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Deorphanization of biogenic amine-responsive G protein-coupled receptors in various model protozoa and platyhelminthes

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Deorphanization of biogenic amine-responsive G protein-coupled receptors in various model protozoa and platyhelminthes

By

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DEDICATION

This dissertation is dedicated to three people who have been my motivation and have shared with me the risks and sacrifices required to complete it; to the memory of my Dad, Mr. Martin D. Y. Agbedanu, who has shown me the joy of intellectual pursuit; to my mum, Mrs. Agnes Agbedanu, who has always been there to pack my bags with foodstuff during all back to school times; made a lot of personal sacrifices that I remember vividly; to my wife, Mrs. Bashiratu Agbedanu, who has been prayerful and supportive in these times and has been the source of inner peace while completing this dissertation.

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sometimes. This dissertation wouldn't have been possible without you all and I am greatly indebted to you than you know.

I remain grateful to my late Dad, Mr. Martin D.Y. Agbedanu, for raising me up so I could stand on mountains. To my Mum, Mrs. Agnes Agbedanu, my Dear sister, Elssie and the Husband, Mr. Jean D. Kpeli and the family at large, thank you for being there in the midst of storm. To my wife, Mrs. Agbedanu, I say a very big thank you for being there all these while. Finally, to all and sundry whose efforts in one way or the other contributed to making this project a success, I say a very big thank you and I apologize I am not able to acknowledge one by one.

CHAPTER 1 GENERAL INTRODUCTION

This dissertation explores the relevance of G protein-coupled receptors in disease causing protozoans and free living flatworms. It explored the biological importance of a catecholamine responsive GPCR in *Tetrahymena* and the physiological importance of serotonergic receptors in the locomotory events of two species of flatworms; *Girardia tigrina* and *Schmidtea mediterranea*. During the preliminary studies, a protozoan GPCR was obtained by genome mining, cloned and heterologously expressed in a yeast expression system, subjected to agonist treatment, and hence, de-orphanized. The serotonin GPCRs in both *Schmidtea mediterranea and Girardia tigrina* were cloned into Gateway vector with which double stranded RNA production was induced in an RNAse III deficient bacterial strain for RNAi studies. Overall, the work has two main goals.

First, a protozoan disease such as tetrahymenosis is an important ailment that affects aquatic life and consequently, Humans. The biology of *Tetrahymena* catecholamine responsiveness and phagocytosis provides a clue to the regulation of *Tetrahymena* survival. Second, the existence of homologues of most flatworm genes in very important Human parasites as well as Humans, explains the rationale behind the study of these cell surface receptors in the phyla Platyhelminthes. A common denominator to these two phyla is their large dependence on monoamines. The future goal is to eventually explore if these gene homologs have similar and exploitable functional roles in higher and parasitic organisms. The entire work is organized into 4 chapters.

1.1   Dissertation organization

Chapter 1 gives the general overview of G protein-coupled receptors (GPCRs) and the families therein, the essential features of GPCRs and their mechanism of activation; a brief description of the various families constituting the GPCRs superfamily, and techniques required in rescuing the supposedly 'perishing' orphan GPCRs. It continues to describe the genesis of an alternative loss-of-function technique that was originally discovered accidentally in petunias and culminating in the streamlining of the mechanism of RNAi. Chapter 2 enumerates the step-by-step processes involved in the deorphanization of a catecholamine-responsive protozoan GPCR involved in bacterial engulfment, dubbed, TetEPI-1.

Chapter 3 describes the characterization of serotonergic GPCRs mediating motility in *Giraria tigrina* and *Schmidtea mediterranea* in a reverse pharmacological process employing an established alternative loss-of-function (small interfering RNA) technique. It identifies the G protein-coupling profiles of the serotonergic receptors by monitoring forskolin-stimulated adenylate cyclase/cAMP levels as important messengers involved in GPCR signaling in response to ligand stimulation. Chapter 4 generally narrows down the main findings of this work and concludes by outlining the big picture of the principles of deorphanization of biogenic amine-sensitive GPCRs mediating planarian motility and protozoan phagocytosis using heterologous expression, alternative-loss-of function technique coupled with transductional coupling determination, and the monitoring of phenotypes.

Appendix A and B constitute two manuscripts to which I have contributed; the first describes the optimization and the establishment of an alternative loss-of-function protocol for the deorphanization of putative GPCRs; the second describes the heterologous expression-based

deorphanization of a protozoan GPCR, EhGPCR-1, relevant to phagocytic pathways of pathogenic *E. histolytica*. These two appendices provide a substantial scope of two established means of deorphanization of GPCRs. Appendix C, D, E and F constitute RNAi protocols, optimized cAMP determination protocols, double T7 dsRNA inducible vector map and target sequences, respectively.

1.2   G protein-coupled receptors defined

GPCRs are also known as heptahelical receptors, serpentine receptors, G protein-linked receptors (GPLR) or 7-transmembrane (7TM) domain receptors. GPCRs are cell surface receptors which serve in the transduction of extracellular stimuli into intracellular signals, *i.e.*, they serve as intermediaries between extracellular stimuli and intracellular signals/mechanisms. They mediate senses such as vision, smell, taste, and pain (Trabanino et al., 2004) and are indispensable among membrane proteins because they constitute the largest and most diverse groups of receptor proteins (Teller et al., 2001). These transmembrane proteins are essential for all multicellular eukaryotes.

In humans, >1000 of 22,000 genes code for GPCRs (Fredriksson and Schiöth, 2005), most of which influence embryogenesis and development by the reception of signals from hormones, neurotransmitters and environmental cues to different magnitudes hence, different degrees of physiological differences observed in infant development (Latronico and Hochberg, 2010). In mice, 392 genes code for GPCRs (Demetrios et al., 2003) and the lack of the G protein-coupling protein, $G\alpha_{13}$, results in embryonic lethality at mid-gestation (Wettschureck and Offermanns, 2005). In colonies of the budding yeast, *Saccharomyces cerevisiae*, 3 of its 5,900 genes encode GPCRs (Overton et al., 2005). The functional stimulation of these 7-transmembrane proteins in yeast is

coupled to their pheromone-response pathway, a well exploited system for ligand identification and characterization of receptor pharmacology and signal transduction (Dowell and Brown, 2009).

Even though they constitute just 3–4% (Schoneberg et al., 2002) of the human genome, defects in GPCR genes contribute to an array of diseases such as allergies, asthma, anxiety, congestive heart failure, glaucoma, heartburn, hypertension, migraine, Parkinson's, psychosis, schizophrenia, ulcers, etc. (Wilson and Bergsma, 2000). It is therefore, evident that GPCRs represent one of the most important families of drug targets. With the establishment of their importance as targets of 30-50% of drugs, their search has been on the increase and even though a number of them are currently identified and paired with known ligands, most still exist with no known ligands.

GPCRs to which there are no known ligands are described as orphan GPCRs (oGPCRs). It has been demonstrated that oGPCRs: may have relevant ligand-independent functions; can modulate the function of GPCRs with known ligands; serve as transporters of neurotransmitter via physical association with these molecules, a phenomenon termed constitutive activity of orphan 7TM proteins (Levoye and Jockers, 2008) discussed later in this chapter. Despite these revelations, the most pursued goal has been to deorphanize these oGPCRs considering the complex nature of investigation of ligand independent functions.

1.2.1 GPCR structure

Structurally, GPCRs have seven transmembrane α-helices forming the TM core. Transmembrane domains are linked by six loops of varying length (Palczewski et al., 2000); three exo-loops and three cyto-loops as shown in Fig. 1.1. GPCRs also bear an extracellular amino-terminal segment and an intracellular carboxy-terminal segment, a topology predicted based on hydropathy profiles and the crystal structure of the class I GPCR, visual pigment rhodopsin (Palczewski et al., 2000). Palmitoylation of the carboxy-terminal segment at a cysteine residue results in the formation of a fourth cytoplasmic loop (Ali et al., 1997). While there is no common ligand binding site, the hydrophobic core formed by the transmembrane provides localized binding sites for small molecular weight ligands, with peptides and protein ligands binding preferentially to N-terminus and the extracellular hydrophobic loops (Gether and Kobilka, 1998). The structure allows glycosylation (at the N-terminus) and phosphorylation at the C-terminal segment relevant for desensitization (Lefkowitz, 1998) and also provides appropriate site of contact for G proteins at the second and third cytoplasmic loops including the C terminus (Gether and Kobilka, 1998).

A TM of 24 in the case of ion channels, results in the selective exclusion of ions greater than 5 Å (Ji et al., 1998) due to the compact nature of the TMs. The TMs are linked extensively with hydrogen bonds (Pebay-Peyroula et al., 1997; Sealfon et al., 1997). There is the view that TM 1, 4 and 7 are more exposed to the lipid bilayer, hence, more hydrophobic compared to the others (Ji et al., 1998). The first two exo-loops are known to contain two conserved Cys residues linked by disulfide bonds in bovine rhodopsin (Ji et al., 1998), for example. Disulfide bonds are historically known for their protective role: promote folding and stability of some proteins especially, proteins secreted to the

extracellular medium (Darby and Creighton, 1995). The existence of the strong disulfide bond, a covalent bond within the structure, partly explains the fixed nature of the bulk of these receptors.

| | | 138 | | 158 |
| Mouse GnRH-R | | DRS | LAITQP | L | AVQSNSKLEQS |

Human m1-R	DRY	FSVTRP	L	SYRAKRTPRR -
Human β₂-R	DRY	FAITSP	F	KYQSLLTKNK -
Serotonin 1d-R	DRY	WAITDA	L	EYSKRRTAGH -
Odorant F3-R	DRY	VAICHP	M	HYTVIMNYK - -
Mouse TRH-R	ERY	IAICHP	I	KAQFLCTFSR -
Human Rhodopsin	ERY	VVVCKP	M	SNFRFGEN - - -
Rat Cannabinoid-R	DRY	ISIHRP	L	AYKRIVTRPK -
Bovine Angiotensin II-R	DRY	LAIVHP	M	KSRLRRTML - -
Rat LH/CG-R	ERW	HTITYA	V	QLDQKLRLR - -
Rat TSH-R	ERW	YAITFA	M	RLDRKIRLR - -

Figure 1.1 Conserved features of typical GPCRs

A two dimensional representation of a GPCR, showing the 7TMs interconnected by the 3 intracellular loops (ICL) and the 3 extracellular loops (ECL). Alignment of amino acid sequences (residues 138 to 158) of intracellular loop II (2i) of the following GPCRs: murine gonadotropin-releasing hormone receptor; human muscarinic acetylcholine receptor (m1-R)(Peralta et al., 1987); human β2-adrenergic receptor (Kobilka et al., 1987); human serotonin 1d receptor (Hamblin and Metcalf, 1991); odorant F3 receptor (Arora et al., 1995); mouse thyrotropin-releasing hormone (TRH) receptor (Straub

et al., 1990); human rhodopsin receptor (Nathans and Hogness, 1984); rat cannabinoid receptor (Matsuda et al., 1990); bovine angiotensin II (Sasaki et al., 1991); rat luteinizing hormone (McFarland et al., 1989) and rat thyroid stimulating hormone (TSH) (Akamizu et al., 1990); shows conserved hydrophobic amino acid residues; e.g. DRY [Aspartate (D), Arginine(R), Tyrosine(Y)] in "vertical box"; Modified from (Arora et al., 1995; Gether and Kobilka, 1998).

1.3   G proteins

G proteins are cytosolic or membrane-associated proteins which exist in their inactive state as a heterotrimeric complex with subunits designated as α, β and γ. They are named G proteins because they bind the guanine nucleotides, GTP and GDP. They are grouped into four families: $G_{s\,(s,\,olf)}$, $G_{i\,(i,\,o,\,z,\,t)}$, $G_{q\,(q,\,11)}$, and $G_{12\,(12,\,13)}$ with respect to sequence homology and functional similarities of their α subunits (Hepler, 2003; Simon et al., 1991; Thomsen et al., 2005). When inactive, GDP remains bound to the Gα subunit as G_α-GDP. When GDP is replaced by GTP, the G protein is activated.

1.3.1   The G_α subunit

The G_α subunits of each of these four families have GTPase, receptor, effector, $G_{\beta\gamma}$ binding domains (Neer, 1995) and a helical GTP binding domain. The helical domain is divergent in the G_α subunit of the four families (Oldham and Hamm, 2006). Unlike the yeast system, the mammalian system has multiple G proteins and regulators of G protein signaling (RGS) (Oldham and Hamm, 2006).

1.3.2   Designing the surrogate yeast system

With a maximum of 3 relevant endogenous GPCRs in yeast, exogenous GPCR expression is exploitable by the elimination of the three endogenous GPCRs. Consequently, GPCR-deleted

recombinant yeast serves as surrogates allowing for heterologous GPCR expression. In order to facilitate the coupling of mammalian GPCRs to the yeast system, the yeast G proteins need to be made to mimic those of the mammalian system. Replacement of the yeast G_{pa1} subunit with the mammalian homologue, G_α, was reported to have limited success in interaction with GPCRs due to low affinity of the mammalian G_α for the yeast $G_{\beta\gamma}$; and producing significant signaling background (Ladds et al., 2005). In order to enhance the yeast "G_α-$G_{\beta\gamma}$" and non-yeast GPCR interaction, a series of chimeric G_α subunits were developed to incorporate receptor binding properties of mammalian G_α subunits into a G_{pa1} subunit (Dowell and Brown, 2002) by the replacement of the distal C-terminus of the yeast G_{pa1} with equivalent residues from various mammalian G_α subunits to yield products called G_α-transplants (Brown et al., 2000). Unlike the G_{pa1} alone, the resulting "yeast- mammalian" G_α chimera, G_{pa1}-G_α, efficiently couples to a variety of non-yeast GPCRs (Ladds et al., 2005). The human B$_2$-adrenergic receptor represents the first heterologously-expressed GPCR that coupled to the pheromone signaling pathway in yeast (King et al., 1990).

1.3.3   Pitfalls

Despite the alterations in the yeast G_α, only about 50% of GPCRs expressed heterologously are able to couple to the pheromone signaling pathway in *S. cerevisiae* (Dowell S.J. and Brown, 2002; Ladds et al., 2005). Apart from the differences in lipid composition of yeast membranes compared to those of mammalian systems, a deficient interaction of the receptor with the G_α subunit following its expression and successful transport to cell surface, could still make the receptor inactive (Ladds et al., 2005; Lagane et al., 2000). Ligand sizes also determine the ability of expressed GPCRs to get activated (Ladds et al., 2005). Ligands with molecular weights (M_r)<1,000 daltons, are generally found to display GPCR activation potencies to similar magnitudes in yeast and mammals (Baranski

et al., 1999; Sachpatzidis et al., 2003), whereas larger ligands (MW>5,000 daltons) are less likely to activate GPCRs heterologously-expressed in the yeast system.

1.3.4   The $G_{\beta\gamma}$ subunit

Various combinations of G_β and G_γ subunit (Table 1.1) results in a number of $G_{\beta\gamma}$ dimers of which not all are able to be activated (Dupré et al., 2009). The $G_{\beta\gamma}$ dimer functions in the inactivation of the G_α subunits, hence, the desensitization of the receptor (Kosaza, 2004; Shenoy and Lefkowitz, 2003). They also modulate Kir3 (G protein-gated inwardly rectifying potassium) channels, muscarinic potassium channels in the heart (Logothetis et al., 1987), voltage-gated calcium channels, phospholipase A$_2$, GPCR kinases (Pitcher, 1998), and MAP kinases (McCudden et al., 2005).

Table 1.1 G protein subunits in mammals

G Protein Subunits	Gα	Gβ	Gγ
# in mammals	21	6	12
Encoding Genes	16	5	12
Size	39-52 kDa	36 kDa	5-10 kDa

1.4   Ligands and regions of interaction

GPCRs recognize a range of ligands including hormones neurotransmitters, odorants, amino acids, chemokines, lipids, and light (GPCRs do not bind light) (Kroeze et al., 2003). Apart from signal transduction via G proteins, some GPCRs are also capable of transducing signals via other signaling molecules such as Jak2 kinase, protein kinase c or phospholipase C_γ (Ji et al., 1998), an indication of the diversity in the GPCR superfamily. While ligands such as biogenic amines, nucleosides,

eicosanoids, and lipid moieties binds exclusively to the TM core, peptides of ≤40 amino acids binds both the TM core and the exoloops; and while polypeptides of >40 but ≤90 amino acids bind to the core exo-loops and N-terminal segment, glycoproteins ≥30 kDa binds exclusively to the N-terminal segment.

In a special instance, protease ligands e.g. thrombin, bind to and cleave the N-terminal sequence, $L^{38}DPRSFLLRNPNDKYEPF^{55}$ of the thrombin receptor at $L^{38}DPR{\downarrow}S^{42}$, yielding the peptide, Met^{1}-Arg^{41}. The resulting shorter N-terminal segment interacts with exoloops to generate a signal, whereas the released peptide binds to platelets and stimulate platelet aggregation (Ji et al., 1998).

1.4.1   Mechanism of activation

Activation of GPCRs ensues following the binding of a ligand to the appropriate domain of the receptor. The basic steps involved in the activation include ligand binding (signal generation), transmembrane signal transduction to cytoplasmic signaling molecules (Ji et al., 1998) via TM hydrogen bond reorganization. Agonist binding induces a conformational change that may involve disruption of the strong ionic interaction between the third and sixth transmembrane helices (Ballesteros et al., 2001; Shapiro et al., 2002) resulting in the activation of the heterotrimeric G protein (by the activated GPCR) and affecting downstream signaling pathways (Neves et al., 2002) via the binding and activation of ubiquitous guanine nucleotide-binding regulatory proteins within the cytosol (Davies et al., 2007).

Agonists bind basically to the long N-terminal tail of the secretin family of receptors to induce receptor activation but extracellular loop (EC) interaction is sometimes required to activate the

receptor (Dautzenberg et al., 1999; Stroop et al., 1996). The EC loops provide the crucial contacts in the activation process (Kubo et al., 1998; Malherbe et al., 2003). Another factor that influences GPCR activation is receptor dimerization, a phenomenon that occurs differently in the different classes of GPCRs suggesting that it is however, not essential for activation of G protein(Cvejic and Devi, 1997; Hebert et al., 1996; Romano et al., 1996).

1.4.2   Signal transduction through G proteins

G proteins transmit signals across the plasma membrane as a result of conformational changes induced by ligand binding to the GPCR. They in turn activate enzymes that regulate protein complexes resulting in the transduction of stimuli into signals affecting neurotransmission, growth, differentiation, or cell death. The GDP bound or basal state (a complex of G_α, G_β, and G_γ subunits) represents the inactive state of the heterodimer, linked to the cytosolic domain of the GPCR. Upon activation, GTP replaces GDP on the G_α subunit, causing it to dissociate from the $G_{\beta\gamma}$ dimer (figure 1.2). The Gα subunit interacts with effector molecules; enzymes such as adenylyl cyclase (AC) and phospholipase C (PLC-β), which in turn produce the second messengers: cAMP and intracellular calcium respectively (Gilman, 1987; Jones et al., 2004).

Receptor downregulation occurs following repeated stimulation of the receptor resulting in signal attenuation or desensitization, via internalization (endocytosis) of the GPCR, a process facilitated by the binding of beta-arrestin (Laporte et al., 2002) to the cytosolic region of the receptor. Multifunctional proteins, designated as regulators of G protein signaling (RGS), regulate G protein signaling by binding to previously activated G_α to accelerate the hydrolysis of GTP to GDP, hence, inactivating the subunit resulting in the termination of signaling (Hollinger and Hepler, 2002).

Approximately 30 of these regulators are currently known with selectivity for G_α subfamilies (Wettschureck and Offermanns, 2005). G protein-coupled receptor kinases (GRKs) that mediate receptor desensitization by phosphorylation of the cytosolic region of the receptor to prevent G protein coupling, however, are not found in yeast (Noble et al., 2003; Penn et al., 2000).
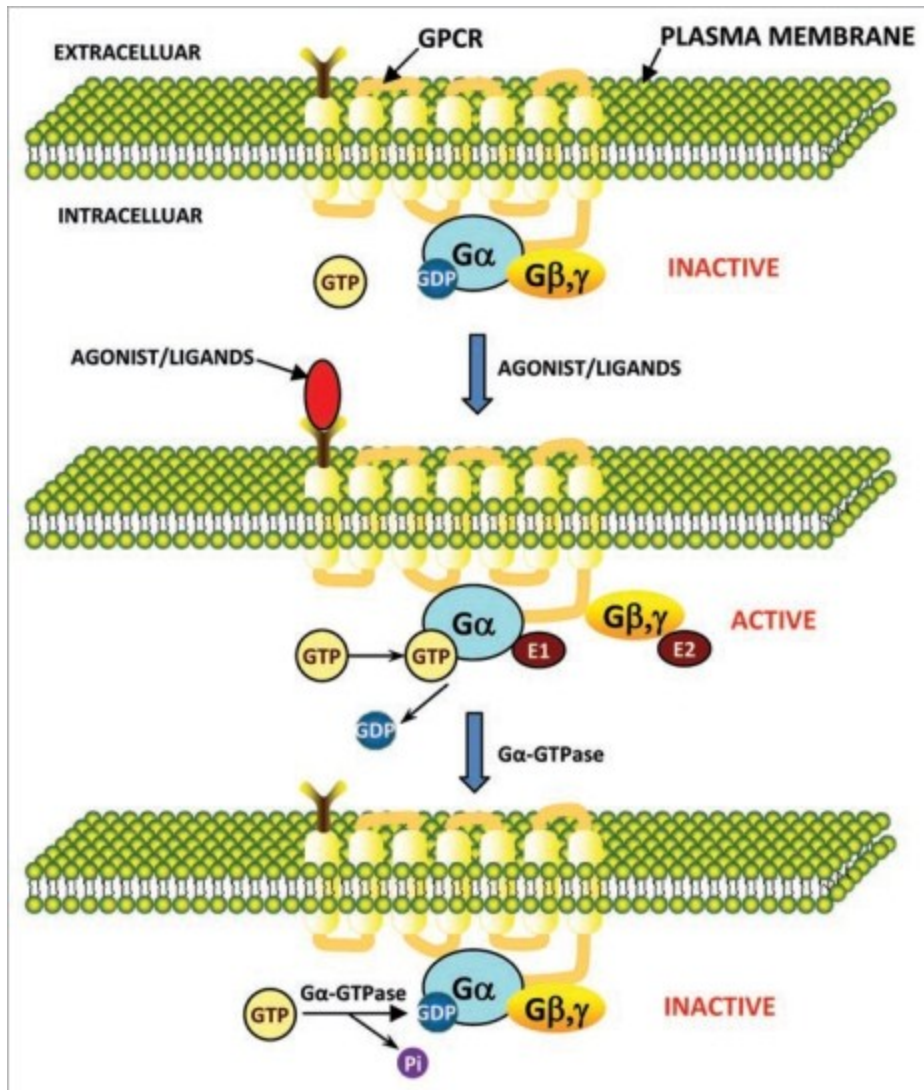
Figure 1.2 Signal transduction through G proteins via GPCRs

A non-stimulated GPCR as well as its coupling proteins (the heterotrimeric G proteins, $G_{\alpha,\ \beta,\ \gamma}$) remain in the inactive state until stimulated by the binding of a ligand to the GPCR. The α subunit

remains bound to a GDP at the inactive state. Upon binding of a ligand to the GPCR, it becomes a guanine nucleotide exchange factor and induces the replacement of GDP with GTP on the G_α-subunit at the cytosolic side of the membrane as a result of the conformational change in the receptor following ligand binding. The result of this nucleotide exchange is the dissociation of the G_α-subunit from the $G_{\beta\gamma}$-dimer subunit. The dissociated G_α then activate other membrane proteins such as adenylyl cyclase, PLC-β, or PKC. After the signal transduction, the inherent GTPase activity of the G_α subunit hydrolyzes the bound GTP to GDP + P_i resulting in inactivation of the of the G_α subunit. It then re-associates with the $G_{\beta\gamma}$ to the form the original inactive heterotrimeric complex $G_{\alpha\beta\gamma}$, completing a cycle of activation and inactivation (Tuteja, 2009). Figure modified from (Tuteja, 2009).

1.4.3 The stimulatory G_α/inhibitory G_α subunits (G_s/ G_i)

The stimulatory G protein-coupled receptors (G_s-coupled receptors) are known to play an important role in proliferation and pluripotency (Layden et al., 2010), differentiation, and development of cells (Rosenberg et al., 2002). Upon binding of an agonist, GPCRs coupled to the Gs subunit of a G protein, activate membrane-associated adenylyl cyclase (AC) (Gilman, 1987) following the exchange of GDP for GTP on the $G_{\alpha s}$ subunit. AC yields cyclic AMP (cAMP) that binds the regulatory subunit and activates cAMP-dependent protein kinase A (PKA), GTP exchange proteins, or ion channels. In addition to two regulatory subunits, PKA has two catalytic subunits which diffuse into the cell nucleus and phosphorylate the transcription factor CREB (cAMP Response Element Binding) at serine residue 133 (Gonzalez and Montminy, 1989). The phosphorylated CREB then binds to CREB-binding protein (CBP) resulting in transactivation and expression of appropriate genes (Gonzalez and Montminy, 1989; Rosenberg et al., 2002). G_s-coupled receptors also stimulate the signal transducer and activator of transcription 3 factor, STAT3 (Liu et al., 2006).

The activation of GPCRs coupled to inhibitory G_α proteins (G_i-coupled receptor) on the other hand results in the inhibition of adenylyl cyclase hence, decrease in cAMP production. They are known to play important roles in the wound healing response of cells (Babbin et al., 2007).

1.4.4   The G_q subunit

The regulatory subunit of G proteins has four classes, namely; $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha 12}$. The $G_{\alpha q}$ class ($G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$, $G_{\alpha 15/16}$) (Simon et al., 1991; Umemori et al., 1997) regulates PLC-β. GPCRs-activating G_q proteins, G_qPCRs, are widely distributed in the CNS and regulate various neuronal processes, neuronal excitability, and synaptic plasticity (Augustine et al., 2003; Berridge, 1998; Billups et al., 2006). Activated PLC-β hydrolyses PIP_2 (phosphatidylinositol 4, 5-bisphosphate) to IP_3 (inositol 1, 4, 5-trisphosphate) and DAG (diacylglycerol). IP_3 binds and activate IP_3 receptors on endoplasmic reticulum resulting in the opening and release of Ca^{2+} from intracellular stores while DAG activates protein kinase C (PKC) (Berridge, 1993; Billups et al., 2006).

G_qPCR signaling is believed to be indirectly effected by membrane depolarization (Billups et al., 2006) as a result of Ca^{2+} influx across the plasma membrane through ion channels. The rationale is that the increase influx of Ca^{2+} results in increased magnitude of Ca^{2+} storage in the endoplasmic stores. The filled endoplasmic Ca^{2+} stores increase the sensitivity of its extracellular IP3 receptors to IP_3 readily available in the cytosol (Nakamura et al., 1999). This in turn stimulates the IP_3 signaling cascade, via positive regulation of PLC-β activity while neuronal cells with dynamic membrane potential however, directly influence G_qPCRs signaling in their plasma membrane (Eberhard and Holz, 1988; Hashimotodani et al., 2005). GPCR Signals transduction via G_q-mediated signaling

(e.g. STR-33) stimulates 5-HT biosynthesis and egg laying and also influences locomotion (Shyn et al., 2003; Tanis et al., 2008) in C. elegans (Lee et al., 2011).

1.4.5   The G_t subunit

The chromosphere, 11-cis retinal, covalently linked to opsin GPCR (rhodopsin), upon the reception of light (ligand), gets converted to all-trans-retinal which changes the conformation of the opsin GPCR (Ridge et al., 2003).   The G_α.tranducin (G_α.gustducin, taste receptor associated) subunit released following the exchange of GDP for GTP, activates the membrane protein cyclic GMP (cGMP) phosphodiesterase which converts cGMP (retina only) to 5'-GMP causing the closure of cGMP-gated cation channels and resulting in cell hyperpolarization, hence the amplification of the light-induced signal (Ridge and Palczewski, 2007).
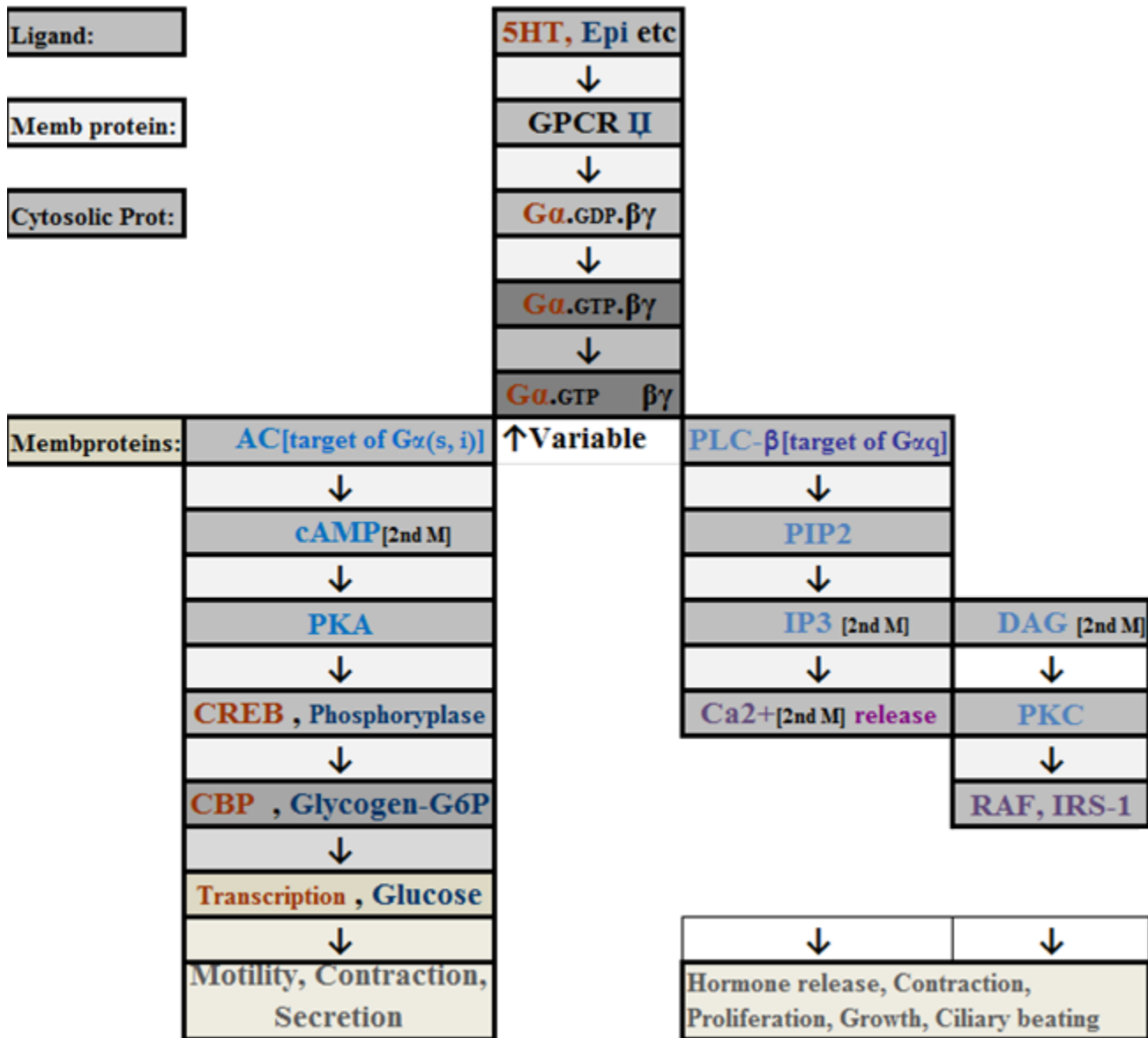
Figure 1.3 Downstream effector proteins on the path of G proteins

G_α subunits released from G proteins following ligand [serotonin (5HT), Epinephrine (Epi)] binding to GPCR are variable and tend to have one of at least three fates: if a stimulatory(s) G_α, they pursue and activate adenylyl cyclase: if an inhibitory (i) G_α, they inhibit adenylyl cyclase resulting in decreased levels of the second messenger, cAMP. The activation and translocation of the catalytic subunit of PKA (protein kinase A) into the nucleus to regulate transcription via CREB and CBP, is

determined by the levels of cAMP with the consequent phenotypic effect of these second messenger release being secretion, contraction, or motility. If the released G_α is $_q$-coupled, it pursues and activates phospholipase C-β, a potent enzyme that cleaves PIP_2 into the second messengers; DAG and IP_3 which in turn stimulate Ca^{2+} release from Ca^{2+} stores to affect hormone release contraction, ciliary beating, and other physiological processes.

1.5   Computational methods for GPCR identification

The challenge with GPCR identifications stems from the inability to effectively crystallize membrane proteins without dissolution in normal solvents (Li et al., 2010; Xiao X. et al., 2009). To date, the only GPCRs structures resolved and available in their 3D conformation are the squid rhodopsin, β1, β2 adrenergic receptors, and the A2A adenosine receptor (Li et al., 2010). However, the availability of amino acid sequence data from genomic sequences of various organisms are on the increase, giving rise to the need for most computational methods for fast and accurate prediction of the structure and function of GPCRs from sequence information.

Some of these methods include the proteochemometric approach (Lapinsh et al., 2005), those based on similarity searches of using primary database search tools (e.g. BLAST, FASTA) coupled with searches of pattern databases (PRINTS)(Lapinsh et al., 2002) and those based on statistical and machine learning method, including support vector machines (SVM) (Bhasin and Raghava, 2004; Gupta et al., 2008; Karchin et al., 2002; Zamanian et al., 2011), hidden Markov models (HMMs) (Eo et al., 2007; Papasaikas et al., 2004; Papasaikas P.K. et al., 2003; Qian et al., 2003), nearest neighbor (NN) (Gao and Wang, 2006; Khan et al., 2008; Li et al., 2010) and covariant discriminant (CD) (Chou and Cai, 2002; Lapinsh et al., 2005).

1.5.1   A typical GPCR sequence structure prediction from genomic data

The Support Vector Machine (SVM) has been used extensively to solve protein secondary structure (Guo et al., 2004; Kumar et al., 2005; Zamanian et al., 2011), subcellular localization (Chou and Cai, 2002; Hua and Sun, 2001), and membrane protein types (Cai et al., 2003). The product of these computational tools is the identification of amino acid sequences of more than a 1000 putative GPCRs. The identification of these 7TM proteins from a given genome is largely dependent on various transmembrane domain protein prediction algorithms with the most used being the hidden Markov model topology prediction server, HMMTOP. It predicts the localization of helical transmembrane segments as well as the topology of transmembrane proteins.

The segment localizations and the topology are not determined by specific amino acid compositions of these helices but by the difference in distributions of the amino acid in various structural windows of these protein sequences (Tusnády and Simon, 1998). Figure 1.4 shows a typical GPCR, a human 5HT4 receptor variant b, with the various TMs colored. The hydrophobic center (HC) for each helix, as calculated by prediction algorithms as the maximum of the peak of hydrophobicity from profile window sizes, determines its localization within the center of the membrane (Donnelly et al., 1993; Trabanino et al., 2004).

