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Serotonin biosynthesis and receptors in helminths

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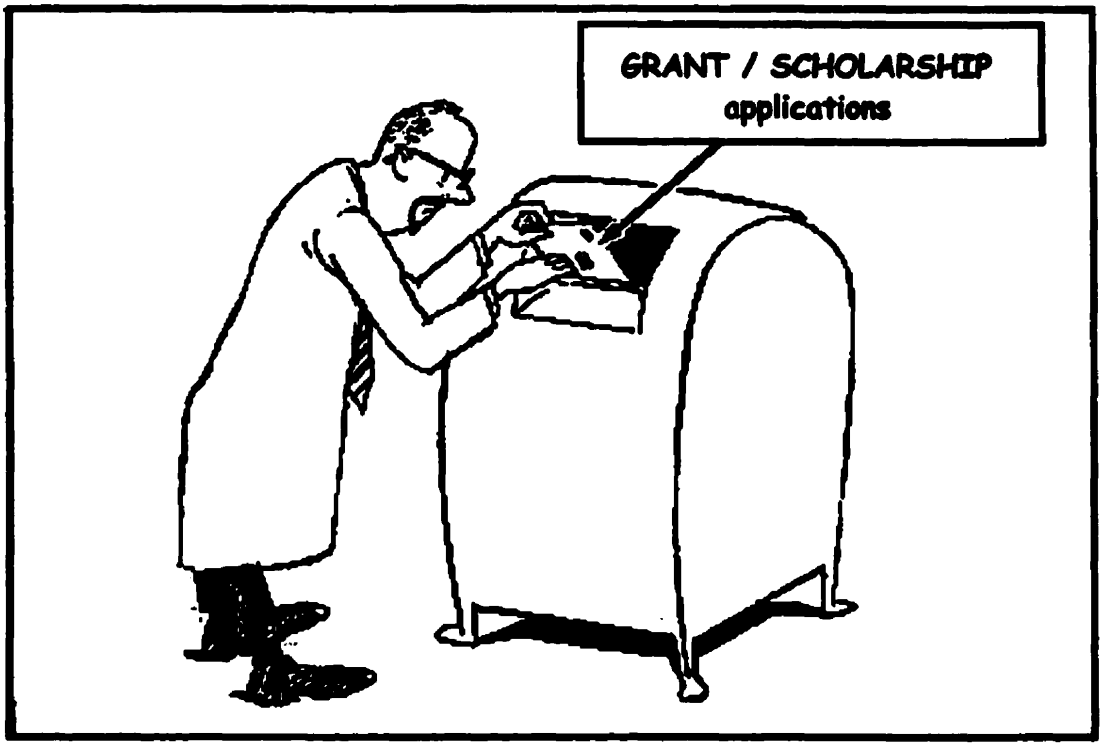
Doctor of Philosophy

A GOOD SCIENTIST IS ALWAYS PUSHING THE ENVELOPE!



(modified from Mark Stivers, *HMS Beagle*, Oct. 1999)

ABSTRACT

Serotonin is a very important neuromodulatory agent that affects many physiological and behavioral responses of both vertebrates and invertebrates. In helminths, especially parasitic ones, not much is known about the biosynthesis and mode of action of serotonin or any of the related biogenic amine neurotransmitters, such as catecholamines (dopamine and noradrenaline). In this study, we cloned two full length cDNAs from *Schistosoma mansoni* encoding tryptophan hydroxylase (TPH) and tyrosine hydroxylase (TH). TPH and TH catalyze the rate limiting steps in the biosynthesis of serotonin and catecholamines, respectively. Both enzymes were expressed in *Escherichia coli* and the purified proteins were shown to have TPH and TH activities. This indicates that *S. mansoni*, and possibly other parasitic helminths, may be capable of synthesizing serotonin and catecholamines endogenously. In the second part of our studies, we looked at the mode of action of serotonin in helminths, in particular the molecular properties of serotonergic G protein-coupled receptors (GPCR). We cloned two helminth GPCRs, one from the free living nematode *Caenorhabditis elegans* and the second from *S. mansoni*. The *C. elegans* receptor (5-HT_{2Ce}) was shown to encode a functional serotonin receptor with structural and signaling properties similar to those of mammalian 5-HT_2 receptors. However, its agonist / antagonist binding profile differed from previously characterized serotonin receptors. The cloned *S. mansoni* receptor (SmGPCRx) was found to represent a new structural class of receptor, which shared about the same level of amino acid sequence homology with various biogenic amines receptors, such as serotonin, catecholamines, and octopamine receptors. Additional sequence analysis and immunolocalization studies confirmed that SmGPCRx possesses structural characteristics of a GPCR. SmGPCRx is the first GPCR ever cloned from a parasitic flatworm. Taken together, these studies mark an important first step towards the biochemical and functional characterization of monoamine neurotransmitters in helminths.

ABREGE

La sérotonine est un neuromodulateur important affectant les réponses physiologiques et comportementales des vertébrés et des invertébrés. On connaît très peu la biosynthèse et le mode d'action de la sérotonine chez les helminthes, en particulier les formes parasitaires. Il en est de même pour certains neurotransmetteurs tels les catécholamines (dopamine et noradrénaline). Au cours de cette étude, nous avons cloné deux cADN codant pour l'hydroxylase de tryptophane (TPH) et l'hydroxylase de tyrosine (TH) provenant de *Schistosoma mansoni*. La TPH catalyse l'étape limitante de la biosynthèse de la sérotonine tandis que la TH catalyse l'étape limitante de la biosynthèse des catécholamines. *S. mansoni* et possiblement d'autres helminthes parasitaires sont donc capables de synthétiser leur propre sérotonine et catécholamine. Dans la deuxième partie de nos travaux, nous avons étudié le mode d'action de la sérotonine chez les helminthes. Nous avons particulièrement porté attention sur les propriétés moléculaires des récepteurs à sérotonine couplés à la protéine G (GPCR). Nous avons cloné un GPCR du nématode *Caenorhabditis elegans* ainsi qu'un second provenant de *S. mansoni*. Le récepteur cloné de *C. elegans* est un récepteur de sérotonine fonctionnel possédant des propriétés structurales et de signalisation semblables aux récepteurs 5-HT_2 que l'on retrouve chez les mammifères. Son profil de liaison d'agonistes et d'antagonistes diffère cependant des autres récepteurs à sérotonine déjà caractérisés. Le récepteur cloné de *S. mansoni* (SmGPCRx) représente une nouvelle catégorie de récepteur car il possède une structure distincte. Il possède une homologie de séquence avec différents récepteurs tels les récepteurs à sérotonine, à catécholamine et à octopamine. Des analyses de séquence ainsi que la localisation par immunofluorescence ont confirmé que le récepteur SmGPCRx possède les caractéristiques structurales d'un récepteur GPCR. Le récepteur SmGPCRx est donc le premier récepteur de type GPCR à être cloné d'un trématode. Ce projet est une première étape importante dans la caractérisation biochimique et fonctionnelle des neurotransmetteurs monoaminés des helminthes.

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Pride, excitement, joy, stress, frustration, hang-overs, and conferences in San Francisco and Hawai'i are some of the emotions that I experienced and the places that I visited during the past five years of my stay at the Institute of Parasitology. Presented here is the final product of these memorable years.

This thesis would not have been possible without the ever-present support and encouragement of my supervisor, Dr. Paula Ribeiro. Paula gave me the freedom to expand my project and investigate new approaches, and was always there to put me back on track whenever I got carried away. In short, it has been a privilege to work with and be mentored by professor Ribeiro.

I would like to thank my labmates, past and present, for providing me with a joyful and relaxing working environment. I am also indebted to Sylvie Labrecque for, as she would say, "helping me to keep a balance between work and fun", and for doing a superb job in translating the abstract of this thesis. Special thanks to the Lebanese brigade at the Institute of Parasitology, including Roni Daoud (a.k.a. Bronzo) and Joseph Nabhan for their valuable friendship and for always making me feel at home. Lots of gratitude is also extended to the rest of my friends and colleagues for making my stay comfortable and enjoyable. I am also thankful to Dr. Robin Beech for always being there to answer some of my questions, especially those related to software problems and Molecular Biology.

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

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STATEMENT OF CONTRIBUTIONS

The experimental work presented in this thesis was designed and performed by the author, under the supervision of Dr. Paula Ribeiro. In manuscript II, the aequorin assays were conducted at the laboratory of Dr. Mark Abramovitz (Merck Frosst, Pointe Claire, CA) with the assistance of Mr. Mark Ungrin.

STATEMENT OF ORIGINALITY

The following findings of this thesis are considered original contributions to the field:

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

In this study we reported the cDNA cloning, expression, purification, and functional characterization of tryptophan hydroxylase (TPH), the enzyme that catalyzes the first and rate-limiting step in serotonin (5-HT) biosynthesis, from the parasitic trematode *Schistosoma mansoni*. Aside from reporting the first TPH to be cloned and characterized from an invertebrate species, this study provides the first molecular evidence that *S. mansoni*, and possibly other parasitic helminths, are capable of synthesizing serotonin endogenously. Cloning of TPH from *S. mansoni* contradicts the earlier notion that TPH and phenylalanine hydroxylase (PAH), a related aromatic amino acid hydroxylase of the same family as TPH, might have diverged sometime after the evolution of insects, and suggests that the separation of TPH from PAH probably occurred before the divergence of platyhelminths. The cloned *S. mansoni* TPH shares overall similar kinetic and regulatory properties to mammalian TPH, except for its increased solubility and much greater enzymatic stability. These properties make the highly active *S. mansoni* TPH a potential model for general studies of TPH structure and function.

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

This study describes the cDNA cloning and functional characterization of a novel serotonin receptor (5-HT_{2Ce}) from the free-living nematode *Caenorhabditis elegans*. This is the first 5-HT_2-like receptor ever cloned and characterized from any helminth. Although the structural and signaling properties of 5-HT_{2Ce} are similar to those of 5-HT_2 receptors, its pharmacology is different and does not conform to any known class of 5-

HT receptor. This receptor displayed a ligand binding profile that could be best described as a mix between that of the 5-HT_1 and the 5-HT_2 class of serotonin receptors. Studies of this nematode 5-HT receptor may provide information to the function of serotonin in related parasitic nematodes.

Manuscript III: Hamdan, F. F. and Ribeiro, P. (1999) Molecular cloning and expression of a novel *Schistosoma mansoni* biogenic amine G protein-coupled receptor (1999) *In preparation*.

In this study, we described the cDNA cloning and expression of a *S. mansoni* G protein-coupled receptor (GPCR) with structural homology to biogenic amine receptors, including serotonin, catecholamines, and octopamine receptors. This is the first GPCR ever cloned from a parasitic platyhelminth. To achieve detectable expression of this receptor in mammalian cells, we re-synthesized the first portion of this receptor sequence using preferred mammalian codon usage. This is the first report of a codon-optimization strategy to enhance the heterologous expression of an invertebrate GPCR in mammalian cells. Such a strategy will be useful for expression of cDNAs from other parasites or invertebrates which have similarly distinctive codon-usage preference.

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

Similar to TPH, the catecholamine-biosynthetic enzyme, tyrosine hydroxylase (TH), is poorly understood in helminths and its presence in these organisms has been questioned. In this study, we cloned, expressed, purified, and functionally characterized a TH (SmTH) from *S. mansoni*. This is the first TH gene to be cloned from an invertebrate, and its presence in *S. mansoni* further suggests that parasitic helminths have the enzymatic capacity to synthesize catecholamines.

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LIST OF ABBREVIATIONS

5-CT	5-carboxyamidotryptamine
5-HT	5-hydroxytryptamine; serotonin
5-HTP	5-hydroxytryptophan
AAADC	Aromatic amino acid decarboxylase
AAAH	Aromatic amino acid hydroxylase
BH_4	(*6R*)-5,6,7,8-tetrahydrobiopterin
CaM-PKII	Ca^{2+} / calmodulin-dependent protein kinase II
CNS	Central nervous cystem
DOB	1-(4-bromo-2,5-dimethoxyphenyl)-2-aminopropane
DOI	2,5-dimethoxy-4-iodophetamine
DOM	1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane
CREB	Cyclic AMP responsive element binding protein
GPCR	G Protein-coupled receptor
8-OH-DPAT	8-hydroxy-2-(di-*n*-propylamino)tetralin
LSD	Lysergic acid diethylamide
MAO	Monoamine oxidase
NO	Nitric oxide
PAH	Phenylalanine hydroxylase
PBS	Phosphate-buffered saline
PCP	*p*-chlorophenylalanine
PLC	Phospholipase C
RACE	Rapid amplification of cDNA ends
SL	Spliced leader
SSC	Saline sodium citrate
SDS	Sodium dodecyl sulfate
TM	Transmembrane domain
TPH	Tryptophan hydroxylase
TH	Tyrosine hydroxylase
UTR	Untranslated region

INTRODUCTION

The monoamine serotonin (5-HT; 5-hydroxytryptamine) is a major neurotransmitter and / or neuromodulator that is widely distributed across phylogeny, and is known to effect a variety of behavioral and physiological responses in different species (Wilkinson and Dourish, 1991; Csaba, 1993; Hen, 1993; Walker et al., 1996; Weiger, 1997). 5-HT exerts its pleiotropic effects by binding to multiple cell surface receptors which belong, for the most part, to the superfamily of G protein-coupled receptors (Gerhardt and van Heerikhuizen, 1997; Barnes and Sharp, 1999). The first and rate-limiting step in the biosynthesis of 5-HT is catalyzed by tryptophan hydroxylase (TPH), the enzyme that hydroxylates tryptophan to 5-hydroxytryptophan, which in turn is decarboxylated to produce serotonin (Mockus and Vrana, 1998). Most of the information available on serotonin biosynthesis and its receptors have come from studies of vertebrates, mainly mammalian. By comparison, very little is known about the properties of 5-HT in invertebrate species.

In helminths, 5-HT has been localized in many tissues of parasitic and free-living worms. In these organisms, 5-HT modulates many behaviors and responses, including motility, carbohydrate metabolism, feeding activity and mating behavior (Davis and Stretton, 1995). The issue of serotonin biosynthesis in helminths has been subject of some controversy, especially with respect to parasitic worms, such as *Schistosoma mansoni*. Some researchers believe that parasitic helminths lack the enzyme TPH necessary to produce 5-HT and, therefore, must rely on the host for a supply of 5-HT (Bennett et al., 1973; Catto and Ottesen, 1979; Cho and Mettrick, 1982; Mansour, 1984; Wood and Mansour, 1986). Although other researchers have provided biochemical evidence for TPH activity in related parasites (Ribeiro and Webb, 1983,1984; Chaudhuri et al., 1988a,b), the question of whether TPH is present in parasitic helminths has not been resolved, especially since no helminth TPH gene has yet been cloned or characterized. As for the case of helminth serotonin receptors, earlier pharmacological work suggested the presence of G protein coupled 5-HT receptors in the tissues of some parasitic helminths, such as, *Fasciola hepatica* (Trematoda), *Hymenolepis diminuta*

(Cestoda), and *Ascaris suum* (Nematoda) (McNall and Mansour, 1984; Ribeiro and Web, 1986,1987; Chaudhuri and Donahue, 1989; Williams, et al., 1992). At the time this study began, however, no 5-HT receptor, or any other monoamine receptor had been cloned from a helminth system, free-living or parasitic. This complete absence of structural information hindered progress in the characterization of these important neuroactive proteins.

This study investigated the molecular properties of 5-HT in helminths, specifically the controversial issue of 5-HT biosynthesis and the properties of the GPCRs that mediate 5-HT signaling. The two main objectives of this project were: 1) to clone and characterize the key enzyme in 5-HT biosynthesis, TPH, from *S. mansoni*, and 2) to clone and characterize 5-HT receptors from two helminthic models, a free-living nematode, *Caenorhabditis elegans* , and a parasitic trematode, *S. mansoni.*

Chapter I provides an overview of the current state of 5-HT research in both vertebrate and invertebrate species, with a focus on helminth studies. This literature review is divided into three parts. The first part deals with the general properties of 5-HT in helminths, its presence, distribution and biological functions. The second part is an overview of the mechanism of 5-HT biosynthesis, in particular the properties of the rate-limiting enzyme, TPH. Finally, the third part of chapter I includes a brief synopsis of GPCRs and a description of what is known about the structural, phamacological, and signaling properties of 5-HT GPCRs. Chapter II describes the cloning and characterization of a stable form of TPH from *S. mansoni.* Chapter III reports the cDNA cloning and functional expression of a novel serotonin receptor from *C. elegans.* Chapter IV describes the molecular cloning and expression of a novel *S. mansoni* GPCR with structural properties related to biogenic amine receptors, such as 5-HT, catecholamine, and octopamine receptors. Chapter V is a final discussion outlining the significance of this study and its contribution to the advancement of the knowledge of the molecular properties of 5-HT in helminths.

In addition to five principal chapters, there are three appendices in this thesis. Appendix I reports the cloning and functional characterization of a *S. mansoni* tyrosine hydroxylase (TH), the enzyme that catalyzes the first and rate-limiting step in the biosynthesis of catecholamine neurotransmitters, such as dopamine and noradrenaline.

TH is structurally and functionally related to TPH, both being members of the family of aromatic amino acid hydroxylases. Similar to TPH, TH in helminths is poorly studied and its presence in these animals has been questioned. The work shown in appendix I represents the first molecular evidence for the presence of a catecholamine-biosynthetic enzyme in any helminth system. Finally, Appendices II and III provide a brief description of the life cycles of the two helminthic models used in this study, *S. mansoni* and *C. elegans*.

CHAPTER I

LITERATURE REVIEW

PART I: SEROTONIN IN HELMINTHS

I.1. Serotonin: history and characteristics - The biogenic amine, serotonin (5-hydroxytryptamine; 5-HT) is a major neuroactive agent that is widely distributed in both vertebrates and invertebrates, including some unicellular organisms such as *Tetrahymena* and amoebas (Csaba, 1993; Hen, 1993; McGowan et al., 1983; Weiger, 1997). In mammals, the aromatic amino acid hydroxylase, tryptophan hydroxylase (TPH) catalyzes the rate-limiting step in the biosynthesis of 5-HT from tryptophan (reviewed in Mockus and Vrana, 1998) (Fig. 1). Historically, the chemical discovery of 5-HT dates back to 1948 when Rapport et al. (1948) reported the isolation of 5-HT and its chemical synthesis as 5-hydroxytryptamine creatinine sulfate (Rapport, 1949). Subsequent investigations led to the conclusion that this active substance was indeed identical to two previously reported agents, a serum-factor that caused vasoconstriction and a gut substance, enteramine, which increased intestinal motility (Erspamer and Aser, 1952). Further studies using fluorescence and immunohistochemical techniques led to the localization of significant amounts of serotonergic neurons in the central nervous system (CNS), which resulted later in the classification of 5-HT as a neurotransmitter (Twarog and Page, 1953) (reviewed in Jacobs and Azmitia, 1992). Subsequently, 5-HT was detected in the nervous systems in many invertebrate (Welsh and Moorehead, 1960). To learn more about the historical aspect of the discovery of serotonin, the readers are referred to a recent article by Rapport (1997).

In the CNS of mammals, the majority of serotonergic neurons have cell bodies that are associated with the raphe nuclei situated along the midline region of the brain stem. These neurons send axonal projections to different parts of the CNS including the cortex, hypothalamus, hippocampus, amygdala, basal ganglia, the superior calculus and the lateral geniculate (Jacobs and Azmitia, 1992; Rubenstein, 1998). This serotonergic transmission has been linked to a wide variety of behaviors and disorders such as schizophrenia, anorexia nervousa, pain perception, aggression, feeding behavior, anxiety, sleep patterns, learning, sexual behavior, and depression (Wilkinson and Dourosh, 1991; Jacobs and Azmitia, 1992; Kroeze and Roth, 1998; Weiger, 1997; Lucki, 1998). In the

periphery, 5-HT is present in the enterochromaffin cells of the gut and in blood platelets (Hen, 1993), where it affects various physiological responses, including vascular and smooth muscle contraction, thermoregulation, respiration, cardiovascular regulation, uterine smooth muscle growth, gastrointestinal contractions, and platelet aggregation (Wilkinson and Dourosh, 1991; Roth, 1994). These diverse serotonergic effects are mediated by multiple cell surface receptors which, in mammals, appear to fall into 7 distinct families and at least 15 different subtypes (Gerhardt and van Heerikhuizen, 1997; Barnes and Sharp, 1999). The pharmacological and signaling properties of the various cloned serotonergic receptors are discussed in the third part of this chapter.

I.2. Serotonin in helminths: function, biosynthesis and receptors

5-HT has been detected in every invertebrate phylum so far investigated and in protozoa such as tetrahymena and amoeba (Welsh and Moorhead, 1960; McGowan et al., 1983; Goldberg and Kater, 1989; Csaba, 1993; Walker et al., 1996; Weiger, 1997). In invertebrates, 5-HT acting through multiple receptors, regulates many behaviors and physiological responses such as motility, aggression, feeding and mating behaviors, circadian rhythms, learning and memory, as well as modulation of different metabolic pathways (Davis and Stretton, 1995; Walker et al., 1996; Weiger, 1997). The following is a brief overview of the general properties of 5-HT in helminths, its localization and functional importance, with greater emphasis on the two helminthic models used in this thesis, namely the parasitic trematode, *Schistosoma mansoni*, and the free-living nematode, *Caenorhabditis elegans*.

Many potential neurotransmitter candidates have been identified in helminths, including 5-HT, acetylcholine, catecholamines (noradrenaline and dopamine), γ-aminobutyric acid (GABA), glutamate, histamine, octopamine, and different neuropeptides (reviewed in Davis and Stretton, 1995). Among these neuroactive agents, 5-HT is the most abundant and functionally diverse. 5-HT has been localized by biochemical, histochemical and / or immunocytochemical techniques in a variety of worms, including nematodes (roundworms), such as *C. elegans* and the pig parasite *Ascaris suum*, and various platyhelminths (flatworms) such as trematodes (*e.g.* the blood

flukes *S. mansoni* and *Fasciola hepatica*) and cestodes (*e.g. Hymenolepis diminuta, Taenia pisiformis*) (Davis and Stretton, 1995).

I.2.1. Serotonin in Platyhelminths - In flatworms, including trematodes and cestodes, 5-HT has been localized in the central (anterior ganglia, transverse and longitudinal nerve cords) and peripheral nervous systems, holdfast structures, body wall muscle, reproductive organs, and some regions of the gut (only in trematodes) (Pax and Bennett, 1991). Earlier work showed that 5-HT causes an increase in motor activity in both trematodes and cestodes (Mansour, 1984; Pax and Bennett, 1991). This modulation of motility is of great importance for worm survival, especially for parasitic species which have to attach to specific sites or move to various locations in the host. In addition to effects on motility, 5-HT plays an important role in the regulation of metabolic processes, especially carbohydrate metabolism, in trematodes and cestodes (Mansour, 1984; Davis and Stretton, 1995). Studies done on the parasitic trematodes *S. mansoni* and *F. hepatica* and the cestode, *H. diminuta*, showed that 5-HT causes an increase in glycolysis and glycogenolysis through activation of relevant key enzymes such as glycogen phosphorylase, phosphofructokinase and adenylate cyclase (Mansour, 1984; Pax and Bennett, 1991).

Little is known about the molecular properties and the heterogeneity of 5-HT receptors in invertebrates, especially in helminths. The first direct evidence for the presence of functional 5-HT receptors in trematodes came from the work of McNall and Mansour (1984), who characterized a specific 5-HT binding site in crude membrane preparations of *F. hepatica*. This 5-HT receptor coupled to the activation of adenylate cyclase and displayed high affinity for the serotonergic agonist, [^{3}H]-*d*-Lysergic acid diethylamide ([^{3}H]LSD; K_D ~ 25 nM). In addition the receptor showed specificity for several classical 5-HT receptor antagonists. Soon after, Ribeiro and Webb (1986,1987) reported the pharmacological and signaling analyses of multiple 5-HT receptors from a crude membrane preparation of *H. diminuta*. One of these receptors showed high affinity for [^{3}H]5-HT and induced activation of adenylate cyclase in response to 5-HT stimulation (Ribeiro and Webb, 1987). Further work (Estey and Mansour, 1987,1988) showed that 5-HT-adenylate cyclase activation in *S. mansoni* was mediated through the activation of

stimulatory G proteins. This response was inhibited by specific 5-HT receptor antagonists in a similar fashion to what was reported for *F. hepatica* (Estey and Mansour, 1988). More recent evidence demonstrated the presence of a mammalian-like stimulatory alpha-subunit ($G_{s\alpha}$) cloned from *S. mansoni* (Iltzsch et al., 1992), suggesting that at least some helminth receptors function by coupling with heterotrimeric GTP binding proteins, just as shown previously for other organisms.

Although there is no binding data for a 5-HT receptor in *S. mansoni*, the above studies are highly suggestive of the presence of 5-HT receptor(s) in this parasite. Furthermore, data available from binding (*F. hepatica*) and signaling studies (*F. hepatica* and *S. mansoni*) showed that the trematode receptors recognized classical serotonergic agonists and antagonists, but the relative affinities for these drugs were different from those of mammalian 5-HT receptors. This difference has led to speculation that helminth receptors have distinctive structural, and possibly functional characteristics.

To date, no 5-HT receptor has yet been cloned and characterized from any platyhelminth. Recently, Saitoh et al. (1997) reported the cloning of several putative G protein-coupled receptors (GPCR) from planaria, two of which showed high homology with other cloned 5-HT receptors. However, no functional studies were undertaken to confirm the identity of these receptors.

I.2.2. Serotonin in nematodes - 5-HT has been identified in many nematodes, both parasitic (*A. suum, Trichostrongylus colubriformis, Nippostrongylus brasiliensis*), and free-living (*C. elegans and Goodyus ulmi*) (Davis and Stretton, 1995; Goudey-Perriere et al., 1997). Most of the 5-HT cellular localization studies in nematodes have been done on *C. elegans* and *A. suum* (Davis and Stretton, 1995). In both nematodes 5-HT was localized to two pharyngeal neurosecretory motor neurons (NSM) and 5 male-specific neurons (CP neurons) in the tail region (Horvitz et al., 1982; White et al., 1986; Loer et al., 1993; Brownlee et al., 1994; Johnson et al., 1996). In addition, 5-HT has been detected in *C. elegans* (but not *A. suum*) in a pair of hermaphrodite-specific motor neurons (HSN) (White et al., 1986; Desai et al., 1988).

Several functional properties of 5-HT have been studied in nematodes. In contrast to the stimulatory effect on motor activity exerted on trematodes and cestodes, 5-HT

either suppresses or completely inhibits nematode motility (Davis and Stretton, 1995; Mansour, 1984). In *C. elegans*, *G. ulmi*, and *A. suum*, 5-HT has been shown to stimulate egg-laying and / or affect mating behavior or posture (Horvitz, et al. 1982; Leach et al., 1987; Loer and Kenyon, 1993; Reinitz and Stretton 1996). In addition, 5-HT was reported to affect other behaviors in *C. elegans* such as pharyngeal pumping and feeding patterns (Horvitz et al., 1982). In *C. elegans*, the NSM have been linked to the modulation of pharyngeal pumping, egg-laying, and motor activity, whereas the CP neurons seem to be more involved in egg-laying and mating behavior (Desai and Horvitz, 1989; Horvitz et al., 1982; Loer and Kenyon, 1993). Recent single cell ablation experiments identified two pairs of *C. elegans* motor neurons HSNL / HSNR and VC4 / VC5 which control egg laying by releasing 5-HT (Waggoner et al., 1998). The majority of the 5-HT-controlled behaviors in *C. elegans* seem to be mediated by the G protein G_o (Mendel et al., 1995; Segalat et al., 1995). The role of 5-HT in the regulation of metabolism is limited to studies of *A. suum* where it was shown to stimulate glycolysis and glycogenolysis and activate adenylate cyclase (Mansour, 1984).

More information is available on the molecular properties of 5-HT receptors and their signaling in nematodes than in other helminths. This is largely due to the recent sequencing of the *C. elegans* genome (Bargmann, 1998) which has unveiled the sequences of a large numbers of potential receptors. Earlier receptor binding studies demonstrated the presence of 5-HT receptors in muscle and intestinal tissues of *A. suum* (Chaudhuri and Donahue, 1989; Williams et al., 1992). The coupling mechanism of the muscle *Ascaris* 5-HT receptor was further shown to involve an increase in cAMP levels, suggesting the activation of the intracellular effector enzyme, adenylate cyclase (Williams et al., 1992). Recently two *C. elegans* and one *A. suum* 5-HT receptors have been cloned and characterized (Olde and McCombie, 1997; Hamdan and Ribeiro, 1999; Huang et al., 1999). These recombinant nematode receptors are described in detail in the third part of this chapter.

I.2.3. Biosynthesis of 5-HT in helminths - According to mammalian studies, 5-HT biosynthesis from tryptophan requires two enzymes, tryptophan hydroxylase (TPH) which catalyzes the first and rate-limiting hydroxylation reaction of this pathway, and an

aromatic amino acid decarboxylase (AAADC) which converts the product of the hydroxylation reaction, 5-hydroxytryptophan (5-HTP) to 5-HT (reviewed in Mockus and Vrana, 1998; Fitzpatrick, 1999). Therefore, for an organism to be able to biosynthesize 5-HT in a similar fashion it should possess the same two enzymes, especially TPH, which sets the pace of the reaction. So far TPH has been cloned only from mammalian species, with the exception of a *Drosophila* phenylalanine hydroxylase (PAH) which showed TPH activity and was thought to represent a PAH/TPH hybrid gene (Neckameyer and White, 1992; Mockus and Vrana, 1998). In addition, earlier preliminary biochemical evidence suggested that another invertebrate, the sea pansy *Renilla koellikeri* (Cnidaria) possessed TPH and was able to carry 5-HT biosynthesis from tryptophan (Pani and Anctil, 1994).

The issue of whether helminths, in particular parasitic ones, are capable of synthesizing their own 5-HT has been the subject of debate. Earlier studies demonstrated that helminths were able to produce 5-HT from 5-HTP, suggesting the presence of the synthetic enzyme AAADC, and possessed the 5-HT degradative enzyme, monoamine oxidase (MAO) (Nimmo-Smith and Raison, 1968; Bennett and Bueding, 1971; Catto, 1981; Mishra et al., 1984). Attempts to show 5-HT biosynthesis and TPH activity in tissue extracts of some parasitic helminths were unsuccessful. This led some researchers to suggest that TPH might be absent in these parasites and that the worms depended on the host for a source of 5-HT (Bennett and Bueding, 1973; Catto and Ottesen, 1979; Mansour, 1984; Wood and Mansour, 1986). Although other researchers challenged these suggestions by reporting preliminary evidence of TPH activity in *H. diminuta* and *A. suum* (Ribeiro and Webb, 1983, 1984; Chaudhuri, 1988a,b), the question of how parasitic helminths obtained 5-HT remained unresolved. Recently, we cloned and characterized a functionally active TPH from *S. mansoni*, thus providing the first molecular and biochemical evidence that *S. mansoni*, and probably other parasitic helminths, are capable of synthesizing their own 5-HT (Hamdan and Ribeiro, 1999) (see chapter II). In addition, other work in our laboratory resulted in the cloning of a full length TPH cDNA from *C. elegans* (Hill and Ribeiro, 1998), indicating that TPH is present in nematodes as well.

PART II: SEROTONIN BIOSYNTHESIS: PROPERTIES OF TPH

II.1. Pterin-dependent aromatic amino Acid hydroxylases

The three aromatic amino acid hydroxylases (AAAHs), tryptophan hydroxylase (TPH; EC1.14.16.4), tyrosine hydroxylase (TH; EC 1.14.16.2), and phenylalanine hydroxylase (PAH; EC 1.14.16.1) comprise a structurally and functionally related family of hydroxylases (reviewed in Hufton et al., 1995; Kappock and Cardonna, 1996; Kaufman and Ribeiro, 1996; Fitzpatrick, 1999). It has been suggested that these three enzymes evolved by gene duplication and divergence from a common ancestral gene (Grenett et al., 1987). TPH catalyzes the hydroxylation of tryptophan to 5-HTP, the initial and rate-limiting step in the biosynthesis of 5-HT. In turn, 5-HT serves as a precursor for the biosynthesis of the pineal hormone, melatonin, whose most important function is the regulation of seasonal rhythms by light-dark cycle (Mockus and Vrana, 1998; Vanecek, 1998). TH similarly catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (L-Dopa), the first and rate-limiting step in the biosynthesis of catecholamines which include dopamine, noradrenaline (norepinephrine) and adrenaline (epinephrine) (reviewed in Kumer and Vrana, 1996). TH-deficiency in the mammalian striatum has been linked to the development of serious neurodegenerative diseases such as Parkinson's (reviewed in Haavik and Toska, 1998; Nagatsu and Ichinose, 1999). In contrast to TPH and TH, which are involved in neurotransmitter synthesis and are primarily associated with neuronal structures, PAH is mainly found in the liver where it catalyzes the hydroxylation of phenylalanine to tyrosine, the rate-limiting step in the obligatory pathway of phenylalanine degradation. Dysfunction of PAH leads to phenylketonuria and hyperphenylalanemia, serious physiological conditions which, if untreated, can often lead to mental retardation (Hufton et al., 1995).

The hydroxylation reactions catalyzed by these different AAAHs have an absolute requirement for a reduced pterin cofactor, tetrahydrobiopterin (BH_4), molecular oxygen and ferrous iron (Fe^{2++}). BH_4 is oxidized during the course of the hydroxylation reaction to free 4a-hydroxy-BH_4 which in turn is converted to quinoid dihydropterin (q-BH_2) either non-enzymatically or through the action of a dehydratase. The enzyme

dihydropteridine reductase (DHPR) catalyzes the reduction of q-BH_2 to BH_4, thus regenerating this essential cofactor (Hufton et al., 1995; Nagatsu and Ichinose, 1999). The different biochemical pathways involving the actions of TPH, TH, and PAH are illustrated in Figure 1.

Structurally, the three enzymes are closely related, all being composed of an N-terminal domain, thought to be regulatory, a conserved central core which includes the catalytic domain and the iron binding site, and a C-terminal region which contains an intersubunit binding domain responsible for holding the enzyme in a tetrameric form (Hufton et al., 1995; Mockus and Vrana, 1998; Fitzpatrick, 1999). Structural information on these enzymes has been derived mainly from *in vitro* site-directed mutagenesis studies and recent analyses of TH and PAH crystal structures (Erlandsen et al., 1997; Goodwill et al., 1997; Fusetti et al., 1998; Fitzpatrick, 1999). At present no crystal structure of TPH is available. In the following section, I will be discussing in more detail the structural, functional, and regulatory properties of TPH with occasional referencing to the other two related hydroxylases

II.2. Molecular cloning of TPH: an evolutionarly perspective – In mammals, TPH is found in serotonergic cells of the brainstem raphe nuclei, the pineal gland, the pancreatic and intestinal enterochromaffin cells, thyroid cells, retina, mast cells, platelets, and in embryonic stem cells (Jacobs and Azmitia, 1992; Mockus and Vrana, 1998; Walther and Bader, 1999). Dysfunction of TPH affects the synthesis of 5-HT and, in the pineal gland, further synthesis of melatonin (Jacobs and Azmitia, 1992). Molecular and biochemical studies of TPH have been hindered by the extreme instability of this enzyme and difficulties in purifying it in large quantities from native or recombinant sources (Kuhn et al., 1980; Vitto and Mandell, 1981; Nakata and Fujisawa, 1982; Park et al., 1994; Tipper et al., 1994; Vrana et al., 1994a; D'Sa et al., 1996a; Banik et al., 1997; Cash, 1998).

TPH has been cloned from many vertebrate species, including rabbit, rat, human, mouse, frog, and chicken (Grenett et al., 1987; Darmon et al., 1988; Boularand et al., 1990; al., Stoll et al., 1990; Green and Besharse, 1994; Florez et al., 1996). In contrast, very few TPH genes have been cloned or characterized from invertebrates.

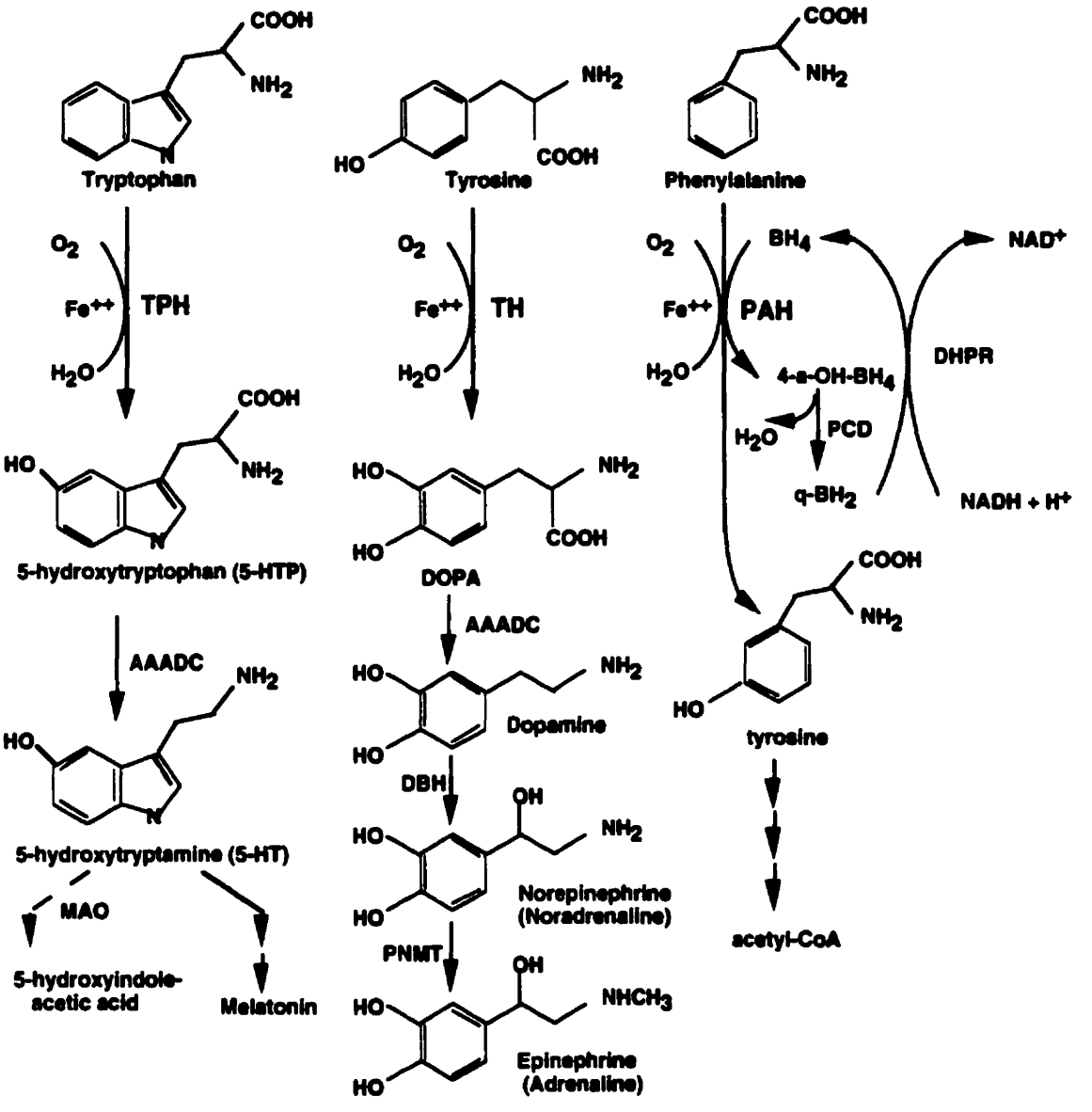

Fig. 1. Enzymatic pathways involved in pterin-dependent hydroxylation of aromatic amino acids. TPH, TH, and PAH catalyze the hydroxylation (rate limiting step) of tryptophan, tyrosine, and phenylalanine to 5-hydroxytryptophan (5-HTP), 3,4-dihydroxyphenylalanine (L-DOPA), and tyrosine, respectively. The enzyme aromatic amino acid decarboxylase (AAADC) converts 5-HTP and L-DOPA, and tyrosine to serotonin (5-HT) and dopamine (which metabolizes to form the two other catecholamines, epinephrine and norepinephrine). 5-HT could be metabolized to produce melatonin or it could be degraded through the action of a monoamine oxidase MAO). The activities of these hydroxylating enzymes require the presence of BH_4, Fe^{2+}, and O_2. Pterin 4a-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) act upon 4a-OH- BH_4 and quinonoid dihydrobiopterin (q-BH_2), respectively, to regenerate BH_4 (modified from Hufton et al., 1995). DBH, dopamine β hydroxylase; PNMT, phenylethanolamine-*N*-methyl transferase.

Neckameyer and White (1992) described the cloning of a *Drosophila* cDNA that encoded a protein with PAH / TPH activity which was assumed to a be a product of a hybrid PAH/ TPH gene. The presence of this hybrid gene in *Drosophila* suggested that the separation of TPH from PAH occurred late in evolution, probably after emergence of insects. Recently, however, we cloned and characterized a functionally active TPH from *S. mansoni* (SmTPH) (Hamdan and Ribeiro, 1999) (see chapter II). This evidence, combined with genomic information of putative TPH, TH, and PAH sequences deposited in the *C. elegans* data base (Bargmann, 1998), argues that the separation of the three AAAHs occurred at an earlier time in evolution before the divergence of flatworms. Indeed, recent phylogenetic analyses distinctly grouped the *Drosophila* PAH / TPH presumed hybrid along with the PAH family, suggesting that the cloned enzyme is structurally related to PAH (Boularand et al., 1998; Hamdan and Ribeiro, 1999). The amino acid sequence relationship between the different cloned AAAHs is illustrated in Figure 2.

II.3. Structural properties of TPH and other AAAHs

Sequence analyses of TPH cDNAs cloned from mammalian and other species revealed that TPH displays high sequence homology (40 – 53 % amino acid identity) with the other two related hydroxylases, TH and PAH (Mockus and Vrana, 1998). *In vitro* deletion mutagenesis and biochemical studies showed that TPH has a similar structural organization to TH and PAH . All three enzymes can be divided into three main domains: an N-terminal domain (regulatory), a catalytic core, and a C-terminal domain (tetramerization domain) (Fig. 3) (Hufton et al., 1995; Mockus and Vrana, 1998; Fitzpatrick, 1999).

II.3.1. TPH N-terminal (regulatory) domain – The boundary separating the N-terminal domain of TPH from its catalytic core has been identified through a series of deletion mutagenesis studies. Previous work showed that deletions of 64, and in some cases up to 106 N-terminal amino acids did not affect the activity of mammalian TPH (Stoll et al., 1990; D'Sa et al., 1996a; Kuhn et al., 1997; Kumer et al., 1997; Moran et al., 1998). Further deletions of the first 116 to 165 N-terminal amino acids greatly reduced

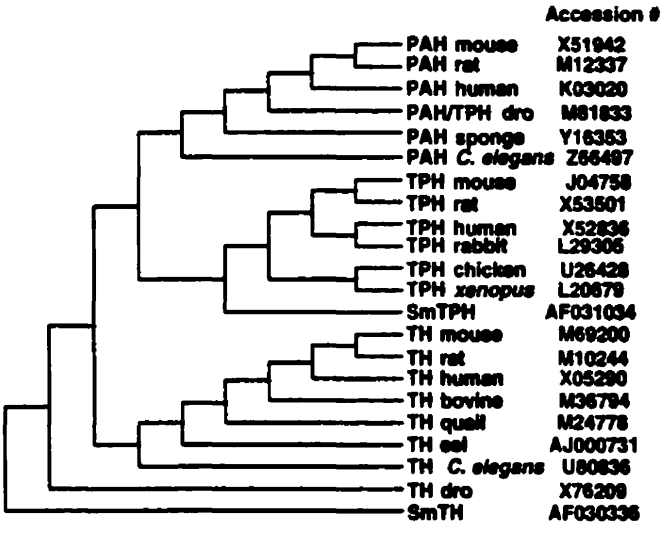

Fig. 2. Dendrogram showing the structural relationship between the various cloned aromatic amino acid hydroxylases. The amino acid sequences of TPHs, THs and PAHs were compared using the program DNASIS vs. 3.7 (Hitachi Software, CA, U.S.A.). The lengths of the horizontal lines connecting one sequence to another are inversely proportional to the percentages of similarity between sequences or group of sequences. Hydroxylase sequences used were from *Homo sapiens* (human), *Bos torus* (bovine), *Oryctolagus cuniculus* (rabbit), *Anguilla anguilla* (eel), *Xenopus laevis*, *Coturnix coturnix* (quail), *Gallus gallus* (chicken), *Rattus norvegicus* (rat), *Mus musculus* (mouse), *Geodia cydonium* (sponge), *Drosophila melanogaster* (dro), *Caenorhabditis elegans*, and *Schistosoma mansoni* (SmTPH and SmTH). The corresponding GenBank accession number is indicated next to each sequence (modified from Hamdan and Ribeiro, 1999)

rabbit TPH activity (Stoll et al., 1990; Yang and Kaufman, 1994; Kumer et al., 1997), suggesting that the region between amino acids 106 and 116 define the boundary between the N-terminal domain and the catalytic core of the enzyme (Mockus and Vrana, 1998). Similarly, for TH and PAH the N-terminal region / catalytic domain boundaries have been previously determined (~ 141 and 165 N-terminal residues for PAH and TH, respectively) (Hufton et al., 1995).

In TH and PAH, the N-terminal domain has been shown to play a regulatory function by tonically inhibiting the activity of the catalytic core (Hufton et al., 1995; Kumer and Vrana, 1996). In the case of TPH less information is available. However, there is enough evidence to suggest that the N-terminal domain may serve a regulatory function as well (Mockus and Vrana, 1998). Deletion of this domain from TH and PAH enhances their enzymatic activities (Hufton et al., 1995; Kumer and Vrana, 1996). For TPH, deletion of 91 to 106 N-terminal amino acids resulted in conflicting reports where the enzymatic activity was found to be either reduced, unchanged, or slightly enhanced (Yang and Kaufman, 1994; D'Sa et al., 1996a; Kumer et al., 1997).

Previous reports indicated that the activity of TPH is increased in response to phosphorylation by the cAMP-dependent protein kinase (PKA) (Garber and Makman, 1987; Makita et al., 1990 Johansen et al., 1996; Banik et al., 1997; Kuhn et al., 1997).

Recently, the N-terminal serine-58 of the rabbit TPH was identified as the phosphorylation substrate site for PKA (Kuhn et al., 1997; Kumer et al., 1997). Additional studies revealed that the regulatory protein 14-3-3 is required for the activation of the phosphorylated enzyme (Banik et al., 1997).

