Functional and structural analyses of *Schistosoma mansoni* nicotinic acetylcholine receptors.

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Statement of contributions

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

Abstract

The objective of this study was to isolate native nicotinic acetylcholine receptors from the adult form of a parasitic trematode, *Schistosoma mansoni*, identify their oligomeric composition and attempt to reconstruct receptors in a heterologous expression model in order to better understand their role in the cholinergic system of the worm.

Using an affinity purification method that exploits binding of α-bungarotoxin to nicotinic acetylcholine receptors, we analyzed column-bound proteins by Mass Spectrometry (MS) and Western blotting. Although, some protein binding was non-specific, MS analyses revealed 3 nicotinic acetylcholine receptor subunits found in 3 independent purifications. Particularly one of them (smp_142690) was consistently found in all three samples sent for MS. The other two (smp_176310 and smp_031680) were identified in two out of three samples. We attempted to immunoprecipitate some of the previously identified receptor subunits by polyclonal antibody against smp_176310 α-subunit. Western blot analyses showed bands of appropriate size when probed with antibodies against smp_176310 and smp_142690, suggesting that they could be interacting.

Two of the identified subunits (176 and 142) were codon-optimized for mammalian cell culture and reconstituted in a heterologous expression system (HEK 293) to be functionally analyzed by a fluorescence-based assay (Premo Halide Sensor, Invitrogen), where yellow fluorescent protein (YFP) can act as an intracellular sensor to monitor anionic flux through the membrane. Successful protein expression in transfected cells was monitored using immunofluorescence technique, with polyclonal antibodies against smp_176310 and smp_142690 subunits. Confocal microscopy of transfected cells showed abundance of protein near plasma membrane. Selection for stable transfection with either one of two subunits proved

to be difficult, as expression levels slowly decreased over the following weeks, potentially indicating cytotoxic properties of our receptors. Unfortunately, initial functional studies proved unsuccessful, as we failed to detect any channel activity. Suspecting that low protein expression and failed membrane targeting could be the cause, we will attempt to further optimise the procedure.

Nearly half of all anthelmintic drugs act by targeting the worm's cholinergic system. Additional research in structural and functional composition of those receptors would greatly aid in future drug development for Schistosomiasis.

Abstract (French)

L'objectif de cette étude était d'isoler les récepteurs nicotinique d'acétylcholine originaire des vers parasites trématodes *Schistosoma mansoni* adultes, identifier leur composition oligomèrique et tenter de les reconstruire dans un modèle d'expression hétérologique afin de mieux comprendre leurs rôles dans le système cholinergique du ver.

En utilisant une méthode de purification par affinité qui exploite la liaison entre les récepteurs nicotinique d'acétylcholine et l'α-bungarotoxine, nous avons réussi à analyser les protéines recueillies par colonne et nous les avons analysés par spectrométrie de masse (SM) et transfert de Western. Bien que quelques protéines se soient liées non spécifiquement, les analyses de SM ont démontré 3 sous-unités faisant partie de la famille des récepteurs d'acétylcholine nicotiniques dans 3 purifications indépendantes. L'une de ces sous-unités en particulier (smp_142690) a été trouvée dans toutes les purifications. Les deux autres sous-unités ont été retrouvées dans 2 des 3 purifications. Nous avons aussi réussi à immunoprecipiter certains des sous-unités précédentes avec des anticorps polyclonaux contre smp_176310 α. Les analyses par transfert de Western démontrent des bandes de tailles appropriées lorsqu'elles furent interrogées avec des anticorps contre smp_176310 et smp_142690, ce qui suggèrerait que ces deux-ci pourraient interagir ensembles.

Deux des sous-unités identifiées (176 et 142) furent optimisées par codons pour les cellules mammaires et reconstituées dans un système d'expression hétérologue (HEK 293) pour

être fonctionnellement analysées par essais basés sur la fluorescence (Premo Halide Sensor, Invitrogen), où la protéine fluorescente jaune (PFJ) agit comme sonde intracellulaire pour surveiller le flux anionique à travers la membrane. Les transfections réussites dans ces cellules ont été observées en utilisant une approche immunofluorescente avec des anticorps polyclonaux contre les sous-unités smp_176310 et smp_142690. Par la microscopie confocale nous avons observé une abondance de ces protéines au niveau de la membrane plasmique des cellules transfectées. Malheureusement, la sélection des cellules transfectées s'est avéré être très difficile, indiqué par une diminution des niveaux d'expression sur une courte période, démontrant ainsi peut-être des propriétés cytotoxiques des récepteurs. Ainsi, la détection d'activité fut sans succès. Nous soupçonnons que la faible expression des protéines ou l'incapacité de la cellule à cibler la protéine à la membrane correctement pourraient être en cause. Une optimization serait nécessaire.

Presque la moitié de toutes les drogues anthelminthiques agissent sur le système cholinergique du ver. Il faudrait étudier davantage les structures et compositions de ces récepteurs pour développer des nouveaux traitements dans l'avenir contre la schistosomiase.

Abbreviations

AbD: Antibody Diluent

ACC: Acetylcholine-gated Chloride Channel subunit

Ach: Acetylcholine

CNS: Central Nervous System

cDNA: Complementary Deoxyribonucleic Acid

DNA: Deoxyribonucleic Acid

ER: Endoplasmic Reticulum

FITC: Fluorescein Isocyanate

GABA: Gamma-aminobutyric Acid

GFP: Green Fluorescent Protein

YFP: Yellow Fluorescent Protein

GluCl: Glutamate-gated Chloride channel

GPCR: G Protein-Coupled Receptor

GSP: Gene Specific Primer

HEK293: Human Embryonic Kidney 293

HRP: Horseradish Peroxidase

IP: Immunoprecipitation

kDa: kilo Dalton

LGIC: Ligand-Gated Ion Channel

mRNA: messenger Ribonucleic Acid

MW: Molecular Weight

nAChR: Nicotinic Acetylcholine Receptor

PBS: Phosphate Buffered Saline

PNS: Peripheral Nervous System

RNA: Ribonucleic Acid

RT: Room Temperature

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrilamide Gel Electrophorasis

TM: Transmembrane

α-BTX: α-bungarotoxin

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CHAPTER 1 – Introduction

1. Background

Schistosomiasis (bilharzia), an infection caused by a group of trematode worms belonging to the *Schistosoma* genus, is one of the most common parasitic infections in tropical parts of the world. Schistosomes are blood-inhabiting parasites that possess unique trait of being dioecious (distinct male and female forms), unlike other trematodes or other flatworms in general. The two sexes show a high level of sexual dimorphism, males being distinctly larger than females. Mature worms inhabit blood vessels associated with intestines or bladder where females produce eggs that are excreted with feces or urine. Exact anatomical localisation within the host is species -dependent.

Schistosomes undergo impressive morphological and physiological changes with each step of their life cycle. Sophisticated adaptations facilitate transition between hosts, including the glycocalyx and tegument for protection from the host, cilia for swimming, gynaecophoric canal for optimal male-female pairing, secretory glands for host penetration and suckers for effective host attachment. As previously mentioned, schistosomes possess a complicated life cycle that requires a freshwater snail as an intermediate host and a mammalian (or avian) definitive host. Infection begins with a free-living, non-feeding, freshwater larva (cercaria). The cercaria life stage of most trematode species swim by sudden bursts of activity and search for a suitable definitive host. Some swims might last for hours until the larva depletes its glycogen reserves. The search is influenced by water turbulence, light and some skin chemicals, including ceramides and linoleic acid. Cercaria has a selection of glands that are fundamental to host infection and contain proteases that help with skin penetration or secrete mucus that aids

attachment to surfaces. Finally, once the cercaria finds a suitable mammalian host, it penetrates the skin layer, where it loses its bifurcated tail and transforms into a schistosomulum.

The schistosomulum remains in the skin for the next 48 hours and completes its transformation from free-living organism into an obligate parasite. The process by which cercaria eventually becomes a schistosomulum is well established and protocols exist that replicate the process in *vitro*, for example by mechanical removal of the tail [1]. *In vitro* transformed schistosomulae are able to grow in culture but they do not reach maturity and egg production has never been achieved.

After penetration of the host and subsequent transformation, the young worm begins its long-lasting migration by traversing the host dermis and venule wall, ultimately entering the blood circulation. First the larvae travel to the pulmonary capillaries, where they enter the systemic circulation. Next they migrate to the hepatic portal vein and continue to mature while feeding on blood. The young adult male and females soon begin to pair-up and migrate to their final destination in the venous plexus near the bladder *(S. haematobium)* or the mesenteric venules near intestines *(S. mansoni, S. mekongi, S. japonicum)*. The maturation process is completed within 4-6 weeks depending on schistosome species, infected host species and its physical condition. Adult female and male worms are closely associated; females take permanent residency within the gynaecophoric canal of males. Some evidence suggests that a degree of molecular signalling is required between two genders for a successful maturation [2]. Production of eggs starts after male-female pairing and is the indicator that worms have reached their adulthood. This process might continue for decades. Female worms produce thousands of eggs per day, most of which leave the host through the gut *(S. mansoni, S. mekongi, S. japonicum)* or the bladder *(S. haematobium)*. The mechanism by which schistosomes manage to

evade host immune response is poorly understood. Adults possess a pair of suckers for attachment, a sophisticated tegument that plays a crucial role in parasite survival due to immune response evasion and well developed reproductive and neural systems.

Upon contact with freshwater, excreted eggs start to hatch and another life stage called miracidium emerges. Miracidia are free-living and non-feeding. Similarly, to cercaria their swimming behaviour is photokinetic and chemokinetic [3]. However, their propulsion is generated by rows of cilia attached to epidermal plates. Unlike cercaria, miracidia are looking for a freshwater snail that would act as an intermediate host. Mechanical movement and proteases are used to enter the snail. Inside the snail, the miracidium loses its ciliated plates and transforms into a mother sporocyst that generates germ-cell derived daughter sporocysts. Required nutrients are absorbed from snail plasma through the sporocyst tegument. In the following days, those sporocysts differentiate into large numbers of cercariae that are eventually released from the snail and the cycle recommences. Detailed mechanism of snail-host immune response evasion is unclear [4,5].

Today, research efforts are largely focused on three species that are responsible for a great majority of schistosomiasis cases: *Schistosoma mansoni, S. haematobium,* and *S. japonicum*. Recent advances in schistosome biology are determined by a crucial need to better understand and control schistosomiasis. Ongoing projects concentrate on identification of new drug candidates and development of vaccines. This is easily explained by numbers, nearly 200 million people are infected around the globe, 120 million suffer from clinical disease and almost a billion are under some risk of infection. Estimated number of deaths is 280 thousand per year in Africa alone. Schistosomiasis is at the top of the list among the most prevalent parasitic diseases of the world. Almost 85% of the infected population reside in Africa and have no access

to any form of treatment [5]. Infection-associated pathology is not caused directly by the adult worms, but rather by large number of eggs that become trapped in host tissue during migration to intestinal lumen. Such migration often leads to formation of fibrotic granulomas and embolism in organs, mostly affecting lungs and liver.

Currently, the treatment of choice for schistosomal infection is praziquantel. Unfortunately, it is also the only one available on the market. Other drugs such as oxamniquine and the anticholinergic drug, metrifonate have been shown to be effective, however they also cause a number of side effects [6]. It is only a matter of time before resistant strains of *S. mansoni* become widespread. Praziquantel resistance has already been generated in laboratory conditions and recent field reports suggest reduced cure rates in sub-Saharan Africa [5,7].

2. Role of Acetylcholine

Acetylcholine (ACh) is a key neurotransmitter involved in the control of muscle contraction in both vertebrates and invertebrates. It can be either inhibitory or excitatory, depending on the species in which it is found and the type of cholinergic receptor involved. ACh is predominantly excitatory in vertebrates, whereas in invertebrates it has both excitatory and inhibitory effects [8]. ACh is produced in the axon terminal of a cholinergic neuron, where choline acetyltransferase transfers an acetyl group to a choline molecule. Once synthesized, ACh is stored in synaptic vesicles and released to synaptic clefts where it binds to specific receptors located post-synaptically on target neurons or neuromuscular junctions, as well as on the surface of blood and other cell types [10].

Acetylcholine is predicted to be an inhibitory neurotransmitter in schistosomes and flatworms in general. There is solid evidence for choline acetyltransferase, the enzyme responsible for acetylcholine synthesis, to be found in schistosomes [12,13,14]. The neuroanatomy of *S. mansoni* has been elucidated through numerous histochemical studies of the acetylcholinesterase (AChE), enzyme responsible for breaking down acetylcholine. When stained for AChE, the most pronounced signal occurs in the central nervous system and associated nerve cords, as well as the suckers and regions presumably associated with neuromuscular innervations [15]. Early studies demonstrated that cholinesterase inhibitors produced paralysis of the worm, highly suggesting deep involvement in motor activity [11]. Because of AChE's important role in the cholinergic system, it is the target of multiple antiparasitic drugs, including metrifonate and lucanthone. Recent studies claim that AChE activity is not restricted to synaptic transmission, but also plays a role in glucose uptake, as both AChE and nAChRs were found on the outer side of the tegumental membrane. Acetylcholine itself was shown to enhance glucose uptake by the parasite [16]. In the past, it has been shown that topical application of cholinergic agonists on cultured *S. mansoni* causes a flaccid paralysis, further suggesting that ACh plays an inhibitory role in the worm's neuromuscular control system [11].

3. Acetylcholine receptors

Among the great variety of neuroreceptors encoded in the genome of *S. mansoni* [12,13], some are members of the Cys-loop ligand-gated ion channel superfamily. Their name originates from the fact that all members contain a conserved disulfide bond between two cysteines separated by 13 amino acids on the N-terminal end. Members of this group build pentameric structures of highly variable composition, function and pharmacology [8,17,18]. The purpose of this structure is to form an ion-conducting pore through the cellular membrane. A pore can be assembled from homomeric or heteromeric subunits. The transmembrane domain of each subunit is composed of four membrane traversing regions (M1, M2, M3 and M4). A peptide motif on the M2 region determines channel selectivity for cations or anions (Table #1). Pro-Ala sequence in the pore-lining domain is suggestive of anion selectivity and a Glu residue usually indicates a cation selective channel [19].

Cys-loop ligand-gated ion channels are directly involved in fast ionotropic neurotransmission in both invertebrate and vertebrate hosts. They can be distinguished based on ion permeability. Some of them are considered excitatory and are permeable to cations, meanwhile others are inhibitory and are permeable to anions. Another way to classify them is based on ligand specificity. Invertebrate Cys-loop ligand-gated ion channels family includes conventional GABA receptors, excitatory and inhibitory nicotinic acetylcholine receptors, range of inhibitory glutamate receptors, biogenic amines and pH-gated receptors [20].