MDKLDANVSSEEGFGSVEKVVLLTFLSTVILMAILGNLLVMVAVCWDRQLRKIKTN
YFIVSLAFADLLVSVLVMPFGAIELVQDIWIYGEVFCLVRTSLDVLLTTASIFHLCCISLDRYYAICCQPLVYRNK
MTPLRIALMLGGCWVIPTFISFLPIMQGWNNIGIIDLIEKRKFNQNSNSTYCVFMVNKPYAITCSVVAFYIPFLLM
VLAYYRIYVTAKEHAHQIQMLQRAGASSESRPQSADQHSTHRMRTETKAAKTLCIIMGCFCLCWAPFFVTNIV
DPFIDYTVPGQVWTAFLWLGYINSGLNPFLYAFLNKSFRRAFLIILCCDDERYRRPSILGQTVPCSTTTINGSTHV
LRDAVECGGQWESQCHPPATSPLVAAQPSDT

Figure 1.4 *Homo sapiens* 5-hydroxytryptamine (serotonin) receptor 4, variant b

Showing the amino acid sequences of its TMs highlighted by the different colors as predicted by HMMTOP; and the conserved "DRY" amino acid residues (motif) of the second intracellular loop (2i: refer figure 1.1) seq :> gi|297206828|ref|NM_000870.5| Source: The Gene DB, ncbi.nlm.nih.gov, 01/15/2012.

1.5.2 The GPCRDB

The GPCRDB is a molecular information system that stores, validates, and disseminates sequence information on experimental data, ligand-binding constants, oligomers, and mutations and multiple sequence alignments of GPCRs (Vroling et al., 2011). This data is automatically updated monthly at http://www.gpcr.org/7tm/. Approximately 50 GPCRs are currently estimated to be targeted by half of all currently marketed drugs whereas at least 300 potentially exploitable ones still remain on bench (Chun et al., 2012; Lagerstrom and Schioth, 2008).

GPCRs can be classified based on ligand specificity or on subfamilies. Database search tools such as BLAST and FASTA (Altschul et al., 1990.) are employed in the classification of GPCRs into subfamilies but are thought to be effective only when the query protein sequence is largely similar to

the database sequences. Others include the Hidden Markov models (Sreekumar et al., 2004) and the G protein and receptor interaction feature finding instrument (GRIFFIN) which employs both HMM and SVM (support Vector Machine) in the prediction of coupling selectivity such that both a ligand and a G protein prediction capability are incorporated, indicating a relationship between the identity of the extracellular ligand and the type of G protein it stimulates (Yabuki, 2005).

1.6   GPCR classification

Among the earliest GPCR classification systems is one introduced by Kolakowski in 1994 (Kolakowski, 1994) in which GPCRs were divided into seven groups, designated A-F and O, based on standard (original) similarity searches. A further development of the system for the GPCRDB database places GPCR into 6 classes namely: Class A Rhodopsin-like (> 80% of all GPCRs in humans); Class B Secretin-like; Class C Metabotropic glutamate receptors; Class D Pheromone receptors; Class E cAMP receptors; and the Class F Frizzled/smoothened family (Table 1.2) (Davies et al., 2007).

While Classes A, B, C, and F are found in mammalian species, Class D proteins are characterized in fungi only and Class E proteins restricted to *Dictyostelium* (Davies et al., 2008). The six classes are further divided into sub-divisions and sub-sub-divisions based on function and ligand specificity. The classes A GPCRs have seven identified subfamilies based on ligands, namely: muscarinic acetylcholine; histamine, serotonin, dopamine, octopamine, adrenoreceptors and trace amines (Horn et al., 2003).

1.6.1   The GRAFS classification

The GRAFS system of classification is an alternative means of GPCR classification that recognizes the adhesion and secretin families as being separate; it distinguishes functional genes from pseudogenes (Davies et al., 2007). It characterizes the GPCR superfamily into the *Glutamates, Rhodopsins*, *Adhesions*, *Frizzled/ Taste 2* and *Secretin* families (GRAFS) (Schiöth et al., 2007). A combination of protein families and protein domains were employed in the human genome project which revealed 616 GPCR sequences forming the A, B, and C classes of GPCRs while 569 GPCR were predicted by a motif based approach coupled with *Interpro*, to belong to the rhodopsin-like GPCRs classes (Davies et al., 2007).

Table 1.2 GPCR classes and families

GPCR Class	GPCR Families
A [I]: Rhodopsin-like	Rhodopsin, Amines (muscarinic, Histamine, Serotonin, Dopamine) Peptide, Purine, Glycoprotein Hormones, Orphans
B[II]: Secretin-like	Calcitonin, Glucagon Latrophilin, Methuselah-like, GnHRH
C [III]: Metabotropic Glutamate-like	Metabotropic glutamate, GABA-b, Taste Receptors, Ca2+-sensing receptors, (CaS receptors)
D[IV]: Pheromone	Pheromone A factor-like (STE2 and STE3), Boss (Bride of sevenless protein), Orphans
E[V]: cAMP	Chemosensory, Odorant, Gustatory
F[VI]: Frizzled/Smoothened	Frizzled

1.6.2   Class A receptors

Rhodopsin belongs to the largest subfamily of the membrane receptors, constituting 90% of all

GPCRs. The crystal structure of rhodopsin forms the basis for the detailed three-dimensional

structural model for a GPCR (Teller et al., 2001). The low resolution (7.5 Å resolutions in the plane

of the membrane and 16.5 Å resolutions, perpendicular to the membrane) three-dimensional study of

rhodopsin indicated the location of the seven rods of density corresponding to transmembrane

helices. This class constitutes a minimum of 286 human non-olfactory class A [I] receptors most of which bind biogenic amines, peptides, or lipid-like substances (Davies et al., 2007).

1.6.3   Class B receptors

The class B receptors constitute molecules believed to mediate intercellular interactions at the plasma membrane. It includes a group of *Drosophila* proteins responsible for the regulation of stress responses and longevity (Nordström et al., 2009). It binds large peptides such as secretin, calcitonin, glucagon, parathyroid hormone, and vasoactive intestinal peptide (VIP) (Cardoso et al., 2005). The secretin GPCR is believed to descend from the a*dhesion* family of GPCRs (Nordström et al., 2009) hence, are both designated as class B in the GPCRDB. Secretin GPCRs have been characterized in mammals, *Caenorhabditis elegans* and *Drosophila melanogaster*, but not in plants, fungi or prokaryotes.

Secretin was the first cloned GPCR of the class II family (Ishihara et al., 1991; Martin et al., 2005). The family has three distinct subfamilies of GPCRs (Harmar, 2001) namely B1, B2 and B3, each of which displayed coupling capabilities to the three G protein families; their activation results in the elevation of cAMP, suggesting G_s coupling (Martin et al., 2005); it has also demonstrated coupling to G_q and G_i proteins. While the B1 Subclass interact with polypeptide hormones (27 to 141 amino acids); secretin, vasoactive intestinal peptide (VIP), glucagon, glucagon-like peptides (GLP-1, GLP-2); glucose-dependent insulinotropic polypeptide (GIP) and growth hormone-releasing hormone (GHRH), the B2 and B3 subclasses interact with epidermal growth factor (EGF), i.e., EGF-7TM (Stacey et al., 2000) and the *D. melanogaster Methuselah* gene product (Harmar, 2001; Lin et al., 1998).

Class B (II) GPCRs are identified with relatively long N-terminus of approximately 120–140 amino acids with a set of six cysteine (Cys) residues linked by three disulphide bonds, a conserved feature in all GPCRs (Bazarsuren et al., 2002; Grauschopf et al., 2000). While class I GPCRs rely on internal hydrophobic sequences for targeting to the plasma membrane, most class II GPCRs have an amino-terminal signal peptide for insertion into plasma membrane but lacks the class I GPCR motif, E/DRY. While class I GPCRs have their IC3 being essential for heterotrimeric G protein interaction, the amino-terminal extracellular domain in class II GPCR is essential for ligand binding. A notable characteristic of class II GPCRs is that most are highly constitutive in their wild-type state while most class I's are not, e.g. point mutation occurring in human PTH receptor resulting in severe hypocalcaemia and hyper-calciuria (Martin et al., 2005; Schipani et al., 1999).

1.6.3.1   Subfamily B1

The Subfamily B1 constitutes classical hormone receptors, encoded by 15 genes in humans. Glucagon, GLP-1 and GLP-2 which are members of the subfamily-B1 are synthesized post-translationally from the single polypeptide precursor, proglucagon. Members of the B1 subfamily regulate intracellular concentrations of cyclic AMP by means of adenylyl cyclase coupling via G_s signaling (Harmar, 2001).

1.6.3.2   Adhesive GPCR Family B2

The adhesive GPCRs, majority of which are still orphans, are designated in the GPCRDB as family B (Bjarnadóttir, 2007) or specifically B2 (Harmar, 2001). They are also called epidermal growth factor-seven span transmembrane (EGF-TM7) receptors (Kwakkenbos et al., 2004), or the long N-

terminal seven transmembrane receptors related to family B (LNB-TM7) (Stacey et al., 2000). The

debate still remains as to whether these adhesive GPCR are functionally coupled to G proteins.

They are however, thought to play a role in cell-cell adhesion just like integrins and cadherins

(Bjarnadóttir, 2007).

In addition to an adhesive functional domain or more, they possess relatively long N-termini of more

than thousand amino acids. These functional domains are generally unique for the Adhesion

members and not found within other GPCR families (Foord, 2002). They are coded by numerous

exons, making their genomic structure complex and difficult to study (Bjarnadóttir, 2007). Some

unique domains characterized in the N-termini of human Adhesion GPCRs using RPS-blast

includes: GPS (GPCR proteolytic site), HBD (hormone binding domain), CA (cadherin repeats)

EGF_Lam (laminin type Epidermal growth factor domain), GBL (galactose binding lectin domain),

Ig (immunoglobulin domain), EGF_CA (epidermal growth factor, calcium binding domain), OLF

(olfactomedin domain), LamG (laminin G domain), and LRR (leucine rich repeat) (Bjarnadóttir,

2007).

1.6.3.3   Subfamily B3

The mutant cell line from *Drosophila*, *methuselah* (*mth*), a gene known to exert an average life span

in *Drosophila*, represents the subfamily B3 of family-B GPCRs. It demonstrates an increase in

average lifespan of approximately 35 percent and shows enhanced resistance to stress conditions

such as high temperature and starvation. There is a minimum of eight paralogs of *methuselah*

encoded within the *Drosophila* genome sequence but no such homologs of *methuselah* are

characterized in the sequences of the human or *C. elegans* genomes (Lin et al., 1998).

1.6.4   Class C GPCRs

The class III (C) GPCRs constitutes eight metabotropic glutamate receptors which are further classified into three main families (Table 1.3) (Pin and Acher, 2002). They possess a large extracellular domain holding the binding sites distal to the 7TM and are capable of forming constitutive dimers with specific activation modes (Rondard et al., 2011). With the high level of expression of these families in the CNS (Chun et al., 2012; Conn and Pin, 1997), they form essential targets for treatment of disorders of the nervous system such as Alzheimer's disease (Marino et al., 2003) anxiety, schizophrenia (Conn et al., 2009), and Parkinson's disease (Johnson et al., 2009).

Group I activate phospholipase C (PLC) and generate intracellular calcium signals via $G_{\alpha q}$ signaling. Both group II ($mGlu_2$ and $mGlu_3$) and group-III ($mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$), apart from adenylyl cyclase inhibition via $G_{\alpha i/o}$ signaling, regulate the activity of some ion channels (Goudet et al., 2004). They have a bilobate extracellular domain, the so-called Venus Flytrap (VFT) module, a domain where endogenous ligands bind and are thought to be activated via an indirect metabotropic process (Pin et al., 2003). The taste receptor classification has three members namely TR-1, TR-2, TR-3 forming a dimer of the Sweet-R (T1R2/T1R3) and the Umami-R (T1R1/T1R3) (Pin and Acher, 2002).

Table 1.3 Metabotropic GPCR groups and members

Metabotropic Glutamate Groups	Members	G Protein Coupling	G Protein target
I	mGlu 1 and 5	Gq/G11	PLC-β
II	mGlu 2 and 3	Gi/o	Ion channel
III	mGlu 4, 6, 7, and 8	Gi/o	Ion channel

1.6.5   Class D

The class D family of GPCRs is composed of pheromone receptors, used for chemical communication (Nakagawa et al., 2005). Some examples of this include fungal pheromone A factor-like (STE2 and STE3), fungal pheromone B-like (BAR, BBR) and fungal pheromone M- and P-factors.

1.6.6   Class E

Cyclic AMP receptors from slime molds are a unique family GPCR which regulate developmental processes in *Dictyostelium*. They are known for the regulation of unicellular aggregation into multicellular organism (Klein et al., 1988).

1.6.7   Frizzled/Smoothened receptors

The Frizzled receptors are involved in *Wnt* signaling as well as mediates hedgehog signaling, a key regulator of animal development (Foord et al., 2002). Wnts are signaling proteins that are secreted to regulate various processes of cell development. Wnt signaling, when mediated by Wnt-10b, act as a molecular switch which regulates adipogenesis; and also act as morphogens forming a spectrum of concentrations that patterns the development of tissues (Coudreuse et al., 2006). The binding of a

Wnt ligand to the frizzled (Fz)-Low density lipoprotein receptor Related Protein (LRP) results in the activation of the protein, designated as "disheveled" found in the cytoplasm (Dsh in Drosophila and Dvl in vertebrates) (Park et al., 2005), in a mechanism suspected to be a possible interaction with phospholipids Cong et al., 2003).

1.7 GPCR deorphanization: scenarios and strategies

1.7.1 Reverse pharmacology

This describes a phenomenon in which sequence identification of a receptor is followed by the discovery of the corresponding ligand prior to pharmacological and physiological investigations, e.g. gene knockout or over-expression of the receptor and its ligand (Libert et al., 1991), a procedure opposite to classical physiology and pharmacology. It constitutes three processes: library-based method, tissue extract based approach and information, or known ligand based approach. In the library-based method, extensive substance libraries are designed to encompass predicted ligands for such receptors in addition to all known 7TM receptor ligands. These are in turn matched with "orphan" receptor libraries, to identify positive hits (Brown et al., 2003).

1.7.2 Orphan receptor strategy

The endogenous ligands of approximately 140 GPCRs are still unknown, resulting in a large group of orphans, which are potentially important sources of drug targets (Tang et al., 2012). In the orphan receptor strategy (tissue extract based approach), ligand screening is carried out by the treatment of expressed receptors with crude tissue extracts followed by the identification of the active compound (Civelli et al., 1999) by gene knockout. The putative or known ligand (information based) approach makes use of reported outcomes of some substances believed to be potential ligands of 7TM

receptors. These putative ligands are used to fish out suspected oGPCRs which are eventually treated with selected ligands (Kotarsky et al., 2003).
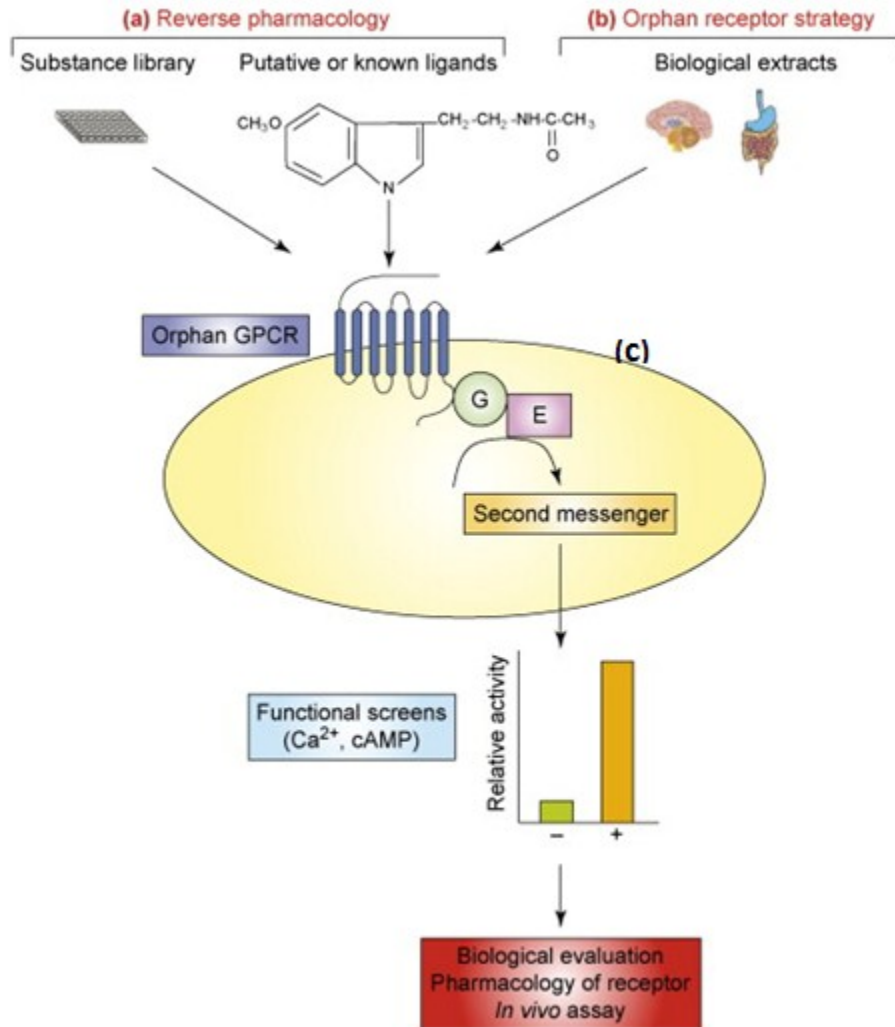
Figure 1.5 A model of the orphan receptor strategy for GPCR deorphanization

The diagram demonstrates methods for GPCR deorphanization**:** (a) shows the method of reverse pharmacology in which cells expressing orphan GPCRs are treated with purified synthetic or natural compounds; and (b) shows the tissue extract based approach (Orphan receptor strategy) in which cells expressing orphan receptors are treated with tissue extracts such that subsequent treatment are administered with further fractions of the same extract, until a characterized distinct and active component is obtained. (C) shows the receptor expression system (e.g. CHO cell, yeast etc.).

Responses are measured by second messenger (cAMP, Ca^{2+}) level monitoring: adopted from Levoye and Jockers (2008).

1.7.3 Factors influencing receptor expression and ligand binding; forms of receptor association

The co-expression of appropriate interacting receptor proteins to enhance either cell surface expression or ligand binding (Levoye and Jockers, 2008) has become the Rosetta stone in the identification of exact ligands for these orphan receptors. While the calcitonin receptor-like receptor (CRLR) for example, requires receptor activity modulating protein (RAMP) for effective translocation to extracellular surface and subsequent binding of ligand (McLatchie et al., 1998), some other GPCRs function following their coexpression with other GPCRs based on the suggestion that GPCRs are capable of forming higher oligomers or dimers (Bouvier, 2001; Milligan, 2006 ; Prinster et al., 2005).

The cell surface translocation of the $GABA_{B1}$ subunit for example, requires the obligatory co-expression with $GABA_{B2}$ subunit, a subunit serving as an orphan 7TM protein lacking a functional GABA orthostatic binding site while the $GABA_{B1}$ subunit is capable of binding to the natural ligand, gamma amino butyric acid (Neuhaus et al., 2005; White et al., 1998). This denotes a classical example of an orphan GPCR association with a non-orphan GPCR. Apart from this form of association, oGPCRs are also known to associate with ion channels, a complex formation occurring constitutively in the absence of ligand activation e.g. the β_2-adrenergic receptor association with the L-type calcium channel (Davare et al., 2001) or with the calcium activated potassium channel (Liu et al., 2004) and the dopamine D5 receptor association with the $GABA_A$-ligand-gated channel. In effect, the $GABA_{B2}$ subunit could be described as an allosteric modulator.

1.7.4   The concept of constitutive activity and inverse agonism

The tendency of oGPCRs to remain active in the absence of ligand binding has been explained in a number of ways. One speculation is the existence of an endogenous ligand that is difficult to isolate (Seifert and Wenzel-Seifert, 2002). The inability to isolate an endogenous ligand is tantamount to no knowledge of its concentration, making the study of constitutively active GPCRs in such systems problematic. Another is the binding of endogenous inverse agonists which possibly results in the masking of the ligand-binding sites (Levoye and Jockers, 2008). The absence of G protein-coupled receptor kinases (GRKs) in yeast needed to regulate inactivation of GPCRs by the prevention of G protein coupling (Noble et al., 2003; Penn et al., 2000) possibly explains the high level of receptor constitutivity in the yeast system.

More than 60 wild-type GPCRs (>40% of all GPCRs) as of 2001, from the families 1-3 of humans and various lab species were found to be constitutively active. Receptors in these families include those for biogenic amines, amino acids, peptides, nucleosides, lipids and proteins (Seifert and Wenzel-Seifert, 2002). Increased constitutive activity has also been identified with various disease-causing GPCR mutants (in equilibrium) compared to wild-types (Seifert and Wenzel-Seifert, 2002). The two-state model of GPCR activation, in attempt to explain GPCR constitutivity, views the TMs to be arranged in a clockwise fashion (Bockaert and Pin, 1999) such that the rotation of TMIII relative to IV constitute a conformational switch of the TM from an inactive R to an active R* isomerization, a state that can occur spontaneously (Seifert and Wenzel-Seifert, 2002) and is conserved among different families (Gether et al., 1997; Sheikh et al., 1999).

In this state, the basal activity of G protein and effector systems increase compared to the absence of GPCR because the state allows the dissociation of GDP from G proteins. Apart from blocking the effects of agonists, full inverse agonists are those ligands that can maximally stabilize the inactive R state and reduce basal GDP/GTP exchange rate, hence, prevent the shift to the active R* state; when the rate of exchange favors GTP association with G protein due to increase spontaneity of R switch to R*, GPCR constitutive activity increases. In such a system, neutral agonists (or antagonists), even though, are unable to alter the equilibrium between the inactive R and active R* states, they are able to block both the inhibitory effects of inverse agonists and the stimulatory effects of agonists (Seifert and Wenzel-Seifert, 2002). This way, receptor constitutivity can be confirmed in the presence of an inverse agonist or an agonist. Agonists, full inverse agonists and antagonists, therefore, form the three basic classes of ligands for the characterization of constitutively active GPCRs in a system believed to contain an endogenous ligand (Morisset et al., 2000; Wieland et al., 2001).

Of the four subtypes of histamine GPCRs; H1, H2, H3 and H4, two are constitutively active: histamine H1 (Leurs et al., 2002) and H3 (Morisset et al., 2000) receptors are GPCRs in which both inactive R and active R* states exist in equilibrium such that even in the absence of histamine, these receptors remain active. This way, histamine, an agonist, stabilizes the active state or promotes the receptor to remain in the "constitutively active" R* state. In the absence or presence of an agonist (histamine), potential inverse agonists (antihistamines) stabilize the receptor in the inactive R state, favoring the association of GDP with G proteins coupling to the receptor rather than GTP. Since all H1 and H3-antihistamines are able to down-regulate such constitutive activity of these two-state receptors, they are classified as inverse agonists.

By coupling to G_q protein subunits, activation of H1-receptors with histamine results in the generation of IP_3, DAG and Ca^{2+} and subsequent activation of NF-kB (Aoki et al., 1998; Bakker et al., 2001; Hu et al., 1999; Leurs et al., 2002), PLD (phospholipase D) and PLA2 (phospholipase A2) (Hill et al., 1997; Leurs et al., 2002). H1-antihistamines successfully down-regulates the levels of NF-kB (Leurs et al., 2002) and diminishes its availability and nuclear translocation for the transcription of inflammation mediators such as interleukins, hence, establishing the therapeutic importance of inverse agonism.

Apart from the use of inverse agonists in the characterization of constitutively active GPCRs, the allosteric inverse agonist, Na+ (as NaCl), also stabilizes the R state (Seifert and Wenzel-Seifert, 2002) by targeting a conserved aspartate residue in TM II of GPCRs (Ceresa and Limbird, 1994) and decreases the basal GDP/GTP exchange rate, hence, favors the association of GDP with G proteins rather than GTP in the absence of ligands. The conditions for the success of this include the check for the presence of Cl^- containing salts (KCl, LiCl and choline chloride). This way, it is easy to eliminate the possibility of the effect of NaCl being due to changes in Cl^- or ionic strength (Costa et al., 1990; Gierschik et al., 1989; Koski et al., 1982; Wenzel-Seifert et al., 1998).

1.8 The genesis of RNAi

RNA interference (RNAi) is a step-by-step process resulting in a post-transcriptional gene silencing (PTGS) in a sequence-specific manner, in both animals and plants. The genesis of RNAi was with petunias in the early 90's when the introduction of extra copies of genes ('transgenes') that codes for deep purple flowers, led to plants with white or patchy flowers (Napoli, 1990; van der Krol et al., 1990) instead of plants with deep purple flowers. At this point, it was assumed that a system was

activated that resulted in the silencing of the transgene coding for purple as well as the native gene of the plant coding for purple. The process was accepted as an evolutionarily ancient method of genome defense in many organisms against foreign genome. The mechanism became clear when Andrew Fire and Craig Mello published their dsRNA-induced gene silencing in nematode worms (Fire et al., 1998) and establishing the mechanism of RNAi using double stranded RNA (dsRNA).

1.8.1   Mechanism of RNAi

The process begins with the injection or uptake of dsRNA that is homologous in sequence to the silenced gene (Fire et al., 1999; Sharp, 2001; Tuschl, 2001). Prior to the sequence-specific dsRNA-mediated native mRNA degradation, the previously injected dsRNA has to be cleaved into short interfering RNA (siRNA) of 21-22 nucleotides in length, by the enzyme, Dicer (ribonuclease III) (Bernstein et al., 2001; Elbashiret al., 2001; Hamilton and Baulcombe, 1999; Zamore et al., 2000). The sense strand (strand with same nucleotides as target gene) of the siRNA (resulting from Dicer cleavage of dsRNA) is removed from the antisense (strand complementary to the target gene). The protein capable of doing the distinction between the two strands of siRNA is the RNA-induced Silencing Complex (RISC). Once the sense RNA is isolated from the antisense, it is destroyed. The remaining antisense strand then serves as a guide to the RISC protein complex, leading them to locate mRNA of target genes to destroy them. This is the case in fruit flies and mammals where the antisense strand gets incorporated into the RISC to target a complementary mRNA by repeated cycles of degradation of specific mRNA; hence, no protein is translated. This way, the gene from which such mRNA is transcribed is unable to get expressed into protein; the gene is therefore, said to be silenced.

In worms and plants on the other hand, the antisense strand of the resulting siRNA might get bound by an RNA-dependent RNA polymerase enzyme (RdRP), forming a complex which pair with a complementary mRNA to synthesize a longer dsRNA. This way, numerous copies of dsRNA are made to be cleaved (by a Dicer) into yet, increased copies of siRNA, whose antisense strands will be specific to different sequences on the same mRNA strand.

1.8.2   Applications

With the advent of RNAi, the classical genetic analysis ('forward genetic analysis') of gene function has taken a different turn called 'reverse genetic analysis' which begins with knocking out (suppression by RNAi as an easier alternative) a specific gene and identifying its function. An important breakthrough with RNAi was its application in the elucidation of genes involved in cholesterol metabolism and heart formation in fruit flies. Genomic libraries of 12,000 different dsRNAs were used to screen *C. elegans* for genes mediating phenotypes such as obesity and ageing (Novina and Phillip, 2004).

While the silencing of *unc-22* gene of *C. elegans* resulted in a phenotype of strong twitchers, the silencing of the *unc-54* gene resulted in paralysis (Fire et al., 1998). Even though the dsRNA was proven to be more effective in producing interference compared to either strands individually (Fire et al., 1998), the large size of the dsRNA makes it an obstacle for uptake by some cells such as Human cells *in vitro*, and are killed mostly by these dsRNAs. This problem was addressed by the use of chemically synthesized short interfering RNA (siRNA; 21-22bp) (Elbashir et al., 2001). The functions of approximately 8000 genes of the human genome were elucidated using siRNA. The hope is to pursue this for application in cancer treatment procedures.

1.8.3   Methods of RNAi and relative potencies

The dsRNA molecule being directed towards target genes to induce suppression can be delivered by injection, soaking of test organism in a solution of dsRNA or by feeding them bacteria expressing the dsRNA. The injection of dsRNA into *C. elegans* resulted in potent and specific interference which were evident in the injected worm as well as its progeny (Fire et al., 1998). The ingestion of dsRNA expressing bacteria is as effective as the injection of dsRNA for RNAi (Kamath et al., 2000).

Feeding has some merits over other forms of dsRNA delivery. First, the process is less labor-intensive, hence, could be carried out on large number of worms when large number of genes needed to be screened. In addition to being able to draw representative statistical conclusion from large sample space, it allows whole genome screen within a relatively short period of time (Kamath et al., 2000). Second, the cost of making dsRNA-expressing bacteria for high-throughput genome-wide RNAi screens is relatively low compared to making dsRNA for injection (Fire et al., 2000) or soaking. The bacterial expressing a specific gene becomes a durable reagent because it could be reused to reproduce an RNAi phenotype at a cheaper cost. Also, the interference effect due to feeding can be titrated to reveal an array of hypomorphic phenotypes due to the absence of any gene (Kamath et al., 2000).

1.8.4   dsRNA-mediated RNAi in mammalian cultures

Even though dsRNA-mediated gene silencing was successful in insect cell lines and worms, the same molecule was unable to induce potent and specific gene silencing in mammalian cells such as Human Embryonic Kidney cells (293), Chinese Hamster Ovary (CHO-K1), Syrian Baby Hamster

Kidney (BHK-21) and Mouse fibroblast (NIH/3T3) (Caplen et al., 2000; Clemens et al., 2000). The application of dsRNA varying in size between 38 and 1,662 base pairs were realized to have elicited a non-specific interferon response such that dsRNA> 30bp binds and activate the protein kinase PKR (Manche et al., 1992) and a synthetase (2,5-oligoadenylate synthetase, 2, 5-AS) (Minks et al., 1979).

The kinase then phosphorylates the translation initiation factors: elF2α and the activated 2, 5 –AS, resulting in non-specific mRNA degradation by the ribonuclease, 2, 5-oligoadenylate-activated ribonuclease L, hence, stalls translation. In order to bypass this interferon response, short interfering RNA (siRNA) with overhanging 3' ends (Base-paired) of 22- nucleotides in length, were used to mediate sequence specific mRNA degradation (Elbashir et al., 2001) resulting in siRNA-mediated gene suppression in mammalian cells.

1.8.5 Planarians as promising research models; models for RNAi studies

Planarians are free-living flatworms in the phylum Platyhelminthes that live mostly in free flowing streams. They belong to the group, Lophotrochozoa, a less studied group of animals that is sister to the Ecdysozoa (e.g. *Drosophila* and *Caenorhabditis elegans*) and the Deuterostomes (e.g. non-mammalian and mammalian vertebrates) (Robb et al., 2008). They possess a synaptic nervous system with cranial ganglia often described as a primitive brain (Abbot and Wong, 2008). They have the ability to regenerate body parts including the central nervous system (CNS), made possible by the possession of a reservoir of adult stem cells (Gentile et al., 2011) called neoblast. In effect, they are close to being "immortal cell lines". Supported by new post genomic technologies and established biological readout systems, the planarian system has become one of the easily available models for the elucidation of gene function for a number of reasons.

First, they can be grown to large populations and maintenance is cost effective. Second, they are easily amenable to RNAi treatments; RNAi can be carried out in them by feeding, injection, or by soaking. Third, they develop phenotypes rapidly. Fourth, some proteins in planaria bear significant similarities to human proteins. Since most planarian genomic regions were found to have significant similarity to human disease-related genes as listed in the paper by Gentile (Gentile et al., 2011), RNAi studies in these can reveal the functions of both known and unknown genes possibly involved in the development and the diseases of man. Also, many planarian genes are homologous to genes in most human parasites such as *Schistosoma mansoni*. The studies and eradication of these human parasites becomes relatively easier having established some degree of functionality of their homologous genes in these planarians.

1.8.6 Promoting planarian research

An aspect of planaria most studied is its impressive regenerative capabilities. As early as the 19th century, Thomas Hunt and Harriet Randolph defined the minimal size of a planarian fragment capable of regeneration to be 1/279th of the intact animal's volume (Morgan, 1901). The use of planarian in current time as a model organism is on the increase. In order to drive research in this area, the 1st International Meeting on Planarian Biology (IMPB) was held in May, 2010 in Münster, Germany, where EuroPlanNet (www.europlannet.org) was lunched as a means to overcoming existing technical limitation in planarian research. It aimed to create a common database that manages integrated genomic and transcriptomic datasets. It will also provide a common infrastructures and a student exchange programme (Gentile et al., 2011). The aim is to promote the use of planarians as model in biomedical research.

2008). Gene-specific knockdown in planarians using RNA interference (RNAi) resulted in the identification of several genes (Newmark et al., 2003; Alvarado and Newmark, 1999).

The *netrin* family of proteins (involved in brain regeneration) acting as chemoattractants or chemorepellents in *Smed* subjected to RNAi, resulted in a disorganized phenotype of axonal projections, hence, their function elucidated to be axon guidance in the process of regeneration (Cebrià and Newmark, 2005). The Fibroblast growth Factor Receptor (FGFR)-related protein, Nou-darake (Ndk), expressed in the head region of the animal relevant for positional identity of the brain, subjected to RNAi, resulted in ectopic brain formation throughout the worm suggesting *ndk* determines and ensures the restriction of the brain to the head region (Cebrià and Newmark, 2005).

1.8.7.2   RNAi in *Girardia tigrina*

Even though the genome (sequencing underway) of the planarian species, *Girardia tigrina*, is yet to be available in its full and annotated form, it has been exploited in other respects; by blind and degenerate amplification of putative genes of planarian origin in studies targeting specific genes; in pharmacological and behavioural studies; and in an array of regenerative studies in the presence of exogenous transmitters. RNAi was applied in *G. tigrina* in the studies of HSP-related genes in the dynamics of neoblasts. The RNAi-mediated silencing of either of the two neoblast-specific HSP members, *Djhsp60* (HSP60 gene family) and *Djmot* (HSP70 gene family), known to be responsible for protection of pluripotent cell system (neoblast) of planarians, resulted in growth arrest in neoblasts and impaired regeneration (Conte et al., 2009; Rossi et al., 2007) of the planaria.

1.8.7.3 RNAi in *Tetrahymena thermophila*

Ciliated protozoans are unicellular eukaryotes most of which have a two nuclear system, somatic macronucleus and a germline micronucleus. *Tetrahymena* is one of such organisms. Both nuclei of *Tetrahymena* originate from the same zygotic nucleus formed during sexual reproduction (Yao and Ju-Lan, 2005). While its macronucleus is responsible for most gene expression functions, the micronucleus endures a faithful genome transmission. RNAi has been well established in the ciliate *Tetrahymena thermophila* and known to have unique consequences on *Tetrahymena* genome different from RNAi consequences in all other organisms.

Instead of causing post-translational gene modification or PTGS, the dsRNA processed into siRNA leads to DNA elimination in *Tetrahymena* (Grewal and Moazed, 2003; Volpe et al., 2002; Yao and Ju-Lan, 2005), hence, an RNA-guided DNA deletion phenomenon. Even though the exact mechanism by which siRNA recognizes the genomic DNA is not yet understood, the discovery supports the contention, that siRNA can specifically recognize DNA sequences. Most importantly, the injection of dsRNA into conjugating cells is enough to induce specific DNA deletion in *Tetrahymena* (Yao et al., 2003), hence, no protein expression. Base pairing was thought to be RNA-DNA pairing through the formation of a d-loop (Yao and Ju-Lan, 2005). The phenomenon of macronuclear or micronuclear reorganization in *Tetrahymena* during vegetative reproduction or conjugation is thought to be governed by this RNAi-guided DNA deletion.

