Characterization of a novel histamine G protein-coupled receptor from *Schistosoma mansoni* (SmGPCR)

Aisha H. Mousa

Institute of Parasitology
McGill University, Macdonald Campus
Ste. Anne De Bellevue, Quebec, Canada

November 2002

A thesis submitted to the Faculty
of Graduate Studies and Research
In partial fulfillment of the requirements of the degree of
Master of Science

Your file Votre référence
ISBN: 0-612-88270-5
Our file Notre référence
ISBN: 0-612-88270-5

ABSTRACT

A G protein-coupled receptor with structural characteristics of a biogenic amine GPCR was cloned from *Schistosoma mansoni* (SmGPCR). SmGPCR was codon-optimized and double-tagged with FLAG and His epitopes at the N- and C-terminal ends, respectively. Immunofluorescence experiments targeting these epitopes revealed that the expression of codon-optimized SmGPCR was highly increased compared to wild-type in mammalian cells. These studies also demonstrated that SmGPCR has a typical GPCR topology, the N-terminus being extracellular and C-terminus intracellular. Functional assays revealed that codon-optimized SmGPCR was responsive only to histamine, which caused a dose-dependent increase in intracellular Ca^{2+} (EC_{50}= 0.54 ± 0.05 μM), but not cAMP, consistent with a G_q pathway of signal transduction. In vitro behavioral studies showed that treatment of *S. mansoni* cercaria with exogenous histamine caused a dose-dependent increase in the motility of the parasite. Various histamine H_1 receptor inhibitors caused a significant decrease in motility leading to complete cercarial immobilization. The paralysis was reversible by addition of excess histamine, consistent with a receptor-mediated effect. In contrast, histamine H_2 and H_3/H_4 receptor blockers had no effect on cercarial motility. Adult schistosomes were also paralyzed by treatment with H_1 receptor antagonists and were unresponsive to an H_2-selective drug. These studies suggest that an H_1-like histamine receptor modulates parasite motility in cercaria and adult worms. Finally, a fragment of SmGPCR-IL_3 was amplified by PCR, expressed in *E. coli* and partially purified for the purpose of raising a polyclonal antibody against SmGPCR. The antibody will be used in future studies for localization of the receptor.

ABRÉGÉ

Un récepteur couplé à la protéine G (GPCR) qui partage des caractéristiques structurelles avec la famille des récepteurs aux amines biogènes a été cloné chez le trématode *Schistosoma mansoni* (SmGPCR). La séquence SmGPCR a été modifiée de manière à optimaliser ses codons. De plus, les épitopes FLAG et HIS ont été ajoutés respectivement à chacune des extrémités N- et C-terminus du récepteur. Les expériences d'immunofluorescence ciblant ces épitopes révèlent que l'expression du récepteur SmGPCR optimalisé, dans des cellules de mammifère, a considérablement augmenté par rapport à l'expression du récepteur sauvage. Ces études ont aussi démontré que le N-terminus est extracellulaire tandis que le C-terminus réside dans le cytosol, une topologie typique des GPCR. Les essais fonctionnels révèlent que le récepteur SmGPCR optimalisé répondait uniquement à l'histamine. L'augmentation des niveaux de calcium intracellulaire, en fonction des concentrations croissantes d'histamine, n'était pas accompagnée de variation du niveau d'AMPc. Ainsi, nous présumons que la voie effectrice mise en jeu implique Gq. Les études physiologiques faites *in vitro* montrent une hausse de la motilité des cercaires de *S. mansoni* en fonction des concentrations croissantes d'histamine. Plusieurs des inhibiteurs du récepteur H_1 à l'histamine qui ont été testés, agissent sur la motilité du parasite et ce, jusqu'à l'immobiliser complètement. La paralysie disparait en présence d'un excès d'histamine suggérant ainsi qu'un récepteur intervient dans la modulation de ce comportement. Par contre, les antagonistes des récepteurs H_2 et H_3/H_4 n'ont pas d'effet sur la motilité des cercaires. Les schistosomes traités avec des antagonistes du récepteur H_1, cessent aussi de bouger et ne répondent pas aux drogues spécifiques à H_2. Ainsi, ces études suggèrent qu'un récepteur vraisemblabement de type H_1 module la motilité du parasite, cercaire ou adulte. La dernière étape du travail consistait à produire un anticorps polyclonal dirigé contre le fragment SmGPCR-IL_3. Le fragment, obtenu par amplification PCR, a été exprimé dans *E. coli* puis, partiellement purifié. L'anticorps servira prochainement à des études pour connaître la distribution du récepteur chez l'animal.

ACKNOWLEDGEMENTS

My experience over the last two years has led me through a rewarding journey that I could not have completed alone. My success is based on the support and encouragement of my supervisor Dr. Paula Ribeiro. Besides giving me the opportunity to excel in a new and exciting field, Paula's teaching style has equipped me with knowledge and concepts that I will apply for years to come; this is a wonderful gift that I have received.

I would like to extend a warm appreciation to my labmates; Joe Nabhan and Christelle for providing me with a joyful and relaxing working environment. Special thanks to Joe for being patient and teaching me "the basics" and I am grateful to Christelle for doing a superb job in translating the abstract of this thesis and for her friendship, which I will always cherish. I am also thankful for having such a wonderful experience at the Institute; the staff and students made my stay meaningful and enriching.

Having come to Canada from overseas, I struggled a great deal before adapting to my new home, my friends Razan, Shereen, Afaf and Jumanah did such a wonderful job by making me feel welcomed and at home, thank you!

My husband Hasan and my sweet darling Rand; thank you for being so patient, I will never forget your love and support throughout these difficult years. You have both made so many sacrifices and I shall always be indebted to you kindheartedness.

Finally, I would like to dedicate this thesis to my family and especially my parents who have provided me with all the emotional support and their prayers; although we were miles apart. I love you more everyday.

THESIS OFFICE STATEMENT

In accordance with the regulations of the Faculty of Graduate Studies and Research of McGill University, the following statement is included in the thesis:

Candidates have the option, **subject to the approval of their Department**, of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). Thesis texts must be bound as an integral part of the thesis.

If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include: (1) A Table of contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rational and the objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, (5) a final conclusion and/or summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extend**. Supervisors must attest to the accuracy of such statements at the doctoral oral defence. Since the task of the examiners is made more difficult in thesis cases, it is in the candiate's best interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.

STATEMENT OF CONTRIBUTIONS

The experimental work presented in this thesis was performed by the author, under the supervision of Dr. Paula Ribeiro. In Chapter II, the codon-optimization was done by Fadi F. Hamdan and the aequorin assay was done in collaboration with Dr. Mark Abramovitz (Merck Frosst, Pointe Claire, CA).

TABLE OF CONTENTS

ABSTRACT---- I
ABREGE---- II
ACKNOWLEDGEMENT---- III
THESIS OFFICE STATEMENT---- IV
STATEMENT OF CONTRIBUTIONS---- V
TABLE OF CONTENTS---- VI
LIST OF FIGURES---- IX
LIST OF TABLES---- X
LIST OF ABBREVIATIONS---- XI
INTRODUCTION---- 1
CHAPTER I: LITERATURE REVIEW---- 3
PART I: *SCHISTOSOMA MANSONI*: THE MODEL SYSTEM---- 4
I.1 *S. mansoni* life cycle---- 5
I.2 Schistosome Biology---- 7
I.2.1 The tegument---- 7
I.2.2 Musculature---- 9
I.2.3 Schistosome Secretory Cells---- 10
I.2.4 Schistosome Digestive system---- 11
I.2.5 Excretory system---- 12
I.3 Schistosomes Nervous System---- 12
PART II: BIOGENIC MONOAMINES---- 14
II.1 General properties of monoamines---- 14
II.2 Histamine---- 17
II.3 Neurotransmitters in Helminths---- 18
II.3.1 Serotonin---- 18
II.3.2 Acetylcholine (Ach)---- 20
II.3.3 Neuropeptides---- 21
II.3.4 Catecholamines---- 23
II.3.5 Histamine---- 24
PART III: HISTAMINE RECEPTORS---- 27
III.1 G protein-coupled receptors: an overview---- 27
III.2 Classification of Histamine receptors---- 30
III.2.1 Histamine H_1 receptor---- 30
III.2.2 Histamine H_2 receptor---- 31
III.2.3 Histamine H_3 receptor---- 32
III.2.4 Histamine H_4 receptor---- 34
PART IV: *SCHISTOSOMA MANSONI* G PROTEIN-COUPLED RECEPTOR (SmGPCR) ---- 35

CHAPTER II: FUNCTIONAL EXPRESSION STUDIES OF A NOVEL *SCHISTOSOMA MANSONI* G PROTEIN-COUPLED RECEPTOR (SMGPCR) ---- 37
CONTRIBUTION ---- 38
ABSTRACT ---- 39
INTRODUCTION ---- 40
EXPERIMENTAL PROCEDURE ---- 42
Codon-optimization of SmGPCR ---- 42
In situ immunolocalization ---- 44
Western Blotting ---- 45
Secreted Alkaline Phosphatase (CRE-SEAP) reporter assays ---- 46
Aequorin Assays ---- 46
RESULTS AND DISCUSSION ---- 48
Heterologous expression of SmGPCR in HEK293 cells ---- 48
Functional assays of SmGPCR ---- 51
REFERENCE ---- 55
CONNECTING STATEMENT I ---- 56
CHAPTER III (Manuscript I): *Schistosoma mansoni*: Effects of histamine and histamine receptor antagonists on parasite motility in *vitro* ---- 57
ABSTRACT ---- 58
INTRODUCTION ---- 59
EXPERIMENTAL PROCEDURES ---- 61
RT-PCR ---- 62
Motility assay of S. mansoni Cercaria ---- 62
Motility assays of adult S. mansoni ---- 63
Drug preparation ---- 64
Statistical analyses ---- 64
RESULTS ---- 65
SmGPCR is expressed in Cercaria ---- 65
Drug effects on cercarial motility ---- 66
Effect of histamine H_1 receptor inhibitors ---- 67
Reversal of cercaria immobilization by histamine ---- 69
Adult motor activity ---- 70
DISCUSSION ---- 74
REFERENCES ---- 78
CONNECTING STATEMENT II ---- 80
CHAPTER IV: *IN SITU* LOCALIZATION OF SMGPCR IN THE INTACT WORM ---- 81
ABSTRACT ---- 82
INTRODUCTION ---- 83
EXPERIMENTAL PROCEDURE ---- 84
Probe design ---- 84
Peptide synthesis ---- 84
Enzyme Linked Immunosorbent Assay (ELISA) ---- 85
Bacterial expression and protein purification of il_3 ---- 86

Western Blot---- 87
RESULTS AND DISCUSSION---- 88
C-terminal peptide antibody---- 88
Purification of IL_3-His---- 89
REFERENCES---- 92
GENERAL DISCUSSION---- 94
GENERAL REFERENCES---- 99
APPENDIX I: Animal Use Protocol-Research ---- 109
APPENDIX II: Biohazards Materials---- 110

LIST OF FIGURES

CHAPTER I: LITERATURE REVIEW
Figure 1: Life cycle of *S. mansoni*---------- 6
Figure 2: Neurotransmitters derived from amino acids---------- 15
Figure 3: *S. mansoni* G protein-coupled receptor (SmGPCR) ---------- 36

CHAPTER II: FUNCTIONAL EXPRESSION STUDIES OF A NOVEL *SCHISTOSOMA MANSONI* G PROTEIN-COUPLED RECEPTOR (SMGPCR)
Figure 1: Construction of codon-optimized SmGPCR expression plasmid 44
Figure 2: Codon-optimization and SmGPCR expression in HEK293(EBNA1) cells---------- 49
Figure 3: Western blot analysis---------- 50
Figure 4: Cellular localization of SmGPCR by immunofluorescence---------- 51
Figure 5: Effect of biogenic amines in an aequorin assay---------- 52
Figure 6: Representative activation curves of codon-optimized SmGPCR----- 53

CHAPTER III (Manuscript I): *Schistosoma mansoni*: Effects of histamine and histamine receptor antagonists on parasite motility in *vitro*
Figure 1: Developmental expression of SmGPCR in *S. mansoni*---------- 65
Figure 2: Histamine and 5-HT activate *S. mansoni* cercarial motility---------- 67
Figure 3: H_1 antagonists immobilize the *S. mansoni* cercaria---------- 68
Figure 4: No effect on cercarial motility in response to histamine H_2 and H_3/H_4 receptor inhibitors---------- 69
Figure 5: Histamine reverses cercarial immobilization---------- 70
Figure 6: Effect of serotonin and histamine on adult schistosomes---------- 71
Figure 7: H_1 antagonists immobilize *S. mansoni* adults---------- 72
Figure 8: H_1 immobilization effect on adult schistosomes is reversible---------- 73

CHAPTER IV: *IN SITU* LOCALIZATION OF SMGPCR IN THE INTACT WORM
Figure 1: Amplification of a fragment from SmGPCR-IL_3---------- 89
Figure 2: Coomassie staining of purified IL_3 portion---------- 91
Figure 3: Western blot of purified protein products---------- 91

LIST OF TABLES

CHAPTER I: LITERATURE REVIEW

Table 1: Summary of neurotransmitters effects on both platyhelminth and nematodes---------- 26

CHAPTER II: FUNCTIONAL EXPRESSION STUDIES OF A NOVEL *SCHISTOSOMA MANSONI* G PROTEIN-COUPLED RECEPTOR (SMGPCR)

Table 1: Oligonucleotides used in constructing the codon-optimized SmGPCR construct---------- 43

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine; serotonin
Ach	Acetylcholine
ANOVA	Analysis of variance
CFA	Complete Freund adjuvant
CNS	Central Nervous System
DA	Dopamine
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle medium
ELISA	Enzyme linked immunosorbent assay
HPLC	High-pressure liquid chromatography
HRP	Horse-radish peroxidase
IFA	Incomplete Freund adjuvant
IL_3	Third intracellular loop
IP_3	Inositol-1,4,5-triphosphate
KLH	Keyhole Limpet Hemocyanin
LB	Luria-Bertani medium
NA	Noradrenaline
NO	Nitric oxide
NPF	Neuropeptide F
PBS	Phosphate-buffered saline
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
TM	Transmembrane domain

INTRODUCTION

Histamine is widely distributed in the animal kingdom, and was first identified as a hormone in 1910 (Barger and Dale, 1910; Liu et al., 2001). Histamine effects are very pleiotropic, and include roles both in pathological and physiological processes, including inflammatory response and gastric acid secretions. In vertebrates histamine functions as a major neurotransmitter in the brain (Panula et al., 2000). All vertebrates examined to date contain histaminergic neurons that innervate most parts of the brain (Eriksson et al., 1996), and regulate various brain activities, including arousal state, thermoregulation, feeding, drinking and sexual behavior (Ghi et al., 1999; Panula et al., 2000, Nonaka et al., 1998). Histamine exerts its effects through binding to histamine receptors. To date, four classes of histamine receptors (H_1, H_2, H_3, H_4) have been identified, all of which are G protein-coupled receptors.

Histamine has a relatively recent history as a neurotransmitter in invertebrates. A role in invertebrate neurotransmission was first suggested following the identification of histaminergic neurons in the slug *Aplysia* (Ono and McCaman, 1980; Weinreich et al., 1975) and in the compound eye and optic lobe of some insects (Elias and Evans, 1983; Maxwell et al., 1978). Moreover, histamine has been identified in the lower invertebrates, mainly helminths, where it is believed to function as a neuromuscular transmitter or modulator. In *Schistosoma mansoni*, histamine and its catabolic enzyme histaminase have been identified (Schwabe and Kilejian, 1968), but the origin of histamine and its mode of action are still unknown. Recently a novel receptor was cloned from *S.*

mansoni (SmGPCR) and shown to have structural characteristic of biogenic amine G protein-coupled receptors, including histamine GPCRs. This provided a foundation for the present study.

The two main objectives of this project were (1) To test the expression of SmGPCR in heterologous environment and identify its natural ligand. (2) To investigate the effect of exogenous histamine and antihistaminic drugs on the parasite in vitro. In addition, I have begun studies aimed at producing an antibody against SmGPCR for future localization of the receptor in the intact worm.

Chapter I provides an overview of the properties of biogenic amines, including histamine in both vertebrate and invertebrate species, with a focus on helminths. The literature review is divided into four parts. The first part focuses on *S. mansoni* as the model system of this study including, the parasite life cycle, biology and the nervous system. The second part is an overview of the properties of biogenic amines and neurotransmitters in helminths. The third part focused on the functional and structural characteristics of histamine receptors. Finally, the fourth part of Chapter I includes a structural description of the *S. mansoni* G protein-linked receptor (SmGPCR). Chapter II describes the expression of SmGPCR in a heterologous environment and functional expression assays. This work identified SmGPCR as a histamine receptor. Chapter III investigates the effect of exogenous histamine and several antihistaminic drugs in the adult and cercaria stages of *S. mansoni* in vitro. The last chapter describes the expression and purification of a fragment of SmGPCR (third intracellular loop) for the purpose of raising polyclonal antibodies against the receptor.

CHAPTER I

LITERATURE REVIEW

PART I: *Schistosoma mansoni*: The model system

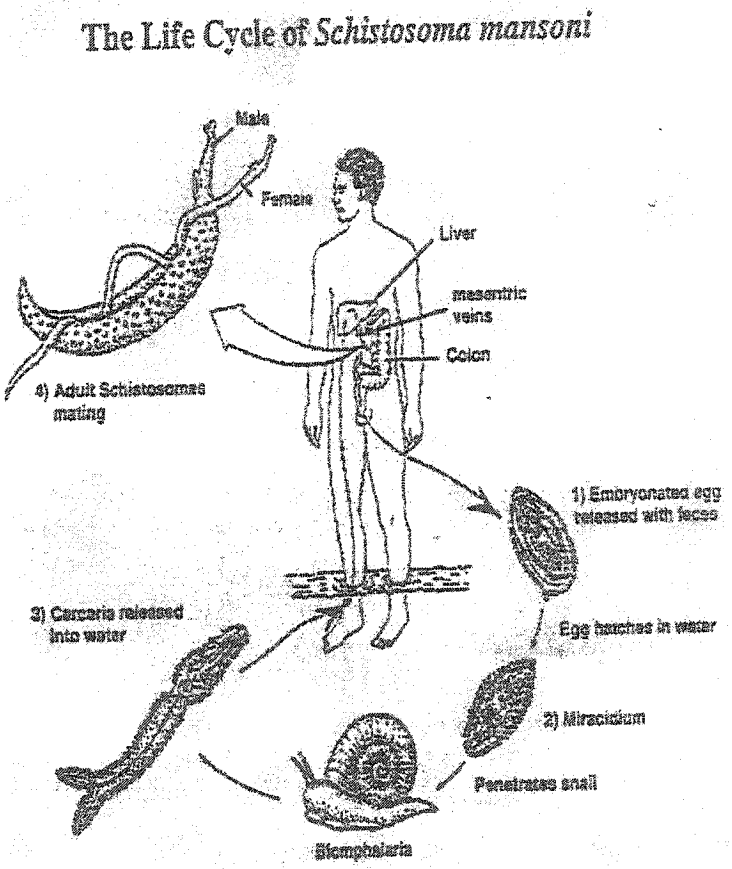
The phylum Platyhelminths (flatworms) is composed of four classes containing about 18,500 species. The class Turbellaria contains the free-living flatworms, whereas Monogenea, Trematoda (flukes) and Cestoda (tapeworms) are entirely parasitic. The focus of this study is on *Schistosoma mansoni,* which belongs to the class Trematoda, and family Schistosomatidae. Flukes are distinctive in that they have no second intermediate host in their life cycles and they mature in the blood vascular system of their definitive hosts. Most species, including species of schistosomes, are dioecous. Schistosoma is an apt name referring to the "split body" (gynecophoral canal) of the male. *S. mansoni* is the causative agent of schistosomiasis, a severe debilitating disease that afflicts more than 200 million people around the world, mostly in developing countries in Africa, Middle East, and South America. Schistosomiasis ranks with malaria and hookworm infections as one of the three great scourges of humans. There are 3 species of schistosomes that are harmful to humans, *S. mansoni*, *S. japonicum* and *S. haematobium*. The pathogenesis of schistosomiasis is almost entirely due to the eggs and not to adult worms. The symptoms are chills and fever, fatigue, headache, malaise, muscle aches, lymphadenopathy and gastrointestinal discomfort. Chronic states are associated with spleenomegaly and bloody diarrhea in the case of *S. mansoni,* or hematouria and pain during urination in *S. haematobium. S. japonicum* causes bloody mucus in the feces and may lead to stenosis of the intestinal tract and papillomas. The drugs of choice for schistosomiasis are praziquantel, oxamniquine and metrifonate (Marquardt and

Demaree 1985; Jordan et al., 1993). I will focus in this study on *S. mansoni* and the following is the life cycle of this parasite.

I.1 *S. mansoni* life cycle

S. mansoni has a complex life cycle that requires an intermediate snail host and a definitive mammalian host (see Fig. 1). The free-swimming stage, miracidium penetrates a snail intermediate host (*Biomphalaria glabrata*) and develops into a sporocyst. Within the host the sporocyst gives rise to a tailed cercaria larva, which becomes the second free-swimming stage in the life cycle. Cercariae pass from the snail into fresh water to infect a mammalian host. Cercariae can live in water for up to three days. If they come into contact with the skin of a human host, they attach and creep about for a time as if seeking a suitable place to penetrate. It has been found that cercaria is chemotactic to the amino acid arginine in the host skin, which stimulates the cercarial postacetabular glands to produce its own arginine and to attract other cercariae to the penetration site (Robert and Janvoy 1996). Upon penetration of the skin, morphological and biochemical changes occur in the cercaria causing its transformation into schistosomula. Schistosomules enter a blood or lymph vessels to reach the right side of the heart and then the lungs. After development in the lungs, they migrate to the liver through the left side of the heart and continue growing (Marquardt and Demaree 1985). Blood-feeding worms develop after 3 weeks in liver sinusoids and then migrate against the blood flow of hepatic portal system to the mesenteric vein for oviposition. Laterally spined eggs will be laid and excreted in host feces, they hatch once reaching the water to release

Fig. 1 Life cycle of *Schistosoma mansoni*. The developmental stages of *S. mansoni* (egg, miracidium, cercaria, and adults) and the intermediate snail host (*Biomphalaria glabrata*) are shown (modified from Robert and Janvoy, 1996).



miracidium and the life cycle continues. Adult schistosomes may live up to 30 years in the host (Mahmoud 2001; Marquardt and Demaree 1985; Jordan et al., 1993).

I.2 Schistosome Biology

Males of *S. mansoni* have a length of 6.4 to 9.9mm long, females 7.2 to 14mm. The males are shorter and stouter than the female. The female worm usually resides in the gynecophoral canal of the male worm, where copulation takes place. The more robust muscles of the males allow the paired worms to work their way "upstream" into smaller veins, where the female deposits eggs (Robert and Janvoy 1996)), Moreover, unpaired female worms do not become sexually mature and have the appearance of starving. Their esophageal musculature is weak and thin, so the muscular action of the clasping male helps the immature female pump blood into her intestine (Stirewalt 1974; Basch 1990). The general body plan of the parasitic flatworms consists of an outer syncytial tegument layer and an inner parenchymal area within which the digestive and reproductive systems are located. Sandwiched between the tegument and the parenchymal layers is the somatic musculature. The following is the description of these components:

I.2.1 The tegument: The external surface of parasitic trematodes, termed the tegument, has a wide range of biological functions. In schistosomes the tegument is about 4um thick (Fried and Graczyk 1997). The basic structure of the tegument is said to be the same in the cercaria, schistosomula and adult worm (Smith et al., 1969; Hockley 1972). The tegument is structurally adapted for

transport, immune evasion and communication with the neuromuscular system (via gap junction). Numerous pits at the surface of the tegument markedly increase the surface area of the parasite which is beneficial for transport function. Tegument consists of double lipid bilayer membrane that allows rapid replacement of old immunologically damaged membrane by new one. It may also permit host antigen, largely blood group glycolipids and glycoproteins, to be incorporated into the surface membrane of the parasite to avoid immune attack (Fried and Graczyk 1997). The tegument contains different components and is part of others such as:-

a) Glycocalyx: is a granular and fibrillar layer up to 0.5um thick overlying the surface membrane. The fibers are branched and interconnected to form a diffuse network (Hockley 1972; Stirewalt 1974). Glycocalyx serves as the interface between the parasite and their environment. The cercarial glycocalyx interact with antibodies in antishistosome serum (CHR) forming a pericercarial seroenvelope (Stirewalt 1974). The source of glycocalyx is unknown (Stirewalt and Walters 1973) but there is evidence to suggest that it is an integral part of the outer membrane of the tegument (Hockley 1972). In schistosomules, the glycocalyx is missing, interrupted or functionally modified. The function of glycocalyx is not fully known but may play a role in adhesion or lubrication, protection against inimical conditions, and physiological adaptation with charging environments, and control surface permeability.

b) Tegument spine: The surface of cercariae and adult schistosomes are spined (long, thin and epically pointed). The spines are about 1um long. The spines are the same in cercariae and adults, but the cercarial spines are smaller than those

of the adult and are not surrounded basally by socket-like invaginations of the basal membrane. Spines in cercaria are found all over the body surface except around the mouth, area surrounding the oral sucker, sensory papillae and around the excretory duct pores at the furcal tips. The spines are longer and more concentrated on the ventral sucker than on the body (Hockley 1968). Spines are composed of tightly packed protein subunits, probably repeating macromolecules, which are not keratinous (Smith et al., 1969). No structural differences have been recorded in the spines on schistosome stages from sporocysts to adults.

c) Sensory papillae: They are not truly tegumentary but a part of the nervous system present on the cercarial surface. These structures projecting from the surface of the body and tail of cercariae are called hairs or setae of 9 to 12 um long. Sensory papillae are recognized as small areas in which underlying cells protrude through the tegument (Smith et al., 1969; Stirewalt 1974). They are nerve cells processes in contact with the environment either by the protruding setae or openings through the tegument and each derived from a single nerve cell process. I will discuss the different type of sensory papillae under the schistosome nervous system.

