expressed in cercariae and first 24 hours of the schistosomula stage [Berriman *et al*., 2009; Protasio *et al*., 2012]. Therefore, transfected schistosomula were assayed for motility within the first 24 hours of transformation. Treatment of schistosomula with SmGAR siRNA caused a hypoactive motor phenotype (70% reduction in body wall contractions) when compared to control animals treated with non-relevant scrambled siRNA.

The hypoactive phenotype resulting from the silencing of SmGAR was surprising, given the hypothesized inhibitory role of ACh receptors in schistosomes. Removal of a direct inhibitor of muscular contraction would most likely lead to an increase in motility.

The decrease in motility observed therefore suggests that SmGAR acts indirectly in the modulation of neuromuscular function rather than directly affecting muscular contraction; a role similar to the regulatory presynaptic M2 receptors of vertebrates [Bellingham and Berger, 1996]. However, further studies are needed to elucidate the exact mechanism by which SmGAR regulates schistosome larval motor function. The reduction in motility caused by silencing SmGAR is also in agreement with evidence linking nematode GARs to the regulation of motor function. In *C. elegans,* suppression of GAR-1 leads to a sluggish, uncoordinated motor phenotype [Keating *et al*., 2003]. GAR-2 has been implicated as a presynaptic regulator in a negative feedback loop controlling worm locomotion [Dittman and Kaplan, 2008]. The results of our RNAi screen suggest that SmGAR may play a similar role in controlling motility during cercarial invasion and the early schistosomula-stage. The generation of a specific SmGAR antibody for use in future immunolocalization experiments would aid in this investigation by determining if SmGAR has the same central nervous system (CNS) expression pattern as GAR-2 [Lee *et al*., 2000; Dittman and Kaplan, 2008].

In the present work, we have described the cloning and functional characterization of a new muscarinic receptor in *S. mansoni*. The results of our functional characterization indicate that SmGAR is a constitutively active muscarinic acetylcholine receptor. It possesses high structural homology to the GAR receptors in *C. elegans* and RNAi experiments indicate that it functions in a similar manner, controlling larval motor behavior. Given these unique qualities and the importance of motor function to schistosome larval survival, we believe that SmGAR merits further investigation as a novel antischistosomal drug target.

## References


1. Ballasteros J, Weinstein H (1995). Integrated methods for the construction of three dimensional models and computational probing of structure-function relations in G-protein coupled receptors. Methods in Neurosciences **25**: 366-428.
2. Barker, LR, Bueding E, Timms AR (1966). The possible role of acetylcholine in *Schistosoma mansoni*. Brit J Pharmacol **26**: 656-665.

3. Bellingham MC, Berger AJ (1996). Presynaptic depression of excitatory synaptic inputs to rat hypoglossal motoneurons by muscarinic M2 receptors. J Neurophysiol **76**(6): 3758-3770.
4. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, et al. (2009). The genome of the blood fluke *Schistosoma mansoni*. Nature **460**: 352–358.
5. Bond RA, Ijzerman AP (2006). Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. Trends in Pharmacol Sci **27**(2): 92-6.
6. Burstein ES, Spalding TA, Brann MR (1998). The second intracellular loop of the m5 muscarinic receptor is the switch which enables G-protein coupling. J Biol Chem **273**: 24322-24327.
7. Butarelli FR, Pontieri FE, Margotta V, Palladini G (2000). Acetylcholine/dopamine interaction in planaria. Comp Biochem Physiol C Toxicol Pharmacol **125**(2): 225-31.
8. Blüml K, Mutschler E, Wess J (1994). Functional role in ligand binding and receptor activation of an asparagine residue present in the sixth transmembrane domain of all muscarinic acetylcholine receptors. J Biol Chem **269**(29): 18870-6.
9. Dale HH (1914). The action of certain esters and ethers of choline, and their relation to muscarine. J Pharmacol Exp Ther **6**:147–190.
10. Day TA, Chen GZ, Miller C, Tian M, Bennett JL, Pax RA (1996). Cholinergic inhibition of muscle fibers isolated from *Schistosoma mansoni* (Trematoda: Digenea). Parasitology **113** (Pt. 1): 55-61.
11. de Ligt RA, Kourounakis AP, IJzerman AP (2000). Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery. Br J Pharmacol **130**(1): 1-12.
12. Dittman JS, Kaplan JM (2008). Behavioral impact of neurotransmitter-activated G-protein-coupled receptors: muscarinic and GABAB receptors regulate Caenorhabditis elegans locomotion. J Neurosci **28**(28): 7104-14.
13. Eglen RM (2012). Overview of muscarinic receptor subtypes. Handb Exp Pharmacol **208**: 3-28.

14. El-Shehabi F, Ribeiro P (2010). Histamine signaling in *Schistosoma mansoni*: Immunolocalization and characterization of a new histamine-responsive receptor (SmGPR-2). Int J Parasitol **40**: 1395-1406.
15. El-Shehabi F, Taman A, Moali LS, El-Sakkary N, Ribeiro P (2012). A novel G protein-coupled receptor of *Schistosoma mansoni* (SmGPR-3) is activated by dopamine and is widely expressed in the nervous system. PLoS Negl Trop Dis **6**(2): e1523.
16. Eswar N, Marti-Renom MA, Webb B, Madhusudhan MS, Eramian D, *et al*. (2006). Comparative Protein Structure Modeling With MODELLER. Current Protocols in Bioinformatics **S15**: 5.6.1-5.6.30.
17. Friedmans D, Fredriksson R, Kappa I, Sciöth HB, Klovins J (2007). Formation of new genes explains lower intron density in mammalian Rhodopsin G protein-coupled receptors. Mol Phylogenet Evol **43**(3): 864-80.
18. Gryseels B, Polman K, Clerinx, J, Kestens L, (2006). Human Schistosomiasis. Lancet **368**(9541): 1106-1118.
19. Hannan F, Hall LM (1993). Muscarinic acetylcholine receptors in invertebrates: comparisons with homologous receptors from vertebrates. EXS **63**: 98-145.
20. Hogger P, Shockley MS, Lameh J, Sadee W (1995). Activating and inactivating mutations in N- and C-terminal i3 loop junctions of muscarinic acetylcholine HM1 receptors. J Biol Chem **270**: 7405–7410.
21. Holden-Dye L, Joyner M, O'Connor V, Walker RJ (2013). Nicotinic acetylcholine receptors: A comparison of the nAChRs of *Caenorhabditis elegans* and parasitic nematodes. Parasitol Int **62**(6): 606-15.
22. Huang Y, Chen W, Wang X, Liu H, Chen Y, *et al* (2013). The carcinogenic liver fluke, *Clonorchis sinensis*: new assembly, reannotation and analysis of the genome and characterization of tissue transcriptomes**.** PLOS One **8**(1): e54732.
23. Hwang JM, Chang DJ, Kim US, Lee YS, Park YS, *et al*. (1999). Cloning and functional characterization of a *Caenorhabditis elegans* muscarinic acetylcholine receptor. Receptors Channels **6**: 415–424.