The dsRNA is transcribed from most deletion elements in the micro or macronucleus and cleaved into sRNA (a species of small RNA, 26 to 28 nucleotides in length) just as in other systems involving a *Tetrahymena* dicer-like gene DCL1 (Mochizuki and Gorovsky, 2005). Finally, the

sRNA becomes the effector RNA in a RISC complex and guides it to genomic regions containing the corresponding sequences where the target DNA is deleted, by a yet-unknown mechanism. This RNAi mediated DNA deletion is evidenced in the fact that silencing of DCL1 or an argonaute protein gene, TWI1, resulted in the blockage of conjugation and DNA deletion in *Tetrahymena* Intraflagellar Transport, IFT, a motor for intraciliary transport in *Tetrahymena* (Awan et al, 2004; Awan et al., 2009), mediated by the kinesin-2 superfamily becomes inhibited when kin5 was silenced by RNAi.
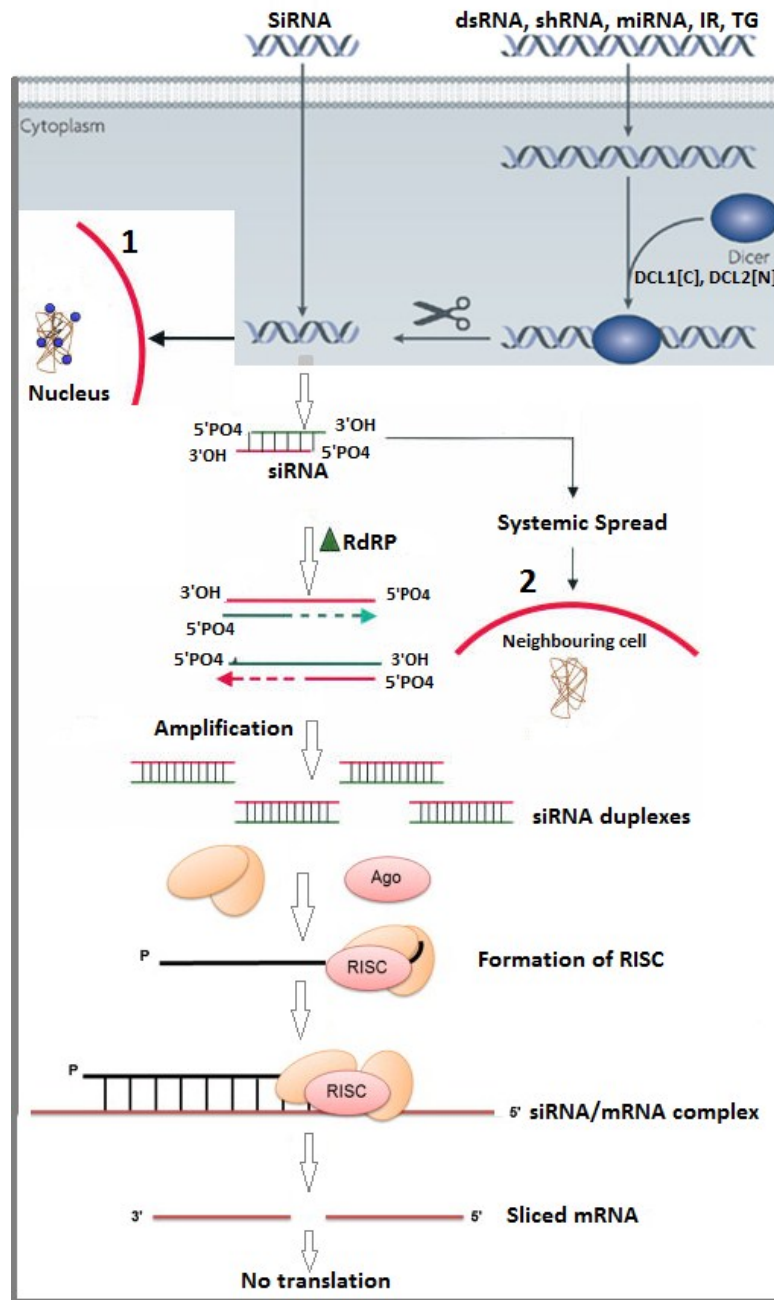
Figure 1.6 A model of the mechanism of double-stranded RNA-induced gene silencing

RNAi begins by the introduction of dsRNA, shRNA, internal repeats or transgenes across the plasma membrane of cells. Within the cell, these molecules are recognized and cleaved by the Dicer enzyme to produce siRNA (short interfering RNA of 21-22 nucleotides in length). 5′ends are

maintained by phosphorylation by a putative kinase. While some of the resulting siRNAs are thought to target *de novo* cytosine methylation events needed for heterochromatin formation (Onodera et al., 2005) (1), in the presence of RNA-dependent RNA polymerase (RdRP) in worms and plants, the antisense strand of other resulting siRNA might get bound by RdRP, forming a complex capable of pairing with a complementary mRNA to synthesize a longer dsRNA which is in turn cleaved by a Dicer into numerous copies of siRNA needed for systemic spread (**2**) and RISC loading. The siRNAs are then loaded onto RISC complexes equipped with a helicase which unwinds the siRNAs such that the antisense strands in the RISC guides the complex to target mRNAs and its cleavage. The numerous antisense components of resulting siRNA will be specific to different sequences on the same mRNA strand. Figure modified from Agrawal et al., 2003; Smith et al., 2012; Whitehead et al., 2009.

1.8.7.4   RNAi, pitfalls

The natural phenomenon of RNAi, however, has some limitations in its occurrence in some species of organisms. Its occurrence is dependent on the existence of the enzyme, RNA-dependent RNA polymerase (RdRP), needed by the antisense strand of siRNA to initiate the amplification of copies of more dsRNA (Novina and Phillip, 2004) enough to "span the genome". In other words, it allows RNAi-mediated gene silencing to spread among non-reproductive tissues by cell-to-cell transfer of dsRNA in plants for example. In an attempt to apply RNAi for disease therapy, the problem of delivery arises, requiring the difficult task of necessary delivery of short dsRNA into specific organs. Another problem of RNAi is the inability to efficiently silence all genes; hence, an RNAi-based screen might evade very important genes. However, it is currently an indispensable complement to classical forward genetics (Fraser et al., 2000).

1.9 General objectives

To pursue and characterize biogenic amine-sensitive GPCRs in two important phyla of organisms in which biogenic amines are indispensable. Previous research identified the relevance of catecholamines and the monoamine, serotonin, in the phyla of Protozoans and Platyhelminthes respectively. However, the mode of reception of these amines hasn't been largely documented. With the current documentation of at least 29 gene homologs of flatworms found in very crucial metabolic disease states of humans and in the native state of important human parasites, and with protozoans being causative agents for a myriad of infectious diseases that are adamant to treatments, it is appropriate first to pursue at the grass roots, the functions of biomolecules that governs the metabolic and physiological processes of these organisms.

Objective 1 To use RNAi coupled with heterologous expression in the deorphanization of protozoan catecholamine responsive GPCR relevant in phagocytic pathways of *Tetrahymena thermophila.*

Objective 2 To use an established loss-of-function GPCR deorphanization protocol coupled with G protein transductional coupling determination and the monitoring of phenotypes in the deorphanization of serotonergic GPCRs in two species of planaria; *Schmidtea mediterranea and Girardia tigrina.*

CHAPTER 2 Catecholamine activation of a putative G protein-couple receptor involved in bacterial engulfment by the protozoan *Tetrahymena*

A paper to be submitted

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2.1   Abstract

Catecholamines are ubiquitous signaling molecules produced and/or recognized by a number of organisms ranging from mammals to plants to protozoa to bacteria. In the protozoan *Tetrahymena*, catecholamines stimulate the phagocytosis of particulates. In other eukaryotes, catecholamines modulate physiologic functions by interacting with membrane-spanning GPCRs on cell surfaces. In this study we investigated three putative *Tetrahymena* GPCRs as transducers of catecholamine-induced bacterial engulfment, a process that is important for both protozoa and bacteria, which may be analogous to the phagocytosis of particulates. RNAi-based studies revealed that knock-down of one of these GPCRs caused diminished bacterial engulfment by *Tetrahymena*. This protein was expressed in an auxotrophic yeast system that enables the deorphanization of GPCRs. The catecholamines epinephrine, norepinephrine, and dopamine activated the receptor in the heterologous expression system while serotonin blocked the activation of this receptor designated as TetEPI-1. Furthermore, epinephrine was shown to stimulate the ability of *Tetrahymena* to engulf bacteria in an axenic culture and this phenomenon was inhibited by RNAi knock-down of TetEPI-1

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and by serotonin. These data demonstrate that TetEPI-1 is an atypical catecholamine-recognizing receptor and this putative GPCR likely facilitates catecholamine-mediated bacterial engulfment in *Tetrahymena*.

2.2 Introduction

Tetrahymena is a free-living protozoan exhibiting phenotypes also observed in pathogenic protozoa, *e.g.*, the engulfment of bacteria (Hirata et al., 2007). Pathogenic protozoa are the causative agents for a large number of infectious diseases that are refractory to treatments, thus the identification of druggable targets is highly desired. Nearly 50% of all marketed drugs target GPCRs (Flower, 1999; Wise et al., 2002), seven transmembrane-spanning receptors that are located on cellular surfaces and serve as transducers of extracellular stimuli into intracellular signals.

Lampert et al. (2011) identified a *Tetrahymena* GPCR and presented evidence for nine GPCR-encoding genes in the *Tetrahymena* database. Another group determined that *Tetrahymena* phagocytosis apparently involves G proteins that couple to GPCRs (Renaud et al., 1995). Candidate ligands for *Tetrahymena* GPCRs include catecholamines since Quiñones-Maldonado et al. (1987) demonstrated that catecholamines stimulate phagocytosis in *Tetrahymena* and since catecholamines are ubiquitous activators of GPCRs in multi-cellular eukaryotes. Additionally, epinephrine and dopamine have been isolated from *Tetrahymena* (Janakidevi et al., 1966) suggesting that these molecules may be intercellular or autocrine-like messengers that coordinate protozoal activity. All together, evidence suggests an associated between phagocytosis, catecholamines, and a GPCR(s) in *Tetrahymena*.

Our laboratory has established that protozoal engulfment of bacteria, which may be analogous to phagocytosis of particulates, is an important phenomenon for bacterial virulence and protozoan physiology (Rasmussen et

al., 2005; McCuddin et al., 2006; Carlson et al., 2007; Brewer et al., 2011). The goal of this project was to explore GPCRs that facilitate bacterial engulfment by *Tetrahymena*. In this study, we targeted three putative GPCRs in *Tetrahymena* and used RNAi to determine if one of these GPCRs is involved in bacterial engulfment by *Tetrahymena*. Because of potential shortcomings of using RNAi in a protozoan with dimorphic nuclei, we also used a heterologous yeast expression system to examine one of the candidate GPCRs. Additional studies evaluated the role of this receptor in catecholamine-activated *Tetrahymena* engulfment of bacteria and it is hence referred to as TetEPI-1, based on its recognition of epinephrine.

2.3   **Materials and methods**

2.3.1   *Tetrahymena* cultures

Tetrahymena thermophilia was obtained from ATCC and axenically grown in the recommended ATCC medium (5.0 g/L proteose peptone, 5.0 g/L tryptone, and 0.2 g/L K_2HPO_4) at 25°C. Media was replaced every four days and cells were diluted 1:14 in fresh media.

2.3.2   **RNAi experiments**

A preliminary GenBank database search identified three putative GPCR genes in *Tetrahymena* [XM_001009792.2 (TetEPI-1), XM_001027519.2, and XM_001010055.2]. siRNA was designed, using the Invitrogen webportal, to silence expression of these three genes in *Tetrahymena*. The sequences GAGATTACTACTAATAGCCTCTCTT, GCTGATTCATTTAATAGCCTTGCTT, and TGGCTCA-GTGTAAGTGACTTAATAT were deemed to be appropriate targets for the three putative GPCRs. A random sequence (CTGACGACAGTTGCATAAAGC) was used as a control. *Tetrahymena thermophila* were grown in 5mL of media to reach confluency (3 x 10^4 cells/mL), and then were harvested by centrifugation at 3,000 x g for 10 min. The pellet was washed twice in 15 mL of deionized water and resuspended in 200μL of deionized water. These cells were then

electroporated (0.2cm electrode gap, 10μF, and 5 milliseconds) with 0.5nM siRNA. Cells were

assessed for bacterial engulfment at 24 hours after electroporation as described previously (Carlson

et al., 2007). Briefly, bacteria were incubated with the protozoa and then *Tetrahymena* cells were

centrifuged at 4,000 x g for 5 minutes. Cells were resuspended in 1mL of Luria-Bertani (LB) broth

containing 108 colony-forming units of a strain of Salmonella that is engulfed by *Tetrahymena* and

survives engulfment (Carlson et al., 2007). Non-engulfed bacteria were then killed with florfenicol

(300μg/mL) and protozoa were lysed with bead-beating. Lysates were recovered and plated on agar

used for enumeration of bacteria engulfed.

2.3.3   RT-PCR experiments

Protozoa were subjected to semi-quantitative evaluation of TetEPI-1 expression. RT-PCR was

performed using oligonucleotide primers specific to TetEPI-1 transcripts (forward, 5'ATGGAC-

CAATCATTTGGAAATCAA3'; reverse, 5'TCAAGTTAGATTTATTTCACGTGAAT3'). RNA

was quantitated (GE Nanovue) and standardized between groups and procedures employed were

similar to those recently described for semi-quantitation of an unrelated transcript (Carlson et al.,

2007). Specifically, 1198bp amplicons were resolved using agarose gel electrophoresis and the

agarose gel-based visual appearance of amplicons was evaluated after every five successive cycles.

2.3.4   Creation of the gene encoding TetEPI-1

RNAi studies identified one of the putative GPCRs [XM_001009792.2 (*i.e.*, TetEPI-1)] as a determinant of

bacterial engulfment. In order to deorphanize this receptor in the yeast heterologous expression system, its

gene must be cloned into a yeast expression vector. Since *Tetrahymena* genes have read-through stop codons

encoding glutamine residues (Adachi and Cavalcanti, 2009), the gene encoding TetEPI-1 was synthesized

(GeneScript) whereby the 13 read-through stops codons (TAA or TAG) were exchanged for glutamine codons (CAA or CAG). Codons were also optimized for expression in yeast.

2.3.5 Creation of the yeast expression vector encoding TetEPI-1

The synthetic TetEPI-1 gene was PCR-amplified with forward (5'GCCATACCATGGACCAATCATTTGGAAATCAA3')

and reverse (5'GCCATAGGATCCTCAAGTTAGATTTATTTCACGTGAAT3') primers which included filler sequences (underlined) and the restriction sites NcoI and BamHI (italicized) incorporated into the 5' and 3' ends of the amplicon, respectively. Purified amplicons and the linearized yeast expression vector Cp4258, which bears a leucine prototrophic marker (Kimber et al., 2009; Wang et al., 2006), were co-digested with NcoI and BamHI restriction endonucleases (New England Biolabs). The digested vector and amplicons were ligated with T4 DNA ligase (New England Biolabs) and the resulting plasmid was transformed into E. coli and individual clones were selected and grown in LB broth overnight. Plasmid DNA was purified using HiSpeed Plasmid Mini Kit (QIAGEN) and inserts were verified using PCR and then sequenced to confirm cDNA orientation and fidelity.

2.3.6 Transformation of yeast with the TetEPI-1 expression vector

Saccharomyces cerevisiae strain CY 19043 (J. Broach, Princeton University, USA) was used as the yeast recipient since these cells are leucine/histidine auxotrophs but exhibit a histidine prototrophic phenotype upon GPCR activation even for exogenous receptors (Kimber et al., 2009; Wang et al., 2006). Non-transformed CY 19043 yeast were grown in YPD media supplemented with all essential amino acids. Cells at mid-log phase (OD600 equal to 0.3 to 0.5) were transformed with 1μg of

Cp4258/TetEPI-1 construct or 1μg empty vector (mock transformants) in the presence of 200μg

salmon sperm DNA (Invitrogen) and 0.1M LiAc (Sigma-Aldrich). Transformed yeast were

incubated at 30oC and then heat shocked at 42oC for 15 minutes. Cells were placed on leucine-

deficient media [1x YNB (Difco), 1x yeast synthetic dropout medium supplement without leucine

(Sigma), 10mM ammonium sulfate (Sigma), and 50% glucose] to select for transformation of

Cp4258 containing the TetEPI-1 insert. Transformants were verified by isolating plasmids

(Promega) and PCR-based detection of the TetEPI-1 insert and its proper orientation.

2.3.7   Ligand assays in yeast transformants

A volume of 3mL leucine-deficient media was inoculated with yeast expressing TetEPI-1, or the mock

controls, and grown at 30°C to an OD_{600} equal to one. Cells were washed three times with leucine/histidine

deficient medium [1x YNB (Difco), 1x yeast synthetic drop out medium supplement lacking leucine and

histidine (Sigma), 10mM ammonium sulfate, 50% glucose, 50mM 4-morpholinepropanesulfonic acid, pH

6.8], then resuspended in 1mL leucine/histidine-deficient media to a density of 15–20 cells/μL.

Approximately 3,000 cells were added to each well of 96-well plates containing the same medium along with

2mM of test agonists in a total volume of 200μL. Cells were grown at 30°C for approximately 24 hours after

which growth was measured spectrophotometrically at OD_{600}. All agonists used in the study were obtained

from Sigma-Aldrich. Mock transformants were used as controls.

2.3.8   Epinephrine and *Tetrahymena* engulfment of bacteria

Tetrahymena thermophila cells were centrifuged at 4,000 x g for 5 minutes and cells were

resuspended in 1mL of LB broth containing 108 colony-forming units of a strain of Salmonella that

is engulfed by *Tetrahymena* and survives engulfment (Carlson et al., 2007) or a strain whose growth

is not stimulated in the presence of catecholamines (McCuddin et al., 2008). Various concentrations

(0.1-200μM) of epinephrine, norepinephrine, dopamine, and serotonin were added and cells were incubated at room temperature for additional 1-16 hours. Percent engulfment was then determined as described previously by killing the extracellular (non-engulfed) bacteria, lysing the protozoa, and enumerating the bacteria liberated from the protozoa (Carlson et al., 2007).

2.3.9   Statistical analyses

Statistical comparisons were made using ANOVA using Bonferonni's correction for multiple comparisons. Prizm 5.0 was the software used.

2.4   Results

2.4.1   RNAi-based identification of a GPCR involved in bacterial engulfment by *Tetrahymena*

Our preliminary database query identified three *Tetrahymena* genes encoding putative GPCRs. In order to assess the association of these GPCRs with bacterial engulfment, we knocked-down receptor expression with siRNA and then evaluated bacterial engulfment in *Tetrahymena*. As shown in Fig. 2.1, bacterial engulfment was significantly hampered in *Tetrahymena* electroporated with one of the siRNA (accession number XM001009792.2). Semi-quantitative RT-PCR experiments revealed that the siRNA dampened expression of this gene. Fig. 2.2 demonstrates the viability of *Tetrahymena* after electroporation, just prior to the engulfment assays.

2.4.2 Deorphanization of the *Tetrahymena* GPCR involved in bacterial engulfment

Studies presented in Fig. 2.1 identified a putative GPCR involved in bacterial engulfment by *Tetrahymena*. In order to deorphanize this receptor, we expressed its codon-optimized cDNA in a yeast heterologous expression system that exploits a histidine prototrophic phenotype upon GPCR activation by a cognate ligand (Kimber et al., 2009; Wang et al., 2006). Previous studies associated catecholamine responsiveness in *Tetrahymena* (Quiñones-Maldonado et al., 1987) thus yeast transformants were exposed to various catecholamines and histidine prototrophism was measured. Control yeast transformants were grown in histidine-deficient media in the absence of a ligand. As shown in Fig. 2.3, epinephrine, norepinephrine, and dopamine elicited moderate increases in yeast growth in the transformants (receptor expression verified by RT-PCR, not shown). Interestingly, serotonin (a non-catecholamine/biogenic monoamine) inhibited the epinephrine-mediated growth of the yeast and it displayed mild inverse agonism of basal receptor activity. Xylazine, an α2-adrenergic receptor agonist, did not stimulate histidine prototrophism in the yeast.

2.4.3 Epinephrine activation of bacterial engulfment by *Tetrahymena*

To confirm the biologic relevance of TetEPI-1 for *Tetrahymena*, we incubated bacteria with an axenic culture of *Tetrahymena* exposed to concentrations of epinephrine that stimulated phagocytosis as per previous studies (Quiñones-Maldonado et al., 1987). Additionally, we incubated bacteria with TetEPI-1 knock-down *Tetrahymena* exposed to concentrations of epinephrine. Bacterial engulfment was then quantitated in these co-cultures. Additionally, serotonin was used in place of epinephrine or with epinephrine. As shown in Figs. 2.4 and 2.5, bacterial engulfment was stimulated by catecholamines in a concentration-dependent manner (EC50= 9-17.5μM) and a time-dependent manner (time to maximal effect ~2hrs). Epinephrine was only able to partially restore

bacterial engulfment in TetEPI-1 knock-down *Tetrahymena*. Serotonin inhibited the effect of epinephrine on phagocytosis (IC50= 32μM) and it inhibited phagocytosis in the absence of epinephrine, suggesting that it has some inverse agonist activity on TetEPI-1.

2.5 Discussion

GPCRs are important membrane proteins because they constitute the largest and most diverse groups of receptor proteins (Teller et al., 2001). With the establishment of GPCRs as targets of numerous drugs, the search for GPCRs has been on the increase. Although *Tetrahymena* has been established as an important model protozoan, there is little information on GPCRs in this organism (Husson et al., 2007; Lampert et al., 2011) and there has been debate regarding their existence in this protozoan (Renaud et al., 1991). Herein, the process of reverse pharmacology was used in the deorphanization of the TetEPI-1 GPCR in *Tetrahymena* as an adrenergic-type receptor that is responsive to catecholamines, and this response is involved in bacterial engulfment. This receptor is likely a GPCR given its ability to stimulate histidine prototrophism in a yeast heterologous expression in which G proteins govern de novo synthesis of histidine following ligand occupancy of a GPCR.

Previous studies have established the production of catecholamines by *Tetrahymena* (Blum et al., 1967; Janakidevi et al., 1966), suggesting that these molecules may serve as intercellular or autocrine-like messengers. However, the mode of catecholamine reception has not been demonstrated in *Tetrahymena*. In the yeast GPCR expression system, the stimulatory effect of the catecholamines on TetEPI-1 was moderate yet consistent. Epinephrine was the most potent of the catecholamines examined, although the potency measurements are reflective of the yeast system that typically diminishes the potency of agonists (Kimber et al., 2009; Wang et al., 2006).

Since catecholamine biology in *Tetrahymena* is biochemically unique, a direct comparison of adrenergic receptor subtypes among eukaryotes is difficult especially considering that TetEPI-1 was expressed in yeast for these studies. Additionally, anabolic and catabolic enzymes govern catecholamine isoforms in *Tetrahymena* (Nomura et al., 1998) although application of exogenous epinephrine led to a stimulatory effect on bacterial engulfment by *Tetrahymena*.

Catecholamine GPCRs have been categorized based on affinities for the ligands. Epinephrine and norepinephrine exhibit high affinity binding to α1-adrenergic receptors with modest affinities for α2- and β-adrenergic receptors (Kroeze et al., 2003). Preliminary data from our studies demonstrated that dobutamine, a synthetic catecholamine with a strong affinity for both β1- and β2-adrenergic receptors (Overgaard and Dzavík, 2008), did not activate TetEPI-1 (data not shown). TetEPI-1 could be best described as an α1-adrenergic receptor but this categorization is currently arbitrary since protozoan receptors will likely have their own classifications as more receptors are characterized. This is especially true given the serotonin-mediated antagonism and inverse agonism of TetEPI-1.

Further studies will be devoted to determining the full array of adrenergic agonists and antagonists of TetEPI-1. It is of note that the activity of this receptor is not completely aligned with a previous study (Quiñones-Maldonado et al., 1987) that identified catecholamine-mediated phagocytosis of particulates. This previous study determined that phagocytosis is activated by lower concentrations of either catecholamine receptor agonists or serotonin receptor antagonists, contrary to the findings in the study presented herein.

In conclusion, we have identified and characterized, for the first time, a unique catecholamine-responsive GPCR in the protozoan *Tetrahymena* thermophilia. The identification of this receptor will serve as model therapeutic target for selectively inhibiting pathogenic protozoa. We have named this unique receptor TetEPI-1, accounting for its recognition of epinephrine.

2.6 Acknowledgments

2.7 Author contributions

SC conceived this study. PA and MB carried out the experiments. PA, SC, MJK, TAD contributed to the writing of the manuscript.

2.8   Figures
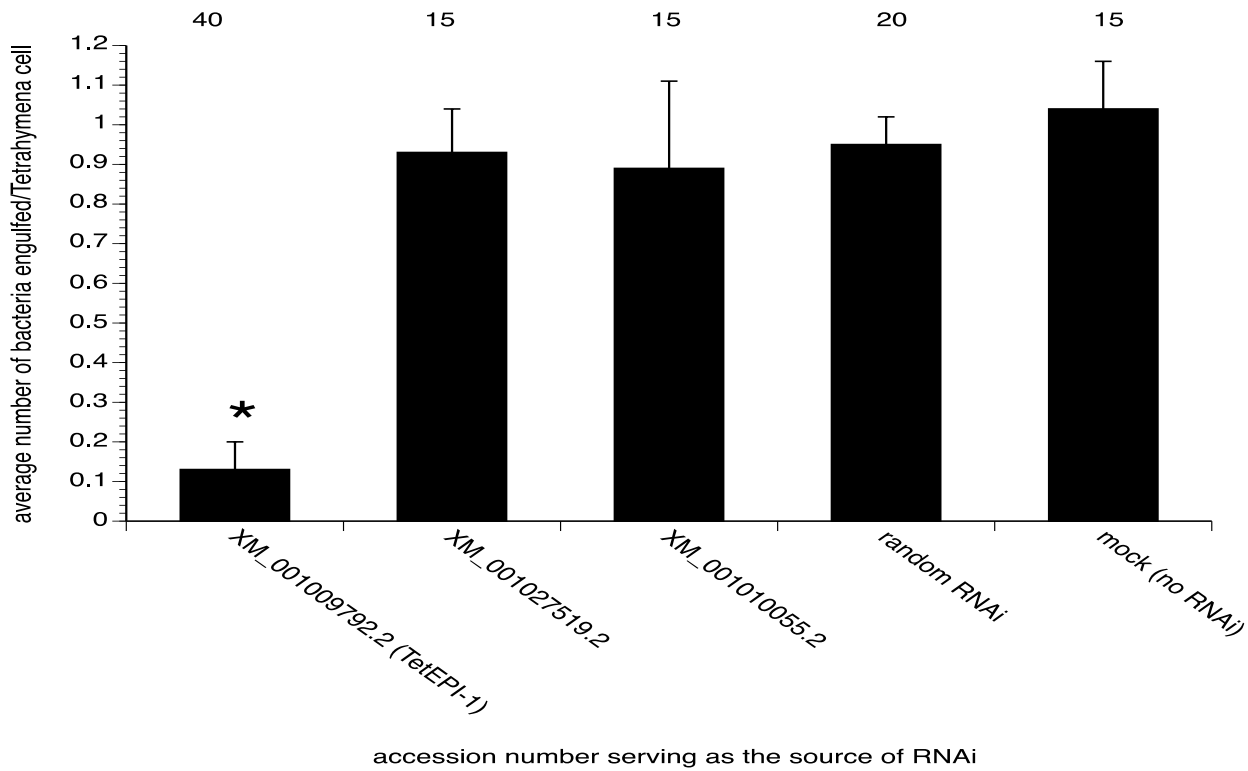
Figure 2.1 Bacterial engulfment and TetEPI-1 expression by *Tetrahymena* electroporated with RNAi corresponding to putative GPCR genes

A preliminary database search identified three putative GPCR-encoding genes (accession numbers provided in the x-axis) in *Tetrahymena* and siRNA was designed based on these sequences. Bacterial engulfment (average number of bacteria engulfed per cell) was determined after electroporation with siRNAs. Random RNAi and mock transformants served as controls. Data presented are the mean $\pm$ sem for three independent experiments performed in triplicate.   *$p<0.05$ versus the rest of the data. Increasing the amount of siRNA 100-fold did not change bacterial engulfment data (not shown). Numbers above each column represent the number of RT-PCR cycles required to yield a visual amplicon for the TetEPI-1 transcript.
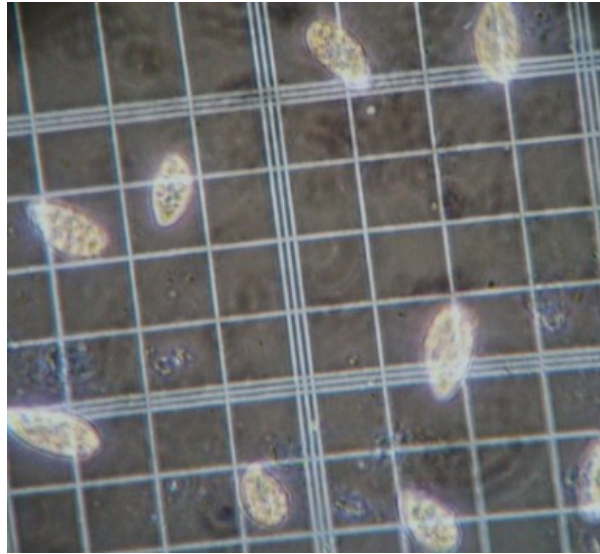
Figure 2.2 Effect of TetEPI-1 gene silencing in *Tetrahymena*

Protozoa were electroporated with siRNA (0.5nM) and cell viability was evaluated microscopically. At 16 hours post-electroporation, cell viability was indistinguishable between mock-electroporated and electroporated *Tetrahymena*, regardless of the siRNA. Magnification = 200X
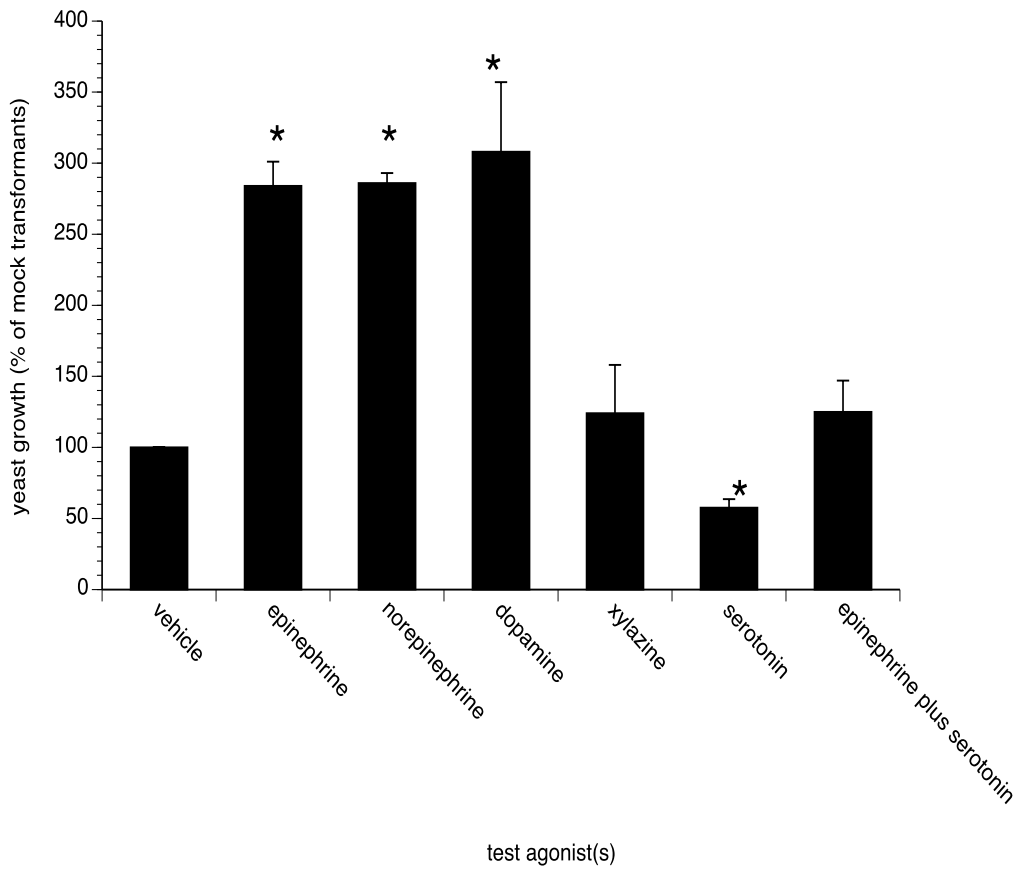
Figure 2.3 Catecholamine-mediated activation of histidine prototrophism in yeast expressing

TetEPI-1

Yeast transformants were exposed to 2mM of the various ligands and yeast growth was measured spectrophotometrically at OD_{600}. To determine background growth, yeast were exposed to ligand-free media lacking the two amino acids (vehicle). Growth is quantitated as compared to growth observed in mock tranformants. Data presented are the mean ± sem for three independent experiments performed in triplicate. *$p < 0.05$ versus vehicle. These effects dissipate when a protease is co-incubated with the ligand (data not shown), suggesting that these effects are transduced by a protein with extracellular domains.
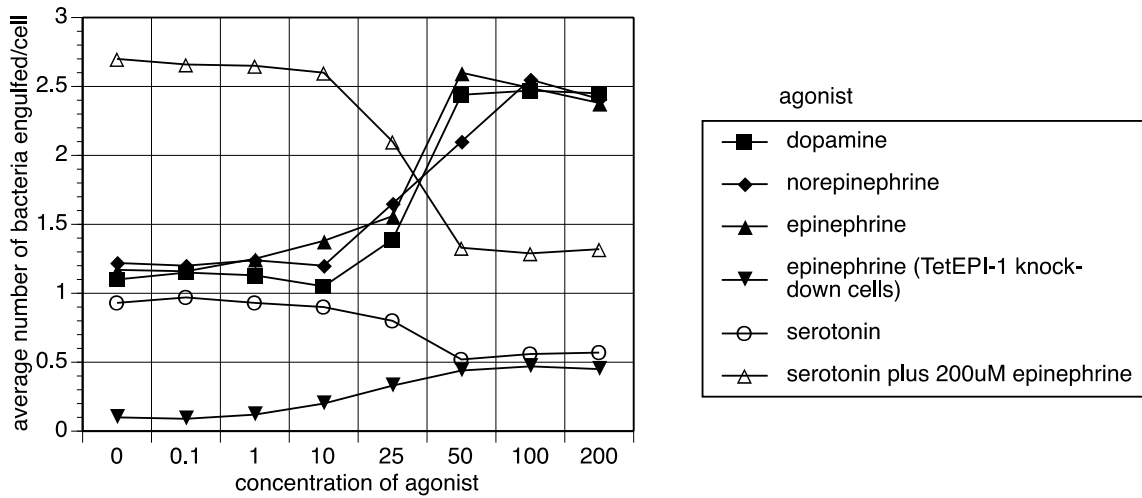
Figure 2.4 Concentration-response curves for bacterial engulfment of *Tetrahymena* exposed to various catecholamines or serotonin

The effect of epinephrine on bacterial engulfment was also assessed in *Tetrahymena* in which TetEPI-1 expression was knocked down by RNAi as per Fig. 1. Bacteria were incubated with *Tetrahymena* and then enumerated after recovery from lysed protozoa. Data presented are the means for three independent experiments performed in triplicate.
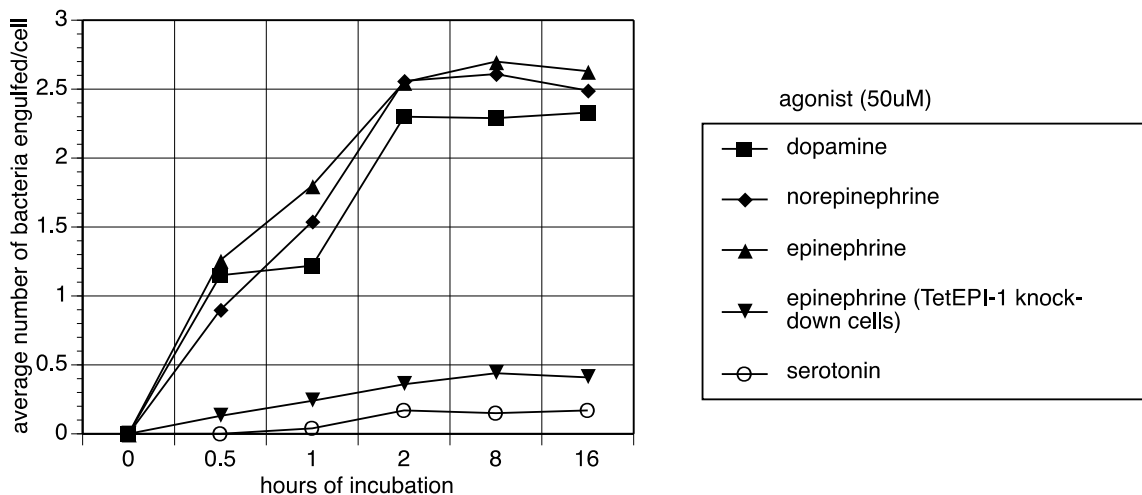
Figure 2.5 Time-dependent assay of bacterial engulfment of *Tetrahymena* exposed to various catecholamines or serotonin [50uM]

The effect of epinephrine on bacterial engulfment was also assessed in *Tetrahymena* in which TetEPI-1 expression was knocked down by RNAi as per Figs. 1 and 2. Bacteria were incubated with *Tetrahymena* and then enumerated after recovery from lysed protozoa. Data presented are the means for three independent experiments performed in triplicate. Similar results (not shown) were observed using a strain of *Salmonella* whose growth is not stimulated in the presence of catecholamines (McCuddin *et al.* 2008).