I.2.2 Musculature: the organization of the musculature is catalogued as a subtegumental system comprising one outer circular and one or more subjacent longitudinal layer of muscle fibers. Cercarial muscle cell or myocytes consist of perikaryon and myofibers. Myofibers may appear coarse as in the musculature of body wall, tail, and oral sucker, or fine as in the parenchymal network. The

myofibers contain a granular sarcoplasm with varying density and a wide distribution of mitochondria, glycogen and sarcoplasmic reticulum. The muscles of specific body organs merit separate description. The musculature of the oral organ is specialized in shape and in the arrangement of its myofibers. It consists of a circular muscle layer positioned between two longitudinal layers. The ventral sucker is provided with muscle fibers that radiate outward, beyond which are circularly arranged muscle fibers. Coarse fibers from the ventral sucker traverse the parenchyma to anchor in the body wall. Myocytes of the parenchymal net and the sheaths of the digestive tract and secretory cells appear similar in structure and differ in the arrangement of the myofibers (Fried and Graczyk 1997; Stirewalt 1974).

I.2.3 Schistosome Secretory Cells: In cercariae, there are at least three different types of large unicellular glands 1) preacetabular gland 2) postacetabular gland. 3) head gland. The contents of these glands are secreted during the emergence process so the glands are empty in the free-living cercaria (Dorsey and Stirewalt 1971). Two pairs of preacetabular glands lie anterior to the ventral sucker and one pair is dorsal to the ventral pair. Three or four pairs of postacetabular are caudal to the ventral sucker. The head gland with its multiple ducts lies completely within the oral sucker and occupied the mid region of this area. The function of the head gland is related to the post penetration adjustment of schistosomules to skin and its secretion provides membranes for repair, replacement and development of the schistosomular oral surface membrane. Postacetabular secretion (mucus) provides adhesion of cercaria to surfaces as

well as protective and enzyme-directing functions. Preacetabular secretions contain calcium and proteolytic enzymes. The calcium may serve as a coenzyme-like activator of proteolytic enzymes, as well as stabilizing agent to the cercarial surface, and is a required factor in the adhesive quality of postacetabular mucus. The proteolytic enzymes are required for penetration by degrading the secreted elements of skin.

I.2.4 Schistosome Digestive system: In adult schistosomes the digestive system consists of a mouth, pharynx, and esophagus that branch to form a pair of intestinal caeca which extend posteriorly and end blindly (Smith et al., 1969; Robson and Erasmus 1970; Fried and Graczyk 1997). The surface of the tegument extends inward to form the wall of the oral cavity and the esophagus, but not the blind cecum (Stirewalt 1974). The muscular housing of the oral cavity and esophagus consists of outer longitudinal and inner muscle fibers. The oral cavity has a very irregularly shaped lumen and is surrounded by the heaviest circular muscle fibers seen along the tract. The blind cecum has a very different structure from that of the oral cavity and esophagus. The caeca consist of a single layer of epithelial cells supported by a thin layer of longitudinal and circular muscle fibers. The epithelial layer is syncytial. The surface of the caeca is amplified up to 100 fold by numerous digitiform microvilli (Fried and Graczyk 1997). The gut epithelial cells contain numerous mitochondria and ribosomes and most the cytoplasm is occupied by endoplasmic reticulum and golgi bodies. A digestive system is also present in the cercaria larval stage but it is considered

to be non-functional for several reasons: a) There is no report of oral ingestion or peristaltic activity. b) No glycogen can be found in the gut sac.

I.2.5 Excretory system: This system comprises of flame cells, primary and secondary collecting tubules, a pair of main collecting tubes in the body and a single tube in the tail, an excretory bladder, an excretory atrium and excretory pores (Stirewalt 1974). The number of flame cells varies from four to six pairs. Flame cells are an irregularly shaped cell embedded in the peripheral parenchymal ground substance. Muscle and nerve cell processes are often seen adjacent to the flame cells. In schistosomules, following the loss of tail, the bladder pore becomes the functional pore and the terminus of the excretory system and the bladder wall becomes continuous with the surface tegument.

I.3 Schistosome Nervous System

The structural unit of the nervous system is the nerve cell consisting of a perikaryon with the cell nucleus, and long extended processes called nerve fibers or axons and dendrons. The central nervous system is composed of a pair of cerebral ganglia connected by a commissure and a variable number of anterior and posterior nerve tracts arising from these ganglia. The cerebral ganglion complex is in the pharynx region. The anterior tracts innervate anterior suckers and other attachment structures, pharynx and portions of the anterior body wall musculature. The major posterior nerve cords run longitudinally in a submuscular position and there are 3 pairs of them in trematodes. The peripheral nervous system consists of transverse commissures which interconnect these cords. The transverse commissures typically occur at regular intervals yielding an orthogonal

arrangement of circular rings along the body length. An intricate meshwork of fine neural processes gives the peripheral nervous system a nerve-like structure. A discrete nerve plexus occurs in association with specific structures that require innervations such as sucker, pharynx, reproductive structures and muscle associated with body wall, glands and tail (Fried and Graczyk 1997; Enasmus 1972). The cercarial nervous system is based on the same pattern as that of the adult. The axons of nerves resemble those of vertebrates except that they are unmyelinated. The nerve processes contain mitochondria and vesicles. The cell bodies of the neurons are connected in the cerebral ganglion and the perikarya show nuclei and golgi bodies. The synapses resemble those described from the mammalian central nervous system. The neuromuscular junctions exhibited closely adherent membranes of nerve and muscle cell and the presence of small vesicles in the axon terminal. Nerve cell processes also appear to provide for the cercaria's contact with environment. Specialized nerve cell processes protrude into and through tegument as described earlier for the sensory papillae.

The functional relationship between the nervous system and the somatic musculature is not very clear. Synaptic structures appear to be relatively abundant at the interface of the cytoplasmic arms and the nervous system while synapses on the myofibril portion of muscles are rather rare (Pax et al., 1996).

Schistosomes have a complete repertoire of neuroactive agents, including biogenic amines, which mediate chemical neurotransmission and neuromodulation in the parasite.

PART II: BIOGENIC MONOAMINES

The biogenic monoamines constitute a group of small neuroactive agents that include serotonin (5-hydroxytryptamine: 5-HT), the catecholamines (dopamine and noradrenaline), octopamine, and histamine. With the exception of histamine, which is derived from the basic amino acid, histidine, all other members of this group are synthesized from aromatic amino acids, mainly tyrosine or tryptophan (Lehninger et al., 1996) (Fig. 2). Monoamines are widely distributed throughout vertebrate and invertebrate phyla and serve a variety of different regulatory functions as neurotransmitters, neuromodulators and neurohormones. The following is an overview of the biology of monoamines with particular emphasis on histamine, which is of interest to this study.

II.1 General properties of monoamines- The biogenic amine, serotonin (5-HT) is a major neuroactive agent that is widely distributed in both vertebrates and invertebrates. Fluorescence and immunohistochemical techniques led to the localization of significant numbers of serotonergic neurons in the central nervous system (CNS), leading to identification of 5-HT as a neurotransmitter (Twarog and Page, 1953). In the CNS of mammals, the majority of serotonergic neurons have cell bodies localized along the midline region of the brain stem. These neurons send long axons throughout the forebrain, the cerebellum, and the spinal cord (Jacobs and Azmita, 1992; Tipper et al., 1994). This serotonergic transmission has been linked to a wide variety of behaviors and disorders such as schizophrenia, anorexia nervosa, pain perception, aggression, feeding behavior, anxiety, sleep patterns, learning, sexual behaviors, depression and

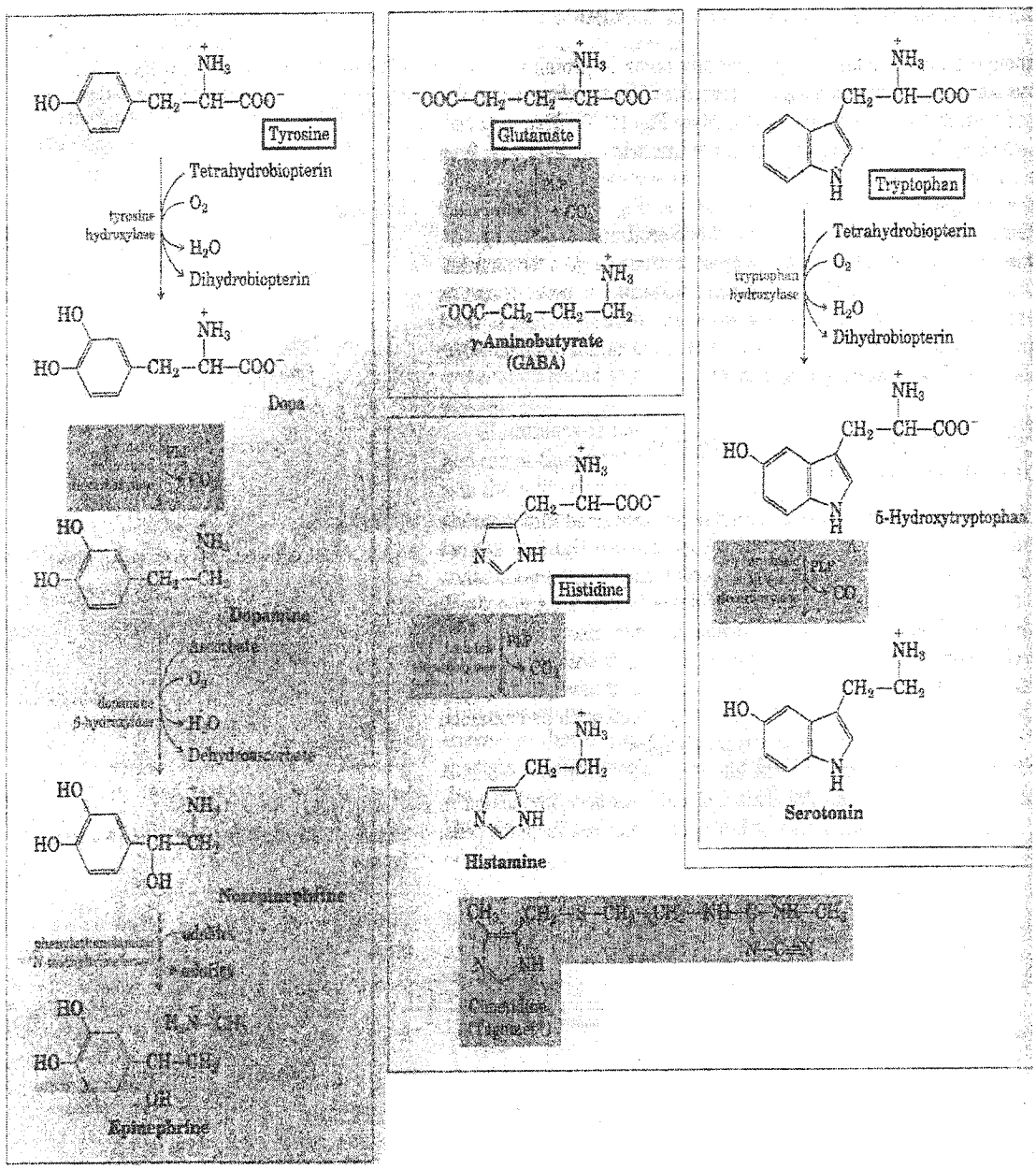


Fig. 2 Neurotransmitters derived from amino acids. The key biosynthetic step is the same in each case: a PLP-dependent decarboxylation (shaded area). Taken from Lehninger et al., 1996.

locomotion. In addition to mammalian systems, 5-HT has been detected in every invertebrate phylum investigated thus far and in protozoa. Among invertebrates, 5-HT regulates many behaviors and physiological responses such as motility, aggression, feeding, mating behavior, circadian rhythm, and modulation of different metabolic pathways (Roth et al. 1998; Angers et al., 1998; Davis and Stretton, 1995).

Octopamine, another monoamine, plays a major role as a neurohormone, neuromodulator and neurotransmitter in various invertebrate species. Octopamine is present only in trace amounts in vertebrates and its role in the mammalian nervous system is presently unclear (Arakawa et al., 1990; Gerhardt et al., 1997). Octopamine has been identified in neural as well as non-neural tissues in invertebrates and modulates almost every physiological process. Among these various functions, octopamine modulates sensory and visual input into the CNS and mediates several behaviors, including submissive postering in lobsters, escape behavior of crayfish, and feeding behavior of blowflies. In *Drosophila*, octopamine has been implicated in locomotion, grooming and olfactory learning. Recently, it has been speculated that human cognition and disorders such as schizophrenia and drug addiction may be related to the octopaminergic system (Han et al., 1998, Roeder, 1999).

The catecholamines, in particular dopamine, play a major role as neurotransmitters both in the central and peripheral nervous systems of verterbrates and invertebrates. Dopamine is involved in a variety of important physiological and behavioral processes, such as neuroendocrine function, emotion, and motor control. Moreover, dopaminergic systems have been

implicated in several neurological disorders, including Parkinson's disease and schizophrenia (Feng et al., 1996; Blakey and Bauman, 2000).

II.2 Histamine

Histamine is derived from histidine through a single decarboxylation reaction catalyzed by the enzyme, histidine decarboxylase. A second enzyme, histaminase, catalyzes the subsequent metabolism of histamine and serves to inactivate the monoamine. Although best known for its activity as a vasodilator and mediator of allergic responses (Thueson et al., 1979; Marshall and Bienenstock, 1994), histamine also functions as a major neurotransmitter and neuromodulator in a wide range of organisms, both vertebrate and invertebrate. Histamine is now a well established neurotransmitter in the brain (Panula et al., 2000). All verterbrates examined to date contain histaminergic neurons that innervate most parts of the brain (Eriksson et al., 1996). In mature mammalian brain, all histaminergic neurons are located in the posterior hypothalamus, where the tuberomammaillary nucleus contains a diffuse group of neurons that project to all major areas of the CNS (Panula et al., 1984, 2000). The histaminergic neuronal system regulates various activities of the brain, including arousal state, thermoregulation, feeding, drinking, sexual behavior, and analgesia (Ghi et al., 1999; Panula et al., 2000; Nonaka et al., 1998). Among the invertebrates, histamine has been implicated as a neurotransmitter of photoreceptors in spiders (Schmid and Becherer, 1999), barnacles (Callaway and Stuart, 1999) and gastropods (Osborne and Patel, 1984). Moreover, histamine has been found in the insect nervous system (locust, cockroach, fruitfly, sphinx moth), the highest levels of histamine being present in the retina and in the lamina neuropil of the

optic lobe (Elias and Evans, 1983; Nassel 1999). This evidence suggests that histamine is a neurotransmitter of photoreceptor cells in all arthropods (Schmid and Becherer, 1999). Histamine has also been identified in the lower invertebrates, mainly helminths, where it is believed to function as a neuromuscular transmitter and / or modulator (see II.3.5)

II.3 Neurotransmitters in Helminths

The ability of a parasite to move to different sites in its host and to maintain its position in host body fluids is crucial to the parasite's survival. Understanding how motility is regulated, particularly at the level of the neuromuscular system, is of great importance both for an understanding of basic parasite behavior and biology and for the development of anthelmintic compounds. Parasitic helminths have several substances that function as a neurotransmitters/ neuromodulators. Among these substances, monoamines function as important neuroactive agents particularly as neuromuscular transmitters and modulators of motor activity, both in platyhelminths (flatworms) (Mellin et al., 1983; Bennett and Gianutsos, 1977; Davis and Stretton, 1995) and nematodes (roundworms) (Sulston et al., 1975; Marr and Muller, 1995). The following is an overview of helminth neuroactive agents with particular emphasis on biogenic monoamines.

II.3.1 Serotonin

In parasitic flatworms, 5-HT serves a variety of functions from control of carbohydrate metabolism to stimulation of motility. 5-HT- like immunoreactivity is found mainly in CNS structures (anterior ganglia, longitudinal and transverse

nerve cords), but it is also seen in a peripheral nexus of small nerve fibers associated with subtegumental muscles, the musculature of the suckers, and muscular lining of reproductive ducts (Marr and Muller, 1995; Pax and Bennett 1991; Pax et al., 1984). 5-HT has been localized in various platyhelminths (flatworms) including, trematodes (e.g. blood flukes, *Schistosoma mansoni* and *Fasciola hepatica*) and cestodes (e.g. *Hymenolepis diminuta* and *Taenia pisiformis*) (Davis and Stretton, 1995). In addition to flatworms, 5-HT has been localized in a variety of nematodes, such as *Caenorhabditis elegans* and the large round worm *Ascaris suum* (Horvitz et al., 1982). In *C. elegans* and *A. suum* 5-HT has been identified mainly in two pharyngeal neurosecretory motor neurons (NSM) and 5 male-specific neurons (CP neurons) in the tail region. In *C. elegans*, 5-HT was also found in a pair of hermaphrodite-specific motor neurons (HSN) (White et al., 1986, Desai et al., 1998).

Earlier work showed that 5-HT causes an increase in motor activity in both trematodes and cestodes and regulates metabolic processes, especially in the regulation of glycogen metabolism and glucose utilization (Mansour, 1984; Pax and Bennett, 1991; Davis and Stretton, 1995; Ercoli et al., 1985). In nematodes, 5-HT suppresses or completely inhibits nematode motility, stimulates egg-laying and contributes to pharyngeal pumping and feeding (Davis and Stretton, 1995).

It has been suggested that parasitic helminths obtained 5-HT from an exogenous source and were unable to synthesize 5-HT endogenously (host) (Bennett and Bueding, 1973). However, a recent study showed that tryptophan hydroxylase (TPH), which catalyzes the rate-limiting step in 5-HT biosynthesis, is present in *S. mansoni* (SmTPH) (Hamdan and Ribeiro, 1999a). This provided the

first evidence that *S. mansoni* and possibly other helminths, are able to synthesize their own 5-HT.

5-HT exerts its effect through serotonergic receptors. These receptors are members of G protein-coupled receptor family and divided into 7 classes in mammals (Angers et al, 1998). Two 5-HT G protein-coupled receptors have been cloned from *C. elegans* (Olde and McCombie 1997; Hamdan et al, 1999b) and one from *A. suum* (Huang et al., 1999). In addition a novel 5-HT-gated Cl^- channel was recently isolated from *C. elegans* (Ranganathan et al., 2000). By comparison much less is known about 5-HT receptors in flatworms. A number of 5-HT binding sites have been described in crude membranes of cestodes and trematodes (Estey and Mansour 1988) but no receptor has yet been cloned from these organisms.

II.3.2 Acetylcholine (Ach)

Ach is a putative inhibitory transmitter in flatworm parasites. Pharmacological studies demonstrated that it inhibits muscle activity, suppressing rhythmical movement and ultimately producing a flaccid paralysis. This occurs both in trematodes *F. hepatica* (Sukhdeo et al., 1986), *S. mansoni* (Pax et al., 1984; Mellin et al, 1983) and in cestodes *H. diminuta* (Sukhdeo et al, 1984).

In contrast, Ach has been identified as an excitatory neurotransmitter in some nematodes, particularly *A. suum* (Walker et al., 1996) causing a body wall muscle contraction (Venter et al., 1988). Biochemical and histochemical studies have shown the presence of Ach esterase in parasitic flatworms within CNS

structures (central ganglia and associated commissures nerve cords), the pharynx, and sucker (Lewert and Hopkins 1965). Staining is also found in fine ramifications throughout the parenchyma/subintegumental muscle regions presumably associated with neuro-muscular innervation, and in muscles lining reproductive structures (Marr and Muller, 1995).

In the mammalian system, Ach has been known to exert its effect by binding two kinds of post synaptic integral membrane proteins, termed nicotinic and muscarinic receptors. Nicotinic receptors are gated cation channels, whereas muscarinic receptors function by activating signal-transducing G-protein and intracellular effectors system (Lee et al., 1999).

Cholinergic receptors in flatworms display a mixture of nicotinic and muscarinic properties and they are pharmacologically different from the mammalian cholinergic receptors (Davis and Stretton, 1995). Nicotinic receptors have been found in *C. elegans* and *H. contortus* and were shown to resembled vertebrate nicotinic receptor. Moreover, a muscarinic acetylcholine receptor was cloned from *C. elegans* (Lee et al., 1999) as well as a vesicular acetylcholine transporter (Alfonso et al., 1993).

II.3.3 Neuropeptides

Neuropeptides function as neurotransmitters and/or neuromodulators in both vertebrates and invertebrates. The presence of a neurosecretory peptidergic component in the nervous system of platyhelminths has long been recognized (Halton et al., 1994; Fairweather et al., 1988). Immunocytochemistry indicated the presence of a wide range of neuropeptides within several parasitic flatworms,

in particular the adult of *S. mansoni* (Gustafsson 1987; Basch and Gupta 1988; Brownlee et al., 1994). These studies revealed the presence of neuropeptides within nerve cell bodies, small and giant nerve processes of the neuropile in the central ganglia and transverse commissure, as well as in the main longitudinal nerve cords (Brownlee et al., 1994).

Flatworms were found to be immunoreactive to pancreatic polypeptide (Maule et al., 1991) and FMRFamide (phe-Met-Arg-phe-amide) related peptides (FaRPs) (Pax et al., 1996). The source of the pancreatic polypeptide immunoreactivity has now been isolated and structurally identified as neuropeptide F (NPF) (Maule et al., 1991), and several FaRPs have also been isolated and structurally characterized from extracts of turbellarians and tapeworms (Mckay et al., 1991). In contrast, no peptides have been isolated or characterized from any of the trematodes.

The biological roles(s) of peptides in flatworms remain unclear. Whole-animal behavioral studies have been generally unsuccessful, (Pax et al., 1996) in part because peptides are unable to penetrate the tegument and reach underlying tissues. Moreover, it is unknown if exogenously applied peptides can survive proteolytic destruction long enough to reach their site of action. To date, the only evidence of peptidergic effects in flatworms has come from studies of isolated muscle strips, where the peptide was applied directly onto exposed muscle. These studies demonstrated a dose-dependent stimulation of muscle contraction by FMRFamide and other FaRPs in schistosomes (Pax et al., 1996). The sensitivity of schistosome muscle to FaRPs suggests that biologically active peptides are present in trematodes and probably act by interacting with

neuromuscular receptors. Among nematodes, there is ample evidence that neuropeptides are present within neurons and have potent effects on behavior. A wide range of peptide-like immunoreactivities has been detected in nematodes. FMRFamide immunoreactivity has been found in neurons of *C. elegans*, *Heterodera glycines* and *A. suum* (Sithigorngal et al., 1990). *A. suum* has a family of FMRFamide-like peptides that can be divided into several subfamilies on the basis of sequence. Measurements of effects on the muscle showed that there are at least four classes of biological activity controlled by these peptides: AF1 (AF=Ascaria FMRFamide-like peptide) and AF2 stimulate contraction and promote rhythmic activity in muscle (Cowden et al., 1989), AF3 and AF4 cause muscle contraction (Cowden and Stretton, 1995); whereas AF11 relaxes muscle (Marr and Muller, 1995). The receptors responsible for these effects have not yet been identified.

II.3.4 Catecholamines

Less work has been done on the role of catecholamines than on 5-HT and Ach or neuropeptides. Biochemical and histochemical studies in helminths have demonstrated the presence of the catecholamines, dopamine (DA) and noradrenaline (NA) in trematodes. In *S. mansoni* NA was detected but no DA, while *F. hepatica* contained DA but not NA (Chou et al., 1972; Bennett and Gianutsos, 1977). The recent identification of the catecholamine biosynthetic enzyme, tyrosine hydroxylase, in *S. mansoni* provided the first evidence that trematodes can produce these substances endogenously (Hamdan and Ribeiro 1998). Catecholamines in *S. mansoni* are inhibitory and cause a lengthening of

the worm but have no obvious effect on motitity (Pax et al., 1984). In the Cestode *D. dendriticum*, catecholamines are found in the CNS but also peripherally in longitudinal and transverse muscle layers.

Some studies emphasize the importance of examining different developmental stages in order to assess the presence of a given neuroactive substance. This is the case with the cestode, *Spirometra mansonoides,* which has catecholamines in the larvae but not the adults (Tomosky-Sykes et al., 1977). It is unknown if this stage-specific difference in neurotransmitter level has biological significance or if it is related to developmental changes in biosynthetic activity. The precise physiological functions of catecholamines in cestodes have not been determined.