24. Keating CD, Kriek N, Daniels M, Ashcroft NR, Hopper NA, *et al.* (2003). Whole-genome analysis of 60 G protein-coupled receptors in Caenorhabditis elegans by gene knockout with RNAi. Current Biol **13**(19): 1715-20.
25. Kenakin, T. (2004). Efficacy as a vector: the relative prevalence and paucity of inverse agonism. Mol. Pharmacol. **65**: 2–11.
26. Kimber MJ, Sayegh L, El-Shehabi S, Song C, Zamanian M *et al*. (2009). Identification of an *Ascaris* G protein-coupled acetylcholine receptor with atypical muscarinic pharmacology. Int J Parasitol **39**(11): 1215-22.
27. Krogh B, Larssen B, von Heijne G, Sonnehammer ELL (2001). Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. J Molec Biol **305**(3): 567-580.
28. Leach K, Simms J, Sexton PM, Christopoulos A (2012). Structure-function studies of muscarinic acetylcholine receptors. Handb Exp Pharmacol **208**: 29-48.
29. Lee YS, Park YS, Chang DJ, Hwang JM, Min CK, *et al*. (1999). Cloning and expression of a G protein-linked acetylcholine receptor from *Caenorhabditis elegans*. J. Neurochem **72**: 58–65.
30. Lee YS, Park YS, Nam S, Suh SJ, Lee J, *et al*. (2000). Characterization of GAR-2, a novel G protein-linked acetylcholine receptor from *Caenorhabditis elegans*. J Neurochem **75**: 1800–1809.
31. Lewis F. (2001). Schistosomiasis. Current Protocols in Immunology **28**: 19.1.1–19.1.28.
32. MacDonald K, Buxton SK, Day TA, Kimber MJ, Ribeiro P (2013). Functional Characterization of a Novel Family of Acetylcholine-gated Chloride Channels in *Schistosoma mansoni*. Submitted.
33. Maule AG, Day TA, Chappell CH (2005). Parasite neuromusculature and its utility as a drug target. Parasitology **131**: S1-S2.
34. Melo F, Sanchez R, Sali A (2002). Statistical potentials for fold assessment. Protein Sci **11**: 430-448.
35. Morariu VI, Srinivasan BV, Raykar VC, Duraiswami R, Davis LS (2008) Automatic online tuning for fast Gaussian summation**.** Advances in Neural Information Processing Systems (NIPS) 2007. Available:

http://books.nips.cc/papers/files/nips21/NIPS2008_0257.pdf. Accessed 13 October 2013.

36. Nabhan JF, El-Shehabi F, Patocka N, Ribeiro P (2007). The 26S proteasome in Schistosoma mansoni: bioinformatics analysis, developmental expression, and RNA interference (RNAi) studies. Exp Parasitol **117**(3): 337-47.
37. Nordhal Peterson T, Brunak S, von Heijne G, Nielsen H (2011). SignalP 4.0: discriminating signal peptides from transmembrane regions. Nature Methods **8**: 785-6.
38. Patocka N and Ribeiro P (2013). The functional role of a serotonin transporter in *Schistosoma mansoni* elucidated through immunolocalization and RNA interference (RNAi). Mol Biochem Parasitol **187**: 32-42.
39. Patocka N, Sharma N, Rashid M, Ribeiro P (2014). Serotonin Signaling in *Schistosoma mansoni*: A Serotonin–Activated G Protein-Coupled Receptor Controls Parasite Movement. PLoS Pathog **10**(1): e1003878.
40. Pei J, Kim BH, Grishin NV (2008). PROMALS3D: a tool for multiple sequence and structure alignment. Nucleic Acids Res **36**(7): 2295-2300.
41. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, *et al*. (2004). UCSF Chimera - A Visualization System for Exploratory Research and Analysis. J Comput Chem **25**: 1605-1612.
42. Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **29**(9): e45.
43. Protasio AV, Tsai IJ, Babbage A, Nichol S, Hunt M (2012). A systematically improved high quality genome and transcriptome of the human blood fluke *Schistosoma mansoni*. PLoS Negl Trop Dis **6**(1): e1455.
44. Reddien PW, Bermange AL, Murfitt KJ, Jennings JR, Sánchez Alvarado A (2005). Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. Dev Cell **8**(5): 635-49.
45. Ribeiro P, El-Shehabi F, Patocka N (2005). Classical transmitters and their receptors in flatworms. Parasitology **131**: S19-S40.
46. Roth B, Kristiansen K (2004). Molecular mechanisms of ligand binding, signaling and regulation within the superfamily of G protein-coupled receptors: molecular

modeling and mutagenesis approaches to receptor structure and function. Pharmacology and Therapeutics **103**: 21–80.

47. Rychlik W (2007). OLIGO 7 Primer Analysis Software. In: Yuryev A, editor. Methods in Molecular Biology Vol. 402: PCR Primer Design. Totowa: Humana Press. pp 35-59.
48. Sabah AA, Fletcher C, Webbe G, Doenhoff J (1986). *Schistosoma mansoni*: chemotherapy of infections of different ages. Exp Parasitol **61**: 294–303.
49. Spalding TA, Burstein ES (2006). Constitutive activity in muscarinic acetylcholine receptors. J Recept Signal Transduction **26**: 61-85.
50. Stevenson BJ, Rhodes N, Errede B, Sprague Jr. GF (1992). Constitutive mutants of the protein kinase STE11 activate the yeast pheromone response pathway in the absence of the G protein. Genes Dev **6**: 1293–1304.
51. Taman A, Ribeiro P (2011). Glutamate-mediated signaling in *Schistosoma mansoni*: a novel glutamate receptor is expressed in neurons and the female reproductive tract. Mol Biochem Parasitol **176**(1): 42-50.
52. Tsai IJ, Zarowiecki M, Holroyd N, Garciarrubio A, Sanchez-Flores A, *et al*. (2013). The genomes of four tapeworm species reveal adaptations to parasitism. Nature **496**(7443): 57-63.
53. Vogel R, Mahalingam M, Lüdeke S, Huber T, Siebert F, *et al*. (2008). Functional role of the "ionic lock"-an interhelical hydrogen-bond network in family A heptahelical receptors J Mol Biol **4**: 648-55.
54. Wang ZX, Broach JR, Peiper SC (2006). Functional expression of CXCR4 in *Saccharomyces cerevisiae* in the development of powerful tools for the pharmacological characterization of CXCR4. In: Ali H, Haribabu B (Eds.), Methods in Molecular Biology, vol. 332: Transmembrane Signaling Protocols, Second Edition. Humana Press Inc., Totowa, NJ, pp. 115–127.
55. Wang W, Wang L, Liang YS (2012). Susceptibility or resistance of praziquantel in human schistosomiasis: a review. Parasitol Res **111**(5): 1871-7.
56. Wess J, Eglen RM, Gautam D (2007). Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. Nat Rev Drug Discov **6**: 721–733.