CHAPTER 3 Identification and functional characterization of serotonin GPCRs mediating flatworm motility

A paper to be submitted

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3.1 Abstract

Planarian flatworms have been rediscovered as important animal models in neuropharmacology and well characterized for developmental biology and regenerative research. With most drugs targeting GPCRs, receptor mediated movement studies in planarians has evolved as a fast growing area of science which can be harnessed into parasite studies to hamper parasite physiology based on characterized receptor homologs shared with these planarians. In a previous study of serotonin pharmacology in the species of planaria; *Schmidtea mediterranea* and *Girardia tigrina*, we've established the ability to silence putative 5HT-receptors and also a neuropeptide receptor using RNA interference. Here, we've investigated putative 5-HT4 receptors that mediate cAMP stimulation and its consequences on motility of planarians due to stimulation by various serotonergic receptor agonists. Even though its actions are normally inhibitory at synapses, serotonin demonstrates an excitatory effect on *S. mediterranea* motility but had moderate inhibitory effect on *G. tigrina* motility. Suppression of both putative 5HT receptors in *S. mediterranea* resulted in significant decrease in worm motility. Suppression of the putative 5-HT4 receptor suspected to mediate

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serotonin-induced moderate decrease in *G. tigrina* motility, resulted in significant decrease in cAMP stimulation (P<0.0001) by serotonin. Forskolin, a potent activator of adenylate cyclase mimics the moderate inhibitory effect of serotonin on *G. tigrina* motility with significant effects at 10μM. Serotonin significantly antagonized the calcium-induced increase in *G. tigrina* motility, further supporting the moderate inhibitory effect of serotonin in this species of planaria. These data demonstrate the relevance of these putative GPCRs in the motility of flatworms. Based on receptor homology, transductional coupling and pharmacology of these putative receptors compared to characterized 5HT4 GPCRs, we conclude these putative GPCRs could be classified as planarian 5HT4 GPCRs, namely *Dtig-ser85, Smed-ser85 and Smed-ser39*.

3.2 Introduction

Serotonin is a biogenic monoamine and a neurotransmitter or neurohormone, found in bilateral animals. In humans, Serotonin signaling is involved in diseases such as anorexia, autism and depression (Schloss and Williams, 1998). In lower organisms, it mediates gut movements and perception of the availability of food resource and controls a range of responses and behaviors, including feeding, locomotion, aggression, temperature regulation, pain perception and sleep (Weiger, 1997). Other factors such as Egg laying, male mating, and pharyngeal pumping are also regulated by serotonin in *C. elegans* (Carre-Pierrat et al., 2006).

The fresh water triclad flat worms collectively known as "planarians" are considered the most primitive example of cephalization of the central nervous system (Ariens-Kappers, 1931) because they are thought as ancestors of bilateria and chordates (Johnson et al., 1995). Early pharmacological studies proved the usefulness of planaria in the elucidation of drug action on the

nervous system (Carolei et al., 1975; Venturini et al., 1989). These investigations resulted in the establishment of behavioral patterns due to drug action on both cholinergic and dopaminergic neurons (Carolei et al., 1975; Caronti et al., 1999). Biogenic amine levels in planaria have been determined (Gustafsson, 1985) and the relevance of these amines; including serotonin in motor activity (Welsh and Williams, 1970) has been established. Serotonin, together with dopamine in this phylum of organisms has been demonstrated to play an indispensable role in the regeneration of their body tissues (Franquinet, 1979).

In mammals, serotonin mediates its effect through seven classes of serotonin receptors (5-HT1-7), six of which are GPCRs (Carre-Pierrat et al., 2006; Hartig, 1997). The 5-HT1 receptor classes A, B, C, D and E subdivisions attenuate adenylate cyclase activity through $G_{i/o}$ protein signaling while the 5-HT5 subclass attenuates adenylate cyclase activity through G_s signaling (Amlaiky et al., 1992; Carre-Pierrat et al., 2006; Maroteaux et al., 1992; Plassat et al., 1992). The 5-HT2 receptor class is made of 3 subclasses; 5-HT2A, 5-HT2B and 5-HT2C, all which couple to $G_{q/11}$ and increase phospholipase C activity (Monsma et al., 1993). Serotonin receptor classes 4, 6 and 7 on the other hand, activate adenylate cyclase activity (Gerald et al. 1995; Meyerhof et al., 1993; Monsma et al., 1993) (Table 1.4).

GPCRs are one of the most important among membrane proteins because they constitute the largest and most diverse groups of receptor proteins (Teller et al., 2001). With the establishment of GPCRs as targets of most drugs, the search for GPCR has been on the increase. Since the inception of the search for GPCRs, most molecules (ligands) that signals by means of a G protein-effector system, has been identified with a cloned GPCR gene. Even though a number of GPCR genes were

currently identified, most still exist in both higher and lower organisms of importance with no known ligands and are so called orphan GPCRs (oGPCRs).

In lower organisms such as planaria, putative receptor identification e.g., 5-HT receptor, by means of degenerate primer polymerase chain reaction was demonstrated (Saitoh et al., 1996). Planarians have been used in a wide range of behavioral and receptor mediated studies of certain phenotypes. The action of D1 or D2 dopamine selective agonists, CY 208243 and SKF 38393 were found to induce screw-like hyperkinesias and "C" like curling respectively in planaria, with the former inhibited by a D1 specific antagonist, SCH 23390 but not by a D2 antagonist, sulpiride (Venturi et al., 1989). While the D2 receptor antagonist, sulpiride decreases spontaneous locomotor velocity of planarians, amphetamines generally increase planarian motility with significant effect at 10 µM (Raffaa et al, 2004). At low doses, cocaine decreases planarian motility but induces a typical D2 agonist response at high doses (Palladini et al., 1996). Both D1 and D2 Dopamine -selective agonists or mixed action agonists were also found to induce a significant increase in cAMP levels that was antagonized by pretreatment with specific DA blocking agent (Venturi et al., 1989).

Planarian response to drug action on its nervous system has been linked to ultrastructural changes (Palladini et al., 1996; Margotta et al., 1997; Caronti et al., 1999). Unlike mammals, planaria lacks blood brain barrier (Caronti et al., 1999), hence, the quantification of serotonin in the CNS is representative of serotonin levels throughout its body. In studying planarian tolerance of addictive drugs, nicotine for example elicited mammalian-like effects, including decreased motility following acute and repeated exposure and subsequent tolerance (Rawls et al., 2011). Planarian locomotor velocities were also found to decrease with increasing concentration of DMSO. When exposed to

0.1-0.3% DMSO, planarians regain their motility in a time-dependent manner but with only those exposed to 0.1% DMSO producing consistent and full recovery of motility (Yuan et al., 2011).

In the process of movement, *G. tigrina,* one of the several species of planarians, derives its major locomotory force from the cilia of its ventral epidermis. Apart from maintaining the flat shape of these worms, the thick layer of the dorsal and ventral body wall muscles play a role in making turning movements. While serotonin pharmacology has been widely studied in a number of species to be excitatory, *G. tigrina* displayed a unique pharmacology in response to serotonin. Serotonin has a moderate inhibitory effect on cilia beating in this species of planarians. Serotonin- mediated increase in cilia beating frequency on the other hand, has been reported in other species but there is no such documentation as receptor mediated locomotion at least in the model organism *S. mediterranea* or *G. tigrina* probably due to limited genomic data for the later.

The activity of relevant membrane protein systems mediating GPCR activity, for example adenylate cyclase, has been determined in *Polycelis tenuis* and enhanced two-fold by serotonin and synergistically enhanced 20-fold by the nucleotide analog guanosine 5′-(ß-γ-imino) triphosphate, Gpp(NH)p (Franquinet, 1979). This serotonin-dependent regulation of adenylated cyclase activity is thought to play a physiological role in these species of organisms. Molecules serving as mediators or messengers of the actions of GPCRs include cAMP, IP_3, DAG and calcium.

Elevated intracellular Calcium levels have inhibitory effects on the cilia of *ciona intestinalis* (Bergle and Tam, 1992) and on the gill cilia of *Aecquipectin irradians* (Stommel *et al*, 1982; Kimberley et al., 1996). The reverse is true in cells such as *Helisoma trivolvis* (Kimberley et al., 1996). Similarly,

increased cyclic AMP has been demonstrated to have cilio-excitatory effects in *Ciona intestinalis* (Bergles and Tamm, 1992), in the tracheal epithelium of rabbit (Tamaoki et al. 1989; Lansley et al. 1992) and also in the nasal epithelium of humans (Di Benedetto et al. 1990). Yet, in this study, the reverse of this was observed to be true in the planarian species, *Girardia tigrina*.

By means of degenerate primers (for *G. tigrina*) and sequence specific primers (for *S. mediterranea*), we've cloned the gene encoding putative 5-HT4 GPCR in *Girardia tigrina* and cloned its homolog, *smed-ser85*, in *S. mediterranea*. A second putative 5HT4 GPCR, *smed-ser39*, was also cloned in *S. mediterranea* based on sequence similarities to 5-HT receptors from other species. Using RNAi, we performed a study of the role of these putative GPCR in serotonin-modulated movement of *G. tigrina and S. mediterranea*. This paper is the first to explore comparatively a serotonin GPCR-mediated planarian motility. It provides a direct comparison of the 5HT pharmacology and functional role of 5-HT receptors in the motility of the two species of planaria, *G. tigrina* and *S. mediterranea*.

3.3 Materials and methods

3.3.1 *Girardia tigrina* cultures

G. tigrina was obtained from Ward Natural Sciences and maintained at 25 degree Celsius, in an aerated media. Their diet is mainly beef liver, with which they were fed twice a week. Worm media was changed daily.

3.3.2　Gene Amplification

The degenerate primers: 82594PC: 5HT4-1F: CCCAGCAACTGGTTGATTTT and 82595PC:

5HT4-1R: ACTGACAAATTCCCGGTTTG; or 82596PC: 5HT4-2F:

ACAAACACCCAGCAACTGGT and 82597PC: 5HT4-2R: GCATCTTCTTGGGCCATATT, were

used to amplify 500 bp fragment GPCRs with the later, from previously prepared cDNA of *G.*

tigrina. Complementary att-B-sites were attached to these primer pairs (attB1 for forward, attB2 for

reverse) to further amplify the previously amplified fragments in a second PCR cycle to attach att-b

sites to these cloned fragments. In a similar fashion, primers with attB1 (on forward; ASMD5939F:

GGGG ACAAGTTTGTACAAAAAAGCAGGCT-AATGCCGGCCTGTTTCCTAT) and attB2 (on

reverse; ASMD5939R: GGGG ACCACTTTGTACAAGAAAGCTGGGT-

AGCATCCTTTTTCTTGTGCG) sites were designed to clone the gene; >mk4.005939.01.01 RNAi

region:AATGCCGGCCTGTTTCCTATGATAAAGTTCCTCTGATAGTGATTAGTACAGTGTTA

ACTTTGCTAAGTGTTGGCACATGCATTGACAATTGTCTCATGATATCGGCCGTAGCTCTT

GTTAAGAAATTACGAACTCCTTGCAACATGTTAATTCTCAACCTTGCAGTTACAGATTTA

TTAGTCGGCACTCTCGTCATACCGTTTGCAAGCATATACCAAATCAAAGGTTACTGGAT

ATTCGATGAGATAGTATGTGATATATTTATTCTTTTTGATGTTTTGCTATGTACCTCATCA

ATACTTAACTTATGTGCAATATCTGTTGATAGATATCTTGTAATTACTCAACCATTTAAA

TATGCTGTGAAACGCACAAGAAAAAGGATGCT

and the primers with attB1 (on forward: ASMD1585F: GGGG-

ACAAGTTTGTACAAAAAAGCAGGCT-CTCCGCTTTTAATTGGAGGA) and attB2 (on reverse:

ASMD1585R:GGGG-ACCACTTTGTACAAGAAAGCTGGGT-CTGTTTCTTTTTCCGGGGAT)

were used to clone the gene >mk4.001585.00.01 RNAi region:

CTCCGCTTTTAATTGGAGGATTCATTGCAGGAGCTTGGATCATATCAGGATTAATTAGC

ATACCACCGGTAATAGTGTGGAAGGAACCCTTTAGACCTGGAACTTGCCAGTTGACGGA

AAATTTGGGATACCAGATATATGCCACTCTTGGTGCCTTCTACATTCCATTAATAATTAT

GTTGGTGCTATATTATCGAATTTTTAAACTAGCAAGAAATATGGCCCAAGAAGATGCCA

AGAGAAAATTAGGTACAGGTCAAATGACTGATGAAGAACAAACTTCATTGCCAAATCA

GTCAGGAAGAACAAATTCCGCTGAAGAAGACAGAAACTTCTTCGGTTTGATCCCACTC

AAAGACCGAGTGAAGGAAATCAAGGTAATGGGTTCGATGTTGAGAAGACTGGAACTGG

ACCTAAAACAAATCCCCGGAAAAAGAAACAG from S. *mediterranea*.

3.3.3 Plasmid preparation: BP-Clonase Reaction

BP-clonase reaction was carried out as per manufacturer's instruction (Invitorgen). Briefly, 2µl of attB-PCR product of 10ng/µl and 1 µl (150ng/µl) of pDONdT7 donor vector with attP-sites were added to 1.5 ml eppendorf tube on ice. Final volume was made to 8 µl with TE buffer, pH 8.0 and 2µl of BP Clonase™ II enzyme mix was added to the reaction, mixed by brief vortexing and incubated at 25°C for 1 hour.

3.3.4 TOP 10 Bacteria transformation

Electrocompetent *TOP 10* bacterial strains were transformed with the BP-Clonase reaction products (*plasmid*) by electroporation. Electroporated cells were resuspended in 500µl of SOC media and incubated at 37 degree Celsius under shaking for 1 hour prior to plating. Colonies were selected, grown in broth and plasmid preps were carried out to isolate perfect clones verified by sequencing to ensure the presence of target gene in vector (plasmid).

3.3.5   *HT115* **transformation**

Freshly plated *HT115* bacteria were obtained from Lisa Timmons, Carnegie Institution of Washington. Bacteria were inoculated in the presence of tetracycline, grown to stationery phase and stored in 80% glycerol. A fraction of overnight cultures grown to OD595= 0.4, were resuspended in 50mM $CaCl_2$ and incubated on ice for 30 minutes to make calcium chloride competent *HT115* cells. Competent cells were stored in 0.1 X $CaCl_2$ solutions and 100µl aliquots used for the transformation of perfect clones from TOP10 transformation of BP-Clonase reaction products (Plasmid).

3.3.6   **Induction of dsRNA production**

Plasmids confirmed by sequencing as perfect clones (*from TOP 10 transformation*) were used to transform chemically competent *HT115* bacterial strains and grown in LB broth overnight in the presence of kanamycin (vector resistance) and tetracycline (bacteria resistance). Cells were scaled up in 2XYT media and grown to OD of 0.3 to 0.4. IPTG (Isopropyl β-D-1-thiogalactopyranoside) were added to a final concentration of 1mM to induce dsRNA production specific to the target gene. Cells were incubated for a further 2 hours under shaking (250 Rev/min) after which cells were placed on ice to inactivate IPTG, spun down (2X) and resuspended in 2XYT and 80% glycerol for storage at minus 80 degree Celsius.

3.3.7   **RNA interference**

Worms were fed HT115 E. coli expressing dsRNA of putative 5-HT4-like GPCR or of a random gene cloned from an unrelated organism. Bacteria expressing dsRNA were mixed with beef liver to aid feeding by the worms. Worms were fed 3-4 times prior to all experiments.

3.3.7 Semi quantitative RT-PCR

Semi quantitative RT-PCR was carried out to investigate the extent of knock down following RNAi.

3.3.8a Membrane preparation

Membrane preparations from worms were obtained as described by Creti et al., (1992). Briefly, Planarians were washed three times in distilled water and homogenized on a previously frozen thin film of sucrose buffer in a motor. The homogenate was transferred into a total volume of 20 ml of cold sucrose buffer (0-4 degree Celsius) made of 5×10^{-3} M Tris-HCl (pH 7.4) and 0.25 M sucrose. Homogenates were centrifuged for 5 min at 1,500xg, the pellet discarded and the supernatant centrifuged for 15 min at 15,000xg. Pellets were re-suspended in 10 ml of the sucrose buffer and centrifuged again at 15,000xg for 15 min. The resulting pellet was re-suspended in cAMP buffer; 50 mM sucrose, 50 mM glycylglycine, 10 mM creatine phosphate, 2 mM $MgCl_2$, 0.5 mM isobutylmethylxanthine (IBMX), 1 mM dithiothreitol (DTT), 0.02 mM EGTA, 10 units/ml creatine kinase, and 0.01% bovine serum albumin suplemented with 0.1 mM ATP and 0.1 mM GTP, and subjected to ligand treatment. Total suspension volume was set at 500 ul/sample, such that each sample would contain cell membranes from approx 3 worms. 500 ul aliquots of this membrane preparation correspond to individual reactions in the cAMP assay.

3.3.8b cAMP assay

Assay was carried out as described by Richards et al., (1979). Briefly, 500µl aliquots each of ligand or non-ligand (control) treated samples are acetylated [12.5µl (of one volume of acetic anhydride to two volume of triethylamine) plus the 500µl cell samples in cAMP buffer]. A 100µl aliquots from the acetylated samples are incubated with 100µl first antibody (CV-27 Pool raised in rabbit by

J.Vaitukaitis, 1:30,000) and labeled I^{125}; Adenosine 3',5'-cyclic phosphoric acid 2'-0-succinyl-3-[^{125}I] iodotyrosine methyl ester, ~20,000 cpm, in centillation viles, mixed and incubated at 4 degree celcius overnight. Alongside these were cAMP standards of concentrations; 4, 8, 16, 32, 64, 128, 256, 512 (fmol/0.1 ml) all acetylated just as the samples and with 100 μl each of first antibody and labelled I^{125} added as previously and incubated overnight. A 100 μl aliquot, each of NRP (Normal Rabbit Plasma) (1:80,000) and second antibody (goat anti-rabbit IgG, 1:40,000) were added and incubated at 25°C for 10 min. A 100 μl aliquot of 50% normal bovine plasma were added followed by 1 ml of ice-cold PEG and samples centrifuged at 3000 rpm (4°C) for 20 min. The supernatant were aspirated and I^{125} levels of pellets of each tube counted with a gamma counter (Packard, B5002). All samples were in triplicates and each member of these triplicates was in turn treated as triplicates.

3.3.8c Motility tracking

Basal motilities of worms were tracked in 4 ml of worm media with no drug for 15 minutes using Ethovision 3.1 video tracking system (Noldus). To determine the effect of serotonin receptor agonists on worm motility, worms were incubated in 4mls of 10^{-4} M serotonin, 5HT [or 8-hydroxy-2, 2-(di-*n*-propylamino) tetralin, 8-OH-DPAT or *meta*-Chlorophenylpiperazine, mCPP)] media for 30-35 minutes prior to tracking. For experiment to investigate second messengers mediating serotonin-induced moderate decrease in *G. tigrina* motility, worms were incubated in 2 mM $CaCl_2$ for 5 minute or in 10 uM forskolin for 30 minutes prior to tracking. For experiments to investigate additive effects of cAMP production due to forskolin and serotonin, 10μM forskolin (Fk) and 10^{-4}M serotonin (5-HT) were co-added to worms and motility monitored. Recoveries from drug effect were monitored by tracking motility after two washout sections at 1 hour intervals.

3.3.9   Statistical analysis

All data were analysed using Prizm 5.0

3.4   Results

3.4.1   Effect of serotonin on planaria motility varies between species

To investigate the pharmacological effects of serotonin and also 8-OH-DPAT, a selective 5HT$_{1A}$

agonist (Jenck, et al., 1989) and mCPP, an agonist with preferential affinity for 5-HT$_{1B}$ and 5-HT$_{1C}$

receptors (Jenck, et al., 1989) and to verify the presence of serotonergic GPCRs in the flat worm and

model organisms, *S. mediterranea* and *G. tigrina*, we monitored motility in the absence and in the

presence of these exogenous serotonin receptor agonists.   Serotonin increased significantly the

motility of *S. mediterranea*.   Contrary to its excitatory effect on *S. mediterranea* (figure 3.1) and

other species as suggested in literature (Hillman & Senft, 1973; Mellin et al. 1983; Holmes and

Fairweather, 1984; Sukhdeo et al. 1984; Maule et al. 1989; Boyle et al., 2000), serotonin has a

minimal *Cilio-inhibitory effect on G. tigrina* basal motility (figure 3.1), suggesting an exception to

the traditional observations of 5HT-induced increase in cilia beating frequency (CBF) as observed in

a number of organisms including *Helisoma trivolvis* (Kimberly et al, 1996).

Both the basal stimulatory and moderate inhibitory effects are reversible in the presence of the

putative serotonin receptors mediating the effect and in the absence of the ligands; demonstrated by

drug washout sections which restore worm motility back to basal levels (figure 3.1). Also, while the

serotonin receptor agonist, mCPP, has cilio-excitatory effect on *S. med* motility, it has no significant

effect on *G. tigrina* motility suggesting at this point, reduced levels of the 5HT1B/C in this species.

8-OH-DPAT alone however, has cilio-excitatory effect on the motility of both species. We know

from our previous study (Zamanian et al, 2012; under review) the extent of stimulation of cAMP levels by serotonin. Since ligand washouts resulted in the restoration of basal motility, we went further to investigate the second messenger mediating this serotonin-induced increase or decrease in the motility of *S. mediterranea* or *G. tigrina* respectively.

3.4.2   Serotonin-induced moderate cilio-inhibitory effect corresponds to increase cAMP levels in *G. tigrina*

The observed moderate decrease in basal motility due to serotonin (figure 3.1) corresponds to the high level of the second messenger, cAMP as stimulated by serotonin in the worm membrane preparation (figure 3.2: *5-HT*) (Data not available for *Smed*). On the other hand, the observed increase in motility due to 8-OH-DPAT (figure 3.1) corresponds to the comparably low levels of cAMP as stimulated by 8-OH-DPAT in the worm membrane preparation (figure 3.2: 8-OH-DPAT). These together suggest an inverse relation between cAMP levels at least in *G. tigrina*. In effect, excessive cAMP levels beyond a threshold induce an inhibitory effect on cilia action in this species of planaria.

3.4.3   5-HT4-like receptor suppression resulted in significantly decreased cAMP stimulation by serotonin but not by 8-OH-DPAT

To investigate the effect of these putative receptors on the level of cAMP production, we silenced the putative 5-HT receptor by feeding bacterial expressing dsRNA specific to this putative 5-HT receptor. By means of semi quantitative RT-PCR, we showed the degree of knockdown of these putative GPCR in *G. tigrina* (Zamanian et al., 2012; under review) and in *S. mediterranea* (figure 3.3), by RNAi. We then subject membrane preparations from these 5-HT4-like GPCR knockout *G. tigrina* worms to exogenous serotonin stimulation, alongside those from control RNAi-fed worms

(figure 3.4). While the extent of serotonin stimulation of cAMP was significantly decreased following knockdown of the putative 5HT4 receptor, the effect of 8-OH-DPAT in the stimulation of cAMP did not change significantly (figure 3.4) relative to control worms.

3.4.4 5-HT4-like receptor suppression resulted in significant decrease in *S. mediterranea* motility but increase in *G. tigrina* motility

To determine if the knockout putative 5-HT GPCRs have any effect on the basal motility of these planarian species, we tracked basal motility of the 5-HT knockout worms vs. control worms. Suppression of both putative 5-HT receptors (*smed-ser85* and *smed-ser39*) in *S. med* (figure 3.3) resulted in significant decrease in worm basal motility (figure 3.5). The suppression of putative *smed-ser85* resulted in the most significant effect on motility. The suppression of its homolog, *Dtig-ser85*, in *G. tigrina* resulted in increased basal motility of worms compared to control RNAi fed worms (figure 3.5). These together, suggest GPCRs even in their homologous forms in different species of planaria could mediate different modes of ligand action.

3.4.5 Comparative effects of agonists on worm motility in the absence of putative GPCRs

In *G. tigrina*, the absence of the putative 5HT4 GPCR has no significant effect on 8-OH-DPAT action on motility (figure 3.6). This is also reflected in the insignificant change in its effect on cAMP levels (figure 3.4) in the presence or absence of the putative GPCR. Even though 8-OH-DPAT is known to be an agonist of 5HT1A, it did not attenuate adenylate cyclase activity in the membrane preparations. Clearly, 8-OH-DPAT activates adenylate cyclase and causes cAMP accumulation in this species. This suggests minimal levels or the absence of 5HT1A receptors at

least in this species of planaria. Stimulation of 5HT5 receptors results in the attenuation of adenylate

cyclase activity (Carre-Pierrat et al, 2006; Amlaiky et al. 1992; Maroteaux et al. 1992; Plassat et al.

1992) just as 5HT1A. 8-OH-DPAT activation of adenylate cyclase in this species suggests the

possible mediation of its action through putative 5HT6/7 receptors but not 5HT4 because

suppression of the putative 5HT4 receptor seems to have no significant effect on the action of 8-OH-

DPAT as mentioned. This suggests the presence of 5HT6/7 receptors as well in planaria.

The absence of the putative 5HT4 GPCR, *Dtig-ser 85* lessened the inhibitory effect of mCPP on

cAMP (figure 3.4) but with no significant effect on motility of *G. tigrina* before (figure 3.1) and

after (figure 3.6) RNAi, suggesting a minimal level of cross talk between the putative 5HT4 receptor

and putative 5HT1B/C in this species. Also, its effect in *S. mediterranea* motility remained fairly

the same before (figure 3.1) and after (figure 3.6) RNAi of putative 5HT receptors, *smed-ser85 and*

smed-ser39.

3.4.6 cAMP mediates moderate serotonin-induced cilio-inhibitory effects on *G. tigrina* motility

In order to confirm the cAMP-mediated serotonin-induced cilio-inhibition resulting in moderate

decrease in *G. tigrina* motility, we added 10μM forskolin, a potent activator of adenylate cyclase

which should accordingly, increase cAMP concentration of treated cells (Kimberley et al, 1996).

Addition of forskolin to worms mimicked the serotonin-induced cilio-inhibition and resulted in

moderate decrease in worm motility (figure 3.7). Since both serotonin and forskolin stimulate

dramatic increase in the level of intracellular cAMP (figure 3.2), we hypothesized that the additive

effect of these two will result in tremendous levels of cAMP. True to our hypothesis, forskolin

together with serotonin resulted in synergistic effect on cAMP production in membrane preparations (figure not shown). Further, we expected this additive increase in cAMP production to reflect in a further inhibition of motility.

To demonstrate this additive effect of cAMP induction by both serotonin and forskolin in intact worms, we co-added these two agents. Contrary to our expectation, the effect of these agents on motility (figure 3.8) as a result of additive cAMP stimulation, was not significantly different from that induced previously by serotonin alone or by forskolin alone (figure 3.7), suggesting there is a limit to cAMP levels required to induce just a significant decrease in worm motility. However, the mode of cAMP stimulation by forskolin in intact worm is not clear. A similar observation was made by Kimberley *et al*, (1996), who previously observed a weak inhibitory effect of cAMP in the regulation of cilia beating frequency (CBF) in early embryos of *Helisoma trivolvis*.

3.4.7   Calcium mediates an increase in *G. tigrina* motility

In order to verify the moderate inhibitory role of cAMP on the motility of *G. tigrina,* we explored the effect of calcium as the second potential second messenger on motility by means of membrane depolarization using $CaCl_2$. Calcium stimulation resulted in a dramatic increase in worm motility (figure 3.7). This first confirms that serotonin stimulation of the putative serotonin GPCRs in these worms does not result in Ca^{2+} release as a second messenger or the putative serotonin GPCRs are not Gq-coupled. Further, the co-addition of calcium and serotonin, resulted in a significant inhibition of the calcium-induced increase in motility (figure 3.7), again suggesting the moderate inhibitory effect of serotonin on *G. tigrina* motility potentially mediated by cAMP.

3.5 Discussion

G. tigrina, formerly, *Dugesia tigrina* is well known for its remarkable regenerative capabilities. Upon division, its anterior portion acts normally while the posterior becomes limited in movement prior to complete regeneration of a new head and cilia. For these reasons, it has been one of the most used planarian species in pharmacological studies and the teaching of biology of regeneration and movement. However, due to limited genomic data of this turbellaria, little is known about receptor mediated mechanisms underlying their movement.

With the advent of genomic data gradually becoming available for some of these planarians including *S. mediterranea*, the study of GPCR-mediated pharmacological events in planarians is on the increase. The reason being that, these flatworms shared receptor homologs with some parasites as well as humans (Gentile et al., 2011). Targets studied in these can be harnessed into parasite studies to hamper parasite movement. In our previous studies (Zamanian et al., 2012; under review), we've demonstrated that exogenous serotonin stimulates increased cAMP levels in planaria to very significant magnitudes. Here in this study, we've investigated the effect of this magnitude of cAMP due to serotonin in the presence or absence of a putative 5-HT4 receptor in *G. tigrina*; and the effect of suppression of homologs of these putative receptors on the motility of *S. mediterranea*.

The moderate decrease in motility of *G. tigrina* observed in the presence of serotonin corresponds to increased cAMP levels due to serotonin. Previous studies by Kimberley et al., (1996), suspected an inhibitory role of cAMP on the movement of *Helisoma trivolvis*, a pond water snail. We have confirmed this observation by treatments of *G. tigrina* with agent known to be indicative of cAMP dependent response e.g. adenylate cyclase activator (Price and Goldberg, 1993), which decreased

worm motility just as serotonin. This is a unique observation in this planarian species considering the fact that the reverse of this is true in cilio-excitation of; the lateral gill cilia of *Mytilus edulis* (Akira, 1987) and the palatine mucosal cilia in frog (Maruyama et al. 1984) such that increase cytoplasmic cAMP levels are thought to mediate these processes.

Further, by means of RNAi, we silenced the putative GPCR, *D.tig-ser85,* resulting in a significant decrease in the level of cAMP when stimulated by exogenous serotonin. Interestingly, the absence of this receptor, hence, significantly decreased cAMP levels due to serotonin, resulted in moderate increases in the basal motility of these worms or no significant decrease in the basal motility of these worms due to serotonin. This suggests that basal cAMP in these worms serves as a check on the rate of movement or acts as a regulator of basal motility of *G. tigrina*. A confirmation of RNAi specificity is evidenced in the observation that upon knockdown of *D.tig-ser85*, 8-OH-DPAT action on the level of cAMP stimulation and on motility remained approximately the same while that of serotonin on the level of cAMP stimulation changed significantly and moderately on worm motility.

3.5.1   5HT4 receptor pharmacology in *G. tigrina*

Pharmacologically, 5HT1A receptors have high selective affinity for 8-hydroxy-2, 2-(di-*n*-propylamino) tetralin (8-OH-DPAT) and results in the inhibition of forskolin-stimulated adenylate cyclase activity (Misane and Ogren, 2000; Leone et al., 2001); our putative receptor demonstrated otherwise; 8-OH-DPAT activated adenylate cyclase. mCPP binds preferentially to 5HT1B/C and inhibits adenylate cyclase activity as observed in figure 3.4, suggesting the presence of 5HT1B/C receptors in *G. tigrina*. While mCPP is also an agonist of 5-HT2A-2C receptors resulting in activation of phospholipase C-β (Leone et al., 2001) with subsequent increase in calcium levels, the

stimulation of *D.tig-ser85* demonstrated otherwise; serotonin or mCPP stimulation did not result in calcium release; rather, exogenous calcium was used to induce increase *G. tigrina* motility. 5HT3 receptors are essentially Na^+ and Ca^{2+} conducting ligand gated ion channels and differ from all other 5HT receptors (Fink et al., 2007; Leone et al., 2001). Our target sequence analysis shows no homology to characterized 5HT3 receptors.

Even though they were first characterized in the CNS, pepripheral 5HT4 receptors are widely spread in higher animals; heart, vasculature, urinary tract and alimentary tract neurons where they facilitate cholinergic transmission and mucosal electrolyte secretion (Hegde and Eglen, 1996). They are G_s-coupled, hence elevate adenylate cyclase activity or increase cAMP levels (Ford and Clarke, 1993). The same effect has been observed upon cDNA transfection and stimulation of both long and short isoforms of the 5-HT4 receptor in cells (Gerald et al., 1995).