In nematodes catecholamines have been reported in *A. suum* and *C. elegans*. In *C.elegans* formaldehyde-induced fluorescence (FIF) revealed eight dopaminergic sensory neurons while in *A. suum* only four of these sensory neurons show DA-like FIF (Sulston et al., 1975). Two behaviors, locomotion and egg laying, are transiently inhibited by exogenous dopamine (Scafer and Kenyon, 1995). Recently a dopamine G protein-coupled receptor has been identified from *C. elegans* by Suo et al., 2002.

II.3.5 Histamine

Histamine and its catabolic enzyme histaminase have been found in the cercariae and adults of the flatworm *S. mansoni* (Schwabe and Kilejian, 1968). One early study reported that histamine may play a role in the modulation of motility in cercaria and schistosomula (Ercoli et al., 1985). The role of histamine

in adult worms is still unknown. Among the tapeworms, significant numbers of histaminergic neurons have been reported in larval *D. dentriticum* (Wikgren *et al*, 1990) and histamine has been detected in tissue extracts of *H. diminuta* (cestoda) (Sukhdeo et al, 1984). High-pressure liquid chromatography (HPLC) analyses of crude tissue extracts demonstrated that *H. diminuta* contained histamine (5pmol/mg). These studies further showed the worm did not synthesize histamine endogenously, but took it up in a manner consistent with simple diffusion (Yonge and Webb, 1992). *Fasciola hepatica* (trematoda) and *Ameiva dorsais* (cestoda) contain relatively low levels of histamine (Eriksson et al., 1996). In contrast, the frog lung parasite, *Haplometra cylindracea* (trematoda), contains neurons which store histamine at high concentration of more than 800 pmol/mg wet weight. Interestingly, the concentration of histamine in this parasite is among the highest reported in the animal kingdom (Eriksson et al, 1996). Recently a novel histamine receptor has been cloned from *S. mansoni.* This is the first helminth histamine receptor and the first member of the G-protein coupled receptor superfamily ever cloned from a parasitic platyhelminth (Hamdan et al., 2002a).

Table 1: Summary of neurotransmitters effects on both platyhelminth and nematodes:

	Effect	
Neurotransmitter	**Platyhelminth**	**Nematodes**
5-HT	- ↑ motor activity - ↑ glycogenolysis - ↑ glycolysis	- Suppress/inhibit motility - Stimulate egg laying - ↑ pharyngeal pumping feeding
Ach	- Inhibit muscle activity - Suppress rhythmical movement - Produce flaccid paralysis	- ↑ muscle contraction
Neuropeptides	- Stimulate muscle contraction	- Stimulate muscle contraction - Promote rhythmic activity in muscle.
Catecholamines	- Inhibitory effect. - Lengthening of the worm. - No effect on motility.	- Inhibit locomotion & egg laying
Histamine	Modulation of motility?	

PART III: HISTAMINE RECEPTORS

I have discussed the general properties of histamine and its presence in the mammalian system as well in invertebrates and helminths. I will now focus on the molecular mechanism of action of histamine as a neurotransmitter. The following is an overview of the structural, pharmacological, and signaling properties of histamine receptors in mammals. Because histamine receptors belong to the superfamily of GPCR, I will start with a brief discussion of the structural and functional properties of the class I (Rhodopsin family) GPCRs.

III.1 G protein-coupled receptors: an overview

G protein-coupled receptors are cell surface receptors that mediate various physiological responses to a remarkably diverse array of signaling molecules, including hormones, neurotransmitters, pheromones, and sensory stimuli (vision, taste, smell) (Hall et al., 1999, Strader et al., 1994, Wess, 1997). In humans, there are different pathologies associated or directly caused by GPCR dysfunction, such as color blindness, retinitis pigmentosa, Parkinson's disease, Alzheimer, asthma, depression, hypertension, sleeping disorders, stress, cardiovascular problems and renal failure (Horn and Vriend, 1998, Alberts et al., 1994). GPCRs are widely present in eukaryotes and it is estimated that more than a thousand different GPCR sequences are present in the human genome (~ 1% of the whole genome) and the genome of the free-living nematode *C. elegans* (~ 5% of the total genes) (Baldwin, 1994, Strader et al.,

1994). Based on amino acid sequence similarity, ligand binding properties and G protein-coupling specificities, the GPCR superfamily can be subdivided into four major classes. The rhodopsin receptor family, which constitutes class I, is considered the largest and the most widely studied of all GPCR classes. Class I contains receptors for sensory stimuli, peptides, glycoprotein hormones, biogenic amines, and others (Horn and Vriend, 1998). Although GPCRs interact with diverse ligands and are different at the amino acid sequence level, their basic organization within each major class is conserved. The global structure of GPCRs consist of seven α-helical hydrophobic transmembrane domains (TM1-TM7) connected by three extracellular loops and three intracellular loops. The NH_2 terminus is extracellular while the carboxyl terminus is intracellular. Palmitoylation at a C-terminal cysteine residue may cause the formation of a forth intracellular loop (Wess, 1998, Baldwin et al., 1997, Gether and Kobilka, 1998, Blakey and Bauman, 2000). Models for structure of GPCRs were initially based on the folding properties of bacteriorhodopsin (*Halobacterium halobium*), analyzed by electron microscopy and high-resolution electron diffraction (Horn and Vriend, 1998, Wess, 1997, Savarese and Fraser, 1992). Recently a high-resolution three dimensional crystal structure of bovine rhodopsin was reported. This evidence shed new light on key residues in the transmembrane domains (TMDs) and the organization of loops, which are critical in GPCR structure (Palczewski et al, 2000).

G protein mediated transmembrane signaling pathways have generated a great deal of attention because of the many physiological and pharmacological events that are mediated by these mechanisms. Upon binding of extracellular

ligands, GPCRs interact with a specific subset of heterotrimeric G-proteins (consisting of α, β, γ subunits) that can inhibit or activate various effectors enzymes or ion channels. For instance, stimulatory G-proteins ($G_{\alpha s}$) catalyze the activation of the intracellular enzyme adenylate cyclase, which stimulates cAMP production and also activates protein kinase A (PKA). The latter, in turn, initiates a cascade of phosphorylation events that lead to cellular responsiveness. On the other hand, inhibitory G proteins ($G_{\alpha i}$) inhibit the activity of adenylate cyclase and thus cause a decrease in cAMP levels and PKA activity. A third major type of G-protein, Gαq, mediates the activation of phospholipase C (PLC), leading to the degradation of the specific membrane phospholipids, phosphatidylinositol 4,5-biphosphate (PIP_2) to yield inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The resulting increase in IP_3 induces Ca^{2+} release from intracellular stores, which in turn activates Ca^{2+} dependent protein kinases, mainly protein kinase C (PKC) and the calmodulin- dependent protein kinase, CaM PKII (Clapham and Neer, 1997, Harnett and Harnett, 1998, Wess, 1998). The three subunits that make up G-proteins are encoded by a diverse number of genes. Moreover, important roles have been proposed for βγ-dimers as multifunctional enhancers or attenuators of intracellular signals (Birnbaumer, 1992, Sternweis, 1994, Clapham and Neer, 1993, 1997), in addition to their role as stabilizing receptors to α-subunits. For instance, βγ-subunits alone have been shown to activate certain PLC isoforms leading to increased Ca^{2+}-mediated signaling. This activity is enhanced by the presence of the G_{α} subunit suggesting that G_{α} and $G_{\beta\gamma}$

act synergistically, or are required for maximal activation of certain PLC isoforms (Dianqing et al., 1993; Selbie and Hill, 1998; Raymond, 1995).

III.2 Classification of Histamine receptors

Histamine receptors are members of Class I GPCR family and are structurally related to the biogenic amine family of receptors. To date, the classification of histamine receptors has been based mainly on rigorous classical pharmacological analysis. The acceptance of additional subtypes still awaits the identification of "sequence differences" within single species and the development of selective agonists and antagonists providing the structural recognition and transductional information necessary for reliable classification (Leurs et al., 1995, Schwartz et al., 1991). Until recently, only three histamine receptors (H_1, H_2, H_3) have been identified, all of which in mammalian systems. In the last year however, new forms have been described: A new histamine receptor H_4, has recently been identified in mammals (Hough, 2001, Zhu et al., 2001). With one exception (see part IV), no histamine G protein-coupled receptors have yet been cloned from invertebrates.

III.2.1 Histamine H_1 receptor

Histamine H_1 receptors have all the structural features of a G-protein coupled receptor and show similarity to other monoamine GPCRs. The highest homology is found with muscarinic m_1 receptor (44%) rather than with the histamine H_2 receptor (40%) (Leurs et al., 1995). In mammals, the histamine H_1 receptor is present in most smooth muscle, endothelial cells, adrenal medulla, heart, CNS and human T-lymphocytes. H_1 receptors are involved in smooth

muscle contraction, increase vascular permeability; stimulate NO formation by stimulating nitric oxide synthase activity (via a Ca^{2+}/calmodulin dependent pathway) and subsequent activation of soluble guanylyl cyclase in a variety of different cell types (Schmidt et al., 1990). The primary mechanism by which histamine H_1 receptors produce functional responses in cells is the activation of phospholipase C via a pertussis toxin-insensitive G-protein that is probably related to the $G_{q/11}$ family of G-proteins. As described above, the activation of $G_{\alpha q}$ leads to an increase in IP_3 and intracellular calcium mobilization. H_1 receptor activation can produce substantial changes in the intracellular levels of cAMP and this does not happen through activation of adenylate cyclase. Instead, the effect on cAMP is due to direct potentiation of other receptors, in particular H_2 and adenosine A_2, which signal through adenylate cyclase. A role for both intracellular calcium ions and protein kinase C has been implicated in this augmentation response (Al-Gadi and Hill, 1987; Garbarg and Schwartz, 1988).

III.2.2 Histamine H_2 receptor

In mammals H_2 receptors are widely distributed in gastric parietal cells, vascular smooth muscle, suppressor T cells, neutrophils, CNS, heart and uterus (rat). Histamine H_2 appears to play a role in regulating intestinal secretion and gastrointestinal motility leading to stimulation of gastric acid secretion, smooth muscle relaxation in airways, uterine and vascular smooth muscle (Del Valle and Gantz, 1997; Hill et al., 1997). H_2 receptors inhibit a variety of functions within the immune system. For example basophils and mast cells have been shown to negatively regulate the release of histamine and can also inhibit antibody

synthesis by lymphocytes, T-cell proliferation, cell-mediated cytolysis and cytokine production. The physiological effects attributed to the H_2 receptor are broad in scope and important. Receptor activation leads to stimulation of cAMP formation in a number of systems (brain slices, stomach, mucosal cells and glands, heart myocytes, basophils and neutrophils). The capability of histamine to stimulate cAMP formation is through direct activation of adenylate cyclase via a guanine-nucleotide-dependent mechanism. Activation of the histamine H_2 receptor has also been associated with Ca^{2+}-dependent signal transduction events (Del Valle and Gantz, 1997). The H_2 receptor leads to an increase in the intracellular concentration of calcium ions but this stimulation effect may be very cell-specific (Hill et al., 1997) and may lead to inhibition of phospholipase A_2 (Del Valle and Gantz, 1997). The ability of H_2 receptor to activate multiple G-proteins fits into the complex paradigm of cross talk between signaling pathways that is now being observed for multiple heptahelical receptors.

III.2.3 Histamine H_3 receptor

Whereas the H_1 and H_2 receptors were cloned nearly a decade ago, the first H_3 receptor was not cloned until 1999 (Hough, 2001). H_3 receptors have been localized in peripheral nerves that innervate heart, lung, gastrointestinal tract, endothelium, enterochromaffin cells, tuberomammilary nucleus and in various brain regions of the guinea pig brain, including cerebral cortex and the basal ganglia (Arrang et al., 1987; Tardivel-Lacombe et al., 2000). H_3 is one of the histamine receptor subtypes that is considered to be responsible for the actions of histamine as a neurotransmitter. H_3 receptors regulate the release of

other important neurotransmitters, such as acetylcholine, dopamine, glutamate, noradrenaline, and serotonin in both the CNS and peripheral nervous system (Drutel et al., 2001). H_3 agonists have also been implicated in the inhibition of gastric acid secretion in dogs (Hill et al., 1997). Recently, the structure of the H_3 receptor in human and other mammalian species was elucidated (Tardival-Lacombe et al., 2000) and new receptor subtypes have been identified. At the genome level, the H_3 receptor gene is interrupted by several introns. Alternative splicing of intron sequences in the primary mRNA transcript results in various H_3 isoforms including 3 variants in rat (H_3A, H_3B, H_3C) (Drutel et al., 2001) and 2 in guinea pigs (H_3L, H_3S) (Tardivel-Lacombe et al., 2000). All splice variants differ in structure of their third cytoplasmic loops but the relevant splicing mechanisms remain uncertain (Hough, 2001; Tardivel-Lacombe et al.; 2000, Drutel et al., 2001). The H_3 receptor isoform that seems to be most predominant in human brain corresponds to the rat H_3A and the guinea pig H_3L. Phylogenetic and homology analysis of the H_3 receptor showed it to be surprisingly different from the previously cloned H_1 and H_2 receptos. The degree of sequence homology between H_3 and other amine GPCRs is only about 30%. Histamine H_3 receptors couple to $G_{i/o}$ (Hough, 2001) leading to inhibition of adenylate cyclase. For H_3 receptor isoforms H_3A, B, C, the inhibition of adenylate cyclase was completely sensitive to pertussis toxin (PTX), consistent with $G_{i/o}$ coupling. Rat H_3 isoforms also activate the MAP kinase-signaling cascade via PTX-sensitive G-proteins. It has been suggested that the different splice variants of H_3 may trigger different signaling mechanisms by coupling to different G-proteins, or the same G-protein with variable efficiencies (Tardivel-Lacombe et al., 2000).

III.2.4 Histamine H_4 receptor

The H_4 receptor was cloned in 2001 and thus is the most recent addition to the histamine receptor family. Localization studies revealed that H_4 is expressed primarily in bone marrow and eosinophils. H_4 is structurally and pharmacologically related to the H_3 receptor subtype (Hough 2001). However, its unique expression profile and physiological role are different from those of other histamine subtypes and thus may represent a therapeutic target for the regulation of immune function, particularly with respect to allergy and asthma (Liu et al., 2001). The H_4 receptor shows 37-43% homology to the H_3 receptor (58% in TM regions). The human H_3 and H_4 receptors possess very similar genomic organization, both having two introns and three exons (Zhu et al., 2001). H_4 receptor seems to couple to $G_{i/o}$ thereby inhibiting cAMP formation. Notably, the distribution of the H_4 receptor shows highest levels in bone marrow and leukocytes (particularly eosinophils and neutrophils), with moderate level in spleen and small intestine and, possibly, mast cells (Hough 2001; Zhu et al, 2001; Liu et al., 2001). No Histamine H_4 receptor subtypes have yet been identified.

PART IV: *Schistosoma mansoni* G protein-coupled receptor (SmGPCR)

This recently cloned receptor from *S. mansoni* is the first member of GPCR superfamily ever cloned form a parasitic platyhelminth (Hamdan et al., 2002a). It is also one of the few biogenic amine receptor sequences obtained from a lower invertebrate. Position specific Iterated BLAST (PSI-BLAST) amino acid sequence of SmGPCR revealed that it is related to biogenic amine and muscarinic acetylcholine receptors. Additional CLUSTAL pairwise comparisons of the conserved TM regions revealed about the same level of homology (~50%) with histamine receptors, serotonin receptors, octopamine receptors, β-adrenergic receptors, dopamine receptors and muscarinic acetylcholine receptors. A dendogram analysis showed that SmGPCR did not cluster exclusively with any of the above receptor groups and thus represents a new structural class of biogenic amine receptor. SmGPCR contains an asparagine residue (Asn^{111}) instead of a highly conserved aspartate in TM3 (Fig. 3). The latter is thought to be involved in the binding of the protonated amine group of all aminergic ligands (Hibert et al., 1991; Oliveira et al., 1994). Mutagenesis studies showed that replacement of this conserved aspartate with an asparagine in various biogenic amine receptors resulted in decreased agonist and antagonist affinities (Van Rhee and Jacobson, 1996). Thus the asparagine substitution of SmGPCR presents a significant change from the typical structural organization of a biogenic amine GPCR and suggests that SmGPCR may have distinctive functional properties.

A major goal of the present study was to identify the ligand for SmGPCR as a first step towards the functional characterization of this receptor. To achieve this goal, it was necessary to optimize conditions for efficient heterologous expression of SmGPCR in mammalian cells and extensive functional assays with all known biogenic amines. As will be shown in the next chapter, these studies identified SmGPCR as a novel histamine receptor of *S. mansoni*.

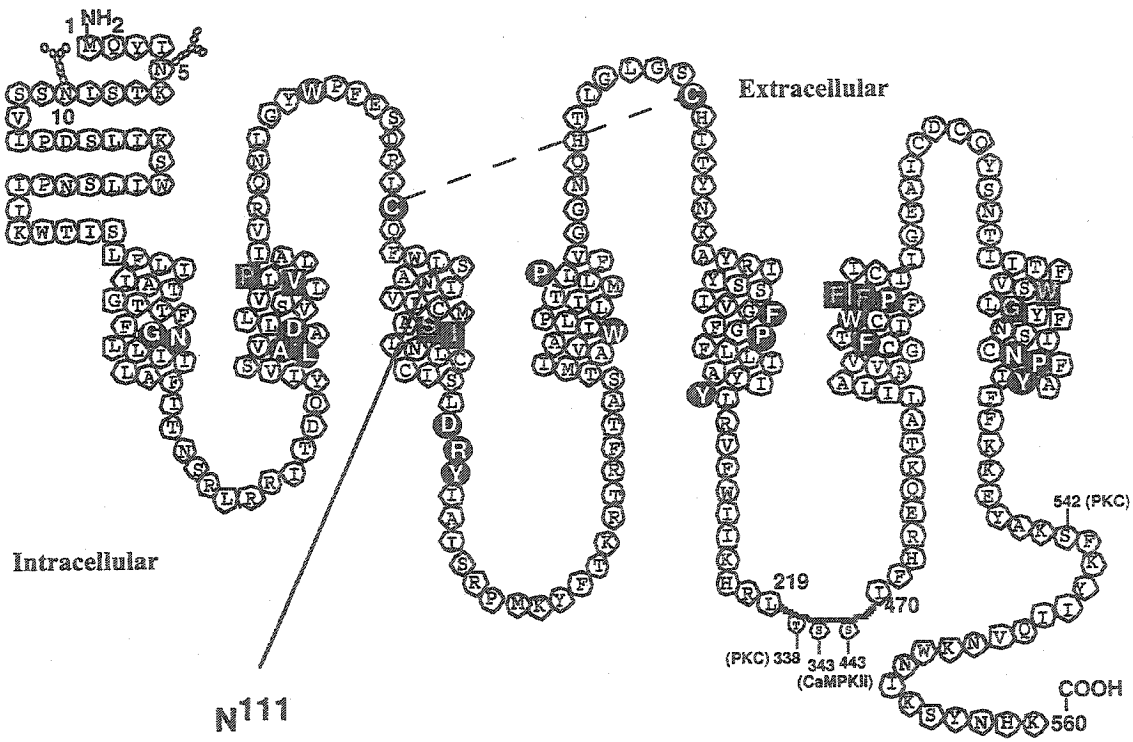


Fig. 3 Schematic model of the predicted protein structures of SmGPCR. SmGPCR topology consists of 7 hydrophobic transmembrane domains connected by 3 extracellular loops and 3 intracellular ones. Amino acid residues that are well conserved among GPCRs are highlighted and encircled. The residues that are conserved mainly in biogenic amine receptors are highlighted in a solid square. The two cysteine residues between the first and second extracellular loop form a disulfide bond. The highly conserved aspartate in TM3 is replaced by asparagines (Asp^{111}--- Asn^{111}) on SmGPCR. Taken and modified from (Hamdan F.F., thesis 1999)

CHAPTER II

Functional expression studies of a novel *Schistosoma mansoni* G protein-coupled receptor (SmGPCR).

CONTRIBUTION

This chapter is an overview of work that was published in Parasitology Research and Molecular Biochemical Parasitology (see full references below). The overview focuses on my own contribution to this work. I performed the in situ immunolocalization and Western blot analysis of wild type and codon-optimized SmGPCR. For the functional assays, I contributed to the CRE-SEAP assays and collaborated with Mark Abramovitz (Merck Frosst) in the aequorin assays. The codon optimization of SmGPCR was done by Fadi Hamdan.

Hamdan FF., **Aisha Mousa**, and Ribeiro P., (2002) Codon-optimization improves heterologous expression of a *Schistosoma mansoni* cDNA in HEK293 cells. *Parasitology Research* **88**, 583-586.

Hamdan FF., Abramovitz M., **Aisha Mousa**, Xie J., Durocher Y., and Ribeiro P., (2002) A novel *Schistosoma mansoni* G protein-coupled receptor is responsive to histamine. *Molecular Biochemical Parasitology* **119**, 75-86.

ABSTRACT

A novel G protein-linked receptor from *Schistosoma mansoni* (SmGPCR) was expressed in mammalian cells and tested for activity. To improve heterologous expression, SmGPCR was codon-optimized according to preferred human codon usage and tagged at both ends with an N-terminal FLAG and C-terminal 6x-his epitopes. Immunofluorescence experiments of transfected mammalian cells (HEK293(EBNA1)) with codon-optimized SmGPCR or wild-type using anti-FLAG antibody revealed increased expression of SmGPCR due to codon-optimization compared to the wild-type. Other in situ immunofluorescence experiments, using both anti-FLAG and anti-His antibodies in HEK293(EBNA1) cells transfected with codon-optimized SmGPCR demonstrated that the receptor is expressed in the cell membrane and the N-terminus is extracellular, whereas the C-terminal terminus is intracellular, consistent with a typical GPCR. Functional expression assay were done by measuring changes in the level of cAMP or Ca^{2+} in cells transiently transfected with codon-optimized SmGPCR. A cAMP-sensitive reporter assay using secreted alkaline phosphatase as a reporter gene (CRE-SEAP) failed to identify an effect of any of the biogenic amines tested on SmGPCR. In contrast, an aequorin assay designed to measure changes in intracellular Ca^{2+} determined that SmGPCR was selectively activated by histamine with an EC_{50} of 0.54 ± 0.05 µM. The same aequorin assay revealed no significant effect of any other biogenic amine on SmGPCR activation. This study indicated that SmGPCR is a functional G protein coupled receptor and is responsive only to histamine.

INTRODUCTION

Parasitic platyhelminths (flatworms) of the genus schistosoma are among the most prevalent causes of human parasitic infection and disease. *Schistosoma mansoni* is the causative agent of schistosomiasis, a severe debilitating disease that afflicts more than 200 million people around the world. Studies of flatworm neurobiology have identified several putative neurotransmitters, including biogenic monoamines, which coordinate all major activities of the parasite in the host. Monoamines are present within the central nervous system and peripheral tissues of *S. mansoni*. Serotonin is the most widely distributed and serves as a positive modulator of motility and metabolic regulator. Unfortunately less is known about the properties of other biogenic amines, particularly histamine. Histamine is known as a mediator of immunity but also functions as an important neurotransmitter in a variety of vertebrate and invertebrate phyla. Histamine has been identified in *S. mansoni*, other trematodes and several tapeworms (cestodes) such as *Hymenolepis diminuta* and *Diphyllobothrium dendriticum*. In situ immunofluorescence studies of the frog trematode, *Haplometra cylindracea* identified histamine neuroactive neurons system which store histamine in very high concentrations. It is unknown if histamine is synthesized endogenously or taken up from the host.

Studies on the function of histamine reported an effect on motor activity both in *S. mansoni* and *H. diminuta*, suggesting a role in neurotransmission or

modulation. However, no histamine receptor has yet been identified in these parasites or any other helminth.

In this study, we report a first invertebrate histamine receptor, a novel *S. mansoni* cDNA (SmGPCR), which has been cloned and has structural characteristics of the amine GPCR family.

EXPERIMENTAL PROCEDURE

Codon-optimization of SmGPCR

The first half of the SmGPCR coding sequence (nucleotides 268 to 1122) was synthesized by one step recursive PCR that contained a total of 16 overlapping mutagenic primers and two external sense and antisense primers that were directed towards the beginning and the end of the codon optimized SmGPCR sequence, respectively (see table 1). The mutagenic primers were designed so as to rewrite the *S. mansoni* cDNA using preferred human codons, as defined in the international DNA sequence data base (Nakamura et al., 1999), without changing the amino acid sequence encoded by SmGPCR. The forward external primer and the first nested mutagenic primer were designed to introduce a *Nhe I* site followed by a kozak sequence (GCCACC) (Kozak 1999), immediately preceding the start ATG and a FLAG epitope (DYKDDDDK) (Hopp et al., 1988). The reverse primer was designed to introduce a flanking *Van 91I* site for the ligation into an expression vector. The remaining half of the SmGPCR coding sequence (1123-1950) was amplified in a standard PCR, using a sense primer that introduced a *Van 91I* site and overlapped with the 3'-end of the above codon-optimized sequence. The antisense primer incorporated a six histidine epitope, followed by a stop codon and a single adenosine immediately after the stop codon (TGAA). The adenosine was added in order to increase translation termination efficiency in the mammalian cells (McCaughan et al., 1995). The reverse primer also introduced a *Not I* restriction site for the subsequent ligation into an expression construct.