57. Willson J, Amliwala K, Harder A, Holden-Dye L, Walker RJ (2003). The effect of the anthelminthic emodepside at the neuromuscular junction of the parasitic nematode *Ascaris suum*. Parasitology **126**: 79-86.
58. Yanai K (2012). Anticholinergic activity of antihistamines. Clin Neurophysiol **123**(4): 633-4.
59. Zamanian M, Agbedanu PN, Wheeler NJ, McVeigh P, Kimber MJ, et al. (2012). Novel RNAi-Mediated Approach to G Protein-Coupled Receptor Deorphanization: Proof of Principle and Characterization of a Planarian 5-HT Receptor. PLoS ONE **7**(7): e40787.

## Figures

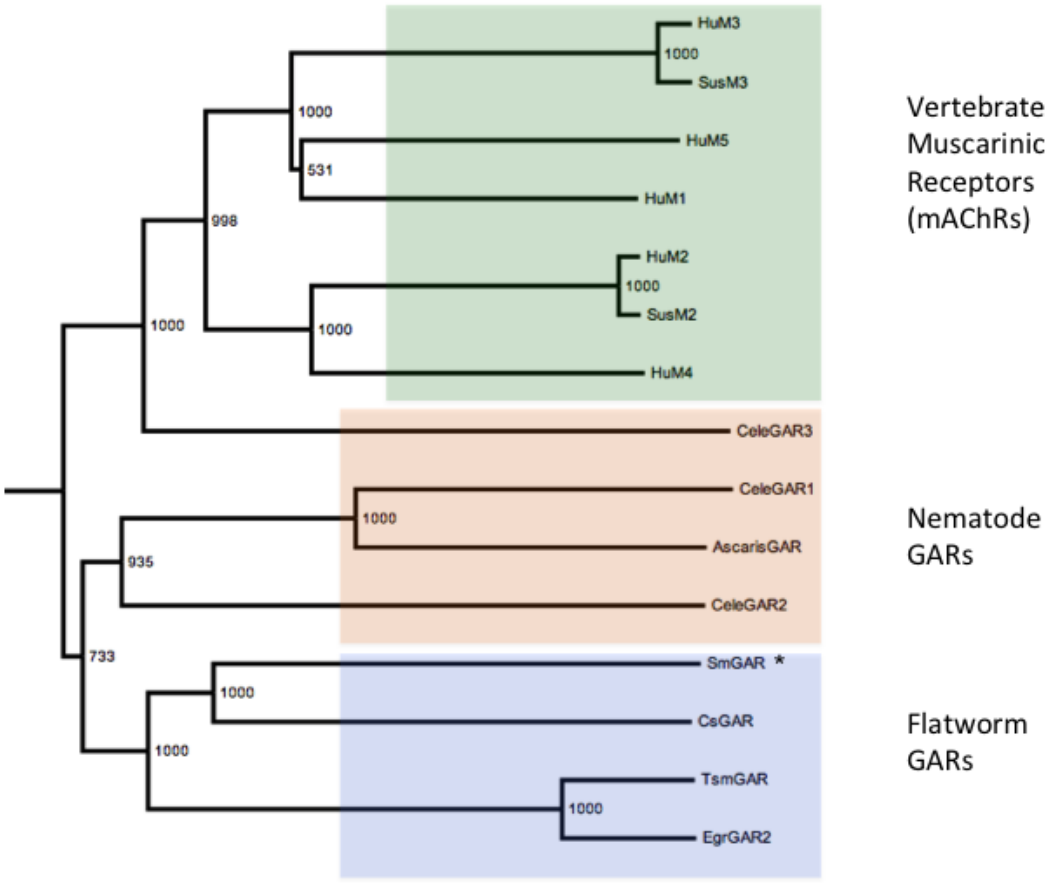


**Figure 1. SmGAR and its homologs form a flatworm-specific clade of GAR-like receptors.** A bootstrapped, neighbor-joined phylogenetic tree was generated from a

PROMALS3D structural alignment of SmGAR and its putative homologs from vertebrates, nematodes and Platyhelminthes (see Table S1 for accession numbers of aligned sequences). The tree is outgroup-rooted to the human 5-HT2 receptor (NCBI accession P28223) and was visualized using FigTree v3.0. Two larger groupings of receptors can be seen. The vertebrate muscarinic acetylcholine receptors (mAChRs) (green box) separate into their canonical subtypes, M1/M3/M5 (top) and M2/M4 (bottom) and also include the closely related *C. elegans* GAR-3 receptor. The remaining invertebrate G-protein-linked acetylcholine receptors (GARs), however, show a further division into the nematode GARs (orange box) and the flatworm GARs (blue box), including SmGAR (starred).