There are 13 Cys-loop subunits located in *S. mansoni* genome, 9 of which belong to nicotinic acetylcholine receptor class characterized by selective activation using nicotine or natural ligand – acetylcholine [12], though, their number may vary depending on how the

bioinformatics search is conducted. Members of this group can be further classified as alpha-α and non-alpha (β, γ, ε, δ) subunits, based on their sequence and associated motifs. Both types share similar molecular structure and physical characteristics, including the Cys-loop motif and the typical topology of four conserved transmembrane regions with extracellular N- and C-termini [8,18]. What makes the alpha-subunit special is the presence of a neurotransmitter binding motif, which constitutes the core of the channel's binding site. Other subunits do not contain that sequence and thus cannot bind ACh. As previously mentioned, Cys-Loop channels are typically pentameric. They can be homomeric assemblies of a single alpha-subunit that is repeated five times or they can have a heteromeric composition with both alpha- and non-alpha subunits in variable combinations. Certain types of pentameric structures, such as the α7 nicotinic receptor in human brain, can bind up to five ACh molecules at once, while others are limited to a single molecule [21,22]. In vertebrates, nicotinic acetylcholine receptors are cation-selective and mediate excitatory responses due to membrane depolarization, whereas invertebrate species have both cation and anion-selective channels [8].

Cys-loop ligand-gated ion channel receptors have been poorly characterised in schistosomes and physiological functions arbitrated by them remain unclear. To date, the only channels to have been cloned are three putative nicotinic acetylcholine receptor subunits from *S. haematobium*, named ShAR1a, ShAR1b, ShAR2b [23] and three glutamate receptor subunits from *S. mansoni* named SmGluCl-1, SmGluCl-2, SmGluCl-3, as well as two acetylcholine receptor subunits from *S. mansoni* named SmACC-1, SmACC-2 [24,25]. That leaves us with almost no information on more than half of Cys-loop subunits in the *S. mansoni* genome.

Previously cloned nicotinic acetylcholine receptors ShAR1a, ShAR1b, ShAR2b found in *S. haematobium* were tested for their ability to form functional channels when expressed from cRNA in *Xenopus* oocytes, but researchers failed to detect evidence of channel activity in response to ACh or nicotine. Immunolocalization of ShAR1a appeared to be localised exclusively to the parasite surface, whereas ShAR1b showed diffuse pattern of fluorescence associated with subtegumental muscle tissue [23,26].

Bioinformatic research into nAChRs of *S. mansoni* has revealed a number of putative subunits found in the genome database (Table #1). Reported structural alignments suggest the presence of both anion and cation-selective schistosome subunits based on M1-M2 linker region of the subunit. A glutamate (E) residue (negatively charged) in the M1-M2 linker confers cation-selectivity, Pro-Ala (P-A) motif in this position suggests anion-selectivity. This was further demonstrated by specific site-directed mutations [27] Immunolocalization studies revealed significant expression of both subunits in the peripheral nervous system of the worm. Some of SmACC-2 immunoreactivity was found on the surface of the parasite. For further analyses, researchers examined the predicted anion-selective nAChR subunits of *S. mansoni* (Smp_157790; Smp_132070; Smp_037960; Smp_176310; Smp_142690), by measuring larval motor phenotype after RNA interference (RNAi) procedure. Larvae treated with nAChR siRNA all showed a significant (at least 2 fold) motor hyperactivity as compared to mock-transfected negative controls. The notion of an anion-selective channel is supported by hyperactive RNAi phenotype due to its consistency with pharmacological studies, where larvae treated with cholinergic antagonists demonstrated similar behaviour [25]. Inactivated by antagonists, anion-selective channels fail to open, thus preventing hyperpolarization and inhibition of action potential, causing hyperactivity in worms.

ID	Receptor	GenBank accession No.	*S. mansoni* GeneDB No.
Cation-selective nAChR	Cys-loop LGIC	AAR84361	Smp_031680
Cation-selective nAChR	Cys-loop LGIC	AAR84362	Smp_139330
Cation-selective nAChR	Cys-loop LGIC	CAZ37489	Smp_180570
Anion-selective nAChR	Cys-loop LGIC	CAZ36947	Smp_176310
Anion-selective nAChR	Cys-loop LGIC	AAX59989	Smp_142690
Anion-selective nAChR	Cys-loop LGIC	CAZ31387	Smp_037960
Anion-selective nAChR	Cys-loop LGIC	CAZ31388	Smp_142700
Anion-selective nAChR	Cys-loop LGIC	CAZ34080	Smp_157790
Anion-selective nAChR	Cys-loop LGIC	CAZ29477	Smp_132070.2
Anion-selective nAChR	Cys-loop LGIC	CAZ29476	Smp_132070.1
GAR	GPCR (class A)	CAZ33193	Smp_152540
GAR	GPCR (class A)	CAZ31912	Smp_145540

Table #1: Predicted acetylcholine neuroreceptors of *Schistosoma mansoni*, obtained from *S. mansoni* genome database [12]. Ion selectivity is determined by M1-M2 linker region of the subunit. A glutamate (E) residue in the M1-M2 linker confers cation-selectivity, Pro-Ala (P-A) motif in this position suggests anion-selectivity. GPCR, G-protein-coupled receptor; LGIC, ligand-gated ion channel; GAR, G-protein-coupled acetylcholine receptor; nAChR, nicotinic acetylcholine receptor. Table adapted from [20] and [25].

It should be mentioned that another family of ACh receptors exist called muscarinic acetylcholine receptors. They belong to G-protein coupled class of receptors (GPCR) and are found in vertebrates and invertebrates alike. These receptors signal through changes in intracellular messenger such as cAMP and are mostly involved in action potential modulation, using positively charged ions such as potassium [28]. Recently, new studies of *S. mansoni* G-protein coupled receptors (SmGAR) provided first evidence of their importance as antiparasitic drug targets. One of the two SmGARs (smp_145540) was cloned and expressed in yeast. Functional studies have shown that cholinergic agonists selectively activate the receptor. To further investigate its activity, researchers studied the role of the receptor *in vivo* using RNAi. The results suggested that SmGAR plays an important role in motor control of young larvae by stimulating their movement. [29]

4. ACh binding sites and α-Bungarotoxin

Research dating back to the 1980s has shown that nicotinic cholinergic receptors have two agonist-binding sites. By occupying these two sites, the agonist stabilises the receptor channel in the open state. With the help of the fluorescence resonance energy transfer method between a receptor-bound fluorescent agonist and a membrane fluorescent probe, sites were estimated to be 25 A below the extracellular apex of the nAChR (Nicotinic acetylcholine receptor). Eventually, electron microscopy results have confirmed the proposed location for the ACh-binding sites at α subunit in the pentameric receptor, and approximately 30 A above membrane surface [30]. Photoaffinity experiments have shown that the ligand-binding site must be at the interface between the α-s and their adjacent subunits.

The ACh-binding site is formed by six loops called A-F. The α-subunit comprises A, B and C loops, while adjacent subunit comprises D, E and F loops. Modelling of three-dimensional structures confirmed that the ACh-binding site must reside at the interface between two subunits [21]. Later X-ray diffraction analysis confirmed the predictions and the existence of the A, B, C, D and E loops [22].

Early work with neuromuscular junctions and ganglionic (neuronal) receptors has shown that significant differences exist between nAChR subtypes. A major criterion is the difference in sensitivity to snake α-bungarotoxin. α-Bungarotoxin is a 74 amino acid neurotoxin derived from snake venom of *Bungarus multicinctus*. It binds to the muscle AChR competitively, inhibiting ACh binding, thereby preventing channel opening and blocking neuromuscular transmission. It was shown that homomeric receptors α7, α8 and α9 are blocked by α-bungarotoxin, whereas heteromeric receptors are insensitive [18,31]. The high-affinity of some nAChRs for bungarotoxin has been explored to purify the native channels from a number of vertebrate species, using standard biochemical methods and, recently proteomics approaches [32]. As discussed below, the same approach will be tested here to isolate nicotinic channels from *S. mansoni*. Based on previously reported results, we know that α-bungarotoxin is capable of blocking ACh induced inhibition of *S. mansoni* muscle fiber contractions [33]. In 1995, α-bungarotoxin binding sites were identified on the parasite surface and presumed to be nicotinic receptors [34]. Few moths later, the cholinergic stimulation of glucose transport in *S. haematobium* was reported to be blocked by nicotinic antagonists such as α-bungarotoxin, by the same research group [16].

5. Potential for anti-schistosomal drug targeting

As with most helminth parasites, the cholinergic system of *Schistosoma mansoni* is an attractive candidate for future drug development [35]. Many current wormicidal drugs function by interacting with cholinergic receptors. By selectively inhibiting those receptors, neuroactive drugs interfere with several vital functions required for survival, reproduction, immune-response evasion or persistence within the host. The recent completion of *S. mansoni* and *S. japonicum* genome projects has greatly stimulated neuronal signalling research in schistosomes [12,13,14]. Early analyses identified an impressive number of receptors and neurotransmitters encoded within the genetic sequence. Some of these receptors might be schistosome specific and represent potential drug targets [20].

The search for anti-schistosomal drug targets has been limited by the lack of suitable high-throughput screening assays to assess functionality and drug binding properties of schistosome receptors [20]. Fortunately, new approaches are starting to emerge that could greatly contribute to development of a new assay and a drug [13, 25, 36]. Most functional studies of Cys-loop channels have relied on classical electrophysiological approaches in *Xenopus* oocytes. Although well established, such procedures are labour intensive, time demanding and are of a low-throughput kind. A number of commercially available fluorescence based assays exist that can be readily adapted to a plate format. These assays compare well with classical electrophysiology studies performed in oocytes and appear to be quite reliable [37, 38]. The high-throughput nature of those assays makes them very appealing for screening of large chemical libraries against parasite receptors. Regrettably, commercial assays are very expensive and have their own limitations such as one-at-a-time delivery method for the reporter and subunit

to be tested. A new type of a high-throughput assay is required that could be easily adapted to the biology of schistosomes but remain relatively cheap and easy to use.

Study rationale and major objectives.

Schistosomiasis (bilharzia), is one of the most common parasitic infections in tropical parts of the world. Nearly 200 million people are infected around the globe, 120 million suffer from clinical disease and almost a billion is under some risk of infection. Mature worms inhabit blood vessels associated with intestines or bladder where females produce eggs that are excreted with feces or urine. Nicotinic acetylcholine receptors are relevant medical targets with potential for anti-schistosomal drug discovery. Ongoing research in structural and functional composition of those receptors lays the foundation of future drug development for Schistosomiasis.

The objective of this study was to identify the oligomeric composition of native nicotinic acetylcholine receptors of *S. mansoni* by affinity purifying them from adult worms and sending eluted samples for Mass Spectrometry. To further investigate their functionality, heterologous expression studies are conducted.

CHAPTER 2 –Affinity purification of native nicotinic LGICs and heterologous reconstitution of anion-selective nicotinic LGICs

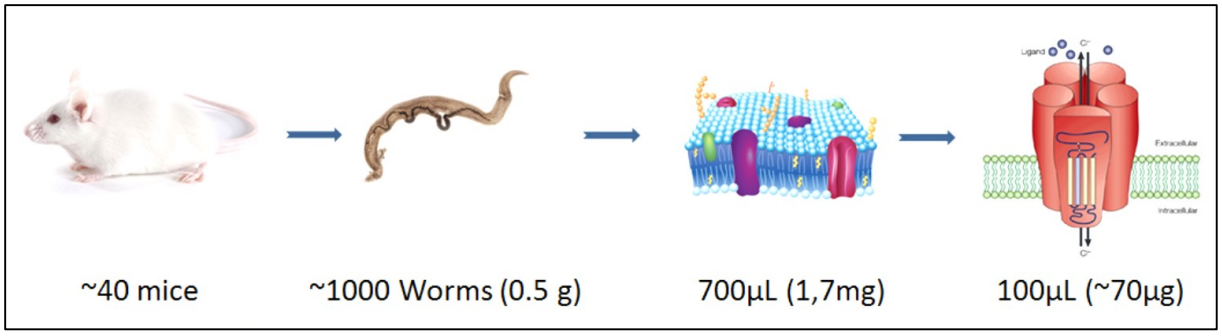
1. Introduction

The neurotoxin, α-bungarotoxin (α-BTX), has been extensively used to localize, identify and purify neuronal α-BTX-sensitive nicotinic acetylcholine receptors [39]. It is well established that that some nicotinic acetylcholine receptors possess high affinity for α-bungarotoxin, most famous being the α7 homomeric channel found in mammalian brain. Previous research has shown that α-BTX binds to putative nicotinic receptors located on the surface of schistosomes [34], suggesting that α-BTX also recognizes at least some of the parasite receptors.

In this study, we attempt to optimize an affinity purification method to selectively purify schistosomal nicotinic acetylcholine receptors based on α-BTX binding. For characterization of schistosome nicotinic acetylcholine receptors, the native channels will be affinity purified directly from worm tissue in the antagonist (α-BTX)-bound state and sent for mass spectrometry analyses.

One of the most widely used tools in biochemistry is the interaction between biotinylated proteins and streptavidin, where streptavidin acts as an immobilized matrix and the biotinylated ligand as a high -affinity bait for the protein of interest. It has been previously shown that biotinylated alpha-bungarotoxin (b- α-BTX) can be used to purify nicotinic receptors by immobilization onto streptavidin beads [32] and therefore we tested this approach to purify native receptors directly from worm tissue. There are several methods available for efficient

elution of biotinylated molecules from streptavidin-coated surfaces. In this study we evaluated and compared several of these methods using biotinylated-BSA and streptavidin- Magne™ beads, following elution with different reagents. These preliminary experiments helped us optimize conditions for subsequent purification of b-α-BTX- labelled worm receptors.



Figure#1: Simplified procedure for receptor purification, along with approximate amount of product generated after each step. Approximately 40 mice are infected through tail with cercaria. Six weeks later, roughly 25 adult worms are harvested from each dissected mouse. During the next step, worms are suspended in lysis buffer, incubated, homogenized and centrifuged multiple times to extract 700µl (1.7mg) of cell membrane proteins. Final product is eluted with 100µl of buffer solutions and contains roughly 70µg of protein.

If successful, this method will allow us to extract nicotinic channels free from contaminants that make studying their structure and function difficult. Extractions from adult worm tissue will provide us with native receptors in natural subunit conformations, something that is hard or even nearly impossible to achieve in heterologous expression systems. Purified samples can undergo mass-spectrometry to verify the identity of the proteins.

Nicotinic acetylcholine receptors (nAChRs) belong to the pentameric ligand-gated ion channel (LGIC) superfamily and can be anionic or cation- selective channels. nAChRs are relevant medical targets with potential for use in drug discovery. Additional research in structural and functional composition of these receptors would greatly aid in future drug development for schistosomiasis [11]. At present, assays that test channel functionality are labour intensive and require specialized electrophysiological equipment. Since LGICs are pentameric pores in the membrane, their structure is defined by five different subunits. Those subunits can be assembled in large number of variations. A new kind of high-throughput assay is required to test if the newly assembled channel is functional and to clarify the exact oligomeric composition of the receptor.