The N-terminal domain sequence is highly divergent among TPH, TH, and PAH (Hufton et al., 1995). It was often thought that this domain was involved in directing enzyme substrate specificity (Abate et al., 1988; Abate and Joh, 1991; D'Sa et al., 1996a). The role of this domain in enzyme specificity was recently investigated by developing TH / PAH and TH / TPH regulatory/ catalytic domains chimera (Daubner et al., 1997; Mockus et al., 1997b). These studies showed that the regulatory domain is not essential for enzyme specificity, however, it might affect the stability of the enzyme (Mockus et al., 1997b).

A very recent study provided a direct evidence linking the N-terminus of TPH to enzyme tetramerization. The authors showed that deletion of a N-terminal 4, 3-hydrophobic motif (residues 21 – 41) of the rabbit TPH abolished its ability to form tetramers (Yohrling et al., 1999). The same α-helix motif is also present in both TH and PAH where it is likely to serve a similar function.

II.3.2. TPH catalytic core – The C-terminal two-thirds of TPH, TH, and PAH displays the highest sequence conservation among the three hydroxylases (Hufton et al., 1995; Mockus and Vrana, 1998). This region has been shown to be responsible for the catalytic activity of these enzymes (Hufton et al., 1995; Kappock and Cardonna, 1996; Mockus and Vrana, 1998). The catalytic core of TPH contains two highly conserved histidine residues (His^{272} and His^{277} of the rabbit TPH) and one glutamate (Glu^{292} of the rabbit TPH) which have been shown, by mutagenesis and crystallization studies of TH and PAH, to constitute a binding site for ferrous iron (Hufton et al., 1995; Erlandsen et al., 1997; Goodwill et al., 1997; Fusetti et al., 1998; Fitzpatrick, 1999). Iron is required for the enzymatic activities of TPH, TH, and PAH (Hufton et al., 1995; Fitzpatrick, 1999). Recently, the iron content of the catalytic core of rabbit TPH was measured *in vitro* by Moran et al. (1998). In addition, Hasegawa et al. (1999) demonstrated that TPH activity depended on the intracellular iron concentration in monolayer cultures of the 5-HT

producing mast cell line, RBL2H3 cells. Other well conserved residues among the catalytic cores of TPH, TH, and PAH, include a stretch of ~ 27 amino acids (His^{252} to His^{277} of the rabbit TPH) which has been shown to constitute the BH_4 binding site of PAH, and possibly of TH and TPH as well (Jennings et al., 1991; Hufton et al., 1995). The location of the substrate binding site has not yet been elucidated for any of the hydroxylases, including TPH (Mockus and Vrana, 1998; Fitzpatrick, 1999).

II.3.3. TPH C-terminal domain – Recent mutagenesis and crystallization studies showed that TH and PAH exist as homotetramers joined by a C-terminal leucine heptad repeat (leucine zipper; tetramerizarion domain) interspersed within a 4,3-hydrophobic motif (Liu and Vrana, 1991; Hufton et al., 1995, 1998; Erlandsen et al., 1997; Goodwill et al., 1997; Fusetti et al., 1998). Similar to TH and PAH, native and recombinant TPH exist as homotetramers (Nakata and Fujisawa, 1982; Hufton et al., 1995; Mockus et al., 1997a; Hamdan and Ribeiro, 1999). TPH sequences also contain the conserved leucine zipper motif at its C-terminus (amino acids 428 – 444 of rabbit TPH) which upon deletion (17 residues) results in an active monomeric form of TPH (Mockus and Vrana, 1998; Moran et al., 1998). Moran et al. (1998) reported that removal of the tetramerization domain of the catalytic core of TPH and subsequent expression in *E. coli* resulted in a highly soluble active enzyme. In contrast to TPH, removal of the tetramerization domain of the C-termini of TH and PAH produces a mixture of predominantly dimeric forms of the enzymes that display reduced activities (Vrana et al., 1994b; Hufton et al., 1998). In addition, Hufton et al. (1998) recently showed that deletion of both the N-terminal and the tetramerization domains of PAH resulted in a predominant monomeric enzyme, whereas, removal of the tetramerization domain alone yielded mainly dimeric forms. This suggested that the N-terminal domain of PAH could play a role in enzyme tetramerization, as has been recently shown for TPH (Hufton et al., 1998; Yohrling et al., 1999).

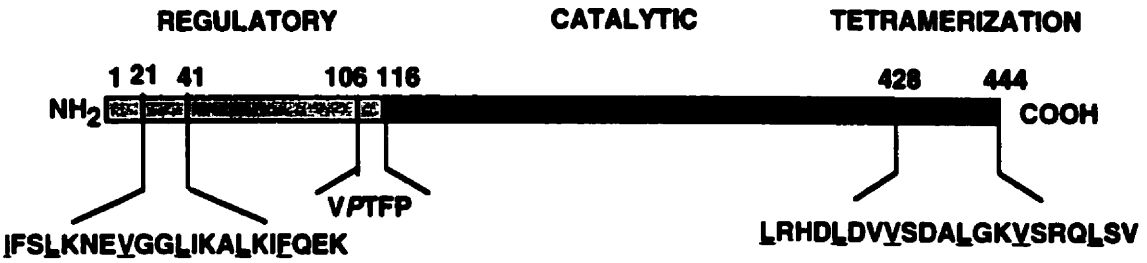

Fig. 3. Structural organization of monomeric TPH. The rabbit TPH is made up of three functional domains: an N-terminal (regulatory) domain, a catalytic core, and a C-terminal (tetramerization) domain. Amino acids 106 to 116 define the boundary between the regulatory and the catalytic domains and mark the beginning of high sequence conservation between TPH and TH. Amino acids 21 to 41 constitute an α-helix motif involved in enzyme tetramerization. The region between amino acids 428 and 444 constitute another tetramerization domain. Residues of the 4,3-hydrophobic motifs (α-helical) are underlined.

II.4. Molecular mechanisms of TPH regulation

TPH and TH share similar regulatory properties, both enzymes being neuronal and involved in neurotransmitter synthesis. The following is a brief overview of the major molecular mechanisms of TPH regulation (for reviews, see Hufton et al., 1995; Kappock and Cardonna, 1996; Kaufman and Ribeiro, 1996; Mockus and Vrana, 1998; Fitzpatrick, 1999), many of which also play a role in the regulation of TH.

II.4.1. TPH activation - Native and recombinant TPH can be activated by several agents including limited proteolysis, polyanions such as heparin, and phospholipids (Friedman et al., 1972; Kuhn et al., 1979; Imai et al., 1989; Yang and Kaufman, 1994; D'Sa et al., 1996a,b). In addition, a large body of evidence shows that TPH phosphorylation is accompanied by increased activity of the enzyme (reviewed in Mockus and Vrana, 1998).

At least two protein kinases, PKA and CaM-PKII, are involved in TPH phosphorylation (Ehret et al., 1991; Vrana et al., 1994a; Johansen et al., 1995, 1996). The phosphorylation site of PKA has been identified as serine-58 which lies in the regulatory domain of rabbit TPH (Kuhn et al., 1997; Kumer et al., 1997). The CaM-PKII phosphorylation site has not yet been identified, but sequence analyses suggest two potential candidates, serine-260 and serine-443, which are part of the catalytic core and the tetramerization domain of rat TPH, respectively (Darmon et al., 1988). Activation of

TPH by PKA or CaM-PKII phosphorylation seems to require the binding of a small cytosolic acidic protein, 14-3-3, which is evolutionarly conserved and highly abundant in the mammalian nervous system (Ichimura et al., 1987, 1988, 1995, 1997; Makita et al., 1990; Isobe et al., 1991; Furukawa et al., 1993; Banik et al., 1997; Kuhn et al., 1997) (for a general review on 14-3-3 see Skoulakis and Davis, 1998). Ichimura et al. (1995, 1997) showed that the C-terminal domain of 14-3-3 (region covering amino acids 171 – 213) is the binding domain for phosphorylated TPH. A recent study by Banik et al. (1997) suggests that 14-3-3 may bind to the regulatory domain of phosphorylated TPH and prevents its dephosphorylation, either by altering the conformation of TPH or hindering the access of phosphatases to the phosphorylation site. This results in increased enzymatic stability since unphosphorylated TPH is less active (Banik et al., 1997). Similarly, 14-3-3 has been previously shown to increase the activity of phosphorylated TH (Ichimura et al., 1987). At present it is unknown if there is a physiological correlation between the role of 14-3-3 and TPH phosphorylation (Mockus and Vrana, 1998).

II.4.2. TPH Inhibition – Several compounds have been shown to inhibit native and recombinant TPH, among them tryptophan, 5-HTP, nitric oxide (NO), catechols, and *p*-chlorophenylalanine (Hufton et al., 1995; Mockus and Vrana, 1998).

TPH inhibition by its substrate, tryptophan, occurs at high substrate concentrations (100 – 200 μM) (Friedman et al., 1972; Yang and Kaufman, 1994; Banik et al., 1997; Moran et al., 1998; Hamdan and Ribeiro, 1999). Substrate inhibition has been also reported for the related hydroxylase, TH (Hufton et al., 1995).

The immediate product of the TPH hydroxylation reaction, 5-HTP, has been shown to inhibit TPH when used in micromolar concentrations (Jequier et al., 1969; Nakata and Fujisawa, 1982; Hamdan and Ribeiro, 1999). When the mechanism of 5-HTP inhibition was investigated as a function of the substrate, tryptophan, it was not consistent with classical product inhibition but was more of a mixed form of inhibition, with predominantly noncompetitive properties (Hamdan and Ribeiro, 1999). Unlike TH, which is inhibited by the products of its hydroxylation reaction, TPH does not undergo feedback inhibition by 5-HT (Nakata and Fujisawa, 1982; Hufton et al., 1995; Hamdan and Ribeiro, 1999).

It was demonstrated earlier that *p*-chlorophenylalanine (PCP) is a potent depletor of brain 5-HT (Koe and Weissman, 1966). Soon after, it was discovered that PCP irreversibly inhibits TPH activity by competing with its substrate tryptophan (Jequier et al., 1967). Inactivation of TPH by PCP has been confirmed in more recent studies done on both tissue-purified (native) and recombinant forms of the enzyme (Nakata and Fujisawa, 1982; Tipper et al., 1994; Hamdan and Ribeiro, 1999)

The three AAAHs, TH, TPH, and PAH, are known to be inhibited by catecholamines (Hufton et al., 1995). Two forms of catecholamine inhibition have been described. At higher concentrations, catecholamines act as competitive inhibitors with respect to the cofactor (BH_4), whereas lower concentrations of catecholamines cause sustained time-dependent inhibition of the enzyme (Kaufman and Ribeiro, 1996). The latter form of inhibition is due to the formation of an inhibitory complex between the catechol (mainly dopamine) and ferric ions (Fe^{3+}) in the enzyme active site (reviewed in Hufton et al., 1995; Kaufman and Ribeiro, 1996; Kumer and Vrana, 1996). Two recent studies suggest that TPH inhibition by dopamine and L-DOPA may also involve the formation of oxidative toxic catechol derivatives, dopamine and L-DOPA quinones, which may alter TPH cysteineyl residues leading to enzymatic inactivation (Kuhn and Arthur, 1998,1999). Inhibition of TPH by dopamine and L-DOPA is thought to be of biological significance in areas of the CNS where serotonergic and catecholaminergic neurons co-exist.

Nitric oxide (NO) has been shown to irreversibly inactivate TPH (Kuhn and Arthur, 1996, 1997a,b). This inactivation was found to be independent of iron and seemed to increase in the presence of the cofactor, BH_4 (Kuhn and Arthur, 1997a,b; Nagatsu and Ichinose, 1999). It has been postulated that NO inactivates TPH by selective attack and modification of sulfhydryl (SH) groups of cysteine residues (Kuhn and Arthur, 1997a). A total of five cysteine residues are conserved between TPH, TH, and PAH (Mockus and Vrana, 1998). However, it remains to be determined whether TH and PAH are similarly subjected to inactivation by NO . These results deduced from *in vitro* studies could be of relevance for the *in vivo* effects of substituted neurotoxic amphetamine drugs, such as 3,4-methylenedioxymethamphetamine (MDMA), which are known to

generate NO and inhibit TPH activity (Stone et al., 1989; Elkins et al., 1993; Gibb et al., 1993; Steele et al., 1994; Seiden and Sabol, 1996).

II.4.3. Regulation of TPH by enzyme stability and alternative splicing – TPH is localized in different tissues in the CNS and the periphery. Earlier studies suggested that TPH expressed in different tissues might differ in enzymatic stability, which could be a form of an *in vivo* regulatory mechanism (Mockus and Vrana, 1998). For example, studies of crude rat tissue extracts showed that TPH from pineal tissue had a much lower half life ($t_{1/2}$ = 75 min) than that found in the brain stem ($t_{1/2}$ = 2 – 3 days) (Meek and Neff, 1972; Sitaram and Lees, 1978). Differences in TPH enzymatic stability have been also reported in the forebrain and midbrain tissues of mice (Knapp et al., 1981). In addition, Hasegawa et al. (1995) recently reported differences in TPH turnover rates between two mastocytoma mouse cell lines. Some antidepressant drugs, such as lithium and chlorimipramine, have been reported to alter TPH stability (Knapp and Mandell, 1983).

One possible explanation for differences in tissue TPH turnover could be the presence of different TPH isoforms. Although TPH is encoded by a single gene (Kim (Boularand et al., 1990; Kim et al., 1991), there is evidence for alternative splicing of the primary TPH transcript. Specifically, human TPH shows an unusual splicing complexity at its 5'-untranslated region (5'-UTR) giving rise to four different transcripts (Delort et al., 1989; Boularand et al., 1995a). Although these different mRNA messages do not produce different proteins, they may be differentially expressed and may differ in their stability and / or translational efficiencies (Mockus and Vrana, 1998). Recently, Wang et al. (1998) reported the isolation of two differentially spliced TPH cDNAs from postmortem human brain tissues. These two splice variants encode two TPH isoforms, one being 22 C-terminal amino acids shorter than the other, and hence may display differences in their biological properties, including enzymatic stability (Wang et al., 1998).

II.4.4. Transcriptional regulation of TPH – Several regulatory elements were identified in the human TPH gene promoter region (Boularand et al., 1995b). Using deletion

mutagenesis and standard reporter gene assays, an inverted CCAAT box motif (McKnight and Kingsbury, 1982) was identified (between nucleotides –73 to –51) and found to be essential for cAMP induction of the human TPH promoter (Boularand et al., 1995b). This inverted CCAAT motif was also shown to bind with high affinity to the transcription factor NF-Y/CBF leading to transcriptional activation of the human TPH gene (Reed et al., 1995; Teerawatanasuk and Carr, 1998). Recently, Teerawatanasuk et al. (1999), through a similar approach, reported that the CCAAT displacement protein (Neufeld et al., 1992) binds to a negative regulatory element (between nucleotides –310 and –220) of the human TPH promoter region. In addition, Ito et al. (1998) demonstrated that MITF transcription factors bind to a CANNTG motif in the mouse TPH promoter (nucleotides –322 to –317; Stoll and Goldman, 1991) and cause its transactivation.

Elevation of intracellular cAMP levels has been previously reported to cause an increase in pineal TPH mRNA (Shein and Wurtman, 1971; Ehret et al., 1991). The rise in TPH mRNA varies according to day / night cycles in both the pineal gland and in the ovine retina (Besancon et al., 1996; Florez et al., 1996; Privat et al., 1999). Interestingly, Besancon et al. (1997) reported that noradrenaline caused an elevation in TPH mRNA in neonatal rat pineal gland. This response is thought to be secondary to the elevation of cAMP due to stimulation of pineal adrenergic receptors. Other agents that are known to modulate TPH mRNA levels include, dihydroxytryptamine, glucocorticoids, and a brain-derived neutrophilic factor (Semple-Rowland et al., 1996; Clark and Russo, 1997; Siuciak et al., 1998).

PART III: SEROTONIN RECEPTORS

Having reviewed the general properties of 5-HT, in particular 5-HT biosysthesis, I will now focus on the molecular mechanism of action of this important neurotransmitter. The following is an overview of the structural, pharmacological, and signaling properties of 5-HT receptors in mammals as well as invertebrates, including helminths. Because the majority of 5-HT receptors belong to the superfamily of GPCR, I will begin with a brief discussion of the structural and functional properties of the class I (rhodopsin family) GPCRs.

III.1. G protein-coupled receptors: an overview

G-protein-coupled receptors (GPCRs) are integral membrane proteins that mediate the activity of a large number of signaling molecules, including hormones, neurotransmitters, growth factors, neuropeptides, and sensory stimuli (vision, taste, smell) (reviewed in Bockaert and Pin, 1999). GPCRs interact with heterotrimeric guanine nucleotide binding proteins (G proteins) which act as signal transducers (reviewed in Wess, 1997,1998). Dysfunction of GPCRs is linked to various diseases such as Parkinsons, color blindness, dwarfism, diabetes, Alzheimer, retina pigmentosa, asthma, depression, schizophrenia, hypertension, sleep disorders, anxiety, stress, cardiovascular problems, renal failure, and others (reviewed in Birnbaumer, 1995; Spiegel, 1996). In addition, recent evidence suggests that chemokine GPCRs are also involved in HIV infection (Feng et al., 1996; Berger et al., 1999). Indeed, it has been estimated that ~ 52 % of all available medicines produced by pharmaceutical companies target this broad class of membrane receptors (Drews, 1996).

GPCRs are widely present in eukaryotes (from yeast to humans) and are well conserved throughout evolution (Vernier et al., 1995; Bockaert and Pin, 1999). GPCR sequences have been also isolated from some viruses (Albrecht et al. 1992; Gao and Murphy, 1994) plants (Josefsson and Rask, 1997; Plakidou-Dymock et al., 1998), protozoa and ancestral metazoa (New and Wong, 1998). It is estimated that more than a thousand different GPCR sequences are present in the human genome (~ 1% of the

genome) and the genome of the free living nematode *C. elegans* (~ > 5% of the total genes), thus constituting one of the largest and evolutionarly conserved protein family (for reviews, see Baldwin, 1994; Strader et al., 1994; Bargmann, 1998; Bockaert and Pin, 1999). Based on amino acid sequence similarity, ligand binding properties, and G protein coupling specificities, the GPCR superfamily can be subdivided into five classes or families. Class I, or the rhodopsin family, contains receptors for sensory stimuli, glycoprotein hormones, peptides, biogenic amines, adenosine, leukotrienes, prostanoids, platelet activating factor, and thrombin receptors. Class II GPCRs includes receptors for large molecular weight hormones such as vasoactive intestinal polypeptide/PACAP, glucagon, secretin, calcitonin and α-latroxin. Class III GPCRs comprises the metabotropic glutamate receptors, calcium sensing receptors, and G_o-phermones. Class IV contains the G_i-vomeronasal pheromone receptors. Class V includes the 'frizzeled' and 'smoothened' receptors involved in embryonic development (Wess, 1998; Bockaert and Pin, 1999). Among these diverse GPCRs, the rhodopsin family (class I), which includes the biogenic amine neurotransmitters, is the largest and the most widely studied. Class I GPCRs will be the main focus of this section.

III.1.1. Structural properties of GPCRs - Although GPCRs interact with diverse ligands and are different at the amino acid sequence level, their basic architecture within each major class is conserved. The global structure of GPCRs belonging to the rhodopsin family consists of seven α-helical hydrophobic transmembrane domains (TMI to TM VII) connected by three extracellular ($el_1 - el_3$) and three intracellular ($il_1 - il_3$) loops. The N-terminus of GPCRs is extracellular while its C-terminus is intracellular (Baldwin, 1993,1994; Van Rhee and Jacobson, 1996; Ji et al., 1998; Bockaert and Pin, 1999). Receptor palmitoylation at its C-terminus (at a cysteine residue) may cause the formation of a fourth intracellular loop (Baldwin, 1994) (Fig. 4). This widely accepted seven transmembrane model is thought to fulfill the minimum requirements necessary to convey functional diversity and structural stability to GPCRs (Ji et al., 1998). To date no high-resolution crystal structure of a complete GPCR has been reported, in part because of the difficulty in crystalizing membrane spanning proteins. The available secondary and

tertiary structural models for GPCRs were initially based on the folding properties of bacteriorhodopsin, a retinal-linked visual pigment, as analyzed by electron microscopy and high resolution electron diffraction (Engelman et al., 1980; Henderson et al., 1990; Pardo et al., 1992). Recently, more three-dimensional information was obtained from low resolution structures of rhodopsin, a model GPCR which functions as a retinal rod cells photoreceptor (Schertler et al., 1993; Baldwin et al., 1997; Unger et al., 1997). These GPCR structural models are further supported by site-directed mutagenesis studies of a large number of GPCRs (reviewed in Savarese and Fraser, 1992; Gether and Kobilka, 1998; Wess, 1997, 1998; Schoneberg et al., 1999).

The regions of highest amino acid sequence homology among GPCRs are located in the transmembrane domains (Probst et al., 1992; Van Rhee and Jacobson, 1996). These TM regions (~ 20 –27 amino acids) are thought to form the ligand binding pocket of the receptor. When viewed from the extracellular surface, the TM domains appear to be arranged in the lipid bilayer as a closed loop in a counterclockwise orientation (Unger et al., 1997). The rest of the GPCR structure, constituting the amino terminus (~ 5 – 230 amino acids), extra and intracellular loops (~ 5 - 230 amino acids), and the carboxyl terminus (~12 – 359 residues) vary in length and show less sequence conservation (Probst et al., 1992; Van Rhee and Jacobson, 1996; Ji et al., 1998). The majority of GPCRs contain consensus *N* -glycosylation sites (NXS/T) at their amino terminus. Glycosylation of the N-terminus of GPCRs is thought to be important for receptor expression and proper receptor targeting to the cell surface (Savarese and Fraser, 1992; Ray et al., 1998; Karpa et al., 1999). The extracellular loops, as well as the N-terminus of GPCRs, are also known to play an important role in ligand binding (Wess, 1997,1998; Colson et al., 1998). On the other hand, the regions that are involved in G protein recognition and activation lie within the cytoplasmic loops (especially il_2 and il_3), intracellular ends of various TM domains, and at the C-terminus of the receptor (Wess, 1997, 1998).

Amino acids that are critical for the folding or overall function of GPCRs seem to be conserved among the various receptor subtypes. On the other hand, residues that are conserved only in specific classes of GPCRs are usually important for ligand binding and signaling events that are unique to this class of receptors (Hibert et al., 1991). The

residues that are conserved among most GPCRs of the rhodopsin family are shown in Figure 4 and have been extensively reviewed elsewhere (Probst et al., 1992; Savarese and Fraser, 1992; Baldwin, 1994,1997; Van Rhee and Jacobson, 1996; Gether and Kobilka, 1998; Wess, 1993,1997, 1998; Schoneberg et al., 1999). Briefly, these residues include a glycine and an asparagine in TM1 (GX_3N or GN), a leucine, alanine and aspartate (LX_2AD) in TM2, and the peptide 'D/HRY' at the C-terminal end of TM3 (TM3/il3). The arginine of the latter motif has been shown to be crucial for G protein coupling (Moro et al., 1993; Scheer et al., 1996; Van Rhee and Jacobson, 1996). Three additional conserved GPCR motifs have been identified in TM4 ($WX_{8/9}P$), TM5 (FX_2PX_7Y), and TM6 (WXP). The highly conserved motif in TM7, NPX_2Y, has been shown to play an important role in receptor activation, sequestration, and signaling specificity (Barak et al., 1994; Mitchell et al., 1998). Mutagenesis studies suggest that the conserved proline residues in TMV, VI, and VII are crucial for proper receptor function and assembly (Wess et al., 1993; Jakubik and Wess, 1999).

Almost all GPCRs contain two cysteine residues, located in the first and the second extracellular loops, which are cross-linked by a disulfide linkage (Probst et al., 1992; Van Rhee and Jacobson, 1996; Zeng et al., 1999a,b). Recent evidence indicates that these two cysteine residues are essential for proper localization of the receptor to the cell surface (Zeng et al., 1999a,b). In addition, most GPCRs contain one or more cysteine residues at the C-terminus which could serve as a target for palmitoylation. Due to its lipohilic nature, the palmitoylated cysteine residue may provide a membrane anchor which produces a fourth intracellular loop (reviewed in Bouvier et al., 1995; Ross, 1995; Morello and Bouvier, 1996). At present, the role of GPCRs palmitoylation is not very clear. However, depending on the type of GPCR, palmitoylation has been linked to various effects, including G protein coupling, receptor trafficking, sequestration, desensitization and phosphorylation (Bouvier et al., 1995; Ross, 1995; Morello and Bouvier, 1996; Wess, 1998, Loisel et al., 1999). Further detailed structural analyses and extensive GPCR amino acid sequence alignments can be retrieved from the GPCR Data Base (GPCRDB) (Horn et al., 1998) (http://www.gpcr.org/7tm).

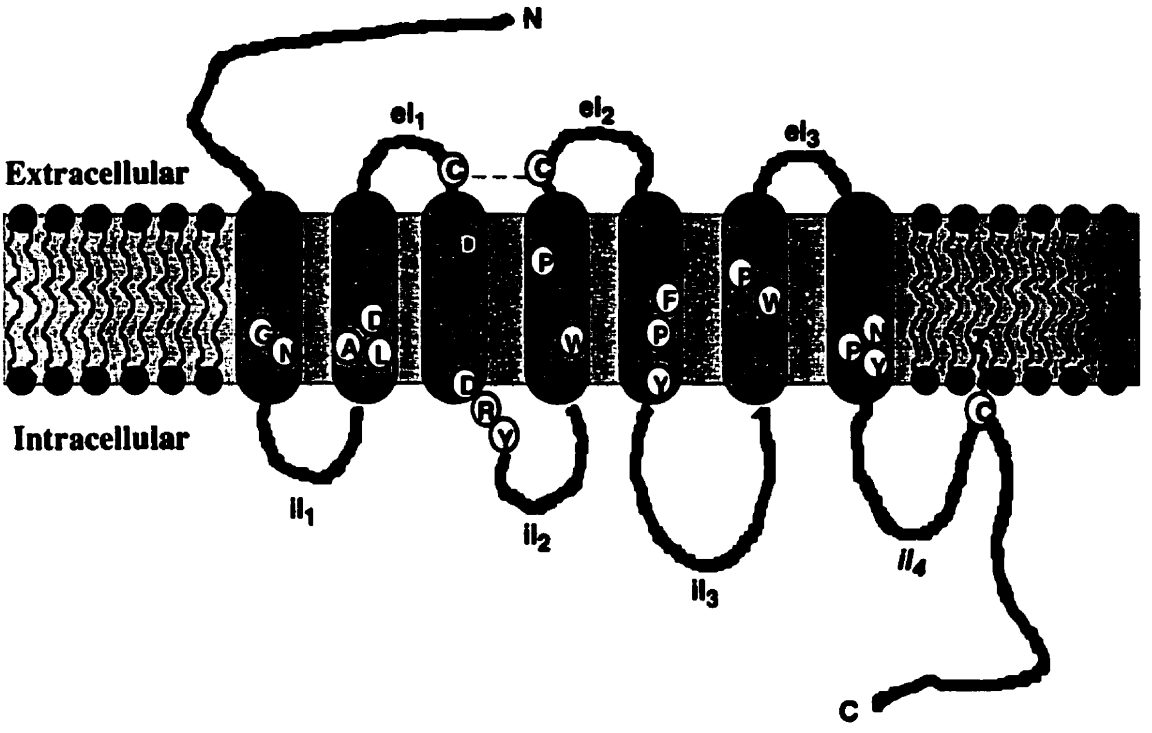

Fig. 4. Schematic two-dimensional structural representation of a class I GPCR. The characteristic seven transmembranes are joined by three extracellular (el_{1-3}) and three intracellular loops (il_{1-3}). The N-terminus is extracellular while the C-terminus is intracellular. Palmitoylation of one or more cysteine residues located within the carboxyl terminus may lead to the formation of a fourth intracellular loop (*il_4*). Encircled amino acid residues are conserved in most rhodopsin-like GPCRs and are important for receptor function. The aspartate with a black background (TM3) is conserved only among biogenic amine neurotransmitter receptors. The two invariant cysteine residues in the first and the second extracellular loops are linked via a disulfide bond (dashed line), an interaction that is crucial for receptor function and surface localization

III.1.2. Signaling pathways mediated by GPCRs - Despite the diversity of GPCRs, their signaling mechanisms are rather similar. Binding of a ligand to the surface of a GPCR induces a conformational change in the receptor which promotes its interaction with an associated intracellular G protein. G proteins are made up of an α subunit and a complex of βand γsubunits. Once activated by the ligand-receptor interaction, GDP bound to the α subunit is exchanged for GTP causing the release of the G protein from the receptor and the concomitant dissociation of the βγ subunits. Both the α–GTP and the βγcomplex are capable of modulating the activity of intracellular effector enzymes (*e.g.* adenylyl cyclase or phospholipase C), ion channels (Ca^{++}, K^{+}), and second messengers

(cAMP, IP_3, Ca^{++}). The result is an activation of protein kinases (PKA, PKC, CaM- PKII) and the onset of a protein phosphorylation cascade that leads to cellular response. The G_α–subunit contains a domain that is involved in binding and hydrolyzing GTP (intrinsic GTPase activity). Consequently, the active α–GTP is changed to an inactive α–GDP which is capable of high affinity binding to free βγ complexes, thus restoring the heterotrimeric G protein to its resting stage and rendering it available for another round of receptor-regulated activation (reviewed in Neer, 1995; Gudermann et al., 1997; Wess, 1997).

Currently, there exists at least 23 different α–subunits, which are organized according to sequence similarity into four main subtypes: $G\alpha_s$, $G\alpha_{i(i,o,t,g,z)}$, $G\alpha_{q(11,\ 14\text{-}16)}$ $G\alpha_{12/13}$. In addition, six β, and 12 γ subunits have been reported (Neer, 1995; Clapham and Neer, 1997). Some of the G_α subunits are sensitive to ADP-rybosylation catalyzed by the A subunits of bacterial toxins such as pertussis toxin (PTX affecting G_i family, except for G_{t2}) or cholera toxin (CTX affecting G_s , and $G_{t1,2}$) (reviewed in Milligan et al., 1995; Fields and Casey, 1997; Harnett and Harnett, 1998). Activated $G\alpha_s$ and $G\alpha_{i/o}$ modulate intracellular cAMP levels by either activating ($G_{\alpha s}$) or inhibiting ($G_{\alpha i/o}$) specific isoforms of the effector adenylate cyclase. A rise in cAMP leads to the activation of protein kinase A (PKA) and subsequent phosphorylation of multiple cellular targets, cytosolic as well as nuclear. An example of the later type of target is the cAMP-response element binding protein (CREB). Phosphorylation of CREB by PKA increases the expression of genes whose promoters include CRE elements (Montminy, 1997; Daniel et al., 1998). Activation of $G\alpha_{q/11}$ subunit leads to an increase in the activity of various isoforms of phospholipase C_β (PLC_β) which, in turn, catalyze the breakdown of a membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, forming two biologically active second messengers, inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The latter activates protein kinase C (PKC) in the presence of calcium, while the former mobilizes Ca^{++} from endogenous stores. One subset of the $G_{i/o}$ family, $G_{\alpha t}$, is known to activate

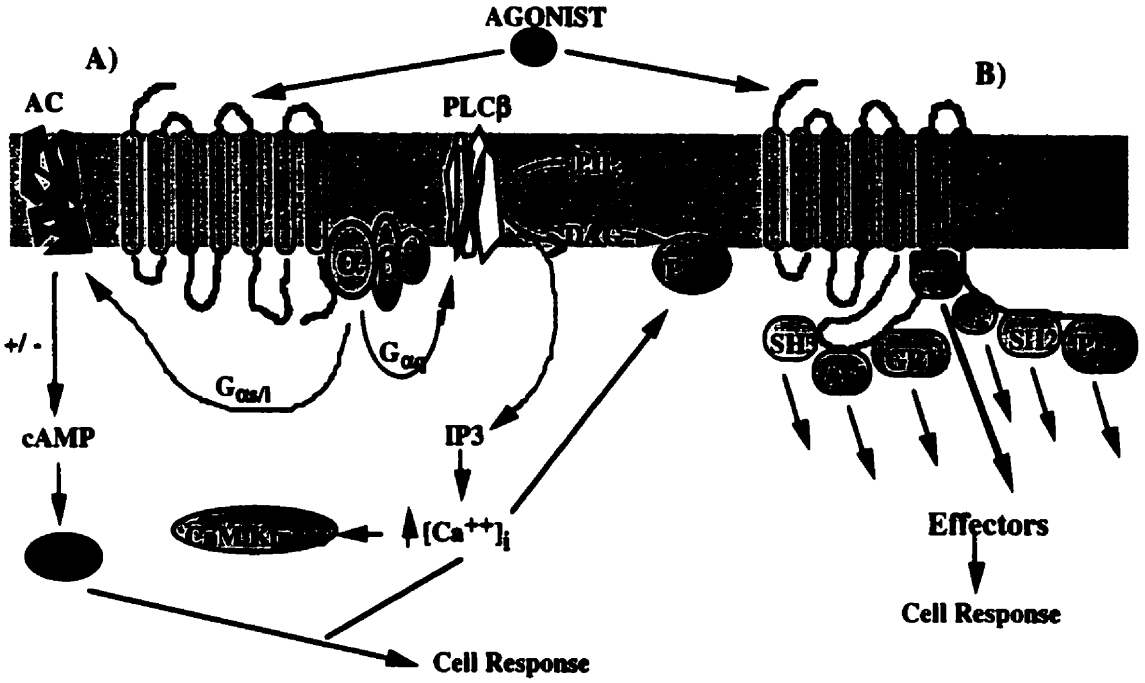

Fig. 5. Signaling pathways of GPCRs. *A*) Examples of the major classical signaling events that result due to agonist activation of a GPCR. This results in the activation of heterotrimeric G proteins, which in turn regulate the activity of cellular effectors (*e.g.* AC, adenylate cyclase, PLC_β). *B*) "Beyond the G protein paradigm". Agonist binding to a GPCR induces the interaction with various intracellular proteins, such as classical G proteins (G), polyproline-binding proteins containing SH3 domains (SH3), arrestins (arr), GPCR kinases (GRK), SH2 domain-containing proteins (SH2), small GTP-binding proteins (g), proteins conatining a PDZ domain (PDZ). As a result multiple signaling pathways are activated leading to specific cellular responses (modified from Hall et al., 1999).

retinal cyclic GMP, another biologically active intracellular messenger causing a decrease in the levels of cGMP phosphodiesterase (Neer, 1995).

Until recently, it was generally believed that the functions of heterotrimeric G proteins, both in signal transduction and GTP hydrolysis, were performed entirely by the alpha subunit, the βγsubunits having mainly a regulatory function. New evidence, however, has shown that the $G_{\beta\gamma}$ subunit complex also functions as a transducer capable of targeting, directly or indirectly, a variety of cellular effectors. Examples of effectors targeted by $G_{\beta\gamma}$ include ion channels, the plasma membrane Ca^{++} pump, phospholipase A_2, PLC_β, adenylyl cyclase, GPCR kinases, MAP kinase cascade, phosphoinositide3 kinase (P13K), and Bruton tyrosine kinase (Btk) (for recent reviews see Clapham and Neer,

1997; Gudermann et al., 1997; Morris and Scarlata, 1997; Hamm, 1998). This multiplicity of targets contributes to the functional diversity of GPCRs.

GPCRs employ a number of strategies to increase the repertoire of cellular targets, thereby increasing functional diversity. There is increasing evidence that GPCRs can couple to more than one type of G protein, which broadens the spectrum of potential protein targets (Gudermann et al., 1997; Hamm, 1998; Wess, 1998; Ghahremani et al., 1999; Liu et al., 1999). In addition, activation of one GPCR can modulate the signaling of other GPCRs, a mechanism referred to as receptor-cross talk (reviewed in Selbie and Hill, 1998). For example, it is known that activation of $G_{\alpha i}$-coupled receptors can increase IP_3 levels resulting from stimulation of $G_{\alpha q}$-coupled receptors (Neer, 1995). Recently, this receptor cross-talk was shown to involve an exchange of $G_{\beta\gamma}$between receptors (Quitterer and Lohse, 1999). Finally, emerging evidence indicates that GPCRs can signal through intracellular transducers other than the classical G proteins. Examples of these alternative signal transducers for GPCRs include polyproline-binding proteins that contain a SH3 domain (SH3), arrestins, G protein-coupled kinases (GRK), SH2 domain-containing proteins (SH2), small GTP-binding proteins, and PDZ domain-containing proteins (PDZ) (Fig. 4B) (reviewed in Gutkind, 1998; Hall et al., 1999).

III.2. Serotonin receptors

5-HT is known to elicit or modulate a diverse array of behavioral and physiological processes by interacting with multiple membrane receptors which are believed to be the product of more than 750 million years of evolution (Peroutka and Howell, 1994). Initially, 5-HT receptors were categorized into three families ($5\text{-}HT_{1\text{-}3}$) according to their pharmacology, as determined by ligand binding studies of crude membrane preparations (Bradley et al., 1986). With the advent of molecular biology and the cloning of a large number of serotonergic receptors, 5-HT receptors have been re-classified according to new criteria that recognize the pharmacological, signaling (coupling to seconday messengers), and primary structural properties of the receptor (Hoyer et al., 1994; Hoyer and Martin, 1997).

The molecular cloning approach has revealed the existence of more than 70 5-HT receptors, which have been grouped into at least 15 different subtypes and classified under seven distinct main families (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7) (for reviews see Boess and Martin, 1994; Hoyer et al., 1994; Saudou and Hen, 1994; Gerhardt and van Heerikhuizen, 1997; Hoyer and Martin, 1997; Kroeze and Roth, 1998; Barnes and Sharp, 1999). With the exception of the 5-HT_3 subtype (Maricq et al., 1991), which is a ligand-gated ion channel, all other 5-HT receptors belong to the superfamily of GPCRs of type I. A dendrogram of representative 5-HT receptors from all major classes and their signaling properties is shown Figure 6.

III.2.1. Mammalian G protein-coupled 5-HT receptors

A large number of mammalian 5-HT receptors have been cloned and characterized in the last decade (Barnes and Sharp, 1999). The nomenclature followed in this section is in compliance with the recommendations of the Nomenclature Committee of the International Union of Pharmacology (NC-IUPHAR) for mammalian 5-HT receptors (Hoyer and Martin, 1997). This committee suggested that newly described mammalian recombinant receptors be represented in low case letters (*e.g.* 5-ht). When enough evidence is provided that a new receptor class is present and elicits a physiological function, then the receptor is designated in upper case (*e.g.* 5-HT). To date, there is no specific nomenclature scheme for invertebrate 5-HT receptors which are of interest to this thesis. What follows is a brief description of the various classes of 5-HT receptors with more emphasis on the 5-HT_2 family and the invertebrate 5-HT receptors. For extensive pharmacological profiles of the various 5-HT receptors the readers are referred to excellent comprehensive reviews by Hoyer et al. (1994), Boess and Martin (1994), Saudou and Hen (1994), Gerhardt and van Heerikhuizen (1997), and Barnes and Sharp (1999).

III.2.1.1. The 5-HT_1 receptor family. The 5-HT_1 family is the largest subclass of 5-HT receptors comprising five subtypes cloned from various species: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E}, and 5-ht_{1F} (Barnes and Sharp, 1999). 5-HT_1 receptors are characterized by their

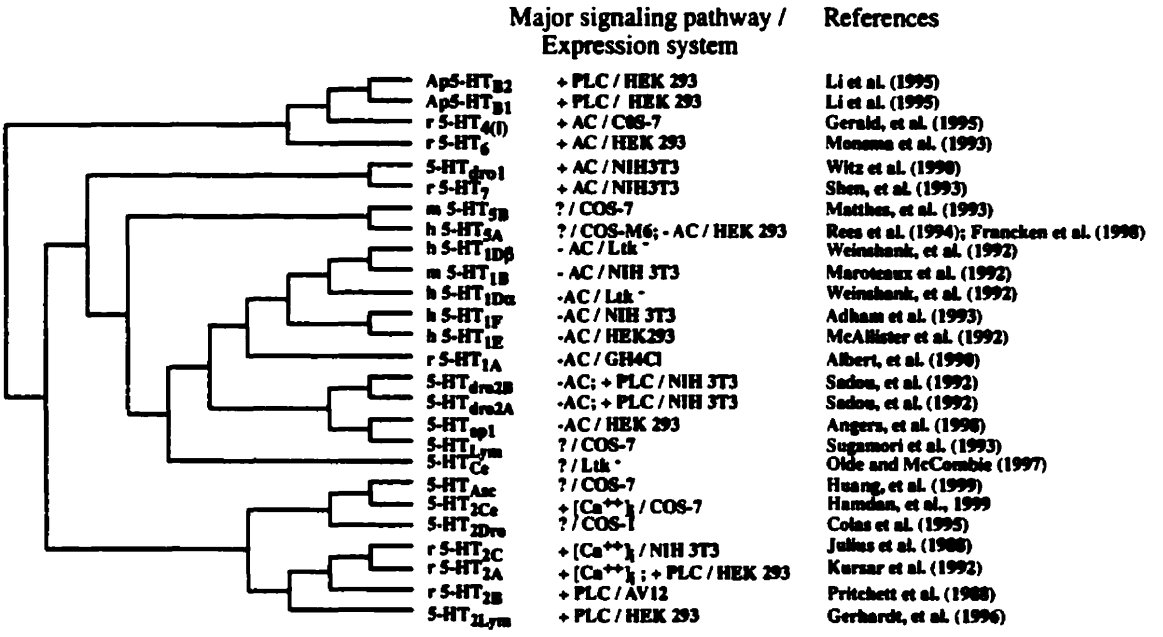

Fig. 6. Dendrogram showing the structural relationship of the various cloned GPCR 5-HT receptors and their major signaling pathway. The amino acid sequences of the various 5-HT receptors were compared and clustered using the program DNASIS vs. 3.7 (Hitachi Software, CA, USA). The lengths of the horizontal lines connecting one sequence to another are inversely proportional to the percentages of similarity between receptors or group of receptors. The 5-HT receptor sequences used were from human (h), rat (r), mouse (m), *Drosophila* (dro or Dro), *Lymnaea* (lym), *Aplysia* (Ap or ap), *C. elegans* (Ce), *A. suum* (Asc). The corresponding signaling pathway (AC = adenylate cyclase; PLC = phospholipase C) and references are shown.

high affinity binding to 5-HT, intronless sequences, and inhibition of adenylyl cyclase by coupling to pertussis toxin sensitive $G_{i/o}$ (Barnes and Sharp, 1999; Gerhardt and van Heerikhuizen, 1997). Initially, these receptors were grouped together due to their high affinity for the agonist 5-carboxyamidotryptamine (5-CT) and the antagonists methiothepin and methysergide (Bradley et al., 1986). However, with therecent cloning of many 5-HT$_1$ subtypes, this classification has been re-addressed taking into consideration the low affinity of 5-ht$_{1E}$ and 5-ht$_{1F}$ for 5-CT and methiothepin (Barnes and Sharp, 1999; Hoyer and Martin, 1997). Several biological and behavioral effects have been attributed to 5-HT$_{1A,B,D}$ receptors, including modulation of sexual behavior, depression, anxiety, movement disorders, food intake, aggressive behavior, and migraine. At present, the physiological roles of 5-ht$_{1E,F}$ remain unclear (Barnes and Sharp, 1999).

III.2.1.2 The 5-HT_4 receptor family - Six subtypes of 5-HT_4 receptors have been identified and characterized so far from rat, mouse, and human (Gerald et al., 1995; Van den Wyngaert et al., 1997; Blondel et al., 1998; Claeysen et al., 1998, 1999): 5-$HT_{4(a)}$ (originally 5-$HT_{4(s)}$), 5-$HT_{4(b)}$ (originally 5-$HT_{4(l)}$), 5$HT_{4(c)}$, 5-$HT_{4(d)}$, 5-$HT_{4(e)}$, and 5-$HT_{4(f)}$. These various 5-HT_4 subtypes are thought to be the product of alternative splicing of pre-mRNA sequences which results in variable C-terminal regions. The different 5-HT_4 splice variants share similar pharmacological profiles, but they differ in tissue distribution and ability to stimulate adenylate cyclase (Barnes and Sharp, 1999). 5-HT_4 can be pharmacologically distinguished by the use of highly specific and potent antagonists, such as GR113808, SB207266, SB204070, RS39604 and SB203186 (Grossman et al., 1993; Medhurst and Kaumann, 1993; Hegde et al., 1995; Wardle et al., 1996). Physiologically, 5-HT_4 receptors seem to affect the functions of the gastrointestinal tract and those of the urinary, endocrine and cardiac systems as well. In addition 5-HT_4 receptors have been associated with modulation of cognitive behavior and anxiety (Bockaert et al., 1998; Barnes and Sharp, 1999).

III.2.1.3. The 5-HT_5 receptor family - The 5-ht_5 family is the least characterized of all the 5-HT receptor families. It includes two subtypes, 5-ht_{5A} and 5-ht_{5B} cloned from mice, rat and humans (Plassat et al., 1992; Erlander et al., 1993; Matthes et al., 1993; Rees et al., 1994). The two 5-ht_5 subtypes share 88 % sequence homology with each other but have very little homology with other 5-HT receptors. The 5-ht_5 receptors contain an intron in the region of the sequence that encodes the third intracellular loop. The two receptors also display similar pharmacological properties characterized by high affinities for 5-CT and ergot derivatives (LSD, ergotamine) but lower affinity for 5-HT itself (Barnes and Sharp, 1999). Recent reports (Francken et al., 1998; Hurley et al., 1998) demonstrated that the signaling of the human recombinant 5-ht_{5A} in HEK293 cells is mediated by the attenuation of adenylate cyclase activity, suggesting that the receptor couples to $G_{i/o}$ proteins. 5-ht_{5B} does not seem to be expressed in humans and, so far, no functional activity has been detected for either the mouse or rat recombinant receptors (Barnes and Sharp, 1999). The physiological functions of the 5-ht_5 receptors awaits further investigation.

III.2.1.4. The 5-HT$_6$ receptor family - 5-ht$_6$ receptors have been isolated and characterized from rat (Monsma et al., 1993; Ruat et al., 1993) and human (Kohen et al., 1996). The two 5-ht$_6$ receptor sequences share 89% sequence homology and each contains one intron. The 5-ht$_6$ receptors were shown to bind with high affinity to tricyclic antipsychotic and antidepressant drugs, such as clozapine and clomipramine, and to signal via the activation of adenylate cyclase in COS7 cells (Barnes and Sharp, 1999). Recently, two selective 5-ht$_6$ receptor antagonists, Ro 04-6790 and Ro 63-0563, have been developed, thus facilitating the pharmacological distinction of this class of 5-HT receptors (Sleight et al., 1998). 5-ht$_6$ receptors have been suggested to play a role in neuropsychiatric disorders and depression (Gerhardt and van Heerikhuizen, 1997; Barnes and Sharp, 1999).

III.2.1.5. The 5-HT$_7$ receptor family - The 5-HT$_7$ family is the most recently characterized among the 5-HT receptors (reviewed by Eglen et al., 1997). It has been cloned from different species, including mouse (Plassat et al., 1993), rat (Lovenberg et al., 1993; Meyerhof et al., 1993; Ruat et al., 1993; Shen et al., 1993), toad (Nelson et al. 1995), guinea pig (Tsou et al. 1994) and human (Bard et al., 1993). This class of 5-HT receptors is thought to play a role in the regulation of circadian rhythms, affective behavior, and neuronal activity (Eglen et al., 1997; Barnes and Sharp, 1999). Alternative splicing of 5-HT$_7$ produces at least four splice variants [5-HT$_{7(a,b,c,d)}$], only three of which could be detected in rat and human tissues (Heidmann et al., 1997, 1998; Stam et al., 1997). The unique pharmacological profile of 5-HT$_7$ receptors is characterized by high affinity to 5-HT and LSD, as well as to some antipsychotics and antidepressants (*e.g.* clozapine). To date, very few selective 5-HT$_7$ receptor antagonists are available (Forbes et al., 1998; Kikuchi et al., 1999). The signaling of 5-HT$_7$ receptors is characterized by positive coupling to adenylate cyclase (Barnes and Sharp, 1999). No major pharmacological or signaling differences were noted between the different 5-HT$_7$ isoforms (Heidmann et al., 1998).

III.2.1.6. The 5-HT_2 receptor family

The 5-HT_2 family currently consists of three subtypes, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, all of which have similar structure, pharmacology, and signaling properties, and have been shown to function as endogenous receptors. Many drugs acting on 5-HT_2 receptors are used to treat several mental illnesses such as schizophrenia, anxiety and depression (Baxter et al., 1995). The following is a brief discussion outlining the structural and functional properties of this family of 5-HT receptors.

III.2.1.6.1. Physiological and structural properties of mammalian 5-HT_2 receptors – The tissue distribution and physiological impact of 5-HT_2 receptors differ according to the receptor subtype. The 5-HT_{2A} receptors are localized in different regions of the mammalian central nervous system (cortical and cerebellar neurons and glial cells) and in the periphery (vascular and uterine smooth muscles, platelets, and fibroblast cells) (Barnes and Sharp, 1999). The 5-HT_{2A} receptor is known to affect many peripheral and central functions, such as motor and sexual activity, platelet aggregation, sleep patterns, neuroendocrine responses and various mental illnesses (*e.g.* depression, migraine, schizophrenia, epilepsy, and hallucinations) (Roth et al., 1998a; Barnes and Sharp, 1999). The 5-HT_{2B} transcript is expressed in many tissues, including stomach fundus, vascular smooth muscle, spinal cord, and in some regions of the brain (Roth et al., 1998b; Barnes and Sharp, 1999). Some of the physiological functions attributed to 5-HT_{2B} receptors include mediation of the rat stomach fundus contraction, relaxation of the rat and cat jugular veins, and possible involvement in 5-HT-induced mitogenic effects during neuronal development (Baxter et al., 1995; Barnes and Sharp, 1999). The 5-HT_{2C} receptor is mainly present in the CNS with highest mRNA and protein levels in the choroid plexus (Abramowski et al., 1995; Barnes and Sharp, 1999). Several behavioral and physiological responses are associated with central 5-HT_{2C} receptors, such as motor behavior, modulation of appetite, penile erection, sleep, and temperature regulation (Koek et al., 1992; Sharpley et al., 1994; Tecott et al., 1995; Barnes and Sharp, 1999).

The first 5-HT_2 receptor to be cloned was 5-HT_{2A} (previously known as 5-HT_2) followed by 5-HT_{2C} (originally named 5-HT_{1C}), and then 5-HT_{2B} (formely known as

5-HT_{2F}) (classification proposed by Humphrey et al., 1993). The members of the 5-HT_2 family have been cloned from many mammalian species such as human, mouse, rat (5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}), and from hamster and pig (5-HT_{2A}) (reviewed in Gerhardt et al., 1997; Barns and Sharp, 1999). Their genomic DNA sequences are recognized by the presence of two (for 5-HT_{2A} and 5-HT_{2B}) or three (5-HT_{2C}) introns at conserved positions in their coding sequences (Gerhardt and van Heerikhuizen, 1997). At the protein level, the mammalian 5-HT_2 receptors encode proteins of ~ 458 to 504 amino acids and share up to 80 % sequence homology within the TM regions (Barnes and Sharp, 1999). Interestingly, 5-HT_{2C} receptors contain an extra hydrophobic stretch (~ 21 amino acids) at the N-terminus which is not present in the other 5-HT_2 receptors (Lubbert et al., 1987; Julius et al., 1988; Saltzman et al., 1991; Yu et al., 1991). Recently, it has been shown that this extra domain plays a role in regulating receptor expression without affecting receptor activity (Hurley et al., 1999). Canton et al. (1996) isolated a non-functional 5-HT_{2C} splice variant, lacking 95 bp (corresponding to the region between il2 to TM IV), that was expressed in brain tissues of mouse, rat, and human. The functional significance of this truncated receptor species remains unknown. No splice variants have been reported for 5-HT_{2A} or 5-HT_{2B}.

Computer modeling and mutagenesis studies of 5-HT_2 receptors, in particular 5-HT_{2A}, provided a wealth of information on structural residues that are important for proper function of these receptors (reviewed in Baxter et al., 1995; Roth et al., 1998b). Table 1 summarizes the key residues that are thought to be crucial for ligand binding and/or G protein coupling of 5-HT_2 receptors.

III.2.1.6.2. Pharmacological and signaling properties of 5-HT_2 receptors – The 5-HT_2 family of receptors is traditionally characterized by a low affinity for 5-HT (high nM to low μM range) and higher affinity for the agonist DOI and its derivatives (DOB and DOM), and for the classical 5-HT_2 antagonists, ritanserin, mianserin, ketanserin, cyproheptadine and spiperone (Barnes and Sharp, 1999; Boess and Martin, 1994). The antagonists ketanserin, cyproheptadine, and spiperone show relatively higher affinity for the 5-HT_{2A} subtype, while 5-HT itself has higher affinity for 5-HT_{2C} receptors (Boess and Martin, 1994). Recently, more selective 5-HT_2 drugs (e.g. MDL100907, SB242084, and

Table 1. Residues important for functional activity of 5HT$_2$ receptors

Receptor	TM / Amino Acid (mutation)	Effect on function /**Remarks**	References
5-HT$_{2A}$	II / **D**120 (N)	↓AG, AN; abolished Emax	1, 2, 3
5-HT$_{2C}$	II / S133	**APR**	4
5-HT$_{2A}$	III / D155 (N)	↓AG, AN; / **APR**	1, 2, 5 - 12
5-HT$_{2C}$	III / D155	**APR**	4
5-HT$_{2A}$	III / S159(A)	↓ AG (5-HT) / **APR**	12 - 14
5-HT$_{2A}$	V / A242(S)	↑mesulergine / **APR**	15, 16
5-HT$_{2A}$	V / S242(A)	↓mesulergine / **APR**	15, 16
5-HT$_{2A}$	VII / Y370(A)	↓AG, Emax / **APR**	10, 17
5-HT$_{2A/C}$	V / F240;F243;F244	**AAM**	1, 4, 10, 13, 18
5-HT$_{2A}$	VI / F340(L)	↓ AG, Emax / **AAM**	17, 19 - 21
5-HT$_{2A}$	IV / W200A	↓ AG, Emax / **AAM**	17
5-HT$_{2A}$	VI / W336A	↓AG, Emax / **AAM**	17
5-HT$_{2A}$	VII / W367A	↓AG, Emax / **AAM**	17
5-HT$_{2A}$	VII / Y370A	↓AG, Emax / **AAM**	10, 17

Increase (↑) or decrease (↓) in agonist (AG) and antagonist (AN) binding affinities are indicated. Emax, agonist efficacy in inducing phosphoinositide hydrolysis; APR, thought to be important in anchoring polar residues of ligands; AAM, thought to be important in anchoring aromatic moieties of ligands. Amino acids in **bold**, are highly conserved among GPCRs, while the underlined ones are mainly conserved among biogenic amine neurotransmitters (Probst et al., 1992; Van Rhee and Jacobson, 1996). References: Edvardsen et al. (1992) (1); Wang et al. (1993) (2); Sealfon et al. (1995) (3); Kristiansen and Dahl (1996) (4); Hibert et al. (1991) (5); Westkaemper and Glennon (1991) (6); Gallaher et al. (1993) (7); Kristiansen et al. (1993) (8); Trumpp-Kallmeyer et al. (1992) (9);Westkaemper and Glennon (1993) (10) Choudhary et al. (1995) (11); Almaula et al., (1996) (12); Zhang and Weinstein (1993) (13); Luo et al. (1994) (14); Kao et al. (1992) (15); Johnson et al. (1994) (16); Roth et al. (1997b) (17); Moereels and Janssen (1993) (18); Choudhary et al. (1993) (19); Roth et al. (1995) (20). Further details on various 5-HT receptors mutagenesis studies are available from the GPCRDP (Horn et al., 1998); http://www.gpcr.org/7tm)

RS102221) have become available, allowing the precise discrimination between the different 5-HT$_2$ subtypes (Sorensen et al., 1993; Baxter, 1996; Bonhaus et al., 1997; Kennett et al., 1997). Table 2 includes a brief list of key 5-HT$_2$ ligands and their corresponding affinities for the different 5-HT$_2$ receptor subtypes.

The major signaling pathway for 5-HT_2 receptors is mediated by pertussis toxin-insensitive $G_{\alpha q}$ and subsequent activation of PLC and increase in IP3 and intracellular Ca^{++} levels (Barnes and Sharp, 1999). This preferred mechanism of action distinguishes the 5-HT_2 family from other 5-HT GPCRs, all of which target adenylate cyclase directly and signal through cAMP. The exact targets for 5-HT_2 signaling remain unclear and may vary according to the tissue distribution of the receptor. In *Xenopus* oocytes stimulation of expressed 5-HT_2 receptors leads to the activation of Ca^{++}-sensitive Cl^-channels, presumably through stimulation of IP_3/Ca^{++} (Pritchett et al., 1988; Yu et al., 1991; Foguet et al., 1992). In other systems, endogenous 5-HT_{2A} receptors have been implicated in the indirect modulation of adenylate cyclase (Berg et al., 1994; Garnovskaya et al., 1995), activation of phospholipase A_2 (Felder et al., 1990) or activation of the Jak/STAT pathway (Guillet-Deniau et al., 1997). Modulation of adenylate cyclase has been shown also in recombinant 5-HT_2 receptors expressed in AV12 cells and was found to be dependent on receptor density (Lucaites et al., 1996). Finally, a recent report by (Launay et al., 1996) showed that stable expression of 5-HT_{2B} in Ltk^- mouse fibroblasts caused the activation of MAP kinase via the Ras pathway which was linked to tumor formation in mice.

III.2.1.6.3. Regulation of 5-HT_2 receptors - The area of receptor regulation has been well investigated for 5-HT_{2A} and 5-HT_{2C}, but not 5-HT_{2B} receptors. Several mechanisms of 5-HT_{2A} regulation have been documented, among them agonist-mediated receptor desensitization, which affects receptor coupling, and modulation of receptor trafficking due internalization, and lysosomal degradation. In addition, there is evidence for long term regulation of 5-HT_{2A} at the level of transcription and mRNA stability, both of which influence the level of receptor expression on the membrane (reviewed in Roth et al., 1998a,b)

5-HT_{2C} receptors are down-regulated in response to prolonged exposure to agonists and antagonists. On the other hand, these receptors are up-regulated when chronically exposed to the antidepressants citalopram and fluoxetine (prozac) (Roth et al., 1998b). This up-regulation is speculated to be secondary to the postulated antagonistic properties of antidepressants (Ni and Miledi, 1997). In addition, emerging evidence

Table 2. Affinity (pK_i) of different ligands for mammalian 5-HT_2 receptors

Receptor / Ligands	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}
5-HT_{2A} receptor			
Spiperone	8.8	5.5 / 7.3[a]	5.9
MDL 100907	9.4	n.d.	6.9
Ketanserin	8.9	5.4	7.0
5-HT_{2B} receptor			
SB 2044741	<5.3	7.8	<6.0
BW 723C86	<5.4	7.9	<6.9
5-HT_{2C} receptor			
SB 242084	6.8	7.0	9.0
RS-102221	6.0	6.1	8.4
RO 60-0175	6.0	5.8	8.8
5-HT_{2B} / 5-HT_{2C} receptors			
SB 200646A	5.2	7.5	6.9
mCPP	6.7	7.4[b]	7.8
SB 206553	5.8	8.9	7.9
Less / non selective			
5-HT	5.5[a]	5.9[a]	7.3[a]
DOI	7.3[b]	7.4[b]	7.8[b]
cyproheptadine	8.5[a]	7.6[a]	7.9[a]
Ritanserin	8.8	8.3	8.9
Mianserin			
LY 53857	7.3	8.2	8.1
ICI 170809	9.1	n.d.	8.3

Data were taken from Sharp and Barnes (1999). [a]Values obtained from Colas et al. (1995). [b] pEC_{50} value for agonist

indicates that 5-HT_{2C} receptors undergo adenosine-to-inosine RNA editing in both rat and humans, which affects G protein coupling (Burns et al., 1997; Niswender et al., 1999). The modified 5-HT_{2C} receptor was shown to have reduced basal activity, decreased agonist affinity and potency, suggesting that RNA editing may play a significant role in the regulation of receptor signal transduction (Herrick-Davis et al., 1999).

III.2.2. Invertebrate 5-HT receptors

According to a recent classification scheme proposed by Gerhardt and van Heerikhuizen (1997), the majority of cloned invertebrate 5-HT receptors can be divided

into three main families: 5-HT_1-like, 5-HT_2-like, and 5-HT_7-like receptors. This grouping is based primarily on similarity of structural and signaling properties with mammalian homologues. Despite the tentative classification, however, the agonist / antagonist preferences of most invertebrate 5-HT receptors differ from those of mammalian counterparts. The pharmacological and signaling profiles of these receptors are summarized in Table 3.

III.2.2.1. 5-HT_1-like invertebrate receptors - To date, five invertebrate 5-HT_1-like receptors have been cloned and characterized. These include two receptors from *D. melanogaster* (5-HT_{dro2A} and 5-HT_{dro2B}) (Saudou et al., 1992), one receptor from the snail pond, *Lymnaea Stagnalis* (5-HT_{lym}) (Sugamori et al., 1993), one receptor from *C. elegans* (5-HT_{Ce}), and a recently isolated receptor from *Aplysia californica* (5-HT_{apl}) (Angers et al., 1998). These receptors have been classified according to sequence homology and signaling similarity with mammalian 5-HT_1 receptors (Fig. 6). In addition to having sequence homology within TM regions, invertebrate and mammalian 5-HT_1 receptors are characterized by a short C-terminus (less than 30 amino acids), a structural property predominantly found in $G_{i/o}$-coupled GPCRs. There are, however, important structural and pharmacological differences between the invertebrate 5-HT_1-like receptors and the mammalian counterparts. Some of these important differences are described below.

The *Drosophila* 5-HT_{dro2B} receptor sequence contains at least four introns within its coding sequence, while all mammalian 5-HT_1 are intronless (Saudou et al., 1992). At present, due to lack of genomic sequence information, it is not known whether the other invertebrate 5-HT_1-like receptors contain introns as well. In addition, the *Drosophila* 5-HT_{dro2A} contains an extra hydrophobic stretch at its N-terminus which is thought to form an additional transmembrane domain (Saudou et al., 1992). This additional sequence is absent in mammalian 5-HT_1 receptors.

There are differences between the binding properties of invertebrate and mammalian 5-HT_1-like receptors (Gerhardt and van Heerikhuizen, 1997). Most notably, recombinant invertebrate 5-HT_1-like receptors have an unusually low affinity for the natural ligand 5-HT. With the exception of the *Aplysia* 5-HT_{apl}, all other invertebrate

receptors in this group have K_D / K_i values for 5-HT in the micromolar range, several order of magnitude higher than what was reported for mammalian 5-HT_1 receptors. Aside from this difference, however, invertebrate and mammalian 5-HT_1-like receptors share similar preferences for agonists and antagonists. In both cases, the receptors show a characteristic preference for ergoline derivatives such as LSD and methiothepin, and much lower affinities for classical 5-HT_2 ligands such as ketanserin, DOI, and cyproheptadine.

Mammalian 5-HT_1 receptors are known to couple to $G_{i/o}$ proteins resulting in inhibition of adenylate cyclase activity. The same signaling pathway has been identified for invertebrate 5-HT_1-like receptors (Gerhardt and van Heerikhuizen, 1997). Agonist stimulation of the *C. elegans* 5-HT_{Ce} and the *Aplysia* 5-HT_{ap1} receptors expressed in mammalian cells (Ltk- and HEK293, respectively) caused attenuation of adenylate cyclase activity, consistent with $G_{i/o}$ signaling (Olde and McCombie, 1997; Angers et al., 1998). In the case of the *Drosophila* 5-$HT_{dro2A,2B}$, the signaling pathway varies according to the cell type. When expressed in NIH3T3 cells, the 5-$HT_{dro2A,2B}$ coupled to pertussis toxin sensitive $G_{i/o}$ protein causing inhibition of adenylate cyclase activity and a small increase (1.5 – 2 fold) in IP_3 production (Saudou et al., 1992). However, expression of the 5-HT_{dro2B} in insect Sf9 cells resulted in a receptor that coupled only to pertussis toxin insensitive $G_{\alpha q}$ protein and induced IP_3 production (Obosi et al., 1996). The authors also showed that $G_{i/o}$ proteins were not expressed in their Sf9 cell line, which could explain why the 5-HT_{dro2B} did not affect the adenylate cyclase pathway. Thus, invertebrate 5-HT_1-like receptors may be capable of coupling to more than one type of G protein, depending on the cellular environment, just as shown previously for mammalian counterparts (Ghahremani et al., 1999; Liu et al., 1999). The signaling pathway for the *Lymnaea* 5-HT_{lym} receptor is yet to be characterized.

III.2.2.2. 5-HT_2-like invertebrate receptors - To date, four 5-HT_2-like invertebrate receptors have been cloned from *D. melanogaster* (5-HT_{2Dro}) (Colas et al., 1995), *L. stagnalis* (5-HT_{2lym}) (Gerhardt et al., 1996), *C. elegans* (5-HT_{2Ce}) (Hamdan et al., 1999), and *A. suum* (5-HT_{2Asc}) (Huang et al., 1999) (see Fig. 6). The *Drosophila* 5-HT_{2Dro} and the *Lymnaea* 5-HT_{2lym} receptors resemble mammalian 5-HT_2 receptors, both

in structure and function. The two receptors share high amino acid sequence homology with mammalian 5-HT_2 receptors (~ 50 % identity within the TM regions) and their coding sequences are interrupted by introns, just like their mammalian counterparts. One striking structural difference between invertebrate and vertebrate 5-HT_2 is the size of the N-terminus region, which is approximately 4 to 5 times longer in the *Lymnaea* and *Drosophila* receptors (~ 280 and 286 amino acids, respectively) compared to mammalian forms (< 80 amino acids). Deletion of the long N-terminus does not seem to affect receptor pharmacology or signaling properties of either 5-HT_{2Dro} or 5-HT_{2lym} (Colas et al., 1995; Gerhardt et al., 1996). In the case of the latter, however, truncation of the N-terminus resulted in increased receptor expression, consistent with what was earlier reported for the *Drosophila* 5-HT_1-like receptors (Witz et al., 1990; Sadou et al., 1992; Gerhardt et al., 1996) . Unlike other invertebrate 5-HT receptors, the pharmacology of 5-HT_{2Dro} and 5-HT_{2lym} agree well with the mammalian 5-HT_2 pharmacology. Specifically, in the case of 5-HT_{2Dro}, which has been more extensively characterized, the agonist / antagonist binding profile correlates well with that of the mammalian 5-HT_{2B} receptor (Colas et al., 1995). Similarly, the 5-HT_{2lym} has binding characteristics of mammalian 5-$HT_{2B,C}$ receptors. Interestingly, 5-HT displays higher affinity for 5-HT_{2Dro} and 5-HT_{2lym} (pK_i = 6.8 – 6.9) than for the invertebrate 5-HT_1-like receptors (see above). Similar to mammalian 5-HT_2 receptors, the 5-HT_{2lym} signals through PLC activation and IP_3 production, suggesting that invertebrate 5-HT_2-like receptors share the same preferred pathway of signal transduction as mammalian counterparts (Gerhardt et al., 1996). The signaling pathway of 5-HT_{2Dro} has not yet been investigated, but *in situ* localization and receptor depletion studies suggest that serotonin signaling through 5-HT_{2Dro} may play a role in *Drosophila* embryogenesis (Colas et al., 1995,1999).

The two most recent additions to the invertebrate 5-HT_2-like receptor family were cloned from nematodes, including *C. elegans* (5-HT_{2Ce}) (Hamdan et al., 1999; see chapter III) and the pig parasite *A. suum* (5-HT_{Asc}) (Huang et al., 1999). The two nematode receptors share high TM amino acid sequence homology with mammalian and invertebrate 5-HT_2 receptors (61% - 71 %) and with each other (81 %) (Fig. 6). Both nematode receptors contain introns in their coding sequences, as observed for other 5-HT_2 receptors. In addition, 5-HT_{2Ce} and 5-HT_{Asc} have short N-termini (< 60 amino

acids) that correlate more with the mammalian 5-HT_2 structure than with the other two invertebrate 5-HT_2-like receptors, 5-HT_{2Dm} and 5-HT_{2lym} (Colas et al., 1995; Gerhardt et al., 1996). In the case of 5-HT_{2Ce}, at least, the 5-HT-induced signaling in transfected COS7 cells resulted in a rise in intracellular Ca^{++} (Hamdan et al., 1999), consistent with the typical mode of action of the 5-HT_2 family. On the other hand, the pharmacological properties of 5-HT_{2Ce} are rather unique. The 5-HT_{2Ce} has a mixed 5-HT_1 / 5-HT_2 pharmacology that does not resemble any known class of 5-HT receptors. We speculated that the "primitive" 5-HT_{2Ce} maybe related to an ancestral 5-HT_1 / 5-HT_2 prototype that existed before the separation of the two classes (for further discussion, see chapter III). The functional data available on the *Ascaris* 5-HT_2-like receptors is limited to LSD and 5-HT binding to membrane preparations of transfected COS7 cells. Altough both the *Ascaris* and the *C. elegans* have similar affinities for ^{125}I-LSD (K_D ~ 0.7 nM and 1.1 nM, respectively), the former shows much higher binding affinity for 5-HT (~ 0.4 µM versus 57 µM). An interesting observation made with the recently cloned *Ascaris* receptor suggest that nematodes may use RNA processing as a means to increase functional diversity of 5-HT_2 receptors. Huang et al. (1999) reported a total of five 5-HT_{Asc} alternatively spliced isoforms which differed in sequence at their C-termini and also appeared to vary with their tissue distribution. It is unknown, at present, if the different splice variants have different functional characteristics.

III.2.2.3. 5-HT_7-like invertebrate receptors - The first invertebrate 5-HT receptor ever cloned, originally named 5-HT_{drol}, showed structural characteristics of mammalian 5-HT_7 receptors (Witz et al., 1990; Gerhardt and van Heerikhuizen, 1997). Unlike most other invertebrate 5-HT receptors, 5-HT_{drol} is encoded by an intronless gene. 5-HT_{drol} has a large N-terminal region which includes an eighth hydrophobic domain, the function of which is unknown. In addition, the N-terminal domain of this receptor is rich in Gly-Ser repeats which are thought to function as glycosaminoglycan attachment sites. Such a motif is usually detected in biological clock genes that regulate circadian rhythm, a function that has been also suggested for the mammalian 5-HT_7 receptor (Gerhardt and van Heerikhuizen, 1997; Barnes and Sharp, 1999). 5-HT_{drol} shares highest sequence

homology with mammalian 5-HT_7 receptors and does not align well with the other 5-HT receptors (Fig. 6). When expressed in mouse NIH3T3 cells and insect Sf9 cells, 5-HT_{dro1} mediated a specific increase in adenylate cyclase activity in response to 5-HT, consistent with the $G_{\alpha s}$-mediated signaling properties of 5-HT_7 receptors (Witz et al., 1990; Obosi et al., 1996).

III.2.2.4. Other invertebrate 5-HT-like receptors - Two closely related 5-HT receptors have been isolated from the sea slug *A. californica* (Ap5-HT_{B1} and Ap5-HT_{B2}) and found to weakly stimulate PLC (1.5 fold above basal level) but not adenylate cyclase, when stably expressed in HEK293 cells (Li et al., 1995). Based on amino acid sequence, the Ap5-HT receptors seem to resemble mammalian 5-HT_4 receptors (Fig. 6). Conclusive classification awaits further functional analyses.

A number of 5-HT-receptor-like sequences have been cloned from invertebrate species, including moths (von Nickisch-Rosenegk et al., 1996), planaria (Saitoh et al., 1997) and balanus (Kawahara et al., 1997). Most of these sequences resemble 5-HT_1 receptors. However, none of these putative receptors has yet been characterized through binding or signaling assays and, therefore, it is unknown whether they encode functional 5-HT receptors.

Finally, pharmacological and signaling studies done on crude tissue extracts have identified the presence of functional 5-HT receptors in other invertebrate phyla, including flatworms (see page 7), *Spisula solidissima* (surf clam) *Renilla koellikeri* (Cnidaria), and *H. medicinalis* (leech) (Krantic et al., 1993a,b; Hajj-Ali and Anctil, 1997; Salzet et al., 1998). The DNA sequences for these receptors have not yet been cloned.

Table 3. Pharmacological and signaling profiles of invertebrate 5-HT receptors

Receptor	Expression System	Binding profile	signaling profile	References
5-HT_{dro2A}	NIH3T3	LSD > dihydroergocryptine > prazosin > *d*-butaclamol > methysergide > 5-HT > yohimbine > 8-OH-DPAT	5-HT: ↓ cAMP; ↑ PLC	Sadou et al. (1992)
5-HT_{dro2B}	NIH3T3	LSD > dihydroergocryptine > *d*-butaclamol > prazosin > methysergide > 5-HT > yohimbine > 8-OH-DPAT	5-HT: ↓ cAMP; ↑ PLC	Sadou et al. (1992)
	Sf9		5-HT: ↓ cAMP; No effect on PLC	Obosi, et al. (1996)
5-HT_{Ce}	Ltk^-	LSD > methiothepin > lisuride > butaclamol > prazosin > clozapine > methylergonovine > methysergide > 1(1-Naphtyl)piperazine > 5-MeO-DMT > (+) propanolol > 5-HT > DOI > 5-MeO-T	5-HT: ↓ cAMP	Olde and McCombie (1997)
5-HT_{ap1}	HEK293	LSD > 5-CT > methiothepin > PAPP > 5-HT > methysergide > clozapine > 8-OH-DPAT > metergoline > yohimbine > mesulergine > ketanserin	↓ cAMP: 5-HT > 5-CT > 8-OH-DPAT	Angers et al. (1998)
5-HT_{dro1}	NIH 3T3	LSD > dihydroergocryptine > d-butaclamol > methysergide > 5-HT > prazosin > yohimbine > 8-OH-DPAT	↑ cAMP: 5-HT > LSD > 5-MeO-T > 2-Me5-HT > 8-OH-DPAT	Witz et al. (1990) Sadou et al. (1992)
5-HT_{lym}	COS-7	methiothepin > LSD > clozapine > methysergide > 5-CT > 8-OH-DPAT > 5-HT > ketanserin		Sugamori, et al. (1993)
5-HT_{2Dro}	COS-1	ritanserin > ketanserin > pizotifen > 5-HT > setoperone > spiperone = N-(3-trifluoromethylphenyl) piperazine = cyproheptadine = mesulergine = N-acetyl-5-HT > methiothepine = methysergide		Colas et al. (1995)
5-HT_{2lym}	HEK293	ritanserin > methsergide > mianserin > yohimbine > ketanserin > spiperone	5-HT: ↑ IP_3	Gerhardt et al. (1996)
5-HT_{2Ce}	COS-7	LSD = lisuride > butaclamol > methiothepin > cyproheptadine > clozapine > metergoline > ktanserin > DOI > 5-HT	5-HT >> lisuride > DOI ; ↑ $[Ca^{++}]_i$	Hamdan et al. (1999)

Pharmacological binding profiles were based on K_i values obtained mainly as a result of competition with radiolabeled LSD (or DOI, in case of 5-HT_{2Dro}) . 5-CT, 5-carboxyamidotryptamine; DOI, 2,5-dimethoxy-4-iodoamphetamine; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propyllamino)tetralin; 5-MeO-T, 5-methoxytryptamine; 2-Me5-HT, 2-methyl-5-hydroxytryptamine; 5-O-MeDMT, 5-methoxy-*N,N*-dimethyltryptamine; PAPP, *p*-aminophenethyl-*m*-trifluoromethylphenyl piperazine; LSD, lysergic acid diethylamide. The signaling profiles were based on EC_{50} values deduced from agonist treatment..

CHAPTER II (MANUSCRIPT I)

Characterization of a Stable Form of Tryptophan Hydroxylase from the Human Parasite, *Schistosoma mansoni*

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ABSTRACT

A cDNA (SmTPH) encoding a protein homologous to tryptophan hydroxylase, the enzyme that catalyzes the rate-limiting step in the biosynthesis of serotonin, was cloned from the human parasite, *Schistosoma mansoni*. Bacterial expression of SmTPH as a histidine fusion protein produced soluble active enzyme, which was purified to apparent homogeneity and a final specific activity of 0.17 μmol / min / mg of protein. The purified enzyme was found to be a tetramer of approximately 240 kDa with a subunit size of 58 kDa. Several of the biochemical and kinetic properties of SmTPH were similar to those of mammalian tryptophan hydroxylase. Unlike the mammalian enzyme, however, SmTPH was found to be stable at 37°C, its $T_{1/2}$ being nearly 23 times higher than that of a similarly expressed rabbit tryptophan hydroxylase. Semi-quantitative RT-PCR showed that the level of SmTPH mRNA in a larval stage of the parasite (cercaria) is 2.5 times higher than in adult *S. mansoni*, suggesting possible differences in the level of enzyme expression between the two developmental stages. This study demonstrates for the first time the presence of a functional tryptophan hydroxylase in a parasitic helminth and further suggests that the parasites are capable of synthesizing serotonin endogenously.

INTRODUCTION

Tryptophan hydroxylase (TPH; tryptophan 5-monooxygenase; EC 1.14.16.4) catalyzes the hydroxylation of L-tryptophan to 5-hydroxy-L-tryptophan (5-HTP). This reaction is the first and rate-limiting step in the biosynthesis of the monoamine neurotransmitter, serotonin (5-hydroxytryptamine: 5-HT) (reviewed in (1)). TPH belongs to a family of aromatic amino acid hydroxylases that also includes the catecholamine biosynthetic enzyme, tyrosine hydroxylase (TH; EC 1.14.16.2) and phenylalanine hydroxylase (PAH; EC 1.14.6.1) (for reviews see (2-4)). The three enzymes catalyze similar hydroxylation reactions and share a distinctive requirement for tetrahydrobiopterin (BH_4) and non-heme ferrous iron as cofactors (2). Studies of TPH have been hampered by the extreme instability of the enzyme (5-11) and the difficulty in obtaining high levels of purified active enzyme from either native or heterologous sources (10-17). Sequence analyses of TPH cDNAs derived from mammalian and other vertebrate species (13,18-23) revealed that TPH shares high overall sequence homology with the other two members of the hydroxylase family (1,2). More recently, deletion mutagenesis studies identified three main functional regions of TPH, including a conserved central core that comprises the catalytic domain (15,16,21,24,25), a C-terminal intersubunit binding region responsible for the formation of enzyme tetramers (26,27) and a divergent N-terminal end. The latter is predicted to have a regulatory function (15,24,28) and may contribute to the instability of TPH (25)

Serotonin, a well known neuroactive agent of the mammalian central nervous system and periphery (29), has been identified in every invertebrate phylum thus far investigated (30). In parasitic flatworms (platyhelminths), including the human bloodfluke, *Schistosoma mansoni*, 5-HT acts as an important regulator of motor activity and carbohydrate metabolism (for review, see (31)) and as such is critical for the survival of the parasite in the host. Immunofluorescence and histochemical studies have localized 5-HT in the central and peripheral nervous systems of the worm, as well as holdfast structures, body musculature, and reproductive structures (31). Earlier studies of 5-HT biosynthesis in parasitic helminths reported conflicting results. The failure to demonstrate TPH activity in crude tissue extracts of *S. mansoni* led some researchers to conclude that

TPH is absent in this animal and that the parasite depends entirely on the host for a source of serotonin (32-36). Although other authors have challenged these studies and presented preliminary evidence of 5-HT biosynthesis in related parasites (37-39), the question of whether TPH is present or absent in parasitic worms, in particular *S. mansoni*, remains largely unresolved.

Here we report the cDNA cloning, purification and functional characterization of tryptophan hydroxylase from *S. mansoni* (SmTPH). This study provides the first evidence that *S. mansoni*, and probably related parasites, possess the endogenous capability for *de novo* synthesis of 5-HT. When expressed in *E. coli*, the purified SmTPH was highly active and, in contrast to the mammalian enzyme, very stable during purification and storage. SmTPH represents a potentially useful model for detailed biochemical studies of TPH structure and function.

EXPERIMENTAL PROCEDURES

Chemicals- L-Tryptophan, 5-hydroxytryptophan (5-HTP), 5-hydroxytryptamine (5-HT), N-acetyl-5-HT, melatonin, p-chlorophenlylalanine (PCP), isopropyl-β-thiogalactoside (IPTG), dithiothreitol (DTT), dihydropteridine reductase, NADH, glycerol, and sephacryl 200HR were purchased from Sigma. Tween 20, ferrous ammonium sulfate, and activated charcoal were from Fisher Scientific. [5-^{3}H]-L-Tryptophan was from Amersham Pharmacia Biotech. (6R)-5,6,7,8-tetrahydrobiopterin (BH_4) and dopamine were from Research Biochemicals International (RBI). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and catalase were from Boehringer Mannheim. All other chemicals were of the highest purity and quality from available commercial sources.

S. mansoni- A Puerto Rican strain of *S. mansoni* was maintained as previously described (40). Crude adult worm tissue extracts were prepared and used directly for TPH activity measurements as described earlier (40). Total RNA was extracted from *S. mansoni* using the TRIzol reagent (Gibco BRL). Poly(A^+) RNA from adult *S. mansoni* was purified from total RNA using oligo-dT-cellulose columns (Amersham Pharmacia Biotech.).

Cloning of full length SmTPH- A partial *S. mansoni* cDNA sequence (576 bp) homologous to other TPH sequences was isolated by homology RT-PCR. Oligonucleotide primers were synthesized based on a predicted genomic TPH sequence from the free-living nematode *Caenorhabditis elegans* (cosmid ZK1290; Genbank accession no. U21308) and used for PCR amplification of adult *S. mansoni* oligodT reverse transcribed cDNA. The sense and antisense primers targeted a region of the predicted catalytic domain that is highly conserved among all aromatic amino acid hydroxylases (see Fig. 3). Primer sequences and RT-PCR conditions are described elsewhere (40).

The 5' end of SmTPH was obtained using a RT-PCR method that targets the conserved 5' end spliced leader (SL) sequence of *S. mansoni* transcripts (41,42). Briefly,

adult *S. mansoni* mRNA (0.5 μg) was reverse-transcribed with an oligo-dT primer and 200 U of murine Moloney leukemia virus reverse transcriptase (Gibco BRL). One tenth of the resulting cDNA was subjected to 30 cycles of PCR (30 sec / 94 °C, 30 sec / 53 °C, 90 sec / 72 °C) in a 50 μl reaction containing 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTPs mix, 0.4 μ*M* of each primer, and 5 U of Taq DNA polymerase (Gibco BRL). Oligonucleotide SmSL (see Fig. 1) which corresponded to nucleotides 9 – 32 of the *S. mansoni* 36-nucleotide SL sequence (41) was used as a sense primer, while oligonucleotide A (see Fig. 1) was used as an antisense primer. An aliquot (2 μl of 1/10 dilution) of the PCR product was similarly subjected to a second PCR reaction (25 cycles; same cycling parameters as above) using the same sense primer (SmSL) and a nested reverse primer (primer B; see Fig. 1). The resulting PCR product was gel-purified, cloned into the vector PCR 2.1 (Invitrogen), and sequenced by the dideoxy chain termination method.

The 3'-end of SmTPH cDNA was amplified from an adult *S. mansoni* cDNA library in pcDNA3.1(+) (Invitrogen). An aliquot of the plasmid library (0.4 μg) was subjected to PCR (30 cycles) using a SmTPH-specific sense primer (primer C; see Fig. 1) and an antisense pcDNA3.1(+) vector-specific primer flanking the multiple cloning site (VSP1: 5'-GGAGGGGCAAACAACAGATGG-3'). The PCR cycling parameters were as above except for an annealing temperature of 55 °C. An aliquot of the PCR product was subjected to a second round of PCR amplification (25 cycles) using a nested SmTPH-specific sense primer (primer D; see Fig. 1) and pcDNA3.1(+) vector-specific-primer (VSP2: 5'-TAGAAGGCACAGTCGAGGC-3'). Amplified products were cloned into pCR2.1 and DNA sequenced as before.

For expression studies, the complete coding sequence of SmTPH was amplified by RT-PCR and subcloned into a T7 polymerase-based pET prokaryotic expression vector (43). OligodT reverse transcribed cDNA was subjected to 35 cycles of PCR with primers that targeted the entire coding sequence of SmTPH (primers S and E; see Fig. 1) and a proofreading DNA polymerase (PWO; Boehringer Mannheim) according to the manufacturer's procedure. To facilitate further subcloning into the expression vector,

enzyme restriction sites *Nde*I and *BamH*I were incorporated at the 5'-end of the sense and antisense primers, respectively. The resulting PCR product was gel-purified, digested with *Nde*I and *BamH*I (Gibco BRL) and ligated into pET15b vector (Novagen) which was linearized by the same two restriction enzymes. Cloning into pET15b introduces an N-terminal oligohistidine fusion tag which adds 20 amino acid residues of vector-derived sequence to the expressed SmTPH product. The final construct was confirmed by DNA sequencing of 3 independent clones and then used to transform *Escherichia coli* host strain BL21(DE3)pLysS (Novagen).

Bacterial Expression and Purification of SmTPH- BL21(DE3)pLysS cells transformed with the SmTPH.pET15b construct were grown at 37 °C in LB-ampicillin-chloramphenicol medium to an OD_{600} of ~ 0.5 to 1.0 (log growth phase). Cultures were supplemented with 0.1 m*M* Ferrous ammonium sulfate, as described previously (13-15), and then induced with 1m*M* IPTG for 2.5 hrs at 30 °C. After induction, the cells were washed once with ice-cold 50 m*M* Tris-HCl (pH 8.0), pelleted by centrifugation and stored frozen at –80°C until used.

For purification of recombinant SmTPH, cell pellets from 100 ml induced bacterial cultures were thawed in 4 ml of 20 m*M* phosphate buffer (pH 7.4) containing 0.5 *M* NaCl, 0.2% Tween 20, 5% glycerol, 10 m*M* imidazole, and a cocktail of protease inhibitors (1 m*M* PMSF, and 50 µg/ml of each leupeptin and aprotinin). To promote cell lysis by the T7 resident lysogen, the cells were subjected to 2 cycles of rapid freeze-thawing followed by sonication on ice (seven pulses of 15 sec separated by intervals of 30 sec) using a vibra cell sonicator (Sonics and Material, Danbury, CT) set at 20 % maximal power. Cell lysates were centrifuged at 12,000 x g for 15 min and 4 °C. The resulting pellet was resuspended in 2.5 ml of the same buffer and similarly sonicated and centrifuged as above. The two supernatants were pooled and used for direct enzymatic assays and for subsequent purification of the expressed enzyme.

Recombinant SmTPH was purified by immobilized metal (nickel) affinity chromatography (44) using the HisTrap kit (Pharmacia Biotech) for purification of

histidine tagged proteins, as described previously (40). Excess imidazole was removed by gel filtration through a Sephadex G-25 column (PD-10; Pharmacia Biotech.) and the purified enzyme was stored in 50 m*M* HEPES (pH 7.5) containing 0.2 *M* NaCl, 10% glycerol, 0.05 % Tween 20, and 1m*M* DTT. Purified enzyme preparations (0.2 mg/ml) were stable for at least 4 days at 4 °C and could be stored at –80 °C for at least one month with no significant loss in activity.

TPH Enzymatic Assays- TPH activity was measured using the tritiated water release method (45,46) with few modifications. The standard assay was performed in a 100 µl reaction of 50 m*M* HEPES (pH 7.5) containing 400,000 cpm of L-[5-^{3}H]Tryptophan and enough unlabelled L-Tryptophan to make a final concentration of 100 µ*M*, 0.2 mg/ml catalase, 0.4 m*M* NADH, 10 mU dihydropteridine reductase, 10 µ*M* DTT, and either purified SmTPH (0.6 µg) or crude adult *S. mansoni* tissue extract (50 - 100 µg of protein), The reaction was started with the addition of 200 µ*M* BH_4, unless indicated otherwise, and the samples were incubated for 10 min at 37°C. Preliminary experiments revealed that TPH activity increased linearly up to 12 min of incubation under these conditions. The reaction was terminated by the addition of 1 ml of activated charcoal [7.5% (wt / volume) in 1 *M* HCl] to each sample. After centrifugation (2000 x g for 10 min), aliquots of the supernatants were radioassayed in 10 ml of scintillation cocktail (ICN). Enzyme activity data were analyzed using Lineweaver-Burk plots or by computer assisted, nonlinear curve fitting to the Michaelis-Menten model. All kinetic parameters [K_m and apparent K_m ($S_{0.5}$)] were determined using the program Enzyme Kinetics (vs. 1.C; DogStar Software) and were obtained from two to three independent experiments, each performed in duplicates or triplicates.

TPH Stability Assay- The stability of SmTPH was assessed in comparison to that of recombinant rabbit brain TPH similarly expressed in *E. coli*. In preparation for these experiments, the complete coding sequence of rabbit brain TPH cDNA (13) was

subcloned into pET15b and expressed as a histidine-tagged protein in BL21(DE3)pLysS *E. coli*. Bacterial cells expressing rabbit TPH or SmTPH were lysed, as described above, and the corresponding soluble fractions containing expressed enzyme were passed through a Sephadex G25 PD10 (Pharmacia) column equilibrated with the same Hepes buffer described earlier. Stability was measured as a function of time according to the procedure of Mockus et al. (25). Briefly, aliquots (100 μg protein) of the crude SmTPH or rabbit TPH extracts were preincubated at 37 °C for varying lengths of time (0, 10, 20, 40, and 80 min) and then assayed for TPH activity. The data were calculated as a percentage of the initial level of activity (t = 0) for each enzyme.

Developmental Expression of SmTPH mRNA in *S. mansoni*- Semiquantitative RT-PCR was employed for the determination of SmTPH mRNA levels. Total RNA (~ 2 μg) from two developmental stages of *S. mansoni* (cercaria and adults) were subjected to DNAse I treatment (amplification grade; Gibco BRL) followed by a standard RT-PCR reaction (1 min / 94 °C, 14 – 36 cycles of: 30 sec / 94 °C, 30 sec / 53.5 °C, 60 sec / 72°C, 7 min / 72 °C) using primers (sense: primer D, antisense: primer F; see Fig. 1) that amplify a 873 bp cDNA product from SmTPH. PCR was standardized by simultaneous amplification of a constitutively expressed control house keeping gene, *S. mansoni* α-tubulin (47,48), as described previously (49). The PCR primers used for the amplification of the 558 bp *S. mansoni* α-tubulin cDNA fragment were as follows: sense: 5'-CTTATCGTCAACTTTTCCATCC-3', antisense:5'-GGAAGTGGATACGAGGATAAG G- 3' (modified from (48)). Standard curves were generated to ensure that the PCR assay was in the exponential phase of synthesis after 34 cycles for SmTPH or 24 cycles for α-tubulin. The resulting PCR products were cloned in PCR2.1 and confirmed by DNA sequencing. Densitometric analysis of the ethidium bromide stained RT-PCR products were performed with the NIH Image program vs. 1.61 (Bethesda, MA).

Other Methods- Size exclusion chromatography of purified SmTPH was performed on a Sephacryl-200HR gel filtration column (10 mm i.d. x 50 cm; Bio-Rad), as described previously (27). Protein concentrations were measured by the method of Bradford (50), using the Bio-Rad protein assay kit and bovine serum albumin (Fischer Scientific) as a standard. Reducing SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (51) using precast 10% acrylamide gels from Novex, Inc. For western blot analysis of SmTPH, aliquots of purified enzyme (0.5 – 1 μg) were electrophoresed as above, transferred onto nitrocellulose (52) and reacted with a sheep polyclonal antibody (1: 500 dilution) raised against rabbit TPH (Chemicon International) followed by a peroxidase - conjugated rabbit anti-sheep IgG (Pierce) as the secondary antibody (1: 1000 dilution).

RESULTS

Cloning of the Full length SmTPH cDNA and Protein Sequence Analyses- A partial TPH sequence was first obtained by RT-PCR using oligodT reverse-transcribed *S. mansoni* cDNA and *C. elegans* primers (40) that targeted a region conserved among all aromatic amino acid hydroxylases. A 576 bp product was sequenced and found to have high homology with TPH sequences from other species. The missing 5' and 3' ends were subsequently obtained by an anchored PCR-based strategy. The 5' end was amplified in a RT-PCR reaction that targeted the conserved spliced leader (SL) sequence of *S. mansoni* (40,41). A 741 bp product corresponding to the 5' end of TPH was sequenced and found to carry a complete *S. mansoni* SL (nucleotides 9 - 36 of the SL sequence; see Fig. 1) including the last four nucleotides which were not part of the SmSL primer used in the anchored PCR reaction. This finding suggests that SmTPH is *trans*-spliced at the 5'-end to the *S. mansoni* SL, just as previously described for several other *S. mansoni* cDNAs (40-42). The 3' end of SmTPH was PCR amplified directly from a *S. mansoni* cDNA plasmid library, using TPH-specific and vector derived primers (see Fig. 1). The resulting product (1000 bp) contains a potential polyadenylation sequence (AATATA) (53) upstream of a polyA tail and thus is presumed to represent the 3' end of the full-length transcript.

Fig. 1 shows the nucleotide and predicted amino acid sequence of SmTPH. The composite cDNA reveals a single open reading frame of 1494 bp encoding a predicted protein of 497 amino acids with a calculated molecular mass of 58 kDa. Protein sequence analysis revealed the presence of two consensus sites (Ser^{151} and Thr^{130}) for phosphorylation by the Ca^{2+} / calmodulin dependent protein kinase type II (CaM PKII) and a consensus leucine zipper motif (SmTPH amino acid pos. 358 to 377). BLAST analysis (54) of the predicted protein sequence indicated that SmTPH is highly homologous to tryptophan hydroxylase from other species. The dendrogram in Fig. 2 shows that SmTPH is more related to TPH sequences than to those of the other two aromatic amino acid hydroxylases, TH and PAH. Based on pairwise CLUSTAL protein alignments (55) SmTPH shares high amino acid sequence homology (65 – 67 %) with vertebrate TPH sequences (13,18-23). In contrast, there is considerably less homology

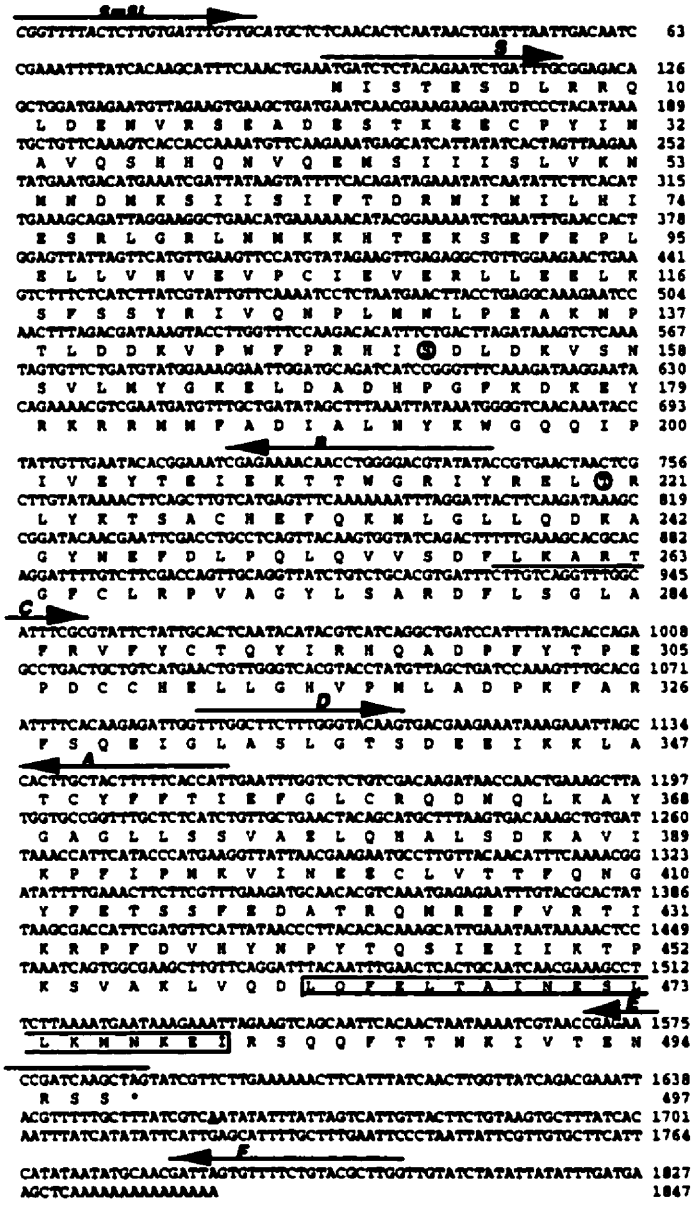

Fig. 1. Complete cDNA and predicted amino acid sequence of SmTPH. Overlined sequences represent sense (SmSL, S, C, and D) and antisense primers (A, B, E, and F) used in RT-PCR and cloning procedures. The conserved *S. mansoni* SL sequence is shown in italics at the beginning of the cDNA sequence. Putative CaMPKII phosphoryation sites are encircled. Boxed amino acid residues represent a predicted tetramerization domain. The potential polyadenylation consensus sequence is underlined and the stop codon (TAG) is indicated by an asterisk.

(57 %) with the only other invertebrate sequence available, a *Drosophila* enzyme that has been described as a TPH / PAH hybrid (56). Structurally, the *Drosophila* enzyme appears to be more closely related to PAH than any of the TPH sequences (Fig.2).

An amino acid sequence alignment of SmTPH and other tryptophan hydroxylase sequences is shown in Fig. 3. A high degree of sequence conservation (up to 82 % homology) is apparent in the carboxy-terminal-two-thirds of the sequence (amino acid positions 143 to 455 of SmTPH), a region that comprises the conserved catalytic domain of TPH (15,16,21,24). Several structural motifs that are characteristic of TPH and other aromatic amino acid hydroxylases are also present in this conserved core region,

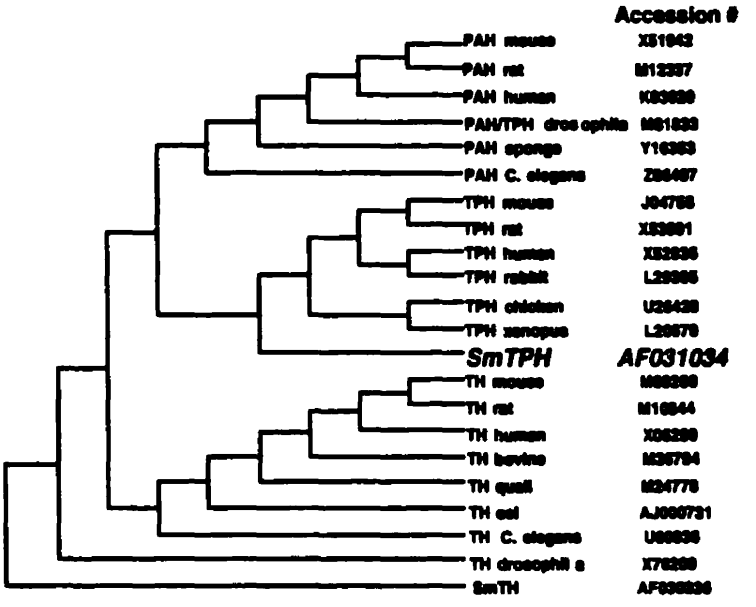

Fig. 2. Dendrogram analysis showing the structural relationship of SmTPH to other cloned aromatic amino hydroxylases. The amino acid sequence of SmTPH was compared to those of TH, PAH, and other TPH sequences using the program DNASIS vs. 3.7 (Hitachi Software, CA, U.S.A.). The lengths of the horizontal lines connecting one sequence to another are inversely proportional to the percentages of similarity between sequences or group of sequences. Hydroxylase sequences used were from *Homo sapiens* (human), *Bos torus* (bovine), *Oryctolagus cuniculus* (rabbit), *Anguilla anguilla* (eel), *Xenopus laevis*, *Coturnix coturnix* (quail), *Gallus gallus* (chicken), *Rattus norvegicus* (rat), *Mus musculus* (mouse), *Geodia cydonium* (sponge), *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Schistosoma mansoni* (SmTPH and SmTH). The corresponding GenBank accession number is indicated next to each sequence.

including a potential iron binding site (SmTPH His^{311}, His^{315}, and Glu^{330}) (2,57,58) and the signature peptide PEPD-CHELLGHVP. This latter is part of a conserved 27-amino-acid sequence (SmTPH Tyr^{289} to His^{315}; see Fig. 3) that forms a cofactor (BH_4) binding site in PAH (59), and possibly TH and TPH as well. Amino acid sequence homology decreases significantly at the amino terminal end (amino acid positions 1 to 142 of SmTPH) and within a C-terminal sequence of approximately 40 amino acids. The latter region contains a potential intersubunit binding domain (SmTPH Leu^{462} to Ile^{480}), consisting of a leucine heptad repeat interspersed by a 4, 3-hydrophobic repeat (26,27) (Fig. 1).

Expression of SmTPH- The complete coding sequence of SmTPH (1494 bp) was amplified directly from *S. mansoni* mRNA by RT-PCR, cloned into the prokaryotic pET15b vector, and expressed in *E. coli* BL21(DE3)pLysS. To ensure maximal TPH enzyme activity, bacterial cultures were supplemented with iron, which is limiting in a

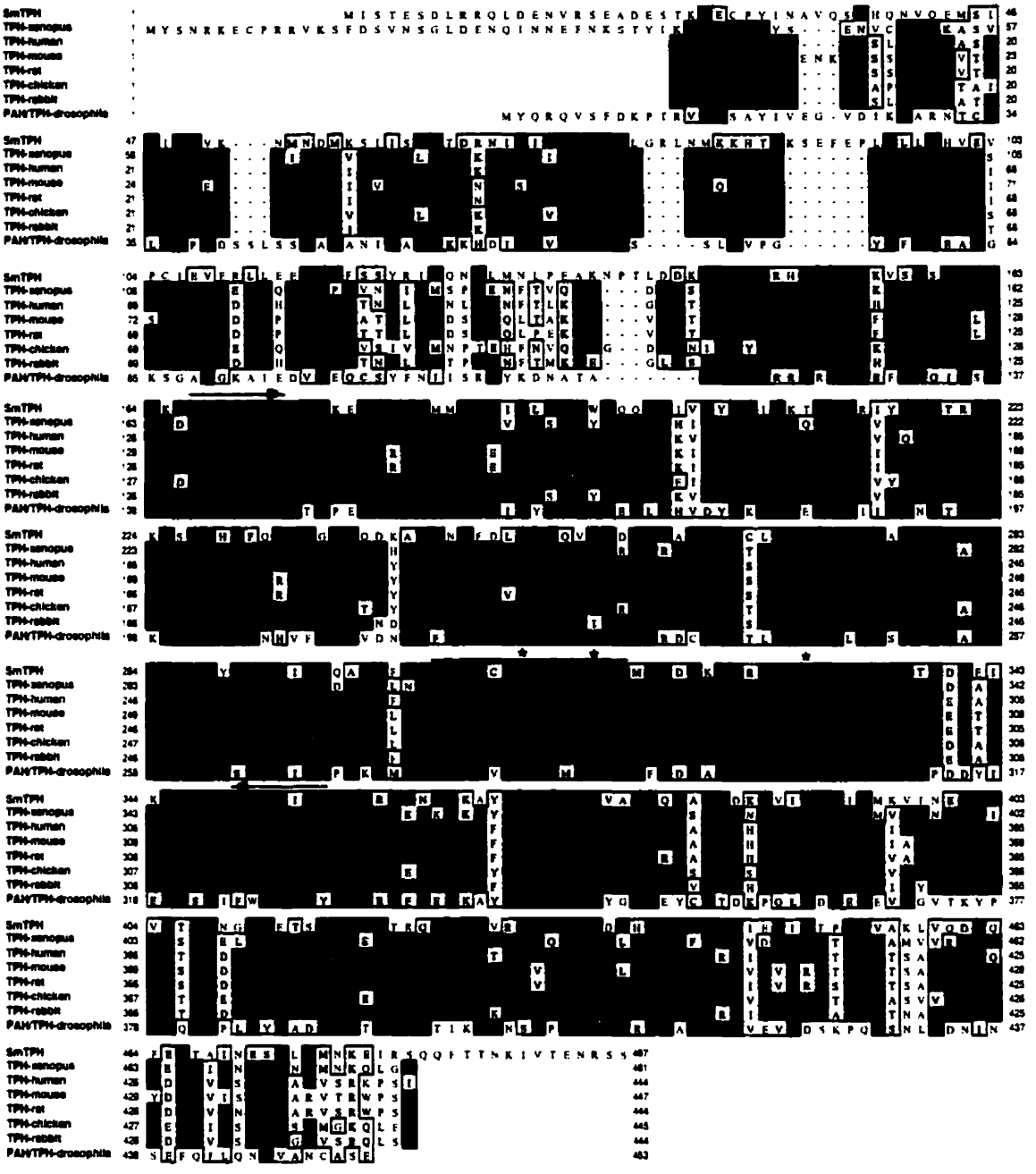

Fig. 3. **Amino acid sequence alignment of SmTPH with other tryptophan hydroxylase sequences.** Protein sequences (refer to Fig. 2 for relevant GenBank accession numbers) were aligned using the program MacVector (vs. 6.5; Oxford Molecular), according to the CLUSTAL method. Predicted conserved residues of the iron binding site are indicated by an asterisk. The overlined sequence represents a conserved signature motif present in all aromatic amino acid hydroxylases. Arrows indicate the conserved regions that were targeted for initial PCR cloning of a partial SmTPH sequence, as described in the experimental procedures section

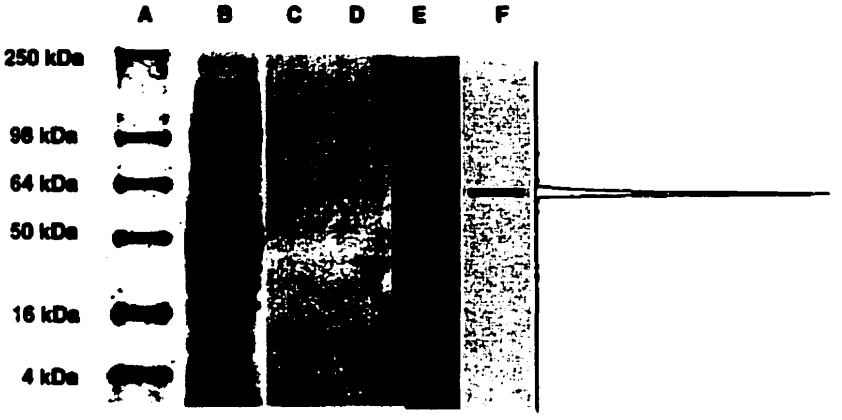

Fig. 4. Purification of recombinant SmTPH. SmTPH was expressed in *E. coli* (BL21) as an N-terminal oligohistidine fusion protein and purified to apparent homogeneity by affinity chromatography. A) Molecular weight standards. B) Coomassie blue stain of a crude lysate of induced BL21(DE3)pLysS cells expressing SmTPH C) Western blot of a crude lysate of induced BL21 cells which were previously transformed with pET15b vector only. D) Western blot of a crude lysate of induced BL21 cells expressing SmTPH. E) Western blot of purified SmTPH. F) Coomassie stain of purified SmTPH and corresponding densitometry profile.

bacterium (13-15). Cloning of SmTPH into pET15b introduces an N-terminal oligo-histidine fusion tag, which permitted the subsequent purification of the enzyme by nickel affinity chromatography. This tag, consisting of six histidines followed by a thrombin cleavage site, added an extra 20 amino acids (~ 2 kDa) to the amino terminus of SmTPH.

Induction of SmTPH-transformed BL21(DE3)pLysS cells produced large amounts of active TPH. Western blot analysis of soluble bacterial protein extracts identified a predominant band of the expected size which reacted with a rabbit anti-TPH antibody (Fig,4). No cross reactivity was detected between the antibody and lysates prepared from *E. coli* transformed with pET15b vector containing no insert (Fig. 4). The average specific activity of SmTPH in crude bacterial extracts was 3.5 ± 0.5 nmoles/min/mg of protein with about 80 % of total enzyme activity being recovered in the soluble fraction. The remaining activity was retained in the pellet in the form of inclusion bodies.

Purification and Macromolecular Structure of SmTPH- SmTPH was purified by nickel affinity chromatography as an N-terminal histidine fusion tag . Based on specific activity measurements, SmTPH was purified about 45 fold to a final specific activity of

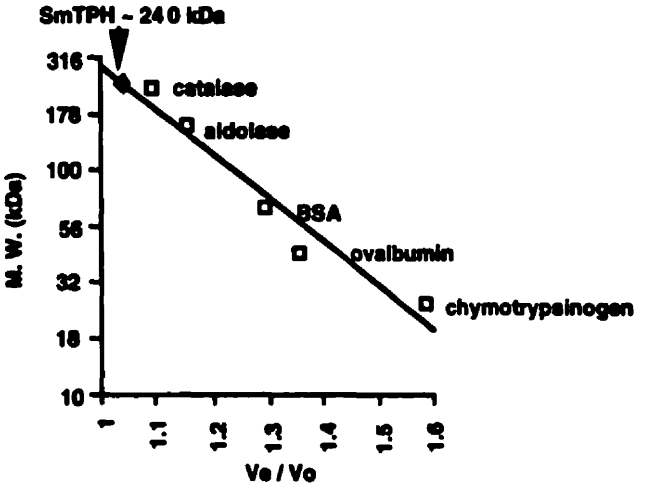

Fig. 5. Molecular weight determination of Purified SmTPH by gel filtration. Purified SmTPH was subjected to size exclusion chromatography using a Sephacryl 200HR resin. The column was calibrated using the following protein standards (Pharmacia): blue dextran (2000 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa). The data are plotted as elution volume (Ve) / void volume (Vo) versus molecular weight (log scale).

170 ± 5.0 nmol/min/mg protein. The yield from 100 ml of induced bacterial culture was 0.66 – 0.8 mg of purified SmTPH. Coomassie Blue staining and densitometric analysis of the purified protein showed a predominant western positive band of ~ 60 kDa, which was consistent with the expected size of the SmTPH monomer (Fig. 4). The purified enzyme was subjected to size exclusion chromatography to determine the oligomeric organization of the protein. Calculations of the molecular weight from a standard Ve/Vo plot suggest that SmTPH exists as a tetramer with approximate molecular weight of ~ 240 kDa (Fig. 5).

Kinetic Properties of Purified SmTPH- All three aromatic amino acid hydroxylases display an absolute requirement for tetrahydrobiopterin as a cofactor (2). Fig. 6A is a representative BH_4 saturation curve showing that the activity of the purified SmTPH is also dependent on its cofactor BH_4. Removing the cofactor abolished all enzymatic activity. The *K*m for BH_4 was measured by varying the concentration of BH_4 (2.5 – 200 μM) and keeping the concentration of the substrate, tryptophan, constant at 100 μM. Purified SmTPH displayed a *K*m for BH_4 of 6.7 ± 0.7 μM (mean ± SEM) and a *V*max value of 163 ± 13 nmol/min/mg protein (mean ± SEM).

The *K*m for the substrate was investigated by varying tryptophan concentration (1 – 250 μM) while fixing the BH_4 concentration at 200 μM (Fig 6B). SmTPH activity was inhibited at high concentrations of tryptophan (> 100 μM), in a similar fashion to what

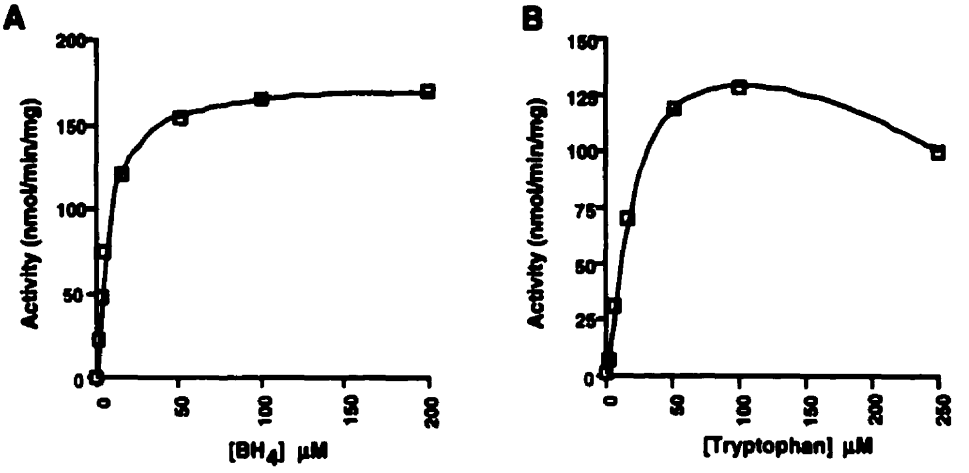

Fig. 6. Activity of purified SmTPH as a function of the cofactor, BH_4, and the substrate, tryptophan. The activity of purified SmTPH was assayed as described under Experimental Procedures, either at variable BH_4 concentrations and a fixed tryptophan concentration at 100 μ*M* (*A*) or at variable tryptophan concentrations and a constant BH_4 concentration of 200 μ*M* (*B*).

was previously reported for mammalian TPH (6,15,17,60). Since an accurate *K*m determination for tryptophan was not possible due to substrate inhibition, an apparent *K*m ($S_{0.5}$) was estimated at (mean ± SEM) 22 ± 2.0 μ*M*.

Several known inhibitors of mammalian TPH, including p-chlorophenylalanine, dopamine and the product of tryptophan hydroxylation, 5-HTP (11,13,61-66) all caused inhibition of purified SmTPH (Table 1). The parasite enzyme was not sensitive to feedback inhibition by 5-HT or by the products of 5-HT metabolism, N-acetyl-5HT and melatonin, even at high concentrations of 0.1 m*M* (Table 1). The inhibition by dopamine was found to be predominantly competitive with respect to the cofactor, BH_4 (Fig. 7A) In the case of 5-HTP, the inhibition was predominantly noncompetitive with respect to tryptophan. The addition of 50 μ*M* 5-HTP caused a marked 2 - 3 fold decrease in *V*max with very little change in the apparent *K*m for the substrate (Fig. 7B).

Low levels of catechols, in particular dopamine, have been shown to cause a sustained time-dependent inhibition of mammalian forms of tyrosine hydroxylase (67-69). To determine if SmTPH is similarly sensitive to this form of dopamine inhibition, aliquots of the purified enzyme (2.8 μ*M*) were pre-incubated with a stoichiometric amount of dopamine for 2, 4, 6, 10, and 15 min at 30 °C and then assayed for TPH

Compounds	IC50 (μM)
p-Chlorophenlyalanine	9
5-HTP	18
Dopamine	22
5-HT	> 100
N-acetyl-5-HT	> 100
Melatonin	> 100

TABLE I. Inhibition of SmTPH by different agents TPH activity assays were performed in the presence of varying concentrations of inhibitors. The assay conditions were as described under Experimental Procedures, except for the inhibition by dopamine where the BH_4 concentration was 25 μM. IC50 values represent the concentrations of inhibitors at which SmTPH activity was reduced by 50 %. Data are averages of 2 to 3 independent experiments each done in duplicate.

activity. No change in SmTPH activity was detected compared to a control sample incubated for the same length of time in the absence of dopamine (data not shown).

Stability of SmTPH- The stability of SmTPH was compared to that of rabbit brain TPH (13) that was similarly subcloned into pET15b and expressed in *E. coli* BL21(DE3)pLysS strain as a histidine-tagged fusion protein. Initial attempts to purify the rabbit enzyme by nickel chelation chromatography yielded a very unstable enzyme ($T_{1/2}$ < 10 min at 37°C) with low specific activity. Therefore, the comparison between SmTPH and the rabbit enzyme was carried out with crude bacterial lysates which had similar specific activities of 3.3 nmol/min/mg protein for SmTPH and 1.49 nmol/min/mg protein for rabbit TPH respectively. The latter value is essentially identical to what was previously reported for similar preparations of this enzyme (13). Aliquots of crude SmTPH or rabbit TPH containing the same amount of protein (~ 100 μg) were preincubated at 37°C for up to 80 min and then assayed for TPH activity. As can be seen in Fig. 8, SmTPH activity remained virtually unchanged over the incubation period, with an estimated half-life ($T_{1/2}$) of ~ 21 hrs. In contrast, the rabbit brain TPH displayed a significantly shorter $T_{1/2}$ of 54 min. This latter value is comparable to the previously reported $T_{1/2}$ for recombinant rabbit TPH (25). The same experiment was repeated with aliquots (1.75 μg) of purified SmTPH. The results (data not shown) produced an estimated $T_{1/2}$ value for the pure enzyme of 99 min at 37°C.

TPH Activity in Crude *S. mansoni* Extracts- Crude tissue extracts of adult *S. mansoni* were prepared and tested directly for TPH enzymatic activity as described above. When

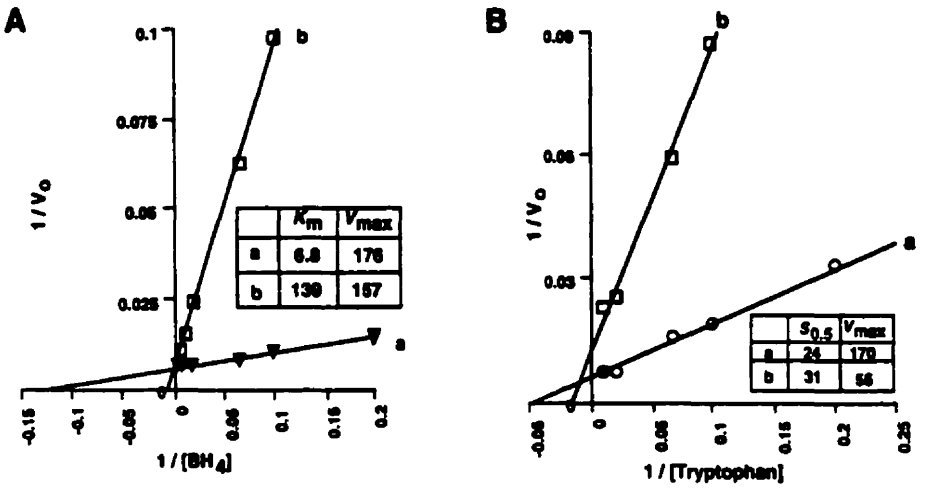

Fig. 7. Inhibition of purified SmTPH by dopamine and 5-HTP. *A*, The activity of purified SmTPH was measured in either the absence (a) or presence (b) of 50 μ*M* dopamine. The BH_4 concentration was varied between 1 to 200 μ*M* while the tryptophan concentration was fixed at 100 μ*M*. *B*, Similarly, SmTPH activity was measured over a concentration range of tryptophan (1 – 250 μ*M*) and a fixed BH_4 concentration (200 μ*M*), in absence (a) or presence (b) of 50 μ*M* of 5-HTP. $1 / V_o$ is the reciprocal of the initial rate measured in nmol/min/mg of purified enzyme. *Insets*, Tables showing the calculated K_m and apparent K_m ($S_{0.5}$) (μ*M*) and V_{max} (nmol/min/mg of protein) values under the different conditions described in *A* and *B*.

compared with a boiled enzyme control, the specific activity level of the native *S. mansoni* TPH was ~ 0.02 – 0.04 nmol/min/mg protein (data not shown). This level of activity is similar to that reported earlier for native TPH measured in rabbit brain extracts (13). When BH_4 was omitted from the assay mixture, no detectable levels of activity were obtained from the worm extracts. This illustrates that the native *S. mansoni* TPH has the same absolute requirement for the biopterin cofactor as the recombinant SmTPH.

SmTPH Developmental Expression in *S. mansoni*- The expression of SmTPH was examined by semiquantitative RT-PCR in two different developmental stages of *S. mansoni*, cercaria and adults. Expression levels were standardized by comparison with a constitutively expressed control gene from *S. mansoni*, α-tubulin (47,48). Fig. 9 indicates that the SmTPH expression level is approximately 2.5 fold higher in the cercarial stage

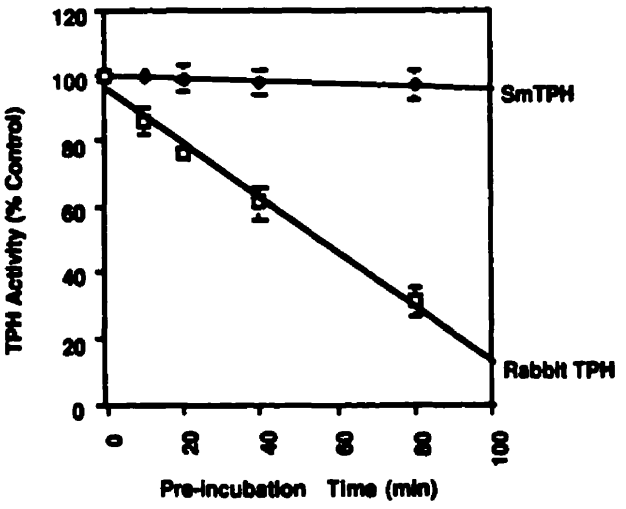

Fig. 8 Stability of SmTPH. Recombinant SmTPH and rabbit TPH were expressed in BL21(DE3)pLysS cells under identical conditions. Aliquots of the supernatants prepared from crude *E. coli* lysates expressing either SmTPH or the rabbit enzyme were preincubated for the indicated periods of time at 37 °C and their specific activities were measured in the presence of 200 μ*M* BH4 and 50 μ*M* tryptophan (25). The data are expressed as the percentage of initial activity for each enzyme measured at t = 0. The initial specific activities (nmol/min/mg protein) for SmTPH and rabbit TPH extracts were 3.3 and 1.46, respectively. All data points represent an *n* = 3. T1/2 values were calculated from linear regression analysis of the data points obtained for each sample.

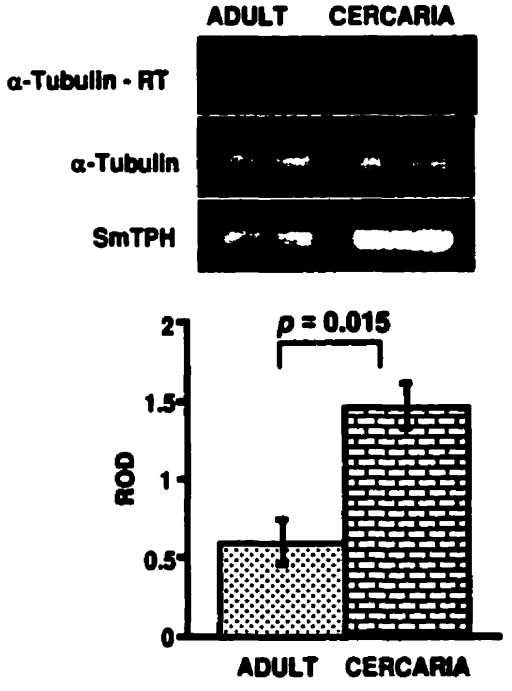

Fig. 9. Developmental expression of SmTPH in *S. mansoni*. Semi-quantitative RT-PCR was performed on total RNA extracted from two different developmental stages of *S. mansoni* (cercaria and adult), as described under Experimental Procedures. The RT-PCR reactions in both developmental stages were standardized by simultaneous amplification of an internal control house keeping gene from *S. mansoni* (α-tubulin). The SmTPH PCR product and that of the α-tubulin control are shown in the top panel. A negative PCR control was done using *S. mansoni* α-tubulin primers on total RNA samples subjected to a mock RT reaction (-RT). The PCR products were analyzed on a 1.2 % agarose gel containing ethidium bromide followed by densitometric analysis. The lower panel shows a bar graph displaying the relative optical density (ROD = optical density of SmTPH PCR product / optical density of α-tubulin control) obtained from adult and cercaria *S. mansoni*. Results are the mean ± SEM of three independent RT-PCR experiments, each done in duplicate. Unpaired *t* test showed that the ROD differences between the adults and cercaria are statistically significant ($p = 0.015$).

than in the adult stage of *S. mansoni*. No α-tubulin PCR products were detected in a control reaction that lacked reverse transcriptase, thus ruling out the possibility of genomic DNA contamination.

DISCUSSION

This study describes the cloning and functional characterization of tryptophan hydroxylase from a lower invertebrate, the parasitic platyhelminth *S. mansoni*. This is the second member of the aromatic amino acid hydroxylase family identified in *S. mansoni*; we recently cloned a functional form of TH from this same parasite (40). The finding of these enzymes in such a primitive invertebrate raises interesting questions about the evolution of the three hydroxylases. There is general agreement that the three enzymes are derived from a common ancestral gene through a series of two gene duplications, the first of which gave rise to TH whereas the second separated TPH from PAH (18,70,71). It has been suggested that the divergence of TPH and PAH occurred late in evolution, possibly after the emergence of arthropods (71). However, as pointed out by Boularand et al (72), recent genome sequencing data have identified distinct predicted genomic sequences for all three enzymes in the nematode, *C. elegans*, suggesting that the two gene duplications occurred earlier than was previously thought. The finding of TPH and TH in *S. mansoni* strengthens this point and further suggests that the divergence of the aromatic amino acid hydroxylases may have occurred before the emergence of platyhelminths.

An alignment of SmTPH with other TPH sequences identified a core of high amino acid sequence identity in the middle to C-terminal region of the enzyme. This stretch of conserved sequence corresponds roughly to the previously defined catalytic domain of TPH (1) and includes several distinctive motifs, including the predicted iron binding site (57,58) and a putative biopterin binding domain (59). The N-terminal region, on the other hand, shows little sequence conservation across the different species of the enzyme and is particularly divergent in SmTPH, with identity scores of 14 – 17 % when compared to cognate mammalian sequences (see Fig. 3). The divergence at the N-terminal end is consistent with the notion that this region lies outside the catalytic domain and may serve a regulatory function (15,21,24,25,28,65), just as shown for other aromatic

amino acid hydroxylases (2,3). The present identification of a putative phosphorylation site for Ca2+ / calmodulin dependent protein kinase II in this N-terminal region of SmTPH (Thr^{130}) gives credence to this notion. At the C-terminal end, the homologous parasite sequence extends to pos. 455 (rabbit TPH pos. 417), which places a tentative C-terminal boundary for the catalytic domain nearly 40 residues prior to the C-terminus. This region shows very little sequence conservation among the different TPH species except for a characteristic intersubunit binding motif (26,27), which is present in all TPH sequences including SmTPH. The conservation of this structural motif in an otherwise divergent C-terminal end supports previous suggestions that the carboxyl region of TPH is not directly involved in catalysis, despite conflicting mutagenesis data (15,27) but rather constitutes a distinctive oligomerization domain (27).

SmTPH was expressed in *E. coli* as a histidine-tagged protein, which was purified and partially characterized. The analysis showed that SmTPH shares many of the characteristics of mammalian TPH, both recombinant and native. Similar to other forms of the enzyme, SmTPH was found to form tetramers of about 240 kDa. In addition, SmTPH showed a characteristic absolute requirement for the reduced pterin cofactor, BH_4. The *K*m for BH_4 was 4 to 7 fold less than what was previously reported for purified mammalian brain TPH tagged to glutathione S-transferase (GST) (16) or maltose binding protein (MBP) (15). It is unknown if this discrepancy is due to the presence of large fusion tags on the two mammalian enzymes, which may have influenced the kinetic determination, or whether the parasite hydroxylase has a significantly higher affinity for the cofactor. With respect to the substrate, tryptophan, SmTPH exhibited a typical kinetic profile, with characteristic substrate inhibition at tryptophan concentrations above 100 μM and an apparent *K*m ($S_{0.5}$) of about 22 μM, similar to what was previously described for mammalian forms of the enzyme (6,15) . Additional characterization of the parasite hydroxylase showed that the enzyme is sensitive to inhibition by the classic TPH inhibitor, p-chlorophenylalanine, as well as the immediate product of the reaction, 5-HTP, but not serotonin or its metabolites, N-acetyl serotonin and melatonin. The lack of inhibition by serotonin was reported previously in crude brain extracts of native TPH (11). It is noteworthy that the inhibition of SmTPH by 5-HTP did not follow classical competitive kinetics, as might have been expected from standard product inhibition.

Instead, 5-HTP inhibition (as a function of tryptophan) showed mixed, predominantly noncompetitive characteristics, with *V*max decreasing nearly 3- fold and the apparent *K*m increasing by about 50 %. The significance of this inhibition profile is unclear, nor is it known if it is unique to the parasite. All available data on product inhibition of mammalian TPH stem from preparations of crude native enzyme (5,11), which is not directly comparable to the purified enzyme preparations used in this study. Additional research on the role of 5-HTP in TPH regulation is needed.

Previous studies have shown that TPH is susceptible to inhibition by catechol products of the TH reaction, in particular dopamine (62-66). Inhibition by dopamine is thought to be biologically relevant in regions of the nervous system where serotonergic and catecholaminergic neurons may interact. A large body of evidence on dopamine inhibition of TH shows the existence of two major mechanisms of hydroxylase inhibition, a time-dependent sustained inhibition seen at low (stoichiometric) dopamine concentrations and competitive inhibition (with respect to the cofactor) which occurs at higher (μM) concentrations of the neurotransmitter (4,73). In the present study, we found that SmTPH is similarly sensitive to inhibition by dopamine, and that the inhibition at μmolar concentrations is essentially competitive with respect to BH_4. We were unable to detect, however, any time-dependent enzyme inhibition at lower concentrations of dopamine. This points to important differences in the responses of TPH and TH to catechol inhibition.

Biochemical studies of TPH have been hindered by the difficulty in obtaining large amounts of purified active enzyme that is suitable for characterization. Even with the advent of molecular biology techniques, researchers have found that recombinant mammalian TPH overexpressed in *E. coli* tends to form inactive inclusion bodies (13,14), unless it is expressed with large fusion partners (15,16), and also becomes unstable upon purification. In contrast to mammalian TPH, however, the parasite enzyme was expressed as a soluble protein, which could be purified and was both active and stable. The specific activity of SmTPH was 2 - 13-fold higher than values reported for purified forms of recombinant mammalian TPH (15-17). In addition, results presented here showed that the half-life of a crude SmTPH extract was about 23 times longer than that of a similar preparation of rabbit TPH also expressed in *E. coli*. After purification, the rabbit enzyme

lost activity very rapidly, whereas SmTPH remained relatively stable, with a half-life at 37°C of 99 min, and could be stored frozen with virtually no loss of activity. The reason for this dramatic difference in enzyme stability is unknown. Recent evidence has suggested that the notorious instability of mammalian TPH (10,11,14,25,63) may be associated, at least in part, with the enzyme's regulatory domain (25,60), roughly the same region that is least conserved in the parasite. This raises the interesting possibility that the stability of SmTPH is related to its distinctive N-terminal domain, which may stabilize activity more effectively than the cognate region of mammalian TPH. A stabilizing effect of the N-terminal regulatory domain on enzyme activity has been reported for the related hydroxylase, TH (25).

The finding of TPH in *S. mansoni* has clarified a long - standing question of how this parasite obtains its serotonin. Just as other parasitic worms, *S. mansoni* has high levels of serotonin within its nervous system and is well known to rely heavily upon serotonin for a wide range of essential activities, among them the regulation of motility and carbohydrate metabolism. Earlier difficulties in identifying TPH activity in tissue extracts of *S. mansoni* led to the generalized belief that the parasite lacked the enzymatic capacity to synthesize serotonin endogenously and thus relied on the human host for a supply of the neurotransmitter. By cloning an active form of TPH from the parasite, and also demonstrating TPH activity in crude worm extracts, the present study has shown clearly that the enzyme is present and active in *S. mansoni.* The earlier negative results were likely due to the paucity of the enzyme in the worm combined with the low sensitivity of the assay (74), both of which would limit the ability to detect TPH activity in the crude worm extracts. It should be noted, however, that the present results do not rule out the possibility that the parasite may recruit some exogenous serotonin from the host, possibly through a tegumental carrier (32,33,36), in addition to synthesizing the neurotransmitter endogenously. In this respect, it is interesting that the levels of SmTPH mRNA in the adult worm, which is strictly parasitic, are nearly 2.5 times lower that in a free-living larval stage (cercaria). Although the difference is small, it is nonetheless surprising in light of the greater development of the serotonergic nervous system in the adult compared to the larva. The possibility exists that the adults rely both on endogenous synthesis and exogenous intake of serotonin, whereas the free-living stage, which must

rely entirely on biosynthetic activity, has proportionally higher levels of TPH. The significance of these results for the development of the parasite and its survival in the host is currently under investigation.

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CONNECTING STATEMENT I

In manuscript I, we looked at the issue of serotonin biosynthesis in helminths, where we described the molecular cloning and characterization of TPH from *S. mansoni.* This study provided the first molecular evidence that parasitic helminths may be able to synthesize serotonin endogenously. In manuscript II, we investigated the mode of action of serotonin in helminths. In this study, a novel 5-HT receptor from *C. elegans* was cloned and its pharmacology and signaling properties were characterized.

CHAPTER III (MANUSCRIPT II)

Characterization of a Novel Serotonin Receptor from *Caenorhabditis elegans*: Cloning and Expression of Two Splice Variants

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ABSTRACT

Serotonin (5-hydroxytryptamine: 5-HT) modulates feeding activity, egg-laying and mating behavior in the free-living nematode,*Caenorhabditis elegans*. We have cloned a novel *C. elegans* receptor cDNA (5-HT_{2Ce}) that has high sequence homology with 5-HT_2 receptors from other species. When transiently expressed in COS-7 cells, 5-HT_{2Ce} exhibited serotonin binding activity and activated Ca^{2+}- mediated signaling in a manner analogous to other 5-HT_2 receptors. However, 5-HT_{2Ce} displayed unusual pharmacological properties, which resembled both 5-HT_2 and 5-HT_1-like receptors, but did not correlate well with any of the 5-HT_2 subtypes. Two splice variants of 5-HT_{2Ce} that differ by 48 N-terminal amino acids were identified. The two isoforms were found to have virtually identical binding and signaling properties but differed in their levels of mRNA expression, the longer variant being four times more abundant than the shorter species in all developmental stages tested. Taken together, the results describe two variants of a novel *C. elegans* serotonin receptor, which has some of the properties of the 5-HT_2 family, but whose pharmacological profile does not conform to any known class of receptor.

INTRODUCTION

Serotonin (5-hydroxytryptamine; 5-HT) is a widely distributed neuroactive agent of vertebrates and invertebrates. In the free-living nematode *Caenorhabditis elegans*, serotonin has been identified within several central and peripheral neurons, including the well characterized pharyngeal neurosecretory motorneurons (NSM), the hermaphrodite-specific neurons (HSN), and the male specific CP neurons (Horvitz et al., 1982; Desai et al., 1988; Loer and Kenyon, 1993). The serotonergic NSM may modulate pharyngeal pumping (feeding), locomotion, and egg laying, while the HSN and CP neurons seem to affect egg-laying and male mating behavior, respectively (Horvitz et al., 1982; Desai and Horvitz, 1989; Loer and Kenyon, 1993). These effects of serotonin are mediated, in part, by G proteins (Bargmann and Kaplan, 1998) and at least one type of G protein-coupled receptor (GPCR), which is negatively linked to adenylate cyclase (Olde and McCombie, 1997). It is unknown at present if there are other receptors and pathways of signal transduction that mediate the multiple effects of serotonin in this animal.

In mammals, where serotonin is a well established neurotransmitter involved in a wide range of physiological activities (Leonard, 1994), as many as seven different classes of serotonin receptors (5-HT_1 - 5-HT_7) have been identified, all of which are further divided into multiple subtypes (Hoyer et al., 1994; Gerhardt and Heerikhuizen, 1997). The vast majority of mammalian serotonin receptors belong to the large GPCR superfamily and couple to adenylate cyclase, either positively (5-HT_4, 5-HT_6 and 5-HT_7) or negatively (5-HT_1). In contrast, the 5-HT_2 family has very distinctive structural properties, and couples to phospholipase C and the phosphoinositol (IP_3) / Ca^{2+}- mediated pathway of signal transduction (Hoyer et al., 1994; Gerhardt and Heerikhuizen, 1997).

Considerably less is known about the molecular properties and diversity of serotonin receptors in invertebrates. Many of the invertebrate receptors that have been cloned and characterized, including the previously described *C. elegans* receptor (Olde and McCombie, 1997), show structural homology with the 5-HT_1 and, to a lesser extent, 5-HT_7 families (Witz et al., 1990; Saudou et al., 1992; Sugamori et al., 1993; Angers et al., 1998). For the most part, these receptors share similar drug binding and signaling properties with their mammalian counterparts (Hoyer et al., 1994; Gerhardt and van

Heerikhuizen, 1997), except for an unusually low affinity (high micromolar K_D) for indolealkylamines, including serotonin, which normally bind to mammalian 5-HT_1 and 5-HT_7 receptors with very high affinity (Hoyer et al., 1994). Recently, two 5-HT_2 receptors have been cloned from *Drosophila* (5-HT_{2Dro}) (Colas et al., 1995), and the pond snail, *Lymnaea* (5-HT_{2Lym}) (Gerhardt et al., 1996). Each of these receptors, in particular 5-HT_{2Dro}, shares structural and pharmacological properties with the mammalian 5-HT_{2B} receptor. 5-HT_{2Lym} has been further characterized as being linked to the hydrolysis of phosphoinositides and the Ca^{2+} signaling pathway (Gerhardt et al., 1996), an indication that 5-HT_2 receptors and their distinctive coupling mechanism are conserved at least in higher invertebrates such as molluscs and insects.

Here we report the molecular cloning and functional characterization of a novel serotonin receptor from *C. elegans* (5-HT_{2Ce}), which has high sequence homology with mammalian and other invertebrate 5-HT_2 receptors, and also appears to be coupled to a Ca^{2+}-mediated signaling pathway. Interestingly, however, 5-HT_{2Ce} has mixed 5-HT_1 and 5-HT_2 ligand binding properties, which do not conform to any other 5-HT_2 receptor, either mammalian or invertebrate. This study identified two splice variants of 5-HT_{2Ce}, which are expressed at different levels in *C. elegans*, but otherwise appear to have similar binding and signaling properties.

EXPERIMENTAL PROCEDURES

Chemicals - The radioisotope ^{125}I-Lysergic acid diethylamide (LSD) (2200 Ci / mmol) was purchased from Mandel (Guelph, ON). Serotonin, histamine, melatonin, n-acetyl-5-hydroxytryptamine (*N*-acetyl-5-HT), and tryptamine were obtained from Sigma (Oakville, ON). Dopamine, octopamine, 2,5-dimethoxy-4-iodoamphetamine (DOI), lisuride, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), ketanserin, metergoline, butaclamol, methiothepin and cyproheptadine were purchased from RBI (Natick, MA). All other chemicals were of the highest reagent grade.

Caenorhabditis elegans - The wild type Bristol N2 strain of *C. elegans* was maintained at room temperature on 100mm NGM agar plates seeded with *E. coli* (OP50 or NA22). To obtain stage- specific worms, *C. elegans* were first synchronized and then purified by floatation on a 30% sucrose gradient as described previously (Lewis and Fleming, 1995). After purification, the worms were pelleted by centrifugation and stored frozen at -80°C until needed. For mRNA extraction, aliquots of frozen adult worms were used directly for poly-A$^+$ RNA purification, using the Fast-Track messenger mRNA isolation kit, according to the recommendations of the manufacturer (Invitrogen, San Diego). Total RNA was isolated from different developmental stages with the use of the TRIzol reagent (Gibco BRL, Burlington, ON).

Cloning of full-length 5-HT$_{2Ce}$ cDNAs - Computer analyses of data from the *C. elegans* genome sequencing project (ACeDB) (Sulston et al., 1992) identified a genomic sequence (cosmid F59C12; GenBank Accession # U41038) which was predicted to encode a protein similar to serotonin receptors from other species. PCR primers targeting this predicted coding region (sense: Primer F; antisense:Primer R ; see Fig.1) were used in an RT-PCR reaction to amplify a putative serotonin receptor cDNA sequence. Briefly, adult *C. elegans* mRNA (0.5µg) was reverse-transcribed with an oligo-dT primer and 200U of murine moloney leukemia virus reverse transcriptase (MMLV) and 1/10 of the resulting cDNA was subjected to 30 cycles of PCR (30 sec / 94°C, 30sec / 54°C, 120 sec / 72°C) in a 50µl reaction containing 0.4 µM of each primer (F and R), according to

standard protocols. A PCR product of 1908 bp was gel-purified, ligated into the PCR 2.1 vector (Invitrogen, San Diego), and sequenced by the dideoxy-chain termination method. The 5' and 3' ends of the receptor cDNA sequence were obtained by standard RACE procedures using the 5'- and 3'-RACE systems from Gibco BRL (Burlington, ON). For 5'-RACE, adult *C. elegans* mRNA (0.5 µg/µl) was reverse transcribed and dC-tailed, prior to PCR amplification (same parameters as above) with the kit's sense oligodG-anchor primer and a 5-HT_{2Ce} gene-specific antisense primer (primer A see Fig.1). An aliquot of the resulting PCR product was subjected to a second PCR reaction using an abridged form of the same anchor primer (also supplied by the RACE kit) and a nested gene-specific antisense primer (primer B; see Fig. 1). For 3'-RACE, reverse-transcription was performed as described above except that an oligodT-adaptor primer (supplied by the 3'RACE kit) was used to prime the reaction. The resulting cDNA was similarly subjected to nested PCR, using two nested gene-specific primers (primers C and D, Fig. 1) and an antisense adaptor primer. The final 5' and 3' RACE products were gel-purified, cloned into PCR 2.1 and confirmed by DNA sequencing.

Construction of long (5-HT_{2CeL}) and short (5-HT_{2CeS}) 5-HT_{2Ce} isoform expression vectors - The complete coding sequences of 5-HT_{2CeL} and 5-HT_{2CeS} were amplified directly by RT-PCR from adult *C. elegans*. RT was performed as described above and the resulting cDNA was PCR amplified using the proofreading DNA polymerase, PWO (Boehringer Mannheim, Laval, Quebec), according to the specifications of the manufacturer. The PCR sense primers were as follows: 5'-*acgcgt***GCCACC**ATGCTCA-TTGAACTCTTCTACAC-3'(for 5-HT_{2CeL}) and 5'-*acgcgt***GCCACC**ATGGGAATCTA-CCATTTCAACGG-3' (for 5-HT_{2CeS}). The antisense primer for both 5-HT_{2CeL} and 5-HT_{2CeS} was: 5'cgagcggccgcTTACAAGAATGTTTCCTTGATGGC-3'. Restriction sites (shown in italics) were added at the 5'-end of each primer to facilitate further subcloning into an expression vector (*Mlu* I for the sense primers and *Not* I for the antisense primer). The forward primers also contained a Kozak motif (shown in bold) before the start ATG (underlined) for optimal translation in mammalian cells (Kozak, 1986). The resulting PCR products were gel-purified, digested with *Mlu* I and *Not* I, and ligated to the eukaryotic expression vector pCI-neo (Promega, Madison) which had been linearized by

the same two restriction enzymes. The final constructs were confirmed by DNA sequencing of at least three separate clones.

Expression of the 5-HT_{2Ce} splice variants in COS-7 cells and membrane preparation- The 5-HT_{2Ce} pCI-neo constructs were transiently transfected into COS-7 cells using Lipofectamine (Gibco BRL, Burlington, ON). Briefly, aliquots of 2.5 µg of plasmid DNA mixed with 20 µl of lipofectamine were added to 10 cm dishes, which had been seeded 10-12 hrs earlier with approximately 10^6 cells. The medium was changed at 5 hrs and 24 hrs post-transfection and the cells were harvested 40 - 48 hrs later. For preparation of crude membrane fractions, transiently transfected COS-7 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then incubated in a hypotonic solution (15 mM Tris-HCL, pH 7.4, 1.25 mM $MgCl_2$, 1mM EDTA) for 10-15 min at 4°C. After swelling, the cells were scraped from the plates, centrifuged at 500 x g for 5 min. and resuspended in 10 ml of ice-cold TEM buffer (50 mM Tris, pH 7.4, 0.5 mM EDTA, 10 mM $MgCl_2$) containing 1 mM of freshly added phenylmethylsulfonyl fluoride (PMSF). The resuspended cells were lysed on ice by sonication (7 pulses of 15 sec on/off) and the lysates were centrifuged at 200 xg for 5 min. The supernatant was centrifuged at 28,000 xg for 20 min at 4°C and the resulting pellet was washed once by resuspension in ice-cold TEM and centrifugation at 28,000 xg. The final crude membrane pellet was stored at -80°C until used in binding assays.

^{125}I-LSD binding assays - Frozen membrane pellets were thawed and resuspended in TEM buffer at a concentration of 0.5 - 1.5 µg protein/µl. Protein was measured according to the method of Bradford (Bradford, 1976), using the BioRad (Mississauga, ON) protein assay kit. ^{125}I-LSD binding was assayed in a 100 µl reaction volume in TEM buffer containing 0.02% ascorbate, COS-7 membranes (5 - 15 µg of protein), ^{125}I-LSD (final concentrations of 0.18 - 5 nM for saturation experiments and 1.5 nM for competition assays), in the presence or absence of various unlabelled drugs . Nonspecific binding was measured in the presence of 10 µM cyproheptadine and was typically less than 10% of total ^{125}I-LSD binding. Incubations were performed at room temperature for 50 min in restricted light. Preliminary experiments indicated that specific ^{125}I-LSD binding

increased linearly up to one hour of incubation under these conditions. Binding reactions were terminated by dilution with ice-cold TEM buffer followed by rapid filtration through Whatman GF/B glass fiber filters which had been previously soaked in 0.2% polyethyleneimine. The filters were washed twice with 4 ml of the same buffer, dried and then radioassayed. All binding data were analyzed by the curve fitting program GraphPad Prism (GraphPad software, San Diego, CA) and were derived from three separate experiments each done in duplicate.

Aequorin Assays - The eukaryotic expression vector (pCDM.AEQ; licensed from the NIH, Bethesda, MD) containing the complete coding sequence of the calcium binding protein, aequorin (Inouye et al., 1985), and either a 5-HT_{2Ce} expressionconstruct or a pCI-neo vector (no insert) control were used in the co-transfection of COS-7 cells. The aequorin assay was performed as described previously (Boie et al, 1997). Briefly, approximately two days after transfection the cells were charged with coelenterazine (8 μM). 96-well plates were prepared containing different concentrations of test drugs and placed singly into a Luminoskan RS luminometer (Labsystems, Neeham Heights, MA) for testing. Charged cells were dispensed at 5 x 10^4 cells per well starting with well A1. Light emission was recorded for the first 30 seconds (Peak 1), after which 100 μl of 0.3% (v/v) Triton X-100 in Hank's balanced salt solution (HBSS) was added to solubilize the cells and the light emission was measured for another 10 seconds (Peak 2). Fractional luminescence was determined at each ligand concentration by dividing the area under Peak 1 by the sum of the areas under Peak 1 plus Peak 2.

Developmental expression of 5-HT_{2CeL} and 5-HT_{2CeS} mRNAs in *C. elegans* - Expression of the two 5-HT_{2Ce} mRNAs was examined by semi-quantitative RT-PCR. Total RNA (2 μg) from three different developmental stages of *C. elegans* (embryos, L2/L3, and adults) was subjected to reverse transcription (as described above) followed by a standard PCR reaction (30 cycles of 45 sec/94°C, 45 sec/60°C, 45 sec/72°C) with primers designed to amplify products of 332 bp and 323 bp for 5-HT_{2CeL} and 5-HT_{2CeS}, respectively. The PCR primers spanned intron regions, so as to prevent genomic DNA amplification, and were selective for the different 5-HT_{2Ce} splice variants. The nucleotide

sequences of the sense primers were (see Fig. 1): 5'CCCTACTATGTACCTGCAA-ATGAATC-3' (primer L for 5-HT_{2CeL}) and 5'-ACCCAAGTTTGAGAATCATTGC-3' (primer S for 5-HT_{2CeS}). The antisense primer (CR: 5'-CTCCATGTCCACACCCCGT-GCC-3') was common for both splice variants. Each PCR reaction was standardized by simultaneous amplification of a control housekeeping gene sequence (*C. elegans* eukaryotic initiation factor 4A homologue; CeIF) (Roussell and Bennett, 1992; Krause, 1995) as described previously (Kinoshita, 1992). Standard curves were generated to ensure that the PCR reaction was in the exponential phase of synthesis after 32 cycles (for 5-HT_{2Ce}) and 21 cycles (for CeIF). The resulting products were subcloned into PCR2.1 and confirmed by DNA sequencing. Densitometric image analyses of RT-PCR products were performed with the NIH Image program vs. 1.61 (Bethesda, MA).

RESULTS

Isolation of two *C. elegans* cDNAs encoding 5-HT$_{2Ce}$ - A computer analysis of genomic sequence from the *C. elegans* Genome Data Base (ACeDB) identified a predicted coding sequence (cosmid F59C12; GenBank Accession #U41038) which showed homology to serotonin receptors from other species. This region was amplified by RT-PCR using reverse transcribed adult *C. elegans* cDNA as a template and primers that targeted the beginning and end of the predicted coding sequence. A 1908 bp product was obtained and confirmed by DNA sequencing of four independent clones. DNA sequence analysis indicated that the amplified PCR product was identical to the predicted sequence obtained from ACeDB, except for a stretch of 57 nucleotides (positions 3967 - 4023 of cosmid F59C12), which had been predicted to constitute an exon region (see GenBank Accession #U41038) but were lacking in all four independent clones of the RT- PCR product. Additional RACE analyses were performed to confirm the 5' and 3' ends of the putative receptor sequence. Interestingly, two 5' RACE products of 460 bp and 546 bp were obtained and found to be identical except for a variable 86 bp region at the 5' end. The two 5' RACE products showed significant sequence overlap with the 1908 bp RT-PCR product described above and thus were assumed to represent the 5' ends of two different transcripts of 5-HT$_{2Ce}$.

The complete cDNA sequence for each 5-HT$_{2Ce}$ transcript and their respective deduced amino acid sequences are shown in Fig. 1. The longest of the two sequences (5-HT$_{2CeL}$) comprises 2358 bp and has an open reading frame which encodes a predicted protein of 683 amino acids with a calculated molecular mass of 76.2 kDa. The 5' end bears a conserved 22 nucleotide spliced leader sequence (SL1) (Krause and Hirsh, 1987), suggesting that 5-HT$_{2CeL}$ is *trans*-spliced at the 5' end to an SL1 sequence, just as described for several other *C. elegans* RNAs (Blumenthal and Stewart, 1997). A short 5' untranslated region (UTR) of 22 bp precedes the predicted start ATG. At the 3' end, the RACE analysis identified a 262 bp UTR, which includes a potential polyadenylation site (GATAAA) (Blumenthal and Stewart, 1997) 21 bp upstream of a poly A tail. The shorter 5-HT$_{2Ce}$ species (5-HT$_{2CeS}$) lacks 86 bp at its 5' end but is otherwise identical to 5-HT$_{2CeL}$.

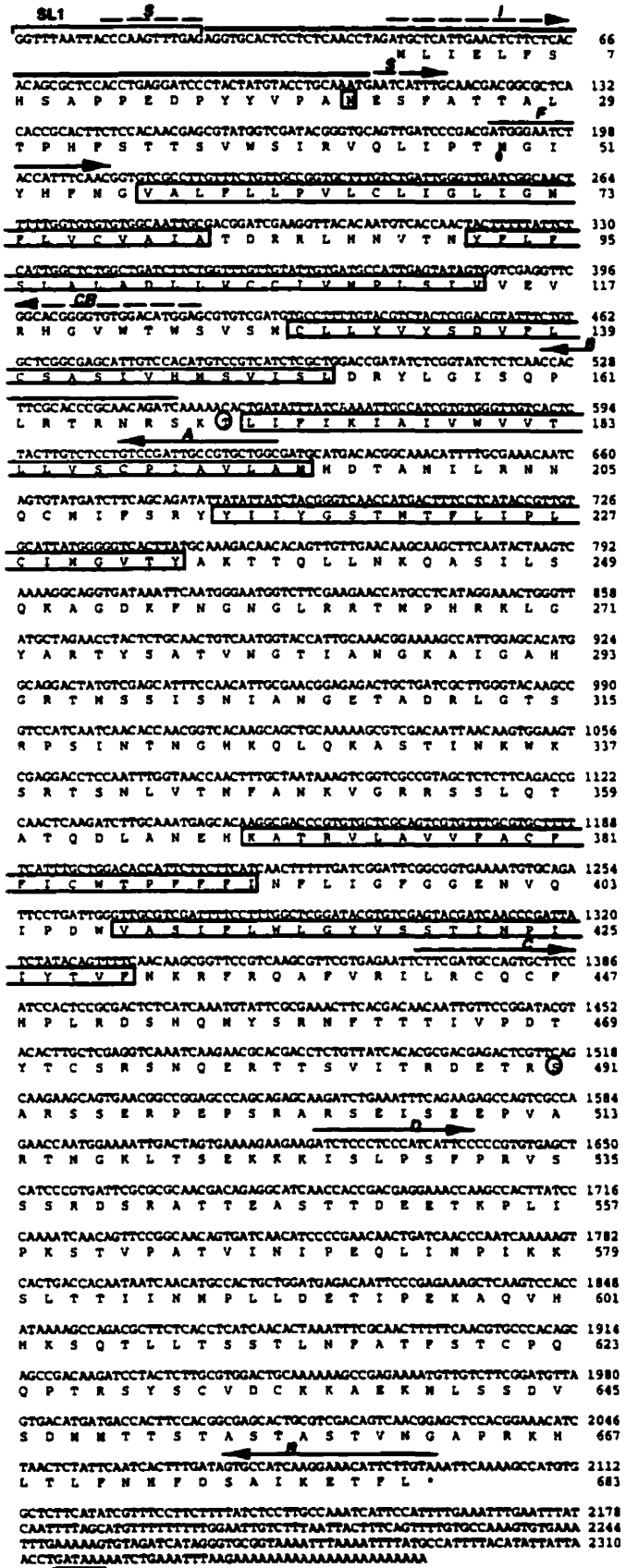

Fig. 1. Complete cDNA and predicted amino acid sequence of the longest 5-HT_{2Ce} splice variant (5-HT_{2CeL}). Nucleotide sequence that is absent in the shorter variant, 5-HT_{2CeS}, is overlined. Each of the two variants is trans-spliced at its respective 5' end to a conserved *C. elegans* spliced leader SL1 sequence. Sense (F, C, D) and antisense (A, B, R) PCR primers used for cloning and RACE analyses are marked by a solid arrow. Selective 5-HT_{2CeL} and 5HT_{2CeS} primers used for semi-quantitative RT-PCR are indicated by dashed arrows (sense: S, L; antisense: CR). The predicted initiation methionine for 5-HT_{2CeS} is marked by a closed circle. Boxed amino acids represent predicted transmembrane domains (TM) which were numbered I through VII. Open squares and circles depict potential N-glycosylation sites and phosphorylation consensus sites, respectively. The position of a potential polyadenylation site (GATAAA) is underlined. The nucleotide sequences for the two splice variants were submitted to GenBank and were designated accession numbers AF031414 (5-HT_{2CeL}) and AF031415 (5-HT_{2CeS}).

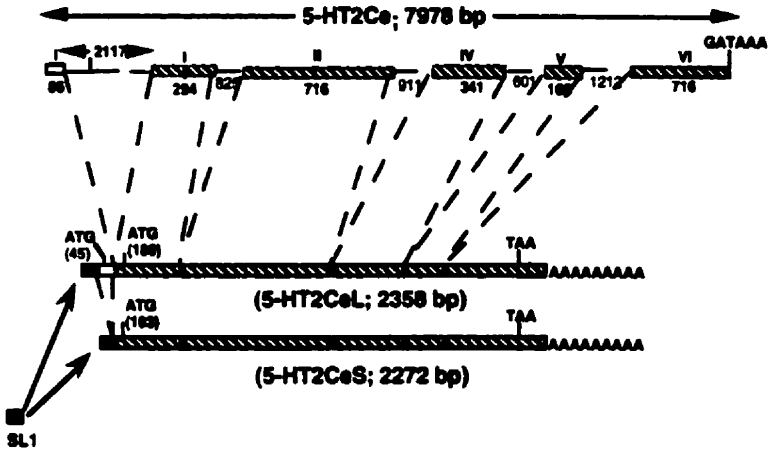

Fig. 2. Genomic organization of 5-HT$_{2Ce}$. The genomic organization of 5-HT$_{2Ce}$ is shown diagrammatically based on genomic sequence from ACeDB and cDNA sequence information, as described in the Results. Hatched boxes indicate exon sequences that are common to both 5-HT$_{2Ce}$ splice variants. The variably spliced exon I, which is present only in 5-HT$_{2CeL}$ transcripts, is represented by an open box. The spliced leader (SL1) sequence which is trans-spliced onto each 5-HT$_{2Ce}$ transcripts is shown by a solid box. Introns are represented by dashed horizontal lines. The relative positions of the two translational start codons, the common stop codon (TAA) and the polyadenylation site (GATAAA) are also indicated.

5-HT$_{2CeS}$ comprises 2272 bp and has a correspondingly shorter ORF which encodes a protein of 635 amino acids with a calculated molecular mass of 70.8 kDa. The predicted start codon for this variant is at nucleotide position 189 of the longer 5-HT$_{2CeL}$ cDNA sequence (Fig. 1). The two proteins vary by 48 amino acids at the N-terminal end. As in the case of the longer species, 5-HT$_{2CeS}$ contains a spliced leader SL1 at its 5' end (Fig. 1), indicating that the shorter variant of 5-HT$_{2Ce}$ is also *trans*-spliced to an SL1 sequence.

A comparison of the cDNA sequences of 5-HT$_{2CeL}$ and 5-HT$_{2CeS}$ with the original genomic sequence from ACeDB provided a genomic map for 5-HT$_{2Ce}$ and also clarified the origin of the two long and short transcripts. The organization of the 5-HT$_{2Ce}$ gene is shown in Fig. 2. The genomic sequence is approximately 8 kb long and spans two overlapping cosmids (K08B5, accession # U41022 and F59C12, GenBank Accession # U41038) of the *C. elegans* genome sequence. The beginning of the sequence was identified at position 31475 of cosmid K08B5 and the potential polyadenylation site is at position 6205 of cosmid F59C12. A total of six exons separated by five introns of variable lengths were identified, including an unusually long intron of 2117 bp which separates the first two exons in the sequence. Further analysis of the genomic sequence identified exon I as the variable 86 bp region of the two 5-HT$_{2Ce}$ species. Whereas the

longer 5-HT_{2CeL} transcript retains all six exons, 5-HT_{2CeS} lacks exon I, though both transcripts are similarly *trans*-spliced at the 5' end to the SL1 sequence. The predicted translational start codons for 5-HT_{2CeL} and 5-HT_{2CeS} are located on exon I and exon II, respectively. The results suggest the long and short species are splice variants of 5-HT_{2Ce} derived from differential splicing of exon I.

Protein sequence analysis of 5-HT $_{2Ce}$ isoforms - Hydropathy analyses of the deduced 5-HT_{2CeL} and 5-HT_{2CeS} amino acid sequences identified seven predicted transmembrane domains (TM) connected by predicted intra- and extracellular loops of variable lengths, an organization that is characteristic of all G-protein coupled receptors. The analyses identified a long third intracellular loop (124 residues), a region that has been implicated in the coupling of GPCRs to intracellular effectors (Wess, 1997), and an unusually long C-terminus (253 amino acid residues) also predicted to be intracellular. In addition, several distinctive GPCR motifs were identified, including the highly conserved "DRY" peptide at the C-terminal end of TM III (Probst et al., 1992; Wess, 1997). These results suggest that the two forms of 5-HT_{2Ce} belong to the GPCR receptor superfamily. 5-HT_{2CeL} and 5-HT_{2CeS} differ only in the length of the predicted N-terminal extracellular domain, which is 48 residues shorter in the latter species. Protein sequence analyses revealed that 5-HT_{2CeL}, but not 5-HT_{2CeS}, carries a consensus site for N-linked glycosylation (position 21) at its extracellular N-terminal domain. In addition, the two isoforms exhibit two consensus phosphorylation sites, one for Ca^{2+}/calmodulin-dependent protein kinase of type II (Thr^{170}) located on the second intracellular loop, and the other for protein kinase C (Ser^{491}), on the predicted intracellular C-terminal tail of the receptor.

BLAST analyses (Altschul et al., 1990) identified high homology to mammalian serotonin receptor sequences from other species, especially those belonging to the 5-HT_2 family. The dendogram in Fig.3 shows that 5-HT_{2Ce} clusters primarily with the two invertebrate 5-HT_2 receptors, *Drosophila* 5-HT_{2Dro} (Colas et al., 1995) and *Lymnaea* 5-HT_{2Lym} (Gerhardt et al., 1996), and the three subtypes of mammalian 5-HT_2 receptors, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} (Hoyer et al., 1994). In addition, 5-HT_{2Ce} is closely related to a putative 5-HT_2-like receptor sequence which was recently cloned from the parasitic nematode *Ascaris suum* (5-HTAsc; Genbank Accession # AF005486). By comparison,

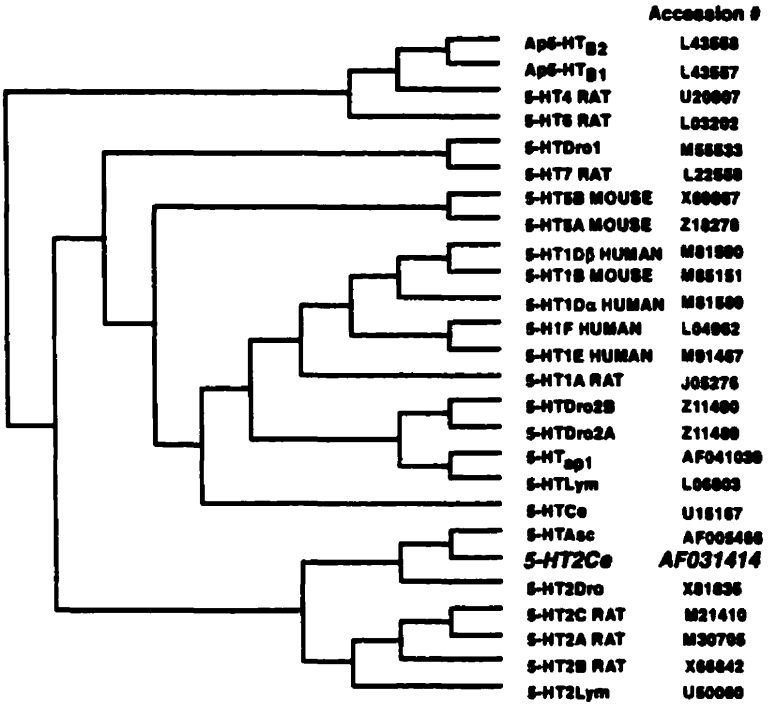

Fig.3. Dendrogram showing the structural relationship of 5-HT$_{2Ce}$ to other cloned G-protein coupled 5-HT receptors. The amino acid sequences of 5-HT$_{2CeL}$ and several serotonin receptors were compared and clustered using the program DNASIS vs. 3.7 (Hitachi Software, San Francisco, CA) . The lengths of the horizontal lines connecting one amino acid sequence to another are inversely proportional to the percentages of similarity between receptors or group of receptors. Receptor sequences used are from human, rat, mouse, *Drosophila* (Dro), *Lymnaea* (Lym), Aplysia (Ap or ap), *C. elegans* (Ce), and *Ascaris* (Asc). The corresponding Genbank accession # is indicated next to each sequence.

5-HT$_{2Ce}$ shows considerably less homology to other 5-HT receptor types, including the previously described 5-HT$_1$-like receptor from *C. elegans* (Olde and McCombie, 1997).

An amino acid sequence alignment of 5-HT$_{2Ce}$ with other members of the 5-HT$_2$ receptor family is shown in Fig. 4. The highest degree of sequence conservation occurs within the predicted transmembrane domains. Based on pairwise protein alignments, 5-HT$_{2Ce}$ shares highest transmembrane domain homology with *Ascaris* 5-HTAsc (81%), followed by *Drosophila* 5-HT$_{2Dro}$ (71%) (Colas et al., 1995), *Lymnaea* 5-HT$_{2Lym}$ (67%) (Gerhardt et al., 1996), rat 5-HT$_{2C}$ (66%) (Julius et al., 1988), rat 5-HT$_{2A}$ (65%) (Julius et al., 1990) and rat 5-HT$_{2B}$ (62%) (Foguet et al., 1992). Several of the amino acid residues that have been implicated in agonist and antagonist binding to mammalian 5-HT$_2$ receptors (Hibert et al., 1991; Choudhary et al., 1992; Wang et al., 1993; Choudhary et al., 1993; Choudhary et al., 1995; Roth et al., 1997) are conserved in 5-HT$_{2Ce}$. In addition, we identified several amino acid residues that distinguish all 5-HT$_2$ sequences, including

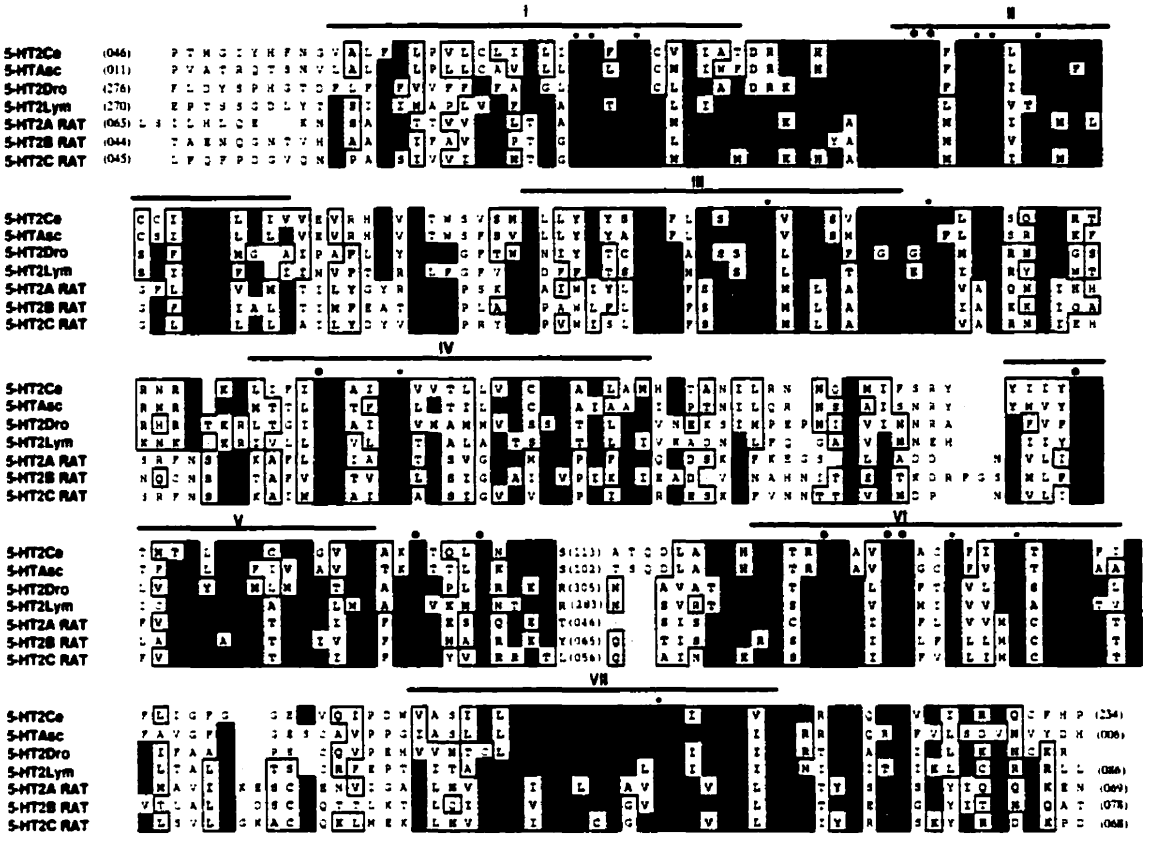

Fig.4. Amino acid sequence alignment. Amino acid alignment of the transmembrane domain regions of 5-HT_{2Ce} with members of the 5-HT_2 family, including rat 5-HT_{2A}, rat 5-HT_{2C}, rat 5-HT_{2B}, *Drosophila* 5-HT_{2Dro}, *Lymnaea* 5-HT_{2Lym} and a putative 5-HT_2 receptor sequence from *Ascaris*, 5-HT_{Asc} (refer to Fig. 3 for relevant GenBank accession numbers). Predicted TM I - VII regions are overlined. Numbers in parentheses correspond to the number of amino acids at the N- and C-termini and the third intracellular loop that are not represented in the alignment. Dark boxes and bold letters represent identical amino acids in at least four of the sequences, while grey boxes indicate conservative amino acid changes. Gaps, indicated by dashes, are included for optimum protein alignment. Amino acid residues that distinguish all 5-HT_2 receptors from other receptor types (see Table 1) are marked by solid circles, while those conserved in all GPCRs are indicated by an asterisk.

5-HT_{2Ce}, from other serotonin receptor types. A list of these characteristic residues is described in Table 1.

Ligand binding properties of 5-HT_{2CeL} and 5-HT_{2CeS} in COS-7 cells - To determine whether the two 5-HT_{2Ce} cDNAs encode functional proteins, constructs of the long and short variants of the receptor were transiently transfected into COS-7 cells for subsequent measurements of receptor binding activity. In preliminary separate experiments, the two expression constructs were modified to introduce a C-terminal FLAG epitope (Hopp et al., 1988), which was subsequently targeted with an anti-FLAG antibody for monitoring

Table 1: Distinctive 5-HT2 residues

Residue No.	Predicted region	5-HT2 receptors	Other 5-HT receptors
93-94	TM2	FL	YL, YF, FF
164	Intracellular loop 2	T,S,F,H,A	Y
175	TM4	K	M, I, L, S
190	TM4	I, V	P
218	TM5	G	S, A, Y, C
237	Intracellular loop 3	T	I, L
241	Intracellular loop 3	L	A
373	TM6	V	T, I, M
377-378	TM6	VF	IT, IL, IM, IV, LI, LL
419	TM7	S,C	N

Analysis based on Clustal alignment of a total of twenty-five 5-HT receptor amino acid sequences, including the seven 5-HT_2-like sequences described in Fig.4 and the following non-5-HT_2 sequences retrieved from Genbank (accession numbers are indicated in Fig. 3): rat 5-HT_7, rat 5-HT_6, mouse 5-HT_{5A}, mouse 5-HT_{5B}, rat 5-HT_4, human 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$, 5-HT_{1E} and 5-HT_{1F}, mouse 5-HT_{1B}, rat 5-HT_{1A}, *Lymnaea* 5-HT_{lym}, *Aplysia* Ap5-HT_{B1}, Ap5-HT_{B2}, and 5-HT_{ap1}, *C. elegans* 5-HT_{Ce}, *Drosophila* 5-HT_{Dro1}, 5-HT_{Dro2A} and 5-HT_{Dro2B}. All amino acid residue numbers refer to *C. elegans* 5-HT_{2CeL} receptor sequence.

expression of fusion 5-HT_{2Ce}-FLAG proteins in COS-7 cells. The results (data not shown) showed intense immunofluorescence mainly on the surface, suggesting that the 5-HT_{2Ce}-FLAG protein was being expressed at high levels and was targeted to the plasma membrane in COS-7 cells. The same results were obtained with the short 5-HT_{2CeS} species similarly fused to the FLAG epitope, whereas no immunofluorescence could be detected in a mock-transfected control.

Membranes of transiently transfected COS-7 cells were isolated and assayed for their ability to bind the serotonergic ligand, ^{125}I-LSD. Membranes containing 5-HT_{2CeL} displayed saturable specific ^{125}I-LSD binding in a concentration range of 0.18 to 5 nM (Fig.5). A computer-assisted Scatchard analysis of 5-HT_{2CeL} saturation isotherms identified a single class of binding sites with an apparent dissociation constant (K_D) of about 1.13 ± 0.18 nM and a binding capacity (B_{max}) of 8.52 ± 0.18 pmol / mg of protein. No specific ^{125}I-LSD binding was observed in untransfected cells or cells transfected with plasmid alone. To determine the pharmacological profile of 5-HT_{2CeL}, we examined the ability of several serotonergic and non-serotonergic ligands to compete for ^{125}I-LSD-labeled receptor (Fig. 6). The resulting K_i values (Table 2) revealed the following order of drug potency: lisuride > butaclamol > methiothepin > cyproheptadine > clozapine >

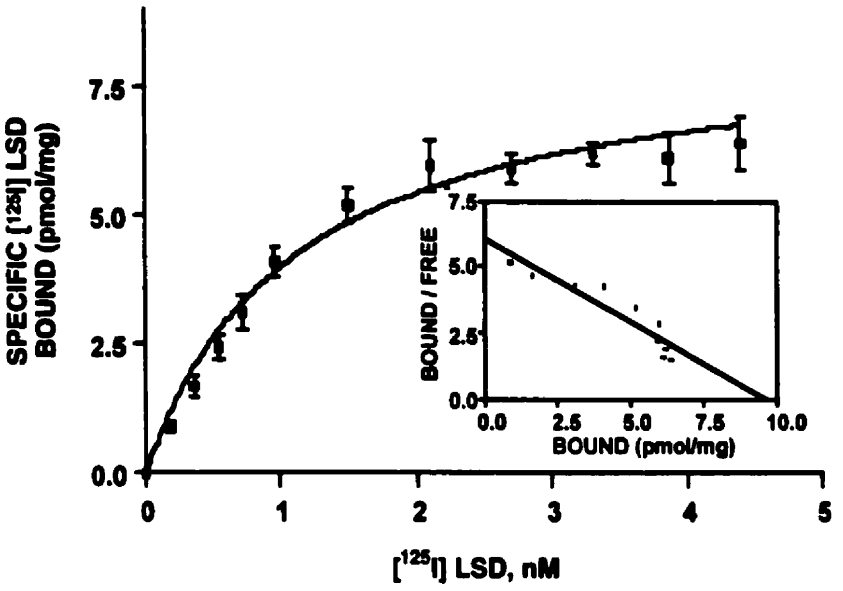

Fig. 5. Saturation and Scatchard analyses of ^{125}I-LSD binding to 5-HT$_{2CeL}$. Saturation curves were obtained by incubating cell membranes from 5-HT$_{2CeL}$-transfected COS-7 cells with increasing concentrations (0.18 - 5 nM) of [^{125}I]LSD, as described in the text. Specific [^{125}I]LSD binding was determined by subtracting total binding from nonspecific binding (measured in the presence of 10 μM cyproheptadine). The data are the means and standard errors of 3 separate experiments, each performed in duplicate. **Inset:** KD (1.13 ± 0.18 nM) and Bmax (8.52 ± 0.5 pmol/mg protein) values were determined by computer-assisted Scatchard transformation of the binding data.

metergoline > ketanserin > DOI > 5-HT. Other biogenic amines, including dopamine, octopamine, histamine and tryptamine were inactive at concentrations of 0.1 mM, as were the products of serotonin metabolism, N-acetyl-serotonin and melatonin, and the mammalian 5-HT$_{1A}$ agonist, 8-OH-DPAT. Similar experiments were repeated with 5-HT$_{2CeS}$ to identify possible differences in the pharmacological profile of the shorter receptor isoform. Competition curves were obtained for serotonin and two of the most effective competitors, methiothepin and cyproheptadine. All three ligands exhibited similar affinities for the short and long forms of the receptor (Table 2), the resulting competition curves being virtually identical (data not shown).

5-HT induces an increase in intracellular Ca^{2+} in 5-HT$_{2Ce}$ transfected COS-7 cells - Agonist activation of serotonergic 5-HT$_2$ receptors is known to cause an elevation in intracellular Ca^{2+}, which serves as a second messenger in 5-HT$_2$-mediated signaling (Hoyer et al., 1994). We used an aequorin-based assay (Boie et al., 1997) to investigate whether 5-HT$_{2Ce}$ mediates a similar $[Ca^{2+}]_i$ increase in COS-7 cells. Cells were transiently

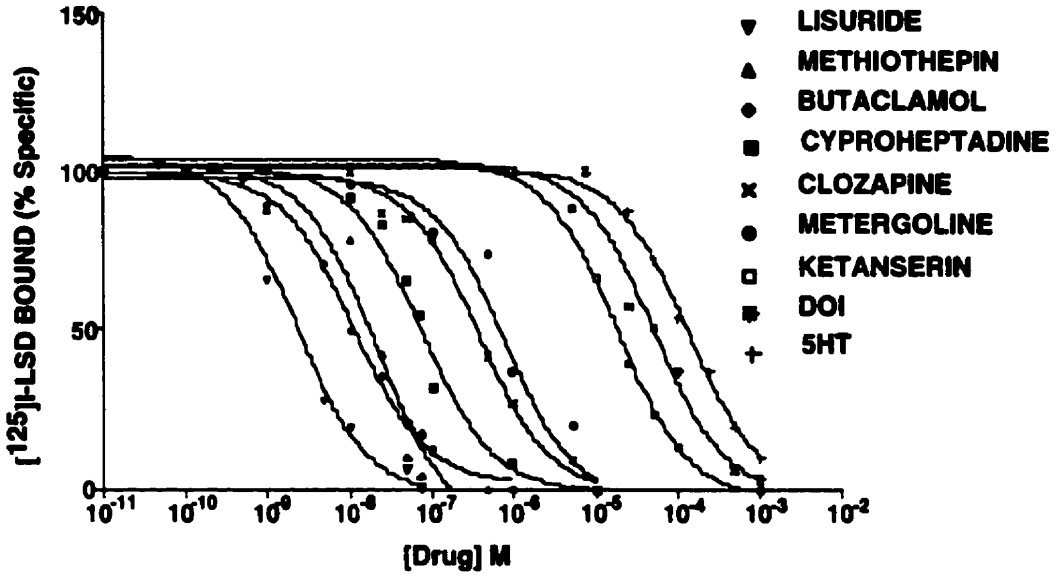

Fig. 6. Inhibition of specific [^{125}I]LSD binding to 5-HT$_{2CeL}$. Membranes of COS-7 cells transiently transfected with 5-HT$_{2CeL}$ were incubated with 1.5 nM [^{125}I]LSD in the presence and in the absence of different competitors. Nonspecific binding was measured in the presence of 10 μM cyproheptadine. The results are shown as a percentage of specific [^{125}I]LSD binding in the absence of a competing drug. The data are the means of 3 separate experiments each performed in duplicate. The corresponding K_i values are shown in Table 2.

co-transfected with an aequorin-expressing plasmid (pCDM.AEQ) and either a 5-HT$_{2Ce}$ expression construct (5-HT$_{2CeL}$ or 5-HT$_{2CeS}$) or vector alone. An increase in aqueorin luminescence, indicative of an elevation in intracellular calcium concentration, was seen after challenge of 5-HT$_{2Ce}$-transfected cells with serotonin or the serotonergic agonist, lisuride. In contrast, the mock-transfected control showed no visible response to either ligand (Fig. 7). Furthermore, no response was detected in 5-HT$_{2Ce}$-transfected cells treated with increasing concentrations (up to 0.1 mM) of dopamine, octopamine, histamine, melatonin and 8-OH-DPAT (data not shown). Serotonin and lisuride elicited identical responses in 5-HT$_{2CeL}$- and 5-HT$_{2CeS}$-transfected cells. The EC_{50} values (mean ± SEM) for serotonin were 0.72± 0.09 μM and 0.80± 0.08 μM for the long and short receptor variants, respectively. The corresponding EC50 values for lisuride were about 3-fold lower (0.20± 0.06 μM and 0.24 ± 0.13 μM for 5-HT$_{2CeL}$ and 5-HT$_{2CeS}$, respectively), but the maximum response was only about 25% of that obtained with serotonin (Fig. 7). Additional experiments with the classical 5-HT$_2$ agonist, DOI, failed to elicit a Ca^{2+}

Table 2. K_i values for ^{125}I-LSD binding to membranes of COS-7 cells transiently transfected with 5-HT_{2CeL} or 5-HT_{2Ces}

Drug	Ki (μM)	
	5-HT_{2CeL}	5-HT_{2CeS}
(+)-Lisuride	0.001	ND
(+)-Butaclamol	0.005	ND
Methiothepin	0.009	0.009
Cyproheptadine	0.032	0.035
Clozapine	0.159	ND
Metergoline	0.340	ND
Ketanserin	7.811	ND
(±)-DOI	21.90	ND
5-HT	57.26	62.28
(±)-8-OH-DPAT	> 100	> 100
N-acetyl-5-HT	> 100	> 100
Melatonin	> 100	> 100
Dopamine	> 100	> 100
(±)-Octopamine	> 100	> 100

K_i values were determined from IC_{50} values, according to the method of Cheng and Prussof (1973). The data are the means of 3 experiments, each in duplicate. Dopamine, octopamine, histamine, N-acetyl-5-HT, melatonin and 8-OH-DPAT had no effect on ^{125}I-LSD binding at a concentration of 100 μM. Standard deviations were < 20 % of the presented values. ND = not determined.

response except for a weak signal at very high concentrations (> 100 μM) of the drug (data not shown). Two classical 5-HT receptor antagonists, methiothepin and cyproheptadine, were tested for their ability to inhibit the serotonin-induced elevation in cellular Ca^{2+}. Each of the two antagonists was able to inhibit the response in the presence of a fixed concentration (10 μM) of serotonin both in 5-HT_{2CeL} - transfected cells (Fig. 7) and cells transfected with 5-HT_{2CeS} (data not shown). The IC_{50} values for methiothepin and cyproheptadine were similar for both the long and short splice variants of 5-HT_{2Ce} with values (mean ± SEM) of 3.62 ± 2.6 μM (long) vs. 2.04 ± 0.45 μM (short) and 5.93 ± 1.4 μM (long) vs. 3.07 ± 0.51 μM (short), respectively. It is noteworthy that methiothepin did not fully reverse the response to serotonin, even at high micromolar concentrations, whereas cyproheptadine caused almost complete inhibition.

5-HT_{2Ce} developmental expression in *C. elegans* - The expression of 5-HT_{2Ce} splice variants was examined by semi-quantitative RT-PCR in three different stages of *C. elegans* (embryos, L2 /L3 and adult). As can be seen in Fig. 8, each of the two 5-HT_{2Ce}

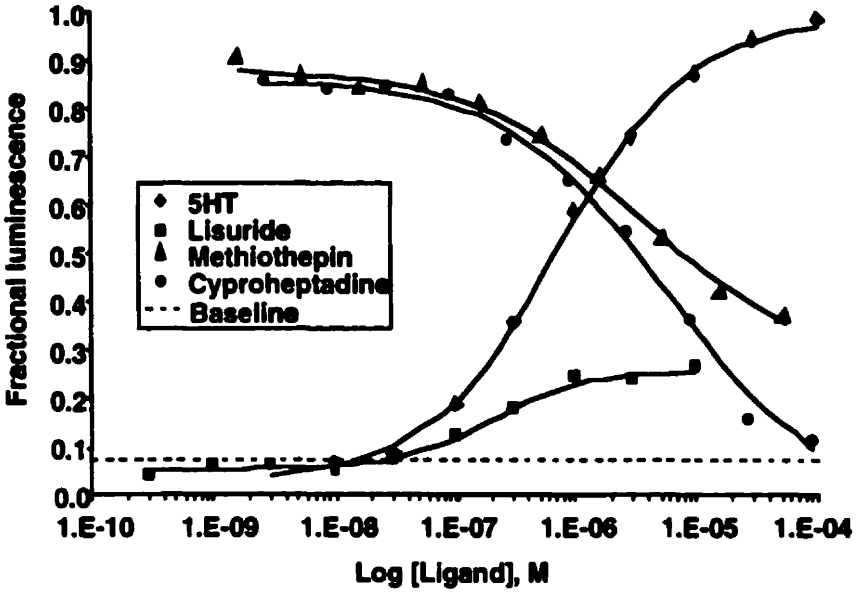

Fig. 7. Agonist and antagonist-induced changes in intracellular Ca^{2+} in COS-7 cells transiently transfected with 5-HT_{2CeL}. Cells transiently co-transfected with 5-HT_{2CeL} and an aequorin-expressing plasmid (pCDM.AEQ) were treated with increasing concentrations of agonists (serotonin and lisuride) and antagonists (cyproheptadine and methiothepin). Antagonism was assayed in the presence of 10 μM serotonin. Fractional luminescence was calculated as described in Experimental Procedures. The data are the means of 3 separate experiments each done in duplicate.

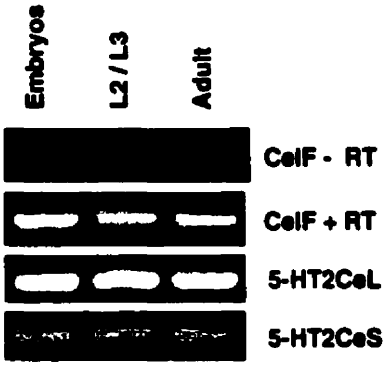

Fig. 8. Developmental expression of 5-HT_{2Ce} splice variants in *C. elegans*. Semi-quantitative RT-PCR was performed on total RNA from different stages of *C. elegans*, using selective PCR primers for the long and the short forms of 5-HT_{2Ce}, as described in the Methods. The reactions in the different stages were standardized by simultaneous amplification of an internal control sequence (CeIF). The two 5-HT_{2Ce} PCR products and the internal control are shown. A negative PCR control was performed using *C. elegans* CeIF primers on RNA samples that were subjected to a mock reverse transcription reaction (-RT). The results are representative of 2 independent RT-PCR experiments each done in triplicate.

splice variants was expressed at approximately the same level in all stages tested. However, the expression level of 5-HT_{2CeL} was consistently 4-fold higher than that of 5-HT_{2CeS} in all three stages. Expression levels were standardized by comparison with a constitutively expressed control gene (CeIF) (Roussell and Bennett, 1992; Krause, 1995). No CeIF PCR products were detected in a negative control that lacked reverse transcriptase thus confirming the absence of contaminating genomic DNA (Fig. 8).

DISCUSSION

This study describes the molecular cloning and functional expression of a novel *C. elegans* receptor (5-HT_{2Ce}) that shows sequence homology with 5-HT_2 receptors from other species. This is the most primitive 5-HT_2-like receptor ever cloned and characterized at the molecular level. With the exception of two receptors from higher invertebrates, *Drosophila* and the pond snail, *Lymnaea* (Colas et al., 1995; Gerhardt et al., 1996), all other 5-HT_2 receptors thus far characterized have been derived from mammalian tissues. A sequence analysis of the predicted protein structure of 5-HT_{2Ce} identified seven transmembrane domains, which is characteristic of all GPCRs, and also several residue patterns that are conserved in all serotonergic GPCRs (Probst et al., 1992). An alignment of 5-HT_{2Ce} with other available 5-HT_2 sequences revealed a surprisingly high level of sequence conservation (> 62%) particularly within the transmembrane regions. Within these highly conserved regions, we identified several residues, or residue patterns, that distinguish the entire 5-HT_2 group from all other receptor types (Table 1). Some of these distinctive 5-HT_2 residues represent significant changes from cognate amino acid acids in other classes of 5-HT receptors. For example, a conserved 5-HT_2 lysine in TM IV (5-HT_{2CeL} pos. 175) corresponds to a hydrophobic or small polar residue in other 5-HT receptors, whereas an invariant TM IV proline of all non-5-HT_2 receptors is modified to a hydrophobic isoleucine or valine (5-HT_{2CeL} pos.190) in the 5-HT_2 sequences. These two residue positions are predicted to face the same side of the TM IV helix, suggesting that this region may be important for 5-HT_2 structure and activity. Similarly, several other distinctive and potentially important 5-HT_2 residues were identified in TM V, VI and VII, as well as the third intracellular loop, a region of the receptor that has been implicated in G protein-coupling (Wess, 1997). The identification of these characteristic residues may be relevant for future mutagenesis studies of 5-HT_2 receptors.

Two variants of 5-HT_{2Ce} (5-HT_{2CeL} and 5-HT_{2CeS}) were identified, a long form of 683 amino acids and a shorter truncated form (635 amino acids) that lacks 48 N-terminal residues in its predicted extracellular domain, but is otherwise identical to the full-length species. A comparison of the two 5-HT_{2Ce} cDNAs with *C. elegans* genomic sequence

available from ACeDB revealed that the 5-HT_{2Ce} gene spans nearly 8.0 kb and is interrupted by five intron regions, including an unusually long 2.1 kb intron that separates the first two exons in the sequence. The presence of introns within the coding region has been reported for other 5-HT_2 receptors and distinguishes this subtype from 5-HT_1 receptors, which are typically intronless (Gerhardt and van Heerikhuizen, 1997). The analysis of genomic sequence also revealed that the variable region between the two species of 5-HT_{2Ce} corresponds to exon I, a stretch of 86 bp which is present in 5-HT_{2CeL} but not 5-HT_{2CeS}. Interestingly, our RACE analyses revealed that the two receptor variants were trans-spliced at their respective 5' ends to a conserved nematode spliced leader sequence (SL1) (Blumenthal and Steward, 1997). *Trans*-splicing of RNAs to an SL sequence is a well known phenomenon in lower invertebrates, including nematodes, where the SL is thought to play a role in the stability of the transcript and the binding to the ribosomal complex, as well as the processing of polycistronic transcripts (Blumenthal and Steward, 1997). The finding that both 5-HT_{2Ce} transcripts are *trans*-spliced to the SL1 suggests that the two variants have been properly processed and thus are not likely to be the product of an aberrant splicing event. The results also suggest that the two receptor variants are probably formed by differential *trans*-splicing of the SL sequence either to the 5' end of the 5-HT_{2Ce} pre-mRNA, leading to formation of the longer variant, or to an internal acceptor site, most likely the 5' end of exon II, which would generate the truncated 5-HT_{2CeS} species. Similar trans-splicing of SL sequences onto internal acceptor sites of immature transcripts has been reported for nematodes and other invertebrates (Davis, 1996; Blumenthal and Steward, 1997). Clarification of the precise nature of these splicing events awaits further investigation.

Ligand binding studies, using a radiolabeled form of the serotonergic ligand, LSD, confirmed that the two 5-HT_{2Ce} clones encode functional serotonin receptors. When transiently transfected into COS-7 cells, each receptor variant was targeted to the cell membrane and was shown to bind ^{125}I-LSD specifically and with high affinity. In addition, serotonin and other known serotonergic agents were able to fully inhibit ^{125}I-LSD binding whereas other related monoamines, such as dopamine and octopamine, which are also present in *C. elegans*, were inactive. These findings demonstrate specificity of 5-HT_{2Ce} for serotonin. Competition studies of the long receptor isoform

revealed some of the binding characteristics of mammalian 5-HT_2 receptors, including a low affinity for the endogenous ligand, serotonin, and comparatively higher affinities for the classic 5-HT_2 antagonists, ketanserin, metergoline and cyproheptadine (Hoyer et al., 1994). However, the present pharmacological characterization also unveiled several surprising properties of 5-HT_{2Ce}, which are inconsistent with those of either mammalian or other invertebrate 5-HT_2 receptors. Most notably, 5-HT_{2Ce} exhibited much higher affinity for ergolines, such as LSD and lisuride, and the nonspecific antagonist, methiothepin, than any of the 5-HT_2 ligands tested. Also surprising was the relative low affinity of 5-HT_{2Ce} for DOI, a selective high-affinity agonist of all known 5-HT_2 receptors, and the lack of discrimination between metergoline, a 5-HT_2 antagonist, and clozapine, a relatively nonspecific antagonist of 5-HT_6 / 5-HT_7 receptors (Hoyer et al., 1994). Taken together, the results indicate that 5-HT_{2Ce} is a novel pharmacological receptor type, which is structurally related to the 5-HT_2 family but has a distinctive drug binding profile.

The binding specificity of 5-HT_{2Ce}, including its apparent preference for ergot derivatives and the exceptionally low affinity for the natural ligand, serotonin, is reminiscent of invertebrate 5-HT_1-like receptors (Gerhardt and Heerikhuizen, 1997) in particular *Lymnaea* 5-HT_{Lym} (Sugamori et al., 1993) and the previously cloned *C. elegans* 5-HT_{Ce} receptor (Olde and McCombie, 1997). In the latter case, the receptor also showed preferential affinity for methiothepin and clozapine, as well as butaclamol, the overall order of drug potency being very similar to that reported here for 5-HT_{2Ce}. These observations suggest that 5-HT_{2Ce} has invertebrate 5-HT_1-like binding determinants, despite the overall lack of structural homology with the 5-HT_1 class. The results also raise the interesting possibility that this most primitive member of the 5-HT_2 family may have retained characteristics of an ancestral 5-HT_1/5-HT_2 prototype that existed before the separation of the two receptor families. Thus 5-HT_{2Ce} maybe be more closely related to an earlier form of 5-HT_2 receptor than any other known 5-HT_2 subtype.

We have shown that serotonin can activate 5-HT_{2Ce} expressed in COS-7 cells and that the activation leads to a rise in intracellular Ca^{2+}. These findings indicate that 5-HT_{2Ce} is able to couple to intracellular effectors in the COS-7 environment and probably acts through the same Ca^{2+}-mediated pathway of signal transduction as other 5-HT_2 receptors.

Although it is unknown if 5-HT_{2Ce} acts in the same manner *in vivo*, it is noteworthy that the key elements needed for 5-HT_2 signaling are present in *C. elegans*, including $G_{\alpha q}$ (Bargmann and Kaplan, 1998), to which 5-HT_2 receptors are known to couple, and a phophoinositide-specific phospholipase C (PLC) (Shibatohge et al., 1998). Additional pharmacological characterization of the 5-HT_{2Ce}-mediated Ca^{2+} response revealed that cyproheptadine and, to a lesser extent, methiothepin are effective antagonists, whereas lisuride has agonist activity and DOI is essentially inactive. The lack of response to the classic 5-HT_2 agonist, DOI, illustrates once again the unusual pharmacological characteristics of this 5-HT_2-like *C. elegans* receptor. It is noteworthy that the effect of lisuride was only about 25% of that obtained with serotonin. Thus, serotonin appears to be a much more efficacious agonist despite its relatively lower affinity for the receptor.

A parallel characterization of 5-HT_{2CeL} and 5-HT_{2CeS} has shown that the two splice variants have essentially identical ligand binding and signaling properties. Serotonin, in particular, displayed the same binding affinity and the same agonist activity at both forms of the receptor. This similarity is not surprising since the two receptor forms differ only in their N-terminal extracellular domain, a region which is not believed to be essential for functional activity of the receptor (Buck et al., 1991). It is noteworthy, however, that the N-terminal region contains a potential N-glycosylation site (pos. 21), which is lacking in the shorter species. Thus, there may be differences in the post-translational modification of the two *C. elegans* receptor isoforms, which have not been identified in this study, but may be of significance *in vivo*. To learn more about these two receptor species, we investigated their relative expression at the mRNA level in different developmental stages of *C. elegans*. The results revealed that the level of expression for the longer receptor variant is about 4-fold higher than that of the shorter species in all developmental stages tested, including embryos, L2/L3 larvae and adults. Additional research is needed to elucidate the biological role of 5-HT_{2Ce} and to determine if differential expression of the two receptor variants has functional significance in *C. elegans*.

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CONNECTING STATEMENT II

In manuscript II, we reported the characterization of a 5-HT receptor isolated from the free-living nematode *C. elegans*. This was the first study to clone and characterize a 5-HT_2-like receptor from a helminth. In manuscript III, we have attempted to characterize a similar receptor, but this time from the parasitic trematode *S. mansoni*. In this study, we reported the cloning and expression of a *S. mansoni* GPCR that has broad structural homology with biogenic amine neurotransmitter receptors, including 5-HT, catecholamines, and octopamine receptors.

CHAPTER IV (MANUSCRIPT III)

Molecular Cloning and Expression of a novel *Schistosoma mansoni* biogenic amine G Protein-Coupled Receptor

Fadi F. Hamdan and Paula Ribeiro

(***In preparation***)

ABSTRACT

This study reports the cloning of a full length *Schistosoma mansoni* cDNA (SmGPCRx) that encodes a protein (560 amino acids) with structural characteristics of a class I G protein-coupled receptor (GPCR). Amino acid sequence analyses indicate that SmGPCRx is related to the biogenic amine family of receptors, displaying similar homology with serotonin, catecholamine, and octopamine receptors (51 – 56 % within the transmembrane domains). However, SmGPCRx did not cluster with any of these receptors in a dendrogram analysis. Furthermore, the fact that SmGPCRx contains an asparagine instead of a highly conserved aspartate in transmembrane domain 3 ($Asp^{3.32}$ to $Asn^{3.32}$; Van Rhee and Jacobson, 1996), suggests that the *Schistosoma* receptor has unique structural characteristics and might be functionally different from previously characterized biogenic amine receptors. Initial expression studies failed to detect significant SmGPCRx production in transfected mammalian cells. Receptor protein expression was dramatically increased, however, when the same cells were transfected with a codon-optimized form of SmGPCRx. Codon optimization was designed to rewrite the first half of SmGPCRx according to mammalian preferred codons, which are translated more efficiently in the human HEK293 cell environment. Immunofluorescence studies demonstrated that SmGPCRx expressed in HEK293 cells is targeted to the plasma membrane. Furthermore, these studies showed that the receptor's amino-terminus is extracellular whereas the carboxyl-terminus faces the inside of the cell, consistent with the classical architecture of GPCR. Ongoing studies are attempting to characterize the identity and signaling properties of this receptor, and to investigate the role of the asparagine / aspartate change in transmembrane domain 3 in receptor expression and signal transduction.

INTRODUCTION

Biogenic monoamines such as serotonin (5-hydroxytryptamine; 5-HT) and catecholamines (dopamine and noradrenaline) are important neuromodulators in both vertebrates and invertebrates (Walker et al., 1996). Monoamines act, primarily, by binding to G protein-coupled receptors (GPCR), a large family of surface receptors that have a characteristic seven transmembrane topology and signal through activation of intracellular heterotrimeric G proteins (for a review see Bockaert and Pin, 1999). In the parasitic trematode *Schistosoma mansoni*, 5-HT is known to increase motility and carbohydrate metabolism of the parasite (Mansour, 1984; Pax et al., 1996). Less is known about the function of catecholamines in trematodes. The limited evidence suggests that catecholamines may play a role as neuromuscular transmitters and modulators of motor activity (Mellin et al., 1983; Pax et al., 1984). These amines are localized in various tissues of *S. mansoni*, including the central and peripheral nervous systems, holdfast and reproductive structures and, in the case of 5-HT, the body wall musculature and the gut as well (Pax and Bennett, 1991). Recently, work in our laboratory provided the first molecular evidence that *S. mansoni* possesses the key hydroxylating enzymes required for biosynthesis of 5-HT and catecholamines (Hamdan and Ribeiro, 1998, 1999).

At present, there are very few studies available on the properties of serotonin and catecholamine receptors in parasitic trematodes. Previous work showed that 5-HT stimulated adenylate cyclase in *S. mansoni* and in the related parasite *Fasciola hepatica* through activation of G protein coupled-receptors (McNall and Mansour, 1984; Mansour and Mansour, 1986; Estey and Mansour, 1987; Estey and Mansour, 1988; Iltzsch et al., 1992). Receptor binding studies on *F. hepatica* tissue extracts indicated the presence a high affinity serotonin binding site with a unique pharmacological profile (McNall and Mansour, 1984). In addition, electrophysiology studies on body wall muscle strips of *F. hepatica* provided evidence for a serotonin receptor whose pharmacology resembled that of mammalian 5-HT_1 class of serotonin receptors (Tembe et al., 1993). These findings suggest multiplicity of 5-HT receptors in *F. hepatica* and probably related trematodes, such as *S. mansoni*. No biochemical studies have yet been done on potential

catecholamine receptors in helminths. Other amine neurotransmitters that have been identified in some platyhelminths (flatworms) including histamine and octopamine (Eriksson et al., 1996; Ribeiro and Webb, 1983), have not yet been identified in *S. mansoni*.

To date, only three 5-HT receptors have been cloned and characterized from helminths, all of which from nematode (roundworm) systems (Olde and McCombie, 1997; Hamdan et al., 1999; Huang et al., 1999). In the present study we report the cloning of a novel *S. mansoni* GPCR (SmGPCRx), which is structurally related to the biogenic amine family of receptors but appears to constitute a new class of receptor. This is the first member of the GPCR superfamily ever cloned from a parasitic platyhelminth. We expressed SmGPCRx in mammalian cells and showed that it is targeted to the plasma membrane such that its N-terminus is extracellular while its C-terminus is intracellular, consistent with a GPCR structural organization. The functional characterization of SmGPCRx is currently ongoing.

EXPERIMENTAL PROCEDURES

S. mansoni - A puerto Rican strain of *S. mansoni* was maintained as described previously (Hamdan and Ribeiro, 1998). Total RNA was isolated from adult *S. mansoni* using the TRIzol reagent (Life Technologies Inc.) and passed through an oligo(dT)-cellulose column (Amersham-Pharmacia Biotech) to obtain purified poly(A^+)mRNA.

Cloning of the full length SmGPCRx – 0.5 - 1 μg of adult *S. mansoni* mRNA was reverse -transcribed with an oligo-$dT_{12\text{-}18}$ primer and 200 U of murine Moloney leukemia virus reverse transcriptase (Life Technologies, Inc). One tenth of the resulting cDNA was used in a PCR reaction (30 cycles: 30 s at 94°C, 30 s at 48°C, and 70 s at 72°C) containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 2 μM of each degenerate primer, and 2.5 U of Taq DNA polymerase (Life Technologies, Inc) in a total volume of 50 μl. The two degenerate PCR primers used in this amplification reaction were designed to target consensus sequences of transmembrane two (TM2) (VA/SVLVMP; sense: 5'-GTTKCIGTIYTKGTNATGCC-3') and TM7 (WLGYFNS; antisense: 5'-GARTTRAARTANCCNARCCA-3') that are well conserved among most biogenic amine GPCRs. An aliquot (1/10) of the PCR product was subjected to another 30 cycles of PCR (identical cycling parameters as above) using the same forward primer with a nested degenerate reverse primer targeting conserved sequences in TM6 (CWL/FPFF; 5'-RAARAANGGRARCCARGA-3'). The final PCR product was directly ligated into a T/A cloning vector (pCR2.1; Invitrogen) and used to transform competent DH5α *E. coli*. Plasmids were purified from a total of 65 different clones and the corresponding inserts were excised by ECoRI digestion and transferred onto a Hybond-N+ filters (Amersham-Pharmacia Biotech). The blots were hybridized, under low stringency, with a ^{32}P-labeled DNA probe (Random Priming; Roche Molecular Biochemicals) corresponding to the transmembrane domain regions of the rat 5-HT_{1A} receptor (Albert et al., 1990). The hybridization conditions were as described previously (Hamdan and Ribeiro, 1998). Four positive clones were obtained and DNA sequenced by the dideoxy chain termination method. One clone, SmGPCRx, displayed amino acid homology with various biogenic amine GPCRs and was selected for further analysis.

To isolate the missing 5'-end of SmGPCRx, we used a RT-PCR approach that targeted the conserved 36 nucleotides 5'-end spliced leader (SL) sequence of *S. mansoni* transcripts (Rajkovic et al., 1990; Davis, et al., 1995), as described previously (Hamdan and Ribeiro, 1998, 1999). The PCR primers (SmSL, A, B) used in these reactions are shown in Fig. 1. The resulting PCR product was cloned into pCR2.1 and DNA sequenced.

The remaining 3'-end of SmGPCRx was obtained by a standard rapid amplification of cDNA ends (RACE) using a 3'-RACE kit (Life Technology, Inc), according to the manufacturer's recommendations. Briefly, RT was performed as described above except that an oligo(dT)-adapter primer (supplied by the 3'-RACE kit) was used to prime the reaction. An aliquot of the resulting cDNA (0.2 µl) was used in a PCR reaction (25 cycles: 30 s at 94°C, 30 s at 53°C, and 90 s at 72°C) (see above for reaction composition) with a SmGPCRx gene-specific sense primer (primer C; see Fig. 1) and an antisense adapter primer (supplied with the 3'-RACE kit). One tenth of the resulting PCR product was subjected to a second round of PCR using a nested gene-specific sense primer (primer D; Fig. 1) and the same antisense adapter primer. The final 3'-RACE product was cloned into pCR2.1 and sequenced.

The complete coding sequence of SmGPCRx was then amplified by RT-PCR using primers that targeted the beginning and the end of the entire coding sequence (primers S and E; see Fig. 1) and a DNA proofreading polymerase (PWO; Roche Molecular Biochemicals), according to the manufacturer's procedure. To facilitate subcloning, *Nhe* I and *Not* I sites were added to the 5'-ends of the sense and antisense primers, respectively. The PCR product was digested with both restriction enzymes and ligated into the pCI-neo vector (Promega) which was previously linearized by the same restriction enzymes. Plasmids from five independent clones were purified and their inserts were DNA sequenced.

Codon-rewriting of SmGPCRx expression constructs – The first 855 bp of the SmGPCRx coding sequence (nucleotides 268 to 1122 of SmGPCRx cDNA; see Fig. 1) were synthesized by a one step recursive PCR reaction (Prodromou and Pearl, 1992). The reaction was designed to rewrite the *S. mansoni* cDNA using preferred mammalian

codons, as defined in the international DNA sequence data base (Nakamura et al., 1999). Briefly, we designed a total of 16 mutagenic oligonucleotides (average length ~ 74 mers) with overlapping regions of 19-21 bases, which spanned the first 855 bp of the SmGPCRx coding sequence. Two additional PCR primers (25 – 34 mers) were synthesized targeting the beginning and the end of the optimized N-terminal portion of SmGPCRx. The forward primer introduced a *Nhe* I site followed by a Kozak sequence (GCCACC) immediately preceding the start methionine (Kozak, 1999) and a 24 nucleotide sequence encoding a FLAG epitope (DYKDDDDK) (Hopp et al., 1988). The reverse primer was designed to introduce a flanking *Van* 911 site for subsequent ligation into an expression construct. Details of primer sequence are shown in Table 1. The PCR reaction (36 cycles) was performed using PWO DNA polymerase, 200 μM of each dNTP, 0.005, 0.01, 0.02, or 0.04 μM of each gene synthesis oligonucleotide and 0.8 μM of each PCR primer. The rest of the SmGPCRx coding sequence (1123 – 1950) was amplified by PCR with a proofreading polymerase using a sense primer that introduced a *Van* 911 site and overlapped with the 3'-end of the above codon-optimized sequence. The antisense primer incorporated a six histidine epitope tag followed by a stop codon and an adenosine (TGAA) to increase translational termination efficiency (McCaughan, et al., 1995), and a *Not* I restriction site. The resulting codon-optimized and wild type PCR products were gel purified and digested with *Nhe* I /*Van* 911 and *Van* 911 /*Not* I, respectively. Subsequently, the two fragments were fused together at the Van 911 site and then ligated into *Nhe* I /*Not* I sites of digested pCEP4 mammalian expression vector (Invitrogen). The resulting construct was confirmed by DNA sequencing.

Cell culture – Human embryonic kidney cells stably expressing the Epstein-Barr nuclear antigen 1, HEK293(EBNA1) (Invitrogen), were maintained in Dulbecco's modified Eagle medium (DMEM) containing 5 % FBS (fetal bovine serum), 1 mM sodium pyruvate, antibiotics (100 U of penicillin, 100 μg / ml streptomycin, and 2.5 μg / ml amphotericin B) and 250 μg/ml geneticin (G418). The cells were cultured at 37°C in a humidified atmosphere of 5 % CO_2/ 95 % air. All cell culture media, sera, and antibiotics were purchased from Life Technologies Inc.

Indirect immunofluorescence - HEK293(EBNA1) cells were seeded into six-well plates (2-3 x 10^5 cells / well) containing sterilized coverslips which were pre-coated with 0.001 % poly-L-lysine (Sigma). Approximately 18 -20 hrs later, the cells were transfected with 1μg of plasmid DNA using the Fugene6 reagent (3μl), according to the manufacturer's recommendations (Roche Molecular Biochemicals). About 48 hrs post-transfection, the cells were cooled on ice (5 min), washed twice with ice-cold PBS, and then fixed on ice for 10 min with 4% paraformaldehyde (Sigma). The cells were washed 3 times (5 min/wash at 4°C) with ice-cold PBS and then incubated for 75 min at 4°C with a monoclonal antibody directed against the FLAG epitope (anti-FLAGM2, Sigma; 5 μg/ml in PBS). The excess unbound antibody was washed off with cold PBS (4 washes, 5 min each) and the cells were incubated for 1 hr at 4°C with a FITC-conjugated goat anti-mouse IgG antibody (Sigma; 1:200 dilution in PBS). Subsequently, the cells were washed as above and coverslips were mounted (PBS / glycerol, 1:1 v/v) on glass slides and examined using fluorescence microscopy.

For experiments using a C-terminal anti-His antibody (Invitrogen), cells were subjected to paraformaldehyde fixation and washed as above, Fixed cells were either used directly or were further permeabilized by treatment with ice-cold methanol for 7 min at –20°C. After washing with PBS at room temperature, the cells were reacted with an anti-His antibody (1:200 in PBS) for 1 hr at 37°C in a humidified chamber. The excess unbound antibody was washed as above and the cells were incubated (1 hr, 37°C) with the FITC-conjugated anti-mouse IgG antibody. The coverslips were washed four times with PBS at room temperature and examined as above.

RESULTS AND DISCUSSION

Cloning of the full length SmGPCRx cDNA – RT-PCR with degenerate oligonucleotides targeting conserved TM sequences (TM2, TM6, and TM7) of aminergic receptors resulted in the amplification of a partial cDNA fragment, SmGPCRx, that cross-hybridized with a rat serotonin receptor 5-HT_{1A} probe (Albert et al., 1990). This cDNA fragment exhibited sequence homology with various biogenic amine receptors, such as serotonin, dopamine, octopamine, and noradrenaline receptors. The remaining 5'-end of SmGPCRx was isolated by RT-PCR in a reaction that targeted the conserved SL sequence of *S. mansoni* transcripts, as described previously (Hamdan and Ribeiro, 1998, 1999). The additional 3'-end sequence was then obtained by standard 3'-RACE (Frohman et al., 1988). The nucleotide and deduced amino acid sequence of SmGPCRx are shown in Fig. 1. The complete cDNA sequence (2002 bp) consists of 267 bp of 5'-untranslated sequence (5'-UTR) starting with the *S. mansoni* SL sequence (Rajkovic et al., 1990), followed by a single open reading frame of 1683 bp (nucleotides 268 to 1950) and a short 3'-UTR of about ~ 52 bp. The latter region included a consensus polyadenylation site (AATAAA) (Birnstiel et al., 1985) upstream of a poly(A) tail, suggesting that SmGPCRx is full length.

Protein sequence analyses - SmGPCRx encodes a predicted protein of 560 amino acids with a calculated molecular mass of ~ 65 kDa. Hydropathy analysis revealed that SmGPCRx is spanned by seven predicted transmembrane domains (TM) joined by three extracellular and three intracellular loops, a structural organization characteristic of G protein-coupled receptors belonging to the rhodopsin family (class I) (Bockaert and Pin, 1999) (Fig. 1). In addition, several functionally important residues that are highly conserved among GPCRs are also present in SmGPCRx, for example, the invariant proline residues of TM5, TM6, and TM7 (SmGPCRx positions 200, 494, and 529, respectively) which are important for proper GPCR function and assembly (Wess et al., 1993; Jakubik and Wess, 1999), and an aspartate in TM2 (pos. 76) that is necessary for agonist activation of biogenic amine receptors (Oliveira et al., 1994; Scheer et al., 1996; Van Rhee and Jacobson, 1996). The "DRY" motif at the intracellular end of TM3,

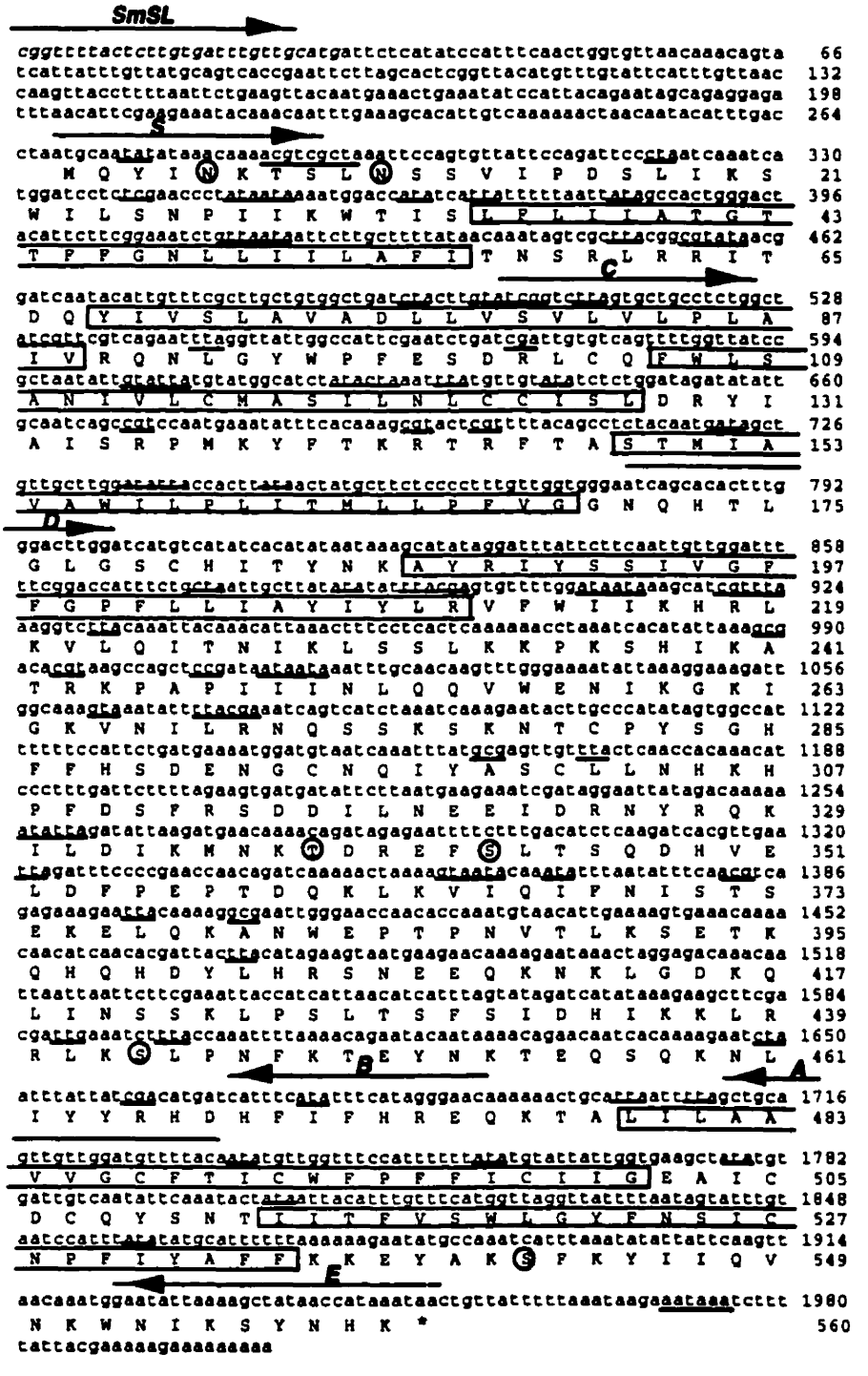

Fig. 1. The SmGPCRx complete cDNA and protein sequences. Transmembrane domains (boxed) were predicted according to the average results of three different algorithms (von Heijne, 1992; Pasquier et al., 1999; Ponting et al., 1999). Codons that are of low usage in mammals are underlined. PCR primers (SmSL, A, B, C, D, S, and E) used for cloning and RACE procedures are marked by a solid arrow. The *S. mansoni* SL sequence is italicized. Consensus *N*-glycosylation (N) and phosphorylation sites (S, T) are encircled. The putative polyadenylation site is underlined and the stop codon is represented by an asterisk.

which is characteristic of most GPCRs, has been shown to be important for G protein-coupling (Moro et al., 1993; Scheer et al., 1996; Van Rhee and Jacobson, 1996). Also present is the "NPX_2Y" motif of TM7, which has been implicated in receptor activation and signaling specificity (Mitchell et al., 1998), as well as agonist-mediated receptor sequestration (Barak et al., 1994). Two cysteines are present in the first and the second extracellular loops (positions 104 and 180). The cognate residues in other GPCRs are linked by a disulfide bond and are necessary for proper cell surface localization of the receptor (Zeng et al., 1999a,b).

Protein sequence analysis revealed the presence of two potential *N*-linked glycosylation sites in the amino-terminal region of SmGPCRx (Asn^5 and Asn^{10}) (Fig.1). Such residues are common at the N-terminus of GPCRs and are thought to be involved in targeting the receptor to the cell surface (Ray et al., 1998; Karpa et al., 1999). In addition, there are several consensus phosphorylation sites present in SmGPCRx, including potential sites for protein kinase C (Thr^{338}, Ser^{542}) and calcium-calmodulin dependent protein kinase type 2 (Ser^{343} and Ser^{443}) (Fig.1). Phosphorylation is known to modulate receptor coupling, G-protein selectivity, and desensitization (for reviews see, Wess, 1998; Bunemann et al., 1999).

Position-Specific Iterated BLAST (PSI-BLAST) (Altschul et al., 1997) amino acid sequence analysis of SmGPCRx indicated that it was related to biogenic amine receptors and muscarininc acetylcholine receptors. Additional CLUSTAL (Thompson et al., 1994) pairwise comparisons of the conserved TM regions revealed moderate sequence homology with serotonin receptors (56 %), octopamine receptors (54 %), β-adrenergic receptors (54 %), dopamine receptors (51%), and muscarinic acetylcholine receptors (51 %) (Fig. 2). However, SmGPCRx did not cluster exclusively with any of the above receptor groups (Fig. 3). Interestingly, SmGPCRx contains an asparagine residue (Asn^{111}) instead of a highly conserved aspartate in TM3 (Fig. 2). The latter is thought to be involved in the binding of the protonated amine group of aminergic ligands (Hibert et al., 1991; Oliveira et al., 1994). Earlier mutagenesis studies showed that replacement of this conserved aspartate with an asparagine in various biogenic amine receptors resulted in decreased agonist and antagonist affinities (Van Rhee and

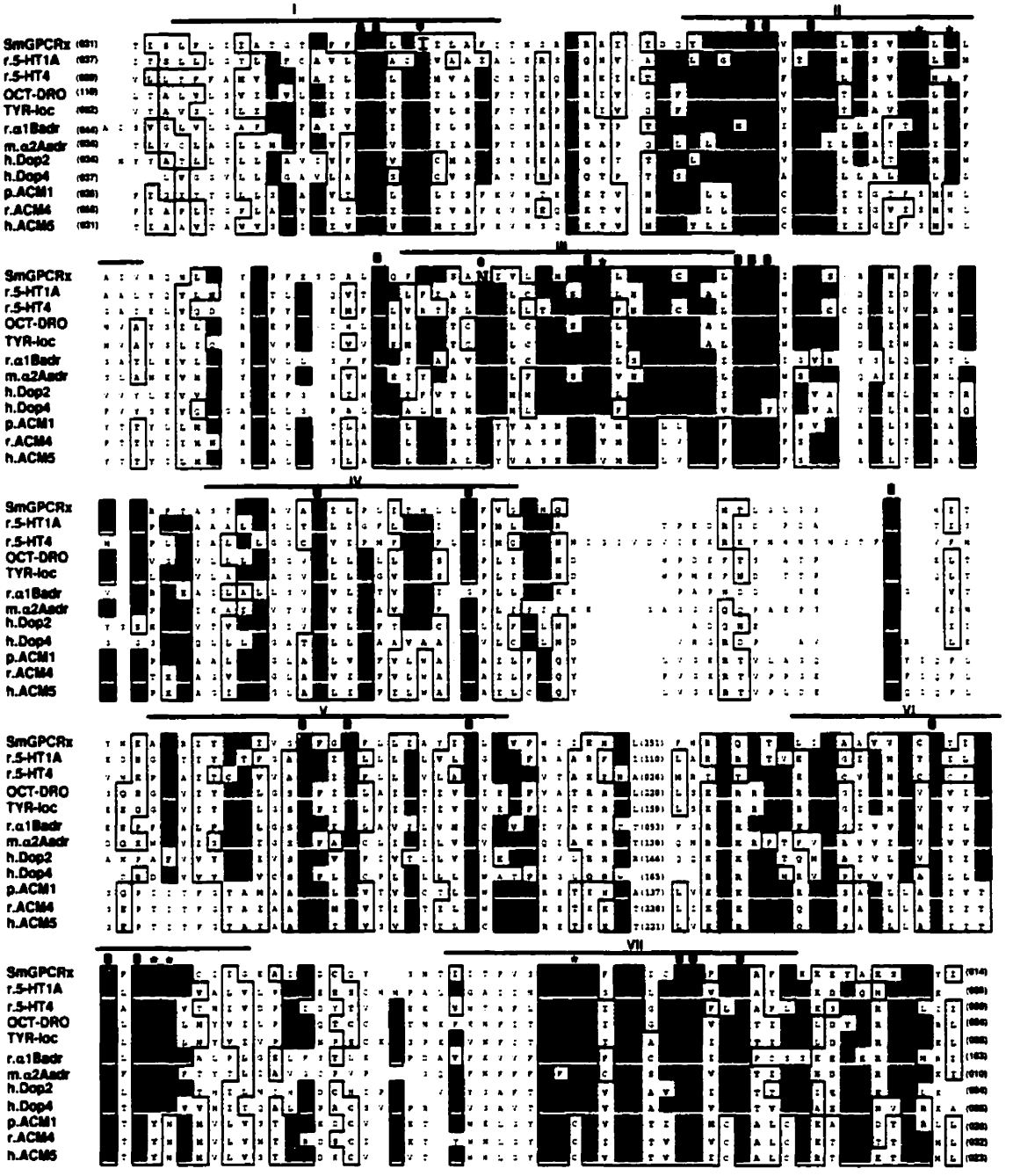

Fig. 2. Amino acid sequence alignment of the TM regions of SmGPCRx and representative sequences of related biogenic amine and muscarinic acetylcholine receptors. Amino acid sequences (see Fig. 3 for abbreviations and Genbank accession numbers) were aligned using the MacVector software (vs. 6.5: Oxford Molecular), according to the CLUSTAL method. Predicted seven TM regions are overlined. Numbers in parentheses respresent the number of amino acids (N- and C-terminal, and in the third intracellular loop) that are excluded in the alignment. Amino acids that are conserved in most class I GPCR are marked by a solid square. Residues that differentiate aminergic receptors from muscarinic receptors are marked by an asterisk. Significant nonconservative substitutions in SmGPCRx are indicated by a solid circle.

Jacobson, 1996). In addition, SmGPCRx has an isoleucine in TM1 (position 51) instead of a highly conserved valine present in aminergic and muscarinic acetylcholine receptors (Fig. 2). The functional importance of this residue in TM1 has not yet been investigated.

Several amino acids that are important for endogenous ligand binding and are thought to distinguish biogenic amine receptors from muscarinic acetylcholine receptors are found in SmGPCRx (Fig. 2) . These include a valine and a proline in TM2, an isoleucine in TM3, two phenylalanines in TM6, and a glycine in TM7 (Kuipers et al., 1997). Since all these functionally important residues are different in muscarinic receptors, it seems likely that SmGPCRx encodes a biogenic amine receptor rather than a muscarinic acetylcholine receptor. Functional assays are needed to confirm the identity of SmGPCRx.

Optimization of SmGPCRx constructs for mammalian expression – Preliminary attempts to express a FLAG-tagged SmGPCRx in mammalian HEK293(EBNA1) were unsuccessful, as determined by immunological techniques (immunofluorescence and western blotting) that targeted the FLAG epitope (Fig. 5A, Wild-type). Similar results were obtained when SmGPCRx (FLAG at C- or N-terminus) was tested for expression in COS-7 cells (data not shown). Since the *S. mansoni* codon usage, which is A/T rich, is known to be different from that of mammals (Nakamura et al., 1999), we re-examined the codon preference of SmGPCRx and compared it to that of mammals. As shown in Fig. 1, a large number of amino acids, especially leucines (T/CTA), isoleucines (ATA), and arginines (CGA), are encoded by codons that are rarely represented in mammals (< 7 %). The majority of these codons (~70 %) are located within the first 850 nucleotides and frequently occur in tandem, consisting of stretches of 2 –3 low-usage codons. Rare codon usage has been associated with poor translational efficiency of overexpressed proteins due to possible movement impairment (jamming) of the translating ribosome, especially if these codons occur in clusters (Kane, 1995). Many researchers have reported this problem when expressing mammalian genes in *E. coli*, which has a similarly distinctive codon usage. In some of these cases, re-writing the cDNA using optimal *E. coli* codons was enough to achieve detectable protein expression (Hernan et al., 1992). Codon

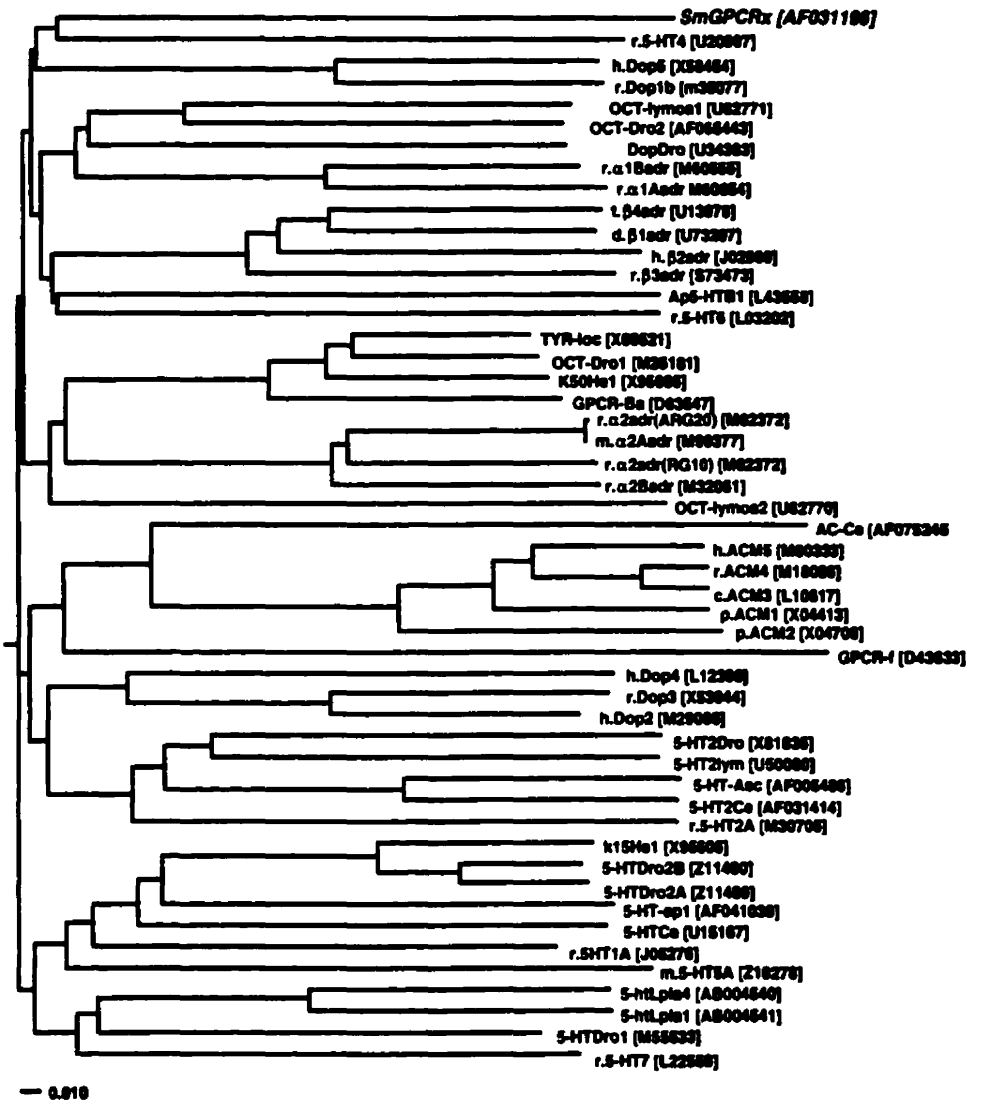

Fig. 3. Dendrogram displaying the structural relationship of SmGPCRx to other members of the GPCR superfamily. Amino acid sequence alignment was performed with the MacVector software (vs. 6.5; Oxford Molecular) and according to the CLUSTALW algorithm. Only the hydrophobic core (TM I to TM V and TM VI to TMVII) of each receptor was included in this analysis. The lengths of the horizontal lines are inversely proportional to the sequence homology between two sequences or between groups of sequences. A reference scale is shown at the bottom of the dendrogram. A value of 0.1 approximately corresponds to a difference of 10 % between two sequences. Receptor sequences that are included here are from human (h), pig (p), chicken (c), rat (r), mouse (m), fish (f), *Drosophila* (Dro), moth (*Heliothis virescens*, He), locust (*Locusta migratoria*, Loc) barnacle (*Balanus amphitrite*, Ba), *Lymnaea* (lym), *Aplysia* (Ap or ap), *C. elegans* (Ce), *Ascaris suum* (Asc), *S. mansoni* (Sm), and planaria (pla). Other abbreviations include: AC or ACM, muscarinic acetylcholine receptor; Dop, dopamine receptor; adr, adrenergic receptor; OCT, octopamine receptor; TYR, tyramine receptor.The corresponding Genbank accession number of each sequence is indicated.

Table 1. Oligonucleotides used in constructing the codon-optimized SmGPCRx construct.

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

Overlapping oligonucleotides (5'-3') spanning a region equivalent to nucleotides 268 to 1121 (~ first half of SmGPCRx coding sequence) of SmGPCRx cDNA (see Fig.1) are numbered 1 to 16 with corresponding length indicated in parentheses. Forward and reverse oligos are indicated by "F" and "R", respectively. PCRF and PCRR are the external forward and reverse PCR primers used to amplify the recursive PCR product. CF and CR are the forward and reverse primers used to amplify the wild type second-half of the SmGPCRx coding sequence. The underlined ATG in 1F and PCRF is the initiation methionine. Underlined TCA in CR represents the reverse complementary sequence of the stop codon, TGA. Nucleotides shown in bold in 1F, PCRF, and CR encode a FLAG epitope, a Kozak sequence, and a six histidine epitope, respectively. Italicized nucleotides in 16R, PCRF, CF, and CR represent *Nhe* I, *Van* 911, *Van* 911, and *Not*I restriction sites, respectively.

optimization also improved the expression of certain invertebrate proteins, such as green fluorescent protein (Yang et al., 1996; Zolotukhin et al., 1996), in mammalian cells.

Therefore, to increase expression of SmGPCRx in mammalian cells we used a recursive PCR approach (Prodromou and Pearl, 1992) to synthesize a new codon-optimized SmGPCRx expression construct, which would be suitable for expression in

mammalian cells. The DNA sequence encoding the first 284 amino acids of SmGPCRx were synthesized, in a single reaction, using 16 overlapping oligonucleotides. The latter were designed to replace *S. mansoni* codons with those most frequently used by humans. The mutagenic PCR reaction was optimized with respect to cycling parameters and primer concentration. We found that the success of the recursive PCR reaction was dependent mainly on the ratio of the external PCR primers (PCRF, PCRR, Table 1) relative to the concentration of internal mutagenic oligonucleotides (primers 1-16, Table 1). A ratio of 0.8 μM of each external primer to 0.02 μM of each mutagenic primer gave the highest specificity and yield (data not shown). In addition, the sequence of each oligonucleotide was carefully designed and checked to avoid stable hairpin formation and the introduction of cryptic splice sites (GTA/TCAG) that may produce truncated PCR products. The resulting codon-optimized first SmGPCRx half was ligated, at a *Van* 911 site, to a cDNA fragment corresponding to the rest of the wild type SmGPCRx sequence. The composite cDNA (chimeric) was then ligated into the mammalian expression vector pCEP4 (Invitrogen) for high level expression in HEK293(EBNA1) cells (Invitrogen) (Fig. 4).

Receptor expression and localization – The optimized SmGPCRx construct included an N-terminal FLAG epitope (Hopp et al. 1988) and a C-terminal six histidine tag to facilitate subsequent immunological detection (Fig. 4). Both epitopes have been previously engineered at the termini of many GPCRs without affecting receptor function (Kobilka, 1995; Robeva et al., 1996; Hamdan et al., 1999; Hoffmann et al., 1999; Xiao et al., 1999). Immunofluorescence studies were used to examine the expression and cellular localization of SmGPCRx in transfected HEK293(EBNA1). Experiments done using the anti-FLAGM2 antibody on unpermeabilized cells showed that SmGPCRx was expressed on the cell surface. This indicates that the receptor is being targeted to the plasma membrane and that the N-terminus, which carries the FLAG epitope, is extracellular. In this case, receptor expression was detected only in cells that were transfected with the codon-optimized construct but not with the initial wild type expression construct or with the vector control (Fig. 5A). This clearly indicated that the codon rewriting strategy was successful in increasing receptor expression to detectable levels. This is the first study to

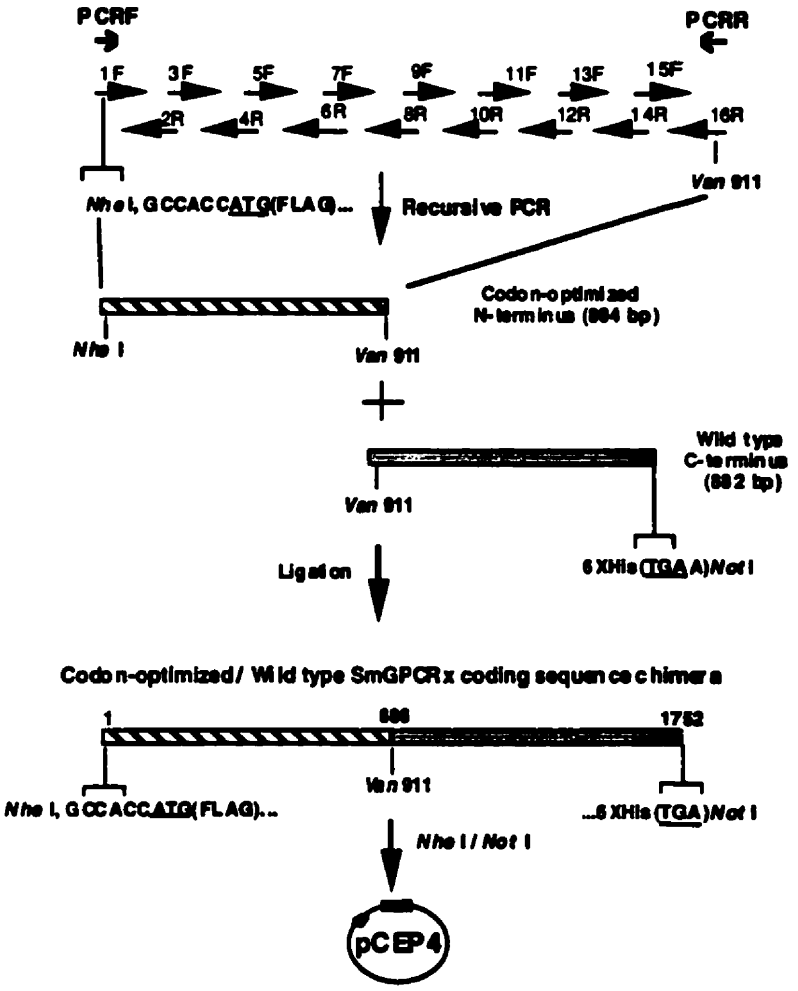

Fig. 4. Construction of the codon-optimized SmGPCRx construct. Nucleotide sequence (positions 268 to 1122 of SmGPCRx cDNA, see Fig. 1) representing approximately the first half of the SmGPCRx coding sequence was synthesized by recursive PCR, using preferred mammalian codons (hatched box). A total of 16 overlapping oligonucleotides and two shorter PCR primers (PCRF and PCRR) used in this reaction are represented by arrows pointing from the 5' to the 3' direction. Underlined ATG or TGA correspond to the initiation methionine and stop codon, respectively. Restriction sites (*Nhe* I, *Van* 911, *Not* I), the Kozak sequence (GCCACC), and the FLAG and histidine epitopes are shown. The codon optimized N-terminal half was ligated to the rest of SmGPCRx coding sequence (nucleotides 1122 to 1967 of SmGPCRx cDNA), at a *Van* 911 site. The resulting chimeric cDNA was then ligated into the pCEP4 vector at the *Nhe* I / *Not* I sites.

describe a codon-optimization approach to circumvent translational problems associated with expression of an invertebrate GPCR in mammalian cells. In addition, we show that recursive PCR can be utilized to synthesize genes or portions of genes as long as 900 bp or even more. This method is potentially useful for overexpression of cDNAs from

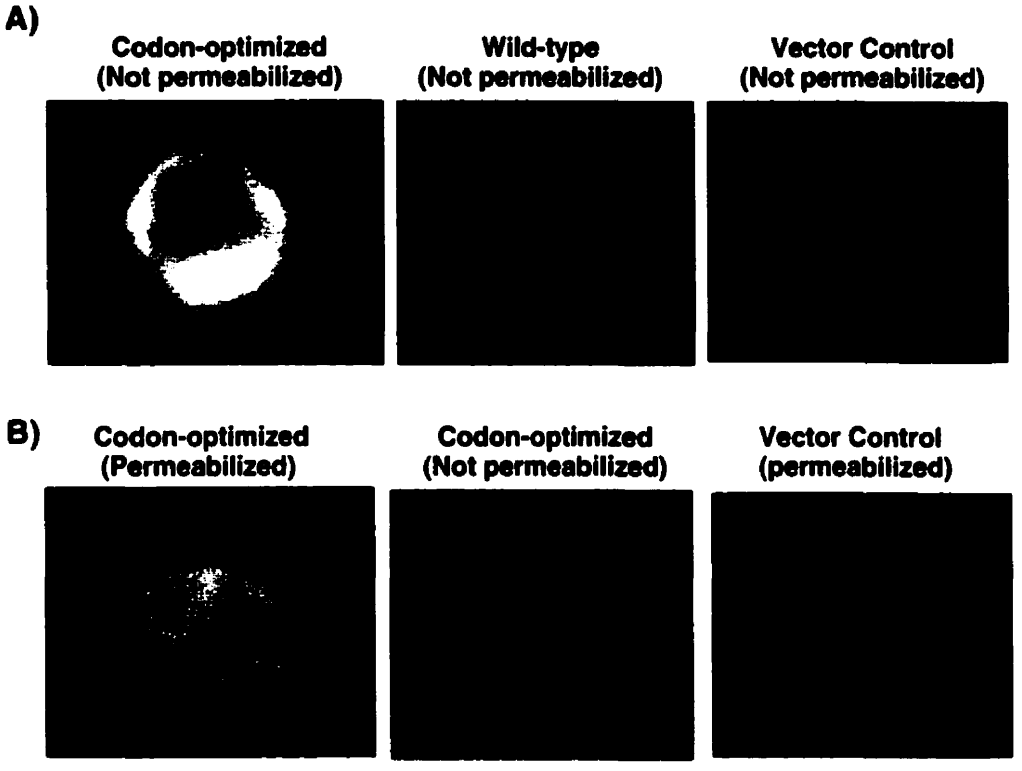

Fig. 5 . Cellular localization of SmGPCRx by immunofluorescence. HEK293(EBNA1) were transfected with the pCEP4 vector alone (vector control) or with the SmGPCRx constructs, either codon-optimized or wild-type. Approximately 48 hrs post-transfection, the cells were fixed with paraformaldehyde (PFA) and subjected to indirect immunofluorescence analysis using either the anti-FLAGM2 (A) or the anti-His (B) and secondary FITC-conjugated antibodies. For analyses of anti-His immunofluorescence, cells were fixed with PFA and either used directly (Not permeabilized) or were permeabilized with methanol prior to incubation with the primary antibody. Transfection efficiency was approximately 30 – 40 %.

other parasites, in particular protozoans which also have very distinctive codon preference.

Immunofluorescence experiments done with the anti-His (C-terminal) antibody revealed detectable expression only in permeabilized cells that were transfected with the codon-optimized construct (Fig. 5B). No anti-His signal was seen in unpermeabilized cells transfected with the same construct or in permeabilized cells transfected with the vector control only (Fig. 5B). This suggested that the C-terminus, which includes the six histidine epitope, is intracellular. Taken together, the results show that the codon-optimized SmGPCRx can be expressed to detectable levels in HEK293(EBNA1) cells, where it seems to be targeted to the cell surface, such that its N-terminus is extracellular while its C-terminus is intracellular, consistent with a classical GPCR architecture.

Studies already in progress are attempting to characterize the functional properties and ligand specificities of SmGPCRx.

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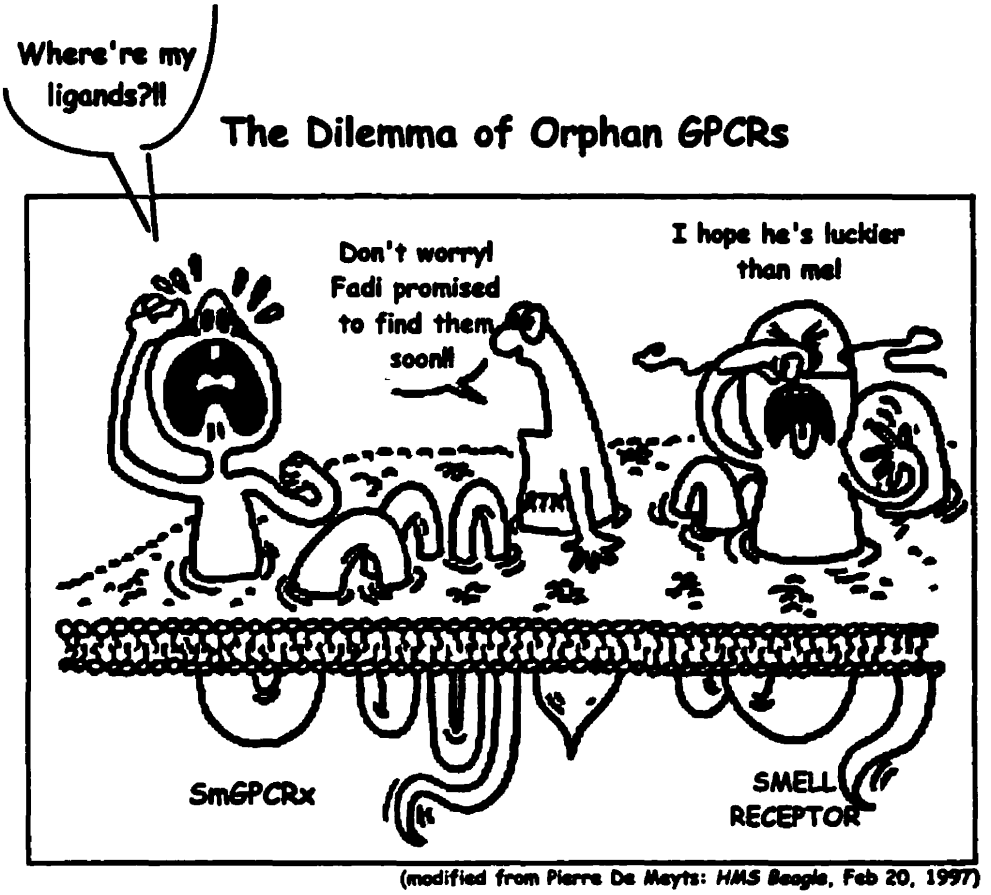
The Dilemma of Orphan GPCRs



(modified from Pierre De Meyts: *HMS Beagle*, Feb 20, 1997)

CHAPTER V

SUMMARY AND CONCLUSIONS

Work presented in this thesis addressed two main questions that are crucial for the understanding of how serotonin functions in helminths. The first question is whether helminths, particularly parasitic ones, are capable of synthesizing their own serotonin. The second question is how serotonin mediates its functions in helminths, specifically what are the molecular properties of its receptors. Our findings support the notion that serotonin in helminths is synthesized endogenously and acts, at least in part, by binding to multiple GPCRs.

The biosynthesis of 5-HT in helminths has been a source of some debate. Two schools of thought have emerged over the last two decades. The first included a group of researchers who were unable to detect tissue TPH activity in several parasitic helminths and, hence, believed that these organisms lacked the capacity for 5-HT biosynthesis. The same researchers proposed that helminths have to rely entirely on the host as a source of serotonin (reviewed in Davis and Stretton, 1995). This notion was supported by other studies which demonstrated active and passive tegumental transport of 5-HT by various parasitic helminths, including *S. mansoni* (Bennett and Bueding, 1973; Catto and ottesen, 1979; Wood and Mansour, 1986), the cestodes, *Mesocestoides corti* and *H. diminuta* (Hariri, 1975; Cyr et al., 1983), and the nematode *A. suum* (Chaudhuri et al., 1988b). The second school of thought challenged this idea and provided biochemical evidence for TPH activity in tissue extracts of *H. diminuta* and *A. suum* (Ribeiro et al., 1983,1984; Chaudhuri et al., 1988a). Aside from conflicting results on the question of TPH activity, there was general consensus that parasitic helminths possess the other biosynthetic enzyme, AAADC, which is required for the decarboxylation of the intermediate, 5-HTP, to serotonin (see Fig. 1, page 13) (Davis and Stretton, 1995).

In manuscript I, we reported the cDNA cloning, purification, and functional characterization of TPH (SmTPH) from *S. mansoni*. This study provided the first molecular evidence that *S. mansoni*, and possibly other parasitic helminths, can synthesize serotonin. We were also able to detect for the first time, measurable TPH activity in crude tissue extracts of *S. mansoni*, indicating that the native TPH enzyme in *S. mansoni* is active. Interestingly, SmTPH mRNA levels were approximately 2.5 fold higher in the free-living cercarial larval stage than in the parasitic adult stage. This

result was rather surprising, especially since the serotonergic nervous system is much more developed in the adult worm. This finding raises the possibility that the adult form of the parasite may utilize two sources of 5-HT, one endogenous and the other from the host. In contrast, the free-living cercaria has to rely solely on endogenous 5-HT synthesis, and therefore, may possess higher levels of TPH transcripts. Utilization of dual sources of 5-HT has been suggested previously for the nematode *A. suum* (Chaudhuri, et al., 1988b).

In addition to providing evidence of TPH function in *S. mansoni*, work in manuscript I contributed greatly to our understanding of TPH structure and biochemistry. This is the first TPH gene ever cloned from an invertebrate. Previously, a TPH-like gene cloned from *Drosophila* was shown to have TPH / PAH activities, and therefore, was described as a TPH / PAH hybrid gene (Neckameyer and White, 1992). This finding led to speculation that TPH and PAH might have diverged sometime after the evolution of insects (Neckameyer and White, 1992). However, the present cloning of *S. mansoni* TPH contradicts this hypothesis and suggests that the separation of TPH from PAH probably happened before the divergence of flatworms.

Structurally, SmTPH has a similar organization to mammalian TPH, consisting of three main domains, N-terminal, catalytic, and C-terminal. The functional analysis also found overall similar kinetic and regulatory properties between the two forms of the enzyme, except for stability of enzymatic activity. When we expressed SmTPH in *E. coli* and compared its enzymatic stability to that of a similarly expressed mammalian rabbit TPH, SmTPH was found to be much more stable with a half-life nearly 23 fold higher than that of mammalian TPH. We have speculated that this increased stability may be due to the unique structure of the N-terminus of SmTPH, whose primary sequence is very different from that of mammalian TPH. This would suggest a role for the N-terminus of TPH in enzymatic stability. Indeed, our findings support an earlier study which showed that replacing the N-terminal domain of rabbit TPH, a notoriously unstable hydroxylase, with that of TH, a relatively more stable enzyme, increased the enzymatic stability of the TPH hybrid. Conversely, substituting the N-terminal domain of TH for that of rabbit TPH significantly decreased the activity of the TH mutant (Mockus et al., 1997). In addition to being stable, SmTPH produced in *E. coli* was

soluble, highly active and could be stored in a purified form at –80°C for up to one month without significant loss of activity. This increased solubility, stability, and specific activity of SmTPH compared to mammalian forms of the enzyme makes SmTPH a potentially useful model for general studies of TPH structure / function.

In addition to TPH, we have cloned a closely related *S. mansoni* hydroxylase, TH, which is required for the biosynthesis of catecholamine neurotransmitters. As with SmTPH, SmTH was overexpressed in *E. coli*, purified and subjected to extensive characterization (Hamdan and Ribeiro, 1998). This represents the first molecular evidence for a catecholamine-biosynthesizing enzyme in any helminth and supports the notion that catecholamines are important neuroactive agents in these animals. Details of this work are described in Appendix I.

Manuscript II described the cloning and functional expression of a novel G protein coupled 5-HT receptor (5-HT_{2Ce}) from *C. elegans* with structural and signaling properties similar to those of 5-HT_2 receptors isolated from other species. This makes 5-HT_{2Ce} the most primitive 5-HT_2-like invertebrate receptor ever characterized at the molecular level. Two splice variants of 5-HT_{2Ce} were isolated and found to differ by a 48 N-terminal amino acids which were lacking from the shorter splice variant (Fig. 1A). Both 5-HT_{2Ce} isoforms had similar functional activities (receptor binding and signaling), although the longer form of the receptor displayed approximately four fold higher expression in the various developmental stages investigated, including eggs, L_2/L_3 larvae, and adults). Structurally, 5-HT_{2Ce} is closely related to the 5-HT_2 family of serotonin receptors and displays highest amino acid sequence homology with a recently cloned *Ascaris* 5-HT receptor (5-HT_{Asc}) (Huang et al., 1999). 5-HT-induced stimulation of COS7 cells transfected with 5-HT_{2Ce} caused an increase in intracellular Ca^{++} levels (EC_{50} ~ 0.7 μM) which was completely antagonized by the serotonergic antagonists, cyproheptadine and methiothepin. This indicated that 5-HT_{2Ce} shares similar signaling properties with other 5-HT_2 receptors, all of which couple to $G_{\alpha q}$ and activate PLC_β, leading to IP_3 production and the release of Ca^{++} from intracellular stores. This exact same signaling pathway was confirmed in a subsequent study of 5-HT_{2Ce} stably expressed in HEK293(EBNA1) cells. Stimulation of these cells by 5-HT caused a 4-5 fold increase in IP_3 levels (EC_{50} ~ 0.8 μM) (Abramovitz, M., personal communication).

This confirms that the stimulation effect of 5-HT on cellular Ca^{++} reported in manuscript II was due to the increased production of IP_3 and not secondary to the activation of surface Ca^{++} channels. This signaling pathway is consistent with the activation of PLC_β via pertussis toxin insensitive $G_{\alpha q}$ proteins, both of which have been previously shown to be present in *C. elegans* (Bargmann and Kaplan, 1998; Shibatohge et al., 1998).

Although 5-HT_{2Ce} has a similar structure and signaling profile as other 5-HT_2 receptors, its pharmacology was different and did not match the profile of any reported 5-HT receptor. This unusual pharmacology can best be described as a mix between that of the 5-HT_1 and the 5-HT_2 families of receptors. This unique pharmacology is characterized by high affinity for ergot derivatives (LSD, methiothepin) and to some 5-HT_2 antagonists (*e.g.* cyproheptadine), but a very low affinity for 5-HT itself. Preferential binding to ergot derivatives and low affinity for 5-HT seem to be a common theme among invertebrate 5-HT_1-receptors, while high affinity binding to cyproheptadine is more consistent with 5-HT_2 receptors (Gerhardt and van Heerikhuizen, 1997). Based on available evidence, we hypothesize that the 5-HT_{2Ce} receptor may be related to an ancestral form of 5-HT_1 / 5-HT_2 receptors that existed before the divergence of the two 5-HT receptor subtypes. At present, not enough pharmacological data is available on the closely related *Ascaris* 5-HT receptor (Huang et al., 1999) in order to compare it with 5-HT_{2Ce}. Both nematode receptors have a similar high affinity for LSD, but the *Ascaris* receptor has a much higher affinity for serotonin, suggesting there may be important structural differences between the agonist binding sites of these two receptors, despite overall high level of sequence homology. The signaling pathway of the *Ascaris* receptor is still undetermined.

Manuscript III described the cloning and expression of a full length *S. mansoni* cDNA (SmGPCRx) which encodes a putative GPCR related to the family of biogenic amine neurotransmitter receptors, including 5-HT, catecholamine, and octopamine receptors (Fig. 1B). This is the first GPCR to be cloned from a parasitic platyhelminth. SmGPCRx shared moderate amino acid sequence homology scores (51 % - 56 % within the TM regions) with various biogenic amine and muscarinic

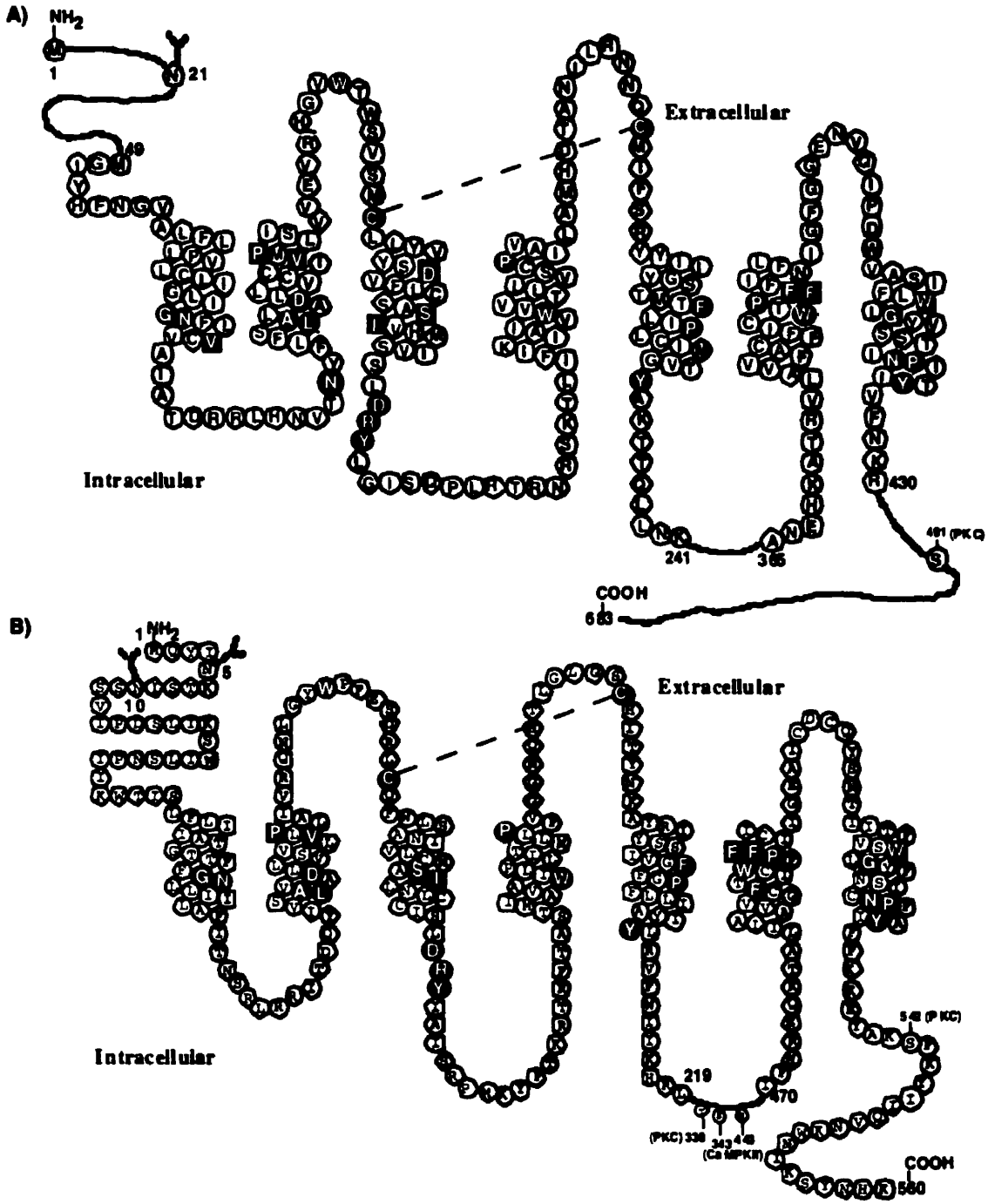


Fig. 1. Schematic models of the predicted protein structures of 5-HT$_{2Ce}$ (A) and SmGPCRx (B). The first 48 amino acids that are missing from the short splice variant of 5-HT$_{2Ce}$ are represented by a thick line. Amino acid residue that are represented in the third intracellular loop and / or the C-terminus of each receptor are also indicated by a thick line. The amino acids that are well conserved among GPCRs are highlighted and encircled. The residues that are conserved mainly in biogenic amine receptors are highlighted in a solid square. The highlighted and underlined serine in TM3 (S) and tryptophan (W) in TM7 are conserved in both aminergic and muscarinic acetylcholine receptors. Putative *N*-glycosylation and phosphorylation sites are shown. The snake-like diagrams were constructed using a software available from the Viseur server (http://brown.incm.u-nancy.fr/viseur/viseur.html).

acetylcholine receptors. However, SmGPCRx did not cluster with any of these receptor groups in a dendrogram analysis. Correlated mutational analysis of functional residues conserved either in biogenic amine receptors or in muscarinic acetylcholine receptors (Kuipers, et al., 1997), showed that SmGPCRx probably encodes an aminergic receptor rather than a muscarinic one. Interestingly, however, SmGPCRx has an asparagine in TM3 in place of a highly conserved aspartate that is crucial for the activity of both aminergic and muscarininc acetylcholine receptors. Previously, Yasuoka et al. (1995), reported the cloning of a fish GPCR which has a similar broad homology with biogenic amine receptors and also has an asparagine in TM3 instead of the conserved aspartate. Although the overall homology between SmGPCRx and the fish GPCR is rather low (46 % within the TM regions), they may be functionally related. Moreover, the presence of an asparagine in the TM3 of the fish GPCR suggests that the aspartate-to-asparagine change has been conserved through the vertebrate line and thus is not likely to represent an aberrant receptor sequence. To date, the ligand and signaling properties of the fish receptor have not been determined. Studying the functions of SmGPCRx may provide clues to the identity of this orphan fish receptor.

Expression of SmGPCRx in mammalian cells proved to be difficult due to codon usage incompatibility between the *S. mansoni* receptor sequence and mammalian cells. Initial attempts to express a FLAG-tagged SmGPCRx in COS7 or HEK cells did not result in detectable expression as tested by immunofluorescence analysis. Since most of the rare codons are clustered within the first half of the SmGPCRx sequence, we re-synthesized this region by recursive PCR (Prodromou et al., 1992), and fused it to the remaining C-terminal SmGPCRx sequence. Expression of this codon-optimized construct in HEK293(EBNA1) cells and subsequent immunofluorescence analysis with antibodies targeting N-terminal and C-terminal receptor epitopes, showed that the SmGPCRx protein was produced and targeted to the cell surface, such that its N-terminus was extracellular while its C-terminus was intracellular. This further confirmed the GPCR architecture of this *S. mansoni* receptor. This is the first study to report a codon-optimization strategy to enhance the expression of a helminth GPCR in mammalian cells. Such an approach may prove useful for expression of cDNAs from other parasites, especially protozoans such as *Plasmodium* and amoeba, which have a

distinctive codon-usage preference. Ongoing studies are attempting to characterize the signaling and pharmacological properties of the codon-optimized SmGPCRx, using a battery of potential test ligands (5-HT, noradrenaline, dopamine, octopamine, tyramine, carabachol (a muscarinic agonist), and histamine). Because of the significance of the aspartate-to-asparagine substitution, I have already generated a mutant form of the codon-optimized SmGPCRx receptor (N111D) which has the asparagine in TM3 replaced by an aspartate, and can be used in future studies for comparative functional assays (data not shown). Taken together, these studies will help to identify the natural ligand for SmGPCRx and will provide information about the functional significance of the N111D mutation.

In conclusion, the findings of this thesis substantiate the importance of serotonin as a neuroactive agent in parasitic helminths and further contribute to the general knowledge of serotonin biosynthesis and mode of action. The demonstration of TPH and TH activities in *S. mansoni*, and their subsequent cloning and characterization, constitute the first molecular evidence that parasitic trematodes, and possibly other helminths are capable of synthesizing serotonin and catecholamines endogenously. This strengthens the notion that serotonin and catecholamines, which are both present in the nervous system of *S. mansoni*, are important neuroactive agents in helminths. In addition, the increased stability and activity of the *S. mansoni* TPH makes it an attractive model for biochemical and structural studies of TPH properties. The isolated *C. elegans* 5-HT receptor represents the first 5-HT_2-like receptor with a mixed 5-HT_1 / 5-HT_2 pharmacology to be characterized from a helminth. Studies of this nematode 5-HT receptor may give insights into the function of 5-HT receptors in related parasitic nematodes, such as *A. suum* and the human parasite, *Ascaris lumbricoides*. Finally, we reported the cloning and expression of the first GPCR from a parasitic platyhelminth. We speculate that this *S. mansoni* receptor may represent a new class of receptor with probable aminergic properties. The structural differences between this receptor and mammalian biogenic amine receptors could be utilized to design potential antihelminthic drugs that preferentially target the parasite receptor.

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APPENDIX I (MANUSCRIPT IV)

Cloning and Characterization of a Novel Form of Tyrosine Hydroxylase from the Human Parasite, *Schistosoma mansoni*

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The nucleotide sequence reported in this paper has been deposited in the GenBank Data Base and was designated accession # AF030336

ABSTRACT

Catecholamines such as dopamine and noradrenaline play important roles as neuromuscular transmitters and modulators in all parasitic helminths, including the human parasite, *Schistosoma mansoni*. We have cloned a novel *S. mansoni* cDNA (SmTH) which shows high homology to mammalian tyrosine hydroxylase, the enzyme that catalyzes the first and rate limiting step in the biosynthesis of catecholamines. Two subsets of SmTH transcripts were identified, one of which carries the *S. mansoni* spliced-leader (SL) sequence at its 5' end, whereas the other does not appear to be *trans* -spliced to the *S. mansoni* SL. The two types of SmTH transcripts encode the same protein of 465 amino acids and a predicted size of 54 kDa. Expression of SmTH as an N-terminal histidine fusion protein in *Escherichia coli* produced an active enzyme which was purified approximately 52-fold to apparent homogeneity and a final specific activity of 0.78 μmol / min / mg. The purified enzyme was found to have the same absolute requirement for a tetrahydrobiopterin (BH_4) cofactor and the same sensitivity to inhibition by high concentrations of the substrate, tyrosine, as the mammalian enzyme. Purified SmTH also showed characteristic inhibition by catecholamine products, although the sensitivity to product inhibition was lower than that of the mammalian enzyme. This evidence indicates that SmTH encodes a functional tyrosine hydroxylase which has similar catalytic properties to the mammalian host's enzyme but may differ in its properties of regulation. This first demonstration of tyrosine hydroxylase in a parasitic helminth further suggests that the parasites have the enzymatic capacity to synthesize catecholamines endogenously.

INTRODUCTION

Catecholamines such as dopamine and noradrenaline are important neuroactive agents in a variety of vertebrates and invertebrates. The first step in the biosynthesis of catecholamines is catalyzed by tyrosine hydroxylase (tyrosine-3-monooxygenase; EC1.14.16.2), the enzyme that converts tyrosine to the short - lived intermediate, 3,4-dihydroxyphenylalanine (L-DOPA), which in turn is rapidly metabolized to produce dopamine, noradrenaline and adrenaline through a multi-step enzymatic pathway. Tyrosine hydroxylase catalyzes the first, rate-limiting reaction in the pathway and as such sets the pace of catecholamine production.

Genes coding for tyrosine hydroxylase have been isolated mainly from mammalian and other vertebrate species (Nagatsu and Ichinose, 1991; Neckameyer and White, 1992). Analyses of these genes and encoded proteins revealed that tyrosine hydroxylase belongs to a family of enzymes that also includes phenylalanine hydroxylase, a key enzyme in the metabolism of phenylalanine, and tryptophan hydroxylase, which catalyzes the rate-limiting reaction in the biosynthesis of another widely distributed neuroactive agent, the indoleamine, serotonin (Kaufman, 1987; Kaufman and Ribeiro, 1996). The three enzymes share catalytic properties, including an absolute requirement for a reduced tetrahydrobiopterin cofactor, and all have a similar structural organization that includes a variable N-terminal third containing the presumed regulatory domain, and a highly conserved catalytic region within the C-terminal two thirds of the protein (Kaufman and Ribeiro, 1996; Kumer and Vrana, 1996). In addition, the two neuronal enzymes, tyrosine and tryptophan hydroxylase, are subject to similar forms of regulation, both at the level of transcription and by phosphorylation at multiple serine residues (Funakoshi et al., 1991; Haycock and Haycock, 1991; Campbell et al, 1986; Kaufman and Ribeiro, 1996; Kumer and Vrana, 1996; Zigmond et al., 1989) .

Catecholamines and serotonin are present in several parasitic helminths, including the human parasite, *Schistosoma mansoni*, where they play several critical roles as neurotransmitters and modulators (Pax and Bennett, 1991; Davis and Stretton, 1995). In *S. mansoni* and the related parasite, *Fasciola hepatica*, dopamine and noradrenaline have been implicated as neuromuscular transmitters and neuromodulators of motor activity

(Hillman and Senft, 1973; Tomosky et al., 1974; Mellin et al., 1983; Pax et al., 1984; Pax and Bennett, 1991; Davis and Stretton, 1995). The two compounds have been identified in the central and peripheral nervous systems, the body wall musculature, holdfast structures and reproductive structures of the parasites (Davis and Stretton, 1995; Pax and Bennett, 1991). Very few studies to date have focused on the biosynthesis of catecholamines in helminths (Ribeiro and Webb, 1983a) and none of the biosynthetic enzymes, including tyrosine hydroxylase, has yet been identified or characterized at the molecular level. In contrast, studies of serotonin biosynthesis in *S. mansoni* and other helminths have reported that tryptophan hydroxylase may be absent in some parasites and that the source of parasite serotonin may be the host (Bennett and Bueding, 1973; Mansour, 1979; Cho and Mettrick, 1982; Hillman, 1983). Though these studies have been challenged by several authors (Chaudhuri et al., 1988; Ribeiro and Webb, 1983b; Ribeiro and Webb, 1984), the question of whether parasitic helminths have mammalian-like aromatic amino acid hydroxylases, including tryptophan or tyrosine hydroxylase, remains largely unresolved.

The present study reports the cloning and functional expression of a tyrosine hydroxylase cDNA from *S. mansoni*, and thus provides the first evidence for the presence of this enzyme in any parasitic helminth. The results show that *S. mansoni* tyrosine hydroxylase (SmTH) shares several of the same structural and catalytic properties as the mammalian enzyme, though the characteristic N-terminal regulatory region of mammalian tyrosine hydroxylase appears to be absent in the parasite. The high degree of conservation of the catalytic domain and similarity of kinetic properties suggests that SmTH plays as important a role in the biosynthesis of catecholamines as does the mammalian enzyme.

EXPERIMENTAL PROCEDURES

Schistosoma mansoni - A Puerto Rican strain of *S. mansoni* was maintained in CD-1 mice and *Biomphalaria glabrata* snails. Mice were infected with 200 freshly shed cercaria and seven to eight weeks later adult worms were recovered by portal venous perfusion (Duvall and DeWitt, 1967), washed three times with RPMI-1640 (Gibco BRL), and stored at -80°C until needed. For preparations of crude worm extracts, approximately 200 mg (wet weight) of adult worms were homogenized in 3 ml of ice-cold homogenization buffer (50 mM HEPES buffer, pH 7.0, 0.2 M NaCl, 1mM EDTA, 0.2% Tween 20, 5% glycerol, 200 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) 5 µg/ ml aprotinin and 5 µg/ml leupeptin) with a hand-held glass homogenizer and centrifuged at 13, 000 x g for 15 min at 4°C. Aliquots (15 - 30 µl) of the resulting crude extract containing approximately 3 mg protein / ml were used directly for measurements of tyrosine hydroxylase activity.

Cloning of a partial SmTH cDNA (C1) - Two PCR primers were synthesized based on nucleotide sequence from a predicted tryptophan hydroxylase gene of the free-living nematode, *Caenorhabditis elegans* (cosmid ZK1290.2; GenBank Accession # U21308). The sense and antisense primers (5'-CTGGACGCTGATCATCCTGGTTT-3' and 5'-AGACCAAATTCAATGGAAAAGAAGTA-3', respectively) targeted a region of the predicted catalytic domain which is conserved in all aromatic amino acid hydroxylases (Kaufman and Ribeiro, 1996). PolyA$^+$ mRNA from adult *S. mansoni* was purified on oligo- dT cellulose columns (Pharmacia Biotech, Inc.) and reverse transcribed (0.5 - 1 µg mRNA / reaction) with an oligo dT primer and 100U of murine moloney leukemia virus reverse transcriptase (MMLV; Gibco BRL). The resulting cDNA was subjected to 10 cycles of touchdown PCR (denaturation: 1 min /94°C; annealing: 1 min / 60 - 54°C at -0.6°C/ cycle; extension : 2 min /72°C) followed by 30 additional cycles of standard PCR (denaturation: 1min /94°C; annealing: 1 min / 54°C; extension : 2 min / 72°C), with 0.5 µM of each primer, according to standard protocols. A PCR product of 576 bp was identified, cloned into pCR2.1 (Invitrogen) and sequenced by the dideoxy-chain termination method. Analysis of the predicted peptide sequence revealed very high

homology to all three mammalian aromatic amino acid hydroxylases(> 60%). This partial sequence was ^{32}P-labelled (Random Priming, Boehringer Mannheim) and used as a probe to screen an adult *S. mansoni* λ-TriplEx cDNA library (kindly provided by Dr. G. O'Neill, Merck Frosst) for SmTH cDNAs. The library was screened under low stringency for 16 - 18 hrs at 42°C in a hybridizing solution of 25% formamide, 6x SSC, 5x Denhardt's, 0.5% SDS, 100μg/ml sheared salmon sperm DNA and 10^6 cpm/ml of the ^{32}P-labelled probe. Filters were washed in 2x SSC / 0.1% SDS twice for 15 min at room temperature and once for 30 min at 42°C. A single hybridizing clone was identified and further purified by two additional rounds of screening under the same conditions. The insert from this positive l clone (C1)was sequenced and identified by BLAST analysis as having high homology to tyrosine hydroxylase.

Rapid Amplification of cDNA ends (RACE) - Analysis of the 5' end of SmTH was performed by two different PCR - based methods: The 5' RACE System from Life Technologies, Inc. and a method based on a variation of anchored PCR which targets the conserved 5' end spliced-leader sequence (SL) of *S. mansoni* transcripts (Rajkovic et al., 1990; Davis et al., 1995). For the 5' RACE reaction, *S. mansoni* mRNA was reverse transcribed and C-tailed, according to the specifications of the manufacturer, prior to PCR amplification with the anchor primer supplied by the RACE kit and an antisense C1-specific primer (primer A; see Fig.1A). An aliquot of the PCR product (0.2 μl) was amplified in a second PCR reaction using an abridged form of the same anchor sense primer along with a nested C1-specific antisense primer (primer B; see Fig.1A). The cycling protocol for each reaction was 30 cycles of 30 sec at 94°C, 30 sec at 53°C and 90 sec at 72°C. For anchored PCR analysis targeting the SL sequence, oligo dT reverse - transcribed cDNA was subjected to a similar nested PCR reaction, using the same two C1-specific antisense primers (primers A and B), but with a sense primer (primer SmSL; see Fig. 1B) that corresponded to nucleotides 9-32 of the *S. mansoni* 36 nucleotide spliced leader sequence (Davis et al., 1995; Rajkovic et al., 1990). The final PCR products were agarose-gel purified, cloned into pCR2.1 (Invitrogen) and confirmed by DNA sequencing of at least two separate clones

Amplification of the complete coding sequence of SmTH Reverse transcribed *S. mansoni* cDNA was subjected to 30 cycles of PCR using primers that targeted the beginning (primer S; see Fig. 1A) and the end (primer F; see Fig.1A) of the predicted coding sequence of SmTH. Enzyme restriction sites *Xho* I and *Bam* HI were incorporated at the 5'end of the sense and antisense primers, respectively, to facilitate further subcloning into an expression vector. PCR reactions were performed with the use of a proofreading DNA polymerase, PWO (Boehringer Mannheim), according to the specifications of the manufacturer. The final PCR product (1399 bp) was gel-purified, digested with *Xho* I and *Bam* HI, and ligated to the prokaryotic expression vector pET15b (Novagen) which had been linearized by the same two restriction enzymes. Cloning into the *Xho* I site of this vector introduces an N-terminal oligohistidine fusion tag, which adds 21 amino acid residues of vector - derived sequence to the expressed SmTH product. The final construct in pET15b was confirmed by DNA sequencing of the entire insert and then used to transform *E. coli* host strain BL21(DE3)pLys (Novagen), according to standard protocols (Sambrook et al., 1989).

Expression and purification of SmTH- Transformed cultures of *E. coli* were grown in LB-ampicillin medium to an OD of approximately 0.6 - 1.0 and then induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 hrs at 37°C. Ferrous sulfate (0.1 mM) was added to expressing cultures to insure that enough iron was incorporated into the recombinant enzyme for subsequent measurements of enzyme activity (Wang et al., 1991). Following induction, the cells were pelleted by centrifugation and washed once in phosphate buffered saline (PBS) prior to freezing at -80°C.

For purification of SmTH protein, cell pellets from 50 ml induced bacterial cultures were thawed and resuspended in 2.5 ml of 20 mM phosphate buffer, pH 7.4, containing 0.5 M NaCl, 0.2% Tween 20, 5% glycerol and a cocktail of protease inhibitors (aprotinin, 50 μg / ml; leupeptin, 50 μg/ml; PMSF, 1 mM). Cells were first subjected to two cycles of rapid freezing and thawing to promote cell lysis by the resident T7 lysozyme, and then sonicated briefly through four cycles of 30 sec pulses separated by periods of 1 min. Cell lysates were centrifuged for 15 min at 13,000 xg and 4°C. The resulting pellet was resuspended in 2.5 ml of the same buffer, sonicated as above and

subjected to a similar centrifugation. The two supernatants were pooled and used for subsequent purification of expressed enzyme. The final soluble fraction was found to contain approximately 60% of total enzyme activity, indicating that a substantial proportion of SmTH protein was retained in the pellet, probably in the form of inclusion bodies (Wang et al., 1991). No further attempts were made to resolubilize active enzyme from the pellet.

Supernatants were loaded onto a 1 ml HiTrap Chelating column (Pharmacia) which had been previously charged with Ni^{2+} ions and equilibrated with 20 mM phosphate buffer, 0.5 M NaCl, 10 mM imidazole, pH 7.4, according to the specifications of the manufacturer. The column was washed first with buffer containing 10 mM imidazole (10 volumes) and then 100 mM imidazole (5 volumes) prior to elution of SmTH protein with 500 mM imidazole in the same buffer. The purified enzyme was immediately concentrated by ultrafiltration with a Centricon-10 microconcentrator (Amicon). To remove excess imidazole, the retentate was washed through two cycles of dilution in storage buffer (50 mM Tris-HCl, 0.2 M NaCl, 10% glycerol, pH 7.2) followed by additional ultrafiltration to a final protein concentration of 0.1 mg / ml. The purified enzyme was stable for up to 24 hrs at 4°C and could be stored at -80°C for at least four weeks with no apparent loss of activity.

Characterization of purified SmTH- Tyrosine hydroxylase activity was measured by the tritiated water release assay (Nagatsu et al., 1964; Reinhard et al., 1986) with the modifications described by Ribeiro et al. (1991). The standard assay was carried out in 100 µl of 50 mM HEPES buffer, pH 6.9, containing 200,000 cpm [^{3}H] tyrosine and enough unlabelled tyrosine to produce a final concentration of 20 µM, 200 units of catalase (Boehringer Mannheim), 5 milliunits of dihydropteridine reductase (Sigma), 0.1 mM NADH (Sigma) and either purified tyrosine hydroxylase (0.1 µg) or crude *S. mansoni* extract (45 - 90 µg protein). The reaction was initiated by the addition of 100 µM (6R)-5,6,7,8 -tetrahydrobiopterin (BH_4) (RBI), unless otherwise indicated, and the samples were incubated for 8 min at 37° C. Preliminary experiments indicated that the reaction was linear up to at least 12 min of incubation under these conditions. To terminate the reaction, 1 ml of activated charcoal (7.5% w/v in 1 M HCl) was added to

each sample and, after centrifugation at 500 xg for 10 min, aliquots of the supernatant containing [^{3}H]OH were radioassayed in 10 ml of scintillation cocktail.

Computer Analyses - Nucleic acid and protein sequences were analyzed and aligned using the MacVector Sequence Analysis vs. 6.0 software package (Oxford Molecular). Oligonucleotide primers for DNA sequencing and PCR were designed with the Oligo 4.0 software (NBI, Plymouth, MN). Protein sequences were compared to sequence data bases at the National Center for Biotechnology (NCBI) using the software BLAST (Basic Local Alignment Search Tool) for protein and nucleotide similarities (Altschul et al., 1990). All enzyme kinetic parameters (Km and apparent Km (S0.5)) were derived from 2 - 3 separate experiments, each in duplicates, and determined by computer-assisted, nonlinear curve - fitting to the Michaelis-Menten model.

Other methods - Recombinant rat PC12 tyrosine hydroxylase was expressed in *E. coli* and purified as described previously (Wang et al, 1991). Protein concentrations were measured by the method of Bradford (Bradford, 1976), using the BioRad protein assay kit and bovine serum albumin as a standard. Reducing SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) using precast 10% acrylamide gels from Novex, Inc. For western blot analysis of SmTH, aliquots of purified enzyme (0.5 - 1 μg) were electrophoresed as above, transferred onto nitrocellulose membranes (Sambrook et al., 1989) and reacted with a sheep polyclonal antibody (1: 500 dilution) raised against recombinant rat PC12 tyrosine hydroxylase (Wang et al., 1991) followed by a peroxidase - conjugated rabbit IgG (1: 1000 dilution) as the secondary antibody.

RESULTS

Isolation of SmTH cDNA clones - Alignment of a predicted *C. elegans* tryptophan hydroxylase sequence with other vertebrate and *Drosophila* aromatic amino acid hydroxylase sequences identified a conserved middle region which was targeted for PCR amplification of a partial hydroxylase sequence from *S. mansoni*. Two PCR primers were designed based on *C. elegans* nucleotide sequence on the assumption that codon usage would be similar in the two closely related organisms. This strategy allowed the use of non-degenerate primers, which in turn improved the specificity of the PCR reaction. A single PCR product of 576 bp was obtained, cloned and sequenced. Analysis of the predicted sequence revealed high level of homology to all aromatic amino acid hydroxylases (> 60%). This partial sequence was subsequently used as a probe to screen an adult *S. mansoni* cDNA library for SmTH cDNAs.

Screening of approximately $5x10^5$ phage clones at low stringency identified one positive clone (C1) of 1444 bp whose predicted coding sequence was found to be highly homologous to tyrosine hydroxylase. However, further sequence analysis revealed that C1 lacked a translational start codon and thus was assumed to be truncated at the 5' end. The missing 5' end was obtained by a standard 5' RACE reaction (Frohman et al., 1988). The RACE procedure generated a 561 bp product which overlapped almost entirely with the C1 clone except for 51 bp of new sequence at the 5' end. Analysis of this new stretch of nucleotides identified an ATG at position 42 which was in translational frame with the remaining SmTH sequence, indicating that the RACE product corresponded to the 5' end of SmTH. The complete cDNA and predicted protein sequences of SmTH are shown in Fig. 1.A. Analysis of the nucleotide sequence identified an open reading frame of 1399 bps which encodes a protein of 465 amino acids. Short predicted untranslated regions can be seen both at the 5' end (41 bps) and 3' end (55 bp). The latter is lacking a polyA$^+$ tail and is presumed to be less than full-length.

In addition to the RACE analysis just described, the 5' end of SmTH was analyzed by a variation on anchored PCR which targets the conserved spliced -leader of *S. mansoni* transcripts (Rajkovic et al., 1990; Davis et al., 1995). The two methods gave different results. Nested PCR with a sense spliced - leader primer (SmSL) and two nested

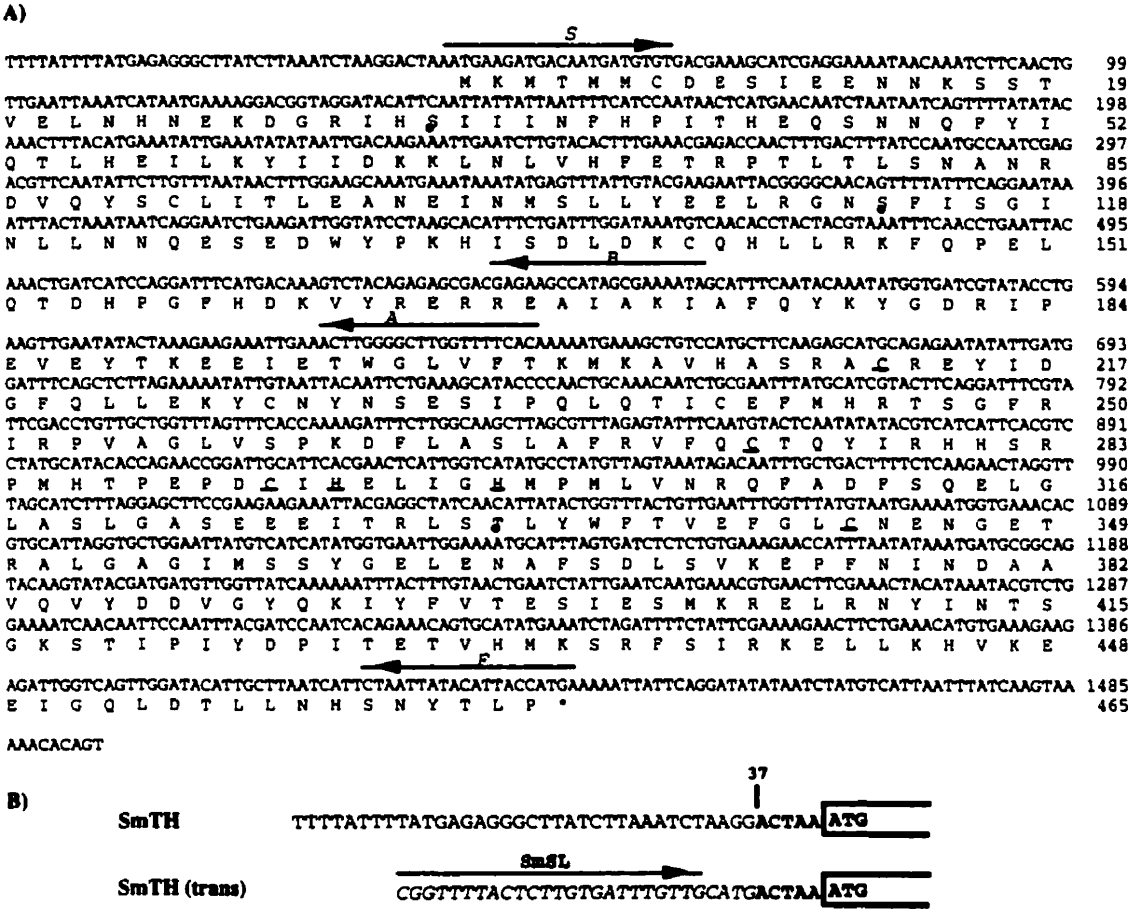

Fig. 1. cDNA sequence and deduced amino acid sequence of SmTH (A) and RT-PCR analysis of the 5' end of SmTH (B). (A) Overlined sequences represent sense and antisense oligonucleotides used in this study. Conserved cysteine and histidine residues are underlined. The black circles denote potential phosphorylation sites. (B) The 5' end of SmTH was obtained by 5' RACE PCR with an anchor primer and antisense primer A followed by a nested PCR with the same anchor primer and antisense primer B. SmTH (trans) is an RT-PCR product amplified with a primer that targets the *S. mansoni* SL sequence (SmSL) and the same two nested antisense primers, A and B. The sequence of the SmSL primer is overlined. The conserved *S. mansoni* SL sequence is shown in italics. Nucleotides that are identical in the two SmTH and SmTH (trans) PCR products are shown in bold. The nucleotide at position 37 marks the point where the two sequences become identical. ATG marks the beginning of the predicted coding sequence.

gene-specific antisense primers (A and B; Fig.1A) generated a shorter 553 bp product. The nucleotide sequence of this product was identical to that described above, except that the first 36 nucleotides were replaced by an SL sequence (Fig. 1B). The latter included the last 4 nucleotides of the conserved SL sequence (positions 32 - 36; CATG) (Davis et al., 1995; Rajkovic et al., 1990) which were not included in the SmSL primer. The splicing event did not alter the translational reading frame of SmTH (Fig. 1B). Taken together, the results are suggestive of two types of SmTH transcripts, one which appears

to be *trans* -spliced to the SL sequence and thus can be amplified by PCR with the SmSL primer, whereas the other, identified by the 5' RACE procedure, does not carry an SL sequence at its 5' end (Fig. 1B). The failure to identify *trans* -spliced species by 5' RACE, which does not target the SL sequence specifically, further suggests that the relative proportion of *trans*-spliced SmTH transcripts is probably small.

Analysis of predicted SmTH protein sequence - SmTH encodes a predicted protein of 465 amino acids with a calculated molecular mass of approximately 54 KDa. Protein subsequence analyses revealed that SmTH contains three consensus sites for phosphorylation by Ca^{2+}-calmodulin dependent protein kinase (amino acid positions Ser^{33}, Ser^{113}, Thr^{332}) and five N-glycosylation sites (amino acid positions 11, 97, 409, 454, 457) (Fig. 1A). BLAST analysis of the predicted protein sequence shows that SmTH is most closely related to tyrosine hydroxylase from other species. Based on pairwise protein alignments, SmTH shares significant homology with tyrosine hydroxylase from rat (61%), mouse (61%), quail (60%), human (59%), and *Drosophila* (59%) (Fauquet et al., 1988; Grima et al., 1985; Grima et al., 1987; Ichikawa et al., 1991; Neckameyer and White, 1992). The degree of conservation is greatest in the mid region of the sequence, particularly from amino acid positions 129 to 363 of SmTH, where homology scores relative to mammalian hydroxylases rise to 72 - 77%. A predicted protein alignment of SmTH with other known tyrosine hydroxylase sequences is shown in Fig. 2. The alignment identified four cysteine residues (SmTH amino acid positions 212, 274, 292, 343) and two histidine residues (SmTH amino acid positions 294 and 299), which are conserved in other species and are thought to play an important role in catalysis (Kaufman and Ribeiro, 1996; Nagatsu and Ichinose, 1991; Kumer and Vrana, 1996; Ramsey et al., 1995). A fifth cystein residue (SmTH Cys^{140}) located at the predicted boundary between the regulatory and catalytic domains of the enzyme (Abate and Joh, 1991; Ribeiro et al., 1993; Walker et al., 1994) is also conserved in the different forms of tyrosine hydroxylase. The iron-binding site of the enzyme, consisting of two histidines and a glutamate residue (Ramsey et al., 1995; Goodwill et al., 1997) is present in SmTH (SmTHHis^{294}, His^{299} and Glu^{339}). In addition, a characteristic signature motif of all aromatic amino acid hydroxylases, the peptide PEPD-CHELLGHVP is present in SmTH

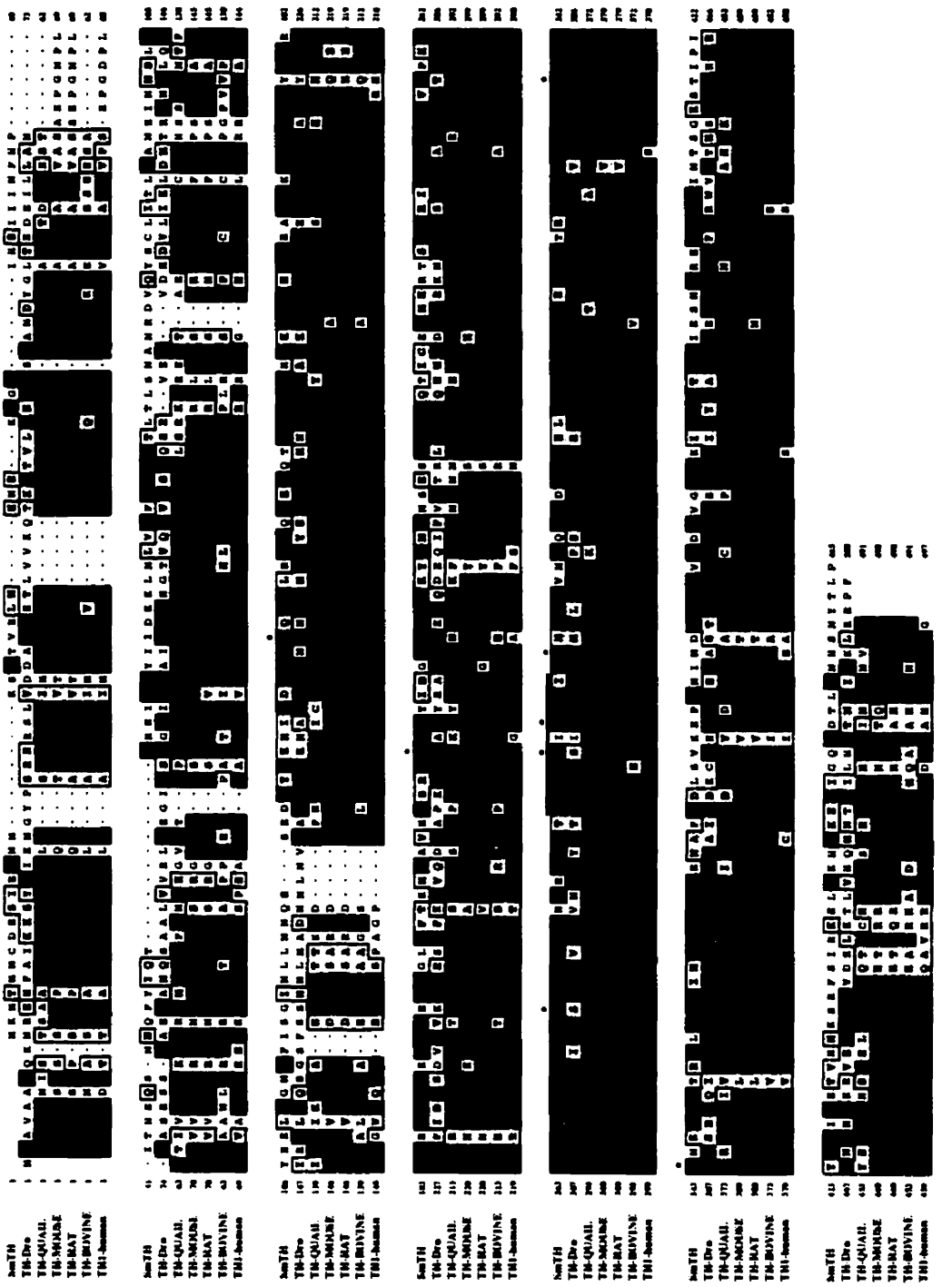

Fig. 2. Alignment of the deduced amino acid sequence of SmTH with other tyrosine hydroxylase sequences. Protein sequences were aligned with the program MacVector, according to the CLUSTAL method. Dark boxes and bold letters represent identical amino acids, while grey boxes indicate conservative amino acid changes. Gaps (-) are inserted for optimum protein alignment. Conserved cysteine and histidine residues that are also present in SmTH are marked by asterisks. The overlined sequence is a conserved signature peptide of all aromatic amino acid hydroxylases. The tyrosine hydroxylase sequences used in the alignment are (GenBank Accession #s) : *Drosophila* (TH-Dro, #U14395), quail (TH-Quail, # M24778), mouse (TH-MOUSE, # M69200), rat (TH-RAT, # M10244), bovine (TH-BOVINE, # M36794), and human (TH1-HUMAN, # X05290).

except that the conserved cysteine and one of the conserved leucine residues are replaced with isoleucines (Fig. 2).

Expression of SmTH in *E. coli* - To establish if SmTH encodes a functional tyrosine hydroxylase, the predicted coding sequence of the cDNA was amplified from reverse transcribed *S. mansoni* cDNA and subcloned into the bacterial expression vector, pET15b. The resulting construct was used to transform *E. coli* strain BL21(DE3)pLys for subsequent induction of SmTH protein expression. Previous studies have shown that *E. coli* is a suitable environment for expression of recombinant mammalian tyrosine hydroxylase, provided that the bacterial cultures are supplemented with iron, which is limiting in the bacterium (Wang et al., 1991). Iron is coordinated to two histidines and a glutamate residue of the enzyme's iron-binding site (Andersson et al., 1988; Ramsey et al., 1995; Goodwill et al., 1997) and is essential for expression of enzyme activity. In the present study, iron was added routinely to expressing cultures of *E. coli* and the conditions for expression of SmTH protein were similar to those established earlier for the mammalian enzyme (Wang et al., 1991).

Cloning of SmTH into the vector pET15b was designed to introduce an N-terminal oligohistidine fusion tag which facilitated subsequent purification of the enzyme by nickel chelation chromatography. The tag, consisting of 10 histidine residues followed by a thrombin cleavage site (Novagen), added approximately 2 kDa of vector-derived sequence to the expressed fusion protein product. Preliminary experiments aimed at removing the histidine tag by cleavage with thrombin resulted in complete loss of enzyme activity. Therefore, no further attempts were made to remove the fusion tag throughout this study.

Induction of transformed *E. coli* with isopropyl β-D-thiogalactopyranoside (IPTG) produced considerable amounts of a protein that reacted with anti- rat tyrosine hydroxylase (Fig.3) and also had tyrosine hydroxylase activity. No western positive protein, or tyrosine hydroxylase activity were detected in cells transfected with vector without insert. The specific activity of SmTH enzyme in crude bacterial extracts was 15 nmol / min/ mg of protein. Similar to rat tyrosine hydroxylase, which forms inclusion bodies when expressed in *E. coli* (Wang et al., 1991), only about 60% of SmTH activity

was detected in the soluble fraction, the remaining enzyme activity being retained in the pellet.

Purification of SmTH - SmTH was purified by nickel chelation chromatography, which selectively binds the N-terminal histidine fusion tag of the expressed protein. Fig. 3 shows a Coomassie stain of a crude extract of induced cells (Fig. 3.A) and purified SmTH after electrophoresis on a 10% SDS-polyacrylamide gel (Fig. 3.C). SmTH appears as the only prominent band on the gel with a corresponding single peak on the densitometry profile. The approximate size of the purified enzyme, determined from comigration of protein standards, was about 55 kDa, which is consistent with the predicted size of SmTH based on sequence analysis. The identity of the purified SmTH was confirmed by western analysis with anti-rat tyrosine hydroxylase (Fig. 3B) and by direct measurements of tyrosine hydroxylase activity. The specific activity of pure SmTH was 0.78 μmol /min / mg and the yield from a 50 ml bacterial culture was 50 - 70 μg of purified enzyme. Based on activity measurements, the expressed SmTH protein represents approximately 2% of total protein in crude extracts of induced bacterial cells.

Partial kinetic characterization of purified SmTH- One of the distinctive characteristics of all three aromatic amino acid hydroxylases is their absolute requirement for a tetrahydrobiopterin cofactor (Kaufman, 1987). SmTH has a similar requirement for BH4; no enzyme activity was detected when the cofactor was omitted from the reaction. The Km for BH_4 was measured in a concentration range of 15 - 200 μM and a fixed tyrosine concentration of 20 μM. Analysis of the resulting saturation curve revealed a Km BH_4 of about 44.8 μM and an average Vmax of 0.87 μmol/min/mg. A representative saturation curve for BH_4 is shown in Fig.4A .

A second distinctive characteristic of mammalian tyrosine hydroxylase is pronounced substrate inhibition at concentrations of tyrosine above 50 μM (Kaufman and Ribeiro, 1996). This was investigated in the present study and the results (Fig.4B) show the same pattern of high substrate inhibition. Kinetics for tyrosine were investigated by varying the amino acid substrate between 5 - 200 μM while maintaining BH_4 at a fixed

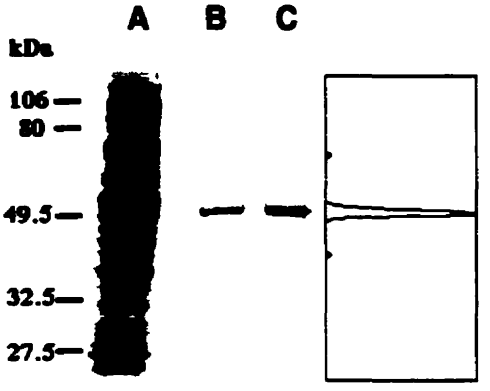

Fig. 3. Purification of recombinant *S. mansoni* tyrosine hydroxylase (SmTH). Recombinant enzyme was expressed in *E. coli* as an N-terminal oligohistidine fusion protein and purified to apparent homogeneity by nickel -chelation chromatography. (A) Coomassie stain of a crude lysate of induced bacteria (B) Western blot of purified SmTH developed with anti-rat PC12 tyrosine hydroxylase. C) Coomassie stain of purified SmTH and corresponding densitometry profile. The relative positions of protein standards are shown.

concentration of 100 μM. Although a proper Km determination was not possible due to substrate inhibition, an apparent Km or S0.5 for tyrosine was estimated at 9.5 μM.

Catecholamines such as dopamine are strong feedback inhibitors of tyrosine hydroxylase. One form of catecholamine inhibition is competitive with respect to the cofactor, the principal effect being a marked increase in the Km for BH_4 (Kaufman, 1987; Zigmond et al, 1989). As shown in Fig.5A, dopamine is also a feedback inhibitor of SmTH activity with an IC_{50} of approximately 10.1 μM. The latter value was determined by varying the concentration of dopamine between 1 - 250 μM at fixed concentrations of BH_4 (100 μM) and tyrosine (20 μM). When the same experiment was repeated with recombinant rat PC12 tyrosine hydroxylase, which had been similarlyexpressed in *E. coli* and purified (Wang et al, 1991), an IC_{50} value of 2 μM was obtained (data not shown), suggesting that SmTH is less sensitive to dopamine inhibition than its mammalian counterpart. To assess whether the inhibition of SmTH by dopamine is competitive with respect to BH_4, saturation curves for BH_4 were repeated in the presence and in the absence of 10 μM dopamine. As shown in Fig.5B, the predominant effect of dopamine is a decrease in the affinity for BH_4, which is consistent with competitive inhibition. The Km for BH_4 in the presence of 10 μM dopamine was 117 μM, nearly 3-fold higher than that measured in the absence of dopamine (117 μM vs 44.8 μM), whereas Vmax was essentially unchanged (Fig. 5B).

In addition to competitive feedback inhibition, low levels of catecholamines cause a time-dependent inactivation of mammalian tyrosine hydroxylase (Okuno and Fujisawa,

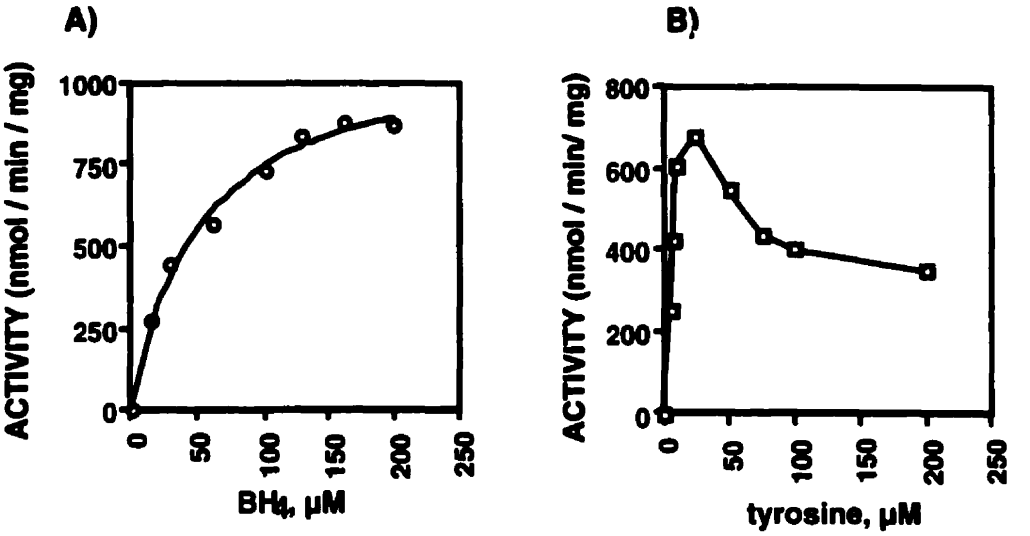

Fig. 4. Activity of purified SmTH as a function of the cofactor, tetrahydrobiopterin (BH_4) and the substrate, tyrosine. Purified SmTH was assayed for tyrosine hydroxylase activity by the tritiated water release method, as described under "Experimental Procedures", either at variable BH_4 concentrations and a fixed tyrosine concentration of 20 µM (A) or at variable tyrosine concentrations and a fixed BH_4 concentration of 100 µM (B). Incubations were carried out for 8 min at 37°C. Enzyme activity is expressed in nanomoles of tyrosine hydroxylated per min / mg of enzyme

1985), which is characterized by a dramatic decrease both in Vmax and the affinity for BH_4 (Daubner et al, 1992; Ribeiro et al, 1992). We tested if low levels of dopamine have a similar time-dependent inhibitory effect on SmTH. Purified SmTH was incubated for 2, 4 and 6 min at 30°C in buffer (50 mM Tris-HCl, 0.2 M NaCl, 10% glycerol, pH 7.2) containing a stoichiometric amount of dopamine (0.5 µM dopamine and enzyme). After incubation, aliquots were immediately assayed for tyrosine hydroxylase activity by the standard tritiated water release method at pH 6.9, using 100 µM BH_4 and 20 µM tyrosine. The results revealed that incubation with dopamine for up to 6 min under these conditions caused a small decrease in SmTH activity of approximately 30% compared to a control sample which was incubated for the same length of time in the absence of dopamine (data not shown). Additional studies revealed that a 6 min incubation of SmTH with a stoichiometric amount of dopamine had no measurable effect on the Km for BH_4 and decreased Vmax by 26% (data not shown). In contrast, a similar treatment of recombinant mammalian tyrosine hydroxylase caused a several fold change both in Vmax and the Km for BH_4 (Daubner et al, 1992; Ribeiro et al, 1992).

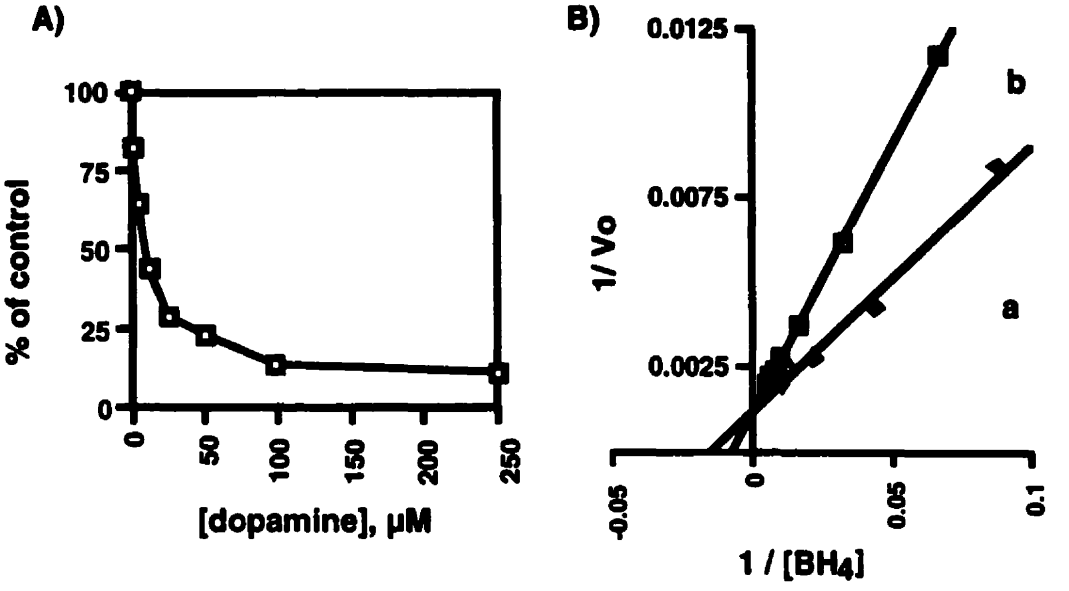

Fig. 5. Product inhibition of purified SmTH by dopamine. (A) Purified SmTH was assayed for tyrosine hydroxylase activity in the presence of 20 μM tyrosine, 100 μM BH_4 and variable dopamine concentrations. The data are expressed as the percentage of a control sample without dopamine (B) The activity of SmTH was measured over a concentration range of 15 - 250 μM BH_4 either in the absence (a) or the presence (b) of 10 μM dopamine. The substrate, tyrosine, was kept constant at 20 μM. 1 / V is the reciprocal of the rate measured in nmol / min / mg enzyme and 1/ [BH_4] is the reciprocal of the concentration of BH_4 in μM.

Characterization of crude *S. mansoni* extracts - The presence of tyrosine hydroxylase in *S. mansoni* was examined further by direct measurements of enzyme activity in a crude worm extract. Measurable tyrosine hydroxylase activity was detected in the extract, approximately 0.02 - 0.03 nmol / min / mg of protein, when compared to a boiled enzyme control. This level of activity is similar to that seen in crude extracts of whole rat brain (Nelson and Kaufman, 1987) or adrenal medulla (Hoeldtke and Kaufman, 1977). No measurable hydroxylase activity was detected in the worm extract when BH_4 was omitted from the reaction mix, indicating that the native enzyme has the same absolute requirement for a reduced pterin cofactor as the recombinant hydroxylase expressed in *E. coli*.

DISCUSSION

This study provides the first molecular characterization of any aromatic amino acid hydroxylase in a parasitic helminth. The present demonstration of a tyrosine hydroxylase homologue, whose structural and catalytic properties resemble those of the mammalian enzyme, provides convincing evidence that in *S. mansoni*, at least, tyrosine hydroxylase is present and likely plays a critical role in the biosynthesis of catecholamine neurotransmitters. The additional finding of tyrosine hydroxylase activity in crude extracts of *S. mansoni* gives further support to this conclusion.

The hydroxylase described in this study is the most primitive form of the enzyme ever cloned or characterized at the molecular level. With the exception of an enzyme sequence from *Drosophila* (Neckameyer and White, 1992), all other tyrosine hydroxylase sequences available have been obtained from vertebrate, for the most part mammalian species (Grima et al., 1985; Grima et al., 1987; Ichikawa et al., 1991; Fauquet et al., 1988; Nagatsu and Ichinose, 1991). Comparisons between the *S. mansoni* hydroxylase and its vertebrate or *Drosophila* counterparts revealed many similarities as well as some interesting differences. Based on sequence analyses, SmTH appears to be more closely related to the mammalian enzymes, particularly bovine and human, than the lower vertebrate (quail) or invertebrate (*Drosophila*) enzyme species. The greatest degree of sequence conservation among all different species of tyrosine hydroxylase, including SmTH, is in the C-terminal half to two-thirds of the protein, a region which is believed to comprise the catalytic domain of the enzyme (Nagatsu and Ichinose, 1991; Kaufman and Ribeiro, 1996; Kumer and Vrana, 1996; Goodwill et al., 1997). Several of the signature motifs of tyrosine hydroxylase are present in the presumed catalytic domain of SmTH, including two conserved histidines, which are thought to be coordinated to iron in the enzyme (Ramsey et al, 1995; Goodwill et al., 1997), and several conserved cysteines believed to play a role in the conformation of the active site. It is noteworthy that one of the most conserved cysteines in vertebrates, a residue which is part of a characteristic motif of all vertebrate aromatic amino acid hydroxylases (PEPD-CHELLGHVP) (Nagatsu and Ichinose, 1991; Kaufman and Ribeiro, 1996) is replaced with an isoleucine in the two invertebrate sequences, *S. mansoni* as well as *Drosophila.* Alhough the

significance of this difference is unknown, the results nonetheless suggest that this cysteine residue is not critical for enzyme activity.

Based on sequence analyses, SmTH diverges from other forms of the enzyme both at its N-terminal and C-terminal ends. Compared with mammalian and quail hydroxylases, SmTH lacks ~ 50 N-terminal amino acid residues and its subsequent amino acid sequence, up to position 129, shows very little homology to other forms of the enzyme. Similarly, SmTH shows divergence within the last 50 C-terminal amino acid residues. These same regions, particularly the N-terminal end, correspond roughly to predicted regulatory domains of mammalian tyrosine hydroxylase (Kaufman and Ribeiro, 1996; Nagatsu and Ichinose, 1991; Kumer and Vrana, 1996). The lack of sequence conservation in these areas suggests that the parasite enzyme may have a different functional organization and / or may be subjected to different forms of regulation.

The present identification of three potential phosphorylation sites for CaMPKII in SmTH (Ser^{33}, Ser^{113} and Thr^{332}) suggests that the parasite hydroxylase may be regulated by multi-site phosphorylation *in vivo*. Although CaMPKII has not yet been identified in *S. mansoni*, there is ample evidence that the parasite has mammalian-like serine / threonine protein kinases, including Ca^{2+}-dependent kinases (Kawamoto et al., 1989; Katsumata et al., 1989; Wiest et al., 1992; Blair et al., 1994; Davies and Pearce, 1995). *S. mansoni* is also known to have calmodulin (Thompson et al., 1986; Siddiqui et al., 1991; Stewart et al., 1992), a further indication that a Ca^{2+}/calmodulin – dependent protein kinase such as CaMPKII may be active in the phosphorylation of SmTH. In mammals, it has been well established that tyrosine hydroxylase is regulated through multi-site phosphorylation at four highly conserved serine (or threonine) residues (Ser/Thr^{8}, Ser^{19}, Ser^{31} and Ser^{40}) located on the regulatory N-terminus of the enzyme. Each phosphorylation site is targeted by a different protein kinase and mediates specific changes in the kinetic behavior of the enzyme leading to an overall increase in activity (Zigmond et al., 1989; Kaufman and Ribeiro, 1996; Kumer and Vrana, 1996). In particular, the phosphorylation of rat and human tyrosine hydroxylase by protein kinase A (Ser^{40}) causes and increase in Vmax, a decrease in the Km for BH_4 and an increase in the Ki for catecholamine inhibition, all of which lead to marked activation of the hydroxylase (Zigmond et al., 1989; Kaufman and Ribeiro, 1996; Kumer and Vrana,

1996). None of these four mammalian phosphorylation sites, including the important site targeted by protein kinase A, are conserved in SmTH. In addition, whereas the mammalian phosphorylation sites are clustered at the N-terminal regulatory end of the enzyme, the three potential phosphorylation sites of SmTH are located both at the N-terminus (Ser^{33}) and in the catalytic domain (Ser^{113}, Thr^{332}). Thus, if SmTH is regulated by phosphorylation, the underlying meachanisms of phosphorylation and probably the corresponding kinetic consequences are likely to be unique to the parasite.

Expression of SmTH in *E. coli* produced an enzyme that had tyrosine hydroxylase activity and shared several of the same kinetic properties as recombinant mammalian enzyme similarly expressed in *E. coli*. As with all other aromatic amino acid hydroxylases, SmTH showed an absolute requirement for a reduced pterin cofactor, the Km for BH_4 being almost identical to that of recombinant rat PC12 tyrosine hydroxylase (Wang et al., 1991). In addition, the two forms of the enzyme shared similar kinetics for the amino acid substrate, tyrosine, including a similar sensitivity to substrate inhibition at tyrosine concentrations above 50 μM (Ribeiro et al., 1993; Wang et al., 1991). On the other hand, SmTH differed from the mammalian enzyme in its response to catecholamine product inhibition. Catecholamines, including dopamine, inhibit mammalian tyrosine hydroxylase in two ways: reversible feedback inhibition, which is competitive with respect to BH_4, and a time-dependent sustained inhibition, which is associated with the tight binding of substoichiometric amounts of dopamine to the enzyme (Andersson et al, 1992; Martinez et al, 1996; Daubner et al, 1992; Ribeiro et al, 1992), and causes a several fold decrease both in Vmax and the affinity for BH_4 (Daubner et al, 1992; Ribeiro et al, 1992). The latter form of inhibition is though to be particularly important *in vivo* because it occurs at physiological levels of dopamine (< 1 μM) and is believed to render the enzyme more sensitive to activation by phosphorylation (Haavik et al, 1990; Okuno and Fujisawa, 1991; Daubner et al, 1992; Ribeiro et al, 1992). In contrast, the present study has shown that SmTH was only weakly inhibited by low (stoichiometric) amounts of dopamine, under conditions where a decrease in enzyme activity of up to 15-fold would be expected (Daubner et al, 1992; Ribeiro et al, 1992). In addition, although SmTH was inhibited by higher concentrations of dopamine, the IC_{50} was approximately 5-fold greater than that for recombinant mammalian hydroxylase similarly expressed in *E. coli*.

Taken together, these results indicate that the parasite is generally less sensitive to dopamine inhibition than its mammalian counterpart. In particular, the weak response to low levels of dopamine suggests that SmTH is not regulated by the same form of sustained catecholamine inhibition that has been described for other forms of tyrosine hydroxylase.

The reason for this diminished responsiveness of SmTH to dopamine cannot be explained at present. Nonetheless, it is noteworthy that the IC_{50} for dopamine inhibition of SmTH is similar to that reported earlier for a deletion mutant of rat PC12 tyrosine hydroxylase, which lacked 157 amino acid residues from the N-terminus and 43 amino acid residues from the C-terminus (Ribeiro et al., 1993), roughly the same two regions which are least conserved in the parasite.This observation gives credence to previous suggestions that these presumed regulatory regions serve to modulate, possibly facilitate the binding of inhibiting catecholamines to tyrosine hydroxylase (Ribeiro et al., 1993), such that a change in their sequence, as in the case of the parasite enzyme, or their removal by mutagenesis, would lead to reduced sensitivity to catecholamine inhibition. Although this hypothesis needs to be confirmed by further experimentation, the present results nonetheless point to SmTH as a potentially useful model for studies of tyrosine hydroxylase regulation by catecholamine products.

Two apparent transcripts for SmTH were identified in this study, one which appeared to be *trans*-spliced at the 5'end to the *S. mansoni* spliced leader sequence (Rajkovic et al., 1990; Davis et al., 1995) and one which was not. *Trans*-splicing to an SL sequence is a well known phenomenon in other lower invertebrates, including trypanosomes and some nematodes, where SL sequences are thought to play a role in the stability of the transcript and the binding to the ribosomal complex, as well as the processing of polycistronic transcripts (Ullu and Nilsen, 1995). In *S. mansoni*, although there is evidence for *trans*-splicing of some RNAs, the relative proportion of *trans*-spliced transcripts is thought to be small. Two studies have suggested that the majority of transcripts in *S. mansoni* and related parasite *F. hepatica* do not undergo *trans*-splicing to the SL sequence, thus questioning the significance of this phenomenon in this class of parasites (Rajkovic et al., 1990; Davis et al., 1995). Our findings that RNAs from the same gene can be processed differently, such that only a subset of transcripts acquires the

SL sequence, raises some interesting questions that deserve further investigation. The *trans*-splicing event appeared to be very precise, since it did not change the translatable reading frame of SmTH, as might have been expected from an aberrant splicing phenomenon. Although the latter cannot be ruled out at present, this finding nonetheless raises the possibility that SmTH transcripts and possibly other parasite mRNAs may undergo some form of differential processing which has functional significance. Additional support for this notion comes from our recent finding of similarly *trans*-spliced and non-*trans*-spliced transcripts for at least three other, unrelated *S. mansoni* genes (author's unpublished data), an indication that differential splicing to the SL sequence may be a common phenomenon in the parasite. The elucidation of these findings awaits further investigation.

In summary, the present study has cloned a parasite cDNA which is homologous to tyrosine hydroxylase and has several of the same kinetic properties as mammalian tyrosine hydroxylase upon expression in *E. coli*. These results provide the first indication that a parasitic helminth has a neuronal aromatic amino acid hydroxylase, and strongly suggests that parasitic helminths are able to synthesize their own catecholamines endogenously, using the same enzymatic pathway as higher organisms. This study also raises an interesting question regarding the presence or absence of the related enzyme, tryptophan hydroxylase, in *S. mansoni*. As indicated earlier, some authors have suggested that *S. mansoni* and other parasites do not have tryptophan hydroxylase and thus must rely upon the host for a supply of serotonin (Bennett and Bueding, 1973; Cho and Mettrick, 1982; Hillman, 1983; Mansour, 1979). Other researchers, however, have questioned this conclusion and presented evidence of serotonin biosynthesis from tryptophan at least in some parasitic helminths (Chaudhuri et al., 1988; Ribeiro and Webb, 1983b; Ribeiro and Webb, 1984). The present demonstration of a closely related aromatic amino acid hydroxylase in *S. mansoni* lends credence to the latter view, and suggests that tryptophan hydroxylase may be similarly present in the parasite. Additional research is needed to clarify this question.

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APPENDIX II

The Life Cycle of *Caenorhabditis elegans*

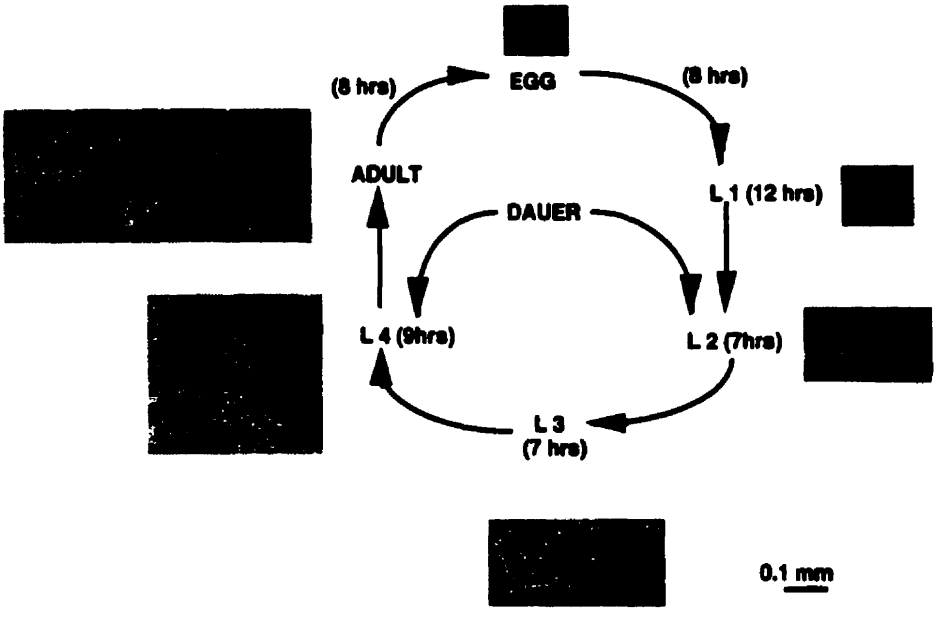

Fig. 1. The life cycle of *C. elegans*. The time needed to reach each developmental stage is indicated in parentheses, as determined from growth (25°C) on petri dishes seeded with OP50 *E. coli* (modified from Lewis and Fleming, 1995).

Caenorhabditis elegans is a transparent free-living nematode that is widely distributed in many regions of the world. This nematode lives in the soil and utilizes bacteria as a source of food and multiplies rapidly, reaching adulthood in approximately 3-4 days. The majority of *C. elegans* worms are hermaphrodites which are capable of producing both sperms and oocytes and, hence, are capable of self-fertilization. *C. elegans* males, which arise spontaneously at a low rate, are capable of fertilizing hermaphrodites. *C. elegans* has a life span of approximately 18-20 days at 20°C. The adult hermaphrodite (~ 1.5 mm in length) sheds the fertilized eggs into the environment. The eggs hatch as L1 larvae which in turn molt into L2, L3, L4, and mature to the adult form (Fig. 1). In cases of starvation and overcrowding, the adult worms die and the L1 larvae develop into a new juvenile stage called the dauer larvae, which can survive without food for several months. When food becomes available, the dauer larvae will develop into the L4 stage which in turn grow to adults.

Within the last 25 years, *C. elegans* has been one of the most studied multicellular organisms. Its cellular anatomy and neuronal systems have been fully mapped, and its genome (~ 8 x 10^4 kb) was recently fully sequenced. The ease of culturing and manipulation combined with a rapid life cycle and wealth of biological information have made *C. elegans* a very popular system to use for biology and genetics studies (Lewis and Fleming, 1995; Bargmann, 1998).

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APPENDIX III

The Life Cycle of *Schistosoma mansoni*

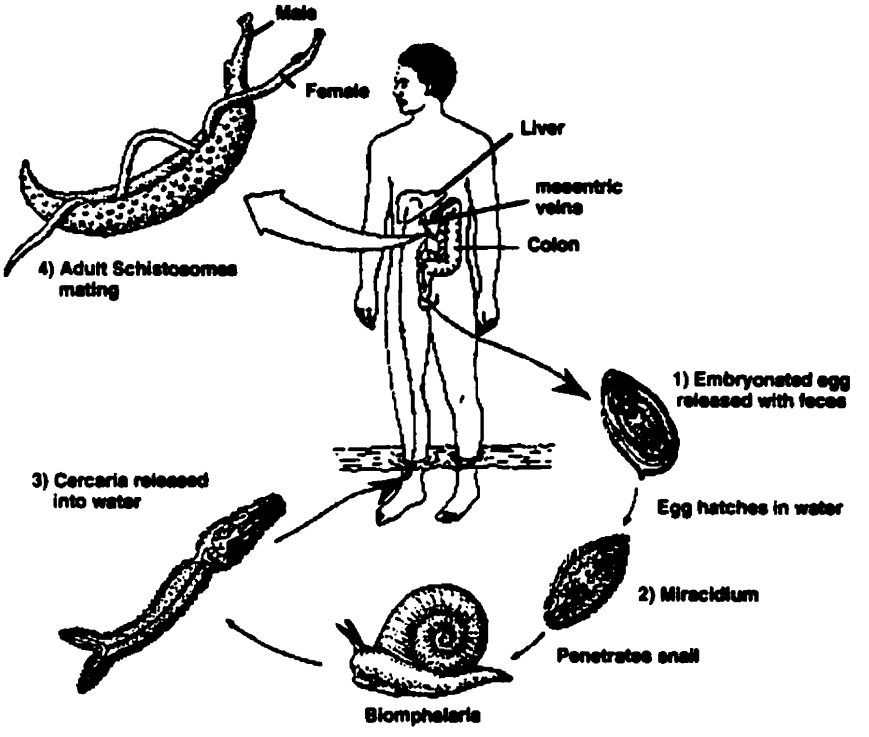

Fig. 1. Life cycle of *S. mansoni*. The developmental stages of *S. mansoni* (egg, miracidium, cercaria, and adults) and the intermediate snail host (*Biomphalaria glabrata*) are shown (modified from Robert and Janvoy, 1996).

Schistosoma mansoni belongs to the phylum Platyhelminthes (flatworms), class Trematoda, and family Schistosomatidae. *S. mansoni* is the causative agent of schistosomiasis, a severe debilitating disease that afflicts more than 200 million people around the world, mostly in developing countries in the Africa, middle east, and South America. *S. mansoni* has a complex life cycle that requires an intermediate snail host, and a definitive mammalian host. In addition, two free-swimming stages exist: miracidia, which infect the snail host (*Biomphalaria glabrata*) and the cercaria, which leave the snail to infect the mammalian host, usually humans. Upon penetrating the skin, morphological and biochemical changes occur in the cercaria causing its transformation into schistosomula. The schistosomula then enter the blood system directly (or indirectly via lymphatics) and pass through the heart to reach the liver via the portal vein. In the

liver, the schistosomula transform into blood-feeding worms and start to grow, pair, and migrate against blood flow of the hepatic portal system to the mesenteric vein for oviposition. The laid eggs are excreted and hatch in water, releasing tiny miracidia, and allowing the cycle to continue (Robert and Janvoy, 1996).

REFERENCE

Robert, L. S. and Janvoy, J. (1996) Digeneans: Strigeiformes, in *Foundations of Parasitology, 5th ed.* (Kemp, M. J., ed.), pp. 233-249. WCB publishers, Boston.