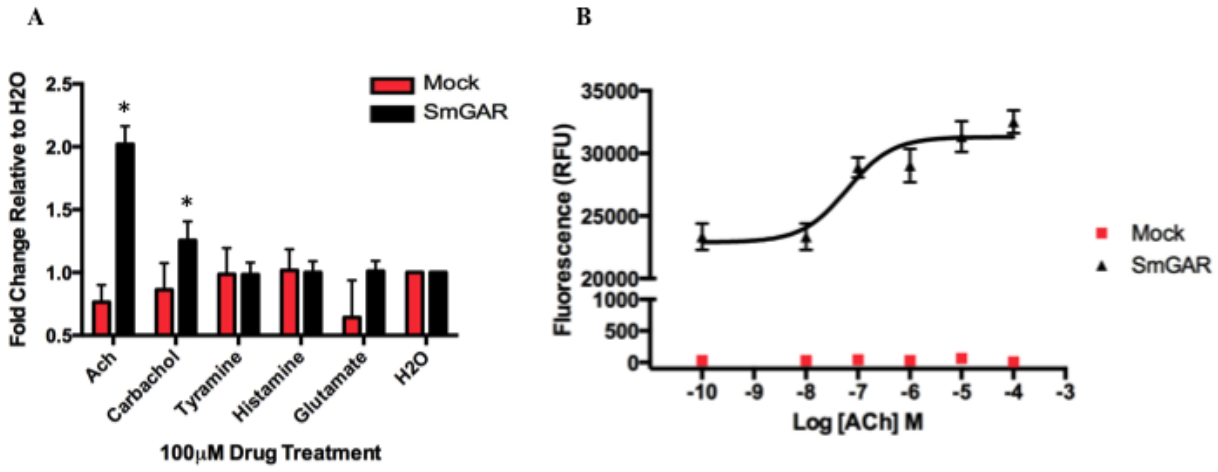


**Figure 2. SmGAR forms a constitutively active receptor that responds selectively to cholinergic compounds.** (A) SmGAR is activated by $10^{-4}$ M acetylcholine (ACh) or $10^{-4}$ M carbachol but not biogenic amines (tyramine, histamine) or glutamate, all at $10^{-4}$ M. Receptor activation was defined as a fold-change in fluorescence compared to water-treated control cells. Cells transformed with empty vector (mock control) showed no significant activation in response to any of the compounds tested. Data are expressed as mean fold-change ±SEM calculated from three separate experiments, each with six replicates. (B) ACh activates SmGAR in a concentration-dependent manner with an $EC_{50}$ = 61 ±1.8 nM and 23.6 ±3.25 $\mu$ M, respectively. There is no growth in the mock-transfected cells at any concentration of

drug added. It is also important to note the high level of agonist-independent growth in SmGAR-expressing cells when compared to the mock control. This high baseline receptor activity is indicative of a constitutively active receptor.

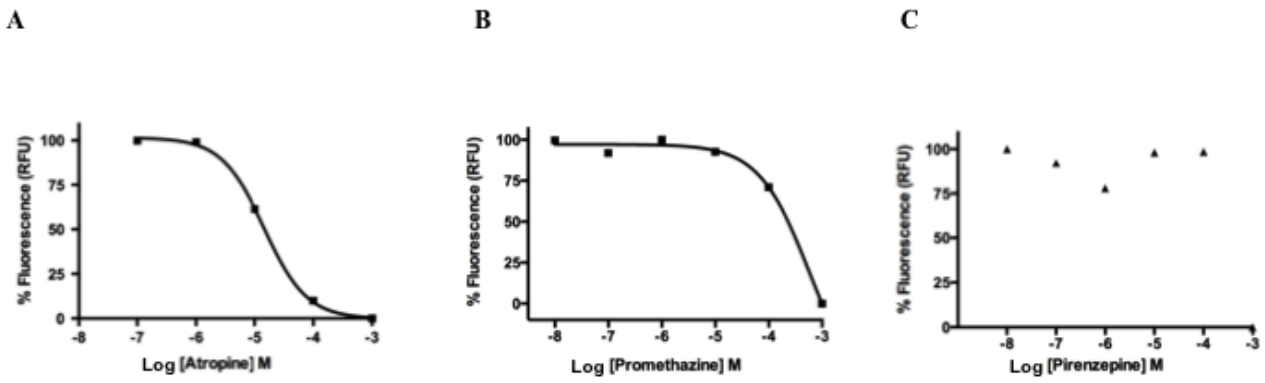


**Figure 3. Atropine and promethazine act as inverse agonists on SmGAR constitutive activity.** SmGAR-expressing yeast were treated with varying concentrations of several anticholinergic compounds (atropine, promethazine, pirenzepine) in order to assay for inverse agonism. (A) The muscarinic agonist atropine was the most potent inverse agonist of SmGAR and reduced the agonist-independent activity in a dose-dependent manner, with an $EC_{50}$ = 14 $\pm$1.3 $\mu$M. (B) The partial muscarinic antagonist promethazine acts as a concentration-dependent inverse agonist of SmGAR, with a calculated $EC_{50}$ = 242 ±6.5 $\mu$M. (C) The M1-selective muscarinic antagonist pirenzepine failed to reduce the constitutive activity of SmGAR at any concentration. Positive control samples containing 1mM of each antagonist in non-selective SC media ($His^+$) and either mock or SmGAR-transformed cells grew normally and indicate the absence of toxicity from the compounds tested.

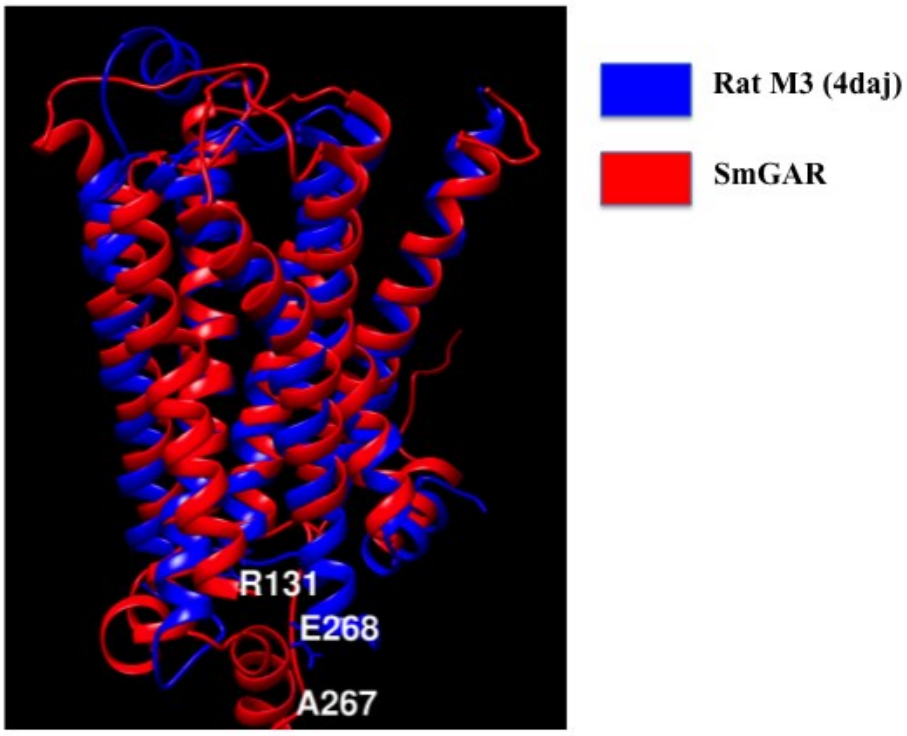


**Figure 4. Homology modeling of SmGAR.** The protein sequence of SmGAR was aligned with the crystal structures of several GPCRs. The highest scoring structure was the rat M3 muscarinic receptor/T4 lysozyme fusion protein (PDB: 4daj), which was subsequently used as a structural template for homology modeling, as described in the Methods. The proposed model of SmGAR (red) was then superimposed over the M3 fusion protein template (blue) and the structures compared. The results show that SmGAR's overall architecture is conserved with other rhodopsin-like GPCRs. SmGAR contains 7 transmembrane domains (TM) and all highly conserved class A GPCR TM domain seed residues are conserved, including Arg131$^{3.50}$ (shown). However, there is also evidence of conformational change-inducing amino acid substitutions present in SmGAR, including Ala267$^{6.30}$, which replaces a highly conserved glutamate (Glu268$^{6.30}$) of the rat receptor (shown). This substitution has been shown in vertebrate mAChRs to break the cytoplasmic ionic lock and induce constitutive activity.