The most definitive classification of receptors is dependent on amino acid sequence, transductional or second messenger coupling and criteria of signaling, pharmacology (Hegde and Eglen, R., 1996). While all the 5-HT receptor subtypes mentioned above have been initially identified by pharmacological approaches and subsequently cloned (Peroutka, 1990), the remaining 5HT5, 5HT6 and 5HT7 were directly characterized by molecular cloning (Boess and Martin, 1994; Branchek and Zgombick, 1997). The use of heterologous expression of recombinant receptors in cell lines has been reported to have unusual drug-receptor interaction behaviors depending on receptor densities transfected or G protein types in a given cells (Kenakin, 1997). An alternative loss-of-function approach validated in our previous studies (Zamanian et al., 2012; under review) now proves promising in the verification of transductional coupling, hence, characterization of novel GPCRs.

Based on each of the definitive steps of receptor classification, we have characterized two homologous serotonin GPCRs in planaria that mediates different serotonin-induced effects on motility. In *G. tigrina*, *Dtig-ser85* mediates serotonin-induced moderate decrease in motility while its homolog in *S. mediterranea* mediates significant increase in motility. We have verified the second messenger coupling of this GPCR in *G. tigrina* to be cAMP (as highlighted in table 3.1) and consequently suggest cAMP mediates moderate serotonin-induced decrease *in G. tigrina* motility. There are three possibilities in literature that explains our finding of serotonin-induced moderate decrease in *G. tigrina* motility.

First, the stimulation of 5-HT4 receptor comprising cholinergic neuron additionally inserted between the serotoninergic and GABAergic neurons modulates GABA release (Fink et al., 2007). Their stimulation results in the increased release of acetylcholine (Ishtiyaque and Ramakrishna, 2011) which, in turn, modulates the release of GABA in bidirectional manner, an example of a more complex regulatory neuronal circuit of serotonin (Fink et al., 2007). Both acetylcholine and GABA were reported to be inhibitory neurotransmitters in planarians (Buttarelli et al., 2008). At high potency activation of 5-HT4 receptors, increased release of GABA is mediated by M1 and/or M3 while the inhibition of GABA release is mediated by M2 receptors at low potency activation of 5HT4 receptors (Fink et al., 2007).

Second, in mammals, 5-HT4 receptor-mediated relaxation of certain gastrointestinal and vascular tissues is attributed to cAMP-induced reduction in intracellular Ca^{2+} levels (Hegde and Eglen, 1996). We have demonstrated in our study that serotonin stimulation of 5HT4 did not result in the elevation

of intracellular calcium; that motility increases only in the presence of exogenous calcium; that the increased levels of cAMP induced by serotonin stimulation of *Dtig-ser85* resulted in intracellular calcium inhibition, hence decrease motility.

Third, the inhibitory response of 5HT4 receptor stimulation in gastrointestinal tract is also believed to be as a result of direct smooth muscle relaxation (Hegde and Eglen, 1996). We've made such similar observation by monitoring turn angle effects in response to serotonin and the two other agonists, where serotonin seem to have negligible contractile effect on muscle cells in this species (data not shown). 5HT4 receptor stimulation results in both excitatory and inhibitory effects (Reeves et al., 1991; Baxter et al., 1991) and the rank order of potency of 5HT4 agonist and antagonists also indicated the inhibitory effects of 5HT on 5HT4 receptors (Hegde and Eglen, 1996). These together or alternatively, possibly play significant roles in our observation of serotonin-induced moderate decrease in G. *tigrina* motility.

3.5.2   5HT4 receptor pharmacology in *S. mediterranea*

In a similar fashion, we have characterized two serotonin GPCRs, *smed-ser85 and smed-ser39* in *Schmidtea mediterranea*. Both receptors mediate increased motility in response to serotonin and serotonin receptor agonists, 8-OH-DPAT and mCPP. Suppression of these two putative GPCRs resulted in significant decrease in worm motility. In mammals, cAMP activation of protein kinase A in myenteric neurons potentially result in closure of potassium channels, with consequent cell excitability and the release of neurotransmitters (Eglen et al., 1995). 5-HT4 receptors in the hippocampus are also largely documented to modulate locomotor activity e.g. in rats (Takahashi et al., 2002).

Even though we were unable to determine the second messenger couplings of these homologs in this species of planaria, coupled with the fact that serotonergic systems in these lower organism might differ to some degree in behavior compared to that in higher organisms, our pharmacological findings agree with previous observations. In terms of transductional coupling, these together or alternatively possibly explain our observation of increase motility in response to serotonin. At this point we conclude based on two of the definitive criteria for GPCR classification; the homology and the 5HT pharmacology of our receptor coupled with physiological consequence of its absence due to RNA interference, in the organism; that these two receptors play a significant role in locomotory behavior of *S. mediterranea.*

3.6 Conclusion

By means of RNAi, we have characterized two novel 5-HT4 GPCRs in the flat worms, *G. tigrina* and *S. mediterranea*. We've shown that, these putative 5-HT4 GPCRs mediates serotonin-induced cilio-excitation in *S. mediterranea* (*smed-ser85, smed-ser39*) and serotonin-induced moderate cilio-inhibition in *G. tigrina* (*Dtig.ser-85*). We demonstrated that the serotonin-induced moderate decrease in motility is mediated by cAMP and not calcium. We've also shown that in the absence of this receptor, cAMP levels due to serotonin significantly decrease; that this decrease in cAMP lessens the inhibition of *G. tigrina* basal motility. Within the context of receptor homology, transductional coupling, 5HT pharmacology coupled with an alternative loss-of-function approach, we conclude these putative GPCRs compares with characterized 5HT4 receptors, hence, could be classified as planarian 5HT4 GPCRs, namely *Dtig-ser85, Smed-ser85 and Smed-ser39*. Future work

aims at investigating the homolog of these receptors in *S. mansoni*, a human parasite largely studied in our lab.

3.6.1   Summary

1. Effect of serotonin on planaria motility varies between species.

2. Serotonin-induced moderate cilio-inhibitory effect corresponds to increase cAMP levels in *G. tigrina*.

3. Putative 5-HT4 (*Dtig-ser85*) knockout results in decrease cAMP stimulation by serotonin but not by 8-OH-DPAT.

4. Putative 5-HT4 (*Dtig-ser85*) knockouts results in significant decrease in *S. mediterranea* motility but moderate increase in *G. tigrina* motility.

5. There are comparative effects of agonists on worm motility in the absence of putative GPCRs.

6. cAMP mediates serotonin-induced moderate cilio-inhibitory effects on *G. tigrina* motility.

7. Calcium mediates an increase in *G. tigrina* motility.

3.7   Acknowledgment

3.8   Author contributions

TAD, MJK and MZ conceived this study. PA and MZ carried out the experiments. MZ and MJK performed the bioinformatics. PA and MZ performed the statistical analysis. PA, MZ, TAD, MJK and SC contributed to the writing of the manuscript.

3.9 Figures and Tables
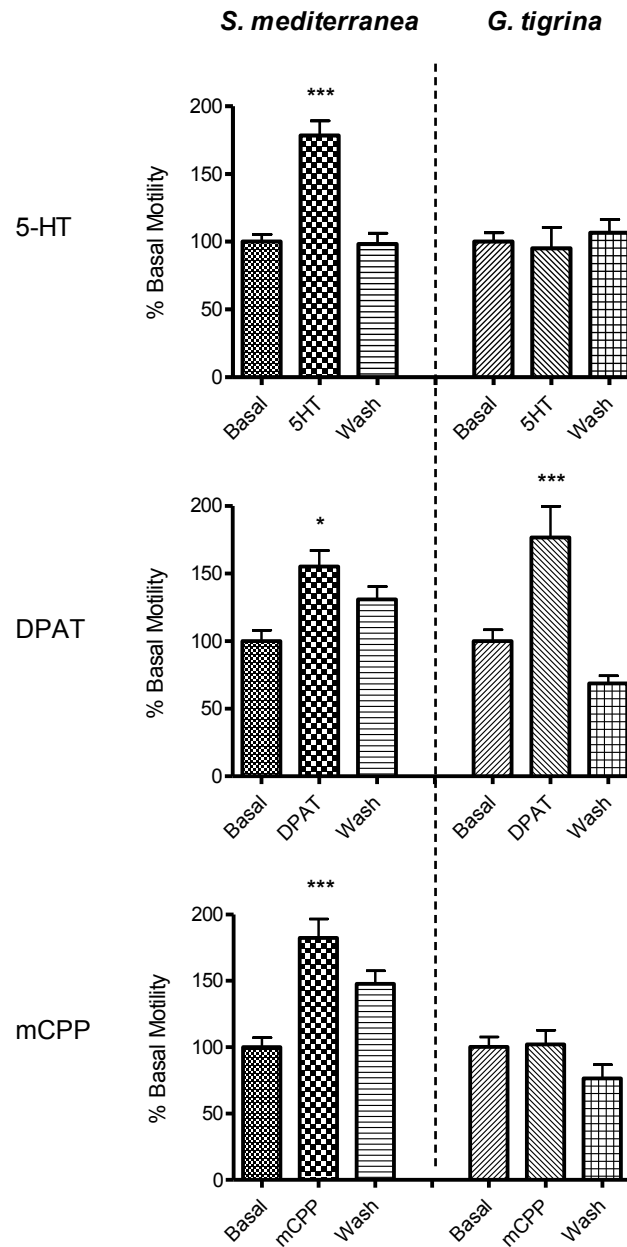
Figure 3.1 Serotonin stimulates homologous receptors in different planarian species differently Addition of serotonin to *S. mediterranea* resulted in significant increased in worm motility but induced a moderate decrease in *G. tigrina* motility. In a similar fashion, the serotonin receptor agonist, mCPP, produced a significant increase in *S. mediterranea* motility but had no significant

effect on *G tigrina* motility. 8-OH-DPAT produced similar effect in these species. These drug effects were reversible in the presence of the receptor and in the absence of agonist (Demonstrated by washout sections). Each bar represents the mean (plus S.E.M.) motility of 18 worms and experiments repeated 3 times.
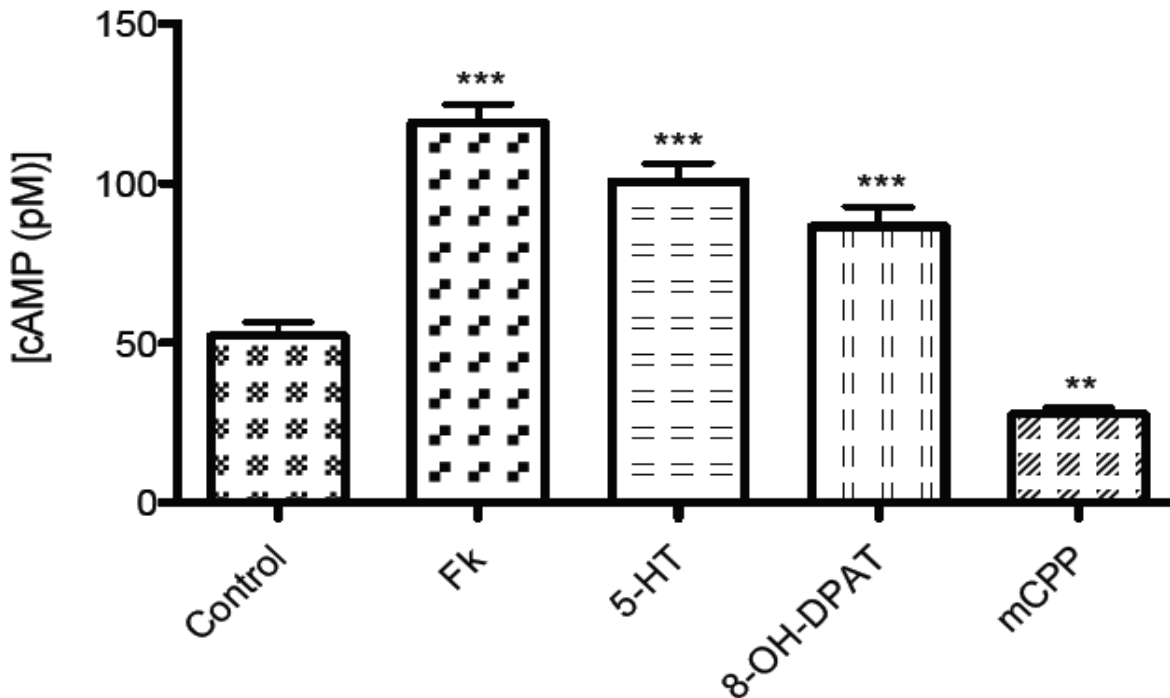
Figure 3.2 Effect of serotonergic agonist on cAMP stimulation in planarian (*Girardia tigrina*) membrane preparation

Serotonin $[10^{-4}M]$ and 8-OH-DPAT $[10^{-4}M]$ stimulate significantly [cAMP] levels compared to basal levels (P<0.0001) while mCPP $[10^{-4}M]$ significantly inhibits basal cAMP levels. Each bar represents the mean (plus S.E.M.) of [cAMP] from 9 samples (due to 3 samples, each triplicated) and experiments repeated 4 times.
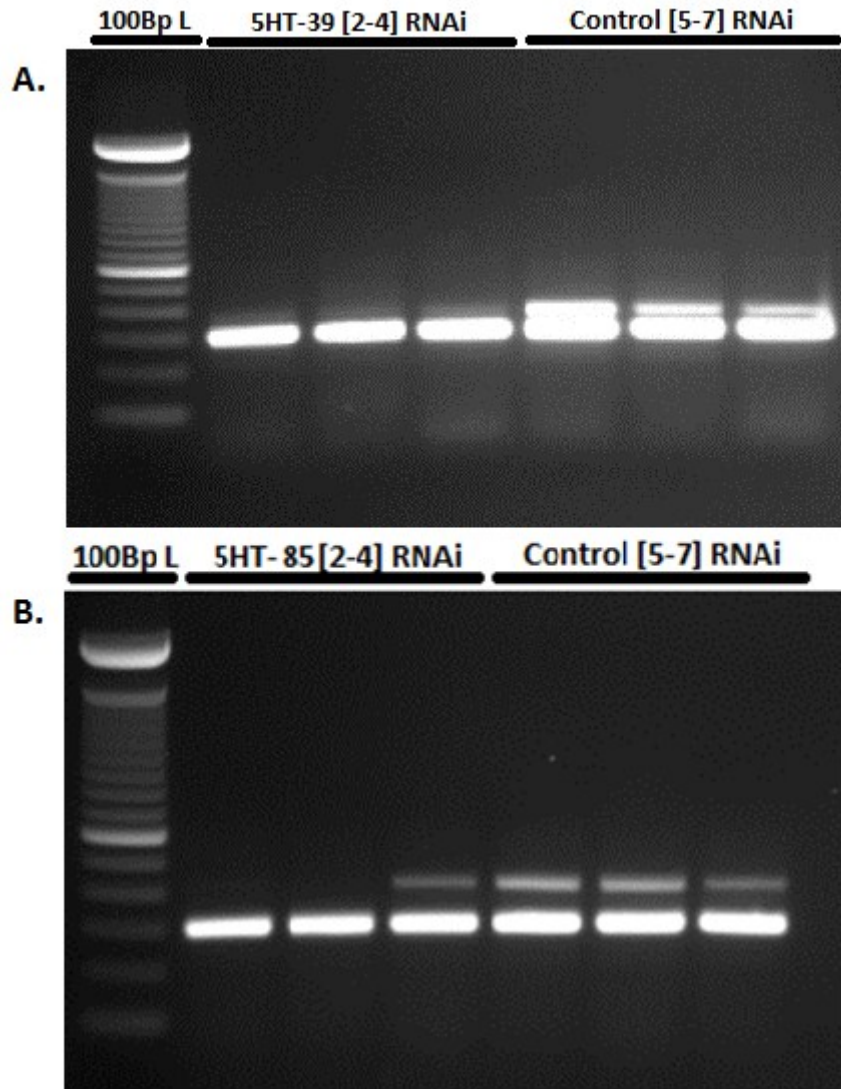
Figure 3.3 Semi quantitative RT-PCR of putative 5HT4 GPCR in S. *mediterranea*
Worms were fed 4 times with bacteria expressing dsRNA specific to cloned putative 5-HT4-Like receptors: **A**. Lane 1: 100bp ladder, Lane 2-4: worms with 5HT39 receptor knocked down. Lane 5-8: Control worms fed with bacteria expressing a random gene. **B**. Lane 1: 100bp ladder, Lane 2-4: worms with 5HT4 (85)-Like receptor knocked down. Lane 5-8: Control worms fed with bacteria expressing a random gene. **C**. **(**Refer C. at figure A.6; Zamanian et al., 2012; under review**).**
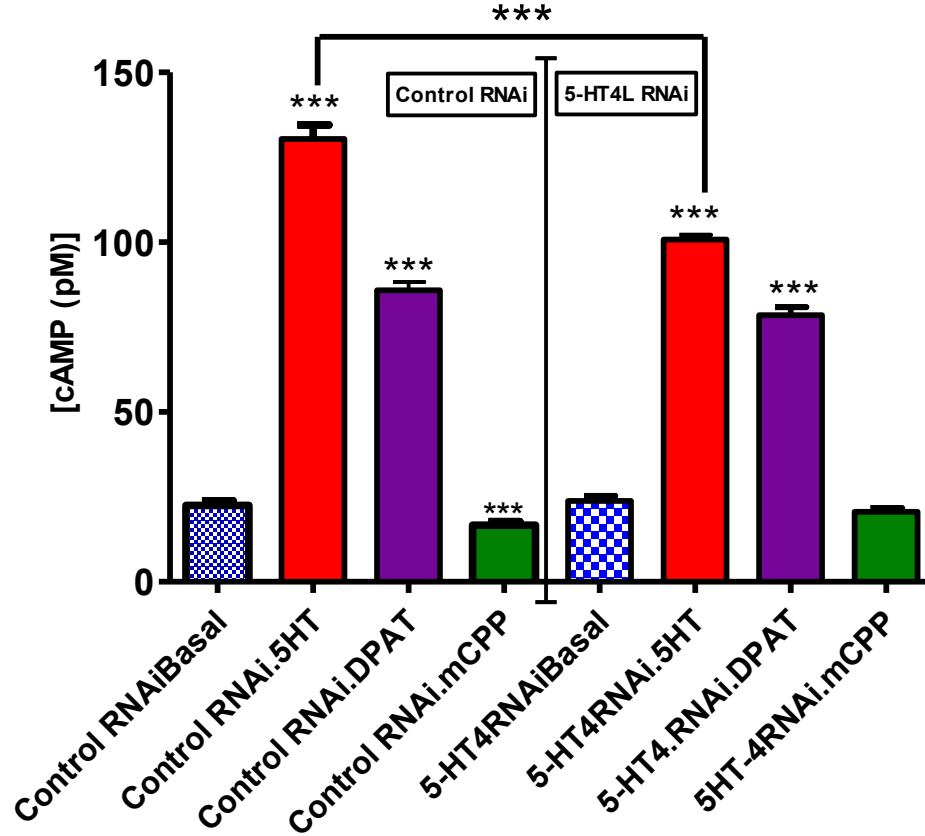
Figure 3.4 Effects of 5HT and 5HT-R agonists on basal intracellular cAMP of planaria subjected to RNAi

Control RNAi: worms fed with bacteria expressing dsRNA of random gene (Control RNAi.Basal vs. Control RNAi.5HT, Control RNAi.8-OH-DPAT, and Control RNAi.mCPP) 5HT4-L RNAi: worms fed with bacteria expressing dsRNA of putative 5-HT4 Receptor (5HT-4 RNAi.Basal vs. 5HT-4 RNAi.5HT, 5HT-4 RNAi.8-OH-DPAT, 5HT-4 RNAi.mCPP). 5HT-4 *silenced* worms shows a dramatic decrease in intracellular cAMP levels ($P<0.0001$) stimulated by serotonin [10^{-4} M] compared to levels stimulated in control RNAi treated worms. 8-OH-DPAT on the other hand, shows no significant change in the extent of cAMP level stimulation after putative 5-HT knockdown (absence of receptor) compared to levels stimulated in controls (in the presence of receptor) suggesting an alternative receptor available for 8-OH-DPAT [10^{-4} M] action (5HT6/7). The inhibitory effects of mCPP [10^{-4} M] on cAMP were lessened in the absence of the putative receptor but had no significant effect on motility before and after RNAi (refer figure 3.1and 3.6 respectively).

Each bar represents the mean (plus S.E.M.) of [cAMP] from 9 samples (due to 3 samples, each triplicated) and experiments repeated 4 times.

S. mediterranea

D. tigrina
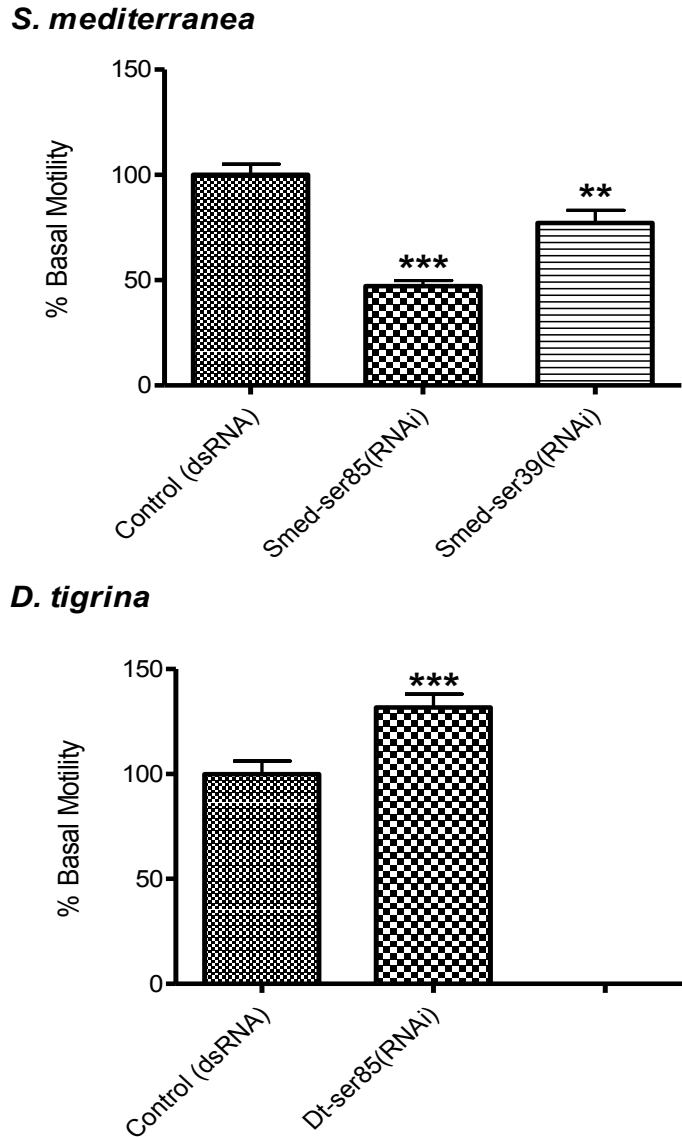
Figure 3.5 Effect of silenced putative serotonin receptors on planaria motility

Putative 5HT-4 GPCRs: *smed Ser85* and *smed Ser39* silenced in *S. mediterranea* by RNAi, resulted in significant decrease in motility ($P<0.0001$). Putative 5HT4 GPCR: *Dt-ser85* silenced in *G. tigrina* by RNAi, resulted in increased basal motility. Each bar represents the mean (plus S.E.M.) motility of 18 worms and experiments repeated 3 times.
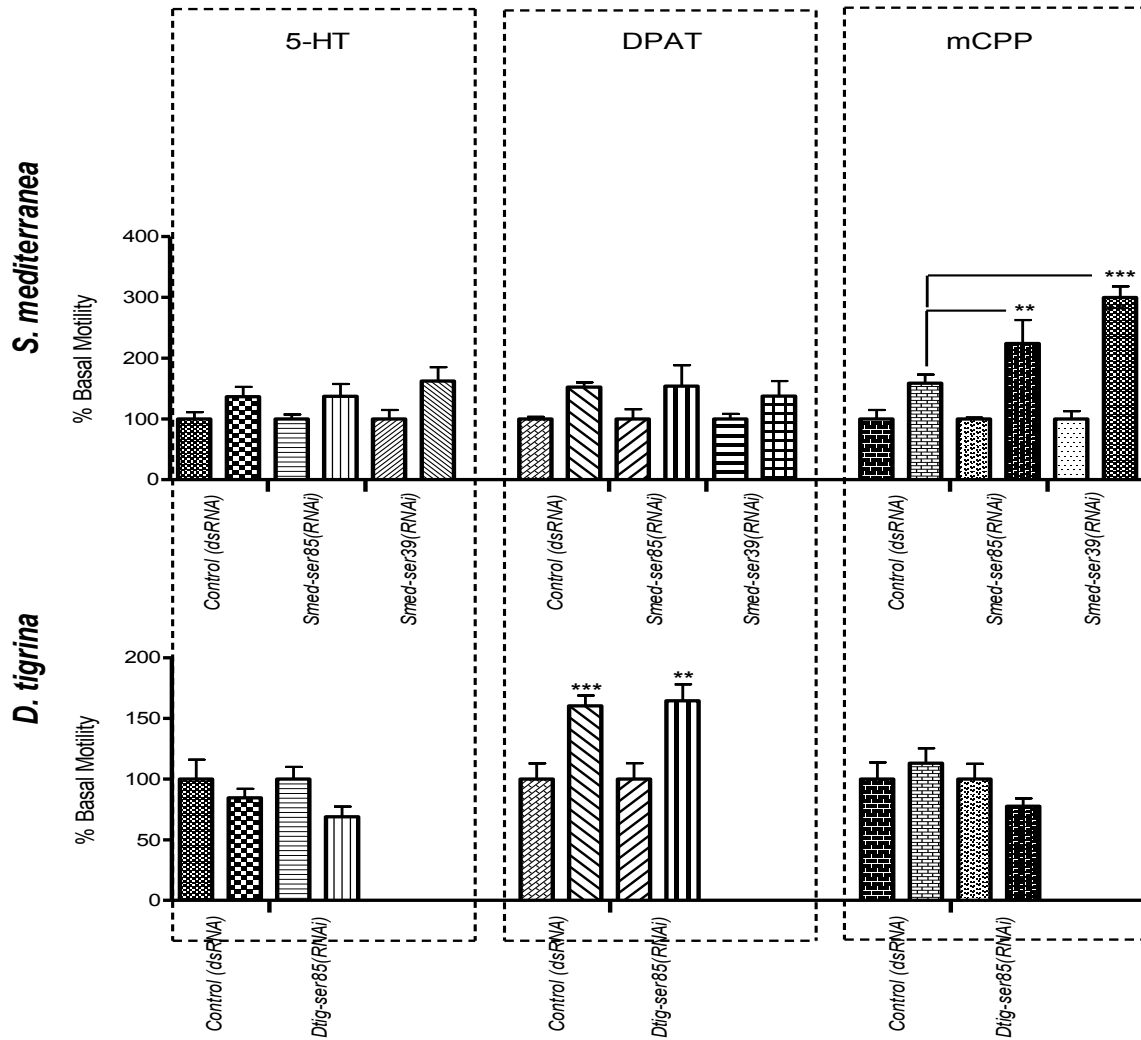
Figure 3.6 The absence of putative serotonin receptors affects the action of serotonin on worm motility

In *S. mediterranea*: silenced putative serotonin GPCRs: *smed-ser85* and smed-ser39 resulted in moderate decrease in the magnitude of both serotonin and 8-OH-DPAT stimulation of motility but enhanced that of mCPP. In *D. tigrina*: silenced putative serotonin GPCR, *D.tig-ser85* resulted in moderate increase on the magnitude of serotonin inhibitory action on the worm motility but no significant change in the effects of 8-OH-DPAT or mCPP. Each bar represents the mean (plus S.E.M.) motility of 18 worms and experiments repeated 3 times.
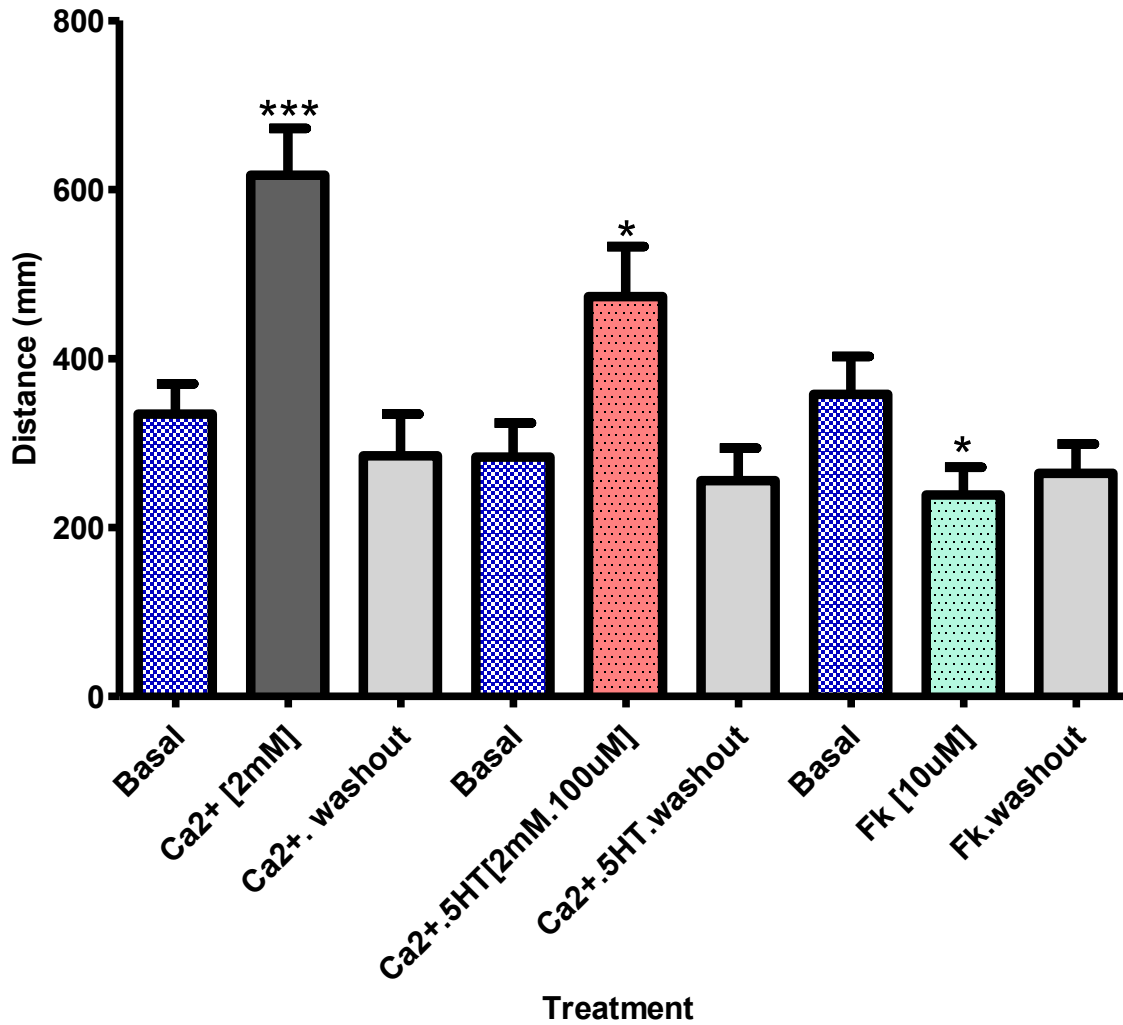
Figure 3.7 Effect of increasing Intracellular calcium on worm motility

Addition of 2 mM CaCl$_2$ resulted in a dramatic increase in worm motility (P<0.0001). Addition of 10μM forskolin (P<0.0001) mimicked 100 μM serotonin-induced moderate decrease in motility. Each bar represents the mean (plus S.E.M.) motility of 18 worms and experiments repeated 3 times.
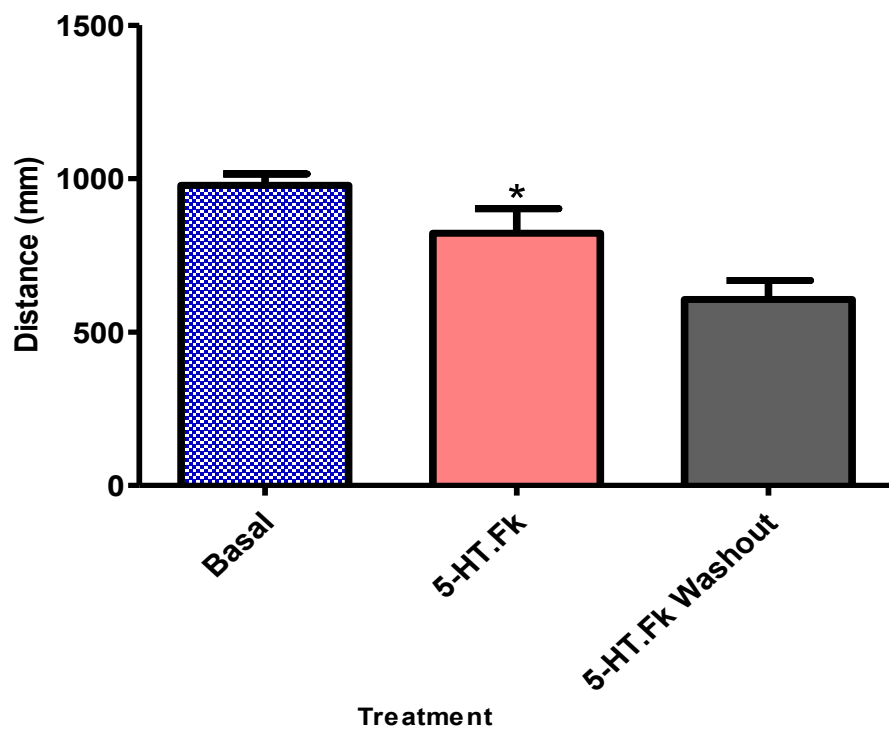
Figure 3.8 Effect of increasing Intracellular cAMP on worm motility

The co-addition of serotonin [10^{-4}M] and Fk [10μM], resulted in a decrease in *G. tigrina* motility to a magnitude not significantly different from that due to 5-HT alone. Each bar represents the mean (plus S.E.M.) motility of 18 worms and experiments repeated 3 times.

Table 3.1 Characterization of 5HT4 in Planaria

5HT Pharm in Mammals			5HT Pharm in *G. tigrina*		DEFINITIVE CHARACTERIZATION				
POTENTIAL GPCRs	AGONIST	COUPLING	COUPLING OBSERVED	POSS AVAI AND STIM.	EFFECT POST-RNAi ON:				
					CAMP	MOTILITY		CONCLUSION	Homology Elim
5HT1A	DPAT	Gi	Gs	N					
5HT1B/C	MCPP	Gi	Gi	Y	↑	↔		**Crosstalk**	
5HT2(A-C)	MCPP	Gq	↓Ca	N					
5HT3	Na$^+$, Ca^{2+}	LGNSCC	↓Ca	N					
5HT4,6,7	5HT	Gs	Gs	Y	↓	↑		Specific	**5HT4**
5HT5	5HT	Gs NEGATV	Gs POSTV	N					

Known agonists and G protein couplings of mammalian serotonin GPCRs even though might have different pharmacological profiles, serve as leads at this point in the streamlining of the functional role of a putative serotonin receptor among an array of characterized ones in planarians. In mammals, DPAT stimulation of 5HT1A results in adenylyl cyclase inhibition (G_i coupling) but resulted in the activation of adenylyl cyclase in *G. tigrina*. It eliminates (N) the possibility of 5HT1A stimulation in *G. tigrina* by DPAT. MCPP stimulation of 5HT1B/C in mammals result in the inhibition of adenylyl cyclase and the same is observed in *G. tigrina*. It accepts (Y) the possibility of 5HT1B/C stimulation in *G. tigrina* by MCPP. By definitive characterization, MCPP increased cAMP levels following RNAi but failed to have physiological bearing on the motility of the worm. It is concluded as crosstalk. MCPP stimulation of 5HT2 (A-C) in mammals results in intracellular calcium increase (G_q-coupled) but the reverse is true in *G. tigrina,* hence, eliminates (N) the possibility of preferential 5HT2 (A-C) stimulation in *G. tigrina* by MCPP. Serotonin stimulation did not result in increased intracellular calcium, hence, eliminates the possibility of 5HT3 stimulation. Exogenous $CaCl_2$ however, increased worm motility. The elimination criterion of 5HT3 at this point is also the fact that our putative serotonin receptor bears no homology to characterized mammalian 5HT3 receptors. Serotonin stimulation of mammalian 5HT5 results in the inhibition of adenylyl cyclase but the reverse is true in *G. tigrina*, eliminating the possible availability and stimulation of 5HT5 in this species. Serotonin stimulation of mammalian 5HT4, 6 and 7 results in the stimulation of adenylyl cyclase as observed in *G. tigrina*. However, based on sequence homology, coupled with characteristic worm response, our putative 5HT-R, *Dtig-ser85 (Gtig-ser85),* represents a 5HT4 receptor rather than 5HT6 and 7.