The second purpose of this research project is to optimize a high-throughput functional assay for anion-selective schistosomal LGICs using fluorescence technology. Recent publications and commercially available products have established that Yellow fluorescent protein (YFP) can act as an intracellular sensor to monitor anionic flux through the membrane [40]. Several mutations have been incorporated into the YFP-protein genetic code to further increase halide sensitivity (H148Q and I152L) [41]. Upon channel activation, anions start to flow through the LGIC and eventually come in contact with YFP, quenching its fluorescence. Such quench is easily measured by a fluorometer and indicates that a functional channel is present or that a new ligand is capable of channel activation (if channel is known to be functional). In theory, a fluorescence functional assay can be performed on whole cells expressing the channel of interest and can measure a fast response.

2. Materials and Methods

2.1 Parasites

Puerto Rican strain of *S. mansoni* was used throughout the study. Parasites were donated by the Biomedical Research Institute, Rockville, Maryland, USA. Cercaria were shed from *Biomphalaria glabrata* snails upon light exposure. 28 days old CD1 mice were infected via the tail. *S. mansoni* adult worms were obtained by portal perfusion and manual extraction after 6 weeks of infection.

2.2 Solubilisation of membrane proteins

This is the first step in the purification of native receptors. A schematic of the complete purification procedure is shown in Figure 2. Solubilized membrane proteins were obtained from adult worms using the Proteoextract® Native Membrane Protein Extraction kit (EMD, Millipore), according to the manufacturer's protocol. About 0.5 g of frozen adult male and female worms is thawed and suspended in 2 ml of lysis buffer (supplied with the kit) containing a cocktail of protease inhibitors at a final concentration of 2 mM (Complete Protease Inhibitor Cocktail and PMSF, Calbiochem). After incubation on ice for 20 min, samples are homogenized by 5 strokes with a hand homogenizer and tight fitting pestle. The lysate is centrifuged for 20 min at 20,000 rpm at 4ºC and the resulting pellets are re-suspended in 2 ml of lysis buffer supplemented with protease inhibitors and centrifuged again. The supernatant, which contains soluble proteins is discarded. The pellets are suspended in 0.7 ml of solubilisation buffer (supplied by the kit) plus protease inhibitors, and rotated for 1 hour. Insoluble material is

removed by centrifugation for 20 min at 20,000 rpm. 25 µg of monobiotinylated alpha-bungarotoxin in PBS (containing 1 mM $CaCl_2$ and 0.5mM $MgCl_2$) is added during the membrane solubilisation step and incubated at room temperature (RT) for 1 hour (2^{nd} step, Figure 2). After incubation, the sample is centrifuged and the supernatant is carefully removed. Protein concentration in the supernatant is measured by using the Pierce™ BCA Protein Assay Kit and following the manufacturer's protocol.

One of the samples (SDS elution) sent for Mass spectrometry was experimentally cross-linked using DTSSP right after labeling with α-bungarotoxin, but before solubilisation step. Membrane protein pellet was resuspended in DTSSP cross-linker solution (2 mM) and incubated on ice for 2 hours. 20 mM stop solution (1M Tris, pH 7.5) was used for 15 minutes to terminate the reaction. Membrane proteins were washed five times with 25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, pH 7.4.

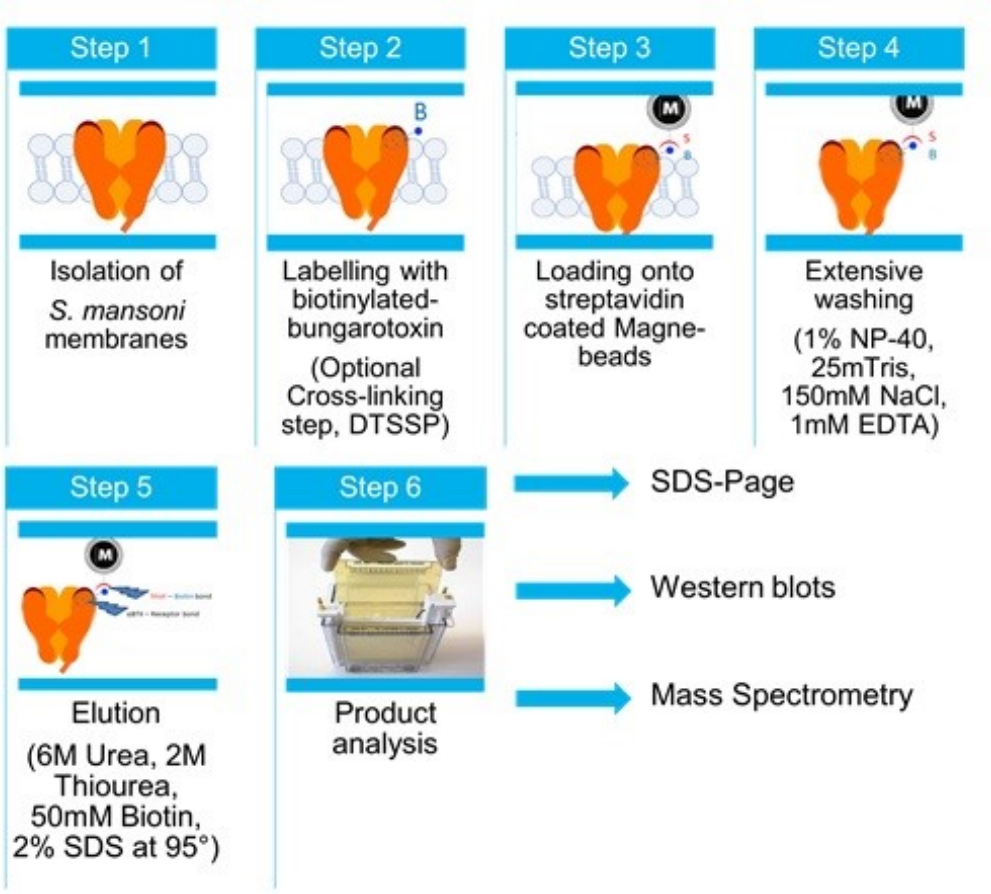


Figure #2: Native receptor purification protocol separated into six distinct steps.

2.3 Channel purification on monomeric streptavidin system

A 1.5 ml column containing Magne™ beads (Promega) linked to streptavidin is made following manufacturer's instructions and equilibrated with PBS. Before use, the irreversible biotin binding sites are blocked by 5 mM d-biotin. Solubilized membrane proteins labelled with mono biotinylated alpha-bungarotoxin are loaded onto the streptavidin beads and incubated at 4°C overnight (3rd step, Figure 2). After washing five times with 1ml of the following buffer: 1% NP-40, 25mTris, 150mM NaCl, 1mM EDTA (4th step, Figure #2), the complex is eluted from the

beads by addition of 100 µl of elution buffer (5th step, Figure 2). Breaking the strong bond between streptavidin and biotin requires harsh conditions and therefore we used an elution buffer that contained 6M Urea, 2M Thiourea, 2% SDS and 30 mM Biotin at 95C. These elution conditions are based on previous studies [36] and were optimized in advance by using biotinylated BSA as a control protein, as described below (see section 2.4). In other experiments, we used a different elution buffer that contained 1M Carbachol. Carbachol is known to bind to nicotinic receptors. When added in excess, carbachol is expected to compete with α-BTX, causing the receptor to dissociate from the beads [42].

2.4 Search for optimal elution buffer to disrupt biotin-streptavidin bond

Biotinylated BSA (b-BSA) was used as a control protein in preliminary experiments to optimize elution of biotinylated proteins from streptavidin beads. Aliquots (500 µL) containing 100 µg of b-BSA were added to 100 µL streptavidin coated magnetic beads, which had been previously washed two times with wash buffer (1% NP-40, 25mTris, 150mM NaCl, 1mM EDTA) and two times with PBS. After 1 hour of incubation, beads were washed 5 times with 1ml of 1% NP-40, 25mTris, 150mM NaCl, 1mM EDTA. The following elution conditions were then tested using the same elution volume of 100µl: (1) 70°C Water; (2) 50mM Biotin; (3) 6M Urea, 2M Thiourea, 50mM Biotin, 2% SDS at 95°C; (4) 6M Urea, 2M Thiourea, 50mM Biotin, 2% Sulfobetaine at 95°C.

2.5 Proteomics analysis

Protein concentrations were measured with Pierce BCA Protein Assay Kit. Samples were dissolved in Invitrosol LC/MS Protein Solubilizer, Invitrogen according to the manufacturer's protocol and diluted five times with 50 mM ammonium bicarbonate (pH 8.5). The protein mix was intensely vortexed and heated to 60ºC for 30 min, reduced with 50 mM DTT (60ºC/30 min) and treated with 5 mM iodoacetamide (room temperature in the dark/30 min). Samples were incubated in Promega Gold Mass Spectrometry Grade Trypsin (1:50 enzyme:substrate) for 14-16 h at 37ºC with shaking. Trifluoroacetic acid was used to stop digestion. Peptide purification was performed with C_{18} Zip-Tip technology (Millipore) and dried in vacuum centrifuge. Peptide sequencing was accomplished using ESI-LC-MS/MS system, LTQ-Orbitrap Elite hybrid mass spectrometer with a nanoelectrospray ion source (ThermoFisher, San Jose, CA). MS spectra were acquiredat a resolution of 60 000 at m/z 400. MS/MS spectra were obtained using collision-induced dissociation for multiply charged ions exceeding a threshold of 10000 counts.

2.6 Protein data analyses

MS data were obtained using Xcalibur (v. 2.0 SR1) and peaks were generated with Mascot distiller (v. 2.1.1, Matrix Science). Searches were performed against *S. mansoni* proteome at National Center for Biotechnology Information using MassMatrix (v. 2.4.2) with the following parameters: Trypsin digestion with two missed cleavages allowed, cysteine carbamidomethylation as a fixed modification, methionine oxidation as variable modification, 0.8 Da tolerance for fragment ion masses, 10 parts per million mass tolerance for precursor ion masses and a minimum peptide length of 6 amino acids. Matched were considered significant if

false discovery rate was less than 5% and if they exceeded the threshold defined by MassMatrix [43, 44]. Protein identification was based on >2 unique matches with greater than 95% confidence and at least 6 amino acids per peptide.

2.7 Antibodies Production, Purification and Western Blots

Polyclonal antibodies against three *S. mansoni* nAChR subunits, smp_176310, smp_142690 and smp_031680 were purchased from 21st Century Biochemicals (Marlboro, MA). Antibody against smp_031680 was produced in rabbits against two synthetic peptides (YSRNGEFHLSGSSVRRYAQRYEC & PYRLYNSVGNFNSKHIQDL) corresponding to positions 205-227 and 566-584 of the protein sequence. Antibody against smp_176310 was produced in rabbits against two synthetic peptides (NAKVNRFGKPHGNKFC & CSKKALSAANAKWNSPLQY) corresponding to the third intracellular loop of the protein. The smp_142690 antibody was raised in rabbits against two synthetic peptides (TDGEAERHIRHEDRVHQLRSVC & LQNINMKQIKLEYKNSLGC) located at the N- and C-terminal ends, respectively. Each peptide was conjugated to ovalbumin as a carrier protein. Peptides were found to be highly specific upon BLASTp analysis against the *S. mansoni* genome [12]. The sera were affinity-purified, using the peptide antigens with the aid of Sulfolink Immobilization kit for peptides (Thermo Scientific) as described in the kit protocol.

In order to perform western blots, solubilized membrane proteins were obtained from adult worms using the Proteoextract® Native Membrane Protein Extraction kit, according to the manufacturer's protocol. Protein assays were performed according to the Bradford method, using the Bio-Rad Protein Assay kit to determine protein concentrations. Approximately 7 µg of

solubilized membrane proteins were resolved in a 4-12% tris-glycine SDS-PAGE gel (Life technologies), transferred onto PVDF membrane and probed with peptide-purified primary antibody (1: 1000) followed by incubation with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit polyclonal secondary antibody (1:10,000) (Life science). A. A mouse monoclonal anti-FLAG M2 antibody was purchased from Sigma-Aldrich.

2.8 Fluorescence microscopy

Confocal microscopy was performed according to the method of Mair et al. (2000) [45] with some modifications. Adult worms were flat-fixed between two glass slides with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS 0.1M, pH 7.4) for 4 hours and washed three times with PBS at 4°C. An additional wash with PBS supplemented with 0.1 M glycine was performed to reduce non-specific background due to unquenched fixation.

Samples were permeabilized with 1% SDS for 20 minutes at room temperature (RT) with end-over-end rotation, washed five times with blocking buffer (0.1 M PBS, pH 7.4, containing 0.1% Triton X-100, 1% bovine serum albumin and 0.1% sodium azide) at RT and then incubated overnight in the same buffer at 4°C. α-Bungaratoxin-FITC was added (1:50 dilution in blocking buffer) and samples were incubated for two days with end-over-end rotation at 4oC. Subsequently, the worms were washed seven times at RT with blocking buffer before examination by scanning confocal microscopy (Nikon LSM 710 with ZEN 2011 software package).

2.9 Immunoprecipitation

Proteins of interest were immunoprecipitated from 0.5ml (0.5mg) of Solubilized membrane proteins previously obtained from adult worms, according to the manufacturer's protocol. The immunoprecipitation was performed using a commercial kit (Thermo Scientific Pierce Direct IP Kit). 8µg of anti-Smp_176310 (anti-ACC-1) polyclonal antibody was desalted and used for agarose column coupling. Column was washed twice with 200µl of IP Washing Buffer (Supplied by the kit). 500µl of membrane proteins were loaded and left to rotate for 1 hour at 4ºC. Sample was washed three times with 200 µl of Tris-Buffered Saline and once with 100µl of 1x Conditioning Buffer (Supplied by the kit). Elution was performed using 100µl of 0.2 M glycine (pH 2.6), incubated for 10 minutes and neutralised with 8µl of Tris (pH 9.5). For the western analyses we used antibodies against ACC-1 (smp_176310) and ACC-2 (smp_142690).