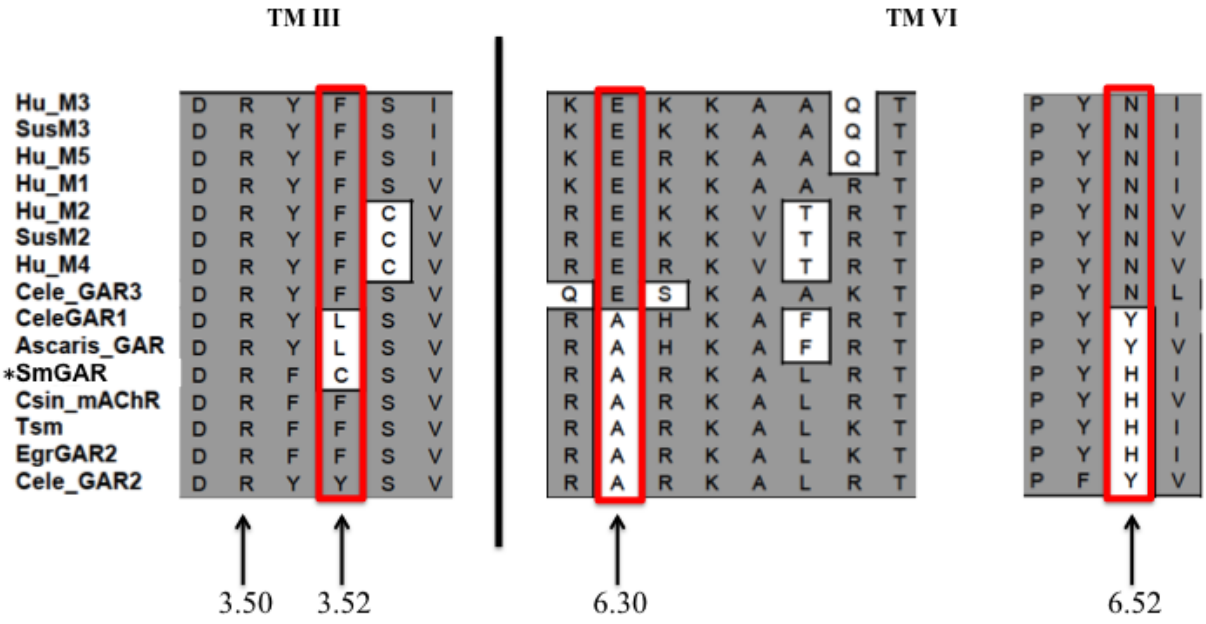


**Figure 5. Sequence analysis of SmGAR identifies several amino acid substitutions that may contribute to constitutive activity.** The structural alignment of muscarinic receptors used to generate the dendrogram comparing SmGAR to vertebrate and invertebrate muscarinic receptors (Figure 1) was examined manually to identify amino acid substitutions that may contribute to the receptor's constitutive activity. (A) Consistent with all class A GPCRs, the third transmembrane domain (TMIII) of SmGAR (starred) contains the highly conserved Asp$^{3.50}$, a key residue for the formation of the cytoplasmic ionic lock. However, SmGAR and two of its nematode homologs (*C. elegans* GAR-1 and *A. suum* GAR-1) substitute the conserved Phe$^{3.52}$ position for a Cys$^{3.52}$ and Lys$^{3.52}$, as identified by the red box. Removal of a hydrophobic amino acid at position 3.52 has been demonstrated to cause constitutive activity in human mAChRs, possibly by structural changes to the G-protein binding pocket or the disruption of ionic lock formation. (B) The sixth TM region (TMVI) of SmGAR contains amino acid substitutions that may contribute to its constitutive activity and unique pharmacology. All invertebrate GAR receptors in the alignment, with the exception of GAR-3 substitute an Ala$^{6.30}$ for the vertebrate conserved Glu$^{6.30}$. The negative charge at this position is necessary for the formation of the cytoplasmic ionic lock and its removal has been shown to

contribute to constitutive activation of receptors. The invertebrate GAR receptors also all display a substitution of the Asn$^{6.52}$ found in mAChRs. Here, the nematode GARs contain a Tyr$^{6.52}$ and the flatworm GARs, including SmGAR, have a His$^{6.52}$. Mutation of this position has been shown to affect the ligand binding, especially antagonists atropine and pirenzepine.