CHAPTER 4 CONCLUSION

This dissertation enumerates the step-by-step identification and characterization of a unique biogenic amine GPCR, as well as a catecholamine-responsive GPCR in the protozoan *Tetrahymena thermophila*. We demonstrated the biological relevance of the *Tetrahymena* receptor (TetEPI-1) as related to bacterial engulfment. Further, in the presence of serotonin, TetEPI-1 was shown to have been stabilized in inactive state, giving the course to suspect serotonin as its potential inverse agonist or antagonist. TetEPI-1 is a potential therapeutic target for selective manipulation of related pathogenic protozoa, especially considering the inverse agonism is the paradigm for antihistamine drugs that stabilize H1 receptors in the absence of histamine during chronic allergic responses of atopy.

Second, by means of alternative loss-of-function technique, this dissertation described the successful characterization of two novel serotonergic GPCR mediating locomotory events in two species of flatworms, *Girardia tigrina* (*Dtig.ser-85*) and *Schmidtea mediterranea* (*smed-ser85, smed-ser39*). Serotonin, by virtue of its dual effects, depending on its targets, demonstrated both cilio-excitatory and moderate cilio-inhibitory effect in these organisms. In the process of characterizing these receptors, this work established flatworm species differences in their response to serotonin. The suppression of these GPCRs demonstrated significant impairment in the motility of *S. mediterranea* while a decrease in cAMP also lessens the moderate inhibitory effects of serotonin on *G. tigrina* locomotion, a clear indication of the relevance of these receptors to these worms. These putative GPCRs compare with characterized 5HT4 receptors, hence, could be classified as planarian 5HT4 GPCRs, namely *Dtig-ser85, Smed-ser85 and Smed-ser39*.

It is worth mentioning that motile effects cannot be totally abolished because a myriad of serotonergic GPCRs possibly exist in these organisms and a specific suppression of one could have negligible effects. A look at the dynamics of second messenger levels (before and after RNAi) and worm locomotion, is tempting to generalize an inverse relation between levels of cAMP and the motility of *G. tigrina* such that increased cAMP levels are inhibitory to worm motility and vice versa.

In effect, this dissertation, by means of an alternative loss-of-function technique (RNAi) and the availability of known ligands for a range of receptors, coupled with transductional coupling determination, has established that the physiological function of a given receptor among a population of receptors can be streamlined. Following the targeting of a given receptor, the determination of G protein transduction coupling is an additional check on the validation of the intact actions of the non-targeted receptors based on response to known ligands and second messenger levels following their stimulation. The effect of these ligands on the phenotypes is yet an additional observation that confirms if the elimination of a target receptor resulting in a cross talk between existing receptors has any physiological bearing on the phenotype of the organism. Based on these observations, future studies first aimed at finding other adrenergic agents capable of agonizing or antagonizing TetEPI-1 and second, investigating the homolog of these characterized serotonergic GPCRs in the human parasite, *schistosoma mansoni*.

APPENDIX A   Novel RNAi-mediated approach to G protein-coupled receptor deorphanization: proof of principle and characterization of a planarian 5-HT receptor

A paper to be submitted

Mostafa Zamanian[1,2*], Prince Agbedanu[2], Nicolas J. Wheeler[2], Michael J. Kimber[1, 2], Tim A. Day[1, 2,*]

Abstract

GPCRs represent the largest known superfamily of membrane proteins extending throughout the Metazoa. There exists ample motivation to elucidate the functional properties of GPCRs given their role in signal transduction and their prominence as drug targets. In many organisms, these efforts are hampered by the unreliable nature of heterologous receptor expression platforms. We validate and describe an alternative loss-of-function approach for ascertaining the ligand and G protein coupling properties of GPCRs in their native cell membrane environment. Our efforts are focused on the phylum Platyhelminthes, given the heavy health burden exacted by pathogenic flatworms, as well as the role of free-living flatworms as model organisms for the study of developmental biology. RNA interference (RNAi) was used in conjunction with a biochemical endpoint assay to monitor cAMP modulation in response to the translational suppression of individual receptors. As proof of principle, this approach was used to confirm the neuropeptide GYIRFamide as the cognate ligand for the planarian neuropeptide receptor GtNPR-1, while revealing its endogenous coupling to $G_{\alpha i/o}$. The

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method was then extended to deorphanize a novel G_s-coupled planarian serotonin receptor, DtSER. A bioinformatics protocol guided the selection of receptor candidates mediating 5-HT-evoked responses. These results provide functional data on a neurotransmitter central to flatworm biology, while establishing the great potential of an RNAi-based deorphanization protocol. Future work can help optimize and adapt this protocol to high throughput platforms as well as other phyla.

Introduction

GPCRs have been the subject of intense research scrutiny due to their central role in eukaryotic signal transduction and their exploitability as drug targets (Flower, 1999; Wise et al., 2002; Lagerstrom and Schioth, 2008). Once identified, GPCRs undergo deorphanization, the process of pairing orphan receptors with their cognate ligands. Current approaches to GPCR deorphanization have severe limitations and are inefficient for large-scale projects. The predominant approaches all require the transient or stable heterologous expression of GPCRs in a surrogate cell system and in most cases, this expression occurs in cells derived from other species and phyla (Tate and Grisshammer, 1996; Mertens et al., 2004; Chung et al., 2008). This has introduced a significant bottleneck in the way of both the pharmacological and structural characterization of GPCRs (Tate and Grisshammer, 1996; McCusker et al., 2007).

The complex regulatory processes that guide the correct folding and export of receptors to the cell membrane (Sexton et al., 2001; Duvernay et al., 2005; Dong et al., 2007; Kobilka, 2007) are not necessarily well-conserved across cell lineages. In the event that a GPCR is successfully expressed on the surface of a host cell, the receptor must operate in conjunction with a foreign complement of accessory and signaling proteins. Further, the structural and functional integrity of receptors can be

altered by local membrane composition (Opekarová and Tanner, 2003; Pucadyil and Chattopadhyay, 2006). The exact post-translational requirements for proper receptor expression and function can vary greatly among receptors, making the task of identifying a suitable heterologous system a receptor-specific process of trial-and-error (Tate and Grisshammer, 1996). Although heterologous expression is not a theoretically challenging feat, individual targets routinely prove to be recalcitrant and consume inordinate effort. In view of these concerns, a simple receptor deorphanization method that could be applied in a native cell or membrane environment could side-step some of these concerns.

Flatworm GPCRs

The phylum Platyhelminthes houses prominent human pathogens as well as tractable model organisms. Flatworm GPCRs represent lucrative anthelmintic targets, as evidenced by the biological activities of their putative ligands (McVEIGH et al, 2005; Ribeiro et al., 2005) and the crucial biological functions of these receptors in other organisms (Wilkie, 2000; Keating et al., 2003). Signaling pathways associated with the GPCR superfamily have been identified as potential targets for life-cycle interruption of flatworm parasites (Fitzpatrick et al., 2009; Taft et al., 2010). The recent availability of platyhelminth genomic data (Berriman et al., 2009; Zhou et al., 2009; Robb et al., 2008) has led to the accumulation of a wealth of receptor and ligand data. A comprehensive in silico protocol revealed over 117 Schistosoma mansoni and 460 *Schmidtea mediterranea* GPCRs, which were classified using phylogenetic, homology-based, and machine-learning approaches (Zamanian et al., 2011). Bioinformatics and proteomics-based studies have similarly led to the expansion of the known set of putative GPCR ligands (McVeigh et al., 2009; Collins et al., 2010).

The pharmacological characterization of orphan flatworm receptors is likely to generate valuable drug discovery leads, while enhancing our understanding of basic receptor biology in this important phylum. Reliance on heterologous expression platforms has hampered efforts to implement functional assays to identify receptor agonists. Only a handful of flatworm GPCRs have thus far been deorphanized, with receptors expressed in such divergent cellular environments as CHO (Omar, 2007), HEK293 (Hamdan, 2002; Taman, 2009), COS7 (Hamdan, 2002), yeast (Taman, 2009; El-Shehabi, 2010), and Xenopus oocyte cells (Nishimura, 2009). We describe a relatively simple loss-of-function deorphanization approach that could be applied in a native cell or membrane environment. This alternative strategy could help catalyze a first-pass mapping of receptors and ligands in this and other phyla.

Inversing the paradigm: RNAi as a deorphanization tool

We validate an RNA interference (RNAi)-based method that allows receptors to undergo deorphanization without the need for full-length cloning and transport to a heterologous expression system. In principle, a collection of putative ligands are screened against membrane preparations to evaluate their effects on second-messengers downstream of GPCR activation. RNAi is then used to assay whether observed responses can be altered or abolished by the knockdown of individual receptors from the membrane preparations. A successful "hit" confirms expression of a given receptor, functionally pairs the receptor with a given ligand, and couples the receptor with a specific G protein signaling pathway. Bioinformatics approaches can be used to help identify receptors as putative targets for a particular ligand, or conversely, to narrow the list of potential ligands for a given receptor.

The primary biochemical endpoints of GPCR activation are typically assayed by recording agonist-evoked changes in cAMP ($G\alpha s$ and $G\alpha i/o$) or Ca^{2+} ($G_{\alpha q}$) levels. A variety of established labeling and detection schemes (e.g. fluorescent, luminescent, and radioisotope) are available for these second messengers (Thomsen et al., 2005). In this study, we focus our efforts on the $G\alpha s$ and $G\alpha i/o$ pathways and employ a radioimmunoassay (RIA) for cAMP detection. Monitoring adenylyl cyclase modulation of cAMP allows us to examine two of the three major GPCR activation endpoints.

While this loss-of-function approach limits pharmacological analysis, it is likely adaptable to high throughput platforms and can serve as an efficient ligand-receptor mapping tool for certain receptor classes. It should be noted that ligands and receptors can display pharmacological promiscuity. Ligands can act through more than one receptor and receptors can respond to more than one ligand, with a range of affinities. Further, receptors responsive to a given ligand do not necessarily share the same G protein coupling profile and are likely to be expressed in different abundances. However, this approach only concerns itself with the contribution of individual receptors to differences between control and RNAi response profiles. The scale and directionality of these differences provide information relevant to ligand responsivity and G protein coupling, respectively. The basic logic of this deorphanization strategy is outlined in figure 1.

Materials and methods

Planarian maintenance

Dugesia tigrina (Ward's Natural Science, Rochester, NY) colonies were maintained in the laboratory in aerated spring water on a regular feeding cycle (3 times per week). Planaria were randomly selected and isolated in approximately 50-worm groupings for RNAi feeding cycles and cAMP assays.

RNA interference

Primer3 (Untergasser et al., 2007) was used to select primers to selectively amplify 400-600 bp fragments of GtNPR-1 and 5HT receptor candidate DtSER. BLAT (Kent et al., 2002) was used to help guard against potential off t-target effects of suppression triggers using the very nearly-related S. *mediterranea* genome. A 465 bp fragment of GtNPR-1 was amplified from a full length clone of Gt-NPR1 housed in pcDNA3.1 (+), with the primers 5'- TGGATCTTTCCAGCGACTCT-3' (forward) and 5'-ATGGTTCGTTCGACGTTTTC-3' (reverse). A 586 bp fragment of DtSER was amplified from D. *tigrina* cDNA isolated using RNAqueous (Ambion) and RETRoscript (Ambion), with a degenerate forward primer: 5'-GGKATGGAAGTATTTCTGGGRAT-3' (forward) and 5'-TGGCATCTTCTTG GGCCATATTTCT-3' (reverse). An RNAi control sequence was amplified from Aedis aegypti cDNA with primers 5'-AATGCCGGCCTGTTTCCTAT-3' (forward) and 5'-AGCATCCTTTTTCTTGTGCG-3' (reverse), corresponding to a putative odorant receptor (VectorBase id: AAEL013422 (Lawson et al., 2007). Second-round PCR was performed for each target sequence using the original gene-specific primers flanked by Gateway Cloning system (Invitrogen) recombination sites: 5'-GGGGattB1- 3' (forward) and 5'-GGGG-attB2-3' (reverse). Entry sequences were subcloned into the pPR244 (pDONRdT7) (Reddien, 2005) destination vector

with corresponding attP1 and attP2 recombination sites using BP Clonase II (Invitrogen). Clones were transformed into TOP10 Electrocompetent E. coli (Invitrogen) and sequence confirmed. RNAi vectors were introduced to HT115 (DE3) cells for transcription of dsRNA, followed by bacterial-mediated feeding per standard protocol.

Semi-quantitative RT-PCR

Total RNA was extracted from individual *D. tigrina* with RNAqueous (Ambion), followed by removal of DNA contaminants with TURBO DNase (Ambion). First strand cDNA synthesis was carried out with the RETROscript kit (Ambion), as part of a two-stage RT-PCR. PCR optimization was carried out with the Quantum RNA 18S Internal Standards kit (Ambion) per manufacturer instructions. 18S ribosomal RNA was used as an endogenous standard for normalizing measures of gene expression and reducing sample-to-sample variation. cDNA samples were used in parallel as templates for multiplex PCR with gene-specific and 18S rRNA primer pairs. PCR reaction products were visualized on 1.2% electrophoretic gel with the Kodak Gel Logic 112 imaging system, and amplicon intensities were analyzed with standard software to derive relative transcript abundances.

Membrane preparation

Planaria were washed twice with cold cAMP buffer containing 50 mM sucrose, 50 mM glycylglycine, 10 mM creatine phosphate, 2 mM MgCl2, 0.5 mM isobutylmethylxanthine (IBMX), 1 mM dithiothreitol (DTT), 0.02 mM EGTA, 10 units/ml creatine kinase, and 0.01% bovine serum albumin. Worms were kept on ice for 5 min and then homogenized on ice for 2 min with a Teflon homogenizer. This preparation was centrifuged at 5,000 X g for 5 min, with the pellet that included cell debris discarded. This centrifugation step was then repeated. The supernatant was centrifuged

at 40,000 X g for 30 min at 4°C. The supernatant was discarded, and the membrane-containing pellet was resuspended via sonication in cAMP buffer suplemented with 0.1 mM ATP and 0.1 mM GTP. Total suspension volume was set at 500 ul/sample, such that each sample would contain cell membranes from 3 worms. 500 ul aliquots of this membrane preparation correspond to individual reactions in the cAMP assay. Samples were incubated with various concentrations (and combinations) of forskolin and/or putative ligands (peptide or biogenic amine) at 37°C for 20 min to stimulate cAMP production. Forskolin and peptide ligands were dissolved in DMSO, with final reaction mixtures containing <0.1% DMSO. DMSO has no measurable effect on cAMP in this range (data not shown). Samples were centrifuged at 3,000 X g for 5 minutes after ligand incubation, and 400 ul of supernatant from each sample (3 samples per treatment) was transferred into a fresh tube for cAMP determination using radioimmunoassay.

cAMP determination

cAMP levels were measured with RIA as previously described (Richards et al., 1979) with minor modifications. 100 µl aliquots from each sample or known standard (standard curve range: 4 - 512 fmol cAMP) were acetylated and incubated overnight at 4°C with primary cAMP antibody (1:30,000) and cAMP [^{125}I] (approx 20,000 cpm). 100 µl of NRP (1:80,000) and secondary antibody (goat anti-rabbit IgG; 1:40,000) were added, followed by incubation at 25°C for 10 min. 100 µl of 50 % normal bovine plasma and 1 ml of ice-cold PEG were added to the scintillation vials. Samples were centrifuged at 3,000 rpm (4°C) for 20 min. The supernatants were aspirated and ^{125}I levels in the pellets were assayed via gamma counter (Packard, B5002). For a given experiment, each sample was assayed in triplicate.

Bioinformatics

HMMER-2.3.2 (Eddy et al., 1998) was used to build a profile HMM for invertebrate 5-HT receptors. Training sequences were procured from GPCRDB and aligned with Muscle 3.6 (Edgar et al., 2004). The profile HMM was constructed with hmmbuild and calibrated with hmmcalibrate. This model was used to search a curated dataset of putative S. *mediterranea* receptors with hmmpfam. The resulting matches were ranked by E-value and the top 20 full-length hits were further examined. Putative hits were matched with their nearest-related S. mansoni and *D. japonica* homologs, and also searched against the NCBI nr database with BLASTp. Maximum parsimony phylogenetic analysis was carried out with the Phylip 3.6 (Retief et al., 2000) package.

Statistical analysis

In cases where a ligand had an overall inhibitory effect on Fk-stimulated cAMP, basal cAMP levels were set as a baseline for individual RIA experiments and cAMP values were normalized with respect to the level of Fk-stimulated cAMP (set at 100%). In cases where a ligand had an overall stimulatory effect on cAMP, cAMP values were normalized with respect to basal cAMP (set at 100%). This allowed us to join datasets from repeated experiments with differing basal cAMP levels due to variance in the quality and yield of individual membrane preparations. One-way analysis of variance (ANOVA) was used with Tukey's post hoc test for multiple comparison analysis of cAMP levels associated with different treatments, for both normalized and raw values. Significances are reported at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

Results and discussion

cAMP assay optimization and ligand screen

A cell membrane preparation protocol was adapted (Humphries et al., 2004) and optimized for planaria, and used to generate samples for treatment with putative GPCR ligands. The downstream effects of ligand incubation on cAMP levels were monitored using a cAMP RIA. A screen was first carried out on *Dugesia* (*Girardia*) *tigrina* membrane preparations with a small number of peptides and biogenic amines. These ligand classes are prominent in platyhelminth biology (Mcveigh et al., 2005; Ribeiro et al., 2005; McVeigh et al., 2009; Collins et al., 2010), and there is a strong likelihood that a subset signal through one or more receptors coupled to either the Gαs or Gαi/o pathways. This would presumably be made apparent by stimulation of basal cAMP levels or inhibition of forskolin (Fk)-stimulated cAMP levels (Insel et al., 2003) as measured by RIA, respectively.

Included in this initial screen were the only two ligands definitively coupled to planarian GPCRs: the neuropeptide GYIRFamide and the biogenic amine serotonin (5-HT; 5-hydroxytryptamine). It was a reasonable assumption that both GYIRFamide and 5-HT would modulate cAMP levels in a whole organism membrane preparation. The *D. tigrina* receptor GtNPR-1 was previously deorphanized, showing a potent dose-dependent response to the neuropeptide GYIRFamide in mammalian cell culture (Omar et al., 2007). Chimeric G proteins ($G_{\alpha qi5}$ and $G_{\alpha qo5}$) were used to divert downstream GtNPR-1 signaling through the $G_{\alpha q}$ pathway, suggesting this receptor is $G_{\alpha i/o}$-coupled in its native environment.

More recently, a *Dugesia japonica* 5-HT GPCR has been deorphanized using Xenopus laevis oocytes (Nishimura et al., 2009) and there is long-established evidence of 5-HT stimulation of cAMP in both S. mansoni (Kasschau et al., 1982; Estey et al., 1987) and other planarian species (Cret`I et al., 1992), suggesting that 5-HT acts through one or more Gs-coupled GPCRs.

Alongside GYIRFamide and 5-HT, we included neuropeptide F (NPF) and octopamine as putative ligands. NPF has been shown to inhibit Fk-stimulated cAMP production in membranes isolated from *S. mansoni* (Humphries et al, 2004). Given the identification of planarian NPF homologues (McVeigh et al., 2009; Collins et al., 2010), we hypothesized that this peptide would have a similar inhibitory effect on cAMP levels. The results of this primary screen show that 10^{-5}M 5-HT drastically stimulates cAMP production, while 10^{-4}M GYIRFamide, 10^{-4}M NPF, and 10^{-4}M octopamine inhibit Fk-stimulated cAMP accumulation in *Dugesia* membrane preparations (figure A.2) to varying degrees. These changes in [cAMP] can be viewed as the additive response profile of each ligand.

We chose to first pursue the response profiles of GYIRFamide, provided that GtNPR-1 is a known target of GYIRFamide in *D. tigrina*. As proof of principle, we investigated whether or not this would be apparent using this loss-of-function assay. Given that the inhibition of adenylate cyclase by GYIRFamide is less potent than that brought on by NPF; this also serves as a more difficult trial for validation of assay sensitivity.

Coupling cAMP assay with RNAi: GtNPR-1 proof of principle

Establishing RNAi-mediated receptor suppression

Double-stranded (ds) RNA was introduced to isolated *D. tigrina* colonies using a bacterial-mediated feeding protocol. Planaria were randomly selected, isolated into treatment groups, and fed either non-flatworm control dsRNA or GtNPR-1 dsRNA. A two-week RNAi feeding cycle consisted of four evenly spaced feedings, followed by a four-day starvation period. Semi-quantitative RT-PCR was used to confirm gene knockdown. A small number of planarians were randomly selected from both experimental and control groups to assay GtNPR-1 suppression, and the remaining planarians were used for membrane assays. Significant GtNPR-1 knockdown (> 80%) is consistent and apparent in the experimental group, while GtNPR-1 expression remains robust in the control group (figure A.3) relative to endogenous standard.

Deorphanization via omparison of response profiles

Membranes were prepared from both control and GtNPR-1 dsRNA-fed planarians, and treated with Fk (10^{-4}M), GYIRFamide (10^{-4}M), and Fk (10^{-4}M) + GYIRFamide (10^{-4}M). RIA was used to assay cAMP levels corresponding to these treatments. Comparison of the response profiles reveals near-complete abolishment of GYIRFamide-evoked inhibition of Fk-stimulated cAMP in the GtNPR-1 knockdown group (figure A.4, Table A.1). Overall, GYIRFamide reduces Fk-stimulated cAMP production by an average of approximately 30% in the control group, and this inhibition was completely abolished by the suppression of GtNPR-1 expression in the RNAi group. These results confirm that GtNPR-1 is agonized by GYIRFamide and further establish that this receptor is natively coupled to the $G_{\alpha i/o}$ signaling pathway.

In silico target selection

The two ligands that most drastically stimulated and inhibited adenlyate cyclase activity in our primary ligand screen were 5-HT and NPF, respectively. We decided to focus on 5-HT in an attempt to deorphanize a Gαs-coupled receptor. To identify and rank 5-HT receptor candidates, a profile HMM was built with sequences procured from GPCRDB (Horn et al., 2003). Training was focused on 62 full-length invertebrate 5-HT and 5-HT-like receptors. This model was used to search against *S. mediterranea* GPCR sequence datasets (Zamanian et al., 2011) and the results were ranked by E-value. The top 20 receptor candidates were used as BLASTp (Altschul et al., 1990) queries against the NBCI "nr" database. This was used to identify receptors displaying 5-HT receptor homology, and to filter against receptors that displayed a non-specific range of biogenic amine receptor-related homology.

Receptors that survived this filter were compared to their nearest-related *S. mansoni* and *D. japonica* homologs (Table A.2). While the bioinformatics evidence suggests multiple receptor targets for 5-HT, we narrowed our list to the best-conserved receptors between parasitic and free-living flatworms and used degenerate PCR to amplify a putative 5-HT receptor from *D. tigrina*. The selection strategy is outlined in Table A.2. The amplified receptor is labeled DtSER and maximum parsimony phylogenetic analysis places this receptor among a group of putative free-living and parasitic flatworm 5-HT receptors that are significantly diverged from those found in other phyla (figure A.5).

RNAi-based deorphanization of planarian 5-HT receptor

DtSER transcript expression was confirmed via PCR, and knockdown was elicited following the protocol described for GtNPR-1. Similar levels of transcript knockdown were obtained (figure A.6). Membranes from control and DtSER dsRNA-fed worms were isolated and treated with 5-HT (10^{-4}M). The response profiles reveal a significant decrease (> 30%) in 5-HT evoked cAMP stimulation in the DtSER RNAi preparations compared to the control preparations. Just as with the neuropeptide receptor knockdown experiments, basal cAMP levels did not differ between control and experimental groups (figure A.7, Table A.3). These results represent the successful deorphanization of DtSER in its native membrane environment. DtSER responds to 5-HT and is coupled to the Gαs pathway. Serotonin receptors are implicated in motility and regeneration due to the phenotypic effects of serotonin in this phylum (Farrell et al., 2008; Saitoh and Yuruzume, 1996). Given that this receptor mediates significant increases in cAMP levels in response to serotonin, it is likely involved in these or other important physiological processes.

Conclusions

This study shows the utility of combining RNAi with biochemical endpoint assays to as a means of deorphanizing GPCRs in their native membrane environment. The approach was first validated using the only deorphanized flatworm neuropeptide GPCR (GtNPR-1), confirming agonism by GYIRFamide while providing information about its endogenous G protein coupling profile. The orphan *D. tigrina* GPCR DtSER was shown to respond to 5-HT, revealing its endogenous G protein pathway and illustrating the utlity of applying an in silico strategy to candidate receptor selection. While these loss-of-function strategy side-steps some of the concerns and difficulties associated with heterologous GPCR expression, there is significant room for improving both the sensitivity and

scalability of this assay. The heavy tissue requirements of the membrane preparation protocols employed introduce a potential rate-limiting step. Further optimizations of membrane or whole cell preparation protocols in this phylum could allow for more efficient and robust pharmacological analysis. This assay could conceivably be adapted to higher-throughput platforms, and extended to include GPCRs that signal through the $G_{\alpha q}$ pathway. Conveniently, establishing receptor-specific RNAi in planaria allows for the accumulation of loss-of-function phenotypic data in parallel to pharmacological data. In this regard, the study of planarians can inform flatworm parasite biology. Biasing the receptor and ligand pool to those best conserved between parasitic and free-living flatworms could shed light on new targets for chemotherapeutic intervention.

Author contributions

MZ, TAD and MJK conceived this study. MZ and PA carried out the experiments. MZ and NJW performed the bioinformatics and statistical analysis. MZ, TAD and MJK contributed to the writing of the manuscript.

Figures

Profile	NT	L	Fk	Fk+L
RNAi-Control	A	B	C	D
RNAi-R	A'	B'	C'	D'

- Apply Ligand (L) to membrane or cell preparation to generate control profile.

I. If $B > A$, L acts through one or more Gαs-coupled receptors.

II. If $D < C$, L acts through one or more Gαi-coupled receptors.

- Apply in silico protocol to predict L-responsive GPCR (R).

- Suppress expression of R with RNAi to generate comparative profile.

- Normalize cAMP data.

I. For stimulatory L: $x* = x/\bar{A}$ and $x* = x/\bar{A}'$

 L: $x* = (x - \bar{A})/\bar{C}$ and $x* = (x - \bar{A}')/\bar{C}'$

- Analyze cAMP data.

I. If $B'* < B*$, R is L-responsive and Gαs coupled.

Alternatively, If $B'* > B*$, R is L-responsive and Gαi coupled.

II. If $D'* > D*$, R is L-responsive and Gαi coupled.

Alternatively, If $D'* < D*$, R is L-responsive and Gαs coupled.

Figure A.1 Logic of RNAi-based deorphanization experiment.

The general set of experimental outcomes for an RNAi-based deorphanization experiment focused on the Gαs and Gαi pathway are shown. Letters $A - D$ and $A0 - D0$ each represent cAMP datasets for particular treatment conditions. Potential results are described with respect to the notion that a given ligand may act on multiple GPCRs that are not necessarily coupled to the same G protein (Gαs

or Gαi). Abbreviations: NT, no treatment; Fk, forskolin; L, ligand; R, receptor; RNAi-control, control membrane preparation; RNAi-R, R-suppressed membrane preparation; x, cAMP measurement variable. Asterisks (*) are used to denote normalized data.
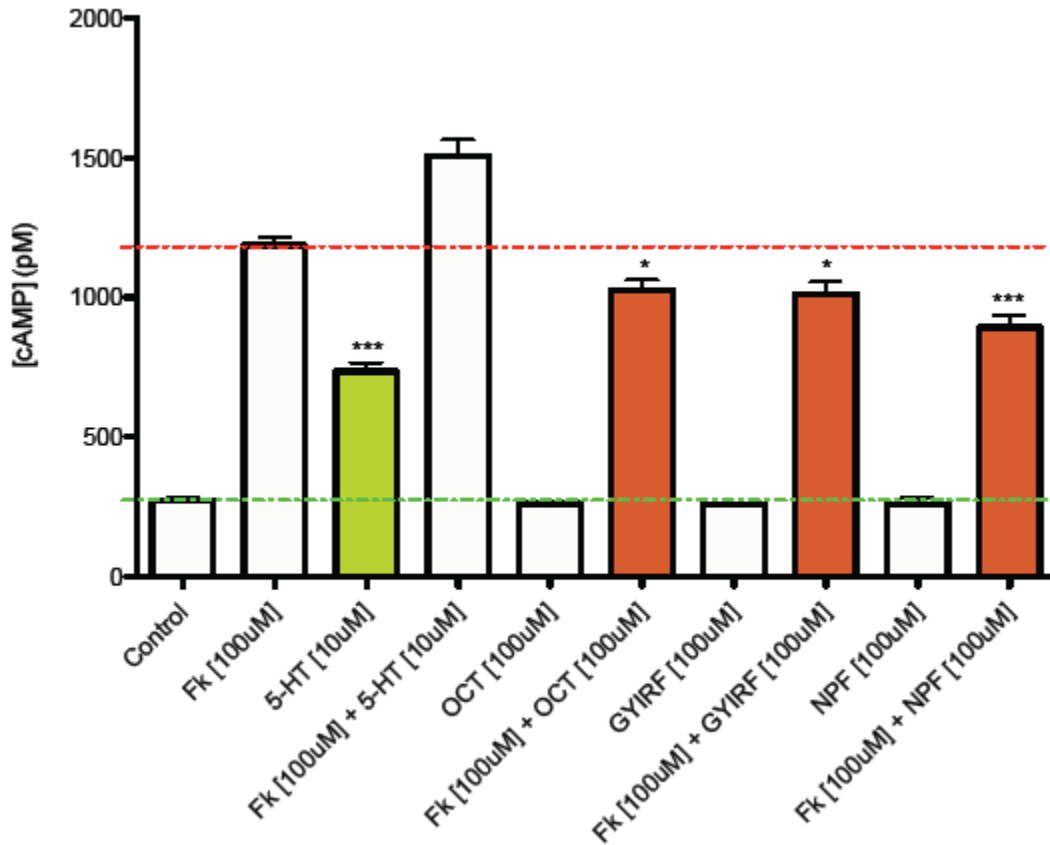
Figure A.2 Peptide and biogenic amine ligand cAMP screen performed against isolated *D. tigrina* membranes

RIA cAMP outputs are shown as mean ± SEM, and asterisks represent statistically significant differences compared with either control or treatment with Fk alone; * $P < 0.05$, *** $P < 0.001$, one-way ANOVA, Tukey post hoc test. Red bars are compared with Fk treatment: octopamine (OCT), GYRIFamide (GYIRF), and neuropeptide F (NPF) all inhibit Fk-stimulated cAMP at 100 uM. The green bar is compared with the control condition: serotonin (5-HT) stimulates basal cAMP. These

changes in cAMP are likely GPCR-mediated, and should therefore be altered in a ligand-specific manner by subtraction of particular receptor targets from cell membranes via RNAi.
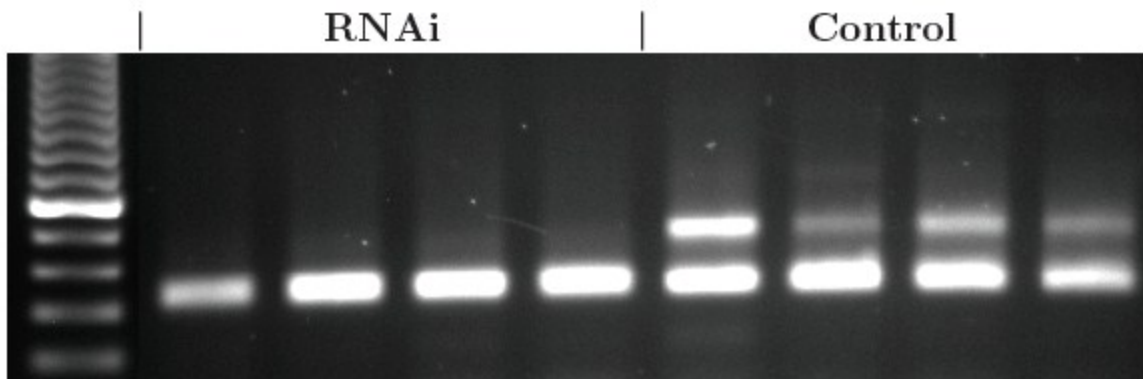
Figure A.3 Semi-quantitative PCR reveals GtNPR-1 knockdown

Lane 1 is a 100 bp DNA ladder; lanes 2-5 represent individual GtNPR-1 dsRNA-fed planarians, and lanes 6-9 represent control dsRNA-fed planarians. The bottom band (~300 bp) is the 18S internal standard, and the top band (~400 bp) shows GtNPR-1 expression. The top band disappears in the experimental group, confirming near abolishment of receptor expression in these worms. Relative band intensities (GtNPR-1/18S rRNA) for GtNPR-1 RNAi group: 0.44 ± 0.15. Relative band intensities for control group (band location manually selected): 0.08 ± 0.02. This corresponds to > 80% knockdown of GtNPR-1 transcript.
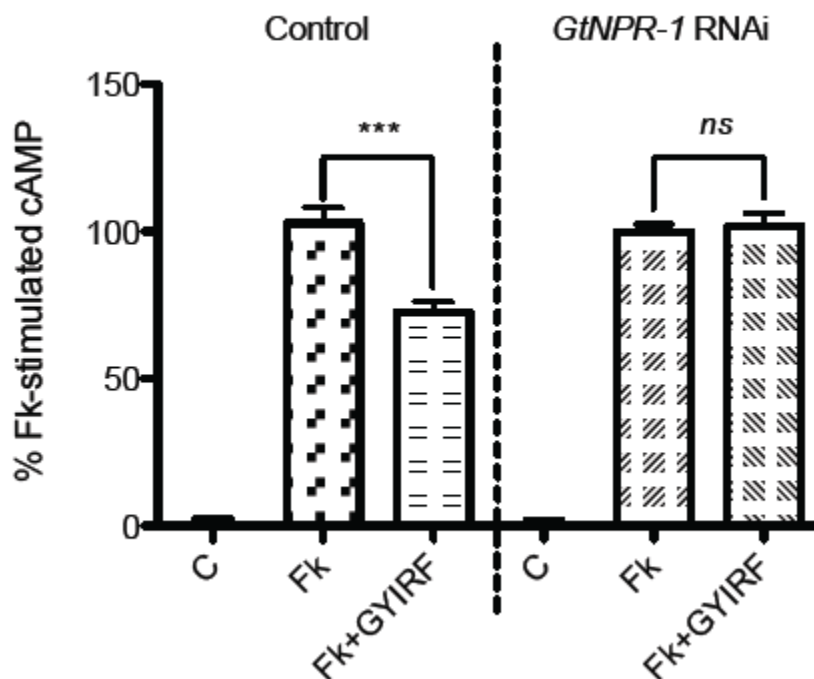
Figure A.4 RNAi-based GtNPR-1 deorphanization