2.10 Plasmid purifications

Plasmid DNA is isolated from previously transformed *E. coli* cells using a commercially available plasmid purification kit (QIA filter Plasmid Midi kit, Qiagen). 25 ml of overnight culture is centrifuged for 15 minutes at 13,000–16,000g. The pellet is completely resuspended in 4ml of P1 buffer (50mM Tris-Cl, pH 8.0, 10mM EDTA, 100ug/mL RNase A). 4ml of P2 solution (200mM NaOH, 1% SDS) is added and the content is mixed by inverting the tube 4-6 times. The tube is incubated for 5 minutes at room temperature. 4ml of P3 buffer (3.0M potassium acetate, pH 5.5) is added and the content is mixed again by inversion. Solution is poured into the barrel of the Qiagen-filter; plunger is used to filter the cell lysate into pre-equilibrated tip. Qiagen-tip should be equilibrated with 4ml of QBT buffer (750mM NaCl,

50mM MOPS, pH7.0, 15% isopropanol, 0.15% Triton X-100). The tip is washed 2 times with QC buffer (1.0M NaCl, 50mM MOPS, pH 7.0, 15% isopropanol) and DNA is eluted with 5 ml of QF buffer (1.25M NaCl, 50mM Tris-Cl, pH 8.5, 15% isopropanol). DNA is precipitated with isopropanol and centrifuged for 30 minutes at 13,000–16,000g. Supernatant is decanted and pellet is washed with 2ml of 70% ethanol. DNA is centrifuged again for 10 minutes and air-dried. Assessments of purity are performed by Nanodrop-1000 spectrophotometer based on 260/280nm light absorption ratio.

2.11 Cell Culture and Transfection

For mammalian expression studies, human codon-optimized constructs of smp_176310 and smp_142690 were synthesized (Genescript, USA) and inserted into the pCi-Neo (Promega) expression vector, using NheI and NotI restriction sites. A C-terminal FLAG tag was also included in the smp_176310-Neo construct to aid in the monitoring of expression. As a positive control we used human Glycine Receptor α1 inserted into the pRK5 expression vector, which is known to form a functional channel [19]. HEK-293 cells are routinely cultured with Dulbecco's Modified Essential Media (DMEM) supplemented with 20 mM HEPES and 10% heat inactivated fetal bovine serum (FBS). Cells were transiently transfected with either the subunit of interest or empty vector (mock transfection), using XtremeGENE 9 transfection reagent (Roche), as recommended by the protocol. For selection of stable transfectants, HEK-293 cells were cultured for 4 weeks in DMEM supplemented with 10% heat inactivated FBS and 1mg/ml G418 Geneticin, starting at 24 hours post-transfection. Protein expression was routinely monitored by in situ immunofluorescence (see section 3.3.1 below). HEK cells were immunoreactive for the

subunits of interest when probed either with specific antibody or anti-FLAG antibody. No immunofluorescence was noted in the negative control cells transfected with empty plasmid.

2.12 YFP-based Assay

For functional assays, transient or stably transfected cells expressing a subunit of interest were transduced with a YFP –like fluorescence sensor (Premo Halide Sensor, Invitrogen), as recommended by the kit protocol, and seeded on a 96-well plates at a density of 100 000 cells per well for the iodide (I-) flux assay. After 8-12 hours incubation at 37°C, 5% CO2, growth media was removed and cells were washed with 150µl of HBSS (Hank's Balanced Salt Solution) once and allowed to equilibrate. YFP fluorescence was measured for 40s before and for 2 minutes after the addition of test compounds with 500nm excitation and 540nm emission settings. 100µl of iodide buffer (140 mM NaI, 20 mM Hepes, 5 mM KCl, 2 mM CaCl2,1 mM MgCl2) containing test compound was added to each well so that the final concentration was 100 µM of agonist in a total volume of 200 µl. Iodide buffer without agonist compound and mock transfected cells treated with the agonist were used as negative control. Channel activity is calculated by measuring the reduction in YFP fluorescence (ΔRF) due to anionic influx over the time of measurement. A fluorescence measurement is taken 40s before the addition of drug (RFinitial) and again after a period of 120s (RFfinal). The RFfinal is subtracted from the RFinitial to generate ΔRF. ΔRF is then divided by the RFinitial and multiplied by 100, resulting in a measurement of %YFP quench. Readings are normalised to water treated controls. Student's t-tests are performed to determine statistically significant differences at $P < 0.05$. Data is acquired for a total of 150 seconds with fluorescence recording at 2 second intervals.

2.13 Confocal microscopy of HEK-293 cells

To test the surface expression of our subunits of interest, we examined the HEK-293 cells by confocal microscopy (Nikon LSM-710) after immunolabeling with specific polyclonal antibodies against smp_142690 (produced in rabbits) and mouse monoclonal anti-FLAG M2 antibody, purchased from Sigma-Aldrich. HEK cells were fixed in 4% paraformaldehyde in PBS buffer (0.01M, pH 7.4) for 30 minutes, washed three times for 10 minutes in the PBS buffer, permeabilized with 0.1% Triton-X for 10 minutes and incubated overnight in PBS supplemented with 3% bovine serum albumin (BSA). The following day cells were incubated overnight at 4oC with the specific antibody (1:100 dilutions for both smp_142690 and anti-FLAG M2) in the BSA supplemented PBS buffer. Cells were then washed three times for 10 minutes at room temperature, again with the BSA supplemented PBS buffer and incubated overnight with secondary antibodies: Alexa Fluor 488 conjugated goat anti mouse IgG and Alexa Fluor 594 conjugated goat anti rabbit IgG both at a dilution of 1:200 in BSA supplemented PBS buffer. HEK cells were then washed 5 times, each for 10 min with PBS at room temperature before examining them by confocal microscopy.

3. Results

3.1 Confocal analysis of adult worms labelled with fluorescent BTX

To test if α-bungarotoxin binds to worm tissue, we labelled adult worms with a commercially available α-bungarotoxin-AlexaFluor488 (ThermoFisher) conjugate and then examined the animals by confocal microscopy. As a counterstain, we used phalloidin- TRITC (tetramethylrhodamine B isothiocyanate), a high-affinity F-actin probe conjugated to red fluorescent dye, to stain muscle fibers of adult worms.

Based on previous studies, we expected α-bungarotoxin to stain the nervous system of the parasite and / or the tegument, where nicotinic receptors are known to be present [24, 34]. Surprisingly, staining was observed only in reproductive structures, more specifically vitelline glands, found exclusively in females (Figure 3). These glands produce yolk cells that surround the newly produced parasite egg. No significant staining was observed in male adults that lack those structures. The unlabelled negative controls showed no visible fluorescence, suggesting the signal is probably not caused by autofluorescence associated with the vitellaria. However, we cannot rule out the possibility that the signal may be non-specific. Attempts to compete the α-bungarotoxin-AlexaFluor488 labeling with a 10-fold excess of unlabelled α-bungarotoxin were unsuccessful, as we failed to observe any reduction in the labeling of the vitelline glands.

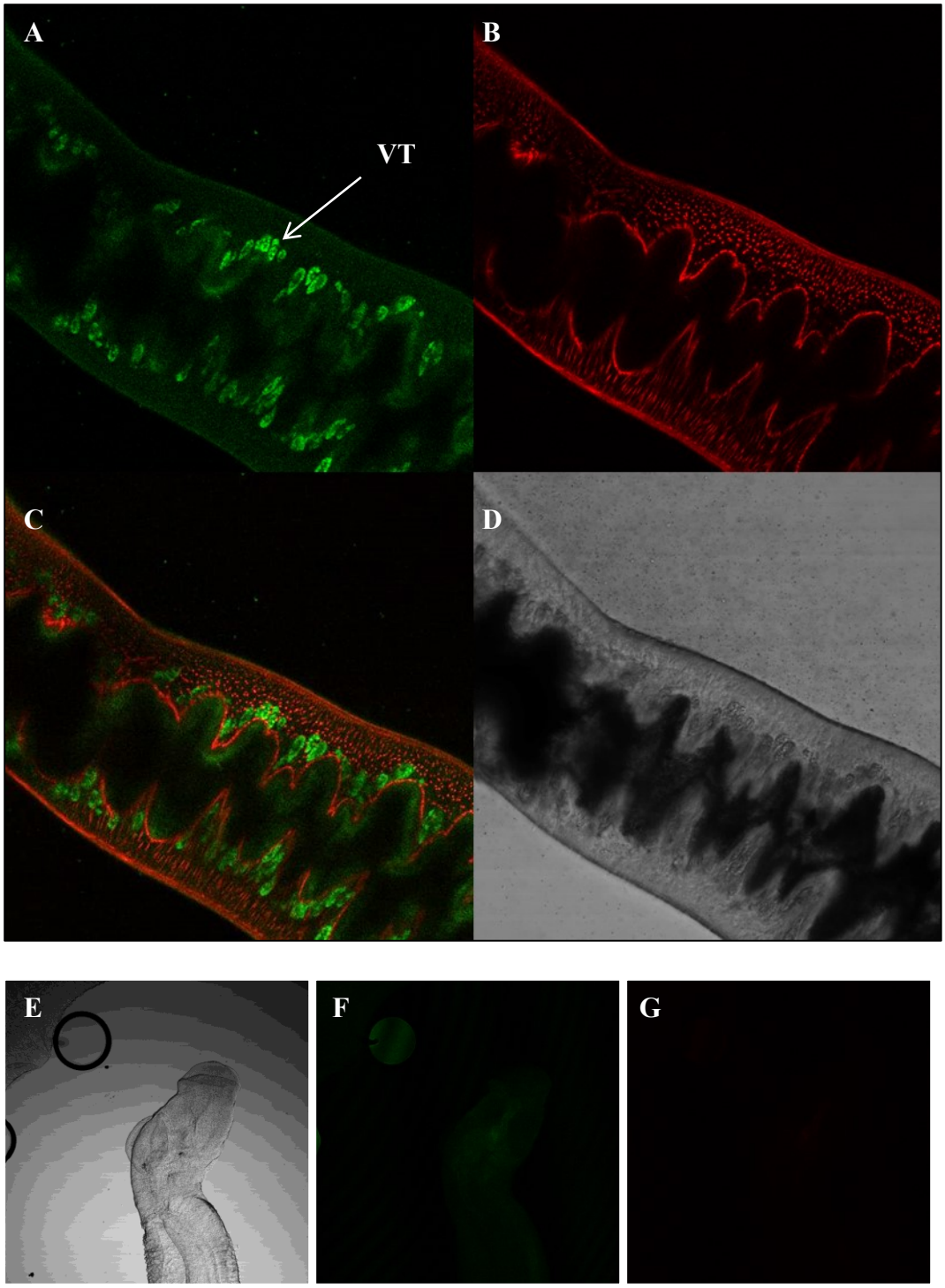


Figure #3: Dual labeling with α-bungarotoxin-AlexaFluor488 and phalloidin-tetramethylrhodamine B isothiocyanate (TRITC). (A) – Midbody of female adult *S. mansoni,* stained with α-bungarotoxin-AlexaFluor488 (ThermoFisher) conjugate. Bright green signal is

observed in vitelline glands (VT). No such pattern is found in male adults (not shown). (B) – Staining with phalloidin- TRITC conjugate shown distinct muscle fiber labeling. (C) – An overlay of (A) and (B) shows non-overlapping expression patterns in adult female worms. (D) – Brightfield transmission image of the same part of female body. (E) – Transmission image of *S.mansoni* head region. (F) – Autofluorescence control picture of (E) in 488nm excitation spectra. (G) – Autofluorescence control picture of (E) in 594nm excitation spectra.

3.2 Purification of native *S. mansoni* nicotinic receptors

3.2.1 Optimization of purification conditions using biotinylated BSA

The method used in this study is based on the labeling of receptors with biotinylated BTX (b-BTX), followed by affinity purification of biotinylated proteins on streptavidin beads. The biotin-streptavidin interaction is one of the strongest non-covalent bonds known, with a dissociation constant (Kd) close to 4×10^{-14} M. The extraordinary strength and specificity of this bond makes it one of the most commonly used in affinity purification assays. However, due to its stability in a wide range of temperatures and pH, the biotin-streptavidin bond can be difficult to break. Recent studies have shown contradicting evidence, with some research groups claiming that the bond can be reliably broken simply by eluting in 70ºC water or buffer containing an excess of biotin, while other researchers claim that very harsh conditions are required, including the addition of detergents, chaotropic agents (e.g. urea) and 95ºC heating [36, 39]. In light of this conflicting evidence, we decided to optimize conditions first with a biotinylated control protein (b-BSA), which was loaded onto streptavidin beads and then eluted with various buffers. The results (Figure 4) show that elution with 70ºC water was ineffective;

there was little protein detected in the 70ºC water eluate (Fig. 5, lane A) whereas a large amount of protein was recovered after a second elution with harsh elution buffer (Fig. 5, lane E), suggesting that most of the protein remained on the streptavidin beads. 50 mM Biotin was more efficient at eluting b-BSA (lane B) compared to water; however, about half of the protein still remained on the beads (lane F). Finally, elution buffers containing detergents and chaotropic agents proved most effective in recovering b-BSA from streptavidin beads. A solution of 6M Urea, 2M Thiourea, 50mM Biotin and 2% SDS, which was heated to 95ºC removed most of the b-BSA added to the beads (Lanes D and G). Replacing SDS with Sulfobetaine (Invitrosol™) under the same elution conditions (6M Urea, 2M Thiourea, 50mM Biotin and heated to 95ºC) produced essentially the same results (Lane C). Seeing how the two latter methods gave us efficient elution, we decided to use these elution buffers for nAchR purification. The SDS containing buffer was used for routine purification experiments and western blot analyses. For proteomics analyses of purified receptors, we used the Sulfobetaine-containing buffer instead, since SDS is not mass-spectrometry compatible [46].

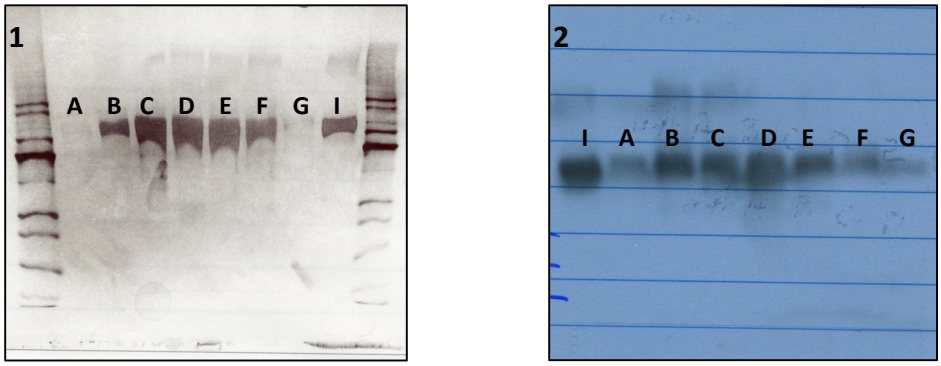


Figure #4: Elution of biotinylated BSA (b-BSA) from streptavidin beads. B-BSA was loaded onto streptavidin beads, washed extensively and eluted under different conditions (1)

– SDS PAGE 4-12% Tris-Glycine gel silver-staining after elution of biotinylated BSA (2) – SDS PAGE 4-12% Tris-Glycine gel probed with Streptavidin-HRP after elution of biotinylated BSA using different conditions. (A) – Biotinylated BSA eluted from streptavidin beads with 70º water. (B) – Biotinylated BSA eluted from streptavidin beads with 50mM Biotin. (C) – Biotinylated BSA eluted from streptavidin beads with 6M Urea, 2M Thiourea, 50mM Biotin, Invitrosol™ (Sulfobetaine), 95ºC. (D) – Biotinylated BSA eluted from streptavidin beads with 6M Urea, 2M Thiourea, 50mM Biotin, 2% SDS, 95ºC. (E) – Elution of remaining Biotinylated BSA from (A) with (D). (F) – Elution of remaining Biotinylated BSA from (B) with (D). (G) – Elution of remaining Biotinylated BSA from (C) with (D). (I) – Loading Control, 1µg of Biotinylated BSA loaded straight into SDS PAGE.