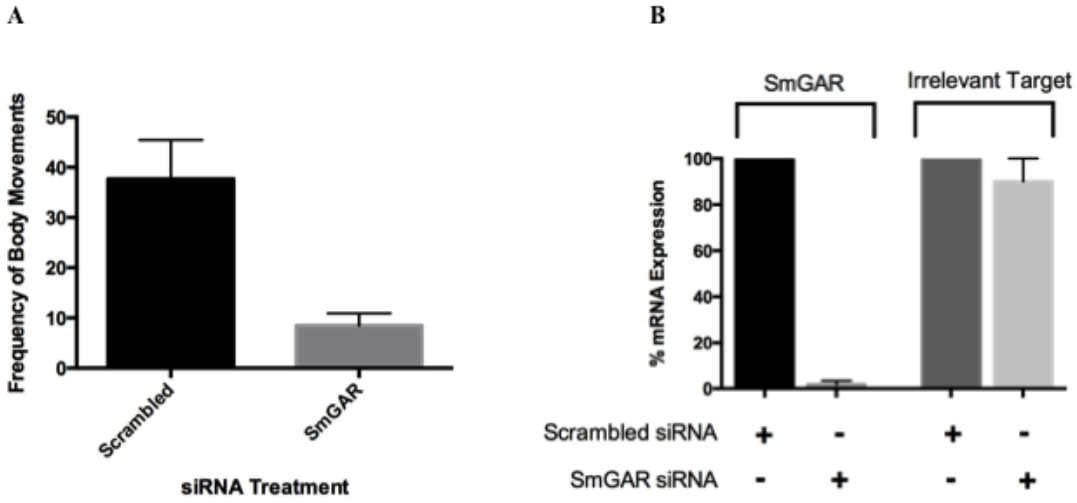


**Figure 6. Silencing of SmGAR affects the motor behavior of early larval schistosomula.** Freshly transformed schistosomula were treated with 50 nM of either SmGAR-specific siRNA or nonsense (irrelevant) scrambled siRNA. 24 hours post-transfection schistosomula were assayed for motor phenotype or collected for confirmation of silencing at the transcript level by qPCR. (A) Suppression of SmGAR in early larval schistosomula induces a hypomotile motor phenotype. Animals treated with SmGAR-specific siRNA show a 70% reduction in the frequency of body movements when compared to the scrambled siRNA negative control. (B) RNA from treated parasites was oligo-dT reverse-transcribed and the resulting cDNA was used as a template for quantitative real-time PCR (qPCR). Primers targeting SmGAR, an off-target (irrelevant) schistosome gene (SmACC-1) and GAPDH were used for PCR amplification. After normalization to the housekeeping gene (GAPDH), relative gene expression of SmGAR and SmACC-1 for siRNA-treated and scrambled control samples using the Pfaffl's method. Treatment with specific siRNA led to complete

silencing of SmGAR at the transcript level when compared to the scrambled control animals. There was no change in the expression level of the irrelevant target, SmACC-1, indicating that silencing of SmGAR was specific. All data are shown as the mean ±SEM of three separate experiments, each containing at least 12 animals.

# Chapter IV- Final Discussion and Conclusions

Emerging praziquantel resistance and the lack of a protective vaccine emphasize the need to develop novel antischistosomal drugs (Greenberg, 2013). Historically, drugs such as pyrantel, metrifonate and ivermectin have been successfully utilized in the treatment of a wide spectrum of helminth infections (Maule *et al.*, 2005). Despite their different modes of action, the common link between these compounds (and PZQ) is that they all target the parasite nervous system. More specifically, these drugs affect the essential biological functions controlled by the parasite neuromusculature. Examples of these include host attachment, feeding and reproduction (Halton, 1996). The neuromuscular system is also vital for invasion and host migration, two processes that are tightly linked to schistosome development (Crabtree and Wilson, 1980).

As a prime source of potential drug targets, numerous schistosome nervous system components, including enzymes involved in neurotransmitter synthesis, degradation and transport have been identified and studied (Hamdan and Ribeiro, 1998, Bentley *et* al., 2005, Ribeiro and Patocka, 2013). The sequencing and annotation of the *S. mansoni* genome has accelerated this process and opened the door for the cloning and characterization of several schistosome neuroreceptors, including GPCRs and ion channels (Ribeiro *et al*., 2013, Dufour *et al*., 2013). Despite this accumulating knowledge of schistosome neurotransmission, the area of schistosome cholinergic biology has remained relatively unexplored. Very early studies reported that schistosomes possess a complete cholinergic system (Bueding, 1952). It has also been noted, however, that ACh seems to elicit inhibitory effects on muscular contraction in schistosomes, most likely by signalling via nicotinic acetylcholine receptors (nAChRs) (Barker *et al*., 1966, Day *et al.*, 1996). This is in stark contrast to vertebrate neuromuscular junction, where ACh is the quintessential excitatory neurotransmitter. Furthermore, it suggests a fundamental, pharmacologically exploitable difference in cholinergic signaling between schistosomes and their human hosts. Although these observations concerning the unique effects of cholinergic drugs on schistosomes have been known for decades, attempts to functionally characterize the receptors responsible for controlling these neuromuscular behaviors were largely unsuccessful (Bentley *et al*., 2004).

In the present work, we provide the first molecular evidence for anion-selective nAChRs mediating inhibitory neuromuscular effects in larval schistosomula. This was achieved using bioinformatics, reverse genetics and cell-based approaches, including a novel fluorescence-based chloride channel assay. We then go on to describe the cloning and functional characterization of a muscarinic acetylcholine receptor (SmGAR), which is the first confirmation of metabotropic cholinergic signaling in flatworms.

The genome of *S. mansoni* is predicted to contain 9 putative nAChR subunits and only one full-length mAChR transcript (Berriman *et al*., 2009, Protasio *et al*., 2012). Earlier studies (see above) have noted that the inhibitory effects of ACh on schistosomes most probably result from the activation of nicotinic acetylcholine receptors. Therefore, we began our analysis of schistosome cholinergic signaling by first examining the amino acid sequences of the putative nAChR receptor subunits. Several (5) of the subunits inspected showed significant homology with cation-selective vertebrate nAChRs but also contained an amino acid substitution in the ion-selectivity (M2) domain of the protein that suggested that the *S. mansoni* subunits were anion-selective (Keramidas *et al*, 2002). Phylogenetic analysis strengthened this prediction, as the schistosome receptors showed high homology with previously characterized nicotinic chloride channels from the snail *Lymnaea* (van Nierop *et al*., 2005) but low similarity to the nicotine-insensitive acetylcholine-gated chloride channels (ACCs) of the nematode *C. elegans* (Putrenko *et al.*, 2005).

We then chose to examine the functional role of the schistosome nAChR chloride channel (SmACC) subunits using RNA interference and immunolocalization. Larval schistosomula were transfected with SmACC subunit-specific siRNA and monitored for motor phenotypes. Knockdown of each subunit elicited a hyperactive motor phenotype when compared to control animals. This agreed with the results of behavioral assays conducted using nAChR antagonists, which also caused similar levels of hypermotility. These data are consistent with the removal of an inhibitory neuromodulator of motor function and implicate anion-selective nAChRs as the receptors responsible for this behavior.

In order to determine whether the SmACC subunits mediate inhibition of muscular contraction directly or indirectly, peptide-derived antibodies against two of the SmACC subunits (SmACC-1 and SmACC-2) were used to localize protein expression in both larval and adult worms. Overall, the expression patterns of both subunits were very similar to that of the nerve plexuses formed by cholinergic neurons in the free-living flatworm *Dugesia japonica* (Nishimura *et al*., 2010). Immunoreactivity against both SmACC-1 and SmACC-2 was present in varicose nerve fibers and neuronal bodies throughout the body and at a depth close to the surface of the parasite. This pattern indicates that these two receptor subunits localize to either the submuscular or subtegumental nerve plexus, structures that innervate and control somatic muscular function (Koopowitz and Chien, 1974, Halton and Gustaffson, 1996). However, unlike the *Dugesia* cholinergic neurons, the SmACC–expressing neurons do not directly innervate the body-wall musculature and therefore most likely mediate their effects in an indirect manner through modulation of neuronal signaling.

