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Molecular characterization of the serotonin and cAMP-signalling pathways in *Echinococcus*

Molekulare Charakterisierung der Serotonin- und cAMP-Signalwege in *Echinococcus*

Inaugural - Dissertation

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1 Introduction

1.1 The small fox tapeworm *Echinococcus multilocularis* and the dog tapeworm *Echinococcus granulosus*

1.1.1 Phylogeny and epidemiology of *Echinococcus spp.*

Echinococcus is the causative agent of echinococcosis, a severe zoonotic disease that is provoked by the metacestode stage of the parasite. Three forms of echinococcosis are known to occur in humans: alveolar echinococcosis, cystic echinococcosis and polycystic echinococcosis. Infection with *Echinococcus multilocularis* leads to alveolar echinococcosis, while infection with *Echinococcus granulosus* causes cystic echinococcosis. The less frequent polycystic echinococcosis is caused by *Echinococcus vogeli* and *Echinococcus oligarthus* (Eckert and Deplazes, 2004).
While *E. multilocularis* is mostly restricted to the northern hemisphere, being distributed in North America, central and northern Eurasia (Craig, 2003), *E. granulosus* can be found worldwide (see Figure 1).

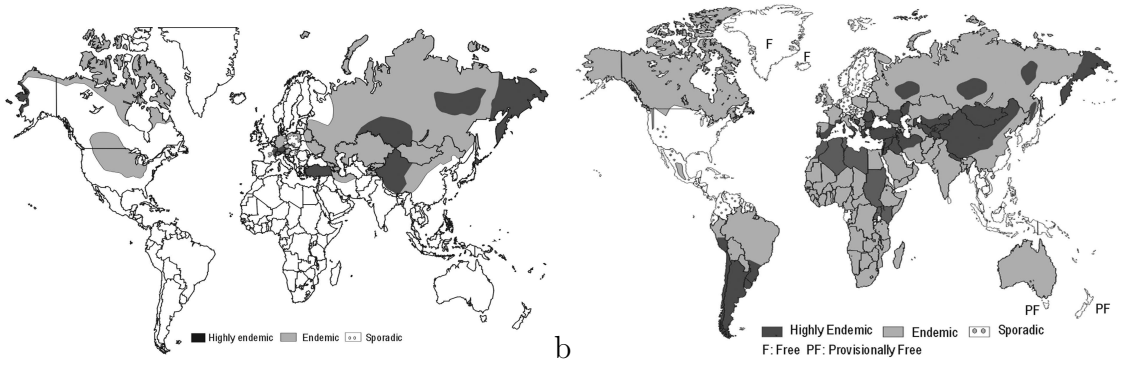


Figure 1: **Approximate global distribution of *E. multilocularis* (a) and *E. granulosus* (b) as of 2002 (Eckert and Deplazes, 2004).** Exact identification of normal and high endemic areas is difficult due to incomplete or lacking data.

Echinococcus vogeli and *Echinococcus oligarthus* on the other hand are restricted to Central and South America (Eckert and Deplazes, 2004). Further members of the genus *Echinococcus* are *Echinococcus equinus* (Le et al., 2002), *Echinococcus felidis*, *Echinococcus ortleppi* and *Echinococcus shiquicus* (Nakao et al., 2007). *Echinococcus* belongs to the family of Taenidae in the order Cyclophyllidea and subclass Eucestoda. Eucestoda in turn are placed in the class Cestoda that is part of the Platyhelminthes (see Figure 2). The phylum Platyhelminthes consists of the free living Turbellaria and the parasitic classes Cestoda and Trematoda (Ehlers, 1986). Important members of the Trematoda are *Schistosoma mansoni* and *Schistosoma haematobium*, the pathogens of schistosomiasis, and *Faciola hepatica*. While Cestoda

and Trematoda are monophyletic, the free living Turbellaria form a paraphylectic taxa (Littlewood et al., 1999). Platyhelminthes are Protostomia and belong to the Lophotrochozoa (Olson et al., 2011). The Protostomia have two main branches, the Ecdysozoa and the Lophotrochozoa (Giribet, 2008). The moulting Ecdysozoa include the Arthopoda like the fruit fly *Drosophila melanogaster*, and the Nematoda that contain important parasitic and non-parasitic worms such as *Ascaris lumbricoides*, *Onchocerca volvulus*, *Loa loa* and *Caenorhabditis elegans*. The non-moulting Lophotrochozoa, on the other hand, include the Mollusca such as *Aplysia californica*, the Annelida and the Platyhelminthes.

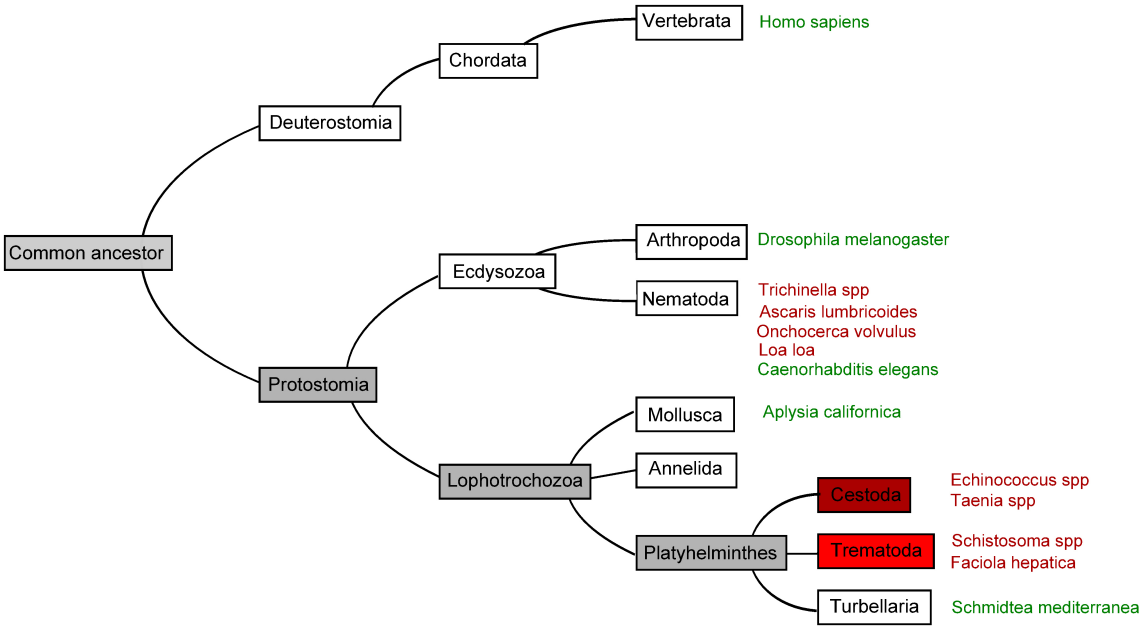
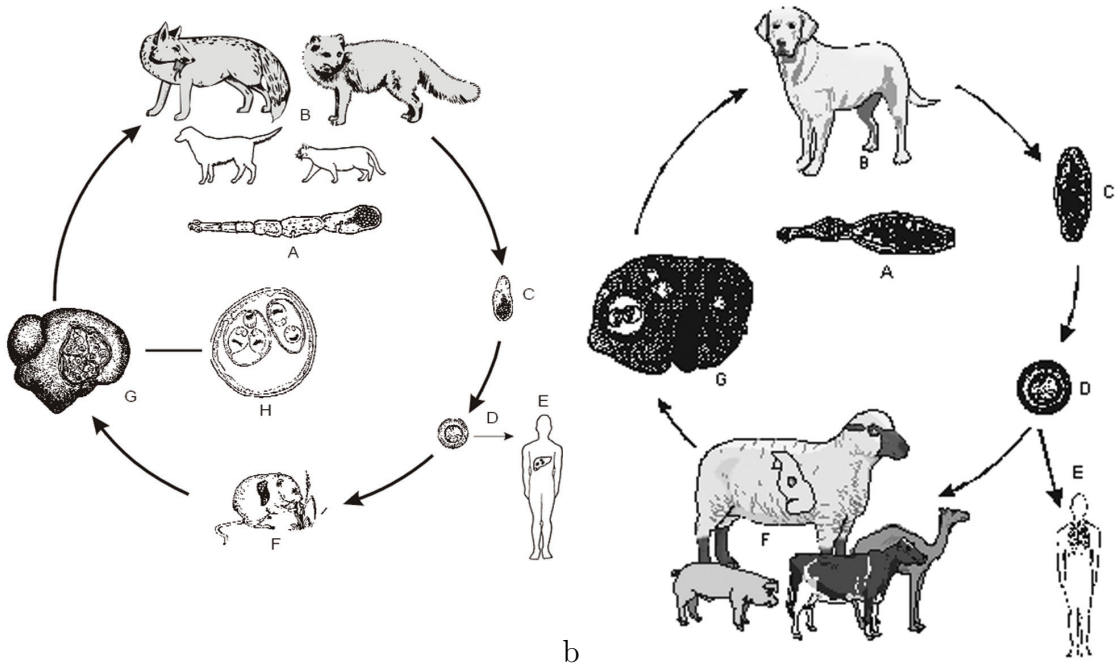


Figure 2: **Phylogenetic position of the tapeworm genera *Echinococcus*** (see Förster, 2012): Parasites are marked in red, non-parasitic organisms in green. The completely parasitic classes Cestoda and Trematoda are shown as red boxes.

1.1.2 Biology and live cycle of *E. multilocularis* and *E. granulosus*

E. multilocularis and *E. granulosus* are small tapeworms with a length of 2 to 7 mm. Though their life cycles are quite similar, they infect different definitive and intermediate hosts. While the adult worm of *E. granulosus* resides in the small intestine of dogs or other canids, the definitive hosts of *E. multilocularis* are foxes. Typical intermediate hosts for *E. granulosus* are ungulates such as sheep, cows, pigs and horses whereas *E. multilocularis* uses small mammals, especially rodents, as intermediate hosts (see Figure 3). Humans are aberrant intermediate hosts both for *E. multilocularis* and *E. granulosus* (Eckert and Deplazes, 2004).



a b

Figure 3: **Life cycle of *E. multilocularis* (a) and *E. granulosus* (b) (Eckert and Deplazes, 2004):** (A) Adult parasite, (B) Foxes/domestic dogs as principal definitive hosts, (C) Protglottid with eggs, (D) Egg with oncosphere, (E) Infection of human, (F) Rodent/sheep as principal intermediate host, (G) Rodent liver with metacestodes/sheep with liver cyst, (H) Single metacestode cyst with protoscoleces.

Infection of the intermediate host is initiated by oral uptake of eggs that harbor the infectious oncosphere. After passage through gut and small intestine the activated oncosphere hatches from the egg and actively penetrates the epithelium of the small intestine. Through transport with the blood flow, the oncosphere gains access to the inner organs and there transforms into the metacestode vesicle (Brehm et al., 2006). The metacestode vesicle consists of an inner cellular layer, the germinal layer, and an outer acellular layer, the laminated layer. The laminated layer is composed of carbohydrates with parasite and host proteins (Brehm, 2010). The inner germinal layer develops protoscoleces inside the metacestode vesicle. While the *E. granulosus* metacestode vesicle forms one massive "hydatid cyst" with a thick laminated layer and grows by size, the *E. multilocularis* metacestode vesicle has a thinner laminated layer. *E. multilocularis* metacestode vesicles not only grow by size but also develop daughter vesicles through budding and form a multi-vesicular parasite tissue which infiltrates the surrounding host tissue (Dixon, 1997).

E. multilocularis and *E. granulosus* have different organ tropism. In humans *E. multilocularis* is almost exclusively found in the liver (99%); *E. granulosus* on the other hand also infects the lungs in 17% (Eckert and Deplazes, 2004). In contrast to *E. granulosus*, *E. multilocularis* has the capacity to metastasize into other organs

like brain, heart and kidney (Brehm, 2010).
When the definite host takes the prey, the invaginated protoscoleces inside the brood capsules of the metacestode vesicle become free and are activated by passage through gut and small intestine. After evagination of the scolex (head region), that is accelerated by the presence of bile, the protoscoleces move inside the small intestine and settle between the villi in the crypts of Liberkühn attaching to the epithelium with its hooks and suckers (Smyth, 1968). After segmentation and self insemination, embryonated eggs containing the 6-hooked oncosphere develop in the last proglottid (Smyth et al., 1967). They are shed with the faces and taken up by the intermediate host; so the life cycle starts anew.
A special characteristic of *Echinococcus* protoscoleces is its ability to re-differentiate. Protoscoleces in the intestine of their definite host develop into a strobilar direction and become adult worms. Protoscoleces in other locations, e.g. the peritoneum, re-differentiate into a vesicular direction. It has been shown that the major factor for development into the strobilar direction is physical contact to a protein containing substrate, otherwise protoscoleces eventually develop into a vesicular direction (Smyth et al., 1966, 1967).

1.2 Alveolar and cystic echinococcosis

Alveolar and cystic echinococcosis are important zoonoses that are caused by the metacestode larval stage of *E. multilocularis* and *E. granulosus*. Alveolar echinococcosis is the most dangerous parasitosis in Europe. Untreated it causes death within 10-15 years after diagnosis in over 90% of the patients (Craig, 2003). Nonetheless echinococcosis is a very rare disease in Europe. Since the beginning of the notification requirement in Germany in 2001 there have been reported about 20 new cases of alveolar echinococcosis and 60 new cases of cystic echinococcosis every year (RKI, Date of inquiry: 15.11.12). Almost all cases of cystic echinococcosis are imported and not aquired in Germany. The highest reported prevalence of echinococcosis today is in the Sichuan Province in China with a prevalence of 3.2% for cystic echinococcosis, 3.1% for alveolar echinococcosis and 0.04% for dual infection (Li et al., 2010).
Humans get infected by the oral uptake of infectious eggs. Like in other intermediate hosts the oncosphere is activated by passage through gut and small intestine, penetrates the epithelium and is carried to the liver with the blood flow. The first manifestation of alveolar echinococcosis almost always is in the liver; the first manifestation of cystic echinococcosis, however, is only in 69% in the liver and in 17% in the lungs (Eckert and Deplazes, 2004). While *E. granulosus* forms one confined

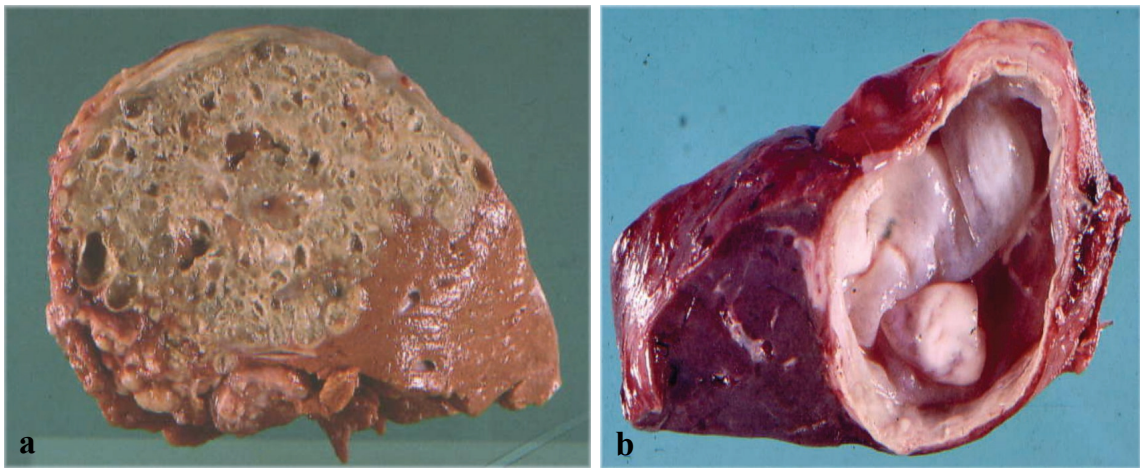


Figure 4: **Liver manifestation of *E. multilocularis* (a) and *E. granulosus* (b) (Eckert and Deplazes, 2004):** (a) Alveolar echinococcosis in a 62-year-old Swiss patient (maximum diameter of single cysts approximately 1.5 cm), (b) Cystic echinococcosis in a patient (endocyst removed; lesion size approximately 3 by 3.5 cm).

cyst, *E. mulitlocularis* can grow infiltratively like a malignant tumor (Dixon, 1997) (see Figure 4).

Cyst growth is generally slow, therefore the latency of echinococcosis is long. It is estimated that first lesions can be detected approximately 5 years after infection. The initial phase of infection is asymptomatic. In a later phase it may cause abdominal pain, nausea, vomiting and liver symptoms such as icterus, cholestasis and thrombosis of the portal vein (Kern, 2010). Rupture of cysts can induce anaphylactic reactions such as urticaria, edema or even anaphylactic shock. Anaphylactic reaction are know to occur in cystic echinococcosis but far less frequent in alveolar echinococcosis (Vuitton, 2004).

Diagnosis of echinococcosis is accomplished with imaging and immunodiagnostic techniques. Ultrasonography is the most wildly used imaging technique due to its availability and effectiveness (Rogan et al., 2006). Other important imaging techniques are computer tomography (CT) and magnetic resonance tomography (MRT) (Moro and Schantz, 2009). Immunodiagnostic techniques include detection of parasite specific antibodies and antigens with ELISA and Western blot. For alveolar echinococcosis a classification system has been developed based on the TNM classification for malignant tumors. The PNM classification of alveolar echinococcosis includes three main factors: the location and extension of the primary parasitic lesion within the liver (P), the involvement of neighbour organs (N) and the presence or absence of metastasis (M) (Kern et al., 2006). The classification system does not only facilitate communication among clinicians but also is an important tool to

make decisions on treatment strategies.
The only cure of echinococcosis is the surgical removal of the complete parasite tissue (Moro and Schantz, 2009). Therefore radical surgery is the first choice whenever a total resection of the lesion is possible. Unfortunately only in 20-30% a curative resection is possible (RKI, 2006). Consequently a livelong medicamentous therapy with benzimidazoles (mebendazole, abendazole) is mandatory for most patients. Benzimidazoles bind to the parasite β-tubulins and inhibit microtubuli formation (Brehm et al., 2000; Jura et al., 1998). However, they are only parasitostatic, not parasitocidal, at applied concentrations and hence have to be taken livelong (Reuter et al., 2004).

1.3 Molecular biology of *Echinococcus* and host-parasite relationship

1.3.1 *In vitro* cultivation of *E. mulilocularis* and *E. granulosus* larval stages

First successful *in vitro* cultivation methods for *E. multilocularis* have been established in the mid-1990s by Hemphill and Gottstein (1995) as well as by Jura et al. (1996). Both the tissue block system by Hemphill and Gottstein and the co-cultivation of homogenized parasite tissue with rat hepatoma cells from Jura et al. rely on the presence of serum and co-cultivation of parasite material with host cells. The presence of serum and host cells is absolutely necessary for parasite growth and development in both systems. Without host cells the parasite material degenerates after short periods of time, indicating that so-called feeder cells secret soluble factors necessary for parasite growth and development.
The problem of all co-cultivation systems is the difficulty to study effects of defined host factors on the parasite due to the continuous presence of the host cells. Therefore an axenic cultivation system was developed by Spiliotis et al. (2004) for cultivation of metacestodes. In this system reducing conditions (nitrogen atmosphere and reducing agents) are of uttermost importance for survival of the parasite. Another key factor is the addition of feeder-cell-conditioned medium. Although reducing conditions and the presence of serum allow the survival of the parasite for several weeks, parasite growth and development depends on addition of conditioned medium, again indicating that soluble factors secreted by the feeder cells are essential for parasite development (Brehm, 2010).
Another great achievement is the isolation and cultivation of *E. mulilocularis* primary cells. primary cells are isolated from metacestode vesicles and cultivated in

co-culture or axenic culture (Spiliotis and Brehm, 2009). In freshly prepared primary cell cultures up to 80% of the cells are germinative cells (Koziol et al., 2014). Within days the primary cells aggregate and form central cavities. First vesicles can be observed after 2-6 weeks of cultivation. The metacestode vesicles have the capacity to develop protoscoleces in culture and when injected into the peritoneum of jirds, lead to infection and growth of parasite material (Spiliotis et al., 2008).
Cultivation of *E. granulosus* is mostly based on protoscoleces. Protoscoleces are obtained from hydatid cysts of naturally infected animals (Fernández et al., 2002) and cultivated as described for *E. multilocularis*. Protoscoleces can re-differentiate into pre-microcysts and microcysts *in vivo* and *in vitro* (Heath and Osborn, 1976; Rodriguez-Caabeiro and Casado, 1988; Walker et al., 2004). Contrary to *E. multilocularis* metacestodes, *E. granulosus* metacestodes increase in size but do not further proliferate *in vitro* once the laminated layer is formed (Hemphill et al., 2010, Brehm, K; pers. commun.).
The fact that *E. multilocularis* proliferates *in vitro* as well as the development of the axenic and the primary cell culture system turn *E. multilocularis* into a suitable model to study host-parasite interactions and molecular developmental processes.

1.3.2 Evolutionarily conserved signalling pathways in *E. multilocularis*

Cell-cell communication and important signalling pathways are well conserved between vertebrates and invertebrates due to their early rise in metazoan evolution (Brehm et al., 2006). The signalling molecules are not only structurally related but often also functionally exchangeable. For example the mammalian insulin can stimulate the insulin receptor of *Drosophila melanogaster* (Fernandez et al., 1995). It also has been shown that the *Echinococcus* insulin receptor binds human Insulin and IGF-I as well as the *Echinococcus* insulin-like peptides and activates the PI3K/Akt signalling cascade of the parasite (Konrad et al., 2003; Hemer et al., 2014). Other components of conserved signalling pathways have already been identified in *E. multilocularis*: the epidermal growth factor receptor (EGFR) (Spiliotis et al., 2003), the MAP kinase cascade (Spiliotis et al., 2006; Gelmedin et al., 2008, 2010) and receptors of the TGF-β and BMP family (Zavala-Gongora et al., 2006). The study of evolutionarily conserved signalling pathways is of great importance for the understanding of host-parasite interactions. Hormonal host-parasite cross-communication might explain how host factors influence survival and development of the parasite and how the parasite in turn influences the immune system of the host. For example, insulin stimulates parasite growth by interaction with *E. multilocularis* insulin receptors (EmIRs) (Hemer et al., 2014).

1.3.3 Molecular and biochemical approaches

One important aim of the studies in the last decades was the identification of specific antigens for diagnosis of echinococcosis (Ito, 2002). *emelp*, coding for an ERM-like protein, was the first chromosomal locus described for *E. multilocularis* (Brehm et al., 1999). Trans-splicing in *E. multilocularis* was reported by Brehm et al. (2000). Trans-splicing is the fusion of two RNA molecules in order to form a mature mRNA. Two forms are distinguished: the genic trans-splicing and the spliced leader (SL) trans-splicing. Genic trans-splicing means that portions of two different RNA transcripts are joined together at splice sides. In spliced leader trans-splicing the donor RNA is a so called spliced leader that is connected to the 5' end exon of a RNA transcript (Hastings, 2005; Lasda and Blumenthal, 2011). In *E. multilocularis* 5 SL-sequences have been identified: SL1, SL2a, SL2b, SL2c and SL2d. Though their gene sequence is slightly variable, no functional preference concerning the genes spliced by each SL could be determined (Tsai et al., 2013, Ference Kiss, pers. commun.). In *E. multilocularis* 13,9% of 10546 predicted genes are presumably trans-spliced. In spliced leader differential display transspliced cDNAs of *E. multilocularis* were amplified with primers against SL1 and poly-A tail sequences (Brehm et al., 2003). This method allowed analysis of gene expression patterns of different larval stages as well as the production of SL based cDNA libraries for *E. granulosus* (Fernández et al., 2002). In 2008 the Wellcome Trust Sanger Institute (Hinxton, Cambridge, UK) started the whole genome sequencing project for *Echinococcus* in cooperation with the groups of Klaus Brehm (Würzburg, Germany) for *E. multilocularis* and Cecilia Fernández (Montevideo, Uruguay) for *E. granulosus*. The genomes of *E. multilocularis* and *E. graulosus* were published in 2013 (Tsai et al.), together with the genomes of two other important tapeworms: *Taenia solium* and the laboratory model *Hymenolepis microstoma*. A transcriptome project of different *E. multilocularis* larval stages is currently in process (Sanger Institute in cooperation with Klaus Brehm). An analysis of the *E. granulosus* transciptome was published in 2012 (Parkinson et al.). The new genome (http://www.genedb.org/Homepage) and transcriptome data not only give insights into the metabolism, detoxification and signalling pathways but provide an important resource for the development of new treatments.

1.4 The cAMP-signalling pathway

1.4.1 G protein coupled receptors

G protein coupled receptors (GPCRs) form the largest protein family in vertebrates and can be found in almost all eukaryotic organisms, which indicates that they are of ancient origin (Römpler et al., 2007). There were more than 800 GPCR sequences identified in the human genome (Fredriksson et al., 2003). GPCRs play an important role in many signal transduction pathways and are important drug targets. They are defined as seven transmembrane proteins that interact with G proteins (Pierce et al., 2002). However, most of the proteins classified as GPCRs have never been shown to interact with G proteins and several GPCRs are known to interact with alternative signalling pathways (Schiöth et al., 2007). GPCRs show great diversity of ligand and G protein binding domains and there is no sequence similarity between all GPCR families except for the seven α-helices that form the central core of the receptor (Bockaert and Pin, 1999). The seven transmembrane domains have been numbered clockwise seen from the cytoplasm (see Figure 5). As the seven transmembrane domains are relatively well conserved, they are used to classify GPCRs (Schiöth and Fredriksson, 2005). The N-termini of GPCRs on the other hand are very diverse. Four of the five main GPCR families have long N-termini with functional or ligand binding domains, e.g. the ligand binding domain of the *Secretin* family is located in the N-terminus (Schiöth et al., 2007).
A conformational change due to ligand binding is responsible for receptor activation; in rhodopsin receptors ligand binding leads to rearrangement of transmembrane domains III and IV (Bockaert and Pin, 1999). Although GPCRs have been considered to be functional as monomers, there is new evidence that at least some GPCRs, e.g. the $GABA_B$ receptor, only function as heterodimers. In other cases receptor dimerization is not necessary for function but might modulate signal transduction (Bockaert and Pin, 1999; Pierce et al., 2002). GPCRs are generally low expressed compared to the average gene expression and the expression level of the five main families is conserved between species (Schiöth et al., 2007).
There are two well know classification systems of GPCRs: the A-F classification system by Kolakowski (1994) and the GRAFS classification system by Fredriksson et al. (2003); Fredriksson and Schiöth (2005). Fredriksson and colleagues collected human GPCR sequences through TBLASTN and hidden Markov model searches and divided them into families using strict phylogenetic criteria (Lagerström and Schiöth, 2008). The GRAFS classification system describes five main families: *Glutamate* (G), *Rhodopsin* (R), *Adhesion* (A), *Fizzled/Taste2* (F)and *Secretin* (S).

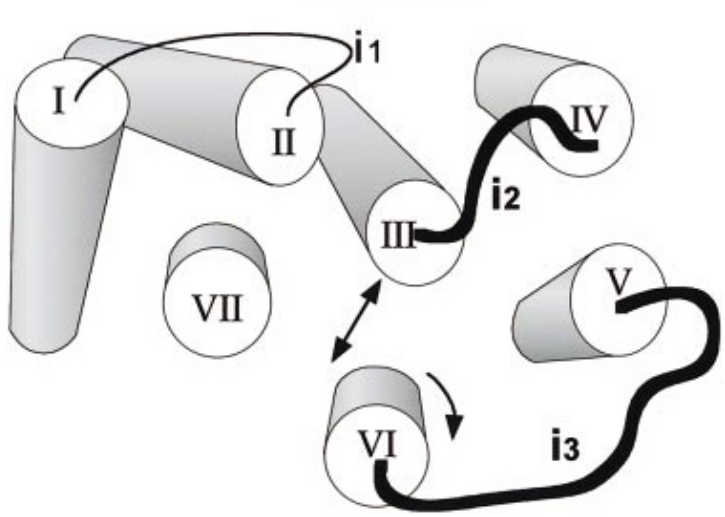


Figure 5: **Illustration of the central core of rhodopsin viewed from the cytoplasm (Bockaert and Pin, 1999, Copyright ©1999 European Molecular Biology Organization):** The structure is deduced from the two-dimensional crystal of bovine and frog rhodopsin and represents the "active conformation". The seven transmembrane domains, that form the central core of the receptor, have been numbered clockwise seen from the cytoplasm. i1, i2 and i3 are intracellular loops.

1.4.2 Adenylate cyclase

Soluble and membrane bound adenylate cyclases convert ATP into the second messenger cAMP. The membrane bound adenylate cyclase (AC) superfamily has nine mammalian isoforms that can be divided into four groups. Group 1 consists of AC1, AC3 and AC8 and is activated by calcium and calmodulin (Hanoune et al., 1997). Group 2, consisting of AC2, AC4 and AC7, is stimulated by the $\beta\gamma$ subunit of the activated G_s-Protein and by phosphorylation with protein kinase C, whereas activity of group 3 ACs (AC5 and AC6) is inhibited by low concentrations of calcium (Hanoune et al., 1997; Gao et al., 2007). The widely expressed AC9 (group 4) can only be activated by the $G_s\alpha$ subunit (Premont et al., 1996). Contrary to most other AC types, AC9 is not limited to certain tissues, but is expressed in most tissues at a high level (Premont et al., 1996). The AC9 sequence is quite divergent compared to other AC types. Another difference is that the mammalian AC9, contrary the other AC types, can not be activated by the diterpene forskolin (Premont et al., 1996; Yan et al., 1997; Pavan et al., 2009).

The adenylate cyclase is presumed to have two transmembrane spans M1 and M2 with each six transmembrane domains and two cytosolic domains C1 and C2, that probably evolved through intragenic duplication (Sossin and Abrams, 2009). The cytosolic domains are further distinguished into C1a, C1b and C2a, C2b (see Figure 6). C1a and C2a are conserved, homologous to each other and catalytically active

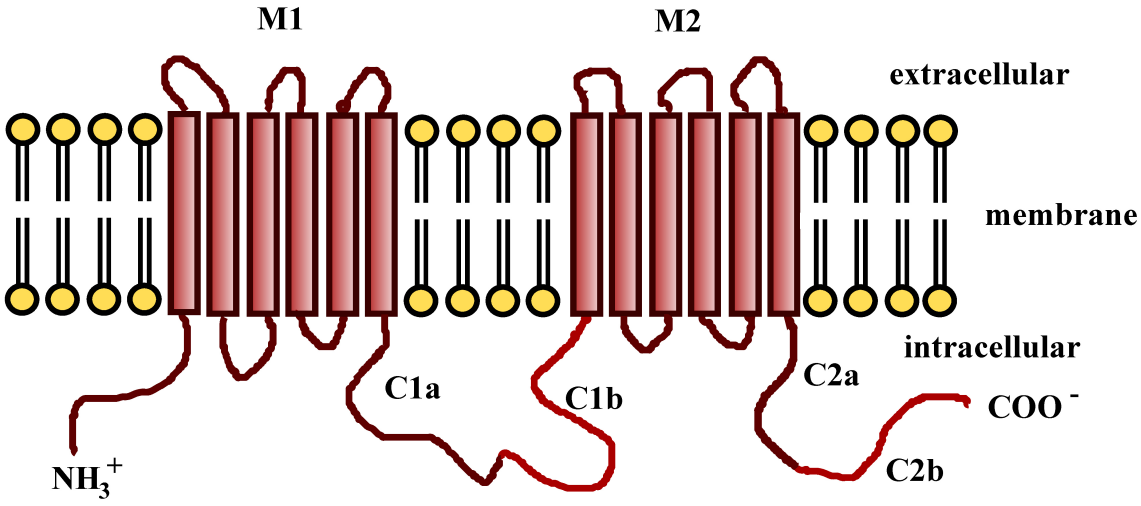


Figure 6: **Structure of membrane bound adenylate cyclases:** Hydropathy predictions of the AC amino acid sequence reveal 12 hydrophobic spans, which are considered to be transmembrane domains. They form two sets, M1 and M2, each consisting of 6 transmembrane domains. C1 and C2 are major hydrophilic regions located in the cytoplasm. C1 and C2 and are further distinguished into C1a, C1b and C2a, C2b. C1a and C2a are required for catalytic activity. Both N-terminus and C-terminus are predicted to be located intracellular (Hanoune et al., 1997).

whereas C1b and C2b are less conserved parts (Tang et al., 1995; Tesmer and Sprang, 1998).

For catalytic activity C1a and C2a form a heterodimer and build the active site. Association of both domains is required for high enzymatic activity (Tang et al., 1991, 1995; Hurley, 1998, 1999). However, only the C2 domain is catalytic active while C1 is presumed to enhance enzymatic activity (Yan et al., 1997).

One molecule forskolin binds to the C1·C2 heterodimer of the AC types 1-8, presumably directly alters conformation of the active site and thus facilitates cAMP synthesis (Tesmer et al., 1997). Mammalian type 9 AC cannot be activated by forskolin due to mutations concerning amino acids that are important for forskolin binding(Yan et al., 1998).

1.4.3 The cAMP signalling pathway

The cAMP signalling pathway is important for quite different biological processes such as metabolic pathways, secretory processes, muscle contraction, gene transcription, differentiation, cell growth, apoptosis and memory (Houslay and Milligan, 1997; Hanoune et al., 1997; Patel et al., 2001).

cAMP was discovered by Sutherland and Rall in 1958. Figure 7 shows the cAMP signalling pathway. GPCRs are stimulated by various ligands, such as hormones,

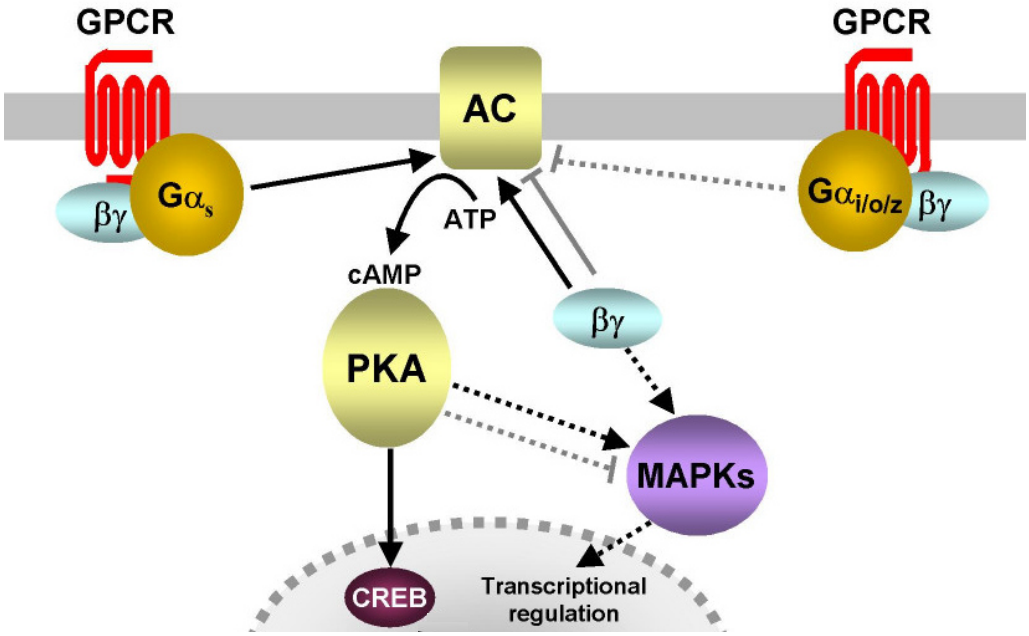


Figure 7: **cAMP signalling pathway (New and Wong, 2007):** Ligand binding to GPCRs leads to a conformational change in the GPCRs that facilitates dissociation of the Gα subunit from the receptor and the βμ subunit. The $G_S\alpha$ subunit has a stimulatory effect on adenylate cyclase which in turn produces cAMP. AC activity is inhibited by the $G_i\alpha$ subunit. The βγ subunit can inhibit or stimulate adenylate cyclase, depending on the adenylate cyclase type, or influence other pathways. cAMP molecules bind to the regulatory subunits of the protein kinase A (PKA) and thereby set the catalytic subunits free. PKA phosphorylates target proteins like the cAMP responsive element-binding protein (CREB). PKA can also influence other pathways such as the MAP kinase pathway.

peptides, lipids, nucleotides etc. (Bockaert and Pin, 1999). The binding of the ligand leads to a conformational change (rearrangements of transmembrane helices) in the GPCRs and permits them to act as guanine nucleotide exchange factors (GEFs) for the bound heterotrimeric G protein (Pierce et al., 2002). The GTP-Gα subunit dissociates from the receptor and the βγ-complex; both the GTP-Gα subunit and the βγ-complex then modulate activity of their various effector proteins (Wettschureck and Offermanns, 2005). Binding of the free $G_S\alpha$ subunit to the adenylate cyclase induces a conformational change in the adenylate cyclase which allosterically stimulates cAMP production (Tesmer et al., 1997); it probably doesn't only "glue" the two catalytic domains together but also has an additional function (Hurley, 1999). The $G_i\alpha$ subunit on the other hand inhibits AC activity (Simonds, 1999).
Two cAMP molecules bind to each regulatory subunit of the protein kinase A. After cAMP binding, the two catalytic subunits are set free from the regulatory ones. PKA phosphorylates a number of different proteins. Among them are GPCR kinases

(GRKs) that phosphorylate the GPCRs, inhibit the receptor Gα subunit interaction (Bohn and Schmid, 2010), facilitate the binding of arrestin to the receptor and promote internalization by clathrin (Billington and Penn, 2003; Billington and Hall, 2012). Tough for a long time this mechanism was considered a negative feed back loop, new studies indicate that certain GPCRs influence cAMP production even after receptor internalization (Jalink and Moolenaar, 2010). Another target of PKA is the cAMP responsive element-binding protein (CREB) which is a transcription factor and induces middle to longtime changes in the cell transcription (New and Wong, 2007). The cAMP stimulation of AC is terminated by the degradation of cAMP through phosphodiesterases (Francis et al., 2011).

1.4.4 The cAMP signalling pathway in invertebrates

Like in mammalians, cAMP signalling regulates a variety of biological processes in invertebrates. Serotonin mediated activation of adenylate cyclase in *S. mansoni* and *F. hepatica* is important for glycolysis and neuromuscular activity (Estey and Mansour, 1987; de Saram et al., 2013). While mammalian adenylate cyclase is only weakly activated by serotonin, adenylate cyclase of adult *S. mansoni* is highly activated by serotonin (Kasschau and Mansour, 1982; Estey and Mansour, 1988). Serotonin and cAMP also regulate developmental processes in *S. mansoni*. Serotonin and forskolin strongly inhibit miracidial transformation, while decrease of intracellular cAMP level, together with an increase in Ca^{2+} mobilization, leads to transformation (Kawamoto et al., 1989; Taft et al., 2010).
Though there are differences, the adenylate cyclase pathway in *S. mansoni* resembles the one found in mammals: it has at least an receptor, a GTP binding regulatory protein complex and a catalytic subunit of adenylate cyclase (Kasschau and Mansour, 1982). GPCRs in *S. mansoni* and *S. mediterranea* have been analysed by Zamanian et al. (2011). They identified 117 GPCRs in *S. mansoni* and 418 in *S. mediterranea*, representing all of the major GPCR families (*Rhodopsin*, *Glutamate*, *Adhesion*, *Secretin* and *Frizzled*), including several serotonin receptors within the *Rhodopsin* family. Following receptor activation, $G_s\alpha$ dissociates from the receptor and activates adenylate cyclase. Various adenylate cyclases have been identified in invertebrates. AC1 is highly conserved with one orthologue in *Aplysia*, **Lottia** and *Drosophila* (Sossin and Abrams, 2009). In *D. melanogaster* AC1 is essential for learning and memory (Iourgenko and Levin, 2000). Most invertebrates appear to have one single orthologue of group 2 adenylate cyclases (AC2,AC4,AC7) (Sossin and Abrams, 2009). AC2 is required for viability in *C. elegans* (Korswagen et al., 1998). Like AC1, AC3 is well conserved and most organisms have an orthologue;

the *D. melanogaster* orthologue is expressed in olfactory tissue and the central nervous system (Iourgenko and Levin, 2000). In *Aplysia*, however, no AC3 orthologue was identified (Sossin and Abrams, 2009). The group 3 adenylate cyclases AC5 and AC6 are closely related; most invertebrates posses a single orthologue of this group, that is well conserved through evolution (Sossin and Abrams, 2009). AC9 on the other hand is most different from the other adenylate cyclases. There has been found a clearly related orthologue in every examined invertebrate (Sossin and Abrams, 2009). While mammalian AC9 are insensitive to forskolin stimulation, the AC9 orthologue of D. melanogaster was shown to be sensitive to forskolin stimulation (Iourgenko et al., 1997). It is also noteworthy that D. melanogaster has an additional AC family. The four AC10 are important for spermatogenesis and not conserved within vertebrates or invertebrates (Cann et al., 2000). The produced cAMP then activates the cAMP dependent protein kinase which is ubiquitous in eukaryotic organisms (Bowen et al., 2006).
In *E. multilocularis* genes for G proteins and protein kinase A have already been analysed. Six Gα, three Gβ and one Gγ subunits of G proteins were identified (Burkhardt, 2011). Four catalytic subunits of protein kinase A and three regulatory subunits were found in the genome(Schilling, 2010). Therefore this work concentrates on the GPCRs and the adenylate cyclases of *E. multilocularis*.

1.5 Serotonin

Serotonin, also called 5-Hydroxytryptamine (5-HT), is a widely distributed neurotransmitter and paracrine hormone that modulates a wide range of biological processes in most of the human organs (Berger et al., 2009; Berumen et al., 2012). Serotonin is mainly found in the the gastrointestinal tract (about 95%) and in the brain. In the gastrointestinal tract serotonin acts both as a neurotransmitter and a paracrine hormone and plays an important role in the peristaltic movement (Lesurtel et al., 2008). In the central nervous system serotonin serves as neurotransmitter and neuromodulator influencing, amongst others, sleep, appetite, sensation of pain, regulation of temperature and mood. Depression may arise due to absence of serotonin or a serotonin precursor and can be treated with serotonin reuptake inhibitors (Terry et al., 2008; Baganz and Blakely, 2013). Serotonin also plays a regulating role in aggregation of platelets and coagulation (Bertaccini, 1960; Yuan et al., 2006; McCloskey et al., 2008), in the immune system (Baganz and Blakely, 2013), in blood pressure (Berger et al., 2009), in remodeling after myocard infarct (Berger et al., 2009), in endocinology and metabolism (Berger et al., 2009).
While platelets are not able to produce serotonin and therefore take it up from

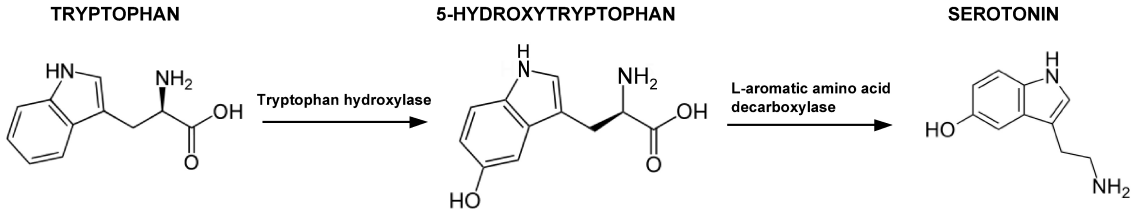


Figure 8: **Biosynthesis of serotonin:** Serotonin is synthesized from tryptophan in two steps: the rate-limiting hydroxylation of tryptophan catalyzed by the tryptophan hydroxylase and the following decarboxylation of the resulting hydroxytryptophan catalyzed by the L-aromatic amino acid decarboxylase.

the blood, enterochromaffin cells and neurons synthesize serotonin from the amino acid tryptophan (Liu et al., 2008; Lesurtel et al., 2008). The rate limiting step in this process is the hydroxylation of tryptophan by the tryptophan hydroxylase (Côté et al., 2003) which is followed by the decarboxylation of hydroxytryptophan (5-HTP) to serotonin (5-Hydroxytryptophan) by the amino acid decarboxylase (see Figure 8). In vertebrates serotonin is mainly degraded by the monoamine oxidase A; to a lesser extend by the monoamine oxidase B (Culpepper, 2012). Another metabolism pathway is the degradation of serotonin to melatonin, a hormone produced by the pineal gland (Klein, 2007).

1.5.1 Serotonin receptors

Serotonin binds to a wide range of receptors that have been divided into seven main types with various subclasses based on binding affinities, structure and transduction mechanisms (Hoyer et al., 1994; Berumen et al., 2012). All serotonin receptors with the exception of the 5-HT_3 receptor are GPCRs of the *Rhodopsin* family and activate different G proteins (Fredriksson et al., 2003; Berumen et al., 2012). 5-HT_1 and 5-HT_5 receptors activate $G_{i/o}$ and therefore inhibit adenylate cyclase, while 5-HT_4, 5-HT_6 and 5-HT_7 receptors activate G_s and in consequence stimulate adenylate cyclase. The 5-HT_2 receptor activates $G_{q/11}$ which leads to hydrolysis of phosphoinositides, producing diacyl gycerol (DAG) and inositol phosphates (IP_3). IP_3 in turn elevates the intracellular calcium level and activates phosphate kinase C (Hoyer et al., 1994; Frazer and Hensler, 1999; Millan et al., 2008; Berumen et al., 2012). The 5-HT_3 receptor is a ligand gated Na^+/K^+ ion channel, whose activation causes neuronal excitation and subsequent neurotransmitter release (Berumen et al., 2012).

The different receptor types and subclasses fulfill a variety of different functions. While all receptor types can be found in the brain, certain receptor types also have specific functions outside the CNS (Terry et al., 2008). 5-HT_{1A} and 5-HT_{2C}

receptors, for example, play a role in pancreatic regeneration(Lesurtel et al., 2008) while 5-HT_{2A} and 5-HT_{2B} receptors are important for liver regeneration after partial hepatectomy (Lesurtel et al., 2006). Serotonin receptors also are may also cause migraine. 5-HT_{1B} and 5-HT_{1D} receptors influence vasodilatation and vasoconstriction of cerebral blood vessels (Berger et al., 2009) which can lead to migraine. Agonists of 5-HT_{1B} and 5-HT_{1D} receptors, e.g. triptans, are used in migraine treatment (Balbisi, 2004; Pascual, 2004). Mitogenic effects in fibroblasts are mediated by 5-HT_{2A} and 5-HT_{2C} receptors (Lesurtel et al., 2008). 5-HT_3 receptors, the Na^+/K^+ ion channels, are responsible for depolarization in myenteric neurons and resulting smooth muscle contraction, the vomiting reflex in the CNS and, together with 5-HT_4 receptors, the peristaltic reflex. Several cancer chemotherapeutic substances, including cisplatin, provoke release of serotonin from enterochromaffin cells which causes nausea and vomiting through stimulation of 5-HT_3 receptors (Lesurtel et al., 2008). 5-HT_4 receptors also may influence cardiac function and ventricular remodeling processes (Berger et al., 2009). The 5-HT_5, 5-HT_6 and 5-HT_7 receptors are predominantly distributed in the brain.

1.5.2 Serotonin transporter

In humans the serotonin transporter plays an important role in terminating transmitter action by removing serotonin from the synaptic cleft and in the active accumulation of plasma serotonin in platelets (Lesurtel et al., 2008). The serotonin transporter represents a molecular target for various antidepressants, such as tricyclic antidepressants or serotonin selective reuptake inhibitors (SSRI) (Horschitz et al., 2001; Neubauer et al., 2006). The serotonin transporter belongs to the solute carrier 6 (SLC6) family, a family of Na^+/Cl^--dependent neurotransmitter transporters, that also includes transporters for γ-aminobutyric acid, norepinephrine, dopamine and glycine (Andersen et al., 2009). SCL6 transporters use the Na^+ gradient as driving force for the substrate transport. Extracellular Na^+ and Cl^-, whose role is not fully understood, are required for translocation of the substrate (Kristensen et al., 2011). A high-resolution X-ray crystallographic structure of a bacterial homologue of the SLC6 transporter, the leucine transporter (LeuT) from *Aquifex aeolicus*, was first reported by Yamashita et al. (2005) and, together with other LeuT structures (Singh et al., 2008), has been used to construct three-dimensional models of SCL6 transporters (Kristensen et al., 2011). The transporter consists of 12 transmembrane domains (TMs), with the first ten forming the protein core. Domains 1-5 are related to domains 6-10 (Yamashita et al., 2005). The transmembrane domains form a cylindrical shape with an interior and an outer part. The inner ring, which holds

the substrate binding side, is formed by TM1, TM3, TM6 and TM6 (see Figure 9) (Kristensen et al., 2011).

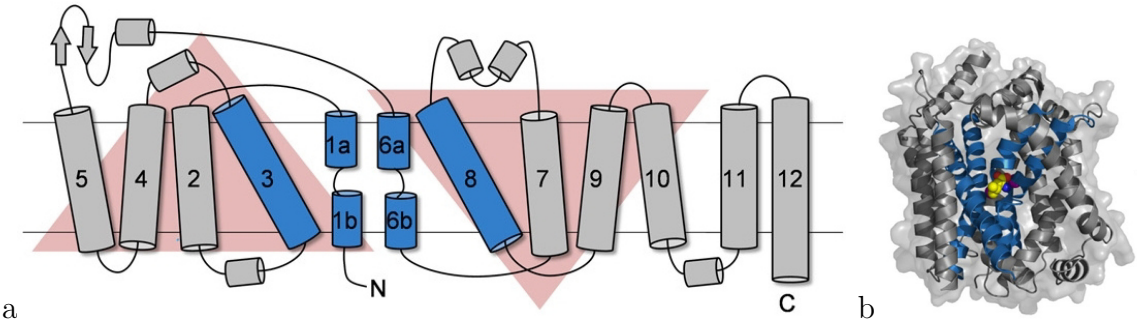


Figure 9: **X-ray crystal structure and topology of LeuT, a bacterial homologue of SLC6 neurotransmitter transporters (Kristensen et al., 2011):** (a) The transporter consists of 12 transmembrane domains, with the first ten forming the protein core. The inner ring (TM1, TM3, TM6, and TM8), that forms the substrate binding site, is shown in blue. The red triangles highlight the 5+5 inverted repeats, formed by TMs 1 to 5 and TMs 6 to 10, that are related by an apparent two-fold symmetry. (b) The structure of LeuT with the TM domains that form the inner ring highlighted in blue is seen from the side. TM10 is omitted for clarity.

1.5.3 Serotonin in invertebrates

Serotonin is a phylogenetically old molecule that is widely distributed in most metazoans and some protozoans (Turlejski, 1996). One of first roles of serotonin was the intracellular regulation of cell activity, followed by its function as a hormone or growth regulating factor. Later serotonin became an important morphogenetic and trophic factor and controlled cell proliferation and differentiation. Its function as neuromodulator and neurotransmitter developed last in evolution (Turlejski, 1996). Serotonergic neurons were found in all investigated arthropods and insects (Turlejski, 1996). Furthermore serotonin is known to influence developmental processes (Turlejski, 1996) and promote sleep states (Flavell et al., 2013) in *Drosophila*. *Drosophila* has three different 5-HT-receptors that act through G proteins and are expressed exclusively in neurons (Saudou et al., 1992). Serotonin is also found in many worm species. It promotes dwelling behavior and food related behavior in *C. elegans* (Flavell et al., 2013) and regulates developmental processes like regeneration in planaians (Franquinet, 1979).

In *S. mansoni* serotonin influences glycolysis, glucose uptake, excretion of lactic acid (Boyle and Yoshino, 2005; Estey and Mansour, 1987; de Saram et al., 2013) as well as developmental processes such as the miracidial transformation (Kawamoto et al., 1989; Taft et al., 2010). Serotonin is important for neuromuscular activity, motility

in the host blood system and is essential for survival of the parasite (Mbah et al., 2012). Forskolin, an adenylate cyclase activator, is able to mimic serotonin effects on muscle contractility in *S. mansoni*, suggesting that serotonin effects motility via a cAMP-dependent pathway (Day et al., 1994). As serotonin-induced sporocyst motility is not inhibited by serotonin reuptake inhibitors, that block the serotonin transporter, and serotonin influences motility without disruption of the worm surface, it is likely that surface exposed serotonin receptors are responsible for motility responses and serotonin uptake is not required to effect motility (Boyle et al., 2000; Boyle and Yoshino, 2005). *S. mansoni* serotonin receptors have a similar pharmaceutical profile, concerning inhibition with antagonists, as *F. hepatica* serotonin receptors but are different compared to mammalian receptors (Estey and Mansour, 1987). Schistosome larvae as well as *Mesocestoides corti* transport exogenous serotonin via a Na^+/Cl^--dependent transporter that is blocked by serotonin reuptake inhibitors that inhibit vertebrate and invertebrate serotonin transporter (Terenina et al., 1995; Boyle and Yoshino, 2005). The schistosome serotonin transporter is expressed both in the free-living cercaria and the parasitic forms but expression level is higher in the parasites where it has been shown to take up serotonin from host blood (Patocka and Ribeiro, 2007). Inhibition of the serotonin transporter with fluoxetine abolishes the stimulation of serotonin on glucose uptake and lactate excretion of S. mansoni (Harder et al., 1987,a).

While a multiplicity of roles have been described for serotonin in *S. mansoni*, there is little information for serotonin in *Echinococcus spp.* beyond the immunocytochemical localization and distribution of serotonin in the nervous system of *E. granulosus* (Brownlee et al., 1994; Fairweather et al., 1994).

1.6 Objectives

Alveolar and cystic echinococcosis are severe zoonotic diseases with limited treatment options. Liver resection and complete removal of the parasite material currently offers the only potential cure. Chemotherapeutic treatment with benzimidazoles, like albendazole or mebendazole, can significantly extend survival times (Craig, 2003). However, therapy with benzimidazoles is merely parasitostatic and often displays strong side effects (Reuter et al., 2004; Rogan et al., 2006; Kern, 2010). Therefore, new drugs are urgently needed.

To discover potential targets for new therapeutic drugs, evolutionarily conserved signalling pathways have been studied in *Echinococcus*. Host factors are known to influence parasite development and survival by interaction with evolutionarily conserved signalling pathways (Brehm, 2010). Targeting components of evolutionarily

conserved pathways has the advantage, that there are already substances available to target these components.

In this work the serotonin- and cAMP signalling pathways are analysed. The cAMP signalling pathway is known to influence a multitude of biological processes, such as metabolic pathways, secretory processes, muscle contraction, gene transcription, differentiation, cell growth and apoptosis (Houslay and Milligan, 1997; Hanoune et al., 1997; Patel et al., 2001). Previous works have shown that the cAMP signalling pathway influences developmental processes and survival of *E. multilocularis* (Schilling, 2010; Burkhardt, 2011). Focus of this work are the G-protein coupled receptors and the adenylate cyclase, their role in the development of different *Echinococcus* larval stages and their influence on parasite survival.

The other part of this work focuses on the role of serotonin in *Echinococcus*. While serotonin is known as an important neurotransmitter in *Echinococcus* (Brownlee et al., 1994; Fairweather et al., 1994; Koziol et al., 2013), there is no information about developmental effects of serotonin. In *S. mansoni* serotonin influences miracidial transformation (Kawamoto et al., 1989; Taft et al., 2010) and parasite survival (Mbah et al., 2012). Furthermore, *S. mansoni* has been shown to take up serotonin from host blood (Patocka and Ribeiro, 2007). Aim of this work is to study the effects of exogenous serotonin on *Echinococcus* development and survival and the role of serotonin transport in *Echinococcus*.

2 Materials and Methods

2.1 Equipment

- Autoclave: Selectomat S2000 (Münchner Medizin Mechanik (MMM),München)
- Centrifuges: Refrigerated Centrifuge 3K30 (Sigma, München), Bench-top Centrifuge Mikro 200 (Hettich, Tuttlingen)
- Developer for radiographic films (Agfa Graphics Germany, Düsseldorf)
- ELISA reader: Multiscan Ex Primary EIA V.2.1-0 (Thermo)
- Gel documentation system: MidiDoc (Herolab, Wiesloch)
- Gelelectrophoresis camber (Bio-Rad, München)
- Heating block: DB-3 (Techne, Cambridge, UK), Heizblock (Liebisch, Bielefeld)
- Heating stirrer: Typ RCT (Jahnke & Kunkel, Staufen i. Br.)
- Incubator: Heraeus (Thermo Electron, Langenselbold)
- Laminar flow hood: BSB 6A (Gelaire Flow Laboratories, Meckenheim), HERA safe Heraeus (Thermo Electron, Langenselbold)
- Micro scales: R160P (Sartorius, Göttingen)
- Microscope: Leica IRB (Leica Microsystems, Wetzlar)
- Mini-PROTEAN II System (BioRad, München)
- Mini Trans-Blot Cell (BioRad, München)
- NanoDrop (PeqLab Biotechnologie, Erlangen)
- Neubauer counting chamber: Neubauer Precicolor, depth 0.1 mm, 0.0025 mm^2
- Pipettes: 0,5-10 µl, 10-100 µl, 1-1000 µl (Eppendorf, Hamburg)
- Protein gelelectrophoresis chamber (Mini-PROTEANR II System BioRad, München)
- Scales: 10-1000 g (Sartorius, Göttingen), R160 (accuracy weighing scale) (Sartorius, Göttingen)
- Shaking incubator: TH30 (Edmund Bijhler, Hartensein, Würzburg), G24 (New Brunswick Scientic, Edison, N.J., USA)
- Spectrophotometer U-2000 (Hitachi, NY, USA)
- Thermocycler: Trio-Thermoblock™ heated lid, Trio-Thermoblock™ oil, T-Gradient (Biometra, Göttingen)
- Vacuum pump (ILMVAC)
- Voltage generator: Power Pack P24 and P25 (Biometra, Göttingen)
- Vortex mixer: L46 (Gesellschaft für Laborbedarf, Würzburg)
- Western blot chambers (Bio-Rad Laboratories, München)

2.2 Consumables

- 12-well plates (Nunc, Roskilde, Dänemark)
- 24-well plates (Nunc, Roskilde, Dänemark)
- Cell culture flasks: 75 cm^2, 175 cm^2 (NUNC, Wiesbaden; Sarstedt, Nümbrecht)
- Nitrocellulose membrane (GE Healthcare, München)
- Radiographic film (Fujifilm Europe, Düsseldorf)
- Safe-lock tubes 0,5, 1,5 and 2 ml (Eppendorf, Hamburg)
- Semi-micro cuvettes (Sarstedt, Nümbrecht)
- Sterile filter (Nalgene, New York, USA)
- Sterile tubes, 15 and 50 ml (Greiner, Nürtingen)
- Syringes and canula, sterile (Braun Melsungen AG, Melsungen)
- Whatman blotting paper (GE Healthcare, München)

2.3 Chemicals and reagents, enzymes, media, kits

Chemicals and reagents

- 2',5'-Dideoxyadenosine (Sigma-Aldrich, München)
- Agarose (ROTH, Karlsruhe)
- Albumin fraction V (pH 7) Blotting grade (BSA) (AppliChem, Darmstadt)
- Ammonium peroxodisulfate (APS), (Carl Roth, Karlsruhe)
- Aqua demin. (VE-water)
- Ampuwa (Fresenius, Bad Homburg)
- Ampicillin (Sigma-Aldrich, München)
- Bathocuproine disulfonic acid (Sigma-Aldrich, München)
- β-Mercaptoethanol (Sigma-Aldrich, München)
- Chloroform (Merck, Darmstadt)
- Citalopram hydrobromide (Sigma-Aldrich, München)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, München)
- dNTP lyophilised (ROTH, Heidelberg)
- Fetal Calf Serum (FCS) (Invitrogen, Darmstadt)
- Forskolin (LC Laboratories, Woburn)
- Glucagon (Sigma-Aldrich, München)
- L-Cystein (Sigma-Aldrich, München)
- Oligonucleotides (Sigma-Aldrich, München)
- Penicillin/Streptomycin (Invitrogen, Darmstadt)
- Polyethylene glycol, average mol wt. 3015-3685 (PEG 3350) (Sigma-Aldrich, München)

- ColorPlus Prestained Protein Ladder, Broad Range (10-230 kDa) (New England Biolabs, Schwalbach)
- Rotiphorese® Gel 30 (37,5:1) (Carl Roth, Karlsruhe)
- Rotiphorese® Gel 40 (Carl Roth, Karlsruhe)
- Serotonin hydrochloride (Sigma-Aldrich, München)
- SmartLadder (Eurogentec, Köln)
- Sodium carbonate (ROTH, Karlsruhe)
- Sodium taurocholate (Sigma-Aldrich, München)
- Tavanic® (Tava, active component levofloxacin, 5mg/ml) (Aventis)
- TEMED (N,N,N',N'-Tetramethylethylenediamine) (Merck, Darmstadt)
- Triton® X-100 (Sigma-Aldrich, München)
- Trizol® Reagent (Invitrogen, Darmstadt)
- Tween® 20 (Merck, Darmstadt)

Enzymes

- Pepsin (Sigma-Aldrich, München)
- T4 DNA Ligase (New England Biolabs, Schwalbach)
- Taq-Polymerase (New England Biolabs, Schwalbach)
- Trypsin/EDTA solution (0,05%/0,02% (w/v) in PBS w/o Ca^{2+}, Mg^{2+}) (Biochrom, Berlin)

Media

- c-DMEM-A (prepared according to Spiliotis and Brehm (2009))
- Dulbecco's Modified Eagle Medium
- NCTC-135 Medium (Sigma-Aldrich)

Kits

- cDNA Synthesis: Omniscript® RT Kit (QIAGEN, Hilden)
- DNA Purification Kits: Easy Pure DNA Purification Kit (Biozym, Hessisch Oldendorf), NucleoSpin® Extract II (Macherey-Nagel, Dijren)
- Plasmid isolation kits: EasyPrep Pro Plasmid Miniprep Kit (Biozym, Hessisch Oldendorf), NucleoSpin® Plasmid (Macherey-Nagel)
- PCR Cloning Kits: PCR Cloning Kits (QIAGEN, Hilden), CloneJET™ PCR Cloning Kit (Fermentas, St. Leon-Rot)
- Protein concentration determination: Pierce® BCA Protein Assay Kit (Thermo Scientific)
- Western blot chemiluminescence: Pierce® ECL Western Blotting Substrate (Thermo Scientific)

2.4 Oligonucleotids

***emac1* specific primers**

Name	Sequence 5' → 3'
1676_0_dw	GGCTCTGACGTTCCCTGTTC
1676_1_dw	GGTGCTCTGGTGGAGTATTG
1676_1_up	CAATACTCCACCAGAGCACC
1676_2_dw	CTGCTTCTCAACTCTTGCTTCTTC
1676_2_up	GAAGAAGCAAGAGTTGAGAAGCAG
1676_2a_dw	GTCACCATAGCTAATCAGATGGAGTC
1676_2a_up	GACTCCATCTGATTAGCTATGGTGAC
1676_3_dw	CAGTCTGTCCTCATCACACTTG
1676_3_up	CAAGTGTGATGAGGACAGACTG
1676_3a_dw	GGATGGTTCATGGGATCTTGTTC
1676_3a_up	GAACAAGATCCCATGAACCATCC
1676_4_dw	GTGACCGATGCTGCTGTTATG
1676_4_up	CATAACAGCAGCATCGGTCAC

***emac2* specific primers**

3320_1_dw	GTTCTGCGTGCGGTAGTCTG
3320_1_up	CAGACTACCGCACGCAGAAC
3320_2_dw	CCATCTGGTCATATTCACATATCC
3320_2_up	GGATATGTGAATATGACCAGATGG
3320_3_dw	CTACACACTGTCCTCGCTGATG
3320_3_up	CATCAGCGAGGACAGTGTGTAG
3320_4_dw	CAGACATTGCCTATCTTCTGCATC
3320_4_up	GATGCAGAAGATAGGCAATGTCTG

***emgpcr1* specific primers**

S1_1_dw	GCTGGCCTCCTGCATTATC
S1_1_up	GATAATGCAGGAGGCCAGC
S1_2_dw	GACAGAGGTGGAACGCTCTAC
S1_2_up	GTAGAGCGTTCCACCTCTGTC
S1_4_dw	GCACTCTGCAGAAGGTACTTCTACG
S1_4_up	CGTAGAAGTACCTTCTGCAGAGTGC

***emgpcr2* specific primers**

S2_0_dw	GGTAAACATGGAGTCACCTTCAAAC
S2_3_dw	GCTCGTAGTCTACGGTCTGACTG
S2_3_up	CAGTCAGACCGTAGACTACGAGC
S2_4_dw	CAAGACATGGACGTCGGTG
S2_4_up	CACCGACGTCCATGTCTTG

***emgpcr3* specific primers**

S3_1_up	CCTGTGCTTGTATTGTCACGG
S3_1_dw	CCGTGACAATACAAGCACAGG
S3_2_dw	CGTACCTTCGGTCTTACTCGAGTG
S3_2_up	CACTCGAGTAAGACCGAAGGTACG
S3_3_dw	CTTCCTCTCTGTTGCACCATCC
S3_3_up	GGATGGTGCAACAGAGAGGAAG
S3_4_dw	CACCAGATTGACGGGTTCTC
S3_4_up	GAGAACCCGTCAATCTGGTG
S3_5_dw	GTGTATCCTAATCCCAAAGTTGTCAC
S3_5_up	GTGACAACTTTGGGATTAGGATACAC

cDNA synthesis primer

cd3rt	ATCTCTTGAAAGGATCCTGCAGGACTTTTTTTTTTTTTTTTTTTTTTTVX

***emelp* specific primers**

Em10 15	AATAAGGTCAGGGTGACTAC
Em10 16	TTGCTGGTAATCAGTCGATC

pDrive specific primers

SP6	CCATTTAGGTGACACTATAGAATAC
T7	GCTCTAATACGACTCACTATAGG

CloneJET1.2 specific primers

pJET1.2 forward sequencing primer	CGACTCACTATAGGGAGAGCGGC
pJET1.2 reverse sequencing primer	AAGAACATCGATTTTCCATGGCAG

pJG4-5 specific primers

JG_4-5-SPR2	CTTATGATGTGCCAGATTATG
JG_4-3_up	TTGGAGACTTGACCAAACCTCT

2.5 Antibodies

Primary antibodies

- anti-Phospho-PKA Substrate (RRXS*/T*): monoclonal antibody against phosphorylated substrates of protein kinase A; 1:1000 for Western blot in 5% BSA-TBST (0,1%), Cell Signaling (#9624)
- anti-β-Actin Antibody: polyclonal antibody against β-actin; 1:1000 for Western blot in 5% BSA-TBST (0,1%), Cell Signaling (#4967)
- anti-Ezrin/Radixin/Moesin Antibody: monoclonal antibody against Elp; 1:1000 for Western blot in 5% skim milk, Cell Signaling (#3142)

Secondary antibodies

- anti-mouse IgG HRP; 1:10.000 for Western blot in 5% skim milk, Jackson, ImmunoResearch
- anti-rabbit IgG HRP; 1:5000 for Western blot in 5% skim milk, Jackson, ImmunoResearch

2.6 Bioinformatical analysis

2.6.1 Identification of homologues in *E. multilocularis* and *E. granulosus*

To identify homologues of proteins involved in cAMP signalling and serotonin pathway, protein sequences from different organisms (see Appendix 9.2.1) were used as query for tblastn searches against the *E. multilocularis* and *E. granulosus* genome (Sanger Institute) and gene predictions (Gene DB) using BioEdit (Hall, 1999). To verify the found sequences, backsearches were performed using blastx at NCBI BLAST and blastx against the SwissProt database to confirm the function of the genes.

For further analysis of adenylate cyclase homologues, the best result of the backsearch analysis was used to repeat the tblastn search against the *E. multilocularis* genome to achieve a better knowledge of the supposed gene structure. Whenever available, ESTs (see Appendix 9.2.2) were used to give further insight in intron-extron structure. To confirm assumed gene ends as well as their intron-exon structure, transcriptome data was visualized by IGV (Robinson et al., 2011).

In order to find homologues of G-protein coupled receptors of the *Secretin* family, predicted protein sequences that contained the 7tm_2 domain (prediction and pfam

analysis by Ference Kiss) were analysied in the same way as homologues for adenylate cyclase.

2.6.2 Gene expression of identified homologues in different *E.multilocularis* stages

Next generation RNA sequencing data (Sanger Institute) of different stages (see Appendix 9.3.1) was mapped against the *E. multilocularis* genome by Ference Kiss using TopHat (Trapnell et al., 2009). The number of reads for adenylate cyclase genes was then calculated using samtools-0.1.18 (Li et al., 2009). The number of reads was normalized to the total number of reads in one data set and the exon length of the gene. Expression was shown in fragments per kilobase of exon per million fragments mapped. Gene expression of genes putatively involved in serotonin pathway and metabolism was calculated with different transcriptome data sets (see Appendix 9.3.2) (Olson et al., 2011; Tsai et al., 2013).

2.6.3 GPCRs in *E. multilocularis*

To identify GPCRs in *E. multilocularis*, all GPCRs found in *S. mansoni* and *S. mediterranea* (Zamanian et al., 2011) were used as query for blastp searches against the predicted protein sequences of *E. multilocularis* (GeneDB, Sanger Institute). For each sequence the best hit as well as all hits with an E-value smaller 10^{-10} were used for further analysis. After the removal of multiple hits, the remaining protein sequences were confirmed to be GPCRs by blastp at NCBI BLAST and analysed with SMART (Schultz et al., 1998; Letunic et al., 2012) and pfam (Punta et al., 2012). To determine the completeness of the protein sequences, the number of transmembrane domains was calculated using HMMTOP (Tusnády and Simon, 1998; Tusády and Simon, 2001) and TMHMM (Krogh et al., 2001). In order to sort GPCRs of *E. multilocularis* into families acording to the GRAFS-system (Fredriksson et al., 2003; Fredriksson and Schiöth, 2005) blastp searches against the GPCRs of *S. mansoni* and *S. mediterranea* were performed.

2.6.4 Phylogeny and binding sites of the *E. multilocularis* serotonin transporter

For phylogenetic analysis of the *E. multilocularis* serotonin transporter (EmSERT), protein sequences of serotonin and dopamine transporters of different organisms (see Appendix 9.4) were aligned using MUSCLE (Edgar, 2004*a*,*b*). The multiple sequence alignment was then used to build a phylogenetic tree with MEGA4 (Tamura et al.,

2007) using the neighbor-joining method (Saitou and Nei, 1987).
In order to learn more about the functional aspects of the *E. multilocularis* serotonin transporter, a sequence alignment with the protein sequences of the human, mouse, drosophila, *S. mansoni* and *E. multilocularis* serotonin transporters was performed and used to identify known binding sites of ligands and specific serotonin reuptake inhibitors, that have been described for the human serotonin transporter (Ravna et al., 2006; Andersen et al., 2009).

2.6.5 Phylogenetic relationship of adenylate cyclases in *E. multilocularis*, *E. granulosus* and *S. mansoni*

In order to gain insights into the origin of *E. multilocularis* adenylate cyclases, adenylate/guanylate cyclase domains in *E. multilocularis* and *S. mansoni* were identified by HMMsearch against the predicted protein sequences of *E. multilocularis* and *S. mansoni* using HMMER-3.0 (Eddy, 2009, 2011; Johnson et al., 2010) with the Hidden Markov model Guanylate_cyc (PF00211) from pfam (Punta et al., 2012) as query.
As there were no protein predictions available for *E. granulosus*, cyclase domains of *E. granulosus* were identified via blast searches against the *E. granulosus* genome using the *E. multilocularis* adenylate cylcase sequences as queries. The coding sequences were deduced on the basis of the *E. multilocularis* sequences and translated into amino acids sequences. As described above, the cyclase domains were identified with HMMsearch.
NCBI BLAST identified cyclase domains as different adenylate and guanylate cyclase types. As adenylate cyclase type IX identified cyclase domains of *E. multilocularis*, *E. granulosus* and *S. mansoni* were used to generate a phylogenetic tree. For this purpose the amino acid sequences of both cyclase domains were stuck together to create a fusion amino acid sequence consisting of the two cyclase domains of one adenylate cyclase type IX. These fusion sequences were aligned by MUSCLE (Edgar, 2004*a*,*b*) and the resulting multiple sequence alignment was used to generate a phylogenetic tree in MEGA4 (Tamura et al., 2007) with the neighbor-joining method (Saitou and Nei, 1987).

2.6.6 Correcting errors in sequenced *E. mulilocularis* cDNA sequences with exonerate

As sequencing of PCR-products may lead to errors in the sequence due to mismatches in reverse transcription or PCR-amplification, cDNA sequences were mapped

against the *E. multilocularis* genome using exonerate (Slater and Birney, 2005) and corrected according to the genome sequence.

2.7 Manipulation of nucleic acids

2.7.1 RNA isolation from parasite material

After isolation and centrifugation, primary cells were resuspended in 500 µl trizol, while protoscoleces and metacestode vesicles were resuspended in 1 ml trizol and incubated for 5 minutes at room temperature. After adding 200 µl chloroform (in case of primary cells 100 µl), the suspension was shaken rigorously and then incubated at room temperature for 2-3 minutes. Centrifuging with 12.000 g at 4 °C for 15 minutes lead to a lower, reddish phenol-chloroform phase, a white interface and a clear upper phase. The upper phase was transfered into a new tube. RNA precipitation was achieved by adding 500 µl isopropyl alcohol and incubating for 10 minutes at room temperature. After another centrifugation (12.000 g, 4 °C, 10 minutes), the supernatant was discarded. Subsequently the pellet was washed with 75% ethanol (DEPC) and centrifuged with 7.500 g at 4 °C for 5 minutes. Again the supernatant was discarded and the pellet was left to dry for 10 minutes. The pellet was resuspended in 15 µl RNase free water by pipetting up and down and incubating at 55 °C for 10 minutes. RNA was stored at -20 °C until use.

2.7.2 Reverse transcription of RNA to cDNA

Reverse transcription of RNA was performed using Omniscript® RT Kit by QIAGEN according to manufacturers' instructions though without using RNase inhibitor. As Oligo-dT primer cd3rt (5'-ATCTCTTGAAAGGATCCTGCAGGACTTTTTTTTT-TTTTTTTTTTTTTTVX-3') was used.
To determine whether the reverse transcription was successful, polymerase chain reaction was performed on the housekeeping gene *emelp* using Em10 15 (5'-AATAAGG-TCAGGGTGACTAC-3') and Em10 16 (5'-TTGCTGGTAATCAGTCGATC-3') as primers (for details of polymerase chain reaction see 2.7.3). Genomic DNA was used as control. PCR with cDNA as template gave rise to a 400 bp product while chromosomal contamination would lead to a 500 bp product.

2.7.3 Polymerase chain reaction with Taq polymerase

The standard polymerase chain reaction (PCR) mixture contained: 10% tenfold Taq buffer, 1% of each primer (100 µM), 1% dNTPs (10 mM), 1% template, 1% Taq polymerase, ad 50 µl H_2O. The following program was used: denaturation at

94 °C for 5 minutes; 35 cycles with 30 seconds of denaturation at 94 °C, 30 seconds at annealing temperatur of the primers and elongation at 72 °C for 1-3 minutes depending on the length of the product; final elongation at 72 °C for 10 minutes, hold at 4 °C. Templates used were either cDNA or cDNA libraries.
For colony PCR the mix contained a final volume of 20 µl, 2 µl of colony water as a template and only 30 cycles were run.

2.7.4 Rapid amplification of cDNA-ends with PCR

To amplify the unknown ends of a gene, rapid amplifications of cDNA-ends with PCR (RACE-PCRs) were performed. cDNA libraries in the vector pJG4-5 were used as template. For amplification of 5'-ends the primer JG_4-5-SPR2 (5'-CTTATGATGT-GCCAGATTATG-3') and an insert specific up-primer were used, while for amplification of 3'-ends the primer JG_4-3_up (5'-TTGGAGACTTGACCAAACCTCT-3') and an insert specific down-primer were used (for details of polymerase chain reaction see 2.7.3).

2.7.5 Gel electrophoresis of PCR products

For gel electrophoresis of PCR products 1% agarose gels were used. In case of more than one distinct band on the gel, gel extraction was performed before purification of PCR products.

2.7.6 Purification of PCR products

PCR-products were purified using NucleoSpin® Extract II from Macherey-Nagel or Easy Pure DNA Purification Kit from Biozym according to manufacturers' protocol.

2.7.7 Ligation of PCR products in pDrive or pJET1.2 Cloning Vector

PCR products were ligated into pDrive Cloning Vector using the QIAGEN® PCR Cloning Kit. The ligation reaction mixture contained 0,5 µl pDrive Cloning Vector, 2,5 µl Ligation Master Mix and 2,0 µl PCR product. After mixing and centrifuging, the reaction mixture was incubated at 4 °C overnight. Alternatively PCR products were cloned into pJet1.2 according to manufacturers' protocol using CloneJET™ PCR Cloning Kit from Fermentas.

2.7.8 Transformation of chemically competent *Escherichia coli* with pDrive or pJET1.2 Cloning Vector

Chemical competent *Escherichia coli*, stored at −80 °C, were thawn on ice. To 50 µl *E. coli* 2,5 µl ligation mixture was added and incubated for 15 minutes on ice. Heat shock was performed at 42 °C for 45 seconds. Then the transformation mixture was incubated on ice for 2 minutes. After adding 200 µl of LB-medium, the mixture was incubated for 45 minutes at 37 °C in the shaker. Subsequently the transformed *E. coli* were plated on LB-ampicillin plates and incubated overnight at 37 °C. In case of transformation with pDrive, 40 µl x-Galactose (40 mg/ml) was applied to LB-ampicillin plates before plating of transformed *E. coli*.

2.7.9 Colony PCR to screen for successful ligations in transformed *E. coli*

As pDrive contained a resistance gene for ampicillin, only *E. coli* that contained the pDrive vector could survive on LB-ampicillin plates. An insertion of the PCR-product in the multiple cloning side lead to destruction of the LacZ α-peptide gene that enabled the production of a blue color in the presence of x-Galactose. Therefore, clones with an insert in pDrive appeared white, while clones without insert produced a blue color.
White clones were picked and solved in 30 µl H_2O. For colony PCR 2 µl colony water and the primers SP6 (5'-CCATTTAGGTGACACTATAGAAT-3') and T7 (5'-GCTCTAATACGACTCACT-ATAGG-3') were used (see section 2.7.3). Clones, whose PCR products gave rise to bands of the expected size on the agarose gel, were used for over night culture. Colony water was stored at 4 °C until use for over night cultures.
In case of transformation with pJET1.2 only clones with insert could survive and were used for colony PCR using pJET1.2 Forward Sequencing Primer (5'-CGACTC-ACTATAGGGAGAGCGGC-3') and pJET1.2 Reverse Sequencing Primer (5'-AAGA-ACATCGATTTTCCATGGCAG-3') (see section 2.7.3).

2.7.10 Over night culture of transformed *E. coli* and plasmid isolation

3-4 µl colony water were given in 5-6 ml LB-medium with ampicillin (1 µl/ml) and incubated at 37 °C overnight in a shaking incubator. Plasmid isolation from over night cultures was performed using EasyPrep® Pro Plasmid Miniprep Kit from Biozym or NucleoSpin® Plasmid from Macherey-Nagel according to manufacturers' instructions. Isolated plasmids were solved in water and stored at −20 °C.

2.7.11 Sequencing inserts of isolated plasmids

For sequencing of the inserts of isolated plasmids, the following sequencing mixture was used: 2 µl of fivefold sequencing buffer, 1 µl of one diluted primer (5 µM), 400 ng of isolated plasmid, add 9 µl H_2O. Each plasmid was sequenced using SP6 and T7 as primers respectively. In case of pJET1.2, pJET1.2 Forward Sequencing Primer and pJET1.2 Reverse Sequencing Primer were used. Sequencing was carried out at the Institut für Hygiene und Mikrobiologie, Universität Würzburg according to Sanger capillary sequencing.

2.8 *In vitro* studies on *E. granulosus* protoscoleces

2.8.1 Substances

Stock solutions were prepared for the adenylate cylcase activator forskolin (12,5 mM in dimethyl sulphoxide and in ethanol, respectively), the adenylate cyclase inhibitor 2',5'-dideoxyadenosine (25 mM in dimethyl sulphoxide), glucagon (250 µM in 0,05 M acetic acid), serotonin (50 mM in 0,1 M hydrochloric acid) and citalopram (10 mM in water) and sterile filtrated. Dilutions were made for each of them (1:10 and 1:100, for serotonin additionally 1:1000), so always the same amount was added for different concentrations used in experiments. The substances and their concentrations were chosen according to Estey and Mansour (1987) and Kawamoto et al. (1989) for forskolin, Désaubry and Johnson (1996) and Nakajima-Shimada et al. (1996) for 2',5'-dideoxyadenosine, Hirai et al. (1987) for glucagon, Boyle et al. (2000), Estey and Mansour (1987), Harder et al. (1987), Harder et al. (1987a), Kasschau and Mansour (1982) and Kawamoto et al. (1989) for serotonin and Ostad Haji et al. (2011) and Patocka and Ribeiro (2007) for citalpram.

2.8.2 Media

For most experiments Dulbecco's modified Eagle medium (DMEM) (4,5 g/l glucose, 3,7 g/l sodium bicarbonate, 4 mM L-glutamine, 10 % fetal bovine serum (FBS), 100 U/ml penicillin and 0,1 g/l streptomycin) was used. pH was controlled to be at 7,4.

For experiments with different media, DMEM and NCTC-135 (2,2 g/l sodium bicarbonate, 0,93 mM L-glutamine, 10 % FBS, 100 U/ml penicillin and 0,1 g/l streptomycin) was used. pH was adjusted to 7,4.

As reducing agents 1 µl/ml L-cysteine (stock solution 100 mM) and 1 µl/ml β-mercaptoethanol (stock solution 1:100, diluted in H_2O) were used.

Media was sterile filtrated prior to use in cell culture. Media was freshly prepared once a week to avoid degradation of L-glutamine (Boxberger, 2007, pp. 91-93).

2.8.3 Isolation and activation of protoscoleces

Protoscoleces were isolated from cysts as described previously (Fernández et al., 2002). Up to use protoscoleces were kept in phosphate buffered saline (PBS) with gentamycin. Pepsin activation of protoscoleces was performed for 30 minutes in 0,05 % pepsin/DMEM (w/o FBS, pH 2, steril) at 37 °C with constant agitation. After washing in PBS three times, protoscoleces were incubated for 3 hours at 37 °C in 0,2 % sodium taurocholate (w/o FBS, pH 7,4, steril) with constant agitation. Protoscoleces were washed in PBS three times prior to use in cell culture experiments.

2.8.4 Effect of cAMP-pathway stimulation and inhibition on protoscolex development and survival

Activated protoscoleces were cultivated in 24-well-plates (about 200-300 per well) with 500 µl DMEM (10% FBS) supplemented with Glucagon (50 nM, 500 nM and 5 µM), serotonin (1 µM, 10 µM, 100 µM and 1 mM), forskolin (1 µM, 10 µM and 100 µM) or 2',5'-dideoxyadenosine (1 µM, 10 µM and 100 µM). Three times a week 250 µl medium was exchanged. The number of developed pre-microcysts and dead protoscoleces was determined by observation with an inverted light microscope. All experiments were performed in triplicate with the dissolvents of each substance as controls.

2.8.5 Effect of serotonin stimulation and serotonin reuptake inhibition on protoscolex development and survival

Experiments were performed on activated protoscoleces cultivated in 24-well-plates (about 300 per well) with 500 µl DMEM (10% FBS). Citalopram was applied to protoscoleces at different concentrations (1 µM, 10 µM, 100 µM) with water as control. To test the effect of citalopram in presence of serotonin, protoscoleces were incubated with 100 µM citalopram for 1 hour before adding 100 µM or 1 mM serotonin respectively. As controls 100 µM citalopram, 100 µM and 1 mM serotonin and media with just the dissolvents were utilized. Three times a week 250 µl medium was exchanged. The number of dead protoscoleces was determined by observation with an inverted light microscope. The experiment was carried out in triplicate.

2.8.6 Effect of different media on protoscolex development and survival

Activated protoscoleces were cultivated in 24-well-plates (about 300 per well) with 500 µl medium. Different media were tested: DMEM with and without FBS (10%), DMEM with FBS (10%) and reducing agents, NCTC-135 with and without FBS (10%).
Experiments were performed both under aerobic and under anaerobic conditions (in a bag filled with nitrogen). Three times a week 250 µl medium was removed and replaced. The number of developed pre-microcysts and dead protoscoleces was determined by observation with an inverted light microscope. Experiments were performed in triplicate.

2.9 *In vitro* drug and inhibitor studies on different larval stages of *E. multilocularis*

E. multilocularis material used in experiments was maintained in Mongolian jirds (*Meriones unguiculatus*) through serial intraperitoneal passages (Spiliotis and Brehm, 2009). Parasite material isolated from jirds was either used for isolation of protoscoleces or *in vitro* co-cultivated with rat Reuber RH- hepatoma cells (Spiliotis et al., 2004).

2.9.1 Isolation of primary cells

For primary cell isolation metacestode vesicles older than 3 months were used. Co-cultivated metacestode vesicles were transfered to axenic cultivation before primary cell isolation. primary cells were isolated as described previously by Spiliotis and Brehm (2009).

2.9.2 Effect of cAMP-pathway stimulation and inhibition and serotonin reuptake inhibition on primary cell development

primary cells of the isolate H95 were cultivated in 24-well-plates in 1 ml c-DMEM-A medium with reducing agents (β-mercaptoethanol, bathocuproine disulfonic acid, L-cystein) under anaerobic conditions (nitrogen) (Spiliotis and Brehm, 2009). 2-3 times a week 500 µl medium was changed.
Substances used in experiments were prepared as described above. Glucagon (50 nM, 500 nM and 5 µM), serotonin (1 µM, 10 µM, 100 µM and 1 mM), the adenylate cylcase activator forskolin (1 µM, 10 µM and 100 µM; solved in ethanol) and the adenylate cyclase inhibitor 2',5'-dideoxyadenosine (1 µM, 10 µM and 100 µM) were

applied to primary cells at indicated concentration and substituted at each change of medium.
Experiments with the serotonin transporter inhibitor citalopram were performed with citalopram alone (1 µM, 10 µM and 100 µM) as well as in presence of serotonin. Therefore, primary cells were preincubated for 1 hour with citalopram (100 µM) before adding serotonin (10 µM and 100 µM).
Aggregation and vesicle formation of primary cells was observed with optical microscope. The number of formed vesicles was counted as an indicator of germinative cell proliferation and differentiation. All experiments were performed in triplicate with the dissolvents of each substance as controls.

2.9.3 Effect of cAMP-pathway stimulation and inhibition and serotonin reuptake inhibition on metacestode vesicle growth and survival

For treatment with glucagon (50 nM, 500 nM and 5 µM), forskolin (1 µM, 10 µM and 100 µM; solved in ethanol) and 2',5'-dideoxyadenosine (5 µM, 50 µM and 300 µM) co-cultivated metacestode vesicles of the isolate H95 were transfered to axenic culture (Spiliotis and Brehm, 2009). Intact vesicles with a diameter of 2-4 mm and without brood capsules were then picked using a truncated blue tip and transfered to a 12-well-plate. About 10 vesicles were used per well. Vesicles were cultivated in 3 ml DMEM (2 % FCS) medium (reducing agents, nitrogen) whereupon 1 ml medium was changed twice a week. Each concentration was applied to three wells. Control cultures were treated with the dissolvents of the substances.
For incubation with citalopram (1 µM, 10 µM and 100 µM) vesicles of the isolate Ingrid were used. Vesicles were incubated in 2 ml c-DMEM-A (Spiliotis and Brehm, 2009) and 1 ml medium was changed twice a week.
Growth and survival of metacestode vesicles was observed with optical microscope.

2.9.4 Effect of serotonin on metacestode vesicle growth

For the growth experiment axenic vesicles of the isolate H95 with a diameter of about 3 mm were transfered to 14 ml tubes, one vesicle per tube. Vesicles were incubated in 2 ml c-DMEM-A (Spiliotis and Brehm, 2009). Medium was changed three times a week by removing with a vacuum pump. Serotonin was added at the following concentrations: 1 µM, 10 µM, 100 µM and 1 mM. 10 vesicles were treated with each concentration. The solvent of serotonin was applied to controls. Vesicle diameter was measured each time the medium was changed.

2.9.5 Forskolin and serotonin stimulation of metacestode vesicles for protein isolation

In order to achieve low basal phosphorylation of protein kinase A substrates, metacestode vesicles were serum starved in 2 ml DMEM (0,02% FCS, reducing agents, nitrogen). Therefore, axenic cultivated metacestode vesicles of the isolate H95 or Ingrid with a diameter of about 3 mm were picked with a tuncated blue tip and transfered to a 12-well-plate with 3-4 vesicles per well. To determine the date of lowest basal phosphorylation of protein kinase A substrates, vesicles were starved for 0, ½, 1, 2 and 4 days before protein isolation.
In the following stimulation experiments vesicles were starved for 1 day (date of lowest basal phosphorylation) before adding forskolin or serotonin to the vesicles. To find out the point of time of the highest phosphorylation after stimulation, vesicles were incubated with forskolin (100 µM) or serotonin (1 mM) for 0, 5, 30, 60 and 120 minutes.

2.9.6 Statistical analysis

Statistical analyses were carried out with Microsoft Excel 2010. To determine significant differences between treated cultures and control cultures, an independent two-sample, two-tailed Student's t-test was performed for each point in time and each applied drug concentration. Differences from control with $P < 0{,}05$ were considered statistically significant.

2.10 Analysis of proteins

2.10.1 Protein lysates from *E. multilocularis* metacestode vesicles

First, metacestode vesicles were washed with PBS. Then vesicles were broken by sucking into a blue tip and transfered into a 1,5 ml tube. Tubes were filled with PBS, containing phosphatase inhibitor sodium fluoride (10 mM) and sodium orthovanadate (1 mM). After centrifugation with 1000 g at 4 °C for 10 minutes, the supernatant was discarded and the pellet resuspended in 100 µl stop-mix (62,5 mM Tris-HCl, pH 6,8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol). To denaturate, proteins were cooked at 100 °C for 5-10 minutes. Denaturation was followed by another centrifugation step (1000 g, 4 °C, 10 minutes). To get rid of remaining unsolved components, the supernatant was transfered into a new tube.

2.10.2 Determination of protein concentration

To determine the protein concentration, Pierce® BCA Protein Assay Kit was used. For working solution, reagents A and B were mixed in a ratio of 50:1. To 200 µl working solution 10 µl bovine serum albumin standards (100, 200, 400, 600, 800, 1000, 1200 µg/ml) or protein solutions of unknown concentrations were added on a 96-well-plate. After 30 minutes of incubation at 37 °C, absorbance was measured at 570 nm in the ELISA reader Multiscan Ex Primary EIA V.2.1-0 (Thermo). Based on the absorbance of the protein standards, the unknown protein concentrations were calculated. Protein standards as well as solutions of unknown concentrations were measured in triplets.

2.10.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

To separate proteins according to their size, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Biorad Mini-Protean system.

Working solutions

PAA	30% Acrylamid/0,8% bis-acrylamid (Roth)
4x Lower-Tris	1,5 M Tris-HCl, pH 8,8 0,4% SDS
4x Upper-Tris	0,5 M Tris-HCl, pH 6,8 0,4% SDS
TEMED	N,N,N',N'-tetramethylethan-1,2-diamin (Merck)
10% APS	0,5 g ammonium persulfate dissolved in 5 ml H_2O
Running buffer	25 mM Tris, pH 8,3 192 mM glycine 0,1% SDS
2x stop mix	125 mM Tris-HCl, pH 6,8 20% glycerol 4% SDS

1,4 ml dH_2O
10% β-mercaptoethanol
some bromophenol blue

Resolving gel composition

	10%
PAA 30%	2 ml
4x Lower Tris	1,5 ml
ddH_2O	2,5 ml

The components of the 10% resolving gel were mixed in a beaker glas and gel polymerisation was started by adding 10 µl TEMED and 30 µl APS. After mixing gently, the gel was poured into the polymerisation chamber. To get a straight rim, the gel was covered with a layer of water. After 30 minutes the water was removed.

Stacking gel composition

PAA 30%	0,375 ml
4x Lower Tris	0,625 ml
ddH_2O	1,625 ml

Polymerisation of the 4% stacking gel was induced by adding 10 µl TEMED and 17,5 µl APS. The stacking gel was then poured on top of the resolving gel and a combs with 15 pockets was inserted into the gel. After polymerisation gels were used for electrophoresis.

Gel electrophoresis Gels were fixed in the electrophoresis chamber and the chamber was filled with running buffer. After loading the gel with the ColorPlus Prestained Protein Ladder, Broad Range (New England Biolabs) as marker for protein sizes and 10 µl of each protein solution (in stop-mix), separation took place at 150 V for about 60 minutes.

2.10.4 Western blot

To transfer proteins from the gel to a nitrocellulose membrane, the Biorad Mini Trans-Blot Cell system was used. The transfer of the separated proteins was carried out at 350 mA for 60 minutes.

Working solutions

Western blotting buffer	192 mM glycine 25 mM Tris base 20% methanol
Blocking solution	5% skim milk (SM) or 5% BSA, dissolved in 1x TBST
Washing buffer (TBST)	20 mM Tris 150 mM NaCl pH 8 0,1% Tween20

After the transfer the nitrocellulose membrane was blocked for 60 minutes in blocking solution. Subsequently it was incubated with the primary antibody over night at 4 °C on the seesaw. After washing three times for 10 minutes with TBST, the membrane was incubated with the secondary antibody for 60 minutes. The washing was repeated and the proteins were visualized by chemiluminescence using Pierce® ECL Western Blotting Substrate and exposure to X-ray films. The films were developed with a Curix 60 automated developer (Agfa).

3 Results

3.1 Genes putatively involved in cAMP-signalling and serotonin pathway in *Echinococcus spp.*

3.1.1 Most genes involved in serotonin pathway and metabolism are encoded on the *E. multilocularis* and *E. granulosus* genome

The *E. multilocularis* and *E. granulosus* genomes were searched for genes that are involved in serotonin pathway and metabolism in other organisms. The results are summarized in table 1.

Predicted function	**Protein prediction** (% of identity between Em and Eg)	**NCBI accession number for the first human hit** (% of identity)	**Swiss-prot accession number for the first hit** (species,% of identity)
Tryptophan hydroxylase (TPH)	**EmuJ_000069500** (95.0)	**AAI14500.1** (54)	**U6IT63_HYMMI** (*H. microstoma*, 71.0)
	EgrG_000069500 (95.0)	**EAW97278.1** (54)	**U6IT63_HYMMI** (*H. microstoma*, 70.0)
Aromatic-L-amino-acid decarboxylase (AADC)	**EmuJ_001173700** (78.0)	**CAG33005.1** (47)	**U6IZW6_HYMMI** (*H. microstoma*, 72.0)
	EgrG_001173700 (78.0)	**3RCH_A** (36)	**U6IZW6_HYMMI** (*H. microstoma*, 57.0)
Putative Serotonin Receptors (5-HT GPCRs)	**EmuJ_000861200** (95.0)	**EAX02623.1** (29)	**H2KUD4_CLOSI** (*C. sinensis*, 49.0)
	EgrG_000861200 (95.0)	**EAX02623.1** (26)	**H2KUD4_CLOSI** (*C. sinensis*, 50.0)
	EmuJ_000867300 (98.0)	**NP_000862.1** (25)	**U6IR94_HYMMI** (*H. microstoma*, 56.0)
	EgrG_000867300 (98.0)	**NP_000862.1** (24)	**U6IR94_HYMMI** (*H. microstoma*, 57.0)
	EmuJ_001050800 (99.0)	**NP_000515.2** (41)	**U6IT32_HYMMI** (*H. microstoma*, 68.0)
	EgrG_001050800 (99.0)	**BAF84118.1** (40)	**U6IT32_HYMMI** (*H. microstoma*, 68.0)
	EmuJ_001171200 (97.0)	**NP_000515.2** (36)	**U6IBG4_HYMMI** (*H. microstoma*, 50.0)
	EgrG_001171200 (97.0)	**NP_000515.2** (36)	**U6IBG4_HYMMI** (*H. microstoma*, 51.0)
Serotonin transporter (SERT)	**EmuJ_000391300** (98.0)	**NP_001036.1** (50)	**U6I323_HYMMI** (*H. microstoma*, 86.0)
	EgrG_000391300 (98.0)	**NP_001036.1** (48)	**U6I323_HYMMI** (*H. microstoma*, 82.0)

Table 1: **Predicted proteins of *E. multilocularis* and *E. granulosus*, homologous to human proteins involved in serotonin pathway and metabolism**: The homologues were identified using reciprocal blast searches. Shown are the identities between *E. multilocularis* and *E. granulosus* homologues, the first human hit in the NCBI protein sequence database and the first hit in the Swiss-Prot protein sequence database.

Reciprocal blast searches revealed the presence of the following genes putatively involved in serotonin biosynthesis: the tryptophan hydroxylase, that catalyses the rate-limiting step in serotonin synthesis, and the aromatic-L-amino-acid decarboxylase; in serotonin transport: the serotonin transporter and in serotonin signalling: G protein coupled serotonin receptors. However, neither the monoamine oxidase (MAO), the enzyme mainly responsible for serotonin degradation in vertebrates, nor the 5-HT_3 receptor, the serotonin gated ion channel, could be detected by reciprocal blast searches. Amino acid sequence identities between proteins of *E. granulosus* and *E. multilocularis* were relatively high (95-98%) with the exception of the aromatic-L-amino-acid decarboxylase (78%). Proteins of *E. granulosus* and

E. multilocularis show higher identities (49-86%) with their flatworm homologues than with human homologues (25-54%), indicating conservation of serotonin pathway genes in flatworms.

RNAseq data of different *E. multilocularis* life stages (Olson et al., 2011; Tsai et al., 2013) indicates that all genes listed in Table 1 are expressed in *E. multilocularis* (see Figure 10).

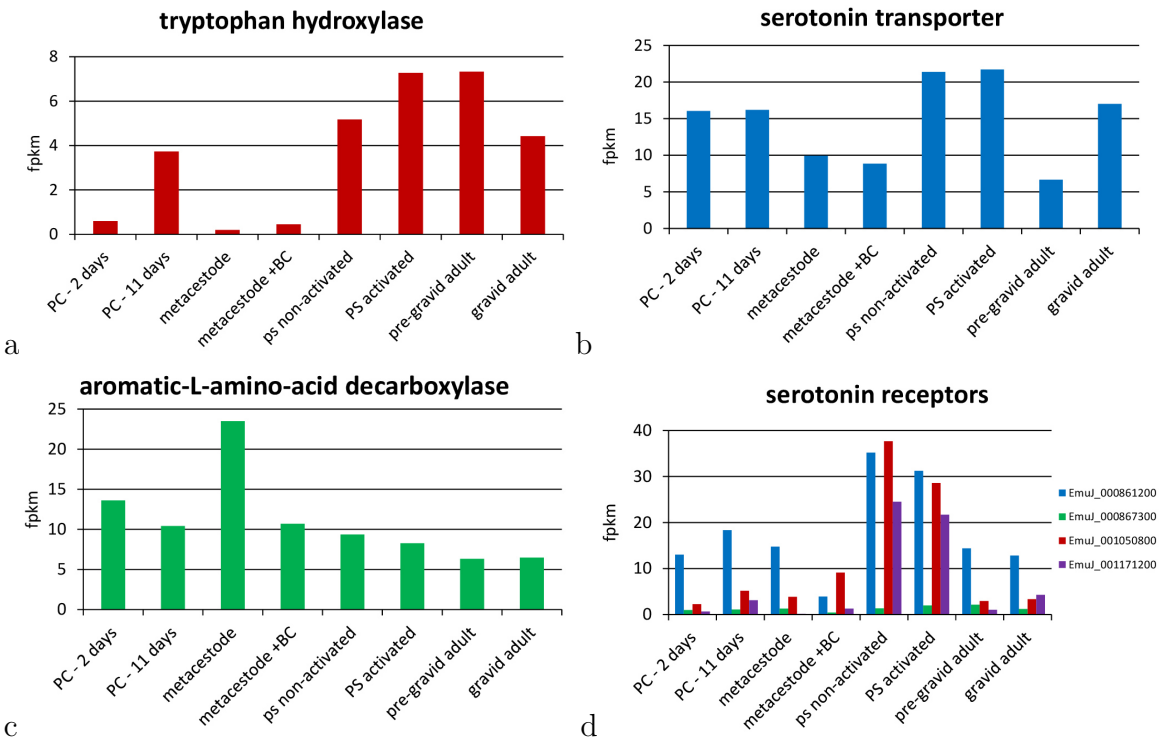


Figure 10: **Gene expression of tryptophan hydroxylase (a), serotonin transporter (b), aromatic-L-amino-acid decarboxylase (c) and serotonin receptors (d):** RNA sequencing data was mapped against the *E. multilocularis* genome and normalized to the number of reads in one data set and to exon length (Olson et al., 2011; Tsai et al., 2013). Expression is shown in fragments per kilobase of exon per million fragments mapped (fpkm). **PC**: primary cells; **PS**: protoscoleces; **BC**: brood capsules

Expression of the tryptophan hydroxylase gene is very low in early primary cells and metacestodes and highest in protoescoleces and adults (see Figure 10a). High expression in protoscoleces and adults could be attributed to high demand of serotonin in the nervous system. The absence of a serotonergic nervous system (Koziol et al., 2013) could account for the low expression in the early primary cell stage and the metacestode stage. However, expression in the late primary cell stage indicates that serotonin synthesis is not restricted to the nervous system. Interestingly, the aromatic-L-amino-acid decarboxylase gene is highest expressed in the metacestode stage without brood capsules (see Figure 10c). However, aromatic-L-amino-acid decarboxylase enzyme has a wide substrate selectivity (Berry et al., 1996) and its gene

expression cannot be attributed to serotonin synthesis alone. The serotonin transporter gene is highly expressed in primary cells, protoscoleces and gravid adults compared to lower expression in metacestodes and pre-gravid adults (see Figure 10b) which indicates expression is not restricted to the nervous system. Serotonin receptor genes are expressed in all stages but expression is highest in protoscoleces (see Figure 10d).

To gain more information about structure and function of the serotonin transporter in *Echinococcus spp.*, the predicted amino acid sequence of the *E. multilocularis* serotonin transporter EmSERT was analysed with HMMTOP (Tusnády and Simon, 1998; Tusády and Simon, 2001) and TMHMM (Krogh et al., 2001) revealing 12 transmembrane domains with both N- and C-termini predicted to be located in the cytosol; a topology that is conserved in N^+/Cl^- dependent neurotransmitter transporters (Barker et al., 1998*a*).
Comparison with the amino acid sequence of the *E. granulosus* serotonin transporter EgSERT showed high similarity. Only 13 amino acids are different in the predicted protein sequences, none of them concerning conserved regions or known binding sites. On the nucleotide level more changes are noticeable. 43 nucleotides have been exchanged and while both genes contain 12 introns, only 6 of them have the same size. 5 vary in size only a few base pairs, but the first intron in *emsert* is 575 bp long compared to 803 bp in *egsert*. Although there are obvious differences in the gene sequences, they do not affect known binding sites.
EmSERT is not only very similar to EgSERT. A MUSCLE alignment showed high conservation of EmSERT to schistosome (81% similarity), *Drosophila* (69% similarity), mouse (68 % similarity) and human (67 % similarity) serotonin transporter (see Figure 11).
The conservation of EmSERT and EgSERT compared to the human serotonin transporter also involves known serotonin and serotonin reuptake inhibitor (SSRI) binding sites. 14 of 19 amino acids of the human serotonin transporter contributing to ligand binding according to Ravna et al. (2006) are conserved in EmSERT and EgSERT (see Table 2), in particular residues in or near TM1 (D98, P106), TM2 (F117, Y121, M124), TM4 (F263, T264), TM7 (S372, G376, F380), TM8 (Y516) and TM11 (F548, F551, I552) of the human serotonin transporter. Although most binding sites are conserved, some amino acids were substituted compared to the human serotonin transporter: Y95F and V102I in TM1, M260L in TM4, T519G in TM10 and L547A in TM11 (see Figure 11).
In contrast, binding sites for selective serotonin reuptake inhibitors (SSRI) according

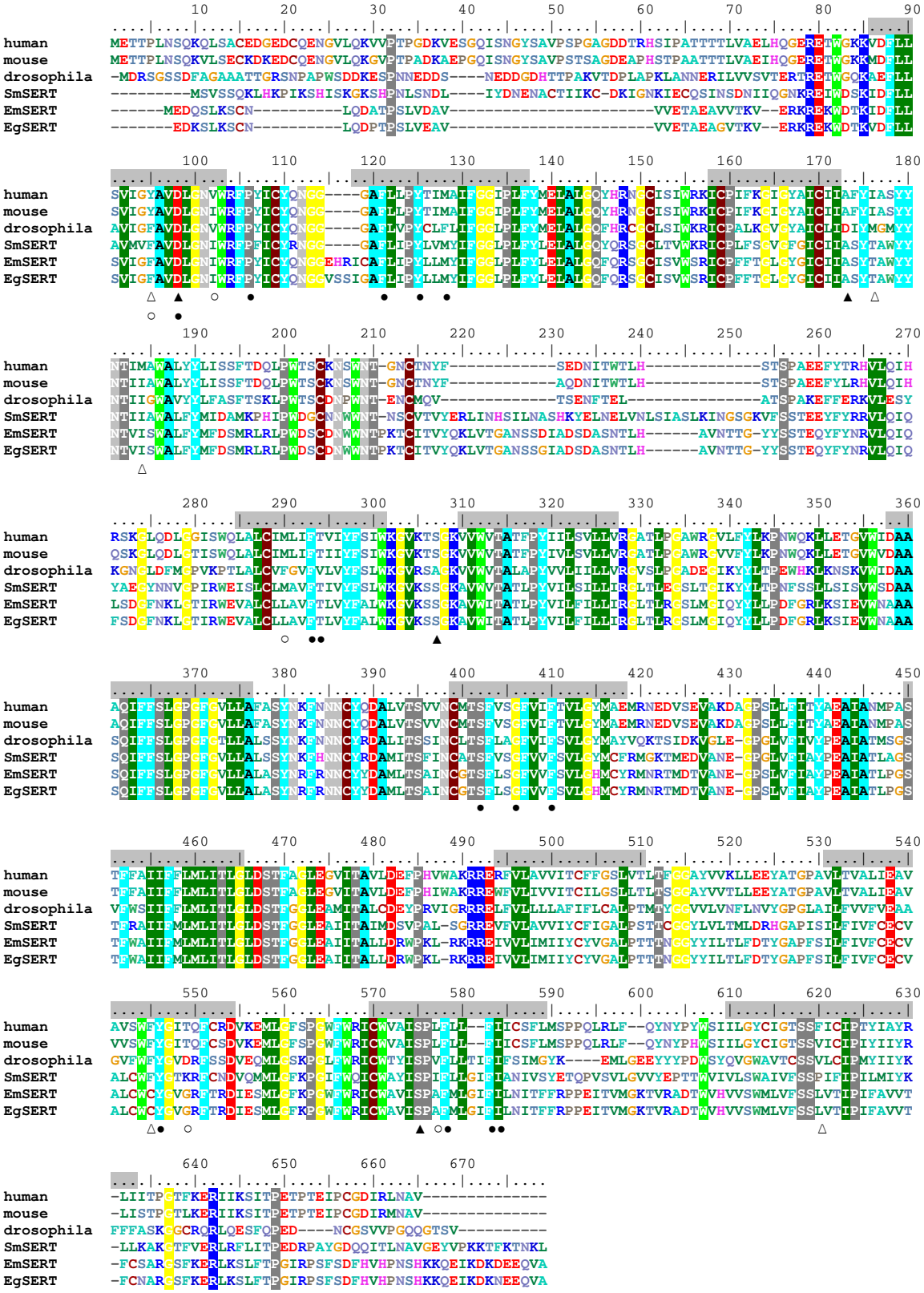


Figure 11: **Comparison of serotonin transporter amino acid sequences:** Shown is a MUSCLE-alignment of the human, mouse, drosophila, *S. mansoni* (SmSERT), *E. multilocularis* (EmSERT) and *E. granulosus* (EgSERT) serotonin transporter visualized by BioEdit. 100% identical amino acids are highlighted. Transmembrane domains of EmSERT according to HMMTOP are shown in gray. Bullets indicate amino acids of human serotonin transporter that contribute to ligand binding according to Ravna et al. (2006) while triangles mark selective serotonin reuptake inhibitors' (SSRI) binding sites (Andersen et al., 2009). Black bullets and triangles show in EmSERT and EgSERT conserved binding sites, while white ones identify substitutions.

	mouse	drosophila	SmSERT	EmSERT	EgSERT
Identities to hSERT	93%	55%	46%	50%	50%
Ligand binding sites	18 of 19	13 of 19	15 of 19	14 of 19	14 of 19
Binding sites for SSRI	7 of 9	3 of 9	5 of 9	4 of 9	4 of 9

Table 2: **Conservation of serotonin and SSRI binding sites in serotonin transporters**: Table shows identities to human serotonin transporter and numbers of conserved amino acid residues contributing to ligand (Ravna et al., 2006) or SSRI binding (Andersen et al., 2009) compared to human serotonin transporter.

to Andersen et al. (2009) are less conserved. Only 4 of 9 amino acids known to be involved in SSRI binding in the human serotonin transporter are conserved in EmSERT and EgSERT: D98 (TM1), A169 (TM2), S276 (TM5) and S545 (TM11) (see Figure 11 and Table 2).
Important substitutions are Y95F (TM1) and I172T (TM3). Double substitution at Y95 and I172 dramatically reduces the inhibitory potency of citalopram (Henry et al., 2006). Additional substitutions in EmSERT and EgSERT compared to human serotonin transporter are M180I (TM3), F513C (TM10) and F586L (TM12). Although EmSERT and EgSERT show high sequence similarity with the human serotonin transporters, important substitution have taken place concerning the binding of SSRIs, particularly citalopram.

To learn more about the phylogenetic relationship of serotonin transporters of different organisms, amino acid sequences of serotonin- and, as comparison, dopamine transporters were aligned using MUSCLE (Edgar, 2004*a*,*b*) and a phylogenetic tree was built from the resulting multiple sequence alignment with the neighbor-joining method (Saitou and Nei, 1987) using MEGA4 (Tamura et al., 2007). The resulting phylogenetic tree (see Figure 12) shows a close phylogenetic relationship between the serotonin transporters of flatworms, while the relationship to the human serotonin transporter is more distant, reflecting the evolutionary distance of the organisms. The dopamine transporters, on the other hand, form a completely independent branch from the serotonin receptors, indicating that similarity of the serotonin transporter between species is greater than the similarity to the dopamine receptor of the same species.

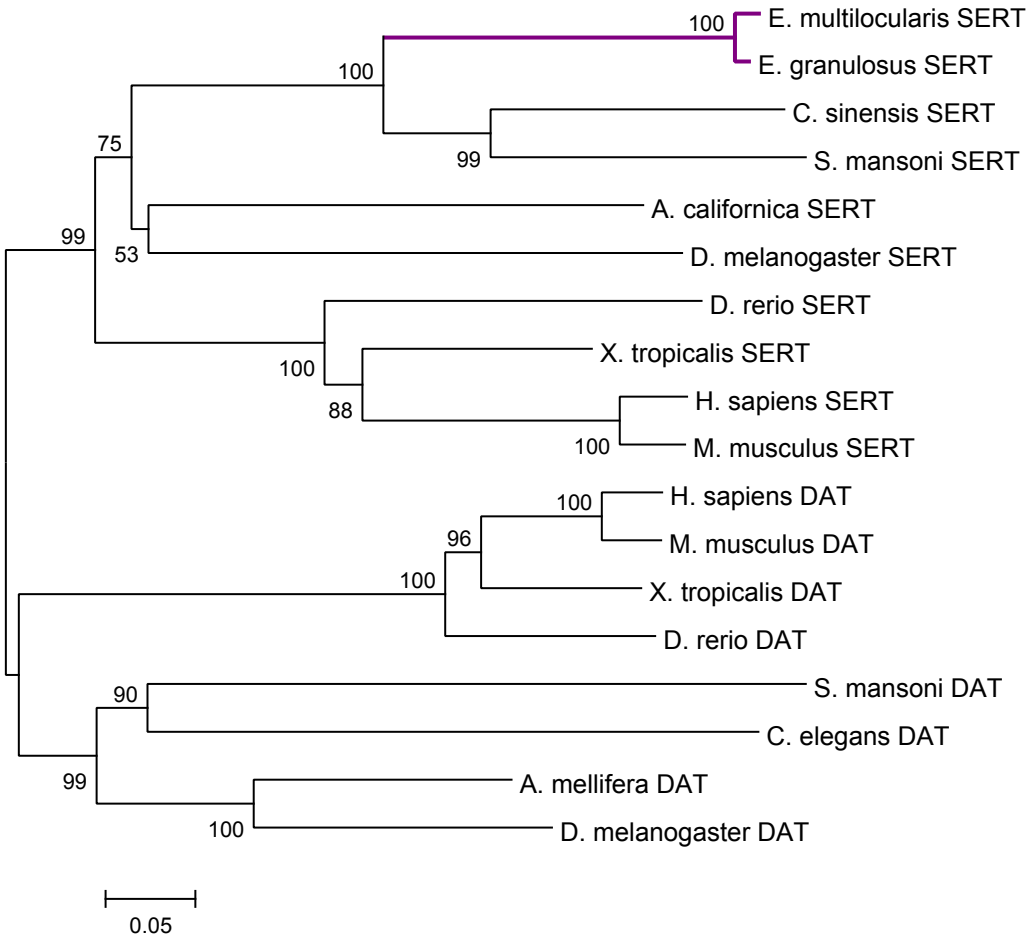


Figure 12: **Phylogenetic analysis of serotonin and dopamine transporters:** Amino acid sequences of serotonin (SERT) and dopamine (DAT) transporters were aligned with MUSCLE (Edgar, 2004*a*,*b*). The resulting multiple sequence alignment was used to build a phylogenetic tree with MEGA4 (Tamura et al., 2007) using the neighbor-joining method (Saitou and Nei, 1987). Numbers are bootstrap values and branch length indicates genetic distance. Sequence sources are listed in Appendix 9.4

3.1.2 Four genes with high homology to adenylate cyclase IX in the *E. multilocularis* and three in the *E. granulosus* genome

Via blast searches four genes homologous to adenylate cyclase type IX were identified in the *E. multilocularis* genome, named *emac1*, *emac2*, *emac3* and *emac4*. ESTs, visualization of transcriptome data with IGV (Robinson et al., 2011) and predicted protein sequences were used to generate a putative gene sequences *in silico*. The coding sequences were cloned and sequenced, using gene specific primers (see Materials and Methods 2.4) designed in conserved regions, from metacestode cDNA libraries or oligo-dT reverse transcribed non-activated protoscoleces' RNA. The 5' and 3' ends were obtained by RACEs from metacestode cDNA libraries (yeast two hybrid libraries). Unfortunately, only the sequence of *emac1* could be obtained completely and a part of *emac2* was sequenced. PCR with *emac3* or *emac4* specific

primers was unsuccessful, probably because of very low gene expression of *emac3* or *emac4* in metacestodes and protoscoleces according RNAseq data (see Figure 14). The final sequence of *emac1* (see Appendix 9.5) contains a short 5' untranslated region, followed by a open reading frame of 3501 bp, a 236 bp untranslated 3' end and a polyA tail. A signal peptide could not be detected. Nor was there any evidence of trans-splicing. The gene sequence contains 7 introns with a size varying from 69 bp to 3282 bp. All introns are located in the first half of the gene.
The partial sequence of *emac2* (see Appendix 9.5) has a continuous open reading frame and encodes the last part of the adenylate cyclase, an adenylate/guanylate cyclase catalytic domain that is followed by a short untranslated 3' end and a polyA tail. In contrast to *emac1*, the predicted sequences of *emac2*, *emac3* and *emac4* have a continuous open reading frame and don't contain introns.
With HMMsearch and blast searches three genes homologous to adenylate cyclase IX were identified in the *E. granulosus* genome: *egac1*, *egac2* and *egac3*. Putative protein sequences were deduced from *E. multilocularis* protein sequences and the *E. granulosus* genome. While EgAC1, EgAC2 and EgAC3 show high amino acid sequence identities with their respective homologue in *E. multilocularis* (>96% identities), no *E. granulosus* homologue could be found for the fourth *E. multilocularis* adenylate cyclase EmAC4 (see Table 3).

Name	**Protein prediction** (% of identity between Em and Eg)	**NCBI accession number for the first human hit** (% of identity)	**Swiss-prot accession number for the first hit** (species,% of identity)
EmAC1	**deduced from sequenced cDNA** (98.4%)	**BAG58443.1** (36)	**U6IWJ2_HYMMI** (*H. microstoma*, 79.0%)
EgAC1	**deduced from EmAC1** (98.4%)	**NP_066193.1** (36)	**U6IWJ2_HYMMI** (*H. microstoma*, 79.0%)
EmAC2	**pathogen_EMU_contig_3320 |846192-849642|g74.t1** (96.4%)	**AAC24201.1** (24)	**H2KR56_CLOSI** (*C. sinensis*, 27.0%)
EgAC2	**deduced from EmAC2** (96.4%)	**NP_640340.2** (28)	**H2KR56_CLOSI** (*C. sinensis*, 27.0%)
EmAC3	**pathogen_EMU_scaffold_007780. g9645.t1 96-1054** (97.2%)	**AAC24201.1** (29)	**G7YC93_CLOSI** (*C. sinensis*, 35.0%)
EgAC3	**deduced from EmAC3** (97.2%)	**AAC24201.1** (30)	**G7YC93_CLOSI** (*C. sinensis*, 34.0%)
EmAC4	**pathogen_EMU_scaffold_007780.g9623.t1**	**AAC24201.1** (29)	**G7YC93_CLOSI** (*C. sinensis*, 34.0%)

Table 3: **Predicted proteins of *E. multilocularis* and *E. granulosus*, homologous to adenylate cyclase IX**: The *E. multilocularis* homologues were identified using reciprocal blast searches. The *E. granulosus* homologues were found with HMMsearch and the sequence was deduced from the *E. multilocularis* protein predictions. Shown are the identities between *E. mulilocularis* and *E. granulosus* homologues, the first human hit in the NCBI protein sequence database and the first hit in the Swiss-Prot protein sequence database.

All *Echinococcus* adenylate cyclases show higher homology among flatworm adenylate cyclases than with human homologues. However, EmAC1 and EgAC1 are evo-

lutionary more conserved, with 79 % identities among flatworms and 36 % identities with human homologues, than the other *Echinococcus* adenylate cyclases with 27-34 % identities among flatworm adenylate cyclases and 24-30 % identities with human homologues (see Table 3).

For insights into the origin of *Echinococcus* adenylate cyclases, adenylate/guanylate cyclase domains in *E. multilocularis*, *E. granulosus* and *S. mansoni* were identified by HMMsearch. Via NCBI BLAST as adenylate cyclases type IX identified cyclase domains were used to generate a phylogenetic tree in MEGA4 (Tamura et al., 2007) with the neighbor-joining method (Saitou and Nei, 1987). The only *S. mansoni* adenylate cyclase type IX clusters with EgAC1 and EmAC1. EmAC3 and EmAC4 are almost identical, they only differ in one amino acid and form a cluster with EgAC3. On the other hand EgAC2 and EmAC2 differ most from the other adenylate cyclases (see Figure 13). The varying numbers and similarity of adenylate cyclases in these organisms indicate gene duplications at different time points in evolution.

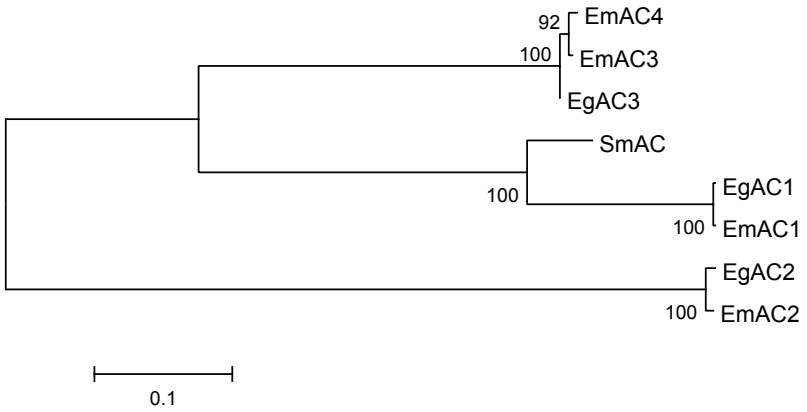


Figure 13: **Phylogenetic relationship of adenylate cyclases in *E. multilocularis*, *E. granulosus* and *S. mansoni*:** Cyclase domains of adenylate cyclases type IX were identified by HMMsearch, confirmed by NCBI BLAST, stuck together to create a fusion amino acid sequence consisting of the two cyclase domains of one adenylate cyclase and aligned with MUSCLE (Edgar, 2004*a*,*b*). The neighbour-joining tree was generated with MEGA4 (Tamura et al., 2007). Numbers are bootstrap values and branch length indicates genetic distance. **Em**: *E. multilocularis*, **Eg**: *E. granulosus*, **Sm**: *S. mansoni*; source for SmAC: emb|CCD58799.1|

Possible structural differences of the four adenylate cyclases type IX in *E. multilocularis* and the three in *E. granulosus* were analyzed in a domain analysis with SMART (Schultz et al., 1998; Letunic et al., 2012). For EmAC1 the amino acid sequence was translated from the sequenced *emac1* while for EmAC2, EmAC3, EmAC4, EgAC1, EgAC2 and EgAC3 the predicted protein sequences were used. Analysis with SMART (Schultz et al., 1998; Letunic et al., 2012) showed that all adenylate cyclases contain two adenylate/guanylate cyclase catalytic domains and two spans of transmembrane domains as is typical for membrane bound adenylate

cyclases (Hanoune et al., 1997). EmAC1, EmAC3, EmAC4, EgAC1 and EgAC3 contain one active (C1) and one inactive (C2) domain. EmAC2 and EgAC2, however, contain two inactive domains without catalytic function (see Figure 14a). Both C1 and C2 are necessary for activity (Tang et al., 1995) although only C2 has catalytic activity. Two amino acids, an asparagine and an arginine, are essential for catalytic activity in C2 domains (Yan et al., 1997) and are substituted in the non-catalytic C1 domains and the inactive C2 domain of EmAC2 and EgAC2. Therefore EmAC2 and EgAC2 consist of two inactive domains and are unlikely to have catalytic activity. Nonetheless, according to *E. mulilocularis* RNAseq data, EmAC2 is expressed at all stages although to a much lower extent than EmAC1. EmAC1 is highest expressed in non-activated protoscoleces and lowest in metacestode vesicles. In comparison to EmAC1, EmAC3 and EmAC4 are expressed quite low and only in the adult worm (see Figure 14b). Note that EmAC3 and EmAC4 not only have almost identical sequences but also a very similar expression profile.

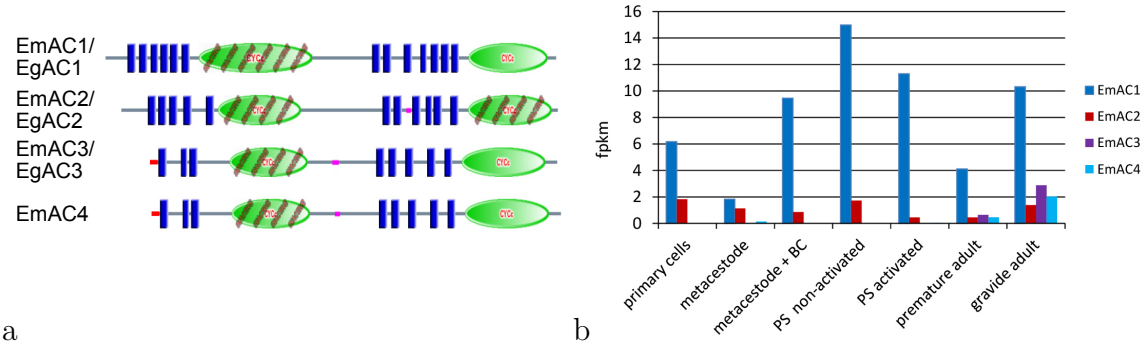


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Figure 14: **Domain analysis and expression of adenylate cyclases:** (**a**) Domain analysis was performed with SMART (Schultz et al., 1998; Letunic et al., 2012). Used sequences are listed in Appendix 9.5. Blue bars indicate transmembrane regions, green Cyc-domains are active or inactive adenylate/guanylate cyclase catalytic domains, presumed signal peptides are marked red. (**b**) Expression was calculated using RNA-seq data and counting the number of reads for each gene. Number of reads was normalized to the total number of reads in one data set and to exon length. Expression is shown in fragments per kilobase of exon per million fragments mapped (fpkm). **PS**: protoscoleces; **BC**: brood capsules

3.1.3 All major G-protein coupled receptor families, but no glucagon receptor, are present in the *E. multilocularis* genome

G-protein coupled receptors (GPCRs) are a large receptor family. They have various functions in cell signalling and often serve as pharmaceutical targets. To get a general overview, GPCRs of *E. multilocularis* were identified via blast analysis using sequences of *S. mansoni* and *S. mediterranea* GPCRs (Zamanian et al., 2011). After confirmation with NCBI BLAST, identified sequences were analysed and sorted

into families according to the GRAFS system (Fredriksson et al., 2003; Fredriksson and Schiöth, 2005). In total 76 GPCRs could be identified, including members of all the major families. Only 55 of the predicted receptors contained all seven transmembrane domains. Protein sequences with 5 or 6 predicted transmembrane domains were considered near full length sequences, while sequences with less than 5 transmembrane domains were classified as partial sequences. The largest family in *E. multilocularis* is the *Rhodopsin* family with 61 members, 42 full length, 11 near full length and 8 partial sequences. The *Rhodopsin* family also contains the predicted serotonin receptors analysied above. The other families are much smaller. 5 *Fizzled*, 4 *Glutamate*, 3 *Adhesion* and 2 *Secretin* GPCRs were identified.

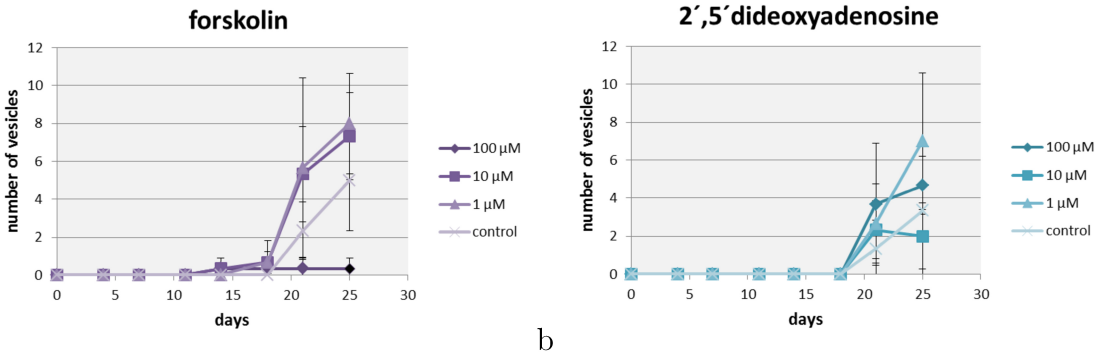
Glucagon receptors are GPCRs of the *Secretin* family. To find out if one of them might be a glucagon receptor, three genes of GPCRs, two of the *Secretin* family (*emgpcr1* and *emgpcr2*) and one of the *Adhesion* family (*emgpcr3*), which at first appeared to belong to the *Secretin* family, were sequenced (see Appendix 9.5) from metacestode cDNA libraries or oligo-dT reverse transcribed non-activated protoscoleces' RNA using gene specific primers (see Materials and Methods 2.4). The 5' and 3' ends were obtained by RACEs from metacestode cDNA libraries (yeast two hybrid libraries). *emgpcr1* has two different splice forms although only one gives rise to a complete protein with 7 transmembrane domains. It was therefore considered to be the correct splice variant while the other form is probably due to irregular splicing. None of the splice forms was trans-spliced nor did encode a protein with predicted signal peptide. The complete splice form is 1455 bp long and contains 10 introns. One of the introns contains a highly expressed unidentified sequence. The amino acid sequence of EmGPCR1 includes a 7-transmembrane domain as well as a hormone binding domain which have been identified by SMART analysis. According to blast analysis the protein is similar to a diuretic hormone receptor. The gene of the other member of the *Secretin* family *emgpcr2* is 2162 bp long and contains only one intron. The complete gene, including the sequence encoding a predicted signal peptide, was sequenced and the amino acid sequence of EmGPCR2 shows similarity to a Methuselah receptor, that is important for life-span regulation (Alic and Partridge, 2007). For *emgpcr3*, a member of the *Adhesion* family, different splice forms could be detected, although, same as for *emgpcr1*, only one appears to be complete. *emgpcr3* is 2002 bp long and contains only one intron. Blast analysis with the amino acid sequence of EmGPCR3 show highest similarity to a latrophilin receptor that binds α-latrotoxin, a presynaptic neurotoxin (Matsushita et al., 1999).

As none of the sequenced GPCRs shows similarity to a glucagon receptor. It is therefore highly unlikely that *E. multilocularis* has a glucagon receptor orthologue.

3.2 *In vitro* drug and inhibitor studies of cAMP-pathway and serotonin reuptake on *E. multilocularis* primary cells

3.2.1 Forskolin stimulate metacestode development from *E. multilocularis* primary cells

It has been shown by Schilling (2010) that cAMP-signalling influences vesicle formation from *E. multilocularis* primary cells, which is an indicator for germinative cell proliferation and differentiation. To further investigate this, axenically cultivated primary cells were treated with 1 µM, 10 µM and 100 µM adenylate cyclase activator forskolin and 1 µM, 10 µM and 100 µM adenylate cyclase inhibitor 2',5' dideoxyadenosine. The with forskolin treated primary cell cultures as well as their controls developed small aggregates after 2 days and formed central cavities after 7 days. After 3 weeks primary cells treated with 1 µM and 10 µM forskolin formed more vesicles than control cultures. Treatment with 100 µM forskolin, on the other hand, significantly (P<0,05) reduced vesicle formation of primary cells after 25 days of incubation (see Figure 15a).



a b

Figure 15: **Vesicle formation of forskolin and 2',5' dideoxyadenosine treated primary cells:** primary cells were cultivated under axenic conditions in c-DMEM-A and incubated with adenylate cyclase activator forskolin (**a**) or adenylate cyclase inhibitor 2',5' dideoxyadenosine (**b**) at indicated concentrations. Formed vesicles were counted as an indicator of germinative cell proliferation and differentiation. Experiments were performed in triplicate. Error bars 1 SD. Data points marked in black indicate treatments found to be significantly different from controls (P<0,05).

Cultures treated with 2',5' dideoxyadenosine and their controls also started to develop small aggregates after 2 days but formed very little central cavities. After 3 weeks vesicles could be seen in all cultures but no clear difference could be observed between cultures treated with different concentrations (see Figure 15b).
These results indicate that stimulation of cAMP-pathway with 1 µM and 10 µM forskolin has a positive effect on vesicle formation while 100 µM forskolin significantly

inhibits vesicle formation. Inhibition of cAMP-pathway with 2',5' dideoxyadenosine has no visible effect on vesicle formation.

3.2.2 Serotonin stimulates metacestode development from *E. multilocularis* primary cells

In order to influence cAMP-signalling in a more physiological way, glucagon and serotonin were used as possible ligands for GPCRs. Primary cell cultures treated with glucagon (50 nM, 500 nM and 5 µM) formed aggregates after 3 days and central cavities could be observed after 7 days. First vesicles appeared shortly afterwards. While cultures treated with 5 µM glucagon formed less vesicles than control cultures, cultures treated with 50 nM and 500 nM formed more vesicles than controls (see Figure 16a).

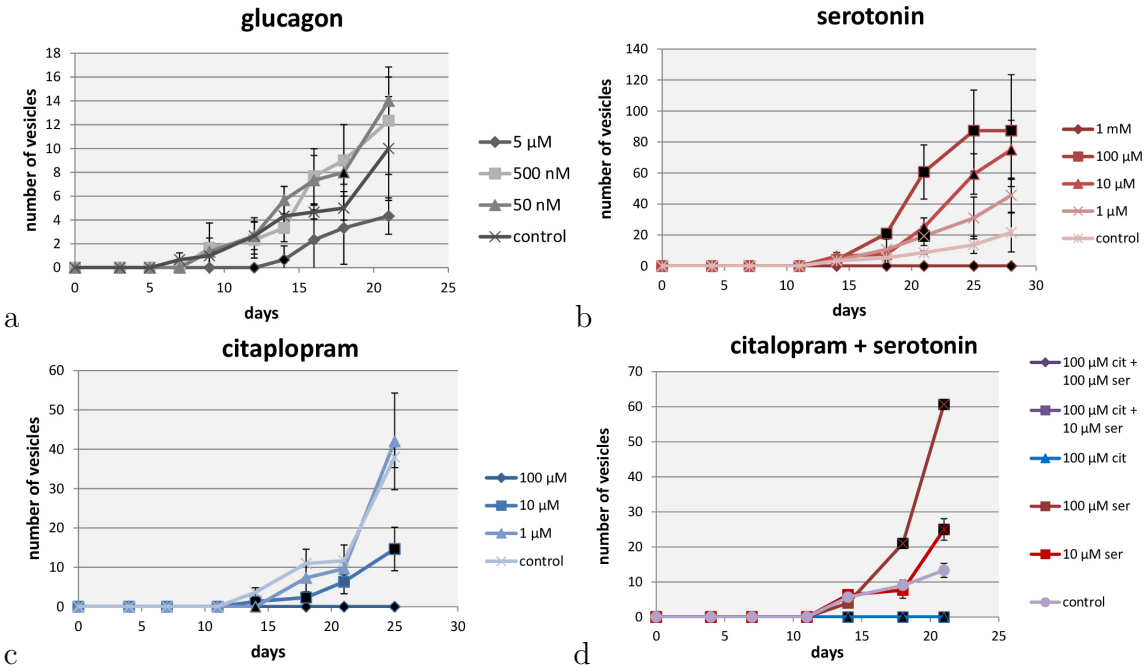


Figure 16: **Vesicle formation after treatment of primary cells with glucagon, serotonin and/or citalopram:** primary cells were cultivated under axenic conditions in c-DMEM-A and incubated with glucagon (**a**) and serotonin (**b**), as possible GPCR ligands, and with citalopram (**c**), an inhibitor of the serotonin transporter. Cultures were also treated with a combination of serotonin and citalopram (**d**). Formed vesicles were counted as an indicator of germinative cell proliferation and differentiation. Experiments were performed in triplicate. Error bars 1 SD. Data points marked in black indicate treatments found to be significantly different from controls (P<0,05).

Serotonin stimulated vesicle formation of primary cells in a dose dependent manner at 1 µM, 10 µM and 100 µM whereas 1 mM serotonin inhibited cell proliferation completely (see Figure 16b). Primary cells aggregated after 2 days and central

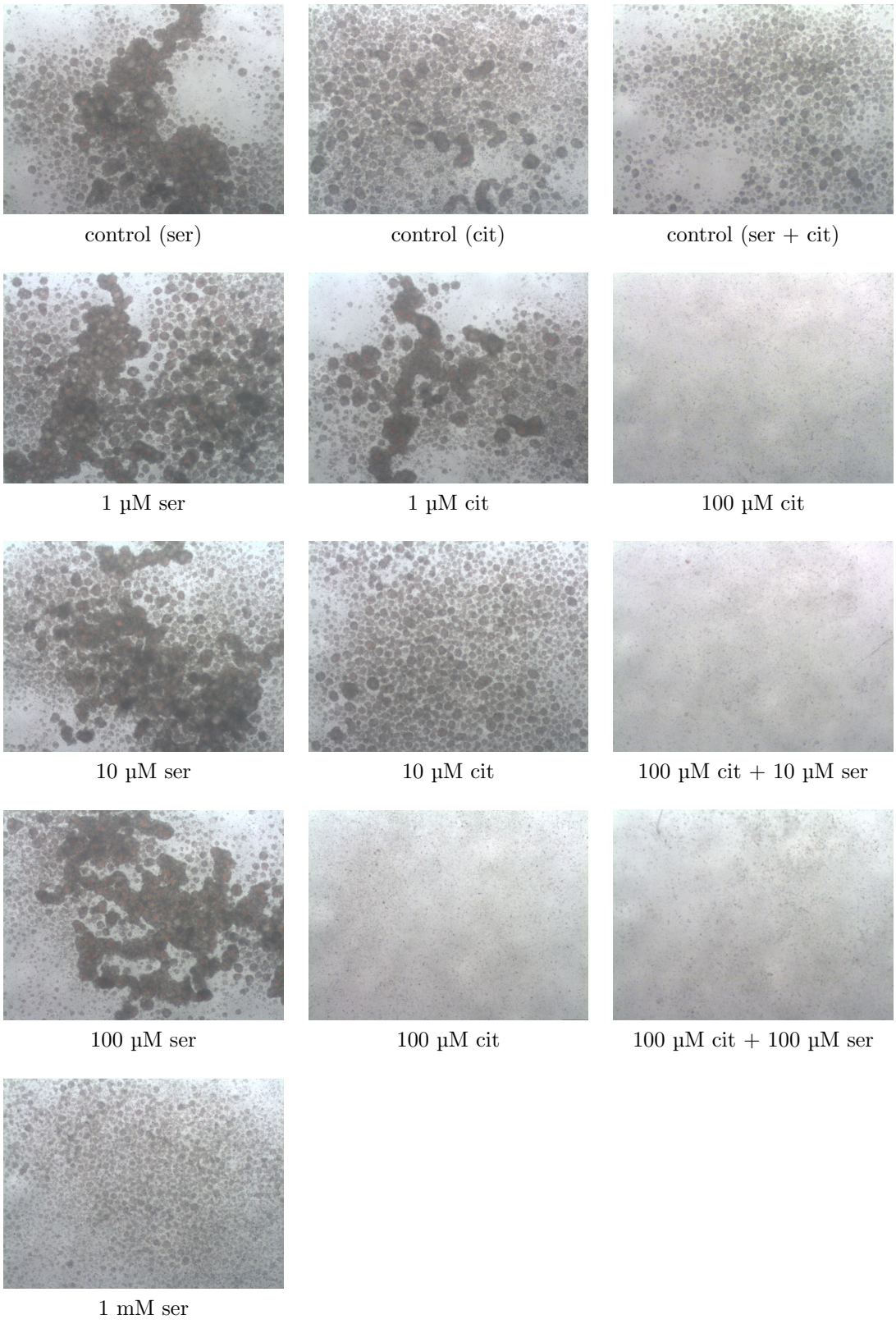


Figure 17: **Aggregation of primary cells after treatment with serotonin and citalopram:** Primary cells were cultivated under axenic conditions in c-DMEM-A. Cultures shown are 7 days old, treated with serotonin **(ser)** and/or citalopram **(cit)** at indicated concentrations. Micrography was obtained with optical microscope.

cavities developed after 7 days. First vesicles could be observed after 2 weeks. After 18 days cultures treated with 1 mM serotonin formed significantly (P<0,05) less vesicles than control cultures. Cultures treated with 1 mM serotonin only formed very small aggregates and did not develop any central cavities or vesicles (see Figure 17). Primary cells treated with 10 µM or 100 µM formed significantly more vesicles than control cultures (P=0,003 after 18 days and P=0,01 after 21 days, respectively) (see Figure 16b).

In contrast, treatment with the serotonin transporter inhibitor citalopram significantly inhibited primary cell aggregation and vesicle formation. In control cultures aggregates formed after 2 days and developed central cavities after 7 days. First vesicles appeared in control cultures after 2 weeks. Treatment with 100 µM citalopram prevented any formation of aggregates and therefore inhibited development of vesicles (see Figure 17). Differences between cultures treated with 100 µM citalopram and control cultures became significant after 14 days of incubation (P<0,01). Though cultures treated with 10 µM citalopram formed aggregates and developed vesicles, after 14 days of incubation significantly (P<0,05) less vesicles could be observed than in control cultures. No difference could be observed between primary cells treated with 1 µM citalopram and control cultures (see Figure 16c).

To determine whether the inhibitory effect of citalopram can be prevented by applying serotonin, with 100 µM citalopram pre-incubated primary cell cultures were then treated with a combination of citalopram and serotonin. Cultures treated with 100 µM citalopram and 100 µM serotonin as well as cultures treated with 100 µM citalopram and 10 µM serotonin did not form aggregates and thus did not develop vesicles (see Figure 17). So there could be no difference observed compared to cultures treated with 100 µM citalopram. Differences between cultures treated with citalopram alone or in combination with serotonin compared to control cultures became significant after 14 days of incubation (P<0,01). In controls without serotonin and citalopram first vesicles were observed after 2 weeks (see Figure 16d).

It could be shown that serotonin significantly induced vesicle formation while inhibition of the serotonin transporter significantly inhibited vesicle formation. The inhibitory effect of citalopram could not be counteracted by adding serotonin.

3.3 *In vitro* drug and inhibitor studies of cAMP-pathway and serotonin reuptake on *E. multilocularis* metacestode vesicles

As alveolar echinococcosis is caused by the metacestode stage of *E. mulilocularis*, it is important to analyse the influence of cAMP-signalling on metacestode development. Therefore metacestode vesicles were treated with forskolin and 2',5' dideoxyadenosine, to influence adenylate cyclase activity directly, as well as with glucagon and serotonin, which act as GPCR ligands.

3.3.1 2',5'-dideoxyadenosine reduces survival of metacestode vesicles

Treatment with the adenylate cyclase activator forskolin slightly reduced survival of metacestode vesicles. After 10 days of incubation 7% of the vesicles treated with 100 µM forskolin were disintegrated, compared to 3% in cultures treated with 1 µM and 10 µM forskolin. All vesicles in contol cultures were still alive after 10 days.
Incubation with the adenylate cyclase inhibitor 2',5'-dideoxyadenosine also reduced survival of vesicles. After 10 days of incubation 25% of the vesicles treated with 300 µM 2',5'-dideoxyadenosine were disintegrated compared to 9% disintegrated vesicles in controls (see Figure 18 a).
These results indicate that activation of adenylate cyclase influences the survival of metacestode vesicles.

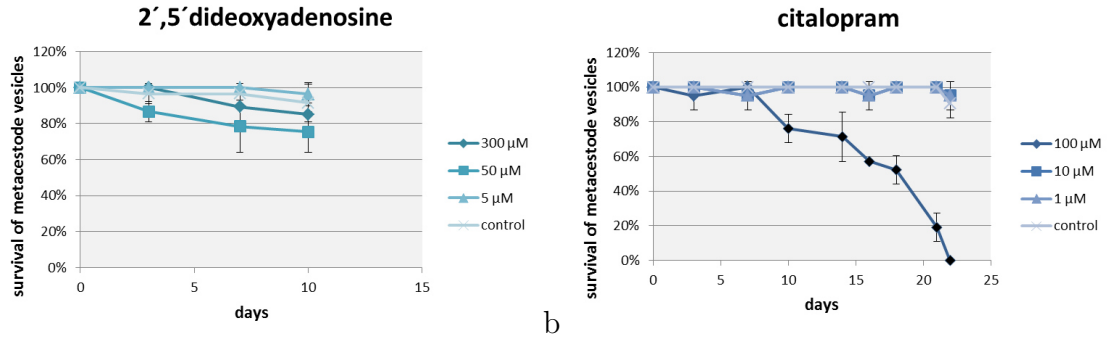


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Figure 18: **Survival of metacestode vesicles treated with adenylate cyclase inhibitor 2',5'-dideoxyadenosine or serotonin transporter inhibitor citalopram:** Vesicles were cultivated under axenic conditions in DMEM (2% FCS)(2',5'-dideoxyadenosine) or c-DMEM-A (citalopram) and incubated with 2',5'-dideoxyadenosine **(a)** or citalopram **(b)**at indicated concentrations. Survival of vesicles was determined by microscopical observation. Experiments were performed in triplicate. Error bars 1 SD. Data points marked in black indicate treatments found to be significantly different from controls (P<0,05).

3.3.2 Citalopram kills metacestode vesicles

Treatment with glucagon (50 nM, 500 nM and 5 µM) had no visible effect on metacestode vesicles. Serotonin, however, influenced vesicle growth. While 1 µM serotonin inhibited vesicle growth, higher concentrations (10 µM, 100 µM and 1 mM) induced vesicle growth in a dose dependent manner.
To learn more about the effect of serotonin, vesicles were incubated with serotonin transporter inhibitor citalopram. After 10 days of incubation with 100 µM citalopram significantly (P<0,05) less vesicles survived than in control cultures. After 3 weeks all vesicles treated with with 100 µM citalopram were disintegrated (see Figure 18b). Vesicles first lost their tension, then collapsed and the germinal layer dissociated from the laminal layer (see Figure 19). In contrast, untreated vesicles and vesicles treated with 1 µM or 10 µM citalopram survived to more than 90% (see Figure 18b).
It could be shown that higher concentrations of serotonin induced vesicle growth while inhibition of serotonin transport resulted in vesicle disintegration.

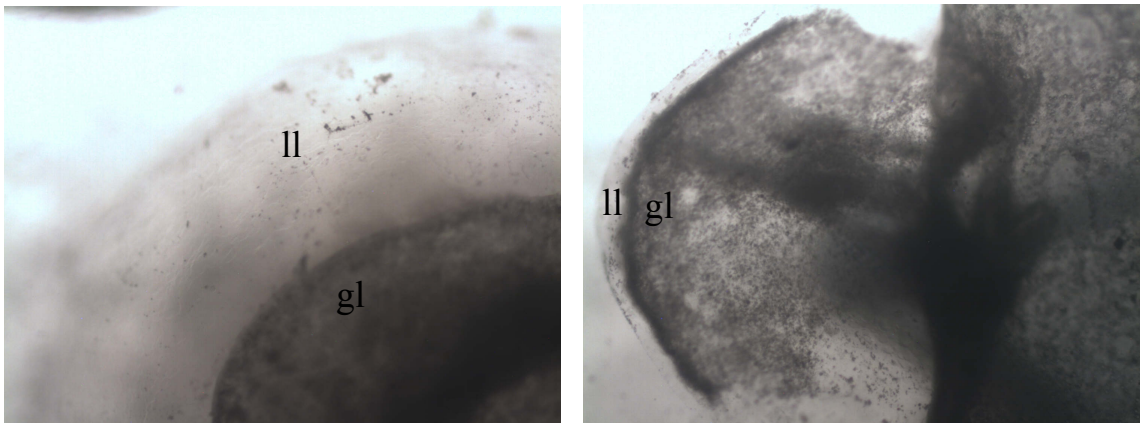


Figure 19: **Metacestode vesicle treated with citalopram:** Vesicles were cultivated under axenic conditions in c-DMEM-A and treated with 100 µM citalopram. Micrography was obtained by optical microscope after 3 weeks of treatment. **ll**: laminated layer, **gl**: germinal layer

3.4 *In vitro* experiments on *E. granulosus* protoscoleces

In order to study the effects of cAMP-signalling in different larval stages, inhibition and stimulation experiments were also performed on *E. granulosus* protoscoleces.
Development of protoscoleces was analysed in respect of re-differentiation and survival of protoscoleces. Re-differentiating protoscoleces vesicularise and become pre-microcysts. Pre-microcysts are completely vesicularised, almost without movement and nearly transparent. In contrast to microcysts they don't possess a laminated

layer (Ponce Gordo and Cuesta Bandera, 1997). Protoscoleces were considered to be dead when they lost their hooks, condensed, lost their defined structure and took on a darker color.

3.4.1 Forskolin influences re-differentiation and survival of *E. granulosus* protoscoleces

To determine the effect of stimulating adenylate cyclase with forskolin as well as to find out if the solvent of forskolin has an effect on protoscolex development, protoscoleces were treated with forskolin solved in ethanol as well as with forskolin solved in DMSO. Both, cultures treated with 100 µM forskolin solved in ethanol and in DMSO for 12 days, developed significantly ($P<0,01$ and $P<0,05$, respectively) more pre-microcysts than their respective control cultures. In contrast, cultures treated with 1 µM and 10 µM forskolin (solved in ethanol or DMSO) showed no difference compared to their respective control cultures concerning re-differentiation of protoscoleces (see Figure 20a,c).

However, protoscoleces treated with forskolin solved in DMSO (see Figure 20c) started to re-differentiate earlier than protoscoleces treated with forskolin solved in ethanol (see Figure 20a). This effect was observed at all concentrations as well as in control cultures.

Treatment with forskolin not only induced re-differentiation of protoscoleces but also caused death of protoscoleces. Protoscoleces treated with 100 µM forskolin solved in DMSO started to die after 12 days of culture and 97% of them were dead after 14 days (see Figure 20d). In comparison, protoscoleces treated with 100 µM forskolin solved in ethanol died later. After 17 days 47% of the protoscoleces were still alive (see Figure 20b). However, after 14 days of incubation, significantly ($P<0,05$) more protoscoleces were dead in all cultures treated with 100 µM forskolin compared to their respective control cultures. Protoscoleces treated with 1 µM or 10 µM forskolin died later than those treated with 100 µM forskolin but earlier than controls. Also at this concentrations, protoscoleces treated with forskolin solved in DMSO died earlier than those treated with forskolin solved in ethanol. Hence, treatment with forskolin induced re-differentiation and caused death of protoscoleces. Treatment with forskolin solved in DMSO induced earlier re-differentiation and death than treatment with forskolin solved in ethanol.

To inhibit adenylate cyclase, the p-site inhibitor 2',5'-dideoxyadenosine was used. Treatment with 100 µM 2',5'-dideoxyadenosine induced re-differentiation of protoscoleces. After 14 days of treatment 14% of the protoscoleces had re-differentiated into pre-microcysts compared to less than 2% in control cultures (see Figure 20e).

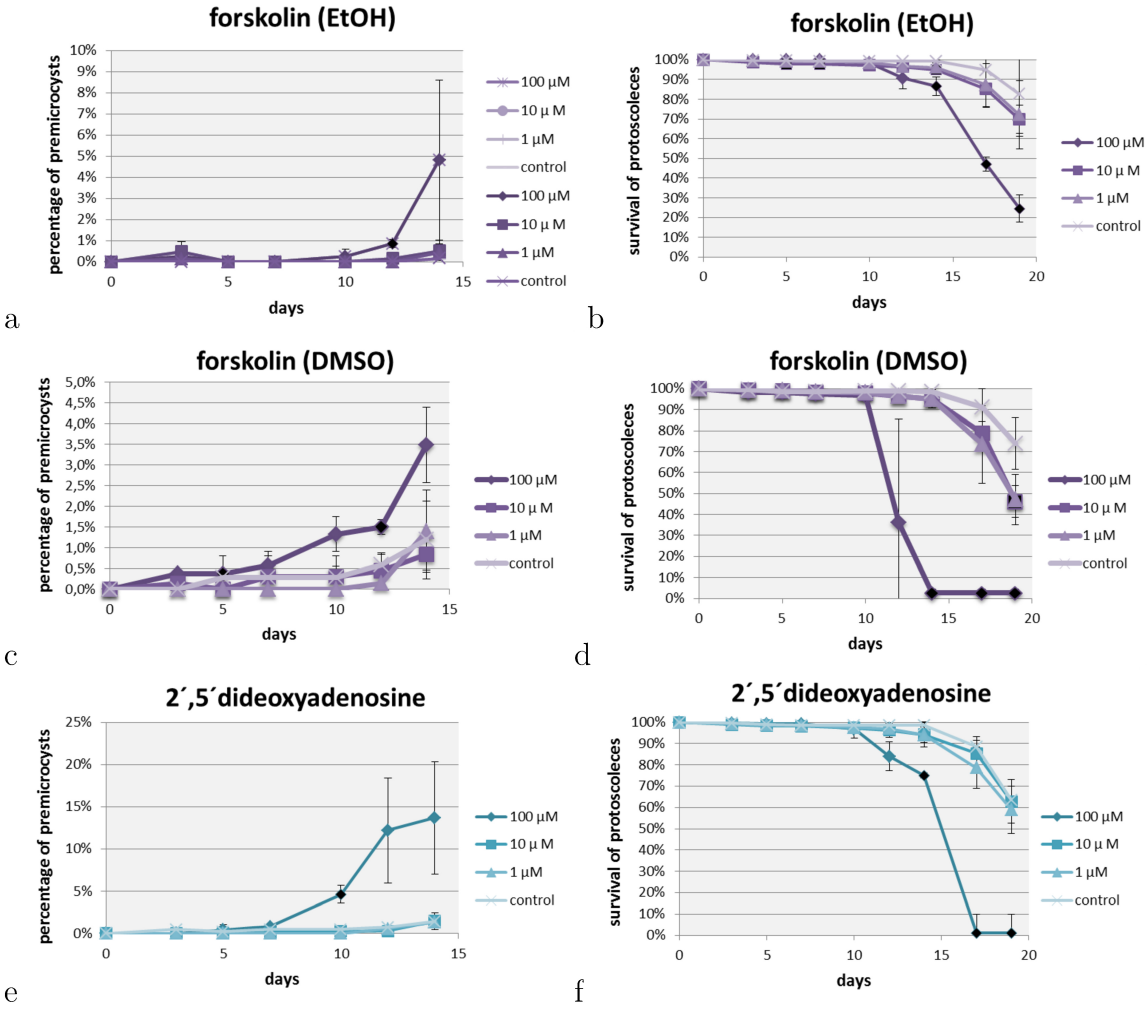


Figure 20: **Treatment of protoscoleces with adenylate cyclase activator forskolin and adenylate cyclase inhibitor 2',5'-dideoxyadenosine:** Protoscoleces were cultivated in DMEM (10% FBS) and incubated with forskolin solved in ethanol **(a,b)**, with forskolin solved in DMSO **(c,d)** and with 2',5'-dideoxyadenosine **(e,f)** at indicated concentrations. Pre-microcysts were counted as an indicator of re-differentiation of protoscolesces (left side). Survival of protoscoleces is shown at the right side. Experiments were performed in triplicate. Error bars 1 SD. Data points marked in black indicate treatments found to be significantly different from controls ($P<0,05$).

Protoscoleces incubated with 1 µM or 10 µM 2',5'-dideoxyadenosine re-differentiated no more than controls. Furthermore, treatment with 100 µM 2',5'-dideoxyadenosine resulted in death of protoscoleces. After 14 days of incubation significantly ($P<0,01$) more protoscoleces were dead in cultures treated with 100 µM 2',5'-dideoxyadenosine compared to control cultures; after 17 days all protoscoleces were dead compared to only 12% dead protoscoleces in controls (see Figure 20f). Treatment with 1 µM or 10 µM 2',5'-dideoxyadenosine had little effect on re-differentiation and death of protoscoleces compared to control.

These results indicate that both the adenylate cyclase activator forskolin and the

adenylate cyclase inhibitor 2',5'-dideoxyadenosine induces re-differentiation and death of protoscoleces.

3.4.2 Citalopram kills protoscoleces

To activate the cAMP-signalling pathway via GPCRs, glucagon and serotonin were used as ligands. No clear difference concerning re-differentiation could be observed between protoscoleces stimulated with glucagon (50 nM, 500 nM and 5 µM) and controls (see Figure 21a). However, protoscoleces stimulated with 50 nM or 500 nM glucagon died earlier than protoscoleces treated with 5 µM glucagon or controls. After 19 days of treatment 42 % of the protoscoleces treated with 50 nM or 500 nM were dead compared to 25% in controls (see Figure 21b).

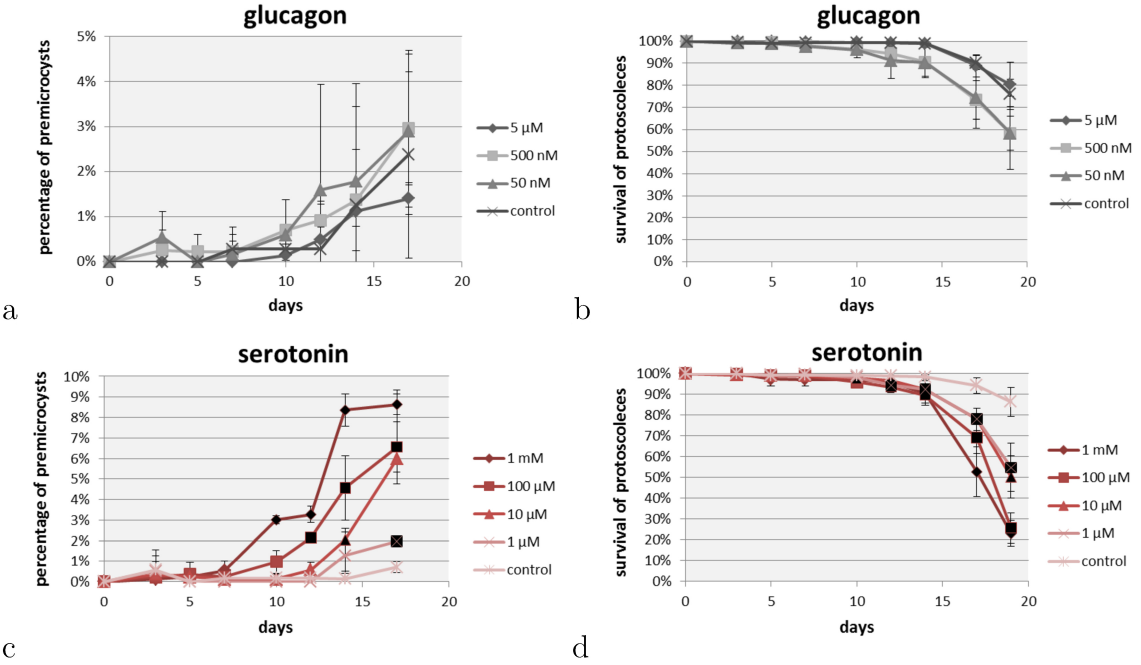


Figure 21: **Treatment of protoscoleces with GPCR ligands glucagon and serotonin:** Protoscoleces were cultivated in DMEM (10% FBS) and incubated with glucagon **(a,b)** and serotonin **(c,d)** at indicated concentrations. Pre-microcysts were counted as an indicator of re-differentiation of protoscoleces (left side). Survival of protoscoleces is shown at the right side.Experiments were performed in triplicate. Error bars 1 SD. Data points marked in black indicate treatments found to be significantly different from controls (P<0,05).

A clearer effect could be observed at protoscoleces treated with serotonin (1 µM, 10 µM, 100 µM and 1 mM). Serotonin induced re-differentiation in a dose dependent manner (see Figure 21c). Protoscoleces treated with serotonin formed significantly (P<0,01) more pre-microcysts than controls after incubation for 10 days (1mM serotonin), 12 days (100 µM serotonin), 14 days (10 µM serotonin) and 17 days (1 µM serotonin), respectively. After 14 days of treatment 8% of the protoscoleces

treated with 1 mM serotonin had formed pre-microcysts compared to less than 1% in controls (also see Figure 22). Treatment with lower concentrations of serotonin lead to a lower re-differentiation rate.

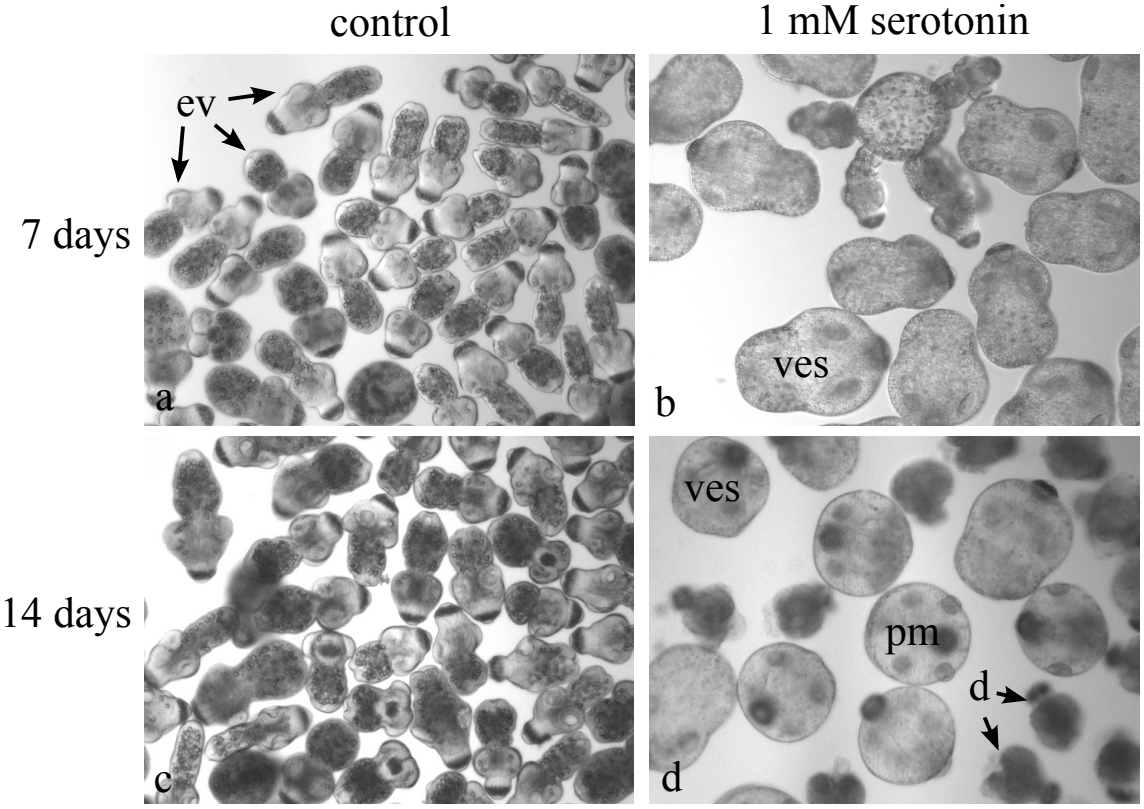
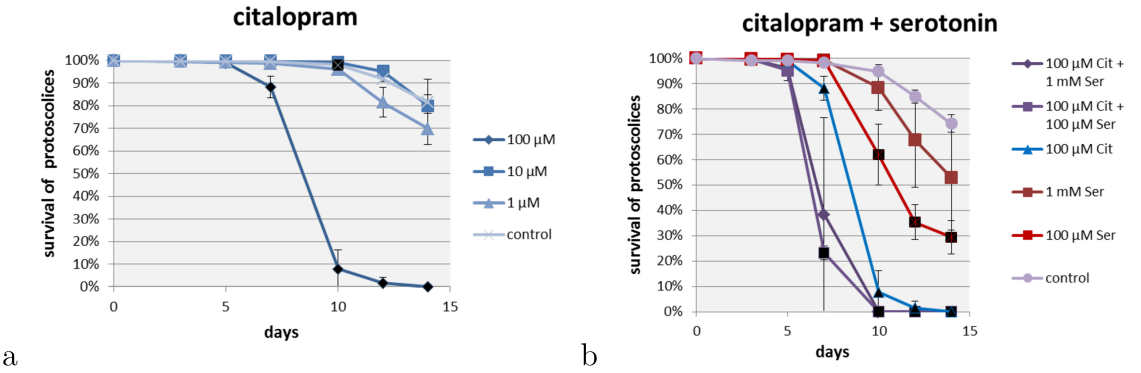


Figure 22: **Treatment of protoscoleces with serotonin:** Protoscoleces were cultivated under axenic conditions in DMEM (10% FBS). Contrary to control **(a)**, protoscoleces treated with 1 mM serotonin **(b)** showed signs of vesicularisation and re-differentiation after 7 days. Prolonged incubation with 1 mM serotonin lead to death of non-re-differentiated protoscoleces **(d)**, compared to control **(c)**. Micrography was optained with optical microscop with hundredfold aumentation. **ev**: evaginated protoscolex, **ves**: different stages of vesicularisation, **pm**: pre-microcyst, **d**: dead protoscolex. Reprinted from Camicia et al. (2013), ©2013, with permission from Elsevier.

Prolonged incubation with serotonin significantly ($P<0,05$ after 17 days incubation for all concentrations) reduced protoscolex viability in a dose dependent manner (see Figure 22c,d). After 19 days of treatment only about 25 % of the protoscoleces treated with 100 µM and 1 mM serotonin survived compared to about 50% surviving protoscoleces in cultures treated with 1 µM and 10 µM serotoin and more than 85 % vital protoscoleces in controls (see Figure 21d). Interestingly, 14 days of treatment with serotonin only lead to death of non-re-differentiated protoscoleces while pre-microcyst were still vital (see Figure 22).

It was shown that treatment of protoscoleces with glucagon had little effect on re-differentiation but could induce death at certain concentrations. In contrast, treatment with serotonin induced both re-differentiation and death of protoscoleces

in a dose dependent manner.



a b

Figure 23: **Treatment of protoscoleces with serotonin transporter inhibitor citalopram and citalopram in combination with serotonin:** Protoscoleces were cultivated in DMEM (10% FBS) and incubated with citalopram **(a)** or with a combination of citalopram and serotonin **(b)** at indicated concentrations. Survival of protoscoleces is shown. Experiments were performed in triplicate. Error bars 1 SD. Data points marked in black indicate treatments found to be significantly different from controls (P<0,05).

In order to study the effect of serotonin transport on protoscolex viability and morphology, protoscoleces were treated with the serotonin transporter inhibitor citalopram. The application of 100 µM citalopram diminished protoscolex viability significantly (P<0,000001), with only 2% vital protoscoles after 12 days compared to more than 90% vital protoscoleces in controls (see Figure 23a). Treatment with 1 µM and 10 µM citalopram had little influence on protoscolex survival. In contrast to other substances, treatment with citalopram did not induce re-differentiation.

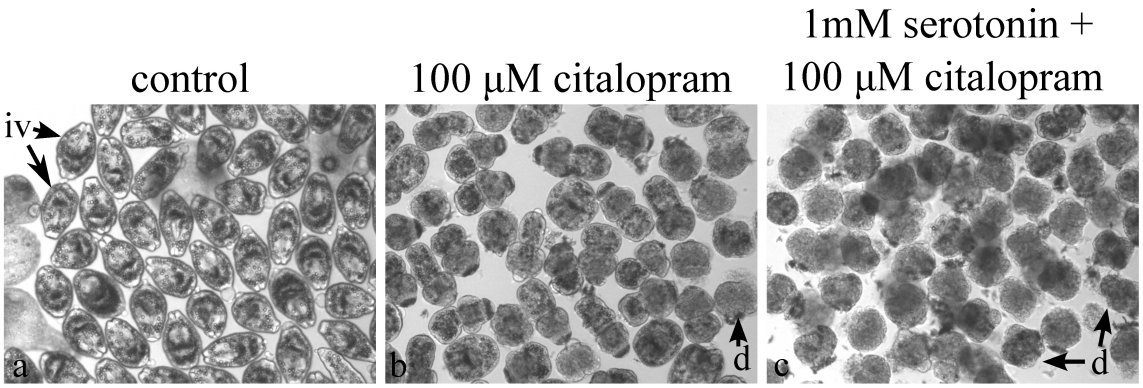


Figure 24: **Treatment of protoscoleces with citalopram:** Protoscoleces were cultivated under axenic conditions in DMEM (10% FBS). In contrast to control **(a)**, after 7 days protoscoleces treated with citalopram **(b)** or a combination of citalopram and serotonin **(c)** stated to die. Micrography was obtained with optical microscop at hundredfold aumentation. **iv**: invaginated protoscolex, **d**: dead protoscolex. Reprinted from Camicia et al. (2013), ©2013, with permission from Elsevier.

To determine whether the killing of protoscoleces by citalpram can be counteracted by additional serotonin, serotonin was added to protoscoleces treated with 100 µM

citalopram. However, protoscoleces treated with additional 100 µM or 1 mM serotonin died even earlier than protoscoleces treated with 100 µM citalopram alone (see Figure 24). Cultures treated with 100 µM citalopram and 100 µM serotonin for 7 days contained significantly (P=0,00003) more dead protoscoleces than cultures treated with 100 µM citalopram alone, 77% dead protoscoleces compared to 12% in cultures treated with 100 µM citalopram alone and 2% in controls (see Figure 23b). Thus death of protoscoleces through treatment with citalopram could not be prevented by adding serotonin. On the contrary, additional serotonin lead to an earlier death than treatment with citalopram alone.

3.4.3 Anaerobic atmosphere and presence of FBS prolong *E. granulosus* protoscolex survival

To find suitable media for further experiments on *E. granulosus* protoscoleces, different media were compared. As in culture of *E. multilocularis* metacestodes the absence of oxygen and the presence of reducing agents is important for survival (Spiliotis et al., 2004), experiments were performed both under aerobic and anaerobic conditions (in a nitrogen environment). Protoscoleces were cultivated in DMEM, DMEM with 10% FBS, DMEM with 10% FBS and reducing agents, NCTC-135 and NCTC-135 with 10% FBS.
Under aerobic conditions protoscoleces incubated with DMEM re-differentiated more than protoscoleces incubated with other media. Protoscoleces incubated with DMEM with FBS or DMEM with FBS and reducing agents formed less pre-microcysts than those incubated with DMEM without FBS. In contrast, protoscoleces incubated with NCTC-135 or NCTC-135 with FBS formed almost no pre-microcysts (see Figure 25a). While protoscoleces incubated with DMEM, NCTC-135 or NCTC-135 with FBS were mostly invaginated, protoscoleces incubated with DMEM with FBS or DMEM with FBS and reducing agents were more evaginated and showed more movement (see Figure 26). Protoscoleces incubated with DMEM or NCTC-135 without FBS started to die after 14 days and were almost all dead after 21 days compared to about 20% dead protoscoleces in cultures with DMEM with FBS and about 10% in cultures incubated with NCTC-135 with FBS (see Figure 25b).

Protoscoleces cultivated under anaerobic conditions had generally a healthier aspect than those cultivated under aerobic conditions: more of them were evaginated, they showed more movement and less of them died. Same as under aerobic conditions, under anaerobic conditions protoscoleces incubated with DMEM re-differentiated more than those incubated with other media. 6% of the protoscoleces incubated with DMEM formed pre-microcysts compared to less than 2% in other cultures.

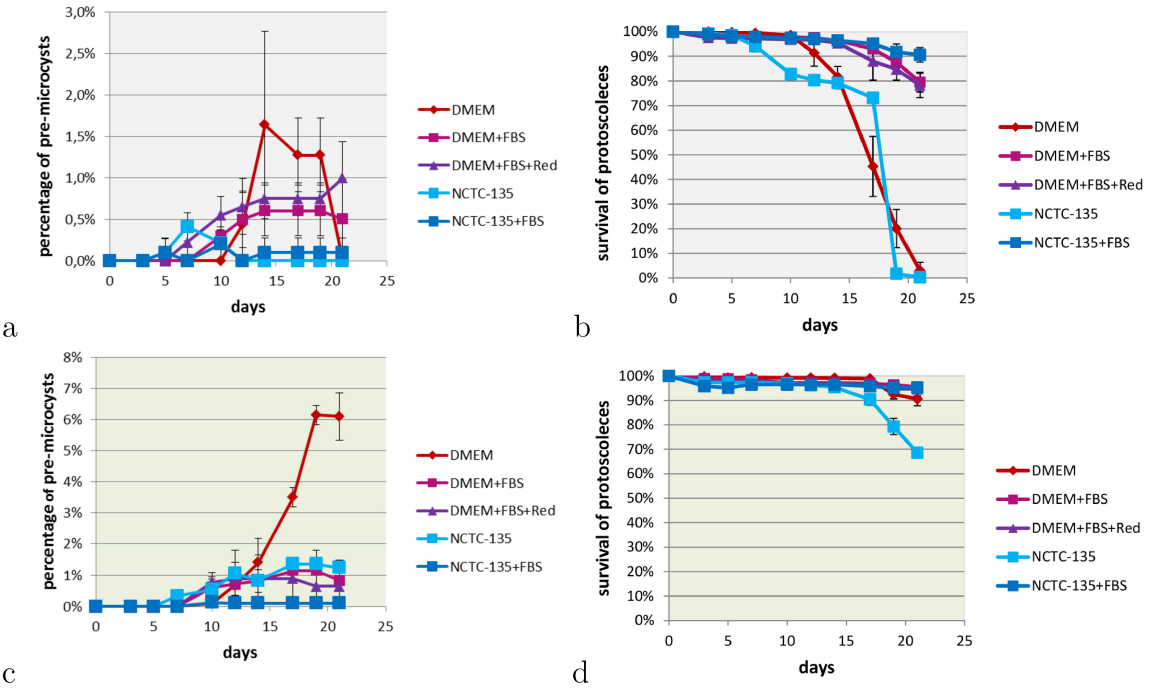


Figure 25: **Culture of protoscoleces with different media:** Protoscoleces were incubated with indicated media and additives under aerobic conditions **(a,b)** and anaerobic conditions **(c,d)**. Pre-microcysts were counted as an indicator of re-differentiation of protoscoleces (left side). Survival of protoscoleces is shown at the right side. Experiments were performed in triplicate. Error bars 1 SD.

Protoscoleces incubated with DMEM with FBS, DMEM with FBS and reducing agents and NCTC-135 without FBS formed less pre-microcysts than those incubated with DMEM without FBS but more than protoscoleces incubated with NCTC-135 with FBS. Protoscoleces incubated with NCTC-135 with FBS showed nearly no sign of re-differencitation(see Figure 25c). They were mostly evaginated and highly active. While protoscoleces incubated with DMEM or NCTC-135 were invaginated, protoscoleces incubated with DMEM with FBS or DMEM with FBS and reducing agents were more evaginated and showed more movement. However, protoscoleces incubated with NCTC-135 with FBS were the most active ones (see Figure 26). Survival of protoscoleces was generally better under anaerobic conditions than under aerobic conditions. After 21 days only 30% of the protoscoleces incubated with NCTC-135 were dead compared to nearly 100% under aerobic conditions. 90 % of the protoscoleces incubated with DMEM were still alive after 21 days under anaerobic conditions compared to less than 5% living protoscoleces under aerobic conditions. About 95 % of the protoscoleces incubated with DMEM with FBS, DMEM with FBS and reducing agents or NCTC-135 with FBS under anaerobic conditions survived 21 days of culture (see Figure 25d).

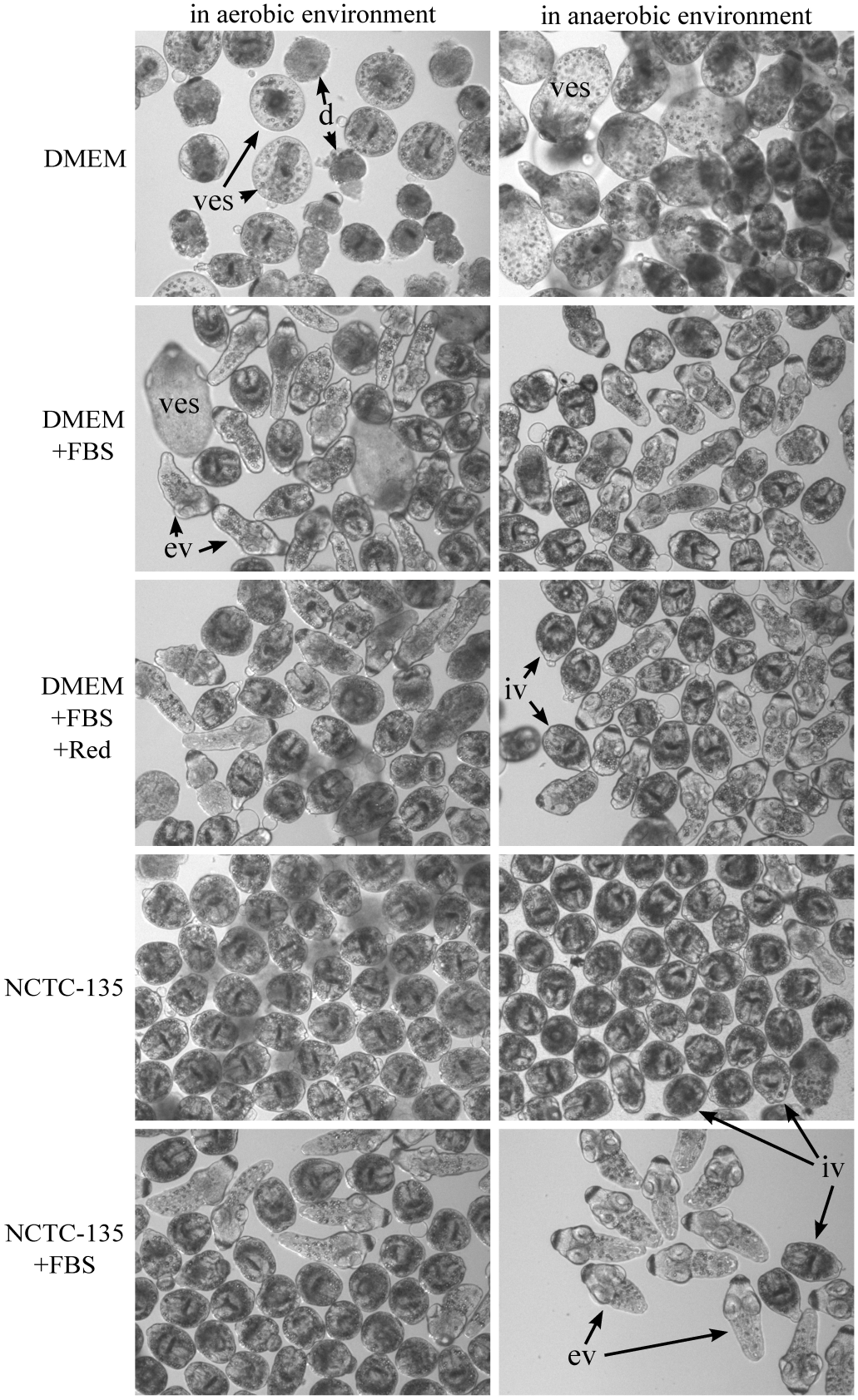


Figure 26: **Protoscolex culture with different media:** Protoscoleces were incubated with indicated media and additives under aerobic conditions (left side) and anaerobic conditions (right side). Micrography was obtained by optical microscope with hundredfold augmentation after 14 days of culture. **ev**: evaginated protoscolex, **iv**: invaginated protoscolex, **d**: dead protoscolex, **ves**: different stages of vesicularisation.

All in all it was shown, that anaerobic conditions and the presence of FBS were important for survival of protoscoleces while the absence of FBS advanced re-differentiation of protoscoleces. The kind of media also influenced re-differentiation, as protoscoleces incubated with DMEM re-differentiated more than those incubated with NCTC-135.

3.5 Forskolin and serotonin increase phosphorylation of protein kinase A substrates in metacestodes

To determine the influence of forskolin and serotonin on the cAMP signalling pathway in *E. multilocularis* metacestodes, phosphorylation of protein kinase A substrates was analysed. To this end, metacestode vesicles were incubated with forskolin or serotonin. After protein isolation, the phosphorylation of protein kinase A substrates was detected by Western blot using an antibody against phosphorylated substrates of protein kinase A (Cell Signaling, #9624).

To determine if incubation in DMEM (0,02% FBS) influenced phosphorylation of protein kinase A (PKA) substrate, metacestode vesicles were incubation in DMEM (0,02% FBS) for ½, 1, 2 and 4 days before protein isolation.

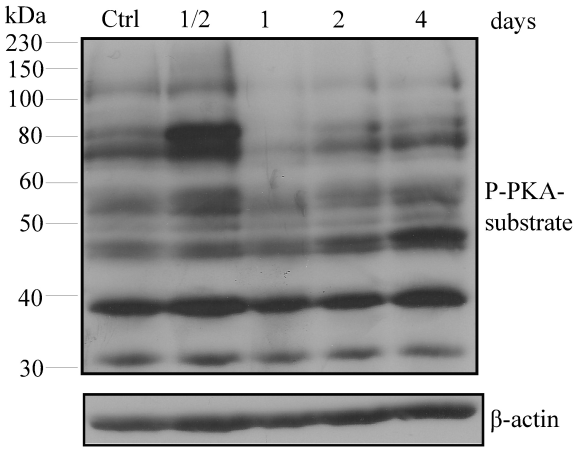


Figure 27: **Activity of protein kinase A after incubation in DMEM (0,02% FBS):** Metacestode vesicles were incubated in DMEM (0,02% FBS) for indicated periods of time. Lysates were produced and separated on a 10% polyacrylamide gel, then transfered to a nitrocellulose membrane and developed with an antibody against phosphorylated substrates of protein kinase A (P-PKA-substrate)(Cell Signaling,#9624). As control for equal protein concentrations an antibody against β-actin (Cell Signaling, #4967) was used. Bands of the protein marker are shown on the left side. Experiment was performed twice.

As shown in Figure 27, after ½ day incubation in DMEM (0,02% FBS), phosphorylation of PKA substrates was higher than without incubation. The lowest level of phosphorylation could be observed after 1 day incubation. After 2 and 4 days,

phosphorylation was about the same level as without incubation. As control for equal protein concentrations, an antibody against β-actin (Cell Signaling, #4967) was used.

As the lowest level of phosphorylation of PKA substrates was after 1 day incubation, for stimulation experiments metacestode vesicles were incubated in DMEM (0,02% FBS) for 1 day before adding the substances. After incubating 1 day in DMEM (0,02% FBS), metacestode vesicles were incubated with 100 µM forskolin or 1 mM serotonin. Both substances increased phosphorylation of PKA substrates. Stimulation with 100 µM forskolin lead to higher phosphorylation after 5 minutes (see Figure 28a). Just as stimulation with forskolin, stimulation with 1 mM serotonin increased phosphorylation of PKA substrates after 5 minutes. The highest level of phosphorylation was achieved by stimulation for 30 minutes. Prolonged incubation with serotonin lead to decreased phosphorylation. Metacestode vesicles stimulated for 120 minutes showed the same phosphorylation level as control (see Figure 28b). It could be shown that stimulation with forskolin and serotonin increased phosphorylation of PKA substrates and influenced cAMP signalling pathway.

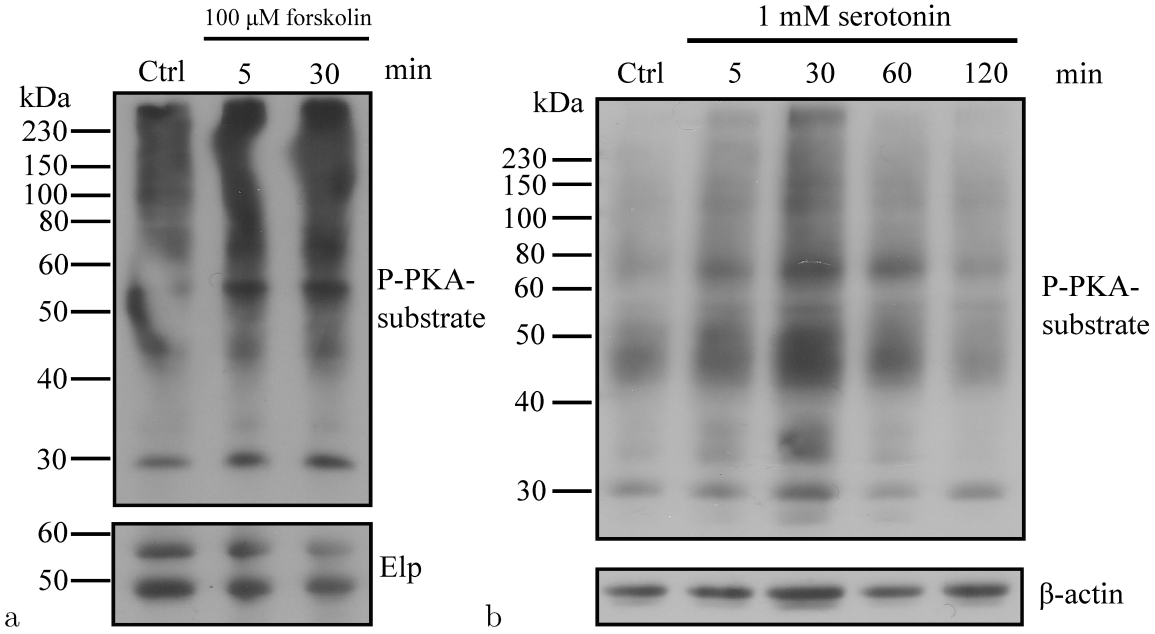


Figure 28: **Activity of protein kinase A after treatment with forskolin and serotonin:** Metacestode vesicles were starved in DMEM (0,02% FBS) for 1 day and then treated with 100 µM forskolin **(a)** or 1 mM serotonin **(b)** for indicated periods of time. Lysates were produced and separated on a 10% polyacrylamide gel, then transfered to a nitrocellulose membrane and developed with