1F(80) - 5' ATGGACTACAAGGACGACGACGAC AAGCAGTACATCAACAAGACCAGCCTGAACAGCAGCGT CATCCCCGATAGCCTGAT - 3'
2R(75) - 5' CAGGAACAGGGAGATGGTCCACTTGATGATAGGGTTGCTCAGGATCCAGCTCTTGATCAGGC TATCGGGGATGAC - 3'
3F(80) - 5' TGGACCATCTCCCTGTTCCTGATCATCGCCACTGGCACCACATTCTTCGGCAACCTGCTGAT TATCCTGGCCTTCATCAC - 3'
4R(82) - 5' GCAGATCAGCCACGGCGAGGGACACGATGTATTGGTCGGTGATTCTCCTCAGTCTGCTGTTG GTGATGAAGGCCAGGATAAT - 3'
5F(85) - 5' CTCGCCGTGGCTGATCTGCTGGTGAGCGTGCTGGTGCTGCCTCTGGCTATCGTGAGACAGAA CCTGGGATATTGGCCATTCGAGA - 3'
6R(72) - 5' GGCCATGCACAGCACGATGTTAGCGCTCAGCCAGAATTGACACAGCCTATCGCTCTCGAATG GCCAATATCC - 3'
7F(53) - 5' CATCGTGCTGTGCATGGCCTCAATTCTGAACCTGTGTTGCATTAGCCTGGACA - 3'
8R(78) - 5' GGCGGTGAACCTGGTCCTCTTTGTAAAATATTTCATGGGTCTAGAGATGGCGATGTATCTGT CCAGGCTAATGCAACA - 3'
9F(78) - 5' GAGGACCAGGTTCACCGCCAGCACCATGATCGCCGTCGCCTGGATCCTGCCTCTGATCACCA TGCTGCTCCCCTTCGT - 3'
10R(84) - 5' CCTATAAGCCTTGTTGTAGGTGATGTGGCAAGATCCCAGGCCCAGTGTGTGTTGATTGCCG CCCACGAAGGGGAGCAGCATGGT - 3'
11F(83) - 5' CCTACAACAAGGCTTATAGGATCTACAGCTCTATCGTCGGATTCTTCGGCCCCTTCCTGCT GATCGCCTACATCTACCTGAGA - 3'
12R(84) - 5' GGCTGCTCAGCTTGATGTTGGTGATCTGCAGGACCTTCAGTCTGTGCTTGATGATCCAGAA CACTCTCAGGTAGATGTAGGCGA - 3'
13F(71) - CCAACATCAAGCTGAGCAGCCTGAAGAAGCCCAAGTCTCACATCAAGGCCACCAGGAAACCCG CTCCCATC - 3'
14R(56) - 5' CCTTGATGTTTTCCCACACCTGCTGGAGGTTGATGATGATGGGAGCGGGTTTCCTG - 3'
15F(56) - 5' GTGTGGGAAAACATCAAGGGCAAAATCGGCAAAGTGAACATCTTGAGGAACCAGAG - 3'
16R(58) - 5' GTGA*CCACTATATGGA*CAGGTGTTCTTAGACTTGCTGCTCTGGTTCCTCAAGATGTTC - 3'
PCRF(34) - 5' ATA*GCTAGCCACC*ATGGACTACAAGGACGACGAC - 3'
PCRR(25) - 5' GTGA*CCACTATATGGA*CAGGTGTTC - 3'
CF(37) - 5' AACACCTGT *CCATATAGTGGC*CATTTTTTCCATTCTG - 3'
CR(58) - 5' AAT *GCGGCCGCTTCA*ATGGTGGTGATGGTGGTG CTTATGGTTGTAGCTCTTAATATTC - 3'

Table 1: Oligonucleotides used in constructing the codon-optimized SmGPCR construct. Overlapping oligonucleotides (5'-3') spanning a region equivalent to nucleotides 268 to 112 of SmGPCR cDNA. The length of 16 mutagenic primers is indicated in parentheses. Forward and reverse oligos are indicated by "F" and "R", respectively. PCRF and PCRR are the external forward and reverse PCR primers used to amplify the recursive PCR product. CF and CR are the forward and reverse primers used to amplify the wild-type second half of SmGPCR coding sequence.

To obtain a complete SmGPCR, the codon optimized and wild-type PCR products were gel purified and digested with *NheI/Van91I* and *Van 91I/Not I* respectively. The two fragments were fused together at the *Van 91I* site and then ligated between *Nhe I / Not I* sites of digested pCEP4 mammalian expression vector or between *EcoR I/ Sma I* sites of digested pCIneo. The two expression constructs, pCEP4-SmGPCR and pCIneo-SmGPCR were confirmed by DNA sequencing of at least two separate clones. A diagramatic representation of the strategy used for codon optimization shown in Fig. 1.

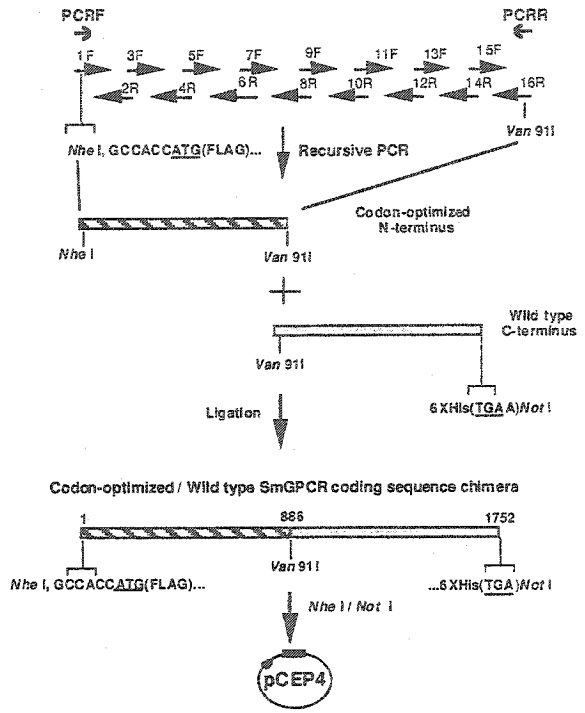


Fig. 1 Construction of a codon-optimized SmGPCR expression plasmid. The first half of SmGPCR cDNA sequence (268-1122; GenBank Accesion No. AF031196) was rewritten according to mammalian preferred codon usage by recursive PCR, using 16 overlapping mutagenic primers and to external primers (PCRF and PCRR) as described in the text. Start and stop codon were added to SmGPCR cDNA sequence (*underlined*). Restriction sites (*Nhe I, Van 91I, Not I*) are shown in *italics*. The Kozak sequence (GCCACC), and the FLAG and oligohexahistidine (6x His) epitopes are shown in boid. *CF* and *CR* are the primers used to amplify the wild-type second half of the SmGPCR sequence cDNA (1123-1950). The two codon-optimized (*hatched box*) and wild-type segments were ligated at a *Van 91I* site. The resulting chimeric cDNA was then ligated into a pCEP4 vector at the *Nhe I/Not I* sites.

In situ immunolocalization

Human Embryonic Kidney cells expressing Epstein-Barr Nuclear Antigen 1, HEK293 (EBNA1), were seeded into six well plates (2-3x10^5 cells per well) containing sterilized coverslips, which were pre-coated with 0.001% poly-L-lysine. Approximately 18-20 hrs later (in a humidified atmosphere of 5% CO_2/95% air at 37 °C), the cells were transfected with 1µg of pCEP4 expression plasmid containing either codon-optimized SmGPCR, wild type SmGPCR, or pCEP4 alone using FuGENE 6 (3µl), according to the manufacturer's recommendations. About 48 hrs post-transfection, the cells were fixed with 4% paraformaldehyde (PFA) and incubated (75 min at 4 °C) with a monoclonal antibody directed against

the FLAG epitope (anti-FLAGM2 : 5µg/µl), followed by a secondary fluorescein isothiocyanate-conjugated antibody for 1 hr at 4 °C (goat anti-mouse IgG, 1:2000 dilution). For experiments using a C-terminal anti-His antibody, cells were fixed with PFA as above and used either directly, or after permeabilization with ice-cold methanol for 7min at -20 °C. Cells were reacted with anti-His antibody (1:200 dilution). Subsequently, the cells were washed in phosphate buffer saline (PBS) and the coverslips were mounted (PBS/glycerol, 1:1 v/v) on glass slides and examined using fluorescence microscopy and conventional visible light, using a Nikon Optiphot-2 fluorescence microscope equipped with Nomarski optics.

Western Blotting

HEK293(EBNA1) cells (1-1.5x10^6 cells) were seeded in 100mm plates and grown in complete media up to about 70-80% confluency. The transfection was performed using the FuGENE 6 transfection kit according to the manufacturer's specifications. The cells were transfected with 3µg each of codon-optimized SmGPCR, wild-type or pCEP4 plasmid alone without insert. About 48 hrs post transfection, transiently transfected cells from each population were rinsed twice with ice-cold lyses buffer (PBS) and then incubated in TEM buffer (10mM $MgCl_2$, 0.5mM EDTA, 50mM Tris-HCl) for 10-15 min at 4 °C. After swelling the cells were scraped off the plates with a rubber policeman, transferred to polycarbonate centrifuge tubes and then sonicated 7 times each for 15 sec separated by 30 sec interval on ice. The resulting lysates were centrifuged at 28,000 xg for 20 min at 4 °C. The membrane protein concentration was measured by the Lowry method, using a kit from Sigma. Aliquots of two forms of the receptor or a control

membrane preparation containing the same amount of total protein (20µg) were resolved on 12% sodium dodecyl sulfate polyacrylamide gel and subsequently electroblotted onto nitrocellulose filters (Novex). Filters were incubated with anti-FLAGM2 antibody (1:1000 dilution) followed by a horse-radish peroxidase-conjugated secondary antibody (1:2000 dilution), according to standard procedures. Western positive bands were detected on film using lumilight (Roche) that were compared to a protein standard.

Secreted Alkaline Phosphatase (CRE-SEAP) reporter assays

HEK293 cells were stably transfected with a secreted form of alkaline phosphatase (SEAP) under the control of a cAMP-responsive element (CRE) (Durocher et al., 2000). CRE-SEAP cells were seeded in 96-well plates (- 10^4 cells per well) and were transiently transfected with expression plasmid, pCEP4-SmGPCR or the vector alone using FuGENE 6. Twenty four hours post-transfection, cells were treated with test agents for 6 or 16 hrs at 37 °C in the presence or absence of forskolin or IBMX, for $G_{i/o}$ or G_s readouts, respectively. Aliquots of the medium containing SEAP were analyzed for enzyme activity by using a colorimetric method and a plate format absorbance reader set at 405nm as described previously (Durocher et al., 2000).

Aequorin Assays

COS7 cells transiently cotransfected with an aequorin expression vector (pCDM.AEQ), and either an SmGPCR expression vector (pCIneo-SmGPCR or pCEP4-SmGPCR) or a vector with no insert. Transfected cells were charged with colenterazine and approximately 48 hrs post-transfection and then dispensed into

96-well plates (5 x 10^4 cells per well) containing different concentrations of drugs. Light emission was recorded with a luminoskan RS luminometer as described (Hamdan et al., 2002a).

RESULTS AND DISCUSSION

Heterologous expression of SmGPCR in HEK293 cells

SmGPCR found to express poorly in transiently transfected mammalian cells. All attempts to optimize the transfection conditions were unsuccessful, suggesting that the problem was due to low translation efficiency of the *S. mansoni* cDNA in the heterologous environment. A close examination of the DNA sequence of SmGPCR revealed that a significant number of amino acid residues were encoded by codons that are rarely represented in mammalian cells, particularly T/CTA (Leu), ATA (Ile) and CGT/A (Arg). The first half of the SmGPCR cDNA sequence contains a majority (~ 70%) of these rare codons compared to mammalian cells and frequently found in clusters of 2-3 consecutive codons. To solve this problem, the *S. mansoni* cDNA was rewritten according to mammalian (human) preferred codon usage. The authors engineered a partially modified cDNA using the original S. mansoni sequence by rewriting the first half of the receptor, according to human preferred codons without changing the protein encoded by SmGPCR. An N-terminal FLAG epitope and a C-terminal oligohexahistidine (6x His) were added to the resulting cDNA (Hamdan et al., 2002b). The receptor expression in transfected HEK293(EBNA1)cells was monitored by in situ immunofluorescence. A comparison of the wild-type and codon-optimized SmGPCR confirmed that the codon rewriting significantly increased expression in the mammalian environment (Fig. 2). Whereas cells expressing the wild-type produced no visible signal, those cells expressing the

codon optimized showed strong anti-FLAG immunofluorescence, indicating high level expression of the receptor.

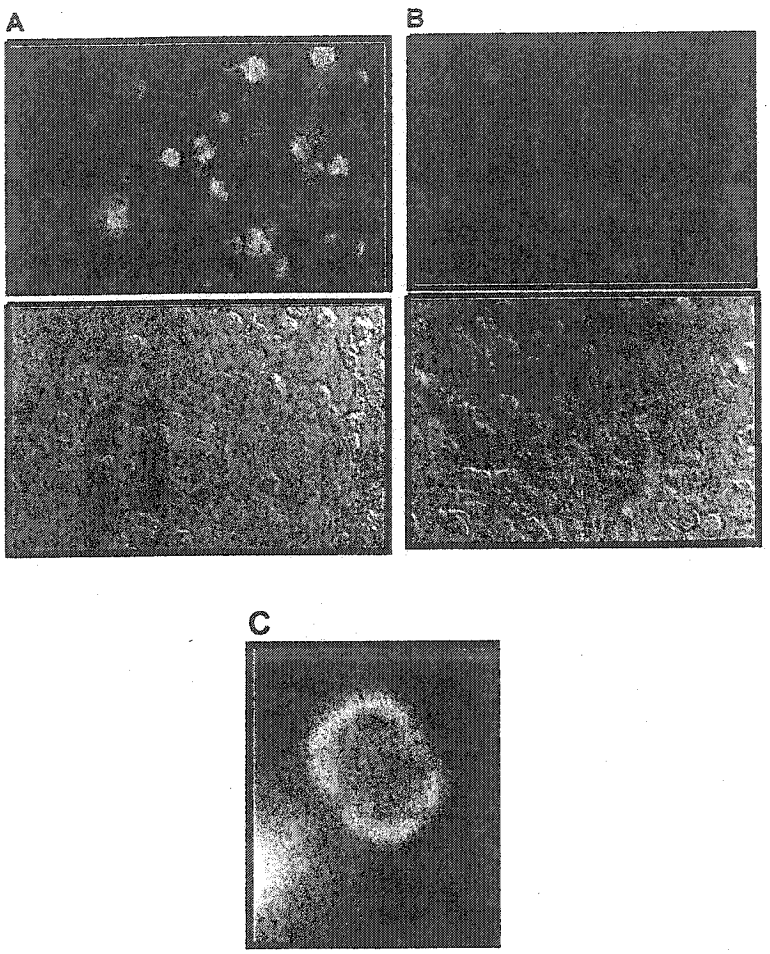


Fig. 2 Codon-optimization and SmGPCR expression in HEK293(EBNA1) cells. Cells were seeded onto coverslips in six-well plates (2-3x10^5 cells/well), and transfected with 1µg of pCEP4 plasmid containing codon optimized SmGPCR (**A**) or wild type SmGPCR (**B**), each fused at the N-terminal end to a FLAG epitope. 48 hrs post transfection, the cells were fixed on ice with PFA and then incubated with a monoclonal antibody targeting the FLAG tag (anti-FLAGM2: 5µg/ml) followed by a secondary fluorescein isothiocyanate antibody (goat anti-mouse IgG; Sigma; 1:2000 dilution). Cells were examined by fluorescence microscopy and conventional visible light. **C**) A closer view of transfected cells shows expression of codon-optimized form of SmGPCR mainly in the cell periphery.

This was confirmed by subsequent Western blot analysis of wild-type and codon-optimized SmGPCR. Aliquots of a 28,000 xg crude membrane fraction containing identical amounts of total protein (20µg) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotted with the anti-FLAG antibody. A strong immunoreactive band corresponding to SmGPCR (~ 65 KDa) was detected in samples transfected with codon-optimized receptor whereas, the

wild-type produced a much weaker band. No signal was detected in the 28,000 xg supernatant of either transfection or in the membranes of control cells transfected with pCEP4 alone (Fig. 3). Taken together, the results show that the codon-optimized SmGPCR was successfully expressed in the mammalian system.

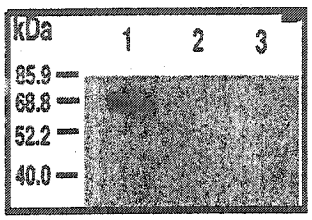


Fig. 3 Western blot analysis: approximately 1-1.5x10^6 HEK293(EBNA1) cells were transfected with 3 µg each with codon-optimized SmGPCR-FLAG (*Lane 1*), wild-type SmGPCR-FLAG (*Lane 2*), or pCEP4 alone without insert (*Lane 3*). Cells were lysed 48 hrs post transfection and a crude 28,000 xg membrane fraction was prepared from each population of cells. 20 µg protein of each population were resolved on 12% SDS polyacrylamide gel and electroblotted onto nitrocellulose filter (Novex). Filters were incubated with anti-FLAGM2 (1:1000 dilution) followed by a horse-radish peroxidase conjugated secondary antibody (1:2000 dilution). Western positive bands were detected on film using lumilight (Roche). The positions relevant protein standards are indicated.

In addition to demonstrating high-level expression, this study confirmed that SmGPCR is properly targeted to the cell membrane in the heterologous environment. Strong immunofluorescence was observed in non-permeabilized cells treated with the anti-FLAG antibody. This is an indication that SmGPCR protein is expressed at high levels on the cell surface and the N-terminus, which carried the FLAG epitope, is extracellular. In contrast, the anti-His antibody produced detectable immunofluorescence only in permeabilized transfected cells that carry the 6x histidine tag in the C-terminus (Fig. 4). The absence of immunoreactivity in nonpermeabilized cells confirms that the C-terminal His epitope is intracellular, consistent with the normal topology of a GPCR. No immunofluorescence was detected in control HEK293(EBNA1) cells transfected with vector alone.

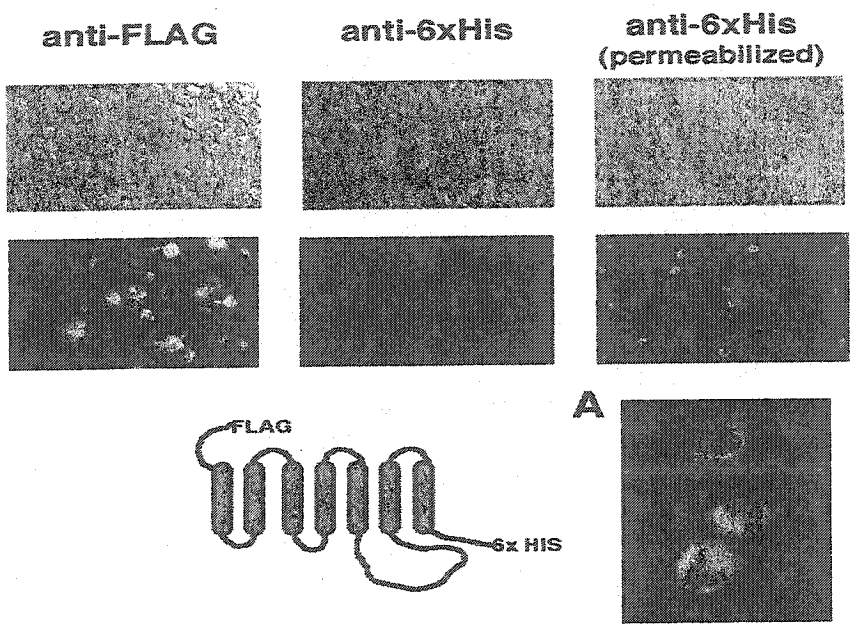


Fig. 4 Cellular localization of SmGPCR by immunofluorescence. HEK293(EBNA1) cells were transfected with a codon-optimized SmGPCR that has a FLAG epitope in the N-terminal and 6x his at the C-terminus. Transfected cells were fixed by PFA and subjected to indirect immunofluorescence using either anti-FLAGM2 or anti-His antibody, followed by incubation with FITC-conjugated secondary antibodies. Fixed cells were used directly or permeabilized with methanol prior to incubation with the primary antibody. Cells were examined by fluorescence microscopy and conventional visible light. No fluorescence was seen in the mock transfected controls with either antibody (data not shown). A) Closer view of permeabilized transfected cells stained with anti-His antibody. Results show strong immunofluorescence on the cell surface, suggesting that SmGPCR was expressed at high level and targeted the cell membrane.

Functional assays of SmGPCR

SmGPCR was initially designated as an 'orphan' receptor because of low sequence homology with other biogenic amine GPCRs. To functionally characterize this receptor a survey of several potential monoamine ligands was a necessity. This work was done in collaboration with Dr. Abramovitz at Merck Frosst. The codon-optimized SmGPCR was transiently transfected both in 293CRE-SEAP (HEK293) and COS7 cells and then tested for signaling activity in response to several amine ligands. The assays were designed to test cAMP or Ca^{2+} pathways with each ligand. 293CRE-SEAP cells were transfected transiently with codon-optimized SmGPCR for measurements of cAMP-mediated signaling.

Transfected cells were treated with various concentrations of test ligands (10^{-9} – 10^{-4} M) including serotonin, histamine, octopamine, tyramine, dopamine, noradrenaline, adrenaline and acetylcholine, in the presence and absence of forskolin. In each case, cAMP responsive reporter (CRE-SEAP) activity was measured as an indirect indicator of changes in cellular cAMP. No significant effect was observed with any of the monoamines tested compared with mock-transfected cells or untreated control. In contrast, a significant response was detected when the receptor was tested in an aequorin assay, which measures receptor-mediated mobilization of intracellular calcium. Cells co-transfected with SmGPCR and an aequorin expression plasmid (pCDM.AEQ) were specifically activated by histamine (Fig. 5).

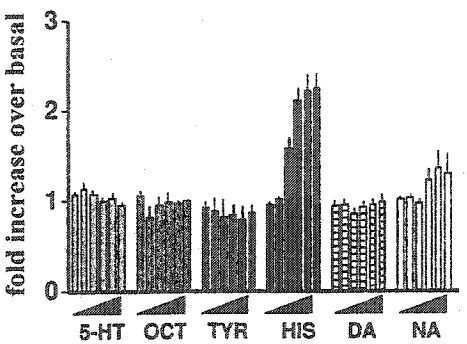


Fig. 5 Effect of biogenic amines in an aequorin assay. Cells were transiently transfected with a codon-optimized SmGPCR (pCIneo-SmGPCR) and co-transfected with aequorin expressing plasmid (pCDM.AEQ) were treated with increasing concentrations of different biogenic amines and the level of aequorin bioluminescence emitted was measured. Each agonist tested at six different concentrations ranging from ($7.5x10^{-9}$ to $5x10^{-5}$ M). The results are shown as fold increase in bioluminescence compared to control (untreated transfected cells). Data are the means and Standard errors of three to five independent experiments, each in duplicate. None of the biogenic amines tested had any effect on control cells. Abbreviations are: 5-HT, serotonin; HIS, histamine; OCT, octopamine; TYR, tyramine; DA, dopamine; NA, noradrenaline.

Histamine elicited a robust, dose dependent increase in aequorin bioluminescence corresponding to an increase in intracellular calcium. Histamine at 50µM concentration showed a nearly 3-fold higher level of luminescence than

an untreated control. None of the other amine had any significant measurable effect on aequorin luminescence. The EC_{50} for SmGPCR was 0.54 ± 0.05 μM (low affinity) based on six independent experiments, each in duplicate Fig. 6.

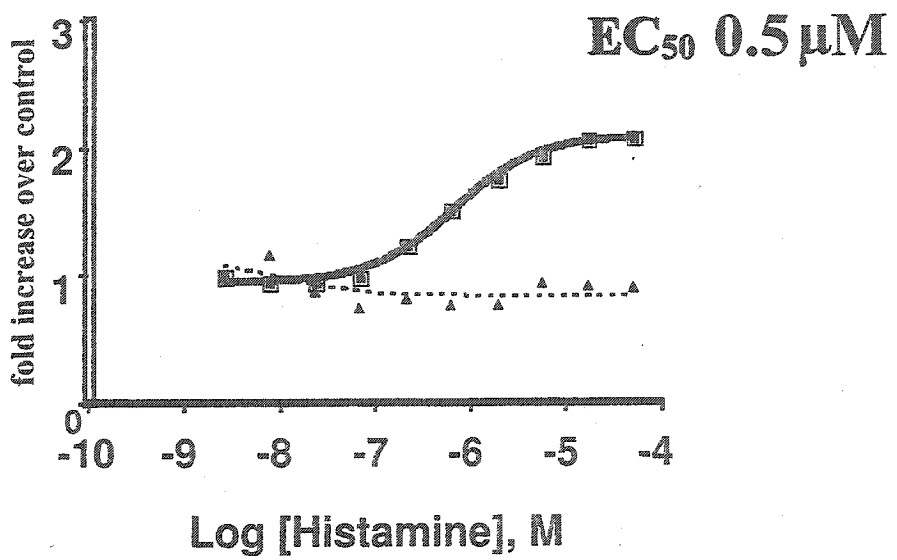


Fig. 6 Representative activation curves of codon-optimized SmGPCR. Cells were transiently transfected with codon-optimized SmGPCR (pCIneo-SmGPCR) co-transfected with aequorin plasmid (pCDM.AEQ) in *solid squares* or pCIneo alone in *broken line*. Histamine effect on transfected cells was measured in bioluminescence emitted over a concentration range of $1.5x10^{-9}$ to $5x10^{-5}$ M in the two populations of cells. Data were standardized relative to basal luminescence in untreated controls. The EC50 for SmGPCR was 0.54±0.05 μM based on the mean and standard errors of six independent experiments, each in duplicate.