Although the RNAi assays and immunolocalization served to elucidate the function of SmACCs at the behavioral level, they provided only limited insight to the pharmacological characteristics of these receptors. The classical method of assaying the functional characteristics of ion channels is heterologous expression in *Xenopus* oocytes. However, our attempts to express SmACC-1 and SmACC-2, either alone or in combination using this system were unsuccessful. One reason for this lack of success was a low level of protein expression, possibly due to the divergent codon usage of flatworms (Hamdan *et al*, 2002). We solved the protein expression issue by obtaining a human codon-optimized SmACC-1 construct. Instead of using the *Xenopus* system, however, we decided to attempt functional assays in a mammalian cell-based expression system using a new fluorescence quench-based anion flux assay. SmACC-1-expressing cells were transduced with a modified anion-sensitive YFP construct and then treated with a panel of drugs. Our results showed that SmACC-1 responds selectively to nicotinic agonists in a concentration-dependent manner but is not activated by other neurotransmitters. Also, the influx of anions and resulting YFP quench caused by the activation of SmACC-1 confirms that SmACC-1 functions as a chloride channel. More importantly, however, the characterization of a parasite ion channel using a

mammalian cell-based expression system represents proof-of-concept for future development of high-throughput functional assays that could be used for screening large compound libraries. A tool such as this would be immensely helpful in the discovery of novel anthelminthics.

After confirming our initial hypothesis that nicotinic chloride channels play an inhibitory role in controlling motor function, we continued our survey of flatworm cholinergic receptors by examining muscarinic signaling. Unlike nAChR-mediated neurotransmission, information concerning the existence and function of mAChRs in flatworms is scarce. Behavioral and pharmacological studies of schistosomes indicate that muscarinic type agonists have little to no effect on schistosome motor behavior (Barker *et al*., 1966, Day *et al*., 1996). Yet, there is also evidence that mAChRs modulate muscular contraction in the trematode *Fasciola heptatica* (Sukhdeo *et al*., 1996). In free-living planarians, mAChRs are active in a variety of important functions, such as muscular contraction, memory and learning (Nishimura *et al*., 2010 and Ramakrishnan *et al*., 2014). The genome of *S. mansoni* is predicted to encode a single mAChR, named SmGAR. Expressional analysis suggests SmGAR is highly up regulated during the cercarial and early schistosomula life stage (Berriman *et al*., 2009), which may indicate its importance in parasite invasion and host migration. We therefore, sought to clone and functionally characterize this receptor.

Initial pairwise and multiple sequence alignments demonstrated that SmGAR shares significant similarity (30-45%) with the GAR receptors of *C. elegans* and other putative flatworm GAR-like receptors but lower homology with mammalian mAChRs. Dendrogram analysis revealed that the flatworm GARs form a unique family of proteins, with sequence features that are distinct from those of nematodes and vertebrates. One such feature is the length of flatworm GARs, which is significantly (2-3 times) longer than those of vertebrates or nematodes.

SmGAR was amplified by PCR using cDNA generated from early stage (<24 hours old) schistosomula. We were unable to amplify sufficient amounts of SmGAR using adult-derived cDNA, which agrees with the predicted transcriptional profile. Expression of SmGAR in a yeast system resulted in a functional receptor that is

moderately activated by cholinergic agonists but not other biogenic amines. Activation of SmGAR by acetylcholine and the muscarinic agonist carbachol was concentration-dependent and yielded $EC_{50}$ values consistent with those found in nematode homologs (Lee *et al*., 2000, Kimber *et al*., 2009). SmGAR also displayed high levels of agonist-independent (or constitutive) activity in the yeast expression system, although we were able to reduce this baseline signaling by using the inverse agonists, atropine and promethazine.

There are several factors that may contribute to the constitutive activity of SmGAR. Heterologous expression of proteins often leads to improper folding and altered G-protein coupling. However, there is also growing evidence for the existence of spontaneously active GPCRs that function as neuromodulators in biological feedback loops (Spalding and Burstein, 2006). These receptors often contain amino acid substitutions at positions important for stabilizing the receptor's inactive conformation, which enable the receptor to spontaneously activate. In order to test whether SmGAR contained any of these features, we conducted an in-depth sequence analysis and performed structural homology modeling. We found several substitutions that may contribute to the constitutive activity of SmGAR. Two of the substitutions ($Cys^{3.52}$ and $Ala^{6.30}$) were localized to the third and sixth TM domains, which form the ionic lock of mAChRs. This lock holds the receptor in the inactive conformation until a ligand binds. Absence of this feature induces constitutive activity in several vertebrate GPCRs and may explain why SmGAR exhibits such high basal activity in the yeast expression system. Interestingly, all invertebrate GAR sequences analyzed contained the $Ala^{6.30}$ substitution. This may suggest fundamental structural differences between vertebrate and invertebrate mAChRs.

Having identified SmGAR as a functional cholinergic receptor, we questioned whether this activity might contribute to regulation of motor function in the worm. This was tested, once again by using an RNAi-based behavioral assay. Suppression of SmGAR expression in larval schistosomula led to a significant reduction in parasite motility. This hypomotile phenotype was observed only in schistosomula that were early stage (<24 hours). Knockdown of SmGAR had no effect on the motor behavior of older

parasites. These results suggest that SmGAR does exert some regulation over early larval motility but the mechanism by which this control takes place is unclear. Removal of an inhibitory neuromuscular signal would be expected to increase worm motility, the opposite of the phenotype observed in SmGAR-silenced parasites. It is possible that SmGAR plays an indirect role in regulating motor function. In vertebrates, M2 mAChRs occupy this role, functioning as presynaptic autoreceptors that control release of ACh from cholinergic neurons (Bellingham and Berger, 1996). A similar role of the GAR-2 receptor has also been suggested in *C. elegans* (Dittman and Kaplan, 2008). Therefore, the reduction of larval motility associated with silencing of SmGAR may be a result of increased ACh-release caused by the removal of an inhibitory-feedback loop.