3.2.2 Purification of b-BTX-labelled *S. mansoni* proteins from streptavidin beads using harsh elution conditions

Solubilized *S. mansoni* membranes (1.7 mg of protein) labelled with b-BTX were loaded onto streptavidin beads and proteins were eluted using the harsh conditions described above. Approximately 70 μg of protein was typically recovered from the beads under these conditions. Initially protein purification was monitored by western blot analysis of the eluates using streptavidin-HRP to detected b-BTX-labelled proteins. Given that b-BTX is likely to dissociate from the receptor due to the harsh elution conditions, blots were routinely treated with an excess of b-BTX and washed extensively to remove unbound BTX prior to probing with streptavidin-HRP. The results show a number of sharp bands appearing on western blots when probed with streptavidin-HRP, most notable a band of approximately 180 kDa and several smaller bands between 64 and 115 kDa (Figure 5). Two different lanes shown on Figure 5 correspond to R – receptor eluted without DTSSP cross-linking step during membrane proteins solubilisation and Cr – receptor eluted after DTSSP cross-linking step (Methods 2.2). Two additional bands are visible on Cr, suggesting the presence of some high molecular weight complexes.

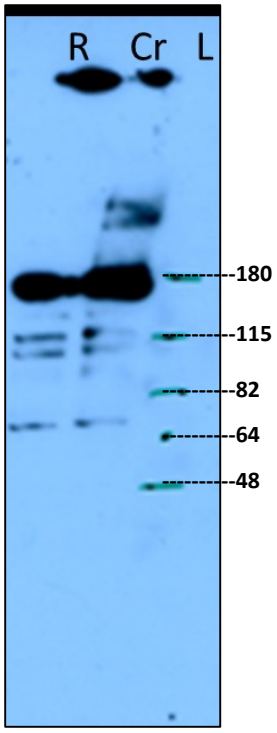


Figure #5: Western blot analysis of b-BTX labelled *S. mansoni* proteins after purification from Streptavidin beads. Blots were probed with Streptavidin HRP to detect biotinylated proteins recovered from the beads (L) – Lane with Pre-stained protein ladder in kDa. (R) - Lane with b-BTX labelled *S. mansoni* proteins eluted with 6M Urea, 2M Thiourea, 50mM Biotin, 2% SDS, 95ºC. (Cr) - b-BTX labelled *S. mansoni* proteins were cross-linked and then purified using the same harsh eluation buffer.

To test if the streptavidin-HRP positive bands correspond to nicotinic receptors, we repeated the western blot analysis with antibodies against specific receptor subunits. b-BTX labelled *S. mansoni* proteins were purified on streptavidin beads as above and eluted with the same harsh buffer (Figure 6). Aliquots of the eluates were resolved by SDS-PAGE and immunoblotted with antibodies against three different nicotinic receptor subunits (Fig. 6, lanes 1-

3) or an irrelevant control (lane 4). The subunit-specific antibodies were previously shown to detect proteins of the expected size: 94 kDa for 176310, 64 kDa for 142690 and 90 kDa for 031680. However, the western blots all produced the same size band, roughly 130 kDa. We also detected a band of about the same size in the irrelevant antibody control (lane 4) suggesting this band is likely non-specific. No bands of the expected subunit sizes were detected in any of the blots.

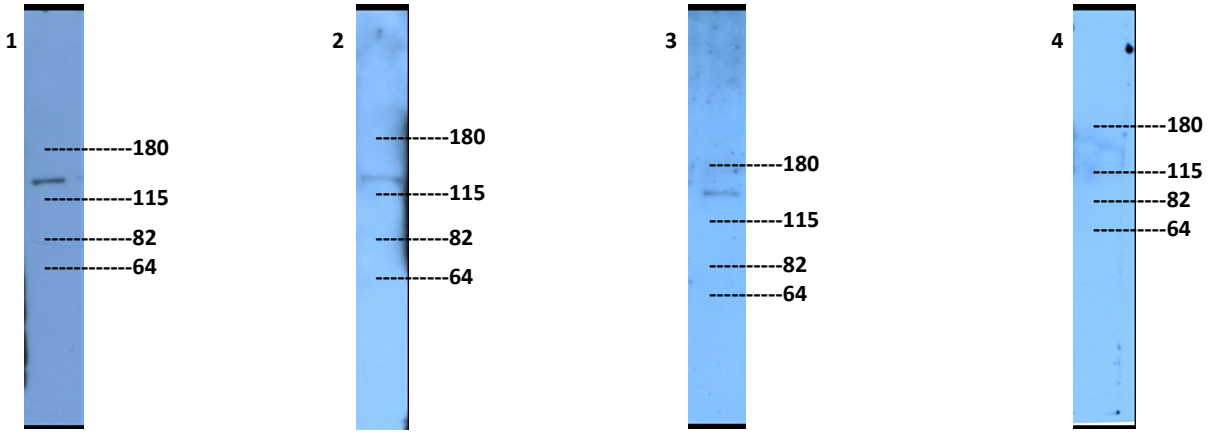


Figure #6: Western blot analysis of purified b-BTX-labelled *S. mansoni* proteins. Proteins were eluted from streptavidin beads using harsh elution conditions (6M Urea, 2M Thiourea, 50mM Biotin, 2% SDS, 95ºC) and probed with antibodies against specific nicotinic receptor subunits (1) – Western blot probed with antibody against 176310 (ACC-1) receptor subunit. (2) – Western blot probed with antibody against 142690 (ACC-2) receptor subunit. (3) – Western blot probed with antibody against 031680 receptor subunit. (4) – Western blot probed with antibody against SmGPR-3 receptor (Irrelevant control).

In other control experiments, we repeated the purification using *S. mansoni* membrane proteins that were labelled with biotin instead of biotinylated BTX (Figure 7, B). Biotin-treated proteins were loaded onto streptavidin beads, washed, eluted under the same harsh conditions and finally tested by western blotting with streptavidin HRP. By replacing b-BTX that binds to nAChRs with biotin, we abolish any possibility for selective purification and instead collect non-specifically interacting proteins. The results show virtually the same pattern of western positive bands in eluates derived from biotin- or b-BTX-labelled proteins (Fig. 7, A). Moreover, when the eluates were probed with subunit-specific antibodies, only the previously described 130kDa band could be consistently detected (Fig. 7, A 2 and 3). Thus we conclude that the bands described in Figures 6 and 7 are due to non-specific interactions of worm proteins with streptavidin beads since they can be seen even in the absence of BTX. The inability to detect receptor subunits in the eluates using specific antibodies is further indication that the purified proteins are probably non-specific. It is possible, however, that BTX-labelled receptors were purified but the levels were too low to be detected by western blotting, or that the antibodies used in this study did not recognize the subunits of the purified channel.

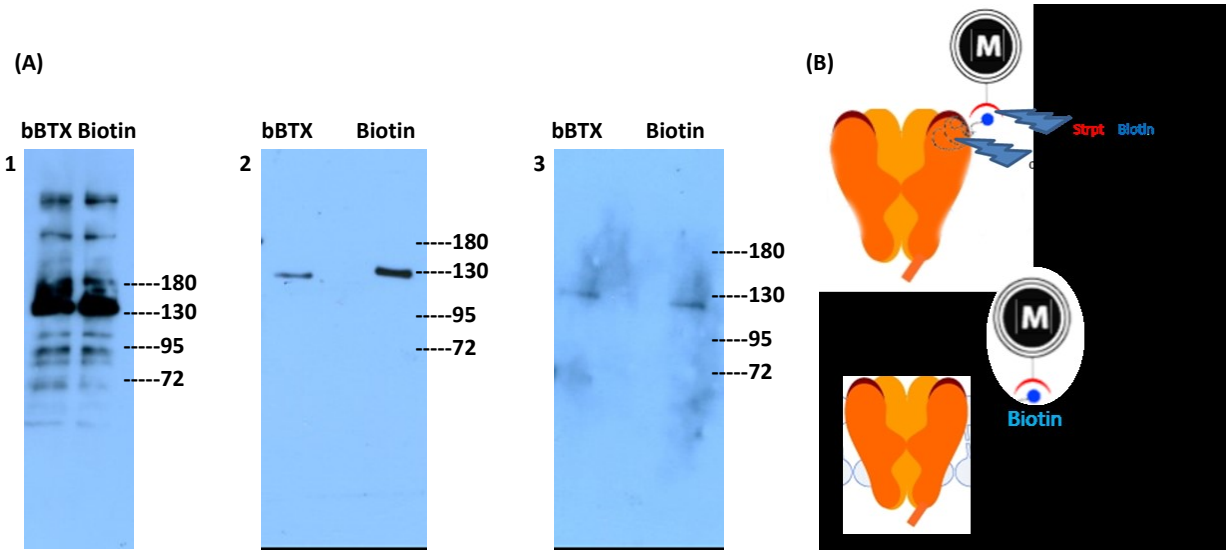


Figure #7: Western blots of control extractions and schematic representation of the concept behind them. (A) – Western blots probed with biotinylated α-bungarotoxin, streptavidin-HRP and antibodies. (1) – Western blot probed with biotinylated α-bungarotoxin and streptavidin-HRP. (2) – Western blot probed with antibody against 176310 (ACC-1) receptor subunit. (3) – Western blot probed with antibody against 142690 (ACC-2) receptor subunit. (B) - Schematic representation of the concept behind control extractions.

3.2.3 Purification of b-BTX-labelled *S. mansoni* proteins from streptavidin beads using an excess of carbachol

The protein complex designed to purify any potential receptor is schematically represented on Figure #7, B and can potentially be eluted by breaking either one of two bonds. However, αBTX – Receptor bond is not as strong as Biotin – Streptavidin and should be our primary target. Required conditions for Biotin – Streptavidin dissociation are extremely harsh

(6M Urea, 2M Thiourea, 50mM Biotin, Invitrosol™ (Sulfobetaine), 95ºC) and could lead to unpredictable receptor denaturation that makes the following analysis difficult.

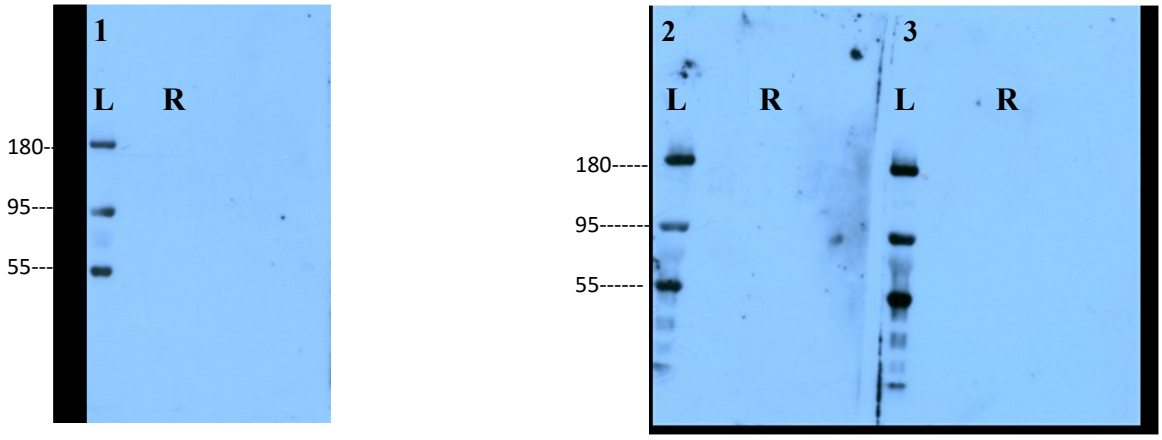


Figure #8: Purification of b-BTX-labelled *S. mansoni* receptors: Elution with competitive cholinergic ligand, carbachol (1M) (L) – Lane with Pre-stained protein ladder in kDa. (R) – Lane with potential receptor in Carbachol eluate. (1) – Western blot probed with antibody against 176310 (ACC-1) receptor subunit. (2) – Western blot probed with streptavidin-HRP. (3) – Western blot probed with biotinylated α-bungarotoxin and streptavidin-HRP.

One option that comes to mind is to elute the receptor by adding an excess of non-biotinylated bungarotoxin (BTX) but that strategy is prohibitively expensive when considering the cost of BTX. Luckily, there's another option that has recently been described [42], and it employs Carbachol that competes with α-BTX for receptor binding site. nAChRs and associated proteins are selectively eluted from streptavidin by one-hour incubation with 100 µl of 1M carbamylcholine chloride (carbacol)and precipitated with acetone for sixty minutes at -20ºC before SDS PAGE. Results of this method are presented in Figure #6. We failed to observe any

bands even on over-exposed western blots (5 minutes), when probed with antibodies or bBTX\Streptavidin-HRP. When quantifying the eluate with Pierce BCA Protein Assay Kit, no protein was detected (<20 µg/mL). It is possible, nonetheless, that resulting amount of eluted receptor is too small to be reliably detected by Western Blots. Original studies that describe this method were isolating α7 nAChR from 1 gram of rat brain tissue, sample that is initially enriched as compared to our experiment were extraction begins with 0.5g of adult worm bodies.

3.2.4 Protein mass-spectrometry data analyses

Three samples were sent for mass-spectrometry analyses: (1) Cross-linked b-BTX-labelled *S. mansoni* proteins eluted from streptavidin beads with 6M Urea, 2M Thiourea, 50mM Biotin, 2% SDS, 95ºC. (2) b-BTX-labelled proteins eluted from streptavidin beads with 6M Urea, 2M Thiourea, 50mM Biotin, 2% SDS, 95ºC; (3) b-BTX labelled proteins eluted from streptavidin beads with 6M Urea, 2M Thiourea, 50mM Biotin, Sulfobetaine (Invitrosol), 95ºC.

In the first sample, DTSSP (3,3'-dithiobis(sulfosuccinimidyl propionate)), a cross-linking reagent with a 8-carbon spacer arm was used after b-BTX labelling and before membrane-protein solubilisation in hopes of binding together receptor subunits and improving chances for successful purification [47]. In the third sample, SDS was replaced by mass-spectrometry compatible Invitrosol (Sulfobetaine), which does not interfere with protein ionisation spectra. Samples that contained SDS went through Tube-Gel Digestion as described by [Lu X et al., 2005],[48], to remove any MS non-compatible detergent.