Histamine had no effect in control cells transfected with pCDM.AEQ and vector alone. These results identify histamine as a selective agonist of SmGPCR and further suggest that the receptor may be coupled through $G_{q/11}$ and the inositol phosphate pathway. SmGPCR has relatively low affinity for histamine, with an EC_{50} in the μM range. Low affinity receptor-ligand interaction appears to be exceptionally prevalent among invertebrates. The vast majority of invertebrate amine GPCRs have μM binding affinities for their respective ligands. It is

unknown if this is due to expression in heterologous environment or if it reflects an intrinsic property of the invertebrate GPCR.

This study raises a number of important questions about the mode of action of histamine in *S. mansoni* and, specifically, the function of SmGPCR in the intact worm. The next chapter will focus on the effect of histamine and antihistaminic drugs on the motility of different parasite stages.

REFERENCES

Durocher Y., Perret S., Thibaudeau E., Gaumond M.H., Kamen A., Stocco R., and Abramovitz M., (2000) A reporter gene assay for high-throughput screening of G protein-coupled receptors stably or transiently expressed in HEK293 EBNA cells grown in suspension culture. *Anal Biochem.* **284**, 316-326.

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

Hamdan F.F., Mousa A., and Ribeiro P., (2002b) Codon-optimization improves heterologous expression of a *Schistosoma mansoni* cDNA in HEK293 cells. *Parasitol. Res.* **88**, 583-586.

Hopp T.P., Prickett K.S., Price V.L., Libby R.T., March C.J., Cerretti D.P., Urdal D.L., and Conlon P.J., (1988) A short polypeptide marker sequence useful for recombinant protein identification and purification. *Biotech.* **6**, 1204-1210.

Kozak M., (1999) Initiation of translation in prokaryotes and eukaryotes. *Gene* **234**, 187-208.

McCaughan K.K., Brown C.M., Dalphin M.E., Berry M.J., and Tate W.P., (1995) Translational termination efficiency in mammals in influenced by the base following the stop codon. Proc. *Natl. Acad. Sci. USA* **92**, 5431-5.

Nakamura Y., Gojobori T., and Ikemura R., (1999) Codon usage tabulated from the international DNA sequence databases; its status 1999. *Nucleic Acids Res.* **72**, 292.

CONNECTING STATEMENT I

Chapter II described the expression of SmGPCR in mammalian cells and identified the receptor as being specific for histamine.

In Chapter III (manscript I), we investigated the effects of histamine and several antihistaminic drugs in two developmental stages of *S. mansoni*, adults and cercaria. In these pharmacological studies we found first that histamine increases cercarial motility in a dose dependent manner in vitro. Second, histamine H_1 receptor antagonists caused a reversible paralysis of both cercaria and adults. On the other hand, histamine H_2 and H_3 receptor blockers had no effect on the parasite in vitro.

CHAPTER III (Manuscript I)

Schistosoma mansoni: Effects of histamine and histamine receptor antagonists on parasite motility in *vitro*.

Aisha Mousa and Paula Ribeiro

(In preparation)

ABSTRACT

In this study we investigated the effects of histamine and several antihistaminic drugs on the motor activity of *Schistosoma mansoni* cercaria and adult in vitro. Exogenously applied histamine caused a dose-dependent increase in the motility of the cercaria, increasing the duration of swimming episodes approximately 2-fold. H_1 receptor inhibitors of different chemical structures, such as promethazine, diphenhydramine and brompheniramine caused a marked decrease in motility, ranging from partial to complete cercarial immobilization. The paralysis was reversible by addition of excess histamine, consistent with a receptor-mediated effect. H_2 and H_3/H_4 receptor antagonists, cimetidine, ranitidine and thioperamide had no effect on cercarial motility. *S. mansoni* adults, collected from infected mice mesenteric system, did not respond to exogenously supplied histamine up to a concentration of 2mM. However, the adult worms reacted to H_1 antihistamines in a manner similar to cercaria, with a dose dependent reduction in motility. The H_2 antagonist, cimetidine, did not immobilize the adult worms. Taken together, the data suggest that a receptor with pharmacological characteristics of histamine H_1 GPCR is present in both *S. mansoni* cercaria and adults and may play a role in modulation of parasite motility.

INTRODUCTION

The parasitic flatworm *S. mansoni* (trematode) is the causative agent of schistosomiasis, a severe debilitating disease that affects over 200 million people in more than 70 countries. Another two billion people are estimated to be at risk (Mellin et al., 1983). The nervous system of this parasite is viewed as a likely point for chemotherapeutic attack. Studies of flatworm neurobiology have identified several putative neurotransmitters, such as biogenic monoamines. Among the monoamines identified in helminths, serotonin (5-HT) is the most widely distributed and functionally diverse as a modulator of motility and a metabolic regulator. 5-HT functions as an excitatory neurotransmitter, causing an increase in the motor activity of *S. mansoni* (Mansour 1984; Pax and Bennet 1991; Davis and Stretton 1995). Acetylcholine is believed to function as an inhibitory neurotransmitter causing a flaccid paralysis (Pax et al., 1984; Mellin et al., 1983). Catecholamines have also been implicated as putative inhibitory neurotransmitters but their specific role is less clear (Hillman and Senft 1973). These amines are localized in various tissues of *S. mansoni*, including the central nervous system, peripheral nervous system, holdfast and reproductive structures and, in the case of 5-HT, the body wall musculature and the gut as well (Marr and Muller 1995). Unfortunately, less is known about the properties of other biogenic monoamines, particularly histamine. Histamine is better known as a mediator of immunity (Thueson et al., 1979), but also functions as an important neurotransmitter in a variety of vertebrate and invertebrate phyla. Histamine has been identified in S. *mansoni* and other trematodes (Eriksson et al., 1996; Ercoli

et al., 1985), as well as several tapeworms (cestodes) (Wikgren et al., 1990; Sukhdeo et al., 1984) such as *Hymenolepis diminuta* and *Diphylobothrium dendriticum*. In situ immunofluorescence studies of the frog trematode, *Haplometra cylindracea,* identified histamine neuroactive neurons which store histamine in very high concentrations (Eriksson et al., 1996). It is unknown if these organisms synthesize histamine endogenously or if it is taken up from the host. There is limited evidence that suggests the involvement of histamine in *S. mansoni* mobility. One early study reported that histamine may play a role in the modulation of motility in cercariae and schistosomula (Ercoli et al, 1985). The role of histamine in adult worms is still unknown. In this study, we have been able to demonstrate an effect of histamine and antihistaminic drugs on the motor activity of *S. mansoni*. Three different classes of histamine antagonists, selective for H_1, H_2, and H_3/H_4 receptors (Hill et al., 1997), were tested in this study. The results showed that histamine H_1 receptor inhibitors immobilized the free-swimming stage cercariae quickly and in a dose-dependent manner, whereas H_2 and H_3/H_4 selective drugs had no effect.

EXPERIMENTAL PROCEDURES

The parasite *S. mansoni* was maintained by cycling through *Biomphalaria glabrata* snails and female CD-1 mice. Each mouse received 120-150 cercariae percutaneously through shaved abdominal skin (Mkoji et al., 1988). Cercariae of *S. mansoni* were obtained for each experiment from 20-40 *Biomphalaria glabrata* infected 40 days earlier with 10 miracida of *S. mansoni* per snail. Snails were dried under light for 30 min. Water was added and the snails were shed within 30 min. Cercariae were washed and finally resuspended in dechlorinated tap water, as described (Basch 1981). Cercaria remained active, with no apparent change in behavior for up to 10 hours under these conditions (Enasmus 1972). Attempts to maintain cercaria in more complex media, including tyrode's saline, phosphate buffered saline (PBS) or spring water all resulted in loss of cercarial motility and thus were not pursued. For isolation of adult *S. mansoni*, mice were first infected with cercaria, as described above, and were sacrified 6-7 weeks post infection by cervical dislocation. After cutting the hepatic vein, the mesenteric system was perfused with sodium citrate saline (150mM NaCl, 25mM Na-Citrate) to recover the adult worms (Mkoji et al., 1988). Worms not recoverable by perfusion were collected with forceps from the mesenteric and portal veins. Adult *S. mansoni* were maintained at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 20mM HEPES for proper pH (7.2). All experiments were carried out within 5-6 hours after the removal of parasite from the mice (Hillman and Senft 1973).

RT-PCR

RT-PCR was employed for the determination of SmGPCR mRNA levels. Total RNA from two developmental stages of *S. mansoni* (cercaria and adults) was isolated using TRizol, as recommend by the manufacture. Aliquots (2µg) of total RNA were further purified on oligo-dt columns, using a commercial kit (Pierce). The resulting polyadenylated mRNA was reverse- transcribed (1 hr / 37 °C) followed by a PCR reaction (5 min / 94°C, 35 cycles of: 60 sec / 94 °C, 60 sec / 52 °C, 60 sec / 72 °C, 7 min / 72 °C) using two sets of primers, one to amplify 558 bp of *S. mansoni* internal control α-tubulin cDNA fragment (sense:5'-CTTATCGTCAACTTTTCCATCC-3', antisense: 5'- GGAAG TGGATACGAGGATAAGG-3') and the other for specific amplification of 373 bp of SmGPCR cDNA using specific primers (sense: 5'-CCGAATTCATGCCCG AACCAACAGA -3'; antisense : 5'- TAGCGGCCGCTG CAGTTTTTTGTTC - 3'). PCR products were resolved by 1% agarose gel electrophoresis, stained with ethidium bromide and analyzed by densitometry.

Motility assay of S. mansoni Cercaria

S. mansoni cercaria were transferred into 24-well (Costar) plates containing 0.5ml of 10mM Hepes buffered water (pH 7.2) and subsequently treated with various concentrations of test drugs or a vehicle at room temperature. Drug effects on cercaria motility were monitored beginning at 2 min post drug addition by direct examination of the parasite under a dissecting microscope. Cercariae display a characteristic swimming pattern consisting of repeated short pulses of swimming activity (forward or backward movement)

interspersed by periods of rest (Enasmus 1972). To quantitate this behavior, we measured the (1) average duration of individual swimming episodes, (2) the frequency of swimming episodes per min and (3) the proportion of parasites displaying swimming activity in the presence and absence of drug. The duration of an individual swimming episode was determined from the average of 3 consecutive measurements for the same organism over a total incubation period of 7 min. Data were obtained from examination of 60-200 parasites, each recorded 3 times. Frequency measurements were done by counting the number of swimming episodes per minute per individual parasite over a total incubation period of 7 min in the presence and absence of test drug. Data were obtained from 3 independent experiments, each involving examination of 20 animals per sample. (3) % motility describes the proportion of animals showing typical swimming activity at different time points during the period of incubation. Cercaria were monitored prior to drug addition and at various times 2,5,10 min up to 50 min following drug addition. Control samples were incubated under the same conditions and for the same length of time in the absence of drug. To test for reversal of drug effects, excess histamine (100 fold), or a vehicle (control) was added after 10 min incubation with test drug and the incubation was extended for an additional 30 min.

Motility assays of adult S. mansoni

Adult worm pairs were placed in 24-well plastic tissue culture plate containing DMEM and 20mM Hepes (pH 7.2). Motility of worms was monitored at room temperature under a dissecting microscope, at 0, 5, 30, 60 and up to 90

min, after addition of test drug or vehicle. Motor activity was scored as 4 (extremely hyperactive), 3 (hyperactive), 2 (normal activity), 1 (hypoactive) or 0 (inactive/paralyzed) as described previously (Mellin et al., 1983). A vehicle treated control group was included in every experiment and monitored at each point during the incubation. Normal motor activity of this control group consisted of waves of peristaltic-like contractions along the full length of the worm, with occasional whipping motion and head portion movement. Paralysis and excitation were readily distinguished from this normal motor behavior.

Drug preparation

All compounds to be tested were dissolved in distilled H_2O at a stock concentration of 10mM and to the final desired concentration in the incubation medium. Stock solutions were made up fresh on the day of each experiment. Serotonin, histamine and the histamine receptor antagonists, promethazine, diphenhydramine, brompheniramine, cimetidine and ranitidine were all obtained from Sigma.

Statistical analyses

All data were analyzed by One Way ANOVA (Analysis Of Variance) using Sigma-Stat software. Data obtained from motility assays of *S. mansoni* cercaria were analyzed using parameteric tests while ANOVA on Ranks (non parametric test) was used with adult's data. Graphs were done using Microsoft Excel and Cricket Graph software.

RESULTS

SmGPCR is expressed in Cercaria

The expression of SmGPCR was examined by RT-PCR in two different developmental stages of *S. mansoni*, cercaria and adults. Expression levels were standardized by comparison with a constitutively expressed control gene from *S. mansoni*, α-tubulin (Hamdan and Ribeiro, 1999a). α-tubulin is known to be expressed at the same level in all stages of the *S. mansoni* life cycle, including adults and cercaria (Webster et al., 1992; Mei and LoVerde 1997) and thus can be used as an internal control. Fig. 1. shows amplification of a SmGPCR product in both developmental stages, suggesting that the receptor is expressed in the adults and larvae at least at the mRNA level. Compared to the α-tubulin internal control, the level of SmGPCR in the cercaria appears to be higher than that in the adults. After standardization for the amount of α-tubulin, the relative density of the cercaria SmGPCR band is approximately two times higher than the corresponding adult band. No bands were observed in a negative control that lacked reverse transcriptase.

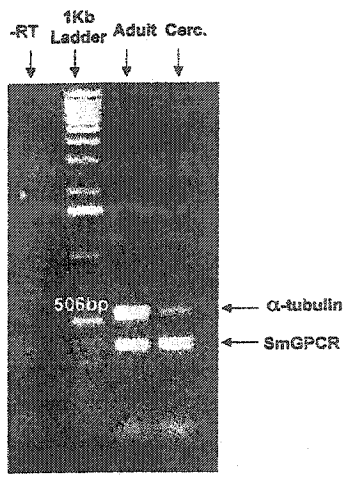


Fig.1. Developmental expression of SmGPCR in *S. mansoni*. RT-PCR was performed with RNA extracted from two developmental stages of *S. mansoni* (cercaria and adult), as described under Experimental Procedures. The RT-PCR reactions in both developmental stages were standardized by amplification of an internal control house keeping gene from *S. mansoni* (α-tubulin). The α-tubulin product is 558 bp whereas the SmGPCR fragment is 373 bp. The PCR products were analyzed on a 1 % agarose gel containing ethidium bromide and analyzed by densitometry.

Drug effects on cercarial motility

S. mansoni cercariae were treated with various concentrations of histamine ranging from 10uM -1mM in *vitro*. The duration of individual swimming episodes was determined by direct examination under a dissecting microscope. Worms were monitored individually in the absence (control) and presence of various concentrations of histamine at room temperature. The average duration of a swimming episode was determined from 3 consecutive measurements for the same worm. Data are the means and standard errors of 60-200 animals, each recorded three times, over 3-5 independent experiments. The results show a stimulatory effect of histamine on cercarial motility up to 2-fold with 100μM histamine (3.08 ± 0.4 sec) compared to the untreated control (1.72 ± 0.05 sec) Fig. 2. The data were analyzed using Microsoft Excel and SigmaStat software and one way ANOVA for multiple group comparison versus the control group. The same test was used to monitor the effect of a known modulator of *S. mansoni* motility, serotonin (Bennett et al., 1969; Bennett and Bueding 1973). Cercariae were similarly treated with serotonin at 10, 100, 500μM or a vehicle and the duration of swimming episodes was measured from analysis of 17-145 animals each recorded 3 times over 1-3 independent experiments. The results show a significantly stimulation of cercarial motility at 500μM, 100μM serotonin but not with10μM compared to the untreated control (1.9 ± .1 sec.) Fig 2. B.

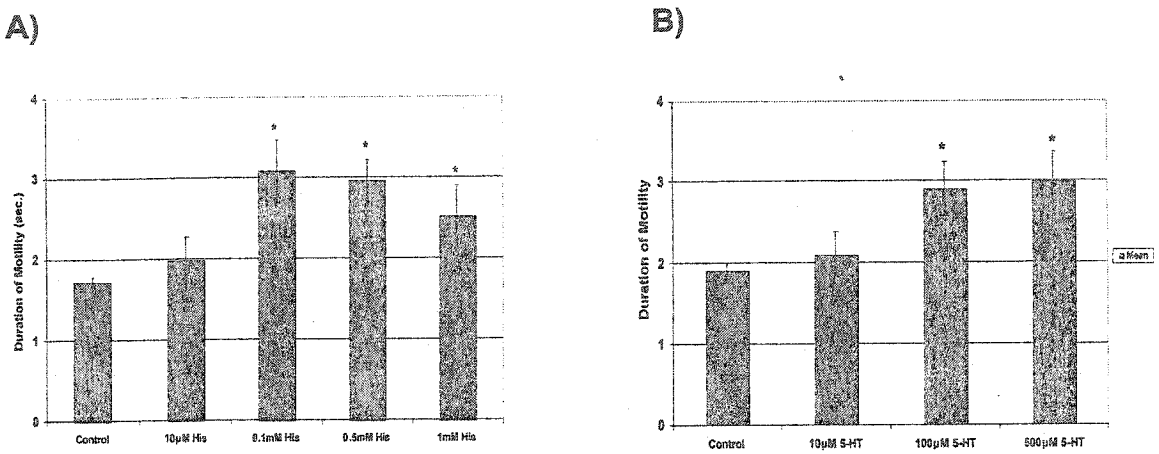


Fig. 2. Histamine and 5-HT activate *S. mansoni* cercarial motility. *S. mansoni* cercariae were shed from infected *Biomphalaria glabrata* snails. Cercariae were incubated in the absence (control) and presence of various concentrations of histamine A) and 5-HT B) at room temperature. Worms were monitored individually after 2 min incubation. The average duration of swimming episodes was determined from 3 consecutive measurements for the same worm. Data are the means and standard errors of minimum 17 and up to 200 animals, each recorded three times, over A) 3-5 B) 1-3 independent experiments. The data were analyzed using Microsoft Excel and Sigma-Stat software. Statistically significant ($P < 0.05$) differences relative to control are marked by asterisks (*).

Effect of histamine H_1 receptor inhibitors

Cercariae were incubated with increasing concentrations of H_1, H_2, and H_3/H_4 histamine receptor blockers or a vehicle. Drug effects were determined by measuring changes in the frequency of swimming episodes measured in one minute incubation. The duration of individual swimming episodes could not be determined because some drugs caused significant inhibition and paralysis of the parasite. The results (Fig. 3. A) show that all H_1 selective drugs, promethazine, diphenhydramine, and brompheniramine significantly inhibited cercarial motility in a dose-dependent manner. Promethazine, the most potent of the three H_1 drugs, caused over 50% decrease in the frequency of motility at a concentration of 10μM and complete paralysis at 100μM. At the same concentration of 100μM, diphenhydramine caused ~ 90% decrease in the frequency of swimming

episodes and brompheniramine caused a smaller but statistically significant decrease ~ 60% (Fig. 3).

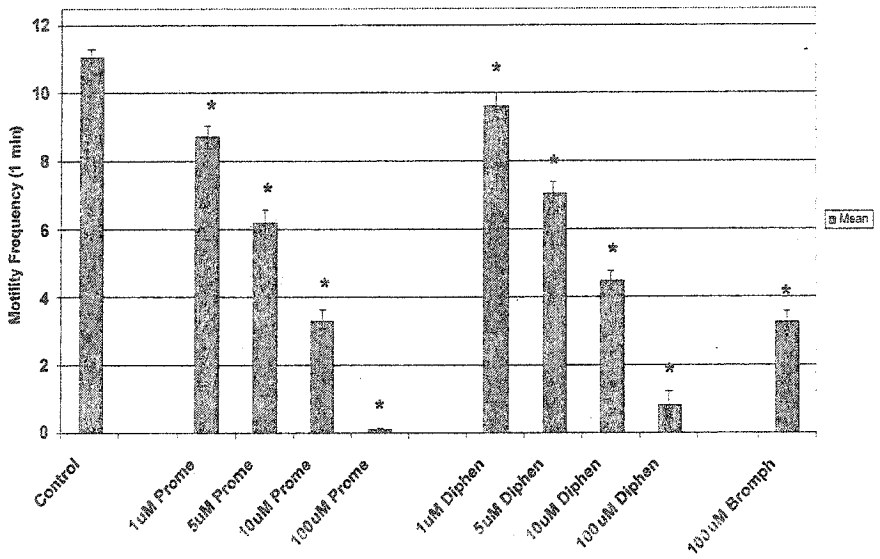


Fig. 3. H_1 antagonists immobilize the *S. mansoni* cercaria. Cercariae were collected from infected snails. Three different H_1 antagonists were tested for their effect on the motility of cercariae (promethazine, diphenhydramine, brompheniramine). Various concentrations of drugs were used ranging from 1μM up to 100μM as indicated. Controls were incubated for the same period of time in the absence of drug. Cercarial motility is expressed as the numbers of swimming episodes per 1 min. Data are the means and standard errors of three independent experiments each with 20 animals. Significantly differences compared to control are marked by asterisks.

In contrast, there was no change in motility frequency after treatment with H_2 receptor inhibitors such as cimetidine and ranitidine (Fig. 4 A), or the H_3/H_4 antagonist, thioperamide (Fig. 4 B). The data was analyzed using Microsoft Excel and one way analysis of variance. All data points were obtained from three independent experiments, each with 20 cercaria per test group.

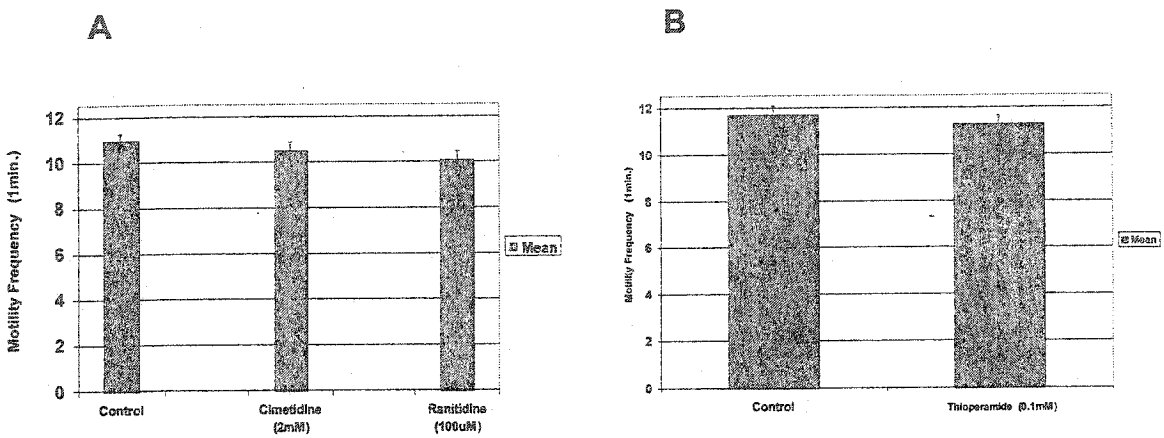


Fig. 4. No effect on cercarial motility in response to histamine H_2 and H_3/H_4 receptor inhibitors. *S. mansoni* cercariae were incubated with H_2 antagonists, cimetidine and ranitidine, **(A)** and with the H_3/H_4 antagonist thioperamide, **(B)** or a vehicle (control). The concentrations of different drugs tested are indicated. Data are the means and standard errors of three independent experiments each with 20 animals.

Reversal of cercaria immobilization by histamine

Cercariae were treated with 50uM promethazine or diphenhydramine for up to 40 min at room temperature. Motility was monitored at different time points by measuring the proportion of animals displaying swimming activity (% motility). The results show that both drugs were able to immobilize all the worms completely within 10 min incubation, promethazine causing complete paralysis after only 2 min. A 100- fold excess histamine or a vehicle was added at 10 min of incubation. The paralyzing effect of H_1 antagonists on the cercaria was partially reversed by addition of excess histamine (Fig. 5). Approximately 43% recovery of motility was observed in sample treated with promethazine after 10min (Fig. 5.A) and about 30% in the case of diphenhydramine (Fig. 5. B). Control untreated cercaria remained active throughout the incubation period with more than 75% of all parasites showing typical swimming activity. For each of the

above experiments data are the mean and standard error of 3-4 independent experiments each with at least 50 worms in average.