The work presented in this thesis serves to clarify our understanding of basic flatworm neurobiology, as well as identifies potential targets for the development of new drugs. Here, we provide the first molecular evidence for the function of a novel family of invertebrate-specific nicotinic chloride channels in schistosomes and have linked these receptors directly to the inhibitory modulation of motor function in larval schistosomula. We have also identified and characterized a schistosome muscarinic receptor that regulates movement in young larvae and therefore may be involved in early parasite migration. The unique qualities possessed by schistosome cholinergic receptors and their key involvement in the modulation of parasite motility nominate both the SmACCs and SmGAR as potential drug targets. Moreover, the high similarity shared between schistosome and other parasitic flatworm cholinergic receptors suggests these may be suitable targets for broad-spectrum anthelminthics. Finally, the mammalian cell-based ion channel assay used in this research serves as a high throughput tool for the investigation of the divergent pharmacology exhibited by many of these parasite receptors.

## References


1. Barker, LR, Bueding E, Timms AR (1966). The possible role of acetylcholine in *Schistosoma mansoni*. Brit J Pharmacol **26**: 656-665.
2. Bellingham MC, Berger AJ (1996) Presynaptic depression of excitatory synaptic inputs to rat hypoglossal motoneurons by muscarinic M2 receptors. J Neurophysiol 76(6): 3758-3770.

3. Bentley GN, Jones AK, Oliveros Parra WG, Agnew A (2004). ShAR1alpha and ShAR1beta: novel putative nicotinic acetylcholine receptor subunits from the platyhelminth blood fluke *Schistosoma*. Gene **329**: 27-38.

4. Bentley GN, Jones AK, Agnew A (2005). Expression and comparative functional characterisation of recombinant acetylcholinesterase from three species of Schistosoma. Mol Biochem Parasitol **141**(1): 119-23.

5. Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, et al. (2009). The genome of the blood fluke *Schistosoma mansoni*. Nature **460**: 352–358.

6. Bueding, E (1952). Acetylcholinesterase activity of *Schistosoma mansoni*. British Journal of Pharmacology and Chemotherapy, 7: 563–566.

7. Crabtree JE, Wilson RA (1980). *Schistosoma mansoni*: a scanning electron microscope study of the developing schistosomulum. Parasitology **81**(Pt 3): 553-64.

8. Day TA, Chen GZ, Miller C, Tian M, Bennett JL, Pax RA (1996). Cholinergic inhibition of muscle fibers isolated from *Schistosoma mansoni* (Trematoda: Digenea). Parasitology **113**(Pt. 1): 55-61.

9. Dittman JS, Kaplan JM (2008) Behavioral impact of neurotransmitter-activated G-protein-coupled receptors: muscarinic and GABAB receptors regulate Caenorhabditis elegans locomotion. J Neurosci 28(28): 7104-14.

10. Dufour V, Beech RN, Wever C, Dent JA, Geary TG (2013). Molecular Cloning and Characterization of Novel Glutamate-Gated Chloride Channel Subunits from *Schistosoma mansoni*. PLoS Pathog **9**(8): e1003586.

11. Greenberg RM (2013). New approaches for understanding mechanisms of drug resistance in schistosomes. Parasitology **140**(12): 1534-46.

12. Halton DW and Gustaffson MKS (1996). Functional morphology of the platyhelminth nervous system. Parasitology **113**: S47–S72.

13. Hamdan FF and Ribeiro P (1998). Cloning and characterization of a novel form of tyrosine hydroxylase from the human parasite, *Schistosoma mansoni*. J Neurochem **71**(4): 1369-80.

14. Hamdan FF, Abramovitz M, Mousa A, Xie J, Durocher Y, Ribeiro P (2002). A novel Schistosoma mansoni G protein-coupled receptor is responsive to histamine. Mol Biochem Parasitol **119**(1): 75-86.

15. Keramidas A, Moorhouse AJ, Pierce KD, Schofield PR, Barry PH (2002). Cation-selective mutations in the M2 domain of the inhibitory glycine receptor channel reveal determinants of ion-charge selectivity. J Gen Physiol **119**(5): 393-410.

16. Kimber MJ, Sayegh L, El-Shehabi S, Song C, Zamanian M *et al*. (2009). Identification of an *Ascaris* G protein-coupled acetylcholine receptor with atypical muscarinic pharmacology. Int J Parasitol **39**(11): 1215-22.

17. Koopowitz H, Chien P (1974). Ultrastructure of the nerve plexus in flatworms. I. Peripheral organization. Cell Tissue Res **155**(3): 337-51.

18. Lee YS, Park YS, Nam S, Suh SJ, Lee J, *et al*. (2000). Characterization of GAR-2, a novel G protein-linked acetylcholine receptor from *Caenorhabditis elegans*. J Neurochem **75**: 1800–1809.

19. Maule AG, Day TA, Chappell CH (2005). Parasite neuromusculature and its utility as a drug target. Parasitology **131**: S1-S2.

20. Nishimura K, Kitamura Y, Taniguchi T, Agata K. (2010). Analysis of motor function modulated by cholinergic neurons in planarian *Dugesia japonica*. Neurosci **168**(1): 18-30.

21. Patocka N and Ribeiro P (2013). The functional role of a serotonin transporter in *Schistosoma mansoni* elucidated through immunolocalization and RNA interference (RNAi). Mol Biochem Parasitol **187**: 32-42.

22. Protasio AV, Tsai IJ, Babbage A, Nichol S, Hunt M (2012). A systematically improved high quality genome and transcriptome of the human blood fluke *Schistosoma mansoni*. PLoS Negl Trop Dis **6**(1): e1455.

23. Putrenko I, Zakikhani M, Dent JA (2005). A Family of Acetylcholine-gated Chloride Channel Subunits in *Caenorhabditis elegans*. J Biol Chem **280**: 6392-6398.

24. Ramakrishnan L, Amatya C, Desaer CJ, Dalhoff Z, Eggerichs MR (2014). Galantamine reverses scopolamine-induced behavioral alterations in Dugesia tigrina. Invert Neurosci Epub ahead of print.

25. Ribeiro P, Gupta V, El-Sakkary N (2012). Biogenic amines and the control of neuromuscular signaling in schistosomes. Invert Neurosci **12**(1): 13-28.

26. Spalding TA, Burstein ES (2006) Constitutive activity in muscarinic acetylcholine receptors. J Recept Signal Transduction 26: 61-85.

27. Sukhdeo SC, Sangster NC, Mettrick DF (1986). Effects of cholinergic drugs on longitudinal muscle contractions of *Fasciola hepatica*. J Parasitol **72**(6): 858-64.

28. van Nierop P, Keramidas A, Bertrand S, van Minnen J, Gouwenberg Y, *et al.* (2005). Identification of Molluscan Nicotinic Acetylcholine Receptor (nAChR) Subunits Involved in Formation of Cation- and Anion-Selective nAChRs. J Neurosci **25**(46): 10617-10626.