Three different subunits were detected based on at least 2 matching peptides. Overall, one subunit (142690 or ACC-2) was detected in all three samples. 176310 or ACC-1 was found in two samples, along with 031680. Protein sequence coverage was relatively low and never exceeded 10%. No other nicotinic receptor subunit could be detected in the three samples. As can be seen on Table 1, cross-linking improved the results somewhat by increasing the number of identified peptides and spectral matches.

Taking these numbers into consideration and looking back at our attempts to detect those subunits by western blots, it is not surprising that we failed to find them. The low number of spectral matches indicates that the subunits were purified at very low levels, likely too low to detect on a gel or by western blotting.

The subunits shown in Table 1 were not the only proteins eluted from the streptavidin beads. The proteomics analysis identified at least 36 other proteins that were present in all 3 samples and had at least 2 matching peptides (data not shown). These included numerous membrane-associated signaling proteins, cytoskeletal proteins and also novel hypothetical proteins. It is unknown at present if these additional proteins were purified through interactions with worm nicotinic receptors (i.e. whether they are part of the receptor's "interactome") or if they bind non-specifically to streptavidin beads.

Crosslink SDS			SDS			Sulfobetaine		
	# of Peptides Identified	**# of Spectral Matches**		# of Peptides Identified	**# of Spectral Matches**		# of Peptides Identified	**# of Spectral Matches**
Smp_142690 ACC-2	7	9	Smp_142690 ACC-2	2	7	Smp_142690 ACC-2	3	3
Smp_176310 ACC-1	3	5	Smp_176310 ACC-1	2	4			
smp_031680	1	1				smp_031680	2	3

Table #2: Summary of protein data analyses and comparison between different elution methods based on the number of identified peptides and spectral matches. Crosslink SDS – Cross-linked receptor eluted from streptavidin beads with 6M Urea, 2M Thiourea, 50mM Biotin, 2% SDS, 95ºC; SDS – Receptor eluted from streptavidin beads with 6M Urea, 2M Thiourea, 50mM Biotin, 2% SDS, 95ºC; Sulfobetaine (Invitrosol) – Receptor eluted from streptavidin beads with 6M Urea, 2M Thiourea, 50mM Biotin, Invitrosol, 95ºC.

3.2.5 Immunoprecipitation of *S. mansoni* nicotinic receptors

In hopes of improving our receptor purification method, we have looked towards immunoprecipitation as an alternative approach to b-BTX-labelling and streptavidin-based purification [49]. Immunoprecipitation (IP) has been extensively used to purify proteins, DNA and other macromolecules from various sources. Fortunately, we have antibodies against some of the potential receptor subunits, which can be used in these studies. Considering how each nAChR is expected to have at least one α-subunit and how ACC-1 (a predicted α-subunit) was

found twice in mass-spectrometry samples, we decided to use anti-ACC-1 (smp_176310) as the primary antibody for the IP. Anti-ACC-1 antibody was first covalently attached to beads, using a commercial kit. Solubilized *S. mansoni* membrane proteins (0.5 mg protein) were loaded onto the column, which was washed extensively, and proteins were eluted with 0.2 M glycine (pH 2.6). Aliquots of the eluate containing 7 µg of protein were then probed with the same anti-ACC-1 antibody or an antibody against a different subunit, ACC-2 (smp_142690) (Fig. 9). The results show immunoreactive bands of the expected size on both western blots. This suggests that IP allows for more efficient purification of nicotinic receptors than the b-BTX-streptavidin system. The fact that IP targeting ACC-1 also recovered ACC-2 suggests these two subunits may interact in the worm and therefore could be components of the same channel.

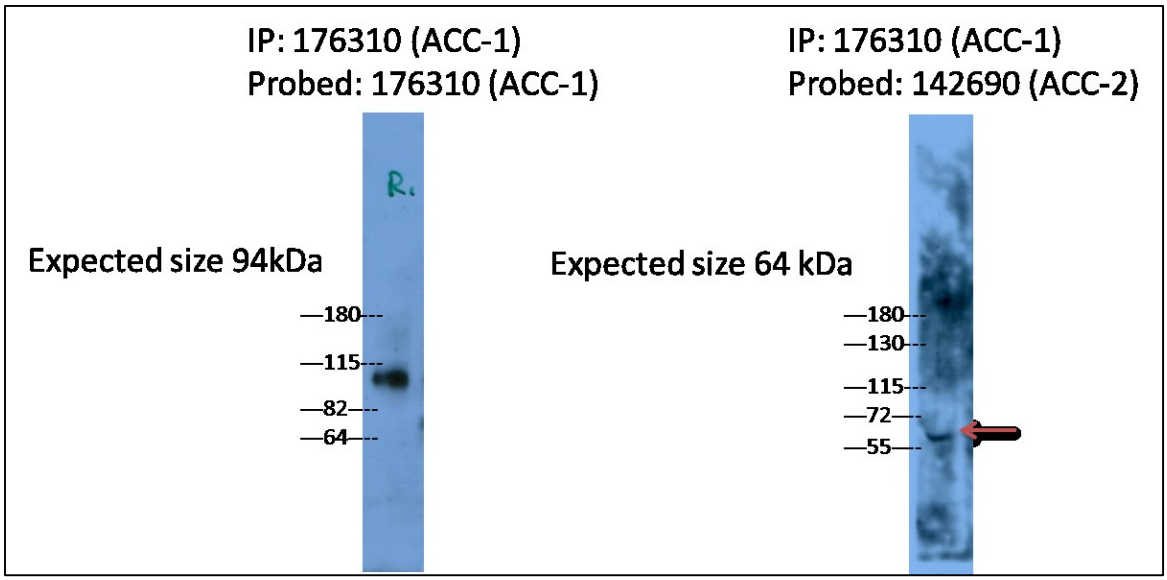


Figure #9: Preliminary results of alternative receptor purification strategy based on immunoprecipitation (IP). IP was performed on beads covalently attached to anti-176310 (ACC-1) antibody. Protein eluted from the beads were analyzed by western blotting with anti-176310

(ACC-1) or anti- 142690 (ACC-2) antibodies. Primary antibody titer 1:500. Secondary antibody titer 1:10000.

3.3 Heterologous expression of *S. mansoni* nicotinic receptors in cultured HEK293 cells

3.3.1 Selection of stable transfectants with G418 Geneticin

During the second part of our project we attempted to express and functionally evaluate some of the subunits found by mass-spectrometry (ACC-1 and ACC-2). HEK293 cells were transfected with codon-optimized (humanized) ACC-1 or ACC-2 and protein expression was monitored by *in situ* immunofluorescence of transfected cells, using polyclonal antibodies against smp_176310 (ACC-1) and smp_142690 (ACC-2). Initially G418 (Geneticin) was used to select HEK-293 cells that stably express either one of two subunits [50]. As can be seen in Figure 10, our attempts to obtain stably transfected lines were not successful. In both cases, after transfection with the subunit of interest, protein expression levels decreased with each passing day despite continuous selection with G418, until almost no expression was visible. A possible explanation is that the expression of these subunits is toxic to the cells such that continuous selection results in progressive loss of those cells expressing the subunits.

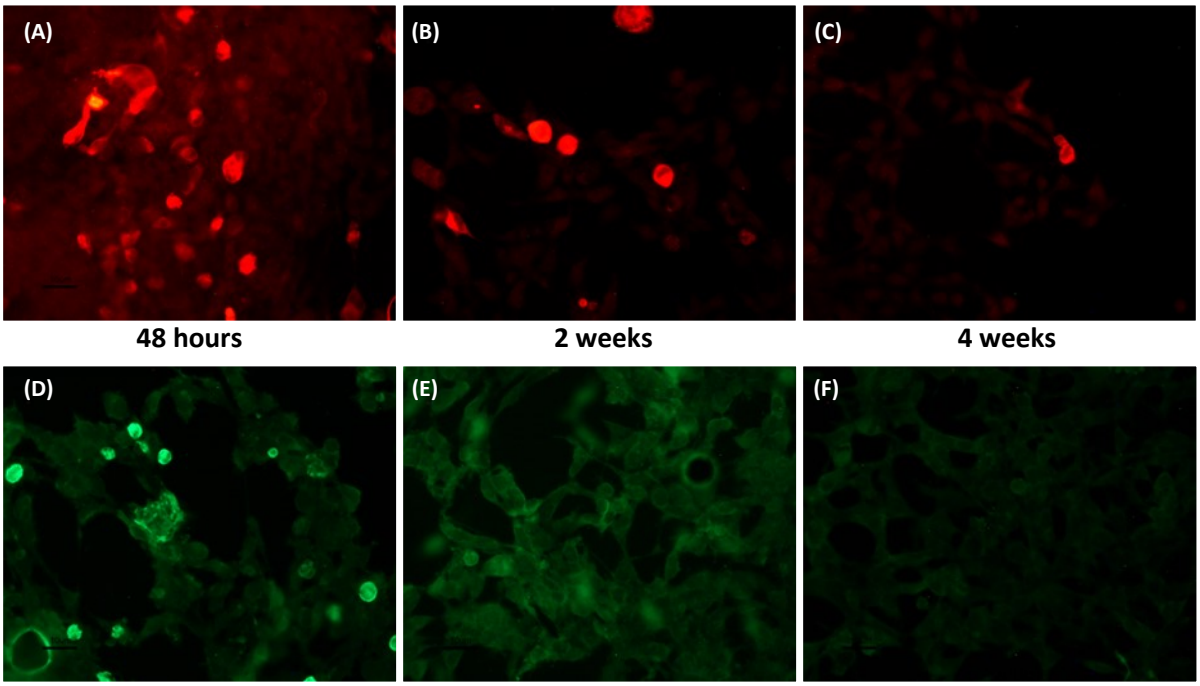


Figure #10: Immunofluorescent antibody assays of HEK-293 cells that underwent selection with G418 Geneticin for stable protein expression of 176310 (ACC-1) or 142690 (ACC-2) receptor subunits. (A) – HEK-293 cells transfected with 142690 subunit after 48 hours of G418 selection. (B) – HEK-293 cells transfected with 142690 subunit after 2 weeks of G418 selection. (C) – HEK-293 cells transfected with 142690 subunit after 4 weeks of G418 selection. (D) – HEK-293 cells transfected with 176310 subunit after 48 hours of G418 selection. (E) – HEK-293 cells transfected with 176310 subunit after 2 weeks of G418 selection. (F) – HEK-293 cells transfected with 176310 subunit after 4 weeks of G418 selection. Assays were performed using polyclonal antibodies against smp_176310 (ACC-1) and smp_142690 (ACC-2).

3.3.2 Transient transfection of HEK-293 cells

HEK-293 cells were routinely passaged and transfected at ~70% confluency with either smp_176310 (ACC-1), smp_142690 (ACC-2) or both in case of confocal microscopy. PciNeo expression vector was used for both constructs. Transfection efficiency can be observed on Figure #11. Cells were stained using polyclonal antibodies against smp_176310 (ACC-1) and smp_142690 (ACC-2). For negative control, ACC-1 transfected cells were stained with anti-ACC-2 antibody and vice versa. No significant non-specific staining could be found in the control sample. Small fraction of transfected cells was highly fluorescent with most of the others being just slightly brighter than control.

Confocal assays have shown that fluorescently labeled protein appears to be near the surface of the cell, just as membrane protein should be (Figure 12). Cells that exhibited higher fluorescence also demonstrated most unhealthy phenotype as compared to non-transfected cells (Figure 12, D). As shown by some previous studies, transmembrane proteins could potentially be cytotoxic and slowly poison the cell by unknown mechanisms or simply by overexpression [51, 52].

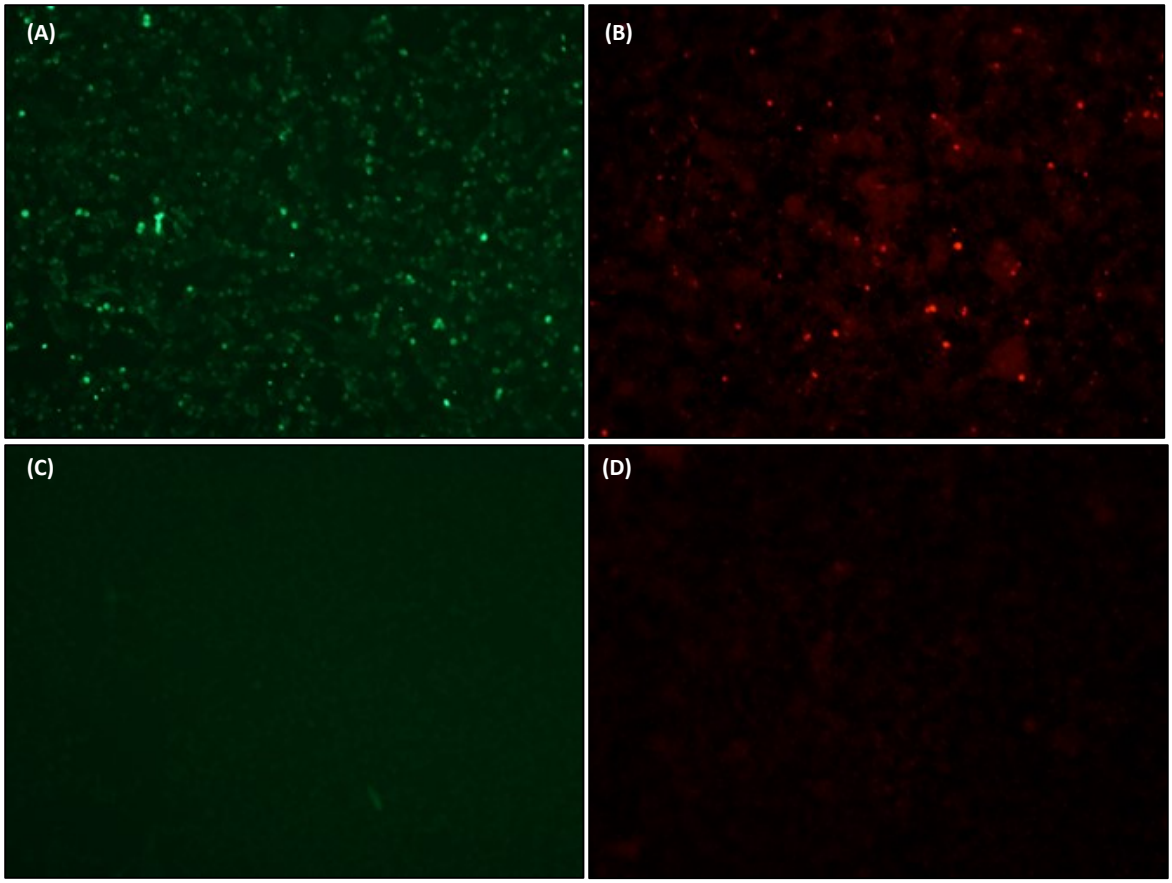


Figure #11: Immunofluorescent antibody assays of HEK-293 cells that have been transfected with 176310 (ACC-1) or 142690 (ACC-2) receptor subunits and corresponding controls. (A) – HEK-293 cells transfected with 176310 subunit after 48 hours, probed with anti-FLAG antibody. (B) – HEK-293 cells transfected with 142690 subunit after 48 hours, probed with anti-142690 subunit antibody. (C) – HEK-293 cells transfected with 142690 subunit after 48 hours, probed with anti-FLAG antibody. (D) – HEK-293 cells transfected with 176310 subunit after 48 hours, probed with anti-142690 subunit antibody.