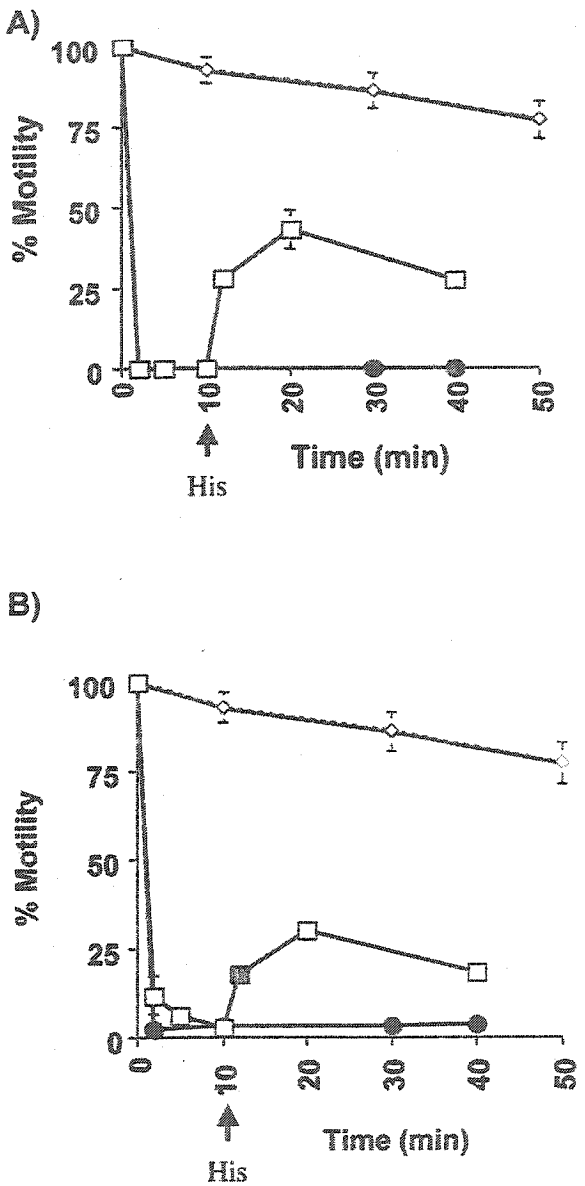


Fig.5 Histamine reverses cercarial immobilization. *S. mansoni* cercariae were treated with 50µM promethazine A) or diphenhydramine B) at room temperature and 100 fold excess histamine (-□-) or a vehicle (-●-) was added after 10 min and the incubation continued for an additional 30 min. A control untreated group broken line (--◊--) was monitored throughout the incubation. % motility describes the proportion of animals moving at each time point throughout the incubation. (His: histamine).

Adult motor activity

Adult *S. mansoni* was incubated with various test drugs and motility was assessed according to a scale of 0 (inactive) to 4 (extremely hyperactive), as

described (Mellin et al., 1983). *S. mansoni* worm motility was strongly stimulated by serotonin (5-HT) at 0.1mM (Fig. 6). This effect was observed in less than 30 min of drug application and served as a term of reference for a grade 4 (extremely hyperactive) response. In addition to serotonin, histamine and antihistaminic drugs were tested on adult *S. mansoni*. The results failed to detect a significant stimulation of adult worm motility by histamine at any of the concentrations tested (0.5mM, 1mM, 2mM), in contrast to effects observed with the cercaria (Fig. 6).

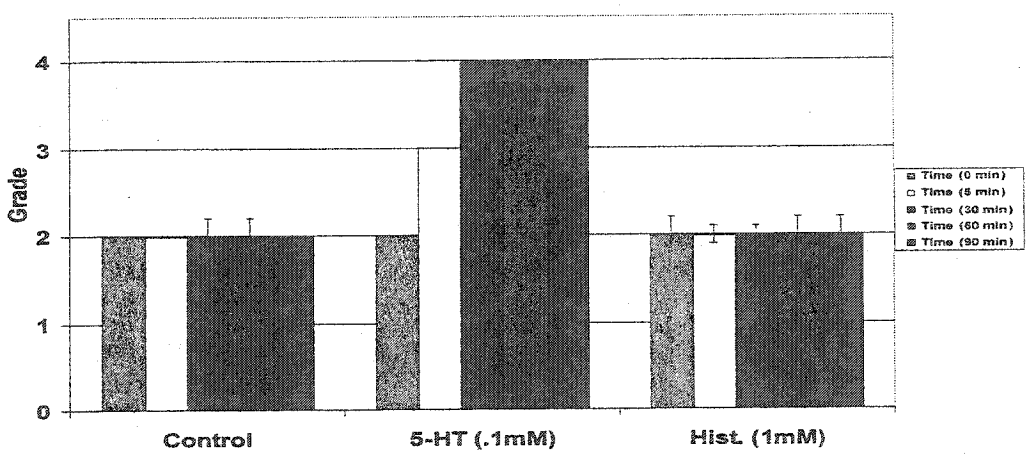
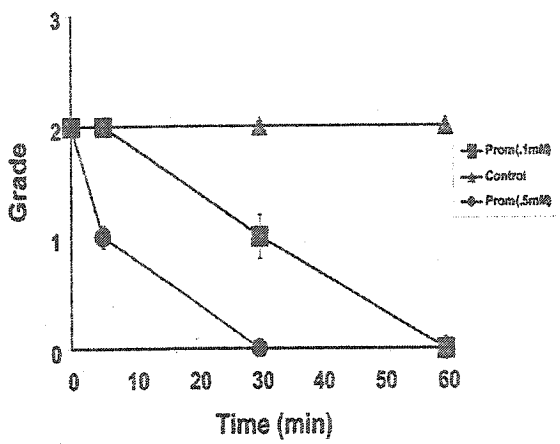


Fig. 6. Effect of serotonin and histamine on adult schistosomes. Adult *S. mansoni* were incubated with various test drugs, serotonin and histamine, or a vehicle as indicated for different periods of time (0, 5, 30, 60, 90 min). The adult worms were monitored for overall motility according to a grading system in vitro. The data are the means and standard errors of 8-10 adult worms.

However, H_1 antagonists (promethazine, diphenhydramine) produced the same paralyzing effect in the adults as they did in the cercaria. Upon addition of 0.5mM of either drug, the motor activity of adult worms decreased gradually until the parasites were completely paralyzed. Diphenhydramine caused complete paralysis faster than promethazine (within 5 min) (Fig. 7), suggesting that the

kinetics of the two drugs are quite different. At a lower concentration (0.1mM), diphenhydramine did not completely paralyze the adult worms within the 60 min incubation (Fig. 7. B), while promethazine caused full paralysis at the same concentration (Fig. 7. A). Basal activity was monitored through the incubation in each experiment and was found to remain constant (Grade 2) for up to 60 min.

A)



B)

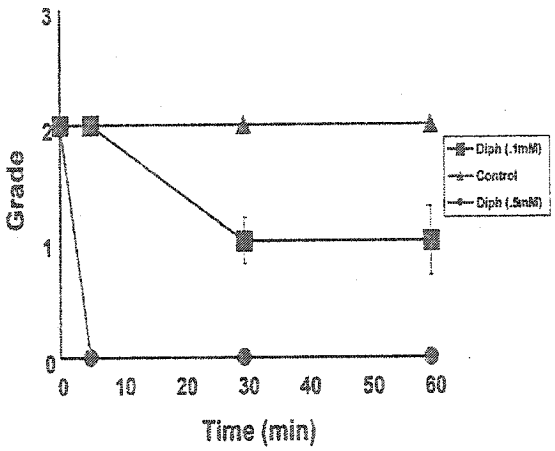


Fig. 7 H_1 antagonists immobilize *S. mansoni* adults. Promethazine **A)** and diphenhydramine **B)** were used at two different concentrations (100µM, 500µM) on adult schistosomes. This is a summary of experiments shown are the means and standard errors of 8-16 adult worms. Basal activity was monitored through the incubation in each experiment. **C)** Representative motility score for adult worms treated with H_1 antagonists (promethazine, diphenhydramine) and an H_2 antagonist (cimetidine) or an untreated control at time =0 and after 5 min and 60 min of incubation.

C)

	Time (0min)	Time (5min)	Time (60min)
Control	2	2	2
Prome (0.5mM)	2	1	0
Diphen (0.5mM)	2	0	0
Cimet (2mM)	2	2	3

To further test if the effect of H_1 antagonists was reversible, adult schistosomes were treated with promethazine or diphenhydramine at 0.5mM concentration for

60 min and the worms were washed twice and resuspended in fresh DMEM. The immobilization effect of diphenhdyramine was fully reversed to normal basal motility once the drug was removed (Fig. 8). The effect of 0.5mM promethazine was not reversible by washing, whereas at a lower concentration of promethazine (0.1mM) the immobilization effect was reversible. The data suggest that H_1 antagonists have an inhibitory effect on the motor activity of adult worms which is both dose-and time-dependent.

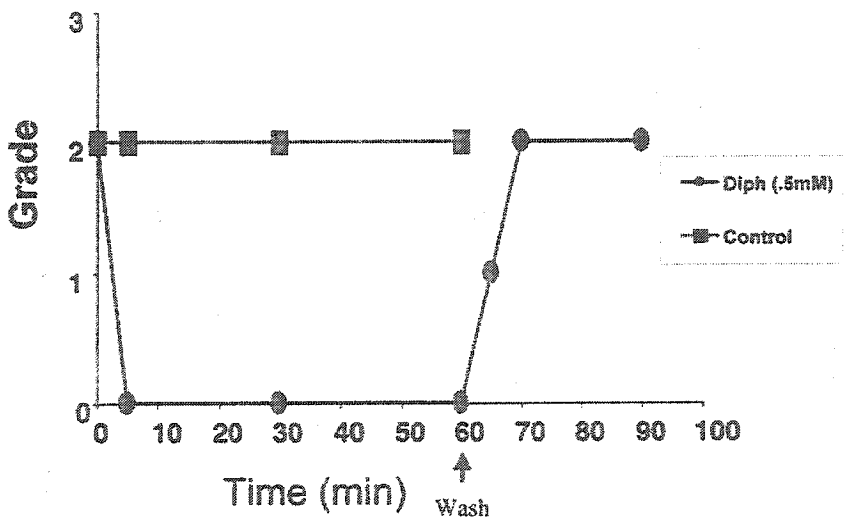


Fig. 8. H_1 immobilization effect on adult schistosomes is reversible. Adults *S. mansoni* were incubated with 500μM diphenhydramine and after 55 min of complete paralysis the worms were washed two times. The results indicate that the drugs effect was washable up to normal basal motility.

In contrast to H_1 antagonists, cimetidine (H_2 inhibitor) caused a slight increase in the adult motor activity at 2mM concentration (Fig. 7.C). Data are the means and standard errors of 9-14 adult worms in 4 independent experiments.

DISCUSSION

Various external stimuli (e.g. heat, light, chemicals) are involved in the penetration of *S. mansoni* cercariae into mammalian hosts (MacInnis, 1969; Cohen et al., 1980). Very little is known about cercarial responses to these stimuli (Cohen et al., 1980), or the mechanisms that modulate the motor activity of the parasite in the host. One earlier study of *S. mansoni* reported that histamine may modulate worm motility in vitro, suggesting that histamine may function as a neuromuscular transmitter or modulator in this parasite (Ercoli et al., 1985). More recently, studies in our laboratory cloned a novel G protein-coupled receptor from adult *S. mansoni* (SmGPCR), which was responsive to histamine (Hamdan et al., 2002a) and thus was postulated to play a role in the modulation of parasite motility. The results presented here support the notion that histamine regulates motility of *S. mansoni* and further suggest that the effect is mediated by a H_1-like receptor.

RT-PCR revealed that SmGPCR is expressed at least at the mRNA level in two developmental stages of the parasite, adults and cercaria. Interestingly, the results also showed proportionally more SmGPCR in cercaria than adults, relative to an internal standard, which was co-amplified by PCR and is known to be constitutively expressed throughout the *S. mansoni* life cycle (Webster et al., 1992). This would suggest that SmGPCR may be expressed at higher level in the cercaria compared to adults, and thus may be particularly relevant to cercarial motility and, possibly, penetration of the host. Additional research is needed to

confirm this observation and elucidate the potential relevance of differential SmGPCR expression in cercariae and adults.

Cercaria and adult *S. mansoni* were each treated with increasing concentrations of exogenously applied histamine in vitro and then monitored for changes in motor activity. The cercarial duration of swimming episodes increased significantly up to 2-fold at a concentration of 0.1mM compared to non-treated animals. On the other hand, histamine did not have any measurable effect when the same test was done with the adult worms. There may be two possible explanations for the absence of response in the adult worms: first, the adult worms may have higher endogenous levels of histamine and increasing this by adding external histamine may not lead to an obvious effect. A second possibility is that the histamine receptor in the adults is not as accessible to exogenous histamine as in the cercaria, and thus may be unresponsive to added histamine. In addition to histamine, this study has tested the effect of added serotonin on the motor activity of both developmental stages. Serotonin strongly stimulated the motor activity of adult worms, consistent with previous observations (Davis and Stretton 1995). A weaker but nonetheless significant stimulatory effect was also observed in the cercaria. This is the first demonstration of an effect of serotonin in cercaria. Previous studies have shown serotonin to be present in cercaria and the enzyme responsible for its synthesis was also identified (Hamdan and Ribeiro 1999a), however, the function of serotonin was unclear. This study suggests that serotonin may contribute to the modulation of cercaria motility but to lesser extent than in the adults.

The finding that histamine stimulates motor activity at least in cercaria prompted us to examine the effects of several classical histamine receptor blockers. Three types of antagonists were used in the study, including drugs which are selective for H_1, H_2, and H_3/H_4 receptors. *S. mansoni* cercariae and adults treated with histamine H_1 antagonists responded in the same way. Both stages showed decreased motor activity, which culminated in complete paralysis. The drug effect was dose dependent and was consistent in all experiments. Moreover, the immobilization caused by H_1 antagonists was reversed by addition of excess histamine in cercaria. In contrast, H_2 and H_3/H_4 histamine receptor blockers did not have the same inhibitory effect on worm motility. Taken together, results suggest that the drug effects were mediated by a specific receptor, since they could be reversed by competition with histamine. The results further suggest that this receptor has pharmacological characteristics of classical histamine H_1 GPCR. Further experiments are needed, however, to confirm that mammalian H_1 antagonists are acting specifically on the *S. mansoni* histamine receptor.

Similar results were obtained with the adult stage of the parasite. H_1 antagonists caused sustained, rapid paralysis of adult worms whereas cimetidine (H_2 antagonist) either had no effect or caused a slight stimulation, depending on concentration used. Although competition studies with histamine could not be performed in the adults, the results nonetheless demonstrated that the effects of H_1 antagonists were not permanent and could be reversed by washing off the drug. This is consistent with H_1 blockers acting through a specific parasite receptor that influences motility.

The question that remains is whether SmGPCR is the receptor responsible for the effects of histamine and antihistaminic drugs on parasite motility. Preliminary results showed that promethazine was able to block the activity of SmGPCR expressed in COS7 cells at very low concentrations (Abramovitz M., personal communication). This observation indicates that SmGPCR is sensitive to the same drug that causes paralysis of the intact organism, suggesting a correlation between the receptor and the effect on motor activity. Experiments are ongoing to elucidate further the role of SmGPCR in vivo and the potential therapeutic effect of H_1 antagonists in *S. mansoni* infections.

REFERENCES

Basch P.F., (1981) Cultivation of *Schistosoma mansoni* in vitro. *J. Parasitol.* **67**, 186-190.

Bennett J., and Bueding E., (1973) Uptake of 5-hydroxytryptamine by *Schistosoma mansoni. Mol. Pharmacol.* **9**, 311-319.

Bennett J.L., Bueding E., and Timms A.R., (1969) Occurance and levels of 5-hydroxytryptamine in *Schistosoma mansoni. Mol. Pharmacol.* 5, 542-545.

Cohen M.L., Neimark H., and Eveland K.L., (1980) Schistosoma mansoni: Response of cercariae to a thermal gradient. J. Parasitol. 66(2), 362-364.

Davis R.E., and Stretton A.O.W., (1995) Neurotransmitters of Helminth, in *Biochemistry and Molecular Biology of Parasites* (Marr J.J., and Muller M. Eds.), 257-287.

Enasmus A.D., (1972) The biology of Trematodes. *Edward Arnold Publishers Ltd (London),* pp. 78-110.

Ercoli N., Payares G., and Nunes D., (1985) *Schistosoma mansoni*: neutrotransmitters and the mobility of cercaria schistosomules. *Exp. Parasitol.* **59**, 204-216.

Eriksson K., Johnston R., Shaw C., Halton D., and Panula P., (1996) Widespread distribution of histamine in the nervous system of a trematode flatworm. *The Journal of Comparative Neurology* **373**, 220-227.

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

Hamdan F.F., and Ribeiro P., (1999a) Characterization of a stable form of tryptophan hydroxylase from the human parasite, *Schistosoma mansoni. J. Bio. Chem.* **274**(31), 21746-21754.

Hill S., Ganellin C., Timmerman H., Schwartz J., Shankley N., Young J., Schunack W., Levi R., and Hass H., (1997) International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacological Review* **49** (3), 253-278.

Hillman G.R., and Senft A.W., (1973) Schistosome motility measurements: Response to drugs. *J. Pharmacol. Exp. Therapeut.* **185**,177-184.

MacInnis A.J., (1969) Identification of chemicals triggering cercarial penetration responses of *Schistosoma mansoni*. *Nature* **224** (225),1221-2

Mansour T.E., (1984) Serotonin receptors in parasitic worms. *Adv. Parasitol.* **23**, 1-36.

Marr J.J., and Muller M., (1995) Biochemistry and Molecular Biology of Parasites. Academic Press, Inc.
Mei H., and LoVerde P.T., (1997) *Schistosoma mansoni*: the developmental regulation and immunolocalization of antioxidant enzymes. *Exp. Parasitol.* **86**(1), 69-78

Mellin T.N., Busch R.D., Wang C.C., and Kath G., (1983) Neuropharmacology of the parasitic trematode, *Schistosoma mansoni*. *Am J. Trop. Med. Hyg.* **32**, 83-93.

Mkoji M.G., Smith M.J., and Prichard K.R., (1988) Antioxidant systems in *Schistosoma mansoni*: correlation between susceptibility to oxidant killing and the levels of scavengers of hydrogen peroxide and oxygen free radicals. *Internat. J. Parasitol.* **18**(5), 661-666.

Pax R., and Bennett J., (1991) Neurobiology of parasitic platyhelminths: possible solutions to the problems of correlating structure with function. *Parasitology* **102**, S31-S39.

Pax A.R.., Siefker C., and Bennett L.J., (1984) *Schistosoma mansoni*: differences in acetylcholine, dopamine, and serotonin control of circular and longitudinal parasite muscles. *Exp. Parasit.* **58**, 314-324.

Sukhdeo M.V.K., Hsu S.C., Thompson C.S. and Fairweather I., (1984) *Hymenolepis diminuta*: behavioral effects of 5-hydroxytryptamine acetycholine histamine and somatostatin. *J. Parasitol.* **70**, 682-688.

Thueson D.O., Speck L.S., Lett-Brown M.A., and Grant J.A., (1979) Histamine releasing activity (HRA): Production by mitogen-or antigen-stimulated human mononuclear cells. *J. Immunol.* **123**, 626-631.

Webster P.J., Seta K.A., Chung S.C., and Mansour T.E., (1992) A cDNA encoding an alpha-tubulin from *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* **51**(1), 169-170

Wikgren M., Reuter M., Gustafsson M., and Lindroos P., (1990) Immunocytochemical localization of histamine in flatworms. *Cell Tissue Res.* **260**, 479-484.

CONNECTING STATEMENT II

In manscript I (Chapter III) we studied the effect of exogenously applied histamine and various histamine inhibitors on the behaviour of *S. mansoni* cercaria and adults.

In Chapter IV, we have begun studies aimed at producing an antibody against SmGPCR. The antibody will be used to localize this new histamine receptor in the intact worm for better understanding of SmGPCR function in vivo.

Chapter IV

In situ localization of SmGPCR in the intact worm

Aisha Mousa and Paula Ribeiro

(In progress)

ABSTRACT

Two divergent regions of the *Schistosoma mansoni* receptor, SmGPCR, have been targetted for the purpose of raising polyclonal antibodies. A peptide (14 a. a.) corresponding to the C-terminal tail of SmGPCR was synthesized, conjugated to KLH, prepared in complete Freund adjuvant, and injected into two rabbits. Three additional boosts were delivered at 21 days intervals. Analysis of immune serum and an immunoglobulin-enriched ammonium sulfate fraction failed to identify specific anti-SmGPCR antibodies either by ELISA or Western blot analysis. The second region targetted for antibody production was the intracellular third loop of SmGPCR. A cDNA corresponding to positions (nucleotide: 1642-1683) of SmGPCR was amplified by PCR and subcloned into prokaryotic expression vector pET-30b expressing a C-terminal 6x-histidine tag. Protein expression was done using BL_{21} rossetta competent cells and induced with IPTG. After induction the soluble fraction IL_3-His was purified using HisTrap chelating column. A positive band of approximate size (~ 17 KDa) was detected in a Coomassie stain and confirmed by Western blot analysis against the C-terminal anti-His tag. The purified IL_3-His protein will be used in future studies for production of a polyclonal antibody against SmGPCR.

INTRODUCTION

S. mansoni has a primitive nervous system like other trematodes. It consists of a simple central and peripheral nervous system, which innervate all parts of the parasite including the sucker, pharynx, reproductive structures and muscles associated with body wall (Fried and Graczyk 1997; Enasumus 1972). Histamine has been identified within the nervous system of several helminthes, including trematodes (Ercoli et al., 1985; Eriksson et al., 1996) and is believed to serve as a neuroactive agent. Histamine has been recognized as a neurotransmitter relatively recently in parasites. In other invertebrates, histamine functions as a central neurotransmitter and a neurotransmitter of photoreceptors in all arthropods (Schmid and Becherer 1999). Studies on the function of histamine reported an effect on motor activity both in *S. mansoni* (Ercoli et al., 1985) and *H. diminuta* (Sukhdeo et al., 1984) suggesting that histamine plays a role as neuromuscular transmitter or modulator. Histamine exerts its effect by coupling to cell surface receptors the majority of which are G protein linked receptors. A novel histamine receptor was recently cloned from *S. mansoni* and shown to encode a functional protein. Our goal this study is to raise a polyclonal antibody against SmGPCR for studies of receptor localization in the intact worm.

EXPERIMENTAL PROCEDURE

Probe design

To design immunochemical probes against a receptor, certain criteria must be followed. First, the region must contain a unique amino acid sequence. A protein sequence alignment of SmGPCR with members of histamine receptors family and other monoaminergic subtypes, revealed a high degree of homology in the transmembrane segments across species and therefore these regions should be avoided. In contrast, the intra/extracellular loop sequences and the carboxyl terminus are non-conserved regions that are ideal targets for probe design. Second, the region should be hydrophilic, as hydrophobic portions tend to produce insoluble fusion proteins upon expression in *E. coli.* Fortunately, the least conserved cytosolic regions of SmGPCR are the most hydrophilic ones. Moreover, highly charged, hydrophilic regions also tend to be the most immunogenic. In the case of SmGPCR the third intracellular loop and carboxyl terminus seem to be the least conserved regions in this receptor and has unique structure. Thus for the purpose of raising polyclonal antibodies, we choose portions of il3 (121 a. a.) and the C-terminal tail (14 a. a.) as target regions.

Peptide synthesis

The last 14 amino acids from the C-terminal region of SmGPCR were synthesized and conjugated with Keyhole Limpet Hemocyanin (KLH) (CQVNKWNIKSYNHK). Two rabbits were immunized with this hemocynin-conjugated peptide (1mg) prepared in complete Freund Adjuvant (CFA) (1:1 v/v) followed by three boosters in incomplete FA a at 21 days intervals, according to

established protocols (Itoh et al., 2001; Kai et al., 1998). Immune serum was collected 7 days following the last boost by cardiac puncture. After ammonium sulfate precipitation (50%) to concentrate antibody fraction presence of anti-SmGPCR, and dialyzed against 3 changes of PBS at 4 °C. The polyclonal antibody was tested by ELISA and Western blot analyses.

Enzyme-linked immunosorbent assay (ELISA)

An Indirect ELISA assay method for antibody binding to synthetic peptides has been previously described (Kidodoro et al., 2002; Baba et al., 2002; Lunardi et al., 2002). Briefly, we used non-conjugated synthetic peptides at 10, 30, and 50 µg/ ml in 0.1% Tri-Fluoro Acetic acid (TFA) and 20% methanol to coat a ninety six well ELISA plate (Nalgene Nunc.). The plate was left overnight to dry at room temperature and then washed once with 1X Phosphate Buffered Saline (PBS). After blocking for 1 hour at 37 °C with PBS containing 5% skim milk and 0.05% Tween-20, we added the antibody fraction at serial dilutions from 1:100 up to 1:10,000 in 1% skim milk in PBS with 0.05% Tween-20, and incubated for 2 hr at 37 °C. We washed the plates three times with 1X PBS with 0.05% Tween-20. Following the last wash, the plates were incubated with Horse-radish peroxidase (HRP) conjugated antiserum goat against rabbit IgG 1:2000 (Chemicon) in blocking buffer (5%skim milk in PBS with 0.05% Tween-20) for 1 hr at 37 °C. After washing, we measured the bound enzymatic activity with Tetramethylbenzidine (TMB; Sigma) after 15 min incubation. Sulfuric acid (2M) was added to stop the reaction, and the optical density was read at 450 nm in a

microtiter reader. Preimmune sera diluted in the same manner as the tested immune serum from rabbits was used as a control.