Treatment groups are Control (control dsRNA) and GtNPR-1 RNAi (GtNPR-1 dsRNA). Treatments are C (control), Fk (10^{-4} M forskolin), and Fk + GYIRF (10^{-4} M forskolin and 10^{-4} M GYIRFamide). Each bar is the mean (± SEM) of 3 individual experiments. Basal cAMP levels were set as a baseline for each individual experiment, and cAMP values were normalized with respect to the level of Fk-stimulated cAMP (set at 100%). This allowed us to join datasets with differing basal cAMP levels, due to variance in the quality and yield of individual membrane preparations. Analysis of the raw cAMP values of individual experiments renders the same results (Table 1). Asterisks indicate significance at $P < 0.001$ (***), and "ns" indicates no significant difference (one-way ANOVA, Tukey post hoc test).
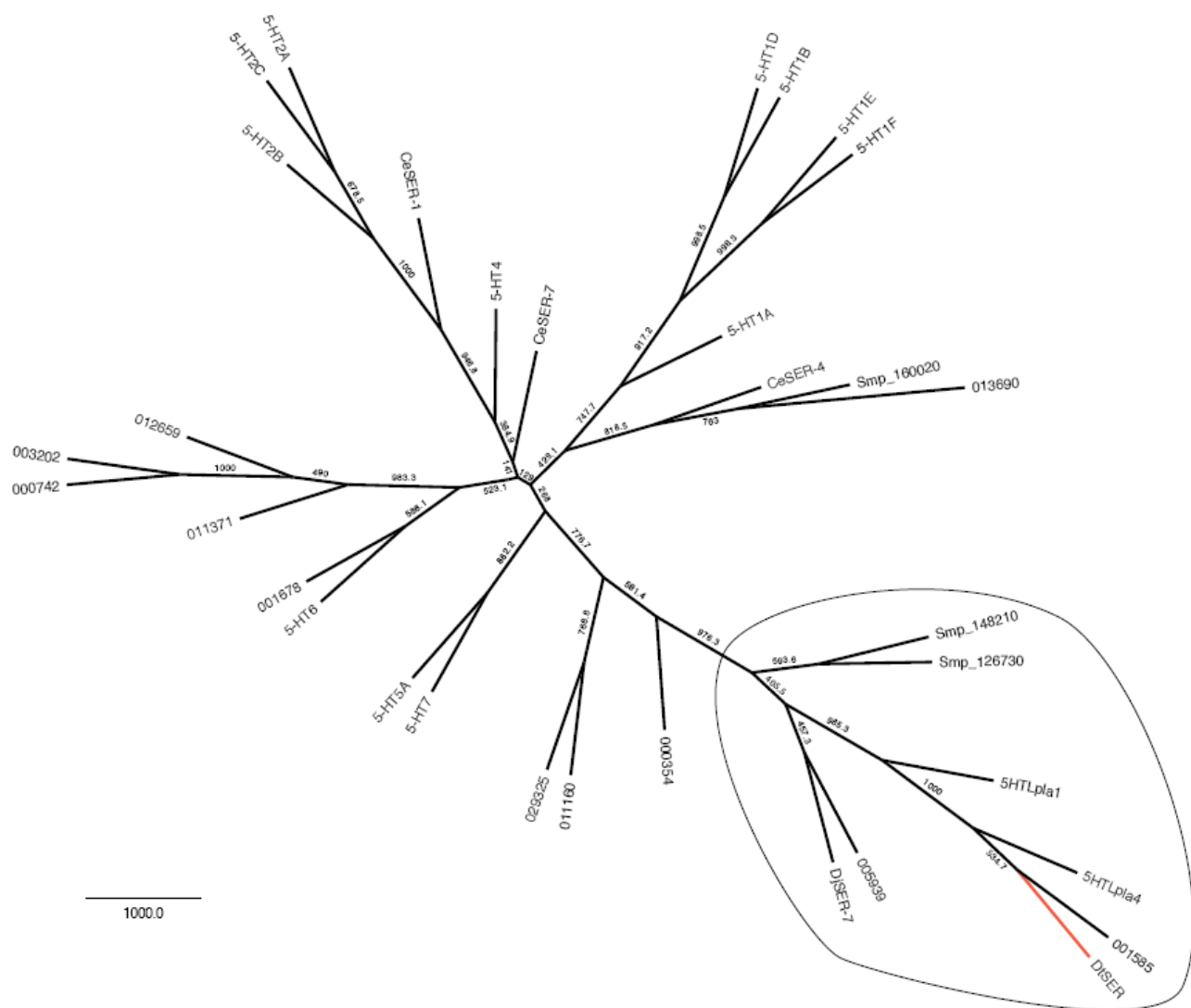
Figure A.5 Maximum parsimony tree of serotonin receptors

Phylogenetic analysis was performed using planarian (*S. mediterranea* and *D. japonica*), parasite (*S. mansoni*), human and C. elegans 5-HT receptors and putative 5-HT receptors. TM domains I-VII were extracted from the alignment for bootstrapping (bootstrap value = 1000). Outlined receptors are significantly diverged from vertebrate and ecdysozoan serotonin receptors. DtSER (red) was amplified using a degenerate PCR strategy and was chosen to undergo RNAi-based deorphanization.
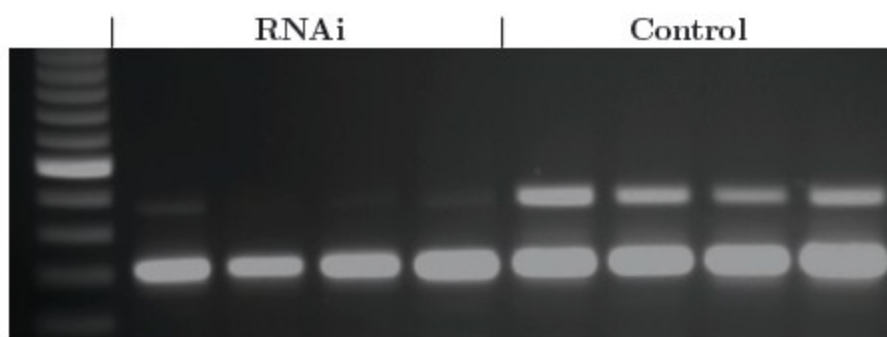
Figure A.6 Semi-quantitative PCR reveals DtSER knockdown

Lane 1 is a 100 bp DNA ladder; lanes 2-5 represent individual DtSER dsRNA-fed planarians, and lanes 6-9 represent control dsRNA-fed planarians. The bottom band (~300 bp) is the 18S internal standard and the top band (~480 bp) shows DtSER expression. The top band disappears in the experimental group, confirming near abolishment of DtSER receptor expression in these worms.
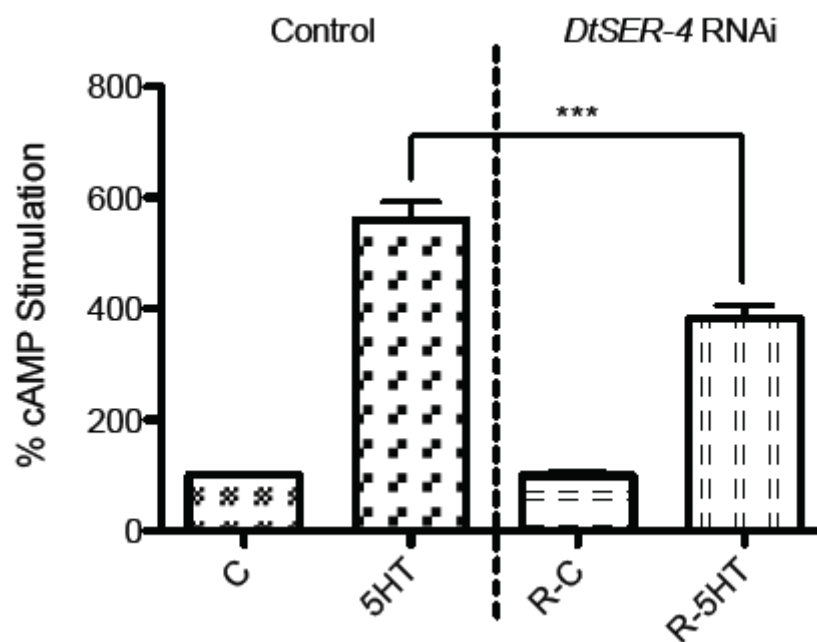
Figure A.7 RNAi-based *DtSER* deorphanization

Treatment groups are Control (control dsRNA) and DtSER RNAi (DtSER dsRNA). Treatments are C (control) and 5-HT (10^{-4} M). Each bar is the mean (± SEM) of 3 individual experiments. cAMP

levels were normalized to basal cAMP levels (set at 100%), and the datasets were joined. DtSER knockdown corresponds to significantly decreased cAMP stimulation (~32%) in response to 5-HT. Analysis of the raw cAMP values of individual experiments renders the same results (Table 3). Asterisks indicate significance at $P < 0.001$ (***), and "ns" indicates no significant difference (one-way ANOVA, Tukey post hoc test).

Tables

Table A.1   RNAi-based GtNPR deorphanization cAMP raw values

EXP	Treatment	Control	*GtNPR1* RNAi
	C	62.05 ± 2.46	60.83 ± 1.91
1	Fk	102.45 ± 4.06	101.47 ± 1.59
	Fk + G	85.02 ± 1.59 ***	103.03 ± 4.27 [ns]
	C	27.88 ± 0.97	33.54 ± 1.27
2	Fk	57.37 ± 2.68	58.78 ± 1.64
	Fk + G	48.67 ± 1.23 **	57.89 ± 0.93 [ns]
	C	81.49 ± 4.06	55.16 ± 1.60
3	Fk	215.96 ± 10.99	129.79 ± 3.61
	Fk + G	195.63 ± 6.17 **	132.60 ± 4.62 [ns]

RIA-determined cAMP values (pM) are provided for three separate experiments (mean $\pm$ SEM). Treatments: C (control), Fk (Forskolin), Fk + G (Forskolin + GYIRFamide). The amount of isolated membrane differs between experiments, as evidenced by basal cAMP levels. This is in part due to differences in the size, number, and feeding behavior of worm batches used for membrane isolation. Analysis (one-way ANOVA, Tukey) of these raw datasets establishes abolishment of cAMP inhibition brought on by GYIRFamide associated with GtNPR-1 suppression. For each experimental grouping, Fk is compared to Fk + G. Asterisks indicate significance at $P < 0.001$ (***), $P < 0.01$ (**), and "ns" means no significant difference.

Table A.2   5-HT receptor candidate selection

S. mediterranea	HMM	BLAST	TM	S. mansoni	D. japonica
mk4.013690.00.01	2.90E-108	+	6	Smp160020 | 8E-45 (157)	
mk4.005939.01.01	**3.90E-85**	**+**	**6**	**Smp148210 | 8E-64 (195)**	**DjSER-7 | 3E-73 (364)**
mk4.011371.00.01	1.20E-71	−	7		
mk4.001585.00.01	3.00E-70	+	5	Smp126730 | 9E-65 (258)	5HTLpla4 | 1E-141 (310)
mk4.007388.02.01	9.60E-69	+	5		5HTLpla1 | 1E-127 (298)
mk4.029325.00.01	2.30E-65	−	7		
mk4.000656.10.01	1.10E-61	−	7		
mk4.004462.02.01	5.10E-51	+	5		DjSER-7 | 1E-115 (227)
mk4.011006.00.01	3.30E-50	+	5		
mk4.003202.01.01	1.10E-49	−	7		
mk4.000943.09.01	1.80E-48	+	5	Smp149770 | 2E-31 (113)	
mk4.000354.16.01	1.70E-47	−	7		
mk4.012659.00.01	5.60E-46	−	7		
mk4.011160.01.01	2.90E-45	−	7		
mk4.000742.09.01	5.00E-44	−	7		
mk4.001678.03.01	1.20E-40	−	7		
mk4.010158.01.01	1.60E-38	+	5		
mk4.013827.00.01	7.80E-35	+	5	Smp126730 | 1E-59 (176)	
mk4.001587.06.01	**1.60E-29**	**+**	**5**	**Smp148210 | 8E-63 (179)**	**5HTLpla1 | 2E-63 (234)**
mk4.000526.00.01	3.60E-28	+	6		
mk4.017426.00.01	-	−	5		
mk4.017583.00.01	-	+	5		
mk4.012214.00.01	-	+	3		
mk4.011860.00.01	-	+	3		
mk4.012270.00.01	-	+	3		
mk4.013819.05.01	-	+	3	Smp149770 | 2E-34 (135)	
mk4.010946.00.01	-	+	5		

5-HT profile HMM hits are ranked by E-value for S. *mediterranea*. Additional sequences were appended via homology searches. This putative list of planarian 5-HT receptors was searched against the NCBI nr database using BLASTp. Receptors that exclusively showed serotonin-related homology in their top returned hits are marked with '+'. HMMTOP (Tusnady et al., 2001) was used to predict the number of TM domains for each sequence. Putative 5-HT receptors from S. mansoni (Zamanian et al., 2011) and D. *japonica* (Saitoh et al., 1997) were searched against the filtered HMM pool. The two nearest-related homologs for each of four S. mansoni receptors are shown, along with E-value and overlap length for each pairing. Similarly, the top pairings for each of three D. *japonica* receptors are shown. Three sequence clusters (bold) show high sequence conservation between parasite and planarian sequences. DjSER-7 has been previously deorphanized (Nishimura et al., 2009) and we therefore excluded this cluster from further consideration. Among the two remaining options, our choice of the sequence cluster highlighted in red is justified as follows: 1) the planarian sequences in this grouping share the highest level of sequence identity with their parasite

sequelog, 2) the presence of two closely-related planarian sequences improves the likelihood of success for degenerate PCR as a strategy to amplify the D. *tigrina* homolog, and 3) deorphanization of a receptor in this cluster will assign a pharmacological identity to a novel subset of GPCRs.

Table A.3   RNAi-based *DtSER* deorphanization cAMP raw values

EXP	Treatment	Control	*DjSER* RNAi	Sig	Δ cAMP stimulation
1	C	29.43 ± 1.26	30.43 ± 2.56	ns	
	5-HT	153.29 ± 4.89	99.18 ± 2.26	***	-37%
2	C	129.33 ± 4.57	114.29 ± 13.22	ns	
	5-HT	498.56 ± 39.86	324.32 ± 42.00	**	-26%
3	C	14.71 ± 0.91	18.02 ± 1.040	ns	
	5-HT	113.18 ± 4.42	96.42 ± 1.69	***	-31%

RIA-determined cAMP values (pM) are provided for three separate experiments (mean ± SEM). Treatments: C (control) and 5-HT (serotonin). Analysis (one-way ANOVA, Tukey) of these raw datasets establishes a significant decrease in 5-HT mediated cAMP stimulation associated with *DjSER* suppression. For each experiment, Control groups and 5-HT treated groups are compared between Control RNAi and *DjSER* RNAi conditions. Asterisks indicate significance at $P < 0.001$ (***), $P < 0.01$ (**), and "ns" means no significant difference.

APPENDIX B   Deorphanization of a bacterial lipopolysaccharide-recognizing G protein-coupled receptor in *Entamoeba histolytica*

A paper to be submitted

Matthew T. Brewer*, Prince N. Agbedanu*, Steve A. Carlson[1,2]

Abstract

Entamoeba histolytica is the causative agent of amebic dysentery, a worldwide protozoal disease that results in approximately 100,000 deaths annually. The virulence of *E. histolytica* may be due to interactions with host bacterial flora whereby trophozoites engulf colonic bacteria as a nutrient source. The engulfment process depends on trophozoite recognition of bacterial epitopes that activate phagocytosis pathways. EhGPCR-1 was previously recognized as a putative GPCR used by *Entamoeba histolytica* during engulfment of bacteria. In the present study, we attempted to deorphanize EhGPCR-1 using a heterologous GPCR yeast system. We determined that bacterial lipopolysaccharide (LPS) serves as an agonist for EhGPCR-1 and that LPS stimulates EhGPCR-1 in a concentration-dependent manner. Additionally, we demonstrated that *Entamoeba histolytica* prefers to engulf bacteria with intact LPS. Thus EhGPCR-1 is an LPS-recognizing GPCR that is a druggable target for treating amebiasis, especially considering the well-established druggability of GPCRs.

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Introduction

Entamoeba histolytica is the causative agent of amebic dysentery, a worldwide protozoal disease that results in approximately 100,000 deaths annually (WHO, 1997). Infection with *E. histolytica* often manifests as colitis but trophozoites can also gain access to the systemic circulation resulting in liver or brain abscesses (Fotedar et al., 2007; Petri and Singh, 1999). The majority of infections are asymptomatic (Huston, 2004) and many of the host factors determining the outcome of infection have not been well characterized.

The virulence of *E. histolytica* may be due to interactions with host bacterial flora. Co-culture with bacteria can restore amoebic virulence in *Entamoeba* cell lines attenuated through serial passage (Bos and Van de Grind, 1977; Wittner and Rosenbaum, 1970) and this effect is related to an up-regulation of genes associated with enhanced phagocytosis (Debnath et al., 2007). Specifically, *E. histolytica* exhibits enhanced adherence and cytotoxic abilities following engulfment of enteropathogenic bacteria (Galvan-Moroyoqui, 2008; Hirata, 2007) Furthermore, phagocytic ability is essential for the pathogenesis of amebiasis and strongly correlated with virulence especially considering that *E. dispar*, a non-pathogenic species, is not able to engulf cells (Hirata, 2007; Rodriguez, 1986).

E. histolytica is also dependent on colonic bacteria as a nutrient source for trophozoites. Prior to the advent of selective medium, trophozoites could only be grown in culture medium containing bacteria (Diamond, 1978). Although trophozoites are now routinely grown in bacteria-free culture medium, exposure to *E. coli* enhances their growth kinetics (Galvan-Moroyoqui et al., 2008).

Phagocytosis is a stepwise process that is initiated by activation of receptors that bind an extracellular target and proceed to activate cytoskeletal rearrangements. While many studies have established the importance of bacterial engulfment by *E. histolytica*, the specific protozoan cell surface receptors that recognize bacteria have not been identified.

In metazoan phagocytes, GPCRs have been found to initiate phagocytosis of bacteria (Soumita et al., 2011). Picazarri *et al.* (2005) described EhGPCR-1, a putative GPCR associated with vesicular trafficking of proteins that localize to phagocytic cups (Picazarri et al., 2005). EhGPCR-1 is highly expressed in pathogenic *E. histolytica* but not in *E. dispar* (unpublished observations**)** possibly accounting for the differential engulfment of bacteria by these species (Pimenta et al., 2002). The ligand for EhGPCR-1 had not been identified prior to the present study.

We hypothesized that since EhGPCR-1 has a putative role in the initiation of phagocytosis, it may recognize bacterial prey that is an essential nutrient source and potentiators of virulence for *E. histolytica*. In the present study, we used a heterologous yeast expression system to screen bacterial components for their ability to activate EhGPCR-1. In addition, we tested the ability of *E. histolytica* trophozoites to selectively predate bacteria based on the presence of a bacterial component putatively identified as a ligand for EhGPCR-1.

Materials and methods

Creation of the yeast expression vector encoding EhGPCR-1

DNA encoding EhGPCR-1 (accession number AY880672) was synthesized by GeneScript using codon optimization for yeast expression. The gene was cloned into the pUC57 vector and the cDNA was amplified with forward and reverse primers adding the restriction sites NcoI and BamHI (5'GCCATACCATGGATCAATCATTCGGTAATCAA3')

and (5'GCCATAGGATCCTTAAGTCAAGTTAATTTCTCTTGAA3') to the 5' and 3' ends of the amplicon, respectively. Purified amplicons and the linearized yeast expression vector Cp4258, which bears a leucine auxotrophic marker (Kimber et al., 2009; Wang et al., 2006), were co-digested with NcoI and BamHI restriction endonucleases. The EhGCR-1 gene was then ligated into Cp4258 using T4 DNA ligase (New England Biolabs). The resulting plasmid was transformed into E. coli and individual clones were selected and aerobically grown overnight at 37oC in Luria-Bertani (LB) broth containing 32μg/mL ampicillin. Plasmid DNA was purified using HiSpeed Plasmid Mini Kit (Qiagen) and inserts were sequenced to confirm cDNA orientation and fidelity.

Transformation of yeast with the EhGPCR-1 expression vector

Saccharomyces cerevisiae strain CY 18043 (J. Broach, Princeton University, USA) was used as the yeast recipient since this strain is a histidine auxotroph that exhibits histidine prototrophism upon GPCR activation even for exogenous receptors (Kimber et al., 2009; Wang et al., 2006). Non-transformed CY 18043 yeast were grown in YPD media supplemented with all essential amino acids. Cells at mid-log phase (OD600 equals 0.3 to 0.5) were transformed with 1μg cDNA construct or 1 μg empty vector (mock transformants) in the presence of 200μg salmon sperm DNA

(Invitrogen) and lithium acetate (100mM, Sigma-Aldrich). Transformed cells were incubated at 30oC and then heat shocked at 42oC for 15 minutes. Cells were placed on leucine-deficient media [1x YNB (Difco), 1x yeast synthetic dropout medium supplement without leucine (Sigma), 10 mM ammonium sulfate (Sigma), and 50% glucose] to select for transformation of Cp4258 with the EhGPCR-1-encoding plasmid. Transformants were verified by isolating plasmids (Promega) and colonies expressing the EhGPCR-1 were verified by PCR prior to the functional assay.

Yeast growth assay

Leucine-deficient media was inoculated with yeast expressing EhGPCR-1 or mock transfected yeast that were grown at 30oC to an OD600 equal to one. Cells were washed three times with leucine/histidine deficient medium [1x YNB (Difco), 1x yeast synthetic drop out medium supplement lacking leucine and histidine (Sigma), 10 mM ammonium sulfate (Sigma), 50% glucose, 50 mM 4-morpholinepropanesulfonic acid, pH 6.8] and resuspended in 1mL leucine/histidine-deficient media, to a density of 15–20 cells/µL. Approximately 3,000 cells were added to each well of 96-well plates containing the same medium along with test agonists in a total volume of 200µL. Cells were grown at 30oC for approximately 24 hours. Initial and final OD600 values were measured with a spectrophotometer to determine growth of the yeast.

Agonist and antibody binding studies

E. coli (K12 strain, Sigma) and rough strain E. coli MG1655 (K12 derivative, N. Cornick, Iowa State University, USA) were grown aerobically overnight in LB broth at 37oC. To produce bacterial lysates, cultures were incubated for 10 minutes at 100oC. Purified lipopolysaccharide (LPS) from E. coli 0111:B4 (Sigma) was also used as a test agonist.

Serial dilutions of whole bacteria, lysate, or LPS were added to leucine/histidine-deficient media for use in the yeast growth assay. Antibody-mediated inhibition of GPCR activation was performed by co-incubating test agonists in 200μL media with 25μL equine anti-LPS antibodies (MG Biologics, Ames, IA; 1:10 titer), in the absence or presence of proteinase K (Qiagen, 50μg/mL, 3 hrs., 37oC).

Bacterial engulfment assay

Bacteria were labeled by growing 8×10^8 CFUs in 1 mL of LB broth containing 10μg FITC. Cells were washed three times with and resuspended in M199 medium (Gibco) supplemented with 25mM HEPES and 5.7mM cysteine (M199s). *E. histolytica* HM1 trophozoites were grown under anaerobic conditions at 37ºC in TYI Medium (Diamond et al., 1978). Trophozoites were harvested by centrifuging for 5 min at 1,000 RPM and 2.5×10^5 trophozoites were added to each well of a 24-well tissue culture dish in 500μL of TYI medium. Cells were allowed to adhere to the wells for 1 hr at 37ºC under anaerobic conditions. Each well was washed twice with pre-warmed M199s medium and inoculated with 8.75×10^6 bacteria in a final volume of 500μL of M199s medium. Bacteria and amoebae were co-incubated at 37ºC for 25 min. The medium was aspirated and 500μL ice-cold 110 mM D-galactose was added to each well to detach trophozoites. Cells were pelleted centrifuging for 5 min at 1,000rpm and washed with 500μL ice-cold 110 mM D-galactose. Cells were then fixed in 4% paraformaldehyde for 20 min at 37ºC. Paraformaldehyde was neutralized with 50mM ammonium chloride, and cells pelleted and resuspended in phosphate-buffered saline. 5μl aliquots were fixed with Fluoromount-G mounting medium (Southern Biotech; Birmingham, AL) on a microscope slide. These specimens were examined by fluorescence microscopy on an Olympus BX51 microscope with a UPlan F1 40x/0.75 objective equipped with an HBO lamp and dichroic FITC illumination filter for visualization of bacterial engulfment by trophozoites.

For flow cytometry experiments, at least 10,000 amoebic cells were analyzed for the presence of internalized bacteria on a Becton Dickinson FACScalibur (excitation 488nm) and fluorescence measured in the FL1 channel. Data was acquired using CellQuest Software (BD Biosciences) and analyzed by Flow Cytometry Analysis software (Tree Star, Inc.; Ashland, OR).

Results

EhGPCR-1 activation by bacterial lysate

To determine if EhGPCR-1 recognizes bacterial components, we monitored the response of the receptor to bacterial lysates using a histidine auxotrophic yeast functional expression assay (Kimber et al., 2009; Wang et al., 2006). The GPCR of interest is expressed in histidine auxotrophic yeast that will grow in histidine-deficient medium when the receptor is stimulated by its cognate ligand or agonist. Thus, in histidine-deficient media, receptor activation induces yeast growth that is quantitated spectrophotometrically. Receptor activation was calculated as increased yeast growth compared to growth of mock-transfected yeast exposed to the same ligand or agonist.

Addition of E. coli K12 lysates to EhGPCR-1-expressing cells resulted in a significant increase in yeast growth (Figure B.1). This effect was markedly attenuated by the addition of anti-LPS antibodies and the effect of the anti-LPS antibodies was abrogated by proteinase K. Addition of a rough strain of E. coli K12, which lacks the outer O-antigen of LPS (Johns et al., 1983), stimulated EhGPCR-1 to a lesser extent. No effect was noted when the EhGPCR-1 expressing yeast were exposed to a panel of catecholamines (data not shown).

Concentration-dependent activation of EhGPCR-1 by LPS

Since EhGPCR-1 was putatively activated by LPS, we examined the ability of LPS to stimulate EhGPCR-1 in a concentration-dependent manner. EhGPCR-1-expressing yeast were incubated with various concentrations of purified LPS isolated from *E. coli* 0111:B4 and histidine prototrophism was measured in the yeast. As shown in Fig. 2, LPS activated EhGPCR-1 in a concentration-dependent manner with an EC_{50} of 15 nM (figure B.2).

Preferential predation of *E. coli* expressing LPS by *Entamoeba histolytica* trophozoites

To determine if *E. histolytica* trophozoites selectively predate bacteria based on the presence of LPS, we compared the engulfment of *E. coli* K12 and a rough strain of *E. coli.* Bacteria were fluorescently labeled with FITC and co-incubated with *E. histolytica* HM1 trophozoites. Trophozoites were washed to remove bacteria that were not engulfed or attached and the number of *E. histolytica* trophozoites containing bacteria was quantitated by flow cytometry. Phagocytosis assays revealed that 23.2% of trophozoites contained *E. coli* K12 while only 3.8% of trophozoites engulfed the rough strain. This represents an 80% reduction in the bacterial engulfment capability of *E. histolytica* (Fig B.3).

Discussion

Previous work indicates that EhGPCR-1 is linked to phagocytic pathways in *E. histolytica* (Picazarri, 2005). The goal of the present study was to deorphanize EhGPCR-1 by determining its cognate ligand and to conduct engulfment assays demonstrating the functional activity of the ligand. GPCRs are cell surface receptors that sense the extracellular environment and are activated by a variety of ligands such as catecholamines, peptides, lipids, carbohydrates, *etc*. While GPCRs have been well

studied in vertebrates, the study of their role in protozoan physiology is only in its infancy. GPCRs are excellent drug targets, reflected by the fact that 30-50% of currently marketed drugs target these receptors (Flower, 1999; Wise et al., 2002). EhGPCR-1 is the first GPCR to be characterized in *E. histolytica* and it may represent an important chemotherapeutic target in this pathogen.

The present study utilized a novel yeast auxotroph assay for screening GPCRs against potential ligands. This approach has recently been applied to GPCRs from parasitic helminths (Kimber et al., 2009; Wang et al., 2006), and may represent a valuable tool for the study of protozoan receptors since culture of these organisms is often difficult. Utilizing this approach, we demonstrated that EhGPCR-1 is activated by a bacterial component of *E. coli*, an effect that was sensitive to anti-LPS antibodies. Purified LPS induced concentration-dependent EhGPCR-1 activation although this response was not as robust as the response to bacterial lysates. Structural variances between LPS from *E. coli* K12 and *E. coli* 0111:B4 might explain differences in receptor activation. Alternatively, additional bacterial components may be required to maximal occupancy of EhGPCR-1. Further research is needed to unveil the unique pharmacology aspects of EhGPCR-1 in *E. histolytica*, e.g. agonist affinity, binding co-operativity, and receptors expressed on each cell.

Based on the results of our yeast expression assay, we hypothesized that *E. histolytica* initiates bacterial engulfment after recognizing bacterial LPS. Phagocytosis assays demonstrated an 80% reduction in the number of trophozoites containing bacteria when the bacterial prey lacked O-antigen, the outermost layer of the LPS. This result is supported by previous research indicating that *E. histolytica* primarily engulfs Gram-negative pathogens (Mirelman et al., 1983). Other investigators have also demonstrated selective predation of bacteria by amoebae based upon

bacterial O-antigen (Wildshutte et al., 2007; Wildshutt et al., 2004). While *E. histolytica* appears to bear a receptor that binds bacterial LPS, it is unclear if other taxa of protozoa possess similar mechanisms for initiation of bacterial engulfment.

In summary, this study demonstrates the utility of a heterologous yeast expression system in the deorphanization of EhGPCR-1, a GPCR used in phagocytosis by pathogenic *E. histolytica*. EhGPCR-1 is activated by bacterial LPS, suggesting that this receptor may be used to initiate phagocytosis upon the recognition of bacterial prey. Functional studies supported the role of LPS in engulfment of *E. coli* by *E. histolytica*. Protozoan GPCRs may represent innovative drug targets and their role in regulating protozoan physiology merits further investigation.

Acknowledgment

Author contributions

SC conceived this study. MB and PA carried out the experiments. MB and SC contributed to the writing of the manuscript.
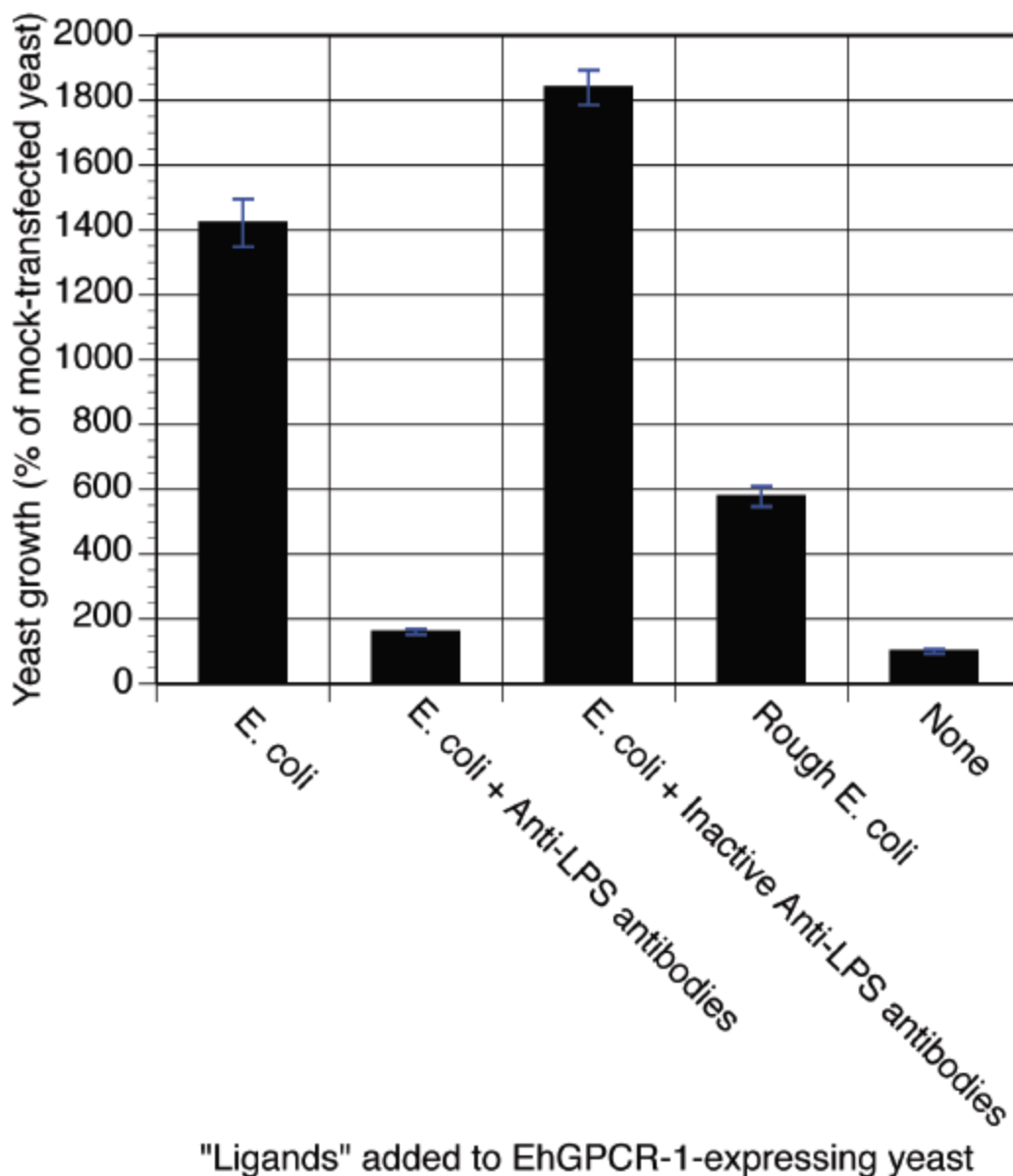
Figures

Figure B.1 *E. coli* K12 lysate stimulation of histidine prototrophism in histidine auxotrophic yeast expressing EhGPCR-1

Yeast growth was measured in histidine-deficient media in the presence of E. coli K12 lysates. Growth was also determines in the presence of these lysates plus intact anti-LPS antibodies or anti-LPS antibodies digested with proteinase K. EGPCR-1 activation was also determines in the presence of a rough strain of *E. coli* K12. Receptor activation is expressed as a percent growth over

mock-transfected yeast. Data are expressed as mean + SEM. For bar with bacteria only, n=6; for antibody experiments n=3
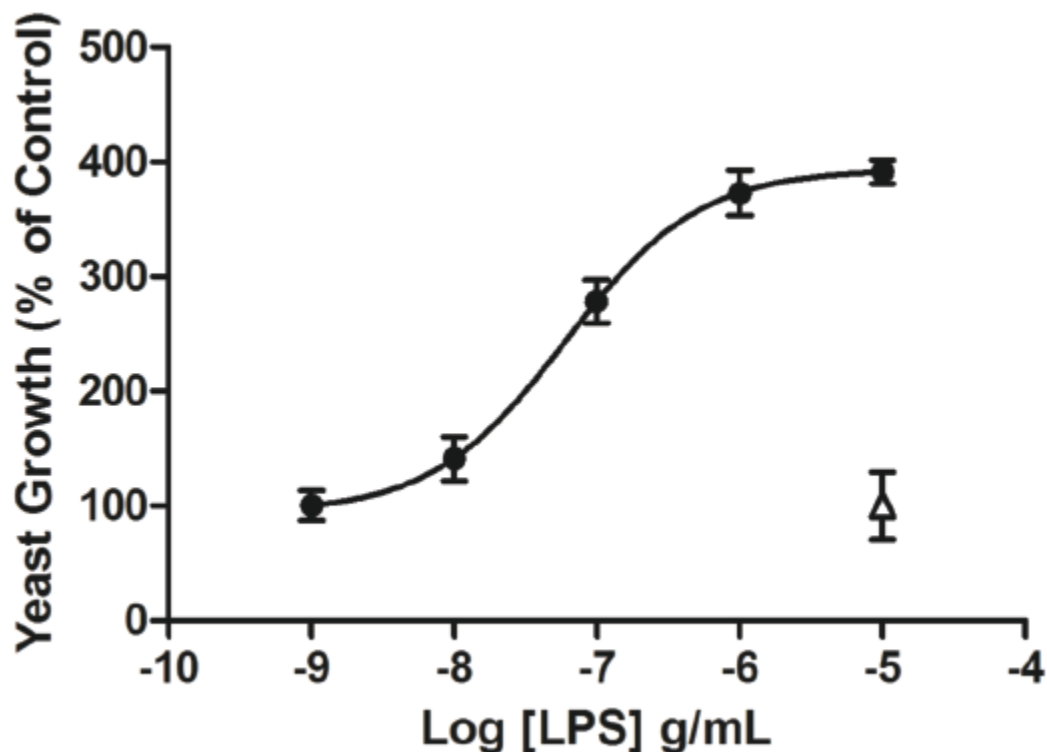
Figure B.2 Concentration dependent activation of EhGPCR-1 by LPS

Activation of EhGPCR-1 by bacterial LPS is concentration dependent with an EC_{50} of 15nM, based on an estimated molecular wright of 2000gm/mole for LPS. The open triangle represents the response of mock-transfected yeast to the highest concentration of LPS used. Each data point represents the mean+ SEM for the three in dependent experiments.
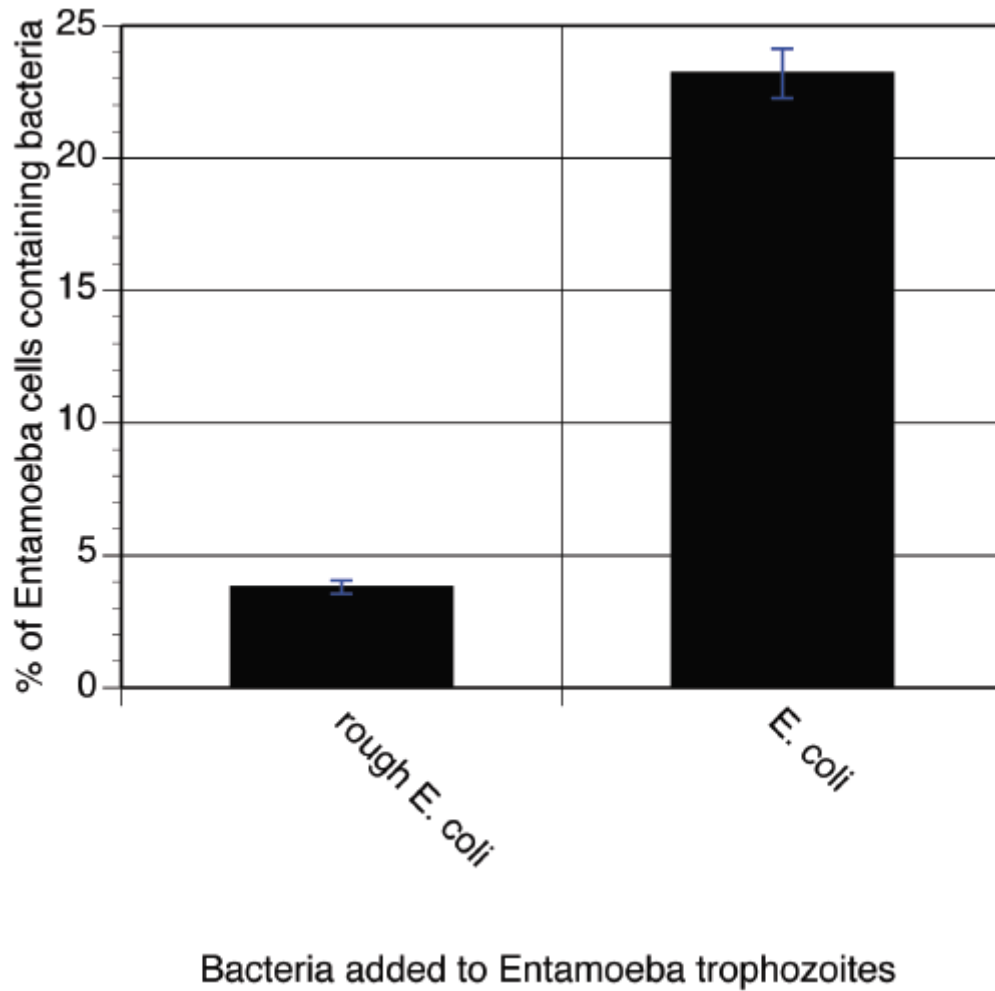
Figure B.3 E. histolytica preferential engulfment of E. coli expressing LPS

E. coli were labeled with FITC and incubated with E. *histolytica*l trophozoites.   Percent of trophozoites harboring bacteria was determined using flow cytometry. Each bar represents the mean + SEM of percent of trophozoites that contained bacteria after 25 min, of co-incubation (n=3, 10,000 trophozoites counted per run).

APPENDIX C   RNAi protocols

Freezing bacterial stocks: (Source: http://www.ciwemb.edu/, 03/31/2012)

Request a freshly plated HT115 bacterial cell from Lisa Timmons, Carnegie Institution of Washington. Inoculate fresh single colony of bacteria into 2.5 ml LB+ antibiotic(s). Grow to early stationary phase. Pipette 0.25 ml 80% glycerol (sterile) and 0.75 ml culture into a sterile screw-cap freezer tube. Mix. Quick freeze on dry ice/ethanol and store at -80C.

Quick procedure for making competent bacterial cells using CaCl2

(http://paramecium.cgm.cnrs-gif.fr/RNAi/BactRNAi_Timmons.html, 03/31/2012)

1. Inoculate overnight culture in LB + antibiotic (TET for HT115 (DE3) strain + antibiotic appropriate for any plasmids in cells) (2-5ml). Shake overnight at 37C.

2. Inoculate 25 ml LB + antibiotic with overnight culture, 1:100 dilution. Grow cells to OD595= 0.4. Can grow cells in 50 ml sterile centrifuge tube.

3. Spin cells 10 min 3000 rpm at 4C.

4. Resuspend pellet in 0.5X original volume cold, sterile 50 mM CaCl2 (12.5ml). Resuspend by GENTLY pipetting up and down a few times with a wide bore pipet--no vortexing.

5. Incubate on ice 30 min.

6. Spin as before at 4C.

7. Resuspend pellet as before in 0.1X original volume CaCl2 (2.5ml). Keep cells cold (4C).

8. Use 50-200 ul for transformation.

BP Clonase reaction

Creating a Gateway® entry clone

(Adupted/ modified from invitrogen BP Clonase Reaction Manual)

1. Add the following components to a 1.5 ml tube at room temperature and mix:

attB-PCR product (=10 ng/µl; final amount ~15-150 ng) 1-7 µl

Donor vector (150 ng/µl) 1 µl

TE buffer, pH 8.0 to 8 µl

2. Thaw on ice the BP Clonase™ II enzyme mix for about 2 minutes. Vortex the BP Clonase™ II enzyme mix briefly twice (2 seconds each time).

3. To each sample (Step 1, above), add 2 µl of BP Clonase™ II enzyme mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly.

4. Return BP Clonase™ II enzyme mix to -20°C or -80°C storage.

5. Incubate reactions at 25°C for 1 hour.

6. Add 1 µl of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37°C for 10 minutes.

Transformation

Transform 6 µl of each LR reaction into 60 µl of One Shot ® Top ten Electrocompetent Cells (Catalog no. C8540-03) by first mixing gently both the Bp Clonase reaction and the Top Ten cells on ice followed by electroporation (0.2 cm electrode gap**,** 10 µF**,** 5 milliseconds). Add 400 µl of S.O.C. Medium and incubate at 37°C for 1 hour with shaking. Plate 20 µl, 50 µl and 100 µl of each transformation onto kanamycin, 100 µg/ml (100 µl / 200 ml agar) selective plates (or the appropriate selection marker for your donor vector). Note: Any

competent cells with a transformation efficiency of $>1.0 \times 10\ 8$ transformants/µg may be used.

Plasmid Preparation

Promega protocol

PCR Check

Using **gene specific** primers **with no attB sites**

<div align="center">

Perfect clone transformation pcr check:

Primer F (no att-sites)	2µl
Primer R (no att-sites)	2µl
10X PCR reaction buffer	2.5µl
dNTP	2µl
Taq Polymerase	1µl
H_2O	38.5µl
Template (prep/colony)	2-3µl
TA (Annealing Temp)	55 deg celcius

</div>

<div align="center">

Sequence confirmation of perfect clone

</div>

Send 20 µl of prep for sequencing at DNA facility, ISU or other

Transformation of chemically competent (CaCl$_2$ Competent) HT115 cell with perfect clone

1. Add 50-200 µl of calcium competent cells to cold, sterile polypropylene tube on ice

2. Add 2 µl of plasmid prep (1-100ng) (perfect clone) and mix gently.

3. Incubate on ice for 30 minute

4. Immerse tube in 37C water bath for 1 min.

5. Incubate tube on ice/water bath for 2 min.

6. Add 1ml sterile SOC media. Incubate 37C with shaking for 1 hour.

7. Plate 10uL, 100uL, 250uL, and remaining culture onto 4 LB + Kanamycin+ tetracycline antibiotic (plasmid resistance) plates. Incubate 37C overnight. (These cells grow slowly, allow 36 hours for colony formation.)
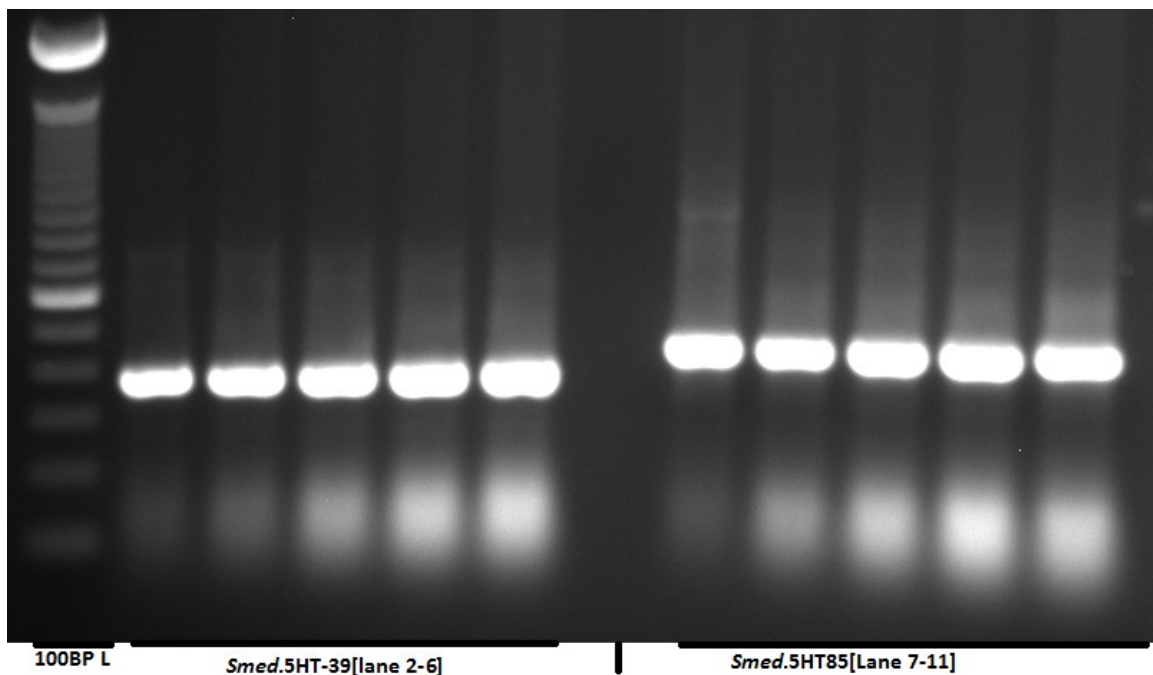
Figure C.1 HT115 bacteria transformed with perfectly cloned targets

Plasmids; of serotonin GPCR targets (*smed*.5HT-39, *smed*.5HT-85) in the donor vector pDONRdT7 were used to transform   the RNase-deficient *E. coli,* HT115 (with IPTG-inducible T7 polymerase). Plasmid prep from the bacteria strain confirmed the presence of receptor targets in the bacteria, a confirmation necessary before the induction of dsRNA.

100BP L   **Acon [Lane 2-5]**
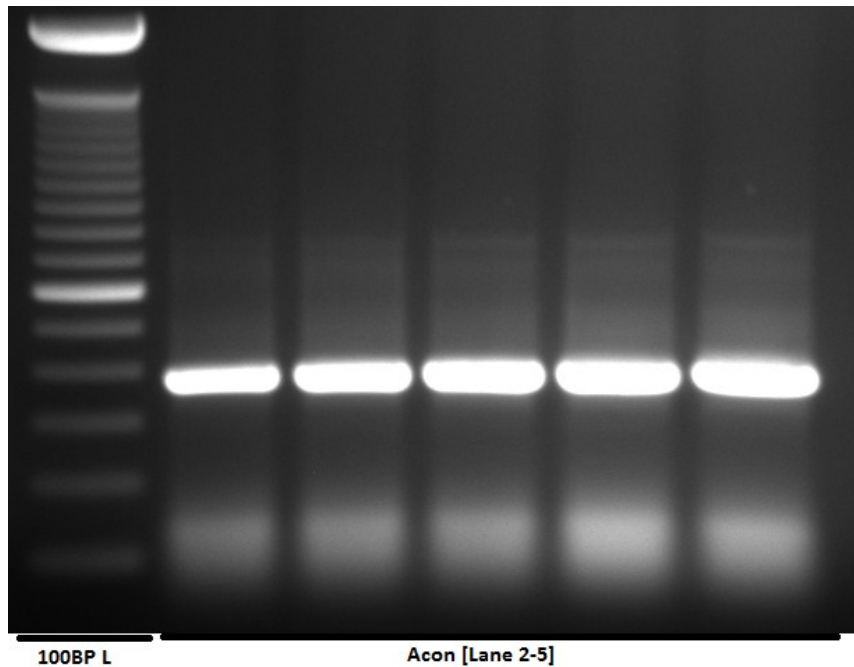
Figure C.2 HT115 bacteria transformed with perfectly cloned Control targets Plasmids; of a random gene (Acon) (source, mosquito) targets in the donor vector pDONRdT7 were used to transform   the RNase-deficient *E. coli,* HT115. Plasmid prep from the bacteria strain confirmed the presence of gene targets in the bacteria, a confirmation necessary before the induction of dsRNA.

Induction of dsRNA in HT115 (DE3) cells + T7 promoter containing plasmid:

Day 1 – Making stocks

1. Inoculate overnight culture of HT115 (DE3) + plasmid in LB+ Kanamycin+ Tetracycline. Incubate 37C with shaking overnight at 250Rev/min. (75-100ug/ml ampicillin for amp-resistant plasmids and 12.5 ug/ml tetracycline)

2.   Make media (500ml final as example, scale as needed).

- 500 ml 2XYT

- 50 µg/ml KAN (2.5 ml KAN / 500 ml 2XYT for 10 mg/ml stock)

- 1 X TET (stock = 1000 X, 500 µl TET stock per 500 ml 2XYT)

Day 2

1. Centrifuge bacteria 10 min at 4000 RPM, 4°C.

2. Decant supernatant, resuspend pellet in 2XYT media.

3. Incubate on shaker, check OD at 1 (alpha 2 may be done already), 1.5, 2 hrs, balancing with ~900 ul 2XYT.

4. At an OD of 0.3 to 0.4, induce dsRNA expression by adding IPTG to a final concentration of 1 mM (100 mM stock = 5 ml stock / 500 ml culture).

5. Incubate a further 2 hr on shaker, remove, cover and put on ice to stop.

6. Spin cultures in 500 ml Beckman centrifuge tubes using SLA 3000 rotor (setting 30) at 4000 RCF for 12 minutes at 4°C (NHH).

7. Decant supernatant and resuspend pellet in 50 ml 2XYT.

8. Centrifuge 10 min at 4000 RPM, 4°C.

9. With 25 ml pipette, remove 40 ml supernatant and resuspend pellet in remaining 10 ml (ex. for 500 ml culture and 25 ul final aliquot).

10. Add 3 1/3 ml glycerol (250 ml glycerol: 750 ml bacteria + media).

11. Add 665 μl of glycerol + bacteria to each microfuge tube.

12. Get dry ice and add EtOH and freeze (keep tubes high enough so no EtOH gets in, low enough so bacteria submerged). Store in minus 80 freezers.

APPENDIX D cAMP membrane preparation protocol optimized
Motor and pestil version

1. Wash motor and pestil several times with distilled water.

2. Then, Wash with Sucrose buffer.

3. Add 2ml sucrose buffer to motor and petil and place in -20 degree Celcius freezer.

4. Fast cool centrifuge to 4 degree celcius.

5. Fill 1 (50ml) Falcon tube with 20 ml cAMP buffer.

6. Add 30ul (stock in minus 20 freezer) of PCK to step 5. Add also GTP (20ul (of 100 mM) [55mg/ml] in 20ml cAMP buffer) and ATP (20ul (of 100 mM) [55mg/ml] in 20ml cAMP buffer) to step 5 and mix well.

7. Wash worms 4 times in their normal media and Transfer worms into 50ml falcon tube.

8. Drain worm media

9. Wash worm 1X with Sucrose buffer.

10. Resuspend worms in 1ml Sucrose buffer

11. Transfer worms into motor and pestil containing frozen layer of sucrose buffer.

12. Homogenize worms with the pestil for approximately 3 min.

13. Transfer homogenate into eppendorf tube in a final volume of 20 ml.

14. Rinse motor to collect excess membrane on the walls of the motor and pestil

15. Place tube on ice for 5 minutes.

16. You may sonicate the lower portion of the sample for 5 seconds.

17. Centrifuge at 1500 g at 1-4 degree celcius for 5 min.

18. Transfer supernatant into fresh eppendorf tube and discard pellets.

19. Spin the supernatant for 15 min at 15,000g.

20. Discard the supernatant and wash the pellets in 10 ml of sucrose buffer: resuspend supernatant in 10 ml of sucrose buffer and centrifuge at 15, 000g for 15min and discard supernatant.

21. Resuspend the pellet in a final volume of 4ml (if worm number equals 40) of cAMP buffer containing Phosphofructokinase (15ul/10ml cAMP buffer), GTP (10ul (of 100 mM) [55mg/ml] in 10 ml cAMP buffer) and ATP (10 ul (of 100 mM) [55mg/ml] in 10 ml cAMP buffer) to the pellet.

22. You may sonicate the lower portion of the sample for 5 seconds

23. Aliquot 400ul samples into 9 (when worm number equals 40) labeled eppendorf tubes for treatments.

24. Arrange samples into consecutive groups of 3 (triplicates to receive the same treatments)

25. Add ligands: 4ul H20 to tubes 1-3 for basal controls; 4ul of 10mM (10^{-2}M) Forskolin to tubes 4-6, 4ul of 10mM (10^{-2}M) 5HT to tubes 7-9, 4ul of 10mM (10^{-2}M) DPAT to tubes 10-12 and 4ul of 10mM (10^{-2}M) 1, 3-CPPH to tubes 13-15, etc...

26. Invert tube several times to mix well.

27. Incubate for 25 min at 35 degree celcius.

28. Centrifuge at 3000 rcf for 5 min.

29. Transfer 350ul of the supernatant into clean tubes.

30. Keep at 4 degree Celcius for cAMP assay.

APPENDIX E   RNAi vector map
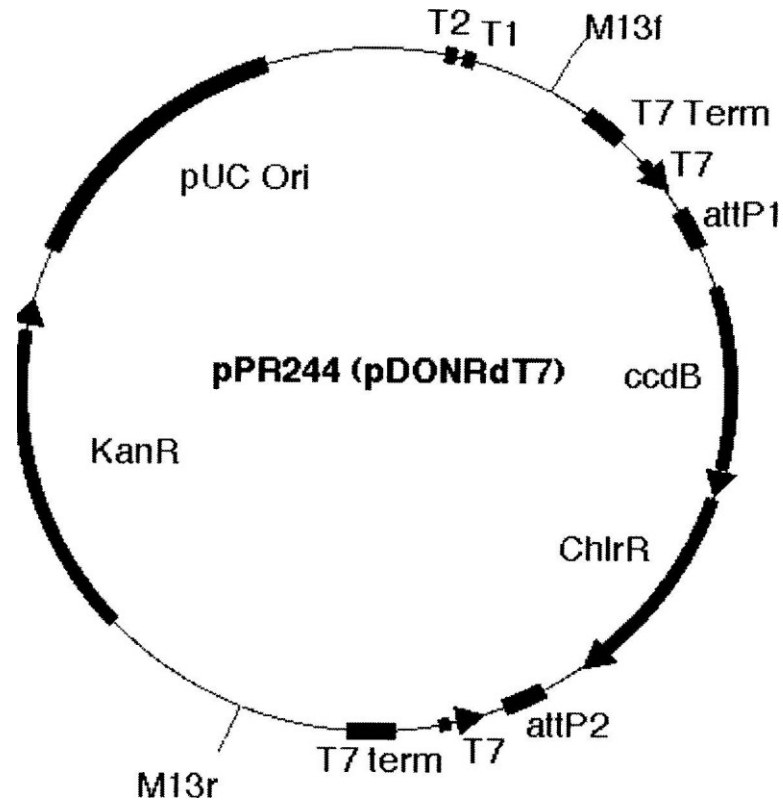
Figure E.1 pDONRdT7 RNAi Vector

A Double T7 Vector with a suicide gene, ccdB. The addition of PCR product of target receptor in the presence of BP-Clonase enzyme, results in the swaping of the region between attP1 and attP2 with the target gene. This way, the transformed vector will not propagate in the presence of Chloramphenicol because the vector loses such resistance after BP- clonase reaction.

>CTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCC

GCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGC

CTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGT

GAGCGCAACGCAATTAATACGCGTACCGCTAGCCAGGAAGAGTTTGTAGAAACGCAAAAAGGCCATCCGT

CAGGATGGCCTTCTGCTTAGTTTGATGCCTGGCAGTTTATGGCGGGCGTCCTGCCCGCCACCCTCCGGGCC

GTTGCTTCACAACGTTCAAATCCGCTCCCGGCGGATTTGTCCTACTCAGGAGAGCGTTCACCGACAAACAA

CAGATAAAACGAAAGGCCCAGTCTTCCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTTCCCTACTCT

CGCGTTAACGCTAGCATGGATGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTCTTAAGCAAAAAA

CCCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGGAAGTAATACGACTCACTATAGGGAGACC

GGCAGATCTGATATCATCGATGAATTCGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTggatccaccggtt

ccatggctagccacgtgacgcgtggatccCCCATCAGCTGGATGGCAAATAATGATTTTATTTTGACTGATAGTGACCTGTT

CGTTGCAACAAATTGATAAGCAATGCTTTCTTATAATGCCCACTTTGTACAAGAAAGCTGAACGAGAAACG

TAAAATGATATAAATATCAATATATTAAATTAGATTTTGCATAAAAAACAGACTACATAATACTGTAAAAC

ACAACATATCCAGTCACTATGAATCAACTACTTAGATGGTATTAGTGACCTGTAGTCGACTAAGTTGGCAG

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GGTGTCCCTGTTGATACCGGGAAGCCCTGGGCCAACTTTTGGCGAAAATGAGACGTTGATCGGCACGTAA

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GGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACTGGATATACCACCGTTGATATATCCCAATGGCATC

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ACGGCCTTTTTAAAGACCGTAAAGAAAAATAAGCACAAGTTTTATCCGGCCTTTATTCACATTCTTGCCCG

CCTGATGAATGCTCATCCGGAATTCCGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGGATAGTGTTC

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GGCCAATATGGACAACTTCTTCGCCCCCGTTTTCACCATGGGCAAATATTATACGCAAGGCGACAAGGTGC

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AACAGTATGCGTATTTGCGCGCTGATTTTTGCGGTATAAGAATATATACTGATATGTATACCCGAAGTATG

TCAAAAAGAGGTGTGCTATGAAGCAGCGTATTACAGTGACAGTTGACAGCGACAGCTATCAGTTGCTCAA

GGCATATATGATGTCAATATCTCCGGTCTGGTAAGCACAACCATGCAGAATGAAGCCCGTCGTCTGCGTGC

CGAACGCTGGAAAGCGGAAAATCAGGAAGGGATGGCTGAGGTCGCCCGGTTTATTGAAATGAACGGCTCT

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GCATGATGACCACCGATATGGCCAGTGTGCCGGTCTCCGTTATCGGGGAAGAAGTGGCTGATCTCAGCCAC

CGCGAAAATGACATCAAAAACGCCATTAACCTGATGTTCTGGGGAATATAAATGTCAGGCTCCCTTATACA

CAGCCAGTCTGCAGGTCGATACAGTAGAAATTACAGAAACTTTATCACGTTTAGTAAGTATAGAGGCTGA

AATCCAGATGAAGCCGAACGACTTGTAAGAGAAAGTATAAGAGTTGTGAAATTGTTCTTGATGCAGATG

ATTTTCAGGACTATGACACTAGCGTATATGAATAGGTAGATGTTTTTATTTTGTCACACAAAAAAGAGGCT

CGCACCTCTTTTTCTTATTTCTTTTTATGATTTAATACGGCATTGAGGACAATAGCGAGTAGGCTGGATACG

ACGATTCCGTTTGAGAAGAACATTTGGAAGGCTGTCGGTCGACTAAGTTGGCAGCATCACCCGAAGAACA

TTTGGAAGGCTGTCGGTCGACTACAGGTCACTAATACCATCTAAGTAGTTGATTCATAGTGACTGGATATG

TTGTGTTTTACAGTATTATGTAGTCTGTTTTTTATGCAAAATCTAATTTAATATATTGATATTTATATCATTT

TACGTTTCTCGTTCTGCTTTTTTGTACAAACTTGGCATTATAAAAAGCATTGCTCATCAATTTGTTGCAAC

GAACAGGTCACTATCAGTCAAAATAAAATCATTATTTGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTC

GTATTACTTCCACATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGAGGATCCCCTATAGTG

AGTCGTATTACATGGTCATAGCTGTTTCCTGGCAGCTCTGGCCCGTGTCTCAAAATCTCTGATGTTACATTG

CACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGT

TATGAGCCATATTCAACGGGAAACGTCGAGGCCGCGATTAAATTCCAACATGGATGCTGATTTATATGGGT

ATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGCTTGTATGGGAAGCCCGATGC

GCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGTCAGACTA

AACTGGCTGACGGAATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTA

CTCACCACTGCGATCCCCGGAAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAGGTGAAAATAT

TGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTCCTTTTAACAGCGA

TCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGTGATTTTGATG

ACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAACTTTTGCCATTCTCACCGGAT

TCAGTCGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATT

GATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGGAACTGCCTCGGTGAGTT

TTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATAAATTGCAGTT

TCATTTGATGCTCGATGAGTTTTTCTAATCAGAATTGGTTAATTGGTTGTAACACTGGCAGAGCATTACGCT

GACTTGACGGGACGGCGCAAGCTCATGACCAAAATCCCTTAACGTGAGTTACGCGTCGTTCCACTGAGCGT

CAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAA

ACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGT

AACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCA

AGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGAT

AAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGG

GGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCA

TTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAAC

AGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCAC

CTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGC

GGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTT

Figure E.2 pDONRdT7 RNAi Vector Nucleotide Sequence

APPENDIX F   target sequences

S. mediterranea; *Smed-ser39*

>mk4.005939.01.01 RNAi region

AATGCCGGCCTGTTTCCTATGATAAAGTTCCTCTGATAGTGATTAGTACAGTGTTAACTTTGCTAAGTG

TTGGCACATGCATTGACAATTGTCTCATGATATCGGCCGTAGCTCTTGTTAAGAAATTACGAACTCCTT

GCAACATGTTAATTCTCAACCTTGCAGTTACAGATTTATTAGTCGGCACTCTCGTCATACCGTTTGCAA

GCATATACCAAATCAAAGGTTACTGGATATTCGATGAGATAGTATGTGATATATTTATTCTTTTTGATG

TTTTGCTATGTACCTCATCAATACTTAACTTATGTGCAATATCTGTTGATAGATATCTTGTAATTACTCA

ACCATTTAAATATGCTGTGAAACGCACAAGAAAAGGATGCT

Original primers:

ASMD5939F

GGGG-attB1-F/primer

GGGG-ACAAGTTTGTACAAAAAAGCAGGCT-AATGCCGGCCTGTTTCCTAT

ASMD5939R

GGGG-attB2-R/primer

GGGG-ACCACTTTGTACAAGAAAGCTGGGT-AGCATCCTTTTTCTTGTGCG

S. mediterranea; *Smed-ser85*

>mk4.001585.00.01 RNAi region

CTCCGCTTTTAATTGGAGGATTCATTGCAGGAGCTTGGATCATATCAGGATTAATTAGCATACCACCGG

TAATAGTGTGGAAGGAACCCTTTAGACCTGGAACTTGCCAGTTGACGGAAAATTTGGGATACCAGATA

TATGCCACTCTTGGTGCCTTCTACATTCCATTAATAATTATGTTGGTGCTATATTATCGAATTTTTAAAC

TAGCAAGAAATATGGCCCAAGAAGATGCCAAGAGAAAATTAGGTACAGGTCAAATGACTGATGAAGA

ACAAACTTCATTGCCAAATCAGTCAGGAAGAACAAATTCCGCTGAAGAAGACAGAAAACTTCTTCGGT

TTGATCCCACTCAAAGACCGAGTGAAGGAAATCAAGGTAATGGGTTCGATGTTGAGAAGACTGGAAC

TGGACCTAAAACAAATCCCCGGAAAAAGAAACAG

Original primers:

ASMD1585F

GGGG-attB1-F/primer

GGGG-ACAAGTTTGTACAAAAAAGCAGGCT-CTCCGCTTTTAATTGGAGGA

ASMD1585R

GGGG-attB2-R/primer

GGGG-ACCACTTTGTACAAGAAAGCTGGGT-CTGTTTCTTTTTCCGGGGAT

Figure F.1 *S. mediterranea; Smed-ser39, Smed-ser39* target sequences and primers

TetEpi-1>

ATGGACCAATCATTTGGAAATTAAACTCATGGATATGAAGAGAATTTGCTGATTATTGAGATTACTACTAA

TAGCCTCTCTTTGATAGGAAGCACTTTTATTGTTTTAATGTACTTATGTAACAAAGATCTCCACACGTTTGC

TTTTAAGCTAGTTTTTTTACTTTCAATTTCAGATATTATTCTTGGATTTGGAAGAATGTTTAACTTTTAATCT

ATTATCACTCAAGATTTCACTCCAGAAAATGGAGTTACATGCTAAGTGTAAAGCTTTTTAGTCACTTTTGGG

GGTCTATCTACAATAGTAACTACTATGGCAATAAGCTGGAGTCTGTGTCAAAGTGTTATCTATGGAATAAA

TAATTTAAATGACTACAGTAAATATTATTATATTGCTATATTTTTATTCCCATTAATTATCTCCATCGTACCC

TTGGCAACTGGAGATTATGGAGTTTCTGGTATTTCTTGTTGGATATATGGCCACGATGATAAGCCTTATGGC

ATTAGAACTATGCTTTGGAGGCTATTCTTATTTTACATACCTCTTTGGCTCTCTGTAATATATAATTCCATAA

ATTATTTCAGGATTAGAAAATTTGTCTACTCCTTATTTGTCGATAACACTGAAGCCTCTAAATAGCACAAAC

AAATAATTAGAAAGCTTACACTATATCCATTGATTATGGTTATTTGTTATTTATTTGCTACAATAAATCGTG

TATATCAATTTTTTGAAGAAAATGAGGTTGAAGCTATAGCGTACTTGCATATATGCTTAGCAGGACTTTAA

GGATTTTTCAATTCTTTAGTCTATGGCTTCAATAAGTAAATTAAAAGTAAAATATGTAAATCATGCATAACT

TAAAAGAAAAAATCTAAAGAATAAGAAGAAAAAGATGTAGTTGAATATTCAAGCAAAAGCAGTATTGAT

GAAAATAGATAGAATGGCTCCAGGTAAGTAACATCTAGTATCGAAAGCAGTATCACAGCAGAGGACGAGT

TTGGCTCAAGTGTATAAAAAGAGATGAGATAAAACTTAGGCAATGATCTTTACCAATATGACTCTCACCCT

ACTTCTAAAATTTATGATAAAGGAATTAATAGGATAGCTCGCTTAAGTGATCTTTCTTCAGTTCATAGCAGT

TCTAGTAATAAAAATCCAAGTGCAAATTCACGTGAAATAAATCTAACTTGA

Prot>

MDQSFGNQTHGYEENLLIIEITTNSLSLIGSTFIVLMYLCNKDLHTFAFKLVFLLSISDI

ILGFGRMFNF-SIITQDFTPENGVTCQVQSFLVTFGGLSTIVTTMAISWSLCQSVIYGIN

NLNDYSKYYYIAIFLFPLIISIVPLATGDYGVSGISCWIYGHDDKPYGIRTMLWRLFLFY

IPLWLSVIYNSINYFRIRKFVYSLFVDNTEASKQHKQIIRKLTLYPLIMVICYLFATINR

VYQFFEENEVEAIAYLHICLAGLQGFFNSLVYGFNKQIKSKICKSCITQKKKSKEQEEKD

VVEYSSKSSIDENRQNGSRQVTSSIESSITAEDEFGSSVQKEMRQNLGNDLYQYDSHPTS

KIYDKGINRIARLSDLSSVHSSSSNKNPSANSREINLT-

Figure F.2 *Tetrahymena* target Sequence (Nucleotide/Protein)

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