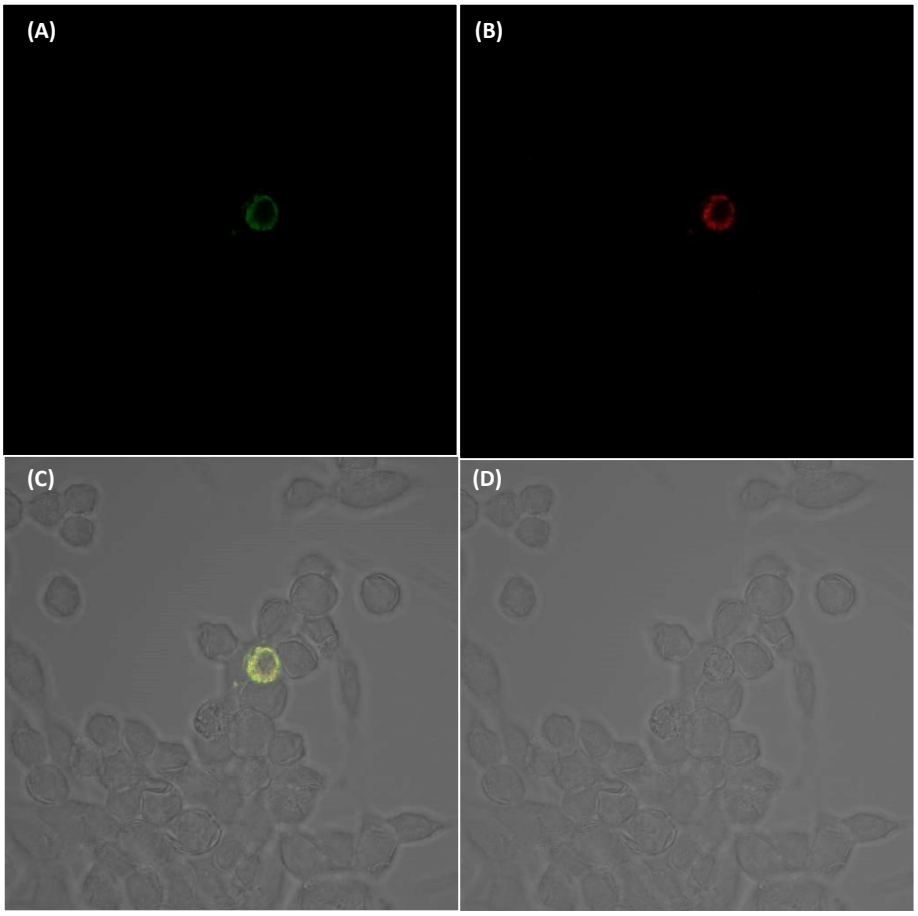


Figure #12: Confocal analysis of immunofluorescent antibody assay of HEK-293 cells that have been simultaneously transfected with 176310 (ACC-1) and 142690 (ACC-2) receptor subunits. (A) – HEK-293 cells transfected with 176310 and 142690 subunit after 48 hours, probed with anti-FLAG antibody. (B) – HEK-293 cells transfected with 176310 and 142690 subunit after 48 hours, probed with anti-142690 subunit antibody. (C) – An overlay of (A) and (B) images. (D) - Brightfield transmission image of the same HEK-293 cells.

3.3.3 YFP-based functional expression assay

Functional expression analysis of *S. mansoni* ACC receptor was carried out in transiently transfected HEK293 cells. A previous study cloned and expressed two nAChR subunits from *S. haematobium* in Xenopus oocytes. However, neither subunit was able to form a functional ion channel either alone or when co-expressed [23]. Our initial attempts to express SmACC-1 failed to produce functional channels in HEK293 cells (Fig 13). Activity assays were performed using the Premo Halide Sensor (Invitrogen). The results of the activity assay do not show significant activation of SmACC-1 by the cholinergic agonist nicotine compared to the water-treated sample. However, these results are based a single transfection; more independent experiments are needed to verify the results and optimize conditions. The immunofluorescence experiments described above showed that ACC-1 appeared to be correctly targeted to the cell surface in transiently transfected HEK292 cells (Figure 12) but the level of protein expression was low in spite of codon-optimisation, which could explain the apparent lack of activity

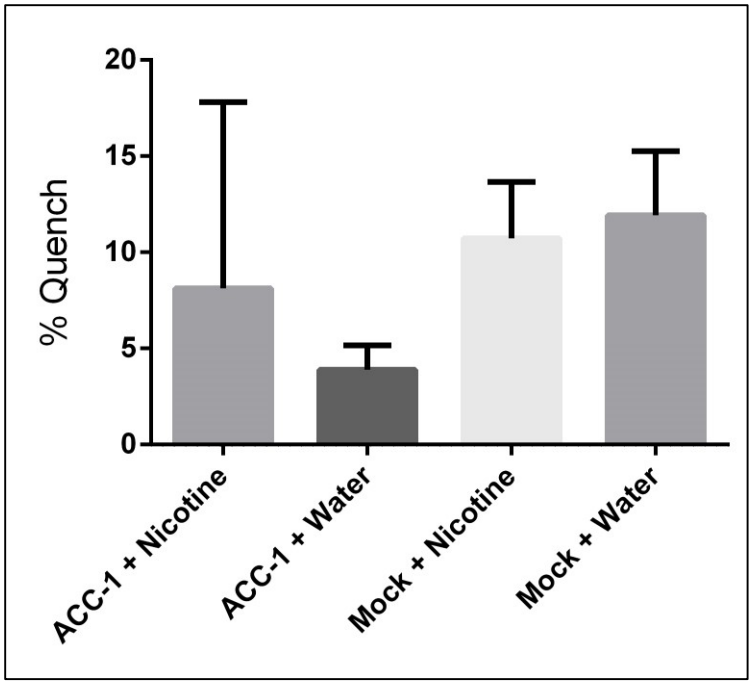


Figure #13: Functional Characterization of SmACC-1 in HEK-293 cells. Treatment of SmACC-1 expressing cells with 100 μM Nicotine did not produce a significant change in YFP fluorescence when compared to both a water-treated negative control and mock-transfected cells treated with 100 μM nicotine. Results are the means and SD from a single transfection performed with 24 technical replicates per treatment.

CHAPTER 3 – Discussion

Schistosomiasis, is one of the most economically important parasitic diseases of the world [4]. All possibilities are explored to find a viable alternative to Praziquantel. Cholinergic receptors are effective drug targets in other helminth parasites [6, 20]. Thus we conducted an investigation of these channels in *S. mansoni*. The major goals of the study were (1) to determine the oligomeric composition of schistosome nAChRs by affinity-purifying the native proteins from adult worms and (2) reconstitute the recombinant channels in a heterologous expression system to assess their functionality.

Acetylcholine is known to be an important excitatory neurotransmitter in vertebrates. Among other activities, vertebrate cholinergic receptors control muscle contractions via depolarization due to an influx of Na^+ and Ca^{2+} [9]. Recently, a new class of anion-selective nicotinic AChRs has been reported in several invertebrates [8]. Unlike the classical cation-selective nAChRs, the new anion-permeable receptors mediate membrane hyperpolarization due to an influx of Cl-, causing an inhibition of action potentials. Possible effects of acetylcholine on muscle functioning of invertebrates depend upon the organism. Classic excitatory effects of acetylcholine are not uncommon among invertebrates, including nematodes [8]. Surprisingly, in trematodes, acetylcholine seems to function in the opposite manner. The application of cholinergic agonists (such as acetylcholine) onto trematodes initiates flaccid paralysis due to relaxation of body muscles [11]. This reaction is quite specific to trematodes and the receptors arbitrating this activity could potentially be a therapeutic target [25]. The sequencing of the *S.*

mansoni genome showed several hypothetic nAChR subunit genes that could play a role in forming anion-selective nAChR receptors [12,13].

Based on previous research, it was estimated that five of the schistosome nAChR subunits (Smp_176310 aka ACC-1, Smp_142690 aka ACC-2, Smp_157790, Smp_037910 and Smp_132070) could potentially be anion-selective and implicated in flaccid paralysis caused by cholinergic compounds. To test this, researchers silenced their function by RNAi in *S. mansoni* schistosomula and measured the effects on larval motor activity. Knockdown of individual subunits resulted in 3-6 fold hypermotile phenotype, supporting the hypothesis. Additional immunolocalization studies showed that two subunits (Smp-176310, ACC-1 and Smp_142690, ACC-2) share similar distribution patterns, suggesting they could be components of the same channel in the worm. ACC-1 was present only in the PNS. ACC-2 was distributed more prominently than SmACC-1 but presented a similar pattern. Counterstaining of both ACCs with the muscular marker phalloidin suggested that neither subunit was localized directly on the body wall musculature [25].

Our work began with fluorescence microscopy studies of adult worms. To test if α-bungarotoxin could be used as a high affinity probe for purification of nAChRs, we labeled the worms with α-bungarotoxin-AlexaFluor488 (ThermoFisher) conjugate. Given that α-BTX binds with high affinity to the α-subunit of nicotinic AChRs [53], we expected to see similar labeling pattern as was previously shown for ACC-1, a predicted α-subunit [25] or labeling in the tegument, as previously described [34]. Unexpectedly, the most significant staining was found in the female reproductive system, specifically in vitelline glands. This signal was not caused by

autofluorescence often associated with female reproductive structures since no comparable signal could be seen in the unlabelled controls. An overlay of α-bungarotoxin-AlexaFluor488 stain with phalloidin-TRITC(muscle fibers) showed no overlapping expression patterns in adult female worms indicating the labeling was not associated with muscle. Our findings did not agree with previous research where scientists described strong labeling of the schistosome tegument with α-bungarotoxin-FITC [34]. However, it is possible that in our case labeling of the tegument (or other organs) was too faint to detect compared to the bright signal coming from vitelline glands.

We proceeded further into purification of native schistosome nAChRs by using a biotinylated form of BTX as bait for affinity purification. In preliminary experiments we optimized the purification by actively searching for an appropriate elution buffer for the streptavidin-biotin system. Intending to make this elution as specific as possible and to avoid possible contamination from other streptavidin interacting proteins, we planned to use carbachol as a competitive cholinergic agonist to elute schistosome nAChRs from the streptavidin beads. Carbachol binds to the same site of nAChR as α-bungarotoxin and therefore was expected to compete with bound BTX when added in excess, allowing the receptor to dissociate from the beads without having to break the much stronger biotin-streptavidin bond. This method was previously described and shown to be effective [42]. Unfortunately, using this method we could not elute any detectable protein based on measurements of protein concentration or western blot analysis. Such results led us to test harsher elution conditions that could denature any non-covalent bond and were designed to break the interaction between the biotinylated BTX and streptavidin. We quickly discovered many scientific papers that seem to contradict one another when it comes to efficient elution of biotinylated proteins from streptavidin beads [36, 39]. Thus

we conducted a preliminary trial to assess various elution buffers in our system, using biotinylated BSA as a test protein. Water heated to 70ºC, claimed by some to be an effective elution buffer [39], produced the worst results by eluting less than half of the loaded b-BSA. Adding an excess of free biotin (50 mM) to displace the biotinylated protein also proved to be innefective. On the other hand, a mixture of 6M Urea, 2M Thiourea, 50mM Biotin, 2% SDS at 95ºC was able to elute nearly all protein. We later introduced a small modification, replacing SDS with Invitrosol(sulfobetaine), which made the eluate Mass-Spectrometry compatible without additional purification steps to remove the detergent, and without changing the effectiveness of the elution buffer.

Using the newly selected buffer, we proceeded to purify b-BTX-labelled schistosome proteins from streptavidin beads. Initial western blot analysis of the eluate revealed many different proteins by probing with streptavidin-HRP. Recognising, that some of those bands could be the result of non-specific interactions with the beads or even BTX, we had predicted that at least some of the recovered proteins would include our receptors of interest. To further test this prediction, we probed the eluted proteins with three subunit-specific antibodies, only to discover that the antibodies all recognized a band of the same molecular weight, which did not correspond to the expected sizes of the subunits. There are several possible explanations for the odd molecular weight of the immunoreactive band. Given that the size (about 130 kDa) was larger than expected for all three subunits, it is possible this was due to post-translational modification of the proteins, for example due to glycosylation [54], an apparent MW shift by carbamylation due to harsh eluting conditionds [55], an apparent MW shift after treatment with a reducing agent, which happens to some proteins [56], or the proteins were recovered as part of a larger complex that resisted denaturation in the SDS-PAGE gel. However, we noted a similar

(albeit fainter) 130 kDa band in the irrelevant antibody negative control, suggesting the band could be non-specific.

To further test for non-specific interactions, we conducted additional control purifications in which biotinylated α-bungarotoxin was replaced with an equimolar amount of free biotin. By eliminating biotinylated α-bungarotoxin, which is responsible for receptor binding to the beads, we did not expect to detect any schistosome nAChRs in the eluate. Any proteins recovered from the beads under these conditions were considered to be non-specific. Unfortunatelly, these experiments revealed essentially the same pattern of protein bands in b-BTX-labelled samples and the biotin-treated negative controls. Combined with the failure to detect subunits of the correct size with specific antibodies, as discussed above, we concluded that the protein bands seen in the gel /western analysys are probably non-specific.

Although the western blots failed to identify specific bands, it is possible that nicotinic receptors were purified at very low levels, too low to be detected by gel analysis or westerns. Thus we questioned whether mass-spectrometry, being more sensitive to low abundance proteins, might be successful in finding small amounts of purified receptor. Three samples of affinity-purified, b-BTX-labelled *S. mansoni* proteins were trypsinized and sent for mass-spectrometry analyses. The results identified three nAChR subunits at least in two out of three samples. These included two anion-selective subunits (smp_176310 (ACC-1) and smp_142690 (ACC-2)) and a predicted cation-selective subunit (smp_031680). The best results, based on the number of spectral matches, were achieved by crosslinking b-BTX-labelled membrane proteins with DTSSP before solubilisation and elution of the complex. Crosslinking is often used to improve purification of oligomeric proteins or to study the interactome of a receptor. In our case,

it may have helped to bind interacting subunits together before purification. ACC-2 (smp_142690) was found in all three samples send for mass spectrometry; it was also the most abundant and with the highest protein scores and sequence coverage. ACC-1 (smp_176310) and smp_031680 were found twice, with the latter having the lowest MS scores.