Bacterial expression and protein purification of IL_3

PCR reaction was done with primers designed to amplify 373 bp the of SmGPCR third intracellular loop (sense: 5'- CCGAATTCATGCCCGAACCA ACAGA -3'; antisense: 5'- TAGCGGCCGCTGCAGTTTTTTGTTC- 3'), and a SmGPCR expression plasmid as a template. The primers were designed to introduce flanking *ECoR I* and *Not I* restriction sites. The resulting PCR product was double digested with *EcoR I* and *Not I* and subcloned into prokaryotic expression vector pET30b, thus fusing a 6XHis tag to its C-terminal extremity. BL_{21} (rossetta) cells transformed with il3 (121 a. a.) in pET30b construct were grown at 37 °C in LB-kanamycin-chloromphenicol medium to an OD_{600} of ~ 0.5-1.0 (log growth phase). The culture was induced with 0.4mM isopropylβ-D-thiogalacto-pyranoside (IPTG) for 3 hours at 37 °C with constant shaking. Following induction, the cells were pelleted by centrifugation for 15 min at 6500 xg and 4 °C and frozen at -80 °C. For extraction of SmGPCR protein, the pellet of a 500 ml induced bacterial culture was thawed and resuspended in 5 ml cold Tris-HCl (pH 7.5) containing 100µg/ml lysozyme, which enhanced cell lysis, and incubated on ice for 30 min.

Cells were sonicated for 3 min with 10 sec bursts on and 10 sec off on ice. The cell lysate were centrifuged at 14,000 xg for 10 min at 4 °C. The resulting pellet was washed twice in 20mM Tris-HCl and resuspended in 1% SDS with

vigorous mixing. Soluble and insoluble fractions were then analyzed by Coomassie staining and Western blotting.

The fusion protein was purified by immobilized metal (nickel) affinity chromatography as described by Kidokoro et al., 2002; Angers et al., 1998 using the HisTrap Kit (*Pharmacia Biotech*) for rapid purification of histidine-tagged fusion proteins as described by Hamdan and Ribeiro, 1998. Briefly, the soluble fraction was loaded onto a 1ml HisTrap chelating column which had been previously charged with Ni^{2+} ions and equilibrated with binding buffer (1X PBS, 10mM imidazole; pH 7.4), according to the specifications of the manufacturer. *E. coli* expressing IL_3-His was lysed in the same binding buffer and centrifuged at 12,000 xg for 10 min at 4 °C.The soluble fraction was loaded onto the HisTrap column by gravity flow. The column was washed with 60mM imidazole (10 volumes) and then 100mM imidazole prior to elution of SmGPCR protein with 500mM imidazole. The purified protein was aliquoted and stored at -80 °C for further analysis by Western blot and Coomassie staining.

Western Blot

Proteins were resolved on 12% sodium dodecyl sulfate polyacrylamide gel and subsequently electroblotted onto PVDF filter. After blocking for 1 hr at room temperature, the filter was incubated with a C-terminus specific anti-histidine antibody (1:2000; Invitrogen) overnight at 4 °C. The blots were washed and incubated with a secondary antibody conjugated to horse-radish peroxidase (HRP; 1:2000), according to standard procedures. Western positive bands were detected on film using lumilight (Roche).

RESULTS AND DISCUSSION

C-terminal peptide antibody

Immune sera from two different rabbits were collected and a partially purified antibody fraction was obtained by ammonium sulfate fractionation. Aliquots of the antibody fraction were tested first by indirect ELISA targeting non-conjugated synthetic peptides coated in 96 well ELISA plates. Plates were treated with secondary anti-rabbit IgG conjugated to HRP enzyme and then read in an ELISA plate reader at 450 nm, according to standard protocols. Unfortunately, the results showed no specific response against our antigen (synthetic peptides) compared to control (preimmune serum) (data not shown). The same negative results were obtained from a Western blot analysis targeting the receptor expressed in mammalian cells (HEK293 EBNA1) and using the immune serum as the primary antibody. No immuno-reactive band of the correct size could be observed in samples reacted with the rabbit's antibody fraction. In contrast, a parallel reaction with anti-FLAGM2 antibody, which targets the N-terminal FLAG epitope, produced a positive band of the predicted size. This confirmed that there was no specific reaction between the rabbit's serum and our receptor (SmGPCR). Thus we have selected another region of SmGPCR as a second candidate for production of a polyclonal antibody.

Purification of IL_3-His

Portion of SmGPCR-IL_3 (373 bp) was amplified using two primers that introduced two restriction sites *EcoR I* and *Not I* (Fig. 1). The PCR product was double digested with these restrictions enzymes and ligated to digested prokaryotic expression vector pET-30b.

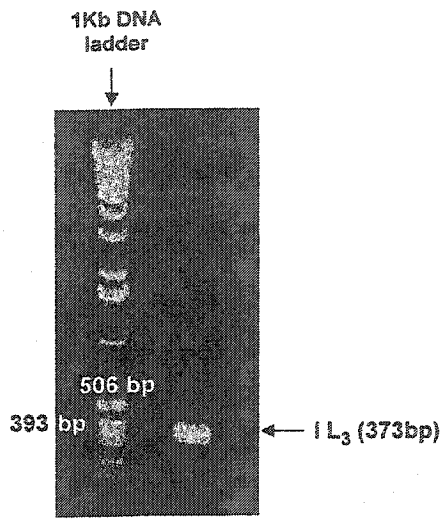


Fig. 1 Amplification of a fragment from SmGPCR-IL_3. PCR reaction was done using two specific primers to amplify a sequence corresponding to 373 bp of SmGPCR-IL_3. The PCR product was analyzed on a 1% agarose gel, stained with ethidium bromide.

The pET-30b construct was transformed using competent cells (BL_{21}) on LB-Kanamycin-Chloromphenicol medium. An isolated colony was grown in LB medium containing the two selective antibiotics, kanamycin and chloromphenicol, and the culture was induced using IPTG, as described in the experimental procedures. SmGPCR-IL_3 fused to C-terminal His tag was then extracted by centrifugation and purified using Nickel chelation chromatography targeting the C-terminal His tag. Three elutions fractions were tested by SDS-PAGE and Western blot analysis. A Coomassie stain (Fig. 2) showed that we were able to express and purify the region of interest corresponding to an approximate mass

of approximately 17 KDa. This is consistent with the expected size of IL_3-His. In addition we identified two other bands on the gel, one with higher and the other lower molecular weight. Most of the protein was recovered in the first eluate (e1) and, to a lesser extent, e2. The results showed little protein loss in the washes, either with 60mM or 100mM imidazole. The identity of the purified protein was further tested by Western blot analysis with C-terminal anti-His antibody (Fig. 3). The ~ 17 KDa band reacted positively with the antibody and thus was confirmed as IL_3-His. Of the other two products, only the lower molecular band was immunoreactive, suggesting that it may be a proteolytic product or truncated translation product of IL_3-His. The remaining high molecular weight band was not immunoreactive and thus seems to be contaminant. Additional optimization is needed to improve the purity of our preparation and increase the amount of protein available for rabbit injection.

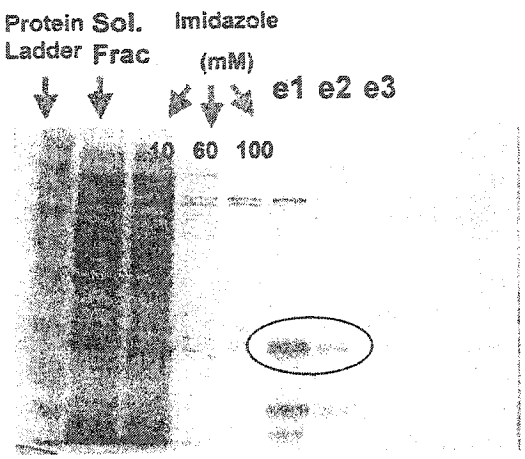


Fig. 2 Coomassie staining of purified il_3 portion. IL_3-His protein was expressed and purified using a HisTrap kit. In this figure the standard protein ladder is in lane one and the soluble fraction of purified protein before loading onto the HisTrap. HisTrap column in lane two. Lanes 3, 4, and 5 are the washes with imidazole binding buffer during protein purification. The protein of interest was found mainly in eluate 1 and some in eluate 2 in lane 6 and 7 respectively.

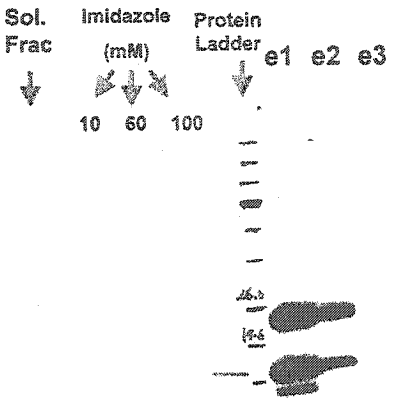


Fig. 3 Western blot of purified protein products. Lane # 1 is the soluble fraction and the next three lanes are the imidazole washes. Lane # 4 is the standard prestained protein ladder. The results showed a positive band corresponded to the targeted protein of SmGPCR and a lower molecular weight band in eluate 1 and 2.

REFERENCES

Angers A., Storozhuk V.M., Duchaine T., Castellucci F.V., and DesGrosesillers L., (1998) Cloning and functional expression of an *Aplysia* 5-HT receptor negatively coupled to adenylate cyclase. *J. Neuroscien.* **18(15)**, 5586-5593.

Baba A., Yoshikawa T., Ogawa S., (2002) *Methods: Journal of the American College of Cardiology* **40**(6), 1153-9.

Enasmus A.D., (1972) The biology of Trematodes. *Edward Arnold Publishers Ltd (London)*, pp. 78-110.

Ercoli N., Payares G., and Nunes D., (1985) *Schistosoma mansoni*: neutrotransmitters and the mobility of cercaria schistosomules. *Exp. Parasitol.* **59**, 204-216.

Eriksson K., Johnston R., Shaw C., Halton D., and Panula P., (1996) Widespread distribution of histamine in the nervous system of a trematode flatworm. *The Journal of Comparative Neurology* **373**, 220-227.

Fried B., and Graczyk K.T., (1997) Advances in Trematode biology. *CRC Press LLC (U.S.A).*

Hamdan F.F., and Ribeiro P., (1998) Cloning and characterization of a novel form of tyrosine hydroxylase from the human parasite *Schistosoma mansoni. J. Neurochem.* **71**(4), 1369-1380.

Itoh M., Odagiri M., Abe H., and Saitoh O., (2001) RGS8 protein is distributed in dendrites and cell body of cerebellar purkinje cell. *Biochem. Biophysic. Res. Communi.* **287**, 223-228.

Kai H., Alexander W.R., Ushio-fukai M., Lyons R.P., Aders M., and Griendling K.K., (1998) G-protein binding domains of the angiotensin II AT_{1A} receptors mapped with synthetic peptides selected from the receptor sequence. *Biochem J.* **332**, 781-787.

Kidokoro M., Aoki A., Horiuchi K., Shida H., (2002) *Methods: Microbes and Infection* **4**, 1035-1044.

Lunardi C., Bason C., Leandri M., Navone R., Lestani M., Millo E., Benatti U., Cilli M., Beri R., Corrocher R., and Puccetti A., (2002) *Methods: Mechanisms of disease* **360**, 915-21.

Schmid A., and Becherer C., (1999) Distribution of histamine in the CNS of different spiders. *Microsc. Res. Tech.* **44** (2-3), 81-93.

Sukhdeo M.V.K., Hsu S.C., Thompson C.S. and Fairweather I., (1984) *Hymenolepis diminuta*: behavioral effects of 5-hydroxytryptamine acetycholine histamine and somatostatin. *J. Parasitol.* **70**, 682-688.

GENERAL DISCUSSION

A major goal of this thesis was to characterize a new G protein-coupled receptor cDNA previously cloned from the bloodfluke, *Schistosoma mansoni* (SmGPCR) (Hamdan et al., 2002a). The first challenge was to express SmGPCR in a heterologous system. It is known that helminth DNA is expressed poorly in heterologous systems such as *Escherichia coli* or mammalian cells. This is due in part to differences in codon usage that significantly decrease translation efficiency and thereby lower protein production (Hernan et al., 1992; Kane 1995). To address this problem, *S. mansoni* cDNA was rewritten according to mammalian preferred codon usage as defined in the international DNA sequence data base (Nakamura et al., 1999), without changing the amino acid sequence encoded by SmGPCR. Codon-optimized SmGPCR was subcloned into an expression vector, and double-tagged with N-terminal FLAG and C-terminal 6x-His epitopes prior to transfection. Immunofluorescence analysis of mammalian cells transfected with codon-optimized SmGPCR targeting the two epitopes revealed a significant increase in receptor expression compared to cells transfected with the wild-type. Expression of codon-optimized SmGPCR in mammalian cells also demonstrated that SmGPCR has a typical GPCR topology, the N-terminal being extracellular and the C-terminus intracellular, consistent with a functional GPCR receptor.

SmGPCR was initially designated as an 'orphan' receptor because of low sequence homology with other biogenic amine GPCRs. Functional expression

assays revealed that codon-optimized SmGPCR was responsive to histamine, which caused a dose-dependent increase in intracellular calcium but not cAMP, as determined by sensitive reporter assays. None of the other monoamines tested had any significant effect on receptor activation by measuring either Ca^{2+} or cAMP-mediated signaling.

A Comparison of SmGPCR to known mammalian histamine receptor classes (H_1, H_2, H_3/H_4) indicated that SmGPCR has relatively low affinity for its natural ligand, histamine, with an EC_{50} in the μM range, which is comparable to mammalian H_2 class (Hill et al., 1997). On the other hand, SmGPCR activation caused an elevation in intracellular calcium, suggesting signaling through $G_{q/11}$ coupling, which is similar to mammalian H_1 histamine receptors. These findings suggest that SmGPCR has some characteristics of both H_1 and H_2 and thus may constitute a new class of histamine receptor.

Work presented in this thesis raises a number of important questions about the mode of action of histamine and the function of SmGPCR in *S. mansoni.* We have begun to address some of these questions by testing the effects of histamine and several antihistaminic agents on the behaviour of the parasite in vitro. RT-PCR showed that SmGPCR was expressed in both adult schistosomes and cercaria, at least at the mRNA level. Thus we selected to use cercaria for the first part of the study. Cercariae are preferable system for behavioral assays because they can be maintained in culture for extended periods of time and they have a well defined swimming behavior that can be easily quantified. Earlier studies had indicated that exogenously applied histamine modulated the motility of *S. mansoni* cercaria (Ercoli et al., 1985),

suggesting that histamine might function as a positive neuromuscular transmitter or modulator in the parasite. The results presented here are consistent with that earlier study. Treatment of *S. mansoni* cercaria with histamine produced a dose-dependent increase in motility up to nearly 2-fold compared to untreated controls. The stimulation was comparable to that obtained with serotonin, which is a known positive modulator of motility in *S. mansoni* (Mansour 1984; Pax and Bennett 1991; Davis and Stretton 1995)). In contrast to the cercaria, the adult form of *S. mansoni* was not responsive to exogenous histamine, even at high concentrations. This lack of effect can not be explained by general unresponsiveness of adult worms in culture, since the same organisms were sensitive to stimulation by serotonin. Instead, the different responses to histamine are likely to reflect differences between the two developmental stages that influence the accessibility and/or level of expression of the histamine receptor. In future research, it will be of interest to determine if the higher sensitivity of the cercaria to histamine is related to the higher expression level of SmGPCR mRNA.

The results showed that histamine H_1 receptor blockers were able to decrease the motility of the parasite and induced complete immobilization in a dose-dependent manner in vitro. Promethazine (H_1 antagonist) was found to be the most potent drug to induce that effect. The parasite immobilization was reversible by addition of excess exogenous histamine in *S. mansoni* cercaria, suggesting that effect of the drug was mediated by a histamine receptor. Similar effects were observed in adult schistosomes. H_1 antagonists (promethazine, diphenhydramine) caused complete paralysis of adult worms. The effect was not

permanent and could be reversed by washing off the drug. Other histamine receptor inhibitors (H_2, H_3/H_4) had no effect on motility, either in cercaria or adult parasites in vitro. These observations suggest the presence of a receptor in *S. mansoni* that has some of the pharmacological properties of mammalian H_1 GPCRs. The question that remains is whether this receptor is SmGPCR. Preliminary results in collaboration with Merck Frosst revealed that SmGPCR expressed in COS7 cells was sensitive to promethazine (H_1 receptor blocker). This suggests sensitivity to H_1 receptor antagonists, which is consistent with the findings seen in the intact animals.

A second important question is how a histamine receptor contributes to the biology of the parasite in vivo, its ability to penetrate the host and subsequent survival. It is unknown if the receptor responds to endogenous histamine, which is required for normal regulation of motor activity, or if the receptor plays some other unknown role related to exogenous histamine. Upon cercarial penetration of human skin, immunological responses lead to degranulation of recruited mast cells and basophils releasing histamine. The possibility exists, therefore, that a parasite histamine receptor may be directed towards that exogenous histamine in the blood circulation, perhaps as a way to evade the host's immune response. It will be of interest to determine where the receptor is localized in the parasite, whether it is present on the surface (tegument) or internally. This will also help to determine if exogenous histamine activates the receptor directly or if it must be transported to the receptor site via specific monoamine transporters. To elucidate the tissue localization of the *S. mansoni* histamine receptor, we began studies aimed at producing an antibody against SmGPCR. The last chapter, we were

able to amplify a fragment of SmGPCR third intracellular loop by PCR. The fragment was subcloned into a prokaryotic expression vector, expressed in *E. coli* as a histidine tagged protein and purified for the purpose of raising polyclonal antibodies in rabbits. Once available, these antibodies will be used to target and thereby localize SmGPCR in the intact worm. Moreover, current research in our lab is attempting to determine if SmGPCR is the receptor responsible for the reported effects of histamine on worm motility in vitro.

GENERAL REFERENCES

Alberts B., Bary D., Lewis J., Raff M., Roberts K., and Watson J., (1994) Molecular Biology of the Cell. 3rd Edition.

Alfonso A., Grundahl K., Duerr JS., Han HP., and Rand JB., (1993) The *Caenorhabditis elegans unc-17* gene: a putative vesicular acetylcholine transporter. *Science* **261**(5121), 617-9.

Al-Gadi M., and Hill S., (1987) The role of calcium in the cyclic AMP response to histamine in rabbit cerebral cortical slices. *Br. J. Pharmacol.* **91**, 213-222.

Angers A., Storozhuk M., Duchaine T., Castellucci V., and DesGroseillers L., (1998) Cloning and functional expression of an *Aplysia* 5-HT receptor negatively coupled to adenylate cyclase. *The Journal of Neuroscience* **18** (15), 5586-5593.

Arakawa S., Gocayne J., McCombie W., Urquhart D., Hall L., Fraser C., and Venter J., (1990) Cloning, localization, and permanent expression of a *Drosophila Octopamine* receiptor. *Neuron* **2**, 343-354.

Arrang J.M., Grabarg M., Lancelot J.C., Lecomte J.M., Pollard H., Robba M., Schwatz M., and Schwartz J.C., (1987) Highly potent and selective ligands for histamine, H_3-receptors, *Nature (Lond.)* **327**, 117-123.

Baldwin J., Schertler G., and Unger V., (1997) An alpha-carbon Template of the transmembrane Helices in the rhodopsin family of G-protein-coupled receptors. *J. Mol. Biol.* **272**, 144-164.

Baldwin J.M., (1994) Structure and function of receptors coupled to G-protein. *Current Opinion Cell Biology* **6**, 180-190.

Barger G and Dale H.H., (1910) Chemical structure and sympathomimetic action of amines. *J. Physiol.* (*Lond*) **41**,19-59

Basch P.F., (1990) Why do schistosomes have separate sexes? *Parasitology Today* **6**, 160-163.

Basch P.F., and Gupta B.C., (1988) Immunocytochemical localization of regulatory peptides in six species of trematode parasites. *Comp. Biochem. Physiol.* **91C**, 565-570.

Bennett J., and Bueding E., (1973) Uptake of 5-hydroxytryptamine by *Schistosoma mansoni. Mol. Pharmacol.* **9**, 311-319.

Bennett J.L., and Gianutsos G., (1977) Distribution catecholamines immature *Fasciola hepatica*: histochemical and biochemical study. *Int. J. Parasitol.* **7**, 221-225.

Birnbaumer L., (1992) Receptor-to-effector coupling through G-protein: Role for βγ-dimers as well as α-subunits. *Cell* **71**, 1069-72.

Blakely R., and Bauman A., (2000) Biogenic amine transporters: regulation in flux. *Current Opinion in Neurobiology* **10**, 328-336.

Brownlee D.J.A., Brennan G.P., Halton D.W., Fairweather I., and Shaw C., (1994) Ultrastructural localization of FMRFamide-and pancreatic polypeptide immunoreactivites within the central nervous system of the liver fluke *Fasciola Hepatica* (Trematoda: Digenea). *Parasitol. Res.* **80**, 117-127.

Callaway J.C., and Stuart A.E., (1999) The distribution of histamine and serotonin in the barnacle's nervous system. *Microsc. Res. Tech.* **44** (2-3), 94-104.

Chou T.C.T., Bennett J., and Bueding E., (1972) Occurance and concentrations of biogenic amines in trematodes. *J. Parasit.* **58**, 1098-1102

Clapham D., and Neer E., (1993) New roles for G-protein βγ-dimers in transmembrane signaling. *Nature Lond.* **365**, 403-406.

Clapham D., and Neer E., (1997) G protein βγ subunits. *Annu. Rev. Pharmacol. Toxicol.* **37**, 167-203.

Cowden C., Stretton A.O.W., and Davis R.E., (1989) AF1, a sequenced bioactive neuropeptide isolated from the nematode *Ascaris*. *Neuron* **2**, 1465-1473.

Cowden C., and Stretton A.O.W., (1995) eight novel FMRFamide-like peptides isolated from the nematode *Ascaria suum*. *Peptides* **16**, 491-500.

Davis R.E., and Stretton A.O.W., (1995) Neurotransmitters of Helminth, in *Biochemistry and Molecular Biology of Parasites* (Marr J.J., and Muller M. Eds.), 257-287.

Del Valle J., and Gantz I., (1997) Novel insights into histamine H_2 receptor biology. *Am. J. Phsiol.* **273**, G987-G996.

Desai C., Garriga G., McIntire S.L., and Horvitz H.R., (1998) A genetic pathway for the development of *C. elegans* HSN motor neurons. *Nature* **336**, 638-646.

Dianqing W., Katz A., and Simon M., (1993) Activation of phospholipase $C\beta_2$ by the α and βγ subunits of trimetric GTP-binding protein. *Proc. Natl. Acad. Sci. USA* **90**, 5247-5301.

Dorsey C.H., and Stirwalt M.A., (1971) *Schistosoma mansoni*: fine structure of cercarial acetabular glands. *Expl. Parasit.* **30**, 199-214

Drutel G., Peitsaro N., Karlstedt K., Wieland K., Smit M., Timmerman H., Panula P., and Leurs R., (2001) Identification of rat H_3 receptor isoforms with different brain expression and signaling properties. *Mol. Pharmacol.* **59**, 1-8.

Elias M.S., and Evans P.D., (1983) Histamine in the insect nervous system: distribution, synthesis and metabolism. *J. Neurochem.* **41** (2), 582-8.

Enasmus A.D., (1972) The biology of Trematodes. *Edward Arnold Publishers Ltd (London)*, pp. 78-110.

Ercoli N., Payares G., and Nunes D., (1985) *Schistosoma mansoni*: neutrotransmitters and the mobility of cercaria schistosomules. *Exp. Parasitol.* **59**, 204-216.

Eriksson K., Johnston R., Shaw C., Halton D., and Panula P., (1996) Widespread distribution of histamine in the nervous system of a trematode flatworm. *The Journal of Comparative Neurology* **373**, 220-227.

Estey S.J., and Mansour T.E., (1988) GTP binding regulatory proteins of adenylate cyclase in *Schistosoma mansoni* at different stages of development. *Mol. Biochem. Parasitol.* **30**, 67-75.

Fairweather I., Macartney G.A., Johnston C.F., Halton D.W., and Buchanan K.D., (1988) Immunocytochemical demonstration of 5-hydroxytryptamine (serotonin) and vertebrate neuropeptides in the nervous system of excysted cysticercoid larvae of the rat tapeworm, *Hymenolepsis diminuta* (Cestoda: Cyclophyllidea). *Parasitol. Res.* **74**, 371-379.

Feng G., Hannan F., Reale V., Yi Hon Y., Kousky C., Evans P., and Hall L., (1996) Cloning and functional characterization of a novel Dopamine receptor from Drosophila melanogaster. *The Journal of Neuroscience* **16** (12), 3925-3933.

Fried B., and Graczyk K.T., (1997) Advances in Trematode biology. *CRC Press LLC (U.S.A).*

Garbarg M., and Schwartz J., (1988) Synergism between histamine H_1-and H_2-receptors in the cyclic AMP response in guinea-pig brain slices:effect of phorbol esters and calcium. *Mol. Pharmacol.* **33**, 38-43.

Gerhardt C., Bakker R., Piek G., Planta R., Vreugdenhil E., Leysen J., and Van Heerikhuizen H., (1997) Molecular cloning and pharmacological characterization of *Molluscan Octopamine* receptor. *Molecular Pharmacology* **51**, 293-300.

Gether U., and Kobilka B., (1998) G protein-coupled receptors. *The Journal of Biological Chemistry* **273** (29), 17979-17982.

Ghi P., Orsetti M., Gamalero S., and Ferretti C., (1999) Sex differences in memory performance in the object recognition test. Possible Role of Histamine receptors. *Pharmacology and Biochemistry and Behavior* **64** (2), 761-766.

Gustafsson K.S.M., (1987) Immunocytochemical demonstration of neuropeptides and serotonin in the nervous system of adult *Schistosoma mansoni. Parasitol. Res.* **74**, 168-174.

Hall R., Premont R., and Lefkowitz R., (1999) Heptahelical receptor signaling: beyond the G protein paradigm. *The Journal of Cell Biology* **145** (5), 927-932.

Halton D.W., Shaw C., Maule A.G., and Smart D., (1994) Regulatory peptides in helminth parasites. *Advances in Parasitol.* **34**, 163-227.

Hamdan F.F., (1999) Serotonin biosynthesis and receptors in helminths. Ph.D thesis.

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

Hamdan F.F., Mousa A., and Ribeiro P., (2002b) Codon optimization improves heterologous expression of a *Schistosoma mansoni* cDNA in HEK293 cells. *Parasitol. Res.* **88**(6), 583-589.

Hamdan F.F., and Ribeiro P., (1999a) Characterization of a stable form of tryptophan hydroxylase from the human parasite, *Schistosoma mansoni. J. Bio. Chem.* **274**(31), 21746-21754.

Hamdan F.F., and Ribeiro P., (1998) Cloning and characterization of a novel form of tyrosine hydroxylase from the human parasite *S. mansoni. J. Neurochem.* **71**(4), 1369-1380.

Hamdan F.F., Ungrin D.M., Abramovitz M., and Ribeiro P., (1999b) Characterization of a novel serotonin receptor from *Caenorhabditis elegans*: Cloning and expression of two splice variants. *J. Neurochem.* **72**, 1372-1383.

Han K.A., Miller N.S., and Davis R.L., (1998) A novel octopamine receptor expressed preferentially in *Drosophila* Mushroom Bodies. *J. Neurosci.* **18** (10), 3650-3658.

Harnett W., and Harnett M., (1998) Heterotrimeric guanine nucleotide-binding proteins in eukaryotic parasites. *Parasitology Today* **14** (1), 27-30.

Hernan R.A., Hui H.L., Andracki M.E., Noble R.W., Sligar S.G., Walder J.A., and Walder R.Y., (1992) Human hemoglobin expression in Escherichia coli: importance of optimal codon usage. *Biochem.* **31**, 8619-8628.

Hibert M.F., Trumpp-Kallmeyer S., Bruinvels A., and Hoflack J., (1991) Three-dimentional models neurotransmitter G-binding protein-coupled receptors. *Mol. Pharmacol.* **40**, 8-15.

Hill S., Ganellin C., Timmerman H., Schwartz J., Shankley N., Young J., Schunack W., Levi R., and Hass H., (1997) International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacological Review* **49** (3), 253-278.

Hockley D.J., (1968) Scanning electron microscopy of *S. mansoni* cercariae. *J. Parasit.* **54**, 1241-1243

Hockley D.J., (1972) *Schistosoma mansoni*; the development of the cercarial tegument. *Parasitology* **64**, 245-252

Horn M., and Vriend G., (1998) G protein-coupled receptors in silico. *J. Mol. Med.* **76**, 464-468.

Horvitz R.H., Chalfie M., Trent C., Sulston J.E., and Evans P.D., (1982) Serotonin and octopamine in the nematode *C. Elegans*. *Science* **206**, 1012-1014.

Hough L., (2001) Genomics meets histamine receptors: new subtypes, new receptors. *Mol. Pharmacol.* **59**, 415-419.

Huang X., Duran E., Diaz F., Xiao H., Messer W.S., Jr. and Komuniecki R., (1999) Alternative splicing of serotonin receptor isoforms in the pharynx and muscle of the parasitic nematode, *Ascaris suum. Mol. Biochem. Parasitol.* 101, 95-106.

Jacobs B., and Azmitia E., (1992) Structure and function of the brain serotonin system. *Physiological Review* **72**, 165-215.

Jordan P., Webbe G., Sturrock F.R., (1993) Human Schistosomiasis. *CAB International (UK).*

Kane J.F., (1995) Effects of rare codon clusters on high-level expression of heterolgous protein identification and purification. *Curr. Opin. Biotech.* 6, 494-500.

Lee YS.., Park YS., Chang DJ., Hwarg JM., Min CK., Kaang BK., and Cho NJ., (1999) Cloning and expression of a G protein-linked acetylcholine receptor from *Caenorhabditis elegans. J. Neurochem.* **72**(1), 58-65

Lehninger L.A., Nelson L.D., and Cox M.M., (1996) principle of biochemistry. 2nd Edition.

Leurs R., Smit M., and Timmerman H., (1995) Molecular pharmacological aspects of histamine receptors. *Pharmac. Ther.* **66** (3), 413-463.

Lewert R.M., and Hopkins D.R. (1965) Cholinesterase activity in *Schistosoma mansoni* cercariae. *J. Parasit.* **51**, 616

Liu C., Ma X-J., Jiang X., Wilson S., Hofstra C., Blevitt J., Pyati J., Li X., Chai W., Carruthers N., and Lovenberg T., (2001) Cloning and pharmacological characterization of a fourth Histamine receptor (H4) expressed in bone marrow. *Mol. Pharmacol.* **59**, 420-426.

Mahmoud AF.A., (2001) Schistosomiasis. *Tropical medicine science and practice* (Volume 3). *Imperial College Press (London).*

Mansour T.E., (1984) Serotonin receptors in parasitic worms. *Adv. Parasitol.* **23**, 1-36.

Marr J.J., and Muller M., (1995) Biochemistry and Molecular Biology of Parasites. Academic Press, Inc.

Marquardt C.W., and Demaree S.R., (1985) Parasitology. *Macmillan publishing company (U.S.A)* Chapter 21: Blood flukes or schistosomes.

Marshal JS. And Bienenstock J., (1994) The role of mast cells in inflammatory reactions of the airways, skin and intestine. *Current Opinion in Immunology* **6**, 853-859.

Maule A.G., Shaw C., Halton D.W., Thim L.D., Johnston C.F., Fairweather I., and Buchanan K.D., (1991) Neuropeptide F: a novel parasitic flatworm regulatory peptide from *Moniezia expansa* (Cestoda: cycloplyllidia). *Parasitol.* **102**, 309-16.

Maxwell G.D., Tait J.F and Hildebrand J.G., (1978) Regional synthesis of neurotransmitter candidates in the CNS of the moth *Manduca sexta. Comp. Biochem. Physiol.* **61C**,109-119.

Mckay D.M., Fairweather I., Johnston C.F., shaw C., and Halton D.W., (1991) Immunocytochemical and radioimmunometrical demonstration of serotonin-and neuropeptide-immunoreactivities in the adult rat tapeworm, *Hymenolepsis diminuta* (Cestoda: Cyclophyllidea). *Parasitol.* **103**, 275-289.

Mellin T.N., Busch R.D., Wang C.C., and Kath G., (1983) Neuropharmacology of the parasitic trematode, *Schistosoma mansoni. Am J. Trop. Med. Hyg.* **32**, 83-93.

Nakamura Y., Gojobori T., and Ikemura R., (1999) Codon usage tabulated from the international DNA sequence databases; its status 1999. *Nucleic Acids Res.* **72**, 292.

Nassel R.D., (1999) Histamine in the brain of insects: A review. *Micro. Res. & Techniq.* **44**, 121-136.

Nonaka H., Otaki S., Ohshima E., Kono M., Kase H., Ohta K., Fukui H., and Ichimura M., (1998) Unique binding pocket for KW-4679 in the histamine H_1 receptor. *European Journal of Pharmacology* **345**, 111-117.

Olde B., and McCombie W.R., (1997) Molecular cloning and functional expression of a serotonin receptor from *Caenorhabditis elegans. J. Mol. Neurosci.* **8**, 53-62.

Oliveira L., Paiva A.C., Sander C., and Vriend G., (1994) A common step for signal transduction in G-protein coupled. *Trends. Pharmacol. Sci.* **15**, 170-172.

Ono J.K., McCaman R.E., (1980) Identification of additional histaminergic neurons in Aplysia: improvement of single cell isolation techniques for in *tandem* physiological and chemical studies. *Neurosci.* **5**, 835-840.

Osborne N.N., and Patel S., (1984) Localisation of histamine-immunoreactivity in specific neurons of the gastropod CNS including a cell which has been identified as histaminergic. *Histochemistry* **81** (2), 207-8.

Palczewski K., Kumasaka T., Hori T., Behnke C., Motoshima H., Fox B., Le Trong I., Teller D., Okada T., Stenkamp R., Yamamoto M., and Miyano M., (2000) Crystal structure of Rhodopsin: a G protein-coupled receptor. *Science* **289**, 739-745.

Ranganathan R., Cannon S.C., and Horvitz H.R., (2000) MOD-1 is a serotonin-gated chloride channel that modulates locomotory behaviour in *C. elegans. Nature* **408**(6811), 470-475.

Panula P., Lintunen M., and Karlstedt K., (2000) Histamine in brain development and tumors. *Cancer biology* **10**, 11-14.

Panula P., Yang HY., and Costa E., (1984) Histamine-containing neurons in the rat hypothalamus. *Proc. Natl. Acad. Sci.* **81**(8), 2572-6.

Pax R., and Bennett J., (1991) Neurobiology of parasitic platyhelminths: possible solutions to the problems of correlating structure with function. *Parasitology* **102**, S31-S39.

Pax A.R., Day A.T., Miller L.C., and Bennett L.J., (1996) Neuromuscular physiology and pharmacology of parasitic flatworms. *Parasitol.* **113**, S83-S96.

Pax A.R.., Siefker C., and Bennett L.J., (1984) *Schistosoma mansoni*: differences in acetylcholine, dopamine, and serotonin control of circular and longitudinal parasite muscles. *Exp. Parasit.* **58**, 314-324.

Raymond J., (1995) Multiple mechanisms of receptor-G-protein signaling specificity. *Am. J. Physiol.* **269**, F141-58.

Robert L.S. and Janvoy J., (1996) Digeneans: Strigeiformes, in *Foundations of Parasitology*. 5th edition (Kamp M.J., ed.), 233-249.

Robson R.T., and Erasmus D.A., (1970) The ultrastructure, based on steroscan observations, of the oral sucker of the cercaria of *S. mansoni*. *Z. Parasitkde* **35**, 76-86

Roeder T., (1999) Octopamine in invertebrates. *Progress in Neurobiology* **59**, 533-561.

Roth B., Willins D., Kristiansen K., and Kroeze W., (1998) 5-Hydroxytryptamine$_2$ family Receptors (Hydroxytryptamine$_{2A}$, Hydroxytryptamine$_{2B}$, Hydroxytryptamine$_{2C}$): where structure meets function. *Pharmacol. Ther.* **79** (3), 231-257.

Savarese T., and Fraser C., (1992) In vitro mutagenesis and the search for structure-function relationships among G protein-coupled receptors. *Biochem. J.* **283**, 1-19.

Scafer WR., and Kenyon CJ., (1995) A calcium-channel homologue required for adaptation to dopamine and serotonin in *C. elegans*. *Nature* **375**, 73-78.

Schmid A., and Becherer C., (1999) Distribution of histamine in the CNS of different spiders. *Microsc. Res. Tech.* **44** (2-3), 81-93.

Schmidt H., Zernikow B., Baeblich S., and Bohme E., (1990) Basal and stimulated formation and release of L-arginine derived nitrogen oxides from cultured endothelial cells. *J. Pharmacol Exp. Ther.* **254**, 591-597.

Schwabe C.W., and Kilejian A., (1968) Chemical aspects of the ecology of platyhelminths in "chemical zoology" (M. Florkin and B.T. Scheer, eds). *Academic Press N.Y.* **2**, 395-466.

Schwartz J.C., Arrang J.M., Garbarg M., Pollard H., and Ruat M., (1991) Histaminegic transmission in the mammalian brain. *Physiol Rev.* **71**, 1-51.

Selbie L., and Hill S., (1998) G-protein-coupled receptor cross-talk: the fine tuning of multiple receptor signaling pathways. *Tips* **19**, 87-93.

Sithigorngal P., Stretton A.O.W., and Cowden C., (1990) Neuropeptide diversity in *Ascaris*: an immunocytochemical study. *J. Comp. Neurol.* **294**, 362-376.

Smith J.H., Reynolds E.S., and Lichtenberg F.Von., (1969) The integument of *S. mansoni*. *Am. J. Trop. Med. Hyg.* **18**, 28-49.

Sternweis P., (1994) The active role of βγ-dimers in signal transduction. *Curr. Opin. Cel. Bio.* **6**, 198-203.

Stirewalt M.A., (1974) *Schistosoma mansoni*: Cercaria to schistosomule. *Adv. Parasitol.* **12**, 115-182.

Stirewalt M.A., and Walters M., (1973) Histochemical analysis of the postacetabular gland secretion of *S. mansoni. Expl. Parasit.* **33**, 56-72

Strader C., Fong T., Tota M., Underwood D., and Dixon R., (1994) Structure and function of G protein-coupled receptors. *Annu. Rev. Biochem.* **63**, 101-32.

Sukhdeo M.V.K., Hsu S.C., Thompson C.S. and Fairweather I., (1984) *Hymenolepis diminuta*: behavioral effects of 5-hydroxytryptamine acetycholine histamine and somatostatin. *J. Parasitol.* **70**, 682-688.

Sukhdeo M.V.K., Sangster N.C., and Mettrick D.F., (1986) effects of cholinergic drugs on longitudinal muscle contractions of *Fasciola hepatica*. *J. Parasit.* **72**, 858-864

Sulston J., Dew M., and Brenner S., (1975) Dopamine neurons in the nematode *Caenorhabditis elegans*. *J. Comp. Neurol.* **163**, 215-244.

Suo S., Sasagawa N., Ishiura S., (2002) Identification of dopamine receptor from *Caenorhabditis elegans. Neurosci. Lett.* **319**, 13-16.

Tardivel-Lacombe J., Rouleau A., Heron A., Morisset S., Pillot C., Cochois V., Schwartz J.C., and Arrang J.M., (2000) Cloning and cerebral expression of the guinea pig histamine H_3 receptor: evidence for two isoforms. *Molecular Neuroscience* **11** (4), 755-759.

Thueson D.O., Speck L.S., Lett-Brown M.A., and Grant J.A., (1979) Histamine releasing activity (HRA): Production by mitogen-or antigen-stimulated human mononuclear cells. *J. Immunol.* **123**, 626-631.

Tipper J., Citron B., Ribeiro P., and Kaufman S., (1994) Cloning and expression of rabbit and human brain tryptophan hydroxylase cDNA in E. coli. *Archives of Biochemistry and Biophysics* **315** (2), 445-453.

Tomosky-Sykes T.K., Mueller J.F., and Bueding E., (1977) Effects of putative neurotransmitters on the motor activity of *Spirometra mansonoides. J. Parasit.* **63**, 492-494.

Twarog B.M., and Page I.H., (1953) Serotonin content of some mammalian tissues and urine and method for its determination. *Am. J. Physiol.* **175**, 157-161.

Van Rhee A.M., and Jacobson K.A., (1996) Molecular architecture of G-protein-coupled receptors. *Drug Development Res.* **37**, 1-38.

Venter C.J., Porzio DI.U., Robinson S.M., Shreeve M.S., Lai J., Kerlavage R.A., Fracek P.S., Lentes K-U, and Fraser M.C., (1988) Evolution of neurotransmitter receptor systems. *Pre. In Neurobio.* **30**, 105-169.

Walker J.R., Brooks L.H., and Holden-dye L., (1996) Evolution and overview of classical transmitter molecules and their receptors. *Parasitology* **113**, S3-S33.

Weinreich D., Weiner C., and MaCaman R., (1975) Endogenous levels of histamine in single neurons isolated from the CNS of *Aplysia californica. Brain Res.* **844**, 342-345.

Wess J., (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *FASEB J.* **11**, 346-354.

Wess J., (1998) Molecular basis of receptor/G-protein-coupling selectivity. *Pharma Ther.* **80** (3), 231-264.

White J., Southgate E., Thomson N., and Brenner S., (1986) The structure of nervous system of nematode *C. elegans. Philos. Trans. R. Soc. Lond. (Biol.)* **314**, 1-340.

Wikgren M., Reuter M., Gustafsson M., and Lindroos P., (1990) Immunocytochemical localization of histamine in flatworms. *Cell Tissue Res.* **260**, 479-484.

Yonge K.A., and Webb R.A., (1992) Uptake and metabolism of histamine by the rat tapeworm *Hymenolepis diminuta*: An in vitro study. *Can. J. Zool.* **70**, 43-50.

Zhu Y., Michalovich D., Wu H.L., Tan K., Dytko G., Mannan I., Boyce R., Alston J., Tierney L., Li X., Herrity N., Vawter L., Sarau H., Ames R., Davenport C., Hieble J., Wilson S., Bergsma D., and Fitzgerald (2001) Cloning, expression and pharmacological characterization of a novel human histamine receptor *Mol Pharmacol.* **59**, 434-441.

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/rgo/animal

Protocol #: 3346
Investigator #: 952
Approval End Date: MARCH 31, 2003
Facility Committee: AGR

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 3346

Title Molecular Biology of Helminths
(must match the title of the funding source application)

1. Investigator Data:

Principal Investigator: Dr. A. Paula Ribeiro Office #:
Department: Parasitology Fax#:
Address: Email: .ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: G. Bingham Work #: Emergency #:
Name: P. Ribeiro Work #: Emergency #:

3. Funding Source:

External ☒
Source (s): NSERC
Peer Reviewed: ☒ YES ☐ NO**
Status: ☒ Awarded ☐ Pending
Funding period: April 1, 2002 - April 1, 2006

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period:

ACTION BY DATE
CC ✓ [illegible]
DS ✓ [illegible]
APPROVED

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed. e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: Date: April 2, 2002

Approval Signatures:

Chair, Facility Animal Care Committee:		Date: May 13, 2002
University Veterinarian:		Date: MAY 15, 2002
Chair, Ethics Subcommittee(as per UACC policy):		Date: 6/20/02
Approved Period for Animal Use	Beginning: April 1, 2002	Ending: MARCH 31, 2003

☐ This protocol has been approved with the modifications noted in Section 13.

MAY 20 2002

APPENDIX II

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

) project should be commenced without prior approval of an application to use biohazardous materials. Submit this plication to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously proved application.

PRINCIPAL INVESTIGATOR: Dr. A. Paula Ribeiro_____TELEPHONE:

ADDRESS:

FAX NUMBER: E-MAIL:

DEPARTMENT: Parasitology_____

PROJECT TITLE: Molecular biology of biogenic amine receptors in helminths_____

FUNDING SOURCE: MRC (CIHR)(x) NSERC NIH ☐ FCAR ☐ FRSQ ☐

INTERNAL ☐ OTHER ☐ (specify) _____

Grant No.: MOP-57709_____Beginning date October 1, 2002_____End date September 30, 2005

dicate if this is

x Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.

Approval End Date: March 31, 2002_____

New funding source: project previously reviewed and approved under an application to another agency.

Agency_____Approval End Date_____

New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed nd certifies with the applicant that the experiment will be in accordance with the principles outlined in the 'Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual". Containment Level (circle 1): 1 (2) 3 4

Principal Investigator or course director:_____ date: 21 08 02
SIGNATURE day month year

Chairperson, Biohazards Committee: [signature] date: 23 08 02
SIGNATURE day month year

Approved period: _____ beginning 01 10 02 ending 30 09 05
day month year day month year

s defined in the "McGill Laboratory Biosafety manual"

2nd REVISION, JANUARY 1996

U Laval

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/rgo/animal

Protocol #: 3346
Investigator #: 952
Approval End Date: MARCH 31, 2003
Facility Committee: AGR

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 3346

Title Molecular Biology of Helminths
(must match the title of the funding source application)

1. Investigator Data:

Principal Investigator: Dr. A. Paula Ribeiro Office #:
Department: Parasitology Fax#:
Address: e-mail:

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: G. Bingham Work #: Emergency #:
Name: P. Ribeiro Work #: Emergency #:

3. Funding Source:

External ☒
Source (s): NSERC
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: April 1, 2002 - April 1, 2006

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period:

ACTION	✓	DATE
CC	✓	June 3 '02
DE	✓	"
APPROVE		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed. e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: Paula Date: April 2, 2002

Approval Signatures:

Chair, Facility Animal Care Committee:		Date: May 13, 2002
University Veterinarian:		Date: MAY 15, 2002
Chair, Ethics Subcommittee(as per UACC policy):		Date: 6/20/02
Approved Period for Animal Use	Beginning: April 1, 2002	Ending: MARCH 31, 2003

☐ This protocol has been approved with the modifications noted in Section 13.

MAY 29 2002

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Dr. A. Paula Ribeiro TELEPHONE:

   ADDRESS:

   FAX NUMBER: E-MAIL:

   DEPARTMENT: Parasitology

   PROJECT TITLE: Molecular biology of biogenic amine receptors in helminths

2. FUNDING SOURCE: MRC (CIHR) (x) NSERC NIH ☐ FCAR ☐ FRSQ ☐

   INTERNAL ☐ OTHER ☐ (specify)

   Grant No.: MOP-57709 Beginning date October 1, 2002 End date September 30, 2005

3. Indicate if this is

   x Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.

   Approval End Date: March 31, 2002

   New funding source: project previously reviewed and approved under an application to another agency.

   Agency Approval End Date

   New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual". Containment Level (circle 1): 1 (2) 3 4

Principal Investigator or course director: date: 21 08 02 (day month year)

Chairperson, Biohazards Committee: (SIGNATURE) date: 23 08 02 (day month year)

Approved period: beginning 01 10 02 (day month year) ending 30 09 05 (day month year)

* as defined in the "McGill Laboratory Biosafety manual"

Sturm, Rosita, Springer

Von: Essenpreis, Alice, Springer
Gesendet: Donnerstag, 14. November 2002 09:15
An: Sturm, Rosita, Springer
Betreff: WG: Asking for permission

Springer-Verlag GmbH & Co. KG
Tiergartenstraße 17
D- 69121 Heidelberg

-----Ursprüngliche Nachricht-----
Von: amousa2 [mailto:amousa2@po-box.mcgill.ca]
Gesendet: Donnerstag, 14. November 2002 06:41
An: Essenpreis@springer.de
Betreff: Asking for permission

Dear Sir/Madame

My name is Aisha Mousa and I am a master's student at McGill University(Montreal-Canada). I will submit my thesis soon (November 18, 2002)which will include one published article from Parasitology Research. I already have the approval of the first and senior authors and I still need a written
permission from the journal in order to include the paper in the thesis.
Please fax the approval to the following number
(Attention: Aisha Mousa)

Article Details:

Hamdan FF., Aisha Mousa, and Ribeiro P., (2002 Jun.) Codon optimization improves heterologous expression of a Schistosoma mansoni cDNA in HEK293 cells.Parasitology Research 88(6):583-6

(10)

Sincerely,
Aisha Mousa

PERMISSION GRANTED

provided:

- Springer-Verlag owns the copyright to the article/book (this is indicated at the bottom/top of the first page or on the imprint page of the book);
- it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required);
- permission is also obtained from the author (address is given on the imprint page, or with the article);
- full credit (Springer-Verlag book or journal title, article title, name(s) of author(s), volume, page, figure number(s), year of publication, copyright notice of Springer-Verlag) is given to the publication in which the material was originally published.

Springer-Verlag GmbH & Co. KG

Date: 26 Nov 02 Rosita Sturm

UNIVERSITY of IOWA HEALTH CARE

Roy J. and Lucille A. Carver
College of Medicine
University of Iowa

Department of Biochemistry

FAX

Date: Nov. 5, 2002

To: Aisha Mousa Fax: _____

_____ Tel: _____

From: John Donelson, editor Tel: _____
Mol. Biochem. Parasitol. Fax: _____

No. of Pages: 2 (including this cover page)

Comments:

Dear Ms./Mr. Mousa,

Mol. Biochem. Pasasitol gives you permission to include the article referenced below as a component of your thesis. I will also communicate this to you via FAX at the number indicated below.

Sincerely yours,

John E. Donelson

John E. Donelson, editor
Mol. Biochem. Parasitol.

----Original Message----
From: Aisha Mousa [mailto:
Sent: Tuesday, November 05, 2002 1:26 PM
To:
Subject: Asking for permission

Dear Dr. Donelson,

My name is Aisha Mousa and I am a master's student at McGill University (Montreal-Canada). I will submit my thesis soon which will include one published article from Molecular and Biochemical Parasitology. I already have the approval of the first and senior authors and I still need a written permission from the journal in order to include the paper in the thesis. Please fax the approval to the following number
(Attention: Aisha Mousa)

Article Details:

Hamdan FF., Abramovitz M. Aisha Mousa, Xie J., Durocher Y., and Ribeiro P., (2002 Jan.) A novel Schistosoma mansoni G protein-coupled receptor is responsive to histamine.
119(1):75-89

Sincerely,
Aisha Mousa