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# Developmental expression analysis and immunolocalization of a biogenic amine receptor in *Schistosoma mansoni*

**Fouad El-Shehabi**[a], **Jon J. Vermeire**[b,1], **Timothy P. Yoshino**[b], and **Paula Ribeiro**[a,*]

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

[b]Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, 2015 Linden Dr., Madison, WI 53706, USA

## Abstract

A *Schistosoma mansoni* G-protein coupled receptor (SmGPCR) was previously cloned and shown to be activated by the biogenic amine, histamine. Here we report a first investigation of the receptor's subunit organization, tissue distribution and expression levels in different stages of the parasite. A polyclonal antibody was produced in rabbits against the recombinant third intracellular loop (il3) of SmGPCR. Western blot studies of the native receptor and recombinant protein expressed in HEK293 cells showed that SmGPCR exists both as a monomer (65 kDa) and an apparent dimer of ≈130 kDa These species were verified by immunoprecipitation of SmGPCR from *S. mansoni* extracts, using antibody that was covalently attached to agarose beads. Further investigation determined that the SmGPCR dimer was resistant to treatment with various detergents, 4 M urea and 0.1 M DTT but could be made to dissociate at acidic pH, suggesting the dimer is non-covalent in nature. Confocal immunofluorescence studies revealed significant SmGPCR immunoreactivity in sporocysts, schistosomula and adult worms but not miracidia. SmGPCR was found to be most widely expressed in the schistosomula, particularly the tegument, the subtegumental musculature and the acetabulum. In the adult stage we detected SmGPCR immunofluorescence mainly in the tubercles of male worms and, to a lesser extent, the body wall musculature. Localization in sporocysts was mainly confined to the tegument and cells within parenchymal matrices. A realtime quantitative reverse-transcription PCR analysis revealed that SmGPCR is upregulated at the mRNA level in the parasitic stages compared to the free-living miracidium and cercariae, and it is particularly elevated during early sporocyst and schistosomula development. The results identify SmGPCR as an important parasite receptor with potential functions in muscle and the tegument of *S. mansoni.*

### Keywords

*Schistosoma mansoni*; Histamine; G-protein coupled receptor (GPCR); Neurotransmitter; Platyhelminth; Biogenic amines; Real-time qPCR; Confocal microscopy

## 1. Introduction

Biogenic amines are small signaling molecules, which are derived from the metabolism of amino acids and include such ubiquitous substances as serotonin (5-hydroxytryptamine: 5HT), catecholamines, phenolamines and histamine (HA). HA is an important neuroactive



*Corresponding author. Fax: +1 514 398 7857. paula.ribeiro@mcgill.ca (P. Ribeiro).
[1]Present address: Department of Pediatrics, Yale University School of Medicine, New Haven, CT 06520, USA.

substance of both vertebrates and invertebrates. In mammals, it functions as a neurotransmitter of the central nervous system (CNS) (Panula et al., 1991; Airaksinen et al., 1991) and also has numerous effects outside the CNS, notably as a regulator of gastric acid secretion, a vasodilator and a mediator of immunity (Marone et al., 2001, 2003). Among invertebrates, HA has been implicated as a neurotransmitter or neuromodulator in the insect eye (Hardie, 1987; Callaway et al., 1989; Nassel et al., 1988) and the somatogastric ganglia of crustaceans (Claiborne and Selverston, 1984). These effects are mediated by cell surface HA receptors, the majority of which are members of the heptahelical G-protein coupled receptor (GPCR) superfamily and are structurally related to rhodopsin. Four different types of histaminergic GPCRs have been identified in mammals (H1–H4), one of which (H3) is alternatively spliced to produce additional variants. These receptors differ in their affinities for HA, signaling mechanisms, tissue distribution and physiological roles (Leurs et al., 1995; Lovenberg et al., 2000; Tardivel-Lacombe et al., 2000; Liu et al., 2000, 2001). Invertebrates have at least one type of HA-activated GPCR that shows modest homology with the mammalian H1 prototype (Hamdan et al., 2002a). In addition, there is increasing evidence that some of the effects of HA in invertebrates are mediated by ionotropic receptors. Studies of arthropods have identified an unusual HA-gated chloride channel of the Cys-loop superfamily that appears to be invertebrate-specific (Hong et al., 2006).

Parasitic flatworms employ a wide range of biogenic amines in their nervous system. Being acoelomates, platyhelminths lack a conventional endocrine system and rely instead on neuronal signaling to coordinate their activities. Much of this signaling is mediated by biogenic amines. The best characterized of these substances, 5HT, has been shown to stimulate muscle contraction and to promote both glycogenolysis and glucose utilization, the overall effect being an increase in motor activity (Boyle et al., 2000; Ribeiro et al., 2005; Boyle and Yoshino, 2005). By comparison, very little is known about the role of HA in flatworms. What information there is available suggests that HA is localized within neuronal structures (Mettrick and Telford, 1963; Wikgren et al., 1990; Eriksson et al., 1996), consistent with a neuroactive role, and is synthesized endogenously (Eriksson et al., 1996), or taken up from the host via tegumental transport (Yonge and Webb, 1992). HA levels vary substantially among parasitic flatworms. Some species, including the bloodfluke *Schistosoma mansoni*, have low tissue levels of HA, whereas other parasites, notably the amphibian trematode, *Haplometra cylindracea* store HA at very high concentrations (Mettrick and Telford, 1963; Schwabe and Kilejian, 1968; Ercoli et al., 1985; Eriksson et al., 1996). The reason for this variation is not clear. A BLAST analysis of the *S. mansoni* genome database identified a potential orthologue of human histidine decarboxylase, the enzyme that synthesizes HA. This suggests that schistosomes have the ability to synthesize HA endogenously, just as they synthesize other biogenic amines (Hamdan and Ribeiro, 1998, 1999). It is possible the rate of synthesis is low or that HA is rapidly degraded once it is released. *Schistosoma mansoni* was reported to possess histaminase (diamine oxidase) activity, an enzyme that breaks down HA (Schwabe and Kilejian, 1968). A high rate of HA catabolism could explain why the basal level of this amine is so low in these animals. Though the function remains unclear, there is evidence to suggest that HA is an important modulator of neuromuscular function and movement among flatworms. Studies of *H. cylindracea* have shown that HA-containing neurons innervate all major bodies of muscle, including the subtegumental (body wall) musculature, ventral sucker and the muscle layers surrounding the alimentary and reproductive tracts (Eriksson et al., 1996). Other studies have shown that exogenously applied HA modulates the frequency of body wall contractions and influences motor activity both in cestodes and trematodes (Sukhdeo et al., 1984; Ercoli et al., 1985). *Schistosoma mansoni* treated with anti-histaminic drugs, such as promethazine, are rapidly paralyzed and the paralysis is reversed by addition of HA (Ercoli et al., 1985), suggesting the amine has a positive effect on motility in this parasite. It is unclear at present whether HA exerts its effects by interacting with receptors located on the

musculature or through some other mechanism that indirectly controls motility. The paralysis produced by the anti-histaminic drugs highlights the importance of HA receptors in these parasites, both with respect to motor control and as potential drug targets.

We have previously cloned a novel *S. mansoni* receptor (SmGPCR) that is specifically activated by HA when expressed heterologously in mammalian cells (Hamdan et al., 2002a, b). SmGPCR shares about the same level of sequence homology with all different types of biogenic amine GPCRs, including the histaminergic H1 type, but has no identifiable mammalian or invertebrate orthologues. This is consistent with the notion that schistosome neuroreceptors are structurally divergent and raises the interesting possibility that SmGPCR may be unique to these parasites. Here we describe a first investigation into the potential function of this receptor in *S. mansoni*. We have examined the pattern of developmental expression and tissue distribution both at the RNA and protein levels, using real-time qPCR, immunofluorescence and confocal microscopy. The results point to SmGPCR as an important schistosome receptor, which is upregulated in the parasitic stages and appears to be enriched in the tegument and neuromuscular structures.

## 2. Materials and methods

### 2.1. Parasites

All parasite stages used in this study were derived from a Puerto Rican (NMRI) strain of *S. mansoni*. Infected *Biomphalaria glabrata* snails were obtained from the Biomedical Research Institute, Rockville, Maryland, USA (Lewis et al., 1986) and were induced to shed cercariae approximately 45 days post-infection by exposure to continuous light for 30 min at room temperature. For cercarial transformation and culturing of schistosomula we modified the original Basch protocol (Basch, 1981) as follows: Cercariae were collected by cooling at 4 °C for 3 h and then vortexed at maximal speed for 2 min to detach the tails (Ramalho-Pinto et al., 1974; Gold and Flescher, 2000). The latter were subsequently removed by adding 70% Percoll (Sigma, Oakville, Ontario, Canada) prepared in minimal essential medium MEM (Gibco, Invitrogen, Canada) followed by centrifugation at 1700 rpm for 10 min at 5 °C. The supernatant containing the tails was discarded and the remaining cercarial bodies were washed twice by repeated cycles of centrifugation (1200 rpm/10 min) and resuspension in MEM supplemented with 1 mg/ml streptomycin (Sigma) and 1000 U/ml penicillin (Sigma) and 0.25 μg/ml Fungizone (Invitrogen, Canada). The bodies were then gradually transferred to RPMI 1640 growth medium (Gibco) through three constitutive washes in a solution containing the same antibiotics as above and a mixture of MEM + − RPMI 1640 at increasing ratios of 3:1, 1:1 and 1:3 v/v. Finally, the cercarial bodies were washed once in RPMI 1640 supplemented with antibiotics and transferred into RPMI 1640 supplemented with 10% heat-inactivated Fetal Bovine Serum FBS (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml Fungizone. The transformed schistosomula were cultured in 24-well plates in a humidified incubator set at 5% $CO_2$/37 °C and supplemented with fresh media every 3–5 days. Animals could be maintained under these conditions for up to 6 weeks with no apparent loss of viability. To obtain adult worms, 28-day old CD1 female mice were infected with freshly shed cercariae (≈150 cercariae/mouse) by active penetration through the skin. Approximately 7–8 weeks postinfection the mice were sacrificed and the adult worms were collected by perfusion of the livers and mesenteric veins (Smithers and Terry, 1965; Carneiro and Lopes, 1986). Miracidia were hatched from eggs collected from the mouse livers approximately 7-week post-infection and transformed into mother sporocysts according to previously described methods (Yoshino and Laursen, 1995). Sporocysts were maintained at 26 °C for 4- or 20-days in complete SM culture medium (Ivanchenko et al., 1999) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 50 μg/ml streptomycin.

### 2.2. Production of a polyclonal anti-SmGPCR antibody

A fraction of the third intracellular loop (il3) of SmGPCR (Accession # AF031196, pos. 1330–1701) was amplified by PCR using primers (sense 5′-*CCGAATTC*ATGCCCGAACCAACAGA-3′ and antisense 5′-*TAGCGGCCGC*TGCAGTTTTTTGTTC-3′) designed to incorporate EcoRI and NotI sites (underlined) at the 5′ and 3′ ends, respectively. The PCR product was cloned between the EcoRI and NotI sites of prokaryotic expression vector pET30a (Novagen, EMD Biosciences, San Diego, CA, USA) and expressed in *Escherichia coli* as an N-terminal 6× Histidine-tagged protein. The resulting SmGPCR-il3 recombinant protein was purified by metal chelation chromatography under denaturing conditions, using a commercial His.Bind kit (Novagen, EMD Biosciences, USA). The purification was verified by SDS–PAGE electrophoresis and Western blot analysis targeting the hexa-histidine tag, according to standard protocols.

To produce the antibody we injected two female adult albino rabbits subcutaneously with 1 ml (2 mg) of the purified His-tagged SmGPCR-il3 mixed in 1:1 ratio with Freund's complete adjuvant (Sigma). Two boosters were done at 2-week intervals with 0.5 ml (1 mg) of the purified recombinant protein and 0.5 ml of incomplete Freund's adjuvant. The animals were sacrificed 2 weeks after the third injection and the serum was isolated. Pre-immune serum was obtained from each rabbit prior to injection of antigen. The antiserum was first evaluated by ELISA against the purified SmGPCR-il3 antigen and the titer was determined to be 1:50,000. The IgG fraction was subsequently purified by protein A sepharose affinity chromatography (Sigma, Canada), dialyzed against PBS, pH 7.4, and the protein concentration was adjusted to 5 mg/ml.

### 2.3. IFA-confocal microscopy of SmGPCR-transfected cells

The anti-SmGPCR-il3 antibody was initially tested in HEK293 (EBNA1) cells that were stably transfected with a codon-optimized SmGPCR cDNA (Hamdan et al., 2002b) tagged to a green fluorescence protein (EYFP) at the C-terminal end (kindly provided by Dr. F. Hamdan, University of Montreal). Cells stably expressing FLAG-SmGPCR-EYFP or untransfected HEK293E cells ($0.5 \times 10^6$/well) were seeded in six well plates and cultured in DMEM containing 5% heat-inactivated FBS, 20 mM HEPES,10 μg/ml Zeocin and 0.25 μg/ml puromycin (Invitrogen, Burlington, Ontario, Canada). For immunofluorescence, the cells were fixed in cold (−20 °C) methanol for 5 min, washed in PBS and blocked in 5% goat serum and 0.5% Triton X-100 in PBS for 1 h at room temperature. Anti-SmGPCR was prepared in the same blocking solution (1:100 dilution) and added for an additional hour at room temperature. This was followed by three washes in PBS and then 1 h incubation with rhodamine-labeled anti-rabbit IgG secondary antibody (1:300 dilution in blocking buffer). Cells were counterstained with 4′,6- diamidino-2-phenylindole DAPI (Sigma, at 1:1000), mounted onto slides and examined using a BIO-RAD RADIANCE 2100 confocal laser scanning microscope equipped with Nikon E800 fluorescence microscope for confocal image acquisition and the LASERSHARP 2000 analyzing software package.

### 2.4. IFA-confocal microscopy of larval and adult stages of S. mansoni

Larval stages (miracidia, sporocysts and schistosomula) and *in vivo*-derived adult worms were washed twice in PBS, pH 7.4 and fixed in freshly prepared 4% paraformaldehyde (PFA; Sigma, Canada) in PBS at 4 °C for 4 h, using end-over-end rotation. The fixative was changed twice after 1 and 3 h of incubation. Following fixation, the worms were washed twice in PBS and treated with 0.1 M glycine for 5–10 min to reduce autofluorescence. Alternatively, a cold acetone fixative was used (Thors and Linder, 2003). The samples were subsequently incubated for 24 h at 4 °C in the same blocking permeabilizing solution described above and treated with anti-SmGPCR IgG (1:100 in blocking solution) for 3– 4

days at 4 °C with gentle rotation, as described previously (Mair et al., 2000, 2003). Animals were washed three times in PBS and incubated in fluorescein isothiocyanate (FITC)-labeled goat antirabbit IgG (Chemicon, Temecula, CA) (1:300 in blocking solution) for 3 days at 4 °C. When phalloidin (0.2 mg/ml) was used as a counter stain, 400 ng of tetramethylrhodamine B isothiocyanate (TRITC)-labeled phalloidin (Sigma, USA) was added 24 h after addition of the secondary antibody and the incubation was continued for two additional days at 4 °C. After washing, the samples were mounted with anti-quench mounting medium (Sigma) and examined by confocal microscopy as described above. The following controls were routinely used in these studies: (1) omission of primary antibody, (2) replacement of primary antibody with pre-immune serum, (3) replacement of primary antibody with an irrelevant IgG and (4) pre-adsorption with an excess of the purified SmGPCR-il3 antigen. The protocol for pre-adsorption was as described previously (Rosin et al., 1998; Coling and Kachar, 2001).

### 2.5. SDS–PAGE and Western blots

Analysis of recombinant SmGPCR were done in HEK293 (EBNA1) cells transiently transfected with an N-tagged FLAG.SmGPCR expression pCEP4 plasmid (Hamdan et al., 2002a). Cells ($10^6$/100 mm dish) were transiently transfected with 3 μg of pCEP4.-FLAG.SmGPCR or empty pCEP4 plasmid (control), using the transfection agent FuGene 6 (Roche, Canada), according to the manufacturer's recommendations. For membrane protein extraction, we followed the protocol of Uberti et al. (2005) with the following modifications: HEK cells were homogenized in PBS containing a protease inhibitor cocktail (1:100 dilution, Sigma) and the homogenate was spun at 30,000*g* for 20 min at 4 °C to isolate a crude membrane pellet. The pellet was solubilized in lysis buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 100 mM $NH_4SO_4$, 10% v/v glycerol and protease inhibitor cocktail) containing 2% CHAPS for 2 h at room temperature with gentle end-over-end rotation and then centrifuged at 16,000*g* for 10 min at 4 °C to remove insoluble material. Aliquots of the resulting supernatant (2.5–3 μg protein) were subsequently prepared in SDS–PAGE sample buffer containing 100 mM DTT and incubated at 37 °C for 20 min prior to loading onto a 4–12% Tris–Glycine precast gel (Invitrogen) for SDS–PAGE. Western blotting was done according to standard protocols, using either polyclonal rabbit anti-SmGPCR-IL3 (1:5000 dilution) or a monoclonal mouse anti- FLAG M2 antibody (1:5000 dilution) (Sigma, USA), followed by the appropriate horseradish peroxidase (HRP)- labeled secondary antibody (1:20,000 dilution). For analyses of schistosomal extracts, we homogenized 30–40 adult *S. mansoni* worms (mixed males and females) in the same 2% CHAPS buffer for 2 min on ice, using a handheld homogenizer. The resulting homogenates were similarly incubated for 2 h at room temperature with end-over-end rotation and centrifuged to remove insoluble material. Aliquots of the supernatant (5–6 μg protein)were subjected toSDS–PAGEand then Western blotted, as described above, using rabbit anti-SmGPCR (1:2500 dilution) and HRP-labeled goat anti-rabbit IgG as the secondary (1:20,000).

### 2.6. Immunoprecipitation of SmGPCR

Immunoprecipitations (IP) were performed with the Seize® Primary Immunoprecipitation kit (Pierce, USA), according to the specifications of the manufacturer. The IP affinity column was prepared first by coupling approximately 400 μg of purified SmGPCR IgG to AminoLink® Plus gel in the presence of sodium cyanoborohydride, followed by extensive washing, as described in the kit protocol. For the IP, a crude homogenate of adult *S. mansoni* was prepared as described above in the same homogenization buffer except that CHAPS was replaced with 1% Triton X-100. Aliquots of the crude extract were diluted with the "Bind" buffer supplied with the kit (1:4 v/v), mixed with the IgG-linked gel and incubated overnight at 4 °C with gentle rotation. After incubation, the gel was washed three to five times with a buffer containing 150 mM NaCl and 0.5% Triton X-100 and the bound

proteins were eluted under acidic conditions (pH 2.8). Unless otherwise specified, aliquots of the IP eluate were immediately neutralized topH≈7.4 by addition of 1 MTris–HCl, pH9.5 (final concentration 50 mM) and then prepared in standard Laemmli SDS–PAGE sample buffer (pH 6.8) supplemented with 100 mM DTT (Laemmli, 1970). The gel and Western blot analyses of IP eluates were performed as described above for the crude schistosomal extracts. For experiments testing the effects of pH, aliquots of the IP eluate were adjusted to the desired finalpHby addition of the appropriate buffer prior to SDS–PAGE. To test the effects of urea (4 M) and Triton X-100 (1%), the test substances were added to aliquots of the neutralized (pH 7.4) IP eluate at the indicated final concentrations. The samples were incubated for 15 min at room temperature after which they were prepared in reducing sample buffer and subjected to gel analysis and immunoblotting as above.

### 2.7. Quantitative PCR analyses

Total RNA was purified from *S. mansoni* cercariae, schistosomulae and adult worms, using RNeasy micro or mini kits (Qiagen, Mississauga, Ontario, Canada). The RNA was quantitated with a Nanodrop ND1000 spectrophotometer (Wilmington, USA) and equal amounts of RNA from the various developmental stages were used for reverse-transcription (RT). The RT was performed according to standard protocols in a 20 μl reaction volume containing purified total RNA (130–180 ng), 200 U M-MLV reverse transcriptase (Invitrogen), 40U RNaseOUT ribonuclease inhibitor (Invitrogen), 0.5 μM oligo (dT)[12–18] (or a gene-specific primer targeting SmGPCR positions 1282–1306: 5′-GAGATGTCAAAGAAAATTCTCTATC- 3′), 0.5 mM dNTPs and 10 mM DTT in 1× first strand buffer (Invitrogen). The real-time qPCR was carried out with the Platinum ® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen) in a final volume of 25 μl containing 2 μl of cDNA and 0.2 μM of each primer. The primers for qPCR were designed so as to amplify approximately 200 bp of either SmGPCR (Accession # AF031196) or *S. mansoni* GAPDH (Accession # M92359), which was used as a housekeeping gene for data normalization. The SmGPCR primer pair was: 5′-CATATTAAAGCGACACGTAAGC-3′ (sense) and 5′-TTG TGGTTGAGTAAACAACTCG-3′ (antisense) and the GAPDH primers were: 5′-GTTGATCTGACATGTAGGTTAG-3′ (sense) and 5′-ACTAAT TTCACGAAGTTGTTG-3′ (antisense). Standards consisting of various concentrations of plasmid pCIneo-SmGPCR were included in each experiment for subsequent quantitation of PCR data. The reactions were performed in a Rotor-Gene RG3000 instrument (Corbbett Research, Australia) and the cycling conditions were as follows: 50 °C/2 min, 95 °C/2 min followed by 45 cycles of 94 °C/15 s; 53 °C/30 s; 72 °C/30 s. For comparison of steady-state SmGPCR transcript levels between miracidia and 4-day/20-day *in vitro* cultured sporocysts a similar procedural approach as that described above was used with the following modifications: After cDNA synthesis, real-time qPCR amplifications were performed in 96-well formatted optical tube strips and caps using a GeneAmp5700 qPCR apparatus (Applied Biosystems, Foster City, CA). SYBR Green reaction mixtures were identical to those described previously (Boyle et al., 2003), with cycle conditions consisting of: 95 °C for 15 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. At the termination of all qPCR reactions, the generation of specific PCR products was confirmed by melting point dissociation curve analyses and DNA sequencing. Expression levels were determined according to the standard curve method (Bustin, 2000) and were normalized to the housekeeping genes GAPDH for cercarial, schistosomula and adult stages, and 18S rRNA for miracidia and sporocysts. The normalized data were then calculated as the fold-change in expression relative to the cercarial or miracidium stage, which was used as an arbitrary term of reference. To verify the quantification and for statistical analysis, the data were re-calculated by the comparative $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001; Cikos et al., 2007) with identical results. For graphic purposes, all qPCR data are represented as fold-difference relative to transcript levels found in the miracidial or cercarial stage.

### 2.8. Other methods

Protein content was measured with a Lowry assay, using a commercial kit (BioRad). Indirect ELISA was performed in 96-well plates coated with purified recombinant SmGPCR il3 protein (0.25–25 μg/ml) and incubated with a serial dilution (1:250,000– 1:50) of rabbit anti-SmGPCR antiserum or pre-immune serum, followed by incubation with a HRP-labeled secondary antibody (goat anti-rabbit IgG, 1:2000), according to standard protocols. Statistical comparisons of qPCR data were done with the Student *t*-test or a one-way ANOVA, followed by a Tukey pairwise comparison. A $P \leq 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Production of the anti-SmGPCR polyclonal antibody

A polyclonal antibody against SmGPCR was produced by immunizing rabbits with a purified fragment corresponding to the receptor's third intracellular loop (SmGPCR-il3, Fig. 1A). This region was selected because it is the most divergent among GPCRs and it has been used successfully to generate antibodies against other biogenic amine receptors (Levey et al., 1991; Rosin et al., 1993, 1998; Schiaffino et al., 1999; Zhou et al., 1999). A BLAST analysis of the SmGPCRil3 fragment against the *S. mansoni*genomedatabase foundnosignificant homology with other schistosome proteins, including other SmGPCR-like receptors. The antibody was first tested against the purified *E. coli*-expressed SmGPCR-il3 fragment by ELISA (not shown) and Western blot analysis (Fig. 1B). The Western blot identified a single band of the correct size, whereas no response could be seen with pre-immune serum or the pre-adsorbed antibody control, showing the interaction was specific. To test if the antibodywas suitable for immunofluorescence analyses, and to verify its specificity, weperformed a confocal analysis of a stably transfected cell line that expresses SmGPCR fused to a green fluorescent tag (EYFP) at the Cterminal end. The results (Fig. 1A and C) show that cells expressing SmGPCR-EYFP (green fluorescence signal) were also labeled by the anti- SmGPCR antibody (red fluorescence), as shown by co-localization of the two signals (yellow fluorescence overlay). No immunolabeling was detected either in untransfected cells (data not shown) or transfected cells treated with antibody pre-adsorbed with purified SmGPCR-il3 (Fig. 1C).

### 3.2. SmGPCR forms multiple species in transfected HEK293 cells and schistosomes

In subsequent studies we performed Western blots of the fulllength SmGPCR expressed in HEK293 cells (Fig. 2A) and the native protein in schistosomal extracts (Fig. 2B). A crude membrane fraction was prepared from cells expressing FLAG-tagged SmGPCR (Hamdan et al., 2002a) and then probed both with anti-SmGPCR and an anti-FLAG antibody. The anti-SmGPCR antibody consistently recognized two prominent bands, one corresponding to the size of the SmGPCR monomer (≈65 kDa) and a larger band at approximately twice that size (≈130 kDa). In addition, we detected a "smear" of very high molecular weight (>180 kDa) species at the top of the gel and, in some samples, a small presumed proteolytic fragment. It must be emphasized that these bands are all SmGPCRspecific, since they were also recognized by the anti-FLAG antibody and none could be seen in the "mock"-transfected control probed with either antibody (Fig. 2A). The Western blot analysis of *S. mansoni* extracts produced a similar pattern of immunoreactive proteins (Fig. 2B). The results show two bands at about 65 and 130 kDa, as well as a larger species that migrated just above the 180 kDa marker.

To verify these results, we immunoprecipitated the native receptor from schistosome extracts, using covalently attached anti- SmGPCR antibody beads. The protein was eluted from the beads under acidic conditions, neutralized and then tested by Western blotting with

anti-SmGPCR. A first test of the neutralized (pH ≈7.4) IP eluate confirmed the existence of multiple SmGPCR species, the monomer (65 kDa) and the dimer (130 kDa) bands being particularly prominent (Fig. 2C). Further analysis of the IP eluate revealed that the 130 kDa species was resistant to treatment with 100 mM DTT, 1% Triton X-100 and, surprisingly, 4 M urea (data not shown) but was sensitive to acidic pH (Fig. 2C). Analysis of the acidified eluate (pH ≈3) showed only one immunoreactive band corresponding to themonomer.Increasing thepHfrom 3 to 7.4 in a stepwise fashion caused progressive appearance of the 130 kDa band and a concomitant decrease in the intensity of the monomer, suggesting the two forms are interconvertible. Though originally described as monomeric, there is increasing evidence that some GPCRs form dimers and even larger oligomeric species that resist denaturation on SDS–PAGE gels (Zhu et al., 2005; Zanna et al., 2008). Our results suggest that SmGPCR exists both as a monomer and an exceptionally stable dimer of about 130 kDa. The dimer is resistant to common denaturing and reducing agents but is sensitive to pH, suggesting it is non-covalent in nature. A larger >180 kDa species could also be detected but the intensity of this band was weaker and more variable. It is unknown if the larger species is produced by non-specific aggregation of SmGPCR during SDS–PAGE, a common problem in gel analysis of integral membrane proteins, or if it represents an oligomeric form of the receptor.

### 3.3. Confocal immunofluorescence analysis of SmGPCR in S. mansoni

To determine the tissue localization of SmGPCR in different life cycle stages of *S. mansoni*, we probed miracidia, sporocysts, schistosomula and adult worms with the polyclonal anti-SmGPCR IgG, followed by a FITC-labeled secondary antibody. Animals were also treated with rhodamine-conjugated phalloidin to label cytoskeletal elements and muscle (Mair et al., 1998, 2000, 2003). Two different types of fixation protocols were tested, one that used cold acetone as a fixative (Avarzed et al., 1998; Guedes et al., 2002) and the standard 4% PFA protocol (Mair et al., 2000, 2003). Acetone fixation was found to provide more detailed structural information but it disrupts phalloidin binding to actin (Bernard-Trifilo et al., 2006) and therefore only those samples treated with PFA were counterstained with phalloidin. An analysis of the early larval stages revealed virtually no SmGPCR in miracidia, whereas sporocysts exhibited distinct green fluorescence localized particularly on and within the surface tegument and cells embedded in the parenchymal matrix. This was seen most clearly in 4-day old sporocysts (Fig. 3A and B) and could not be detected in any of the controls tested, including the pre-adsorbed antibody control (Fig. 3C and D). Schistosomulae exhibited strong and surprisingly widespread SmGPCR fluorescence staining in the tegument, acetabulum, anterior muscle cone, parenchyma and, less consistently, within the esophagus (Fig. 3E and G). To test whether SmGPCR was associated with muscle, we repeated the experiment in schistosomulae that were counterstained with TRITC-conjugated phalloidin. Intense phalloidin staining was clearly seen in the schistosomula mainly as striated muscle bands (Fig. 3I). An overlay of SmGPCR fluorescence (green) with phalloidin (red) produced regions of intense yellow fluorescence, suggesting that SmGPCR co-localizes with muscle. The strongest area of co-localization was in the musculature of the anterior cone, the acetabulum and the subtegumental musculature (Fig. 3I–K). Finally, in adult *S. mansoni*, SmGPCR was widely expressed in the tubercles of the male tegument (Fig. 4A, C and D). There was no comparable pattern of fluorescence in any of the male controls tested (Fig. 4B). Counterstaining with TRITC-conjugated phalloidin revealed a surface pattern of green fluorescent tubercles surrounded by red spines, with no apparent co-localization. Aside from the tubercles, significant SmGPCR fluorescence was detected in the subtegumental musculature, particularly in the anterior end and head region (Fig. 4E). Female worms exhibited strong fluorescence in the reproductive organs (not shown) but this was presumed to be an artifact caused by autofluorescence, since it could also be seen in the negative controls.

### 3.4. Quantitative RT-PCR analysis of SmGPCR mRNA expression

The confocal IFA analysis detected SmGPCR in all parasitic stages tested, including sporocysts, schistosomula and adults, but not miracidia. We also noted that the extent of immunolabeling varied among the parasitic stages, with the schistosomula showing widespread fluorescence, whereas in the adults the receptor was more restricted to the tegument and subtegumental region. This led us to question whether SmGPCR expression might be developmentally regulated in *S. mansoni*. To address this question, we started by comparing the level of SmGPCR protein in different stages of *S. mansoni* by means of Western blot analyses. We detected the monomer and dimer in all the stages tested (cercariae, schistosomula and adults) but the analysis was not sufficiently quantitative to assess differences in expression among these stages. As an alternative strategy, we measured expression at the mRNA level, using real-time quantitative RT-PCR. Expression levels were compared first in *S. mansoni* cercariae, schistosomula and adult worms. In a separate experiment SmGPCR transcript levels were compared between miracidia and *in vitro* cultured sporocysts. The qPCR data were standardized relative to housekeeping genes (GAPDH or 18S rRNA) and the differences in expression were calculated, using standard curve and/or comparative $\Delta^{Ct}$ methods (Bustin, 2000; Cikos et al., 2007; Livak and Schmittgen, 2001), with similar results. Results of the analysis of the mammalian host stages suggest that SmGPCR is upregulated immediately after cercarial transformation (Fig. 5A). We detected a strong ≈10-fold increase ($P < 0.01$) in SmGPCR expression in newly transformed schistosomulae (S0) compared to cercariae. SmGPCR levels increased further at 7 days post-transformation ($P < 0.0001$) and then returned to S0 level in older (14-day) schistosomula and the adult worms. To test if SmGPCR is upregulated in the snail parasitic stage, we performed a second comparative RT-qPCR analysis of *S. mansoni* sporocysts and miracidia. The results showed that SmGPCR mRNA levels were increased 20-fold in 4-day old sporocysts ($P < 0.01$) and greater than 200-fold in 20-day old sporocysts ($P < 0.001$) compared to miracidia (Fig. 5B).

## 4. Discussion

Nearly 50 years after the first discovery of biogenic amines in schistosomes, very little is known about their mode of action. One of difficulties is the continuing lack of molecular information about amine receptors in any of the parasitic flatworms. Aside from the prototype, rhodopsin (Hoffmann et al., 2001) and SmGPCR (Hamdan et al., 2002a), only one other member of this receptor superfamily has yet been cloned from these animals (Pearson et al., 2007). SmGPCR was previously shown to be activated by HA when expressed in mammalian cells (Hamdan et al., 2002a). The response to HA was dose-dependent and produced an intracellular calcium response, suggesting this receptor may be coupled to Gq and the $Ca^{2+}$/phosphoinositol signaling pathway. Importantly, the receptor could not be activated by any of the other known biogenic amine transmitters, suggesting it is selective for HA. We cannot, however, rule out the possibility that there may be an unknown, structurally related amine in schistosomes that can activate the receptor *in vivo*. SmGPCR is unusual in that it shows about the same level of homology with all different types of biogenic amine receptors, including the histaminergic receptors, and so cannot be identified by sequence analysis. The most surprising feature of this receptor is that its predicted binding pocket lacks a highly conserved TM3 aspartate ($Asp^{3.32}$), which is replaced with an asparagine. $Asp^{3.32}$ is believed to be directly involved in the binding of biogenic amines and is present in every other aminergic GPCR cloned to date, both vertebrate and invertebrate (Roth and Kristiansen, 2004; Roth, 2006). In SmGPCR, however, the Asp → $Asn^{3.32}$ substitution does not hinder HA-induced activity (Hamdan et al., 2002a), suggesting the conformation of the binding pocket is quite different in this receptor. Following the completion of the *S. mansoni* genome project, we can see at least two closely related homologues of SmGPCR in the SchistoDB genome database, both of

which have the same unique Asp → Asn$^{3.32}$ substitution in TM3. This raises the possibility that SmGPCR is part of a cluster of amine receptors that diverged early in evolution and may be unique to the parasites. It remains to be determined if these other SmGPCR-like sequences also encode functional receptors and if they are similarly activated by HA.

To further characterize this receptor, we began by raising polyclonal antibodies to a portion of the third intracellular loop (il3) of SmGPCR. This is the most divergent region among GPCRs and as such is often targeted for antibody production. A first evaluation based on ELISA, *in situ* immunofluorescence and Western blotting indicated that the antibody was of high titer and could recognize the full-length 65 kDa protein both in transfected HEK293 cells and schistosomal extracts. We noted, however, that the antibody also recognized a band of about 130 kDa as well as a mixture of high MW species that migrated at the top of the gel. To assess the specificity of the signal, we used a FLAG-tagged SmGPCR expressed in HEK293 cells and repeated the Western analysis with a different antibody targeting the FLAG epitope. The results showed virtually the same pattern of immunoreactive species with both anti-SmGPCR and anti-FLAG antibodies, whereas no signal could be detected in the mock-transfected cells, cells treated with pre-immune serum or the il3-preadsorbed antibody control. Thus we concluded that the multiple bands are all derived from SmGPCR and represented different forms or aggregates of the receptor. There are many reports of GPCRs forming dimers and higher order species that resist denaturation on SDS–PAGE gels (Bai et al., 1998; Bouvier, 2001; Romano et al., 1996; Romano et al., 2001; Balasubramanian et al., 2004; New et al., 2006). The higher MW species could be artifacts caused by membrane solubilization and aggregation of the receptor in the SDS-rich environment of the gel, a common problem in studies of membrane proteins. GPCR dimers, on the other hand, appear to be physiologically relevant. GPCR dimerization has been reported to play an important role in the regulation of ligand specificity, binding affinity and conformational activation (Bouvier, 2001). The exact chemical nature of these dimers is not known but there is increasing evidence they can involve intermolecular disulfide linkages, non-covalent interactions, or both (Hebert et al., 1996; Bai et al., 1998; Romano et al., 1996; Romano et al., 2001; Franco et al., 2007; Dalrymple et al., 2008). The 130 kDa species described here is most likely a non-covalent dimer of SmGPCR. This is suggested by the fact that it is resistant to reducing agents such as DTT but can be made to dissociate at acidic pH. That it was seen consistently in preparations of recombinant and native receptor suggests the dimer is a biologically relevant species. The higher MW forms are harder to interpret at this point. Their large size, variable intensity and the fact they were more abundant in transfected cells, where the receptor is overexpressed, all suggest these are SDS-induced aggregates that formed during the gel analysis. We cannot rule out other explanations, however, including the possibility of receptor oligomerization and/or heavy glycosylation producing these larger forms of the receptor.

The anti-SmGPCR antibody was subsequently used to investigate the developmental stage-specific expression and tissue distribution of the receptor in *S. mansoni*. HA has been implicated in the control of neuromuscular function in these animals (see Ribeiro et al., 2005) and therefore we had expected SmGPCR to be associated with neuronal and/or muscle structures that are innervated by HA-containing neurons. It, therefore, was surprising to find that SmGPCR immunoreactivity was found predominantly in the “parasitic” stages (sporocysts, schistosomulae and adults), compared to the free-swimming miracidial and cercarial larval forms. This finding was corroborated by SmGPCR gene expression data demonstrating significant receptor gene upregulation in parasitic vs. freeswimming stages, suggesting a critical role of SmGPCR in the establishment and/or maintenance of parasitism within their hosts. To date, however, that role remains unclear. Histaminergic neurons have proven difficult to visualize in *S. mansoni* but in another trematode (*H. cylindracea*) they were shown to innervate the musculature of several tissues, including the body wall muscles

and the acetabulum, as well as the cerebral ganglia and major nerve cords of the CNS (Eriksson et al., 1996). The pattern of HA innervation in the musculature correlates well with that of SmGPCR described here. The results showed significant co-localization of SmGPCR immunofluorescence and phalloidin in the subtegumental musculature, particularly the outer layer beneath the tegument, both in schistosomula and adult worms. In the schistosomula, we also detected significant expression in the musculature of the suckers, in particular the acetabulum. These results are consistent with a role for SmGPCR in neuromuscular transmission or modulation. That the receptor is expressed in the body wall musculature further suggests that SmGPCR mediates at least some of the effects of HA on schistosome motility. One obvious difference between the tissue distribution of this receptor and the previously described pattern of HA innervation is in the CNS, which was shown to be rich in HA fibers (Eriksson et al., 1996) and yet did not express SmGPCR. If the same neuronal architecture exists in schistosomes, this suggests that there may be other HA receptors that mediate neuronal signaling within the CNS.

Aside from the musculature, the most conspicuous site of SmGPCR expression was the tegument. We detected consistent immunoreactivity in the tegument of all parasitic stages tested, including sporocysts, schistosomula and the adult worms. Adult males, in particular, showed very robust expression in the tubercles of the outer tegument. The discovery of a signal transducing receptor on the surface of schistosomes is surprising but not unprecedented. In recent years, researchers have identified an acetylcholine (nicotinic) receptor (Camacho et al., 1995; Bentley et al., 2004), receptor tyrosine kinases (Davies et al., 1998; Forrester et al., 2004; Osman et al., 2006) and at least one other GPCR of unknown function (Pearson et al., 2007) in the tegument of *S. mansoni* or *S. hematobium*. Being situated on the surface, these receptors are not likely to be part of an endogenous signaling system. Rather, they are believed to be activated by exogenous signals and to mediate some form of host–parasite communication. The presence of SmGPCR on the surface suggests that it too is activated by an exogenous substance, presumably HA or a HA-like substance that is present in host blood. There is evidence that the parasite stimulates HA production to facilitate passage through blood vessels during the initial migration (Catto et al., 1980; Boros, 1989; Rao et al., 2002). A tegumental HA receptor may be part of a system that allows the parasite to respond to a change in environmental HA, for example by increasing motility or some other unknown behavior. Given that SmGPCR is clustered in the tubercles, which are enriched in sensory nerve endings (Gustafsson, 1987), this receptor could be acting through chemosensory circuits that originate at the parasite surface. The neuronal processes that supply the tubercles connect to peripheral elements and ultimately the CNS, so that any signaling through these circuits could have profound effects on worm behavior.

These results raise new questions about the role of HA in *S. mansoni*. Since it was first described nearly four decades ago, there have been conflicting reports about the biological relevance of HA in these parasites. On the one hand, the tissue level of HA in *S. mansoni* appears to be low, lower than that of other parasitic platyhelminths (Schwabe and Kilejian 1968; Perez-Keep and Payares 1978; Eriksson et al., 1996). This has made it difficult to visualize histaminergic neurons *in situ*. On the other hand, there is evidence that HA has effects on parasite motility (Ercoli et al., 1985; Ribeiro et al., 2005) and the existence of SmGPCR suggests these are receptor- mediated effects. The present results reinforce the notion that HA signaling is important in schistosomes. Based on the distribution of SmGPCR, we suggest there may be two HA systems operating in these animals, an endogenous system that controls primarily the musculature, and an exogenous one located on the tegument that is probably activated by host-derived amine. The data also suggest these systems are differentially expressed during the course of the life cycle. Sporocysts show robust SmGPCR expression both at the RNA and protein levels and the receptor

appears to be localized mainly to the tegument and its surface. However, we could not detect evidence of association with muscle fibers at this stage. In contrast the schistosomula and adult worms showed a more widespread distribution that included the musculature and other tissues, in addition to the tegument. These observations reinforce the previous hypothesis of a dual system of HA signaling involving direct receptor communication through the tegument (perhaps with exogenous or environmental HA) and a neuronal receptor-HA interactions involving regulation of motility, muscle activity and the like.

As mentioned previously, SmGPCR was found to be more widely expressed in the parasitic sporocyst and young schistosomula than other stages, including adult worms. At the RNA level, we found that SmGPCR was upregulated several fold in day 7 schistosomula compared to older larvae (14 days) or adults. The timing of this upregulation suggests that HA signaling could be particularly important during initial larval development and the lung stage, which occurs roughly at 7–8 days post-infection. The most striking upregulation was seen in sporocysts. SmGPCR expression levels increased >200-fold in 20-day old *in vitro* cultured sporocysts compared to miracidia. At this time, the sporocysts have increased in length approximately two- to threefold and contain numerous embryonic cell masses, which are destined to become the next generation of motile daughter sporocysts (Yoshino and Laursen, 1995). The coincidence of this increased growth and cellular differentiation capacity with SmGPCR expression strongly suggests a possible regulatory role of this receptor in sporocyst growth and development. HA in sporocysts may be obtained through endogenous synthesis or more likely, may be contributed by the snail host itself. The freshwater gastropod, *Lymnaea stagnalis*, possesses abundant central and peripheral histaminergic neurons (Hegedus et al., 2004) that could serve as sources of free HA in innervated tissues or fluid spaces, as has been suggested for other biogenic amines (e.g., 5HT, dopamine) found in *B. glabrata*, intermediate host of *S. mansoni* (Manger et al., 1996; Boyle and Yoshino, 2002). Unfortunately, initial attempts to silence SmGPCR expression by RNAi have not been successful either in sporocysts or schistosomula (data not shown), even under conditions known to effectively silence other schistosome targets (Boyle et al., 2003; Nabhan et al., 2007). Thus the biological relevance of this upregulation remains unclear. More research is needed to elucidate the function of HA signaling and SmGPCR in these parasites.

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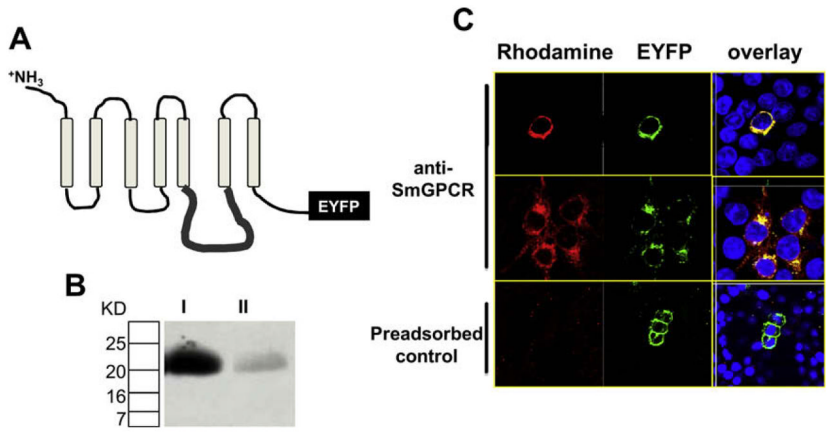


**Fig. 1.**

Production of an anti-SmGPCR polyclonal antibody. A portion of the third intracellular loop (il3) of SmGPCR (pos. 355–449) was expressed in *E. coli* as a recombinant His-tagged protein and purified to homogeneity prior to injecting into rabbits to produce anti-SmGPCR antibody (A) Schematic representation of SmGPCR showing a typical seven transmembrane topology and the il3 region targeted for antibody production. (B) Anti-SmGPCR antibody (lane I) or antibody that was pre-adsorbed with an excess of purified il3 antigen (Lane II) were tested against the purified il3 protein by Western blot analysis. The results show a strong, single immunoreactive band of the correct size in samples probed with anti-SmGPCR IgG but only a weak band in the pre-adsorbed control. (C) The antibody was tested first in HEK293E cells that were stably transfected with SmGPCR fused at the C-terminal end to EYFP. Cells were incubated with anti-SmGPCR antibody followed by a rhodamine-conjugated secondary antibody. An overlay of rhodamine (red) and EYFP (green) produced bright yellow fluorescence, indicating co-localization of the two signals. No rhodamine fluorescence could be detected in cells incubated with pre-immune serum (not shown) or anti-SmGPCR antibody that was pre-adsorbed with purified il3 antigen (pre-adsorbed control) Cells were counterstained with Dapi (blue) to visualize the nucleus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

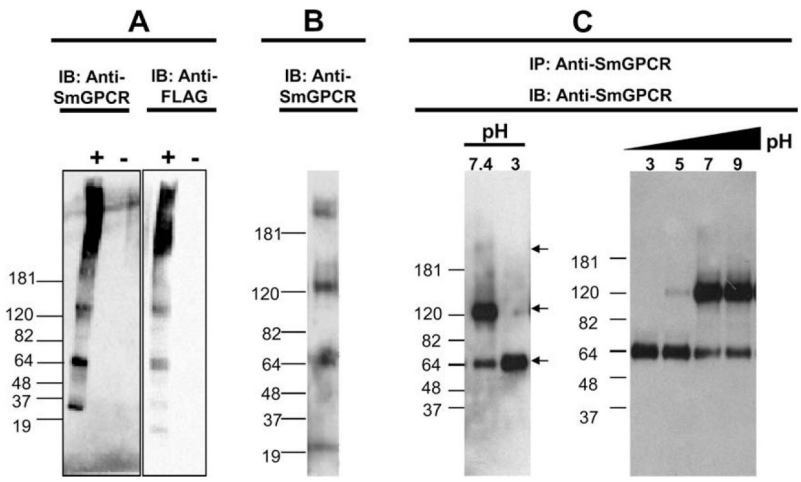


**Fig. 2.**

Western blot and immunoprecipitation analyses of SmGPCR. (A) HEK293E cells were transfected with a FLAG-tagged SmGPCR expressing vector (+) or empty vector (−). Cells were homogenized and aliquots of a solubilized crude membrane fraction were immunoblotted either with anti-SmGPCR IgG or with a monoclonal anti-FLAG antibody. The sizes of the protein ladder are indicated. (B) A representative Western blot analysis of an adult *S. mansoni* extract probed with anti-SmGPCR antibody. (C) SmGPCR was immunoprecipitated from a crude extract of *S. mansoni*, using beads covalently coupled to anti-SmGPCR IgG and then immunoblotted with the same antibody. The receptor was eluted from the antibody beads under acidic conditions and immediately neutralized to pH 7.5. At neutral pH we see three bands corresponding to the monomer (65 kDa), dimer (130 kDa) and a faint high MW species (>180 kDa) but only the monomer can be detected when the sampled was acidified to pH 3 prior to immunoblotting (left panel). A stepwise increase in pH (pH 3–9) caused progressive dimerization of the receptor (right panel). IB, immunoblotted; IP, immunoprecipitated.

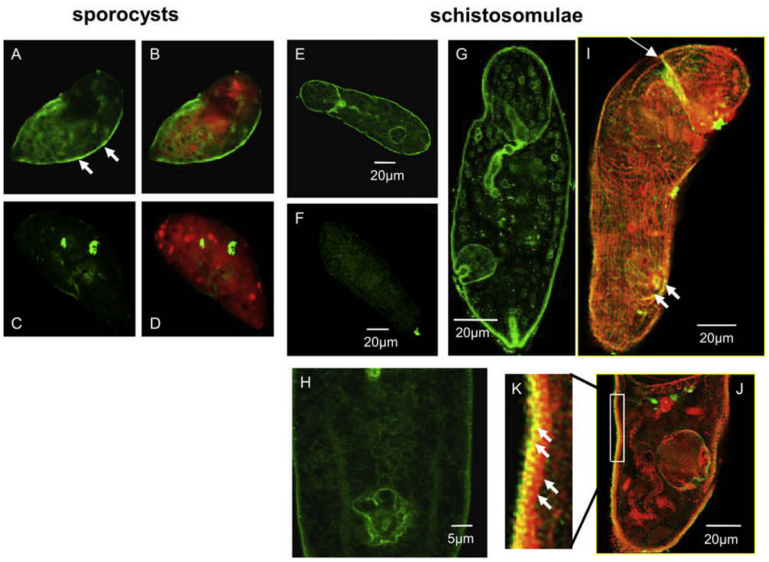


**Fig. 3.**

Localization of SmGPCR in larval stages of *S. mansoni*. Samples were fixed with 4% PFA or ice-cold acetone, permeabilized with 0.5% Triton X-100 and then probed with anti-SmGPCR IgG followed by a FITC-labeled 2ry antibody. Red TRITC-phalloidin was used as a counterstain to visualize the musculature. Sporocysts: SmGPCR immunofluorescence (green) was detected on the tegument (arrows) and within the parenchyma of 4-day-old *in vitro* cultured sporocysts incubated with anti-SmGPCR (A and B) but not the pre-adsorbed anti-SmGPCR IgG control (C and D). An overlay of the phalloidin (red) and SmGPCR (green) signals showed no apparent co-localization (B). Schistosomula: *In vitro* transformed schistosomula were cultured for 4 days (E) or 8 days (G) and then probed with anti-SmGPCR antibody. Fluorescence can be seen in the acetabulum, the tegumental region, the esophagus and parenchyma cells. A close-up of the posterior end of a 8-day animal shows a distinct pattern of SmGPCR fluorescence in the acetabulum (H). No significant immunoreactivity could be detected in the negative controls treated with il3-preadsorbed antiserum (F), pre-immune serum or when the primary antibody was omitted (not shown). 28-day-old schistosomula were probed with anti-SmGPCR antibody (green) and phalloidin (red) to test for possible colocalization of the receptor with the musculature. Red phalloidin labeling of the major longitudinal, circular and oblique body wall muscles is clearly visible (I). The overlay identified significant co-localization (yellow fluorescence) in the muscle cone of the head region (arrow), musculature of the acetabulum (arrow head) and the subtegumental musculature (box) (I and J). A close-up of the parasite body wall shows co-localization of SmGPCR and phalloidin in the outer layer of the musculature (yellow fluorescence) (K).

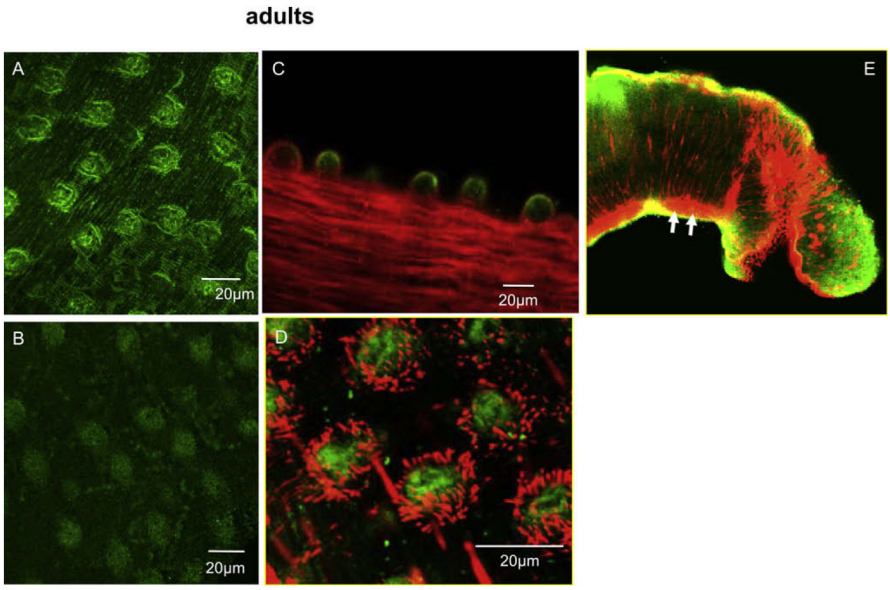


**Fig. 4.**

Localization of SmGPCR in adult worms. Adult male *S. mansoni* were probed with anti-SmGPCR IgG or a pre-adsorbed anti-SmGPCR IgG control, followed by green FITC labeled secondary antibody. Red TRITC labeled phalloidin was used to label the muscles and the tegumental spines. Animals incubated with antiserum show strong immunoreactivity in the tubercles of the dorsal tegument (A and C), whereas only background fluorescence could be detected in the negative control (B). Co-labeling with anti-SmGPCR and phalloidin produced a distinctive pattern of green immunoreactive tubercles surrounded by red phalloidin–labeled tegumental spines (D). Co-localization of SmGPCR with phalloidin was detected in the subtegumental musculature, particularly near the anterior head region (E, yellow fluorescence).

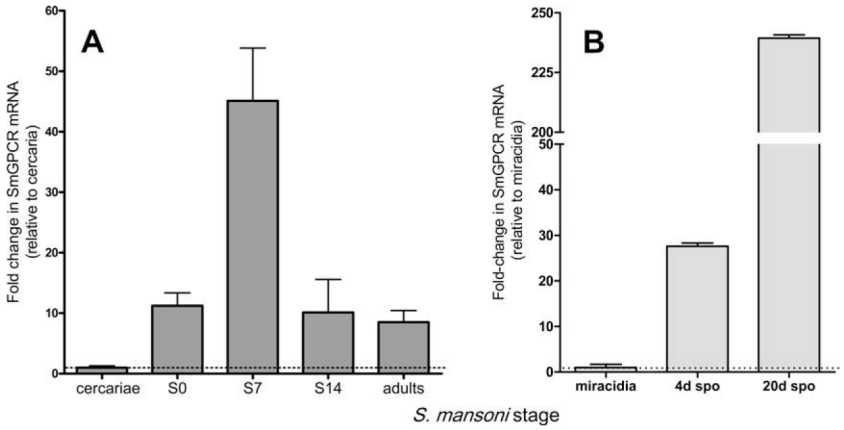


**Fig. 5.**

Developmental expression of SmGPCR in *S. mansoni*. Quantitative PCR was performed on reverse-transcribed RNA from *S. mansoni* cercariae, adult worms and *in vitro* transformed schistosomula harvested immediately after transformation (stage 0, S0) and at 7 days (S7) or 14 days (S14) post-transformation (A) and from miracidia and 4- day and 20-day cultured sporocysts (B). The qPCR data were standardized by simultaneous amplification of internal housekeeping controls (GAPDH or 18S rRNA) and differences in expression data were calculated according to the comparative $\Delta\Delta C_T$ method. The results are shown as the fold-change in SmGPCR expression relative to the cercariae (A) or miracidia (B) and are the means ± SEM of a minimum of three experiments, each in triplicates. S, schistosomula; spo, sporocyst.