The presence of ACC-1 and ACC-2 in the affinity-purified samples raises the interesting possibility that these proteins could be subunits of the same heteromeric nicotinic channel. This would also explain why ACC-1 and ACC-2 have similar RNAi phenotypes and similar immunolocalization patterns in schistosomes [25]. The notion that ACC-1 and ACC-2 are interacting subunits is further supported by the immunoprecipitation data presented in this study. Western blot analyses detected bands with molecular weights that match those predicted for SmACC-1 (smp_176310) and SmACC-2 (smp_142690) subunits.

The third subunit detected by mass-spectrometry analysis, Smp_031680, is a predicted alpha nAChR subunit and a closely related homologue of the previously described *S. haematobium* ShAR receptor [23]. Although this protein co-purified with ACC-1 and ACC-2, it is unlikely they are components of the same channel. First, the ion-selectivity is different, the ACCs being anion-selective whereas Smp_031680 is predicted to be cation-selective. In addition, recent functional studies of this protein showed that the immunolocalization pattern differs from that of the two ACCs and the RNAi phenotype is also different (Rashid and Ribeiro, manuscript in preparation), suggesting these are likely to form different channels. Smp_031680 is structurally related to the vertebrate alpha7 nicotinic channel, which is homomeric and BTX-sensitive. Being alpha-like, it is possible Smp_031680 forms a similar homomeric channel in *S. mansoni* but this has yet to be tested experimentally.

Our next step was to conduct functional expression analyses of ACC receptors in a heterologous expression system. The goal was to express ACC-1 and ACC-2 both individually and in combination to see if they formed a channel together and how that interaction might affect channel activity. Initial attempts to express SmACC-1 failed to produce functional channels. However, when the sequence was codon-optimized (humanized) to improve protein expression, significant receptor activity was detected in transfected HEK293 cells [25]. Thus we purchase a codon-optimized ACC-2 to test to together with codon-optimized ACC-1 in functional expression studies. Activity assays were conducted using the Premo Halide Sensor (Invitrogen) fluorescence-based assay, which is specifically designed to measure chloride channel activity.

In preparation for these studies, we began by transfecting HEK 293 cells with each codon-optimized subunit and then selecting for stable expression with G418. The results showed high level protein expression for both subunits during the early stages of selection but protein levels decreased steadily as selective pressure with G418 continued. Neither protein could be detected after about 4 weeks of selection. Though the reason for these results is unknow, we speculate that ACC-1 and ACC-2 may be toxic to the cell when expressed at high levels and therefore the G418 selection may, over time, cause preferential loss of ACC-expressing cells. As an alternative, we used transiently transfected HEK293 cells instead, which were harvested and tested 48 hr post-transfection with each subunit. Subunits expression levels were relatively low in the transiently transfected HEK cells but the proteins seemed to be correctly targeted to the cell surface, as determined by confocal immunofluorescence analysis.

A preliminary channel activity assay was performed on transiently transfected HEK cells expressing codon-optimized ACC-1. Heterologously expressed ACC-1 forms a homomeric channel that can be activated by nicotine [25]. Unfortunately, our preliminary experiments did

not detect a significant response to nicotine in the ACC-1-transfected cells. This could be due to low expression of the protein, lack of appropriate folding in the heterologous environment, or some other unknown factor. More experiments from multiple transfections are needed to verify these results. We would also suggest the use of a positive control to help optimize the activity assay. We recently obtained a construct that expresses a human Glycine receptor, which is a known ligand-gated chloride channel [19]. This construct will be used in future experiments as a positive control for functional expression studies of schistosome ACCs. Trying to improve protein expression levels and/or co-transfecting chaperone proteins such as ric-3 [57, 58] along with the subunit in question are alternative possibilities worth pursuing. Fluorescence-based functional analysis of ACCs is an interesting alternative to the more classical electrophysiological method. Although electrophysiology is the gold standard for evaluation of channel activity, this method is labour-intensive and generally unsuitable for testing multiple agonists when de-orphanizing a channel, or for large-scale screens of compound libraries in drug discovery. Expression of ion channels in mammalian cells allows a high-throughput measurement of ion fluxes using fluorescence –based assays, not only the premo halide assay described here but others as well, for example by using fluorescent dyes that are sensitive to membrane potential. Various commercially available assays exist for different channel types.

In conclusion, we add to growing evidence that acetylcholine is an important neurotransmitter in *S. mansoni*. We describe two purification methods for native *S. mansoni* nAChRs and provide evidence that two anion-selective nAChR subunits, ACC-1 and ACC-2 are co-purified based on mass spectrometry and immunoprecipitation data. Combined with previous studies [25] these results suggest that ACC-1 and ACC-2 may interact as subunits of a

heteromeric channel in the worm. Sadly, we have not yet been able to verify this hypothesis by co-expressing the two subunits *in vitro* and testing for activity. We have, however, established conditions for transient expression of the two subunits in HEK 293 cells and showed that the subunits appear to be targeted to cell surface based on *in situ* immunofluorescence. These are important steps that provide a foundation for future functional analyses of schistosome ACCs.

Bibliography

1. Milligan JN, Jolly ER. Cercarial transformation and in vitro cultivation of *Schistosoma mansoni* schistosomules. J Vis Exp. 2011;(54)

2. Fitzpatrick JM, Hoffmann KF. Dioecious *Schistosoma mansoni* express divergent gene repertoires regulated by pairing. Int J Parasitol. 2006;36(10-11):1081-9.

3. Kassim O, Gibertson DE. Hatching of *Schistosoma mansoni* eggs and observations on motility of miracidia. J Parasitol. 1976;62(5):715-20.

4. Walker AJ. Insights into the functional biology of schistosomes. Parasit Vectors. 2011;4(1):203.

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

6. Harder A. Chemotherapeutic approaches to schistosomes: current knowledge and outlook. Parasitol Res. 2002;88(5):395-7.

7. 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.

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

9. Millar NS. Assembly and subunit diversity of nicotinic acetylcholine receptors. Biochem Soc Trans. 2003;31(Pt 4):869-74.

10. Minna JD. Nicotine exposure and bronchial epithelial cell nicotinic acetylcholine receptor expression in the pathogenesis of lung cancer. J Clin Invest. 2003;111(1):31-3.

11. Barker LR, Bueding E, Timms AR. The possible role of acetylcholine in *Schistosoma mansoni*. Br J Pharmacol Chemother. 1966;26(3):656-65.

12. 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.

13. Protasio AV, Tsai IJ, Babbage A, et al. A systematically improved high quality genome and transcriptome of the human blood fluke *Schistosoma mansoni*. PLoS Negl Trop Dis. 2012;6(1):e1455.

14. The *Schistosoma japonicum* genome reveals features of host-parasite interplay. Nature. 2009;460(7253):345-51.

15. Arnon R, Silman I, Tarrab-hazdai R. Acetylcholinesterase of *Schistosoma mansoni*--functional correlates. Contributed in honor of Professor Hans Neurath's 90th birthday. Protein Sci. 1999;8(12):2553-61.

16. Camacho M, Agnew A. Schistosoma: rate of glucose import is altered by acetylcholine interaction with tegumental acetylcholine receptors and acetylcholinesterase. Exp Parasitol. 1995;81(4):584-91.

17. Tsetlin V, Kuzmin D, Kasheverov I. Assembly of nicotinic and other Cys-loop receptors. J Neurochem. 2011;116(5):734-41.

18. Itier V, Bertrand D. Neuronal nicotinic receptors: from protein structure to function. FEBS Letters. 2001;504(3):118-125.

19. 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.

20. Ribeiro P, Geary TG. Neuronal signaling in schistosomes: current status and prospects for post genomics Can. J. Zool. 2010;88(1):1-22.

21. Le novère N, Corringer PJ, Changeux JP. Improved secondary structure predictions for a nicotinic receptor subunit: incorporation of solvent accessibility and experimental data into a two-dimensional representation. Biophys J. 1999;76(5):2329-45.

22. Brejc K, Van dijk WJ, Klaassen RV, et al. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. Nature. 2001;411(6835):269-76.

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

24. Dufour V, Beech RN, Wever C, Dent JA, Geary TG. Molecular cloning and characterization of novel glutamate-gated chloride channel subunits from *Schistosoma mansoni*. PLoS Pathog. 2013;9(8):e1003586.

25. Macdonald K, Buxton S, Kimber MJ, Day TA, Robertson AP, Ribeiro P. Functional characterization of a novel family of acetylcholine-gated chloride channels in *Schistosoma mansoni*. PLoS Pathog. 2014;10(6):e1004181.

26. Bentley GN, Jones AK, Agnew A. ShAR2beta, a divergent nicotinic acetylcholine receptor subunit from the blood fluke Schistosoma. Parasitology. 2007;134(Pt 6):833-40.

27. Galzi JL, Devillers-thiéry A, Hussy N, Bertrand S, Changeux JP, Bertrand D. Mutations in the channel domain of a neuronal nicotinic receptor convert ion selectivity from cationic to anionic. Nature. 1992;359(6395):500-5.

28. Ishii M, Kurachi Y. Muscarinic acetylcholine receptors. Curr Pharm Des. 2006;12(28):3573-81.

29. Macdonald K, Kimber MJ, Day TA, Ribeiro P. A constitutively active G protein-coupled acetylcholine receptor regulates motility of larval *Schistosoma mansoni*. Mol Biochem Parasitol. 2015;202(1):29-37.

30. Miyazawa A, Fujiyoshi Y, Stowell M, Unwin N. Nicotinic acetylcholine receptor at 4.6 A resolution: transverse tunnels in the channel wall. J Mol Biol. 1999;288(4):765-86.

31. Samson AO, Levitt M. Inhibition mechanism of the acetylcholine receptor by alpha neurotoxins as revealed by normal-mode dynamics. Biochemistry. 2008;47(13):4065-70.

32. Liu S, Babcock MS, Bode J, et al. Affinity purification of a chimeric nicotinic acetylcholine receptor in the agonist and antagonist bound states. Protein Expr Purif. 2011;79(1):102-10.

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

34. Camacho M, Alsford S, Jones A, Agnew A. Nicotinic acetylcholine receptors on the surface of the blood fluke Schistosoma. Mol Biochem Parasitol. 1995;71(1):127-34.

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

36. Rybak JN, Scheurer SB, Neri D, Elia G. Purification of biotinylated proteins on streptavidin resin: a protocol for quantitative elution. Proteomics. 2004;4(8):2296-9.

37. 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

38. Johansson T, Norris T, Peilot-sjögren H. Yellow fluorescent protein-based assay to measure GABA(A) channel activation and allosteric modulation in CHO-K1 cells. PLoS ONE. 2013;8(3):e59429.

39. Holmberg A, Blomstergren A, Nord O, Lukacs M, Lundeberg J, Uhlén M. The biotin-streptavidin interaction can be reversibly broken using water at elevated temperatures. Electrophoresis. 2005;26(3):501-10.

40. Galietta LJ, Haggie PM, Verkman AS. Green fluorescent protein-based halide indicators with improved chloride and iodide affinities. FEBS Lett. 2001;499(3):220-4.

41. Rhoden KJ, Cianchetta S, Stivani V, Portulano C, Galietta LJ, Romeo G. Cell-based imaging of sodium iodide symporter activity with the yellow fluorescent protein variant YFP-H148Q/I152L. Am J Physiol, Cell Physiol. 2007;292(2):C814-23.

42. Paulo JA, Brucker WJ, Hawrot E. Proteomic analysis of an alpha7 nicotinic acetylcholine receptor interactome. J Proteome Res. 2009;8(4):1849-58.

43. Xu H, Yang L, Freitas MA (2008) A robust linear regression based algorithm for automated evaluation of peptide identifications from shotgun proteomics by use of reversed-phase liquid chromatography retention time. BMC Bioinformatics 9:347

44. Xu H, Freitas MA (2009) Mass Matrix: a database search program for rapid characterization of proteins and peptides from tandem mass spectrometry data. Proteomics 9(6):1548-1555

45. 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.

46. Zhang N, Li L. Effects of common surfactants on protein digestion and matrix-assisted laser desorption/ionization mass spectrometric analysis of the digested peptides using two-layer sample preparation. Rapid Commun Mass Spectrom. 2004;18(8):889-96

47. Rohila JS, Chen M, Cerny R, Fromm ME. Improved tandem affinity purification tag and methods for isolation of protein heterocomplexes from plants. Plant J. 2004;38(1):172-81.

48. Lu X, Zhu H. Tube-gel digestion: a novel proteomic approach for high throughput analysis of membrane proteins. Mol Cell Proteomics. 2005;4(12):1948-58.

49. Marritt AM, Cox BC, Yasuda RP, et al. Nicotinic cholinergic receptors in the rat retina: simple and mixed heteromeric subtypes. Mol Pharmacol. 2005;68(6):1656-68.

50. Capelli AM, Castelletti L, Chen YH, et al. Stable expression and functional characterization of a human nicotinic acetylcholine receptor with α6β2 properties: discovery of selective antagonists. Br J Pharmacol. 2011;163(2):313-29.

51. Gubellini F, Verdon G, Karpowich NK, et al. Physiological response to membrane protein overexpression in E. coli. Mol Cell Proteomics. 2011;10(10):M111.007930.

52. Andréll J, Tate CG. Overexpression of membrane proteins in mammalian cells for structural studies. Mol Membr Biol. 2013;30(1):52-63.

53. Ringler P, Kessler P, Ménez A, Brisson A. Purification of the nicotinic acetylcholine receptor protein by affinity chromatography using a regioselectively modified and reversibly immobilized alpha-toxin from Naja nigricollis. Biochim Biophys Acta. 1997;1324(1):37-46.

54. Dacosta CJ, Kaiser DE, Baenziger JE. Role of glycosylation and membrane environment in nicotinic acetylcholine receptor stability. Biophys J. 2005;88(3):1755-64.

55. Kollipara L, Zahedi RP. Protein carbamylation: in vivo modification or in vitro artefact ? Proteomics. 2013;13(6):941-4.

56. Zhao DS, Gregorich ZR, Ge Y. High throughput screening of disulfide-containing proteins in a complex mixture. Proteomics. 2013;13(22):3256-60.

57. Roncarati R, Seredenina T, Jow B, et al. Functional properties of alpha7 nicotinic acetylcholine receptors co-expressed with RIC-3 in a stable recombinant CHO-K1 cell line. Assay Drug Dev Technol. 2008;6(2):181-9

58. Williams ME, Burton B, Urrutia A, et al. Ric-3 promotes functional expression of the nicotinic acetylcholine receptor alpha7 subunit in mammalian cells. J Biol Chem. 2005;280(2):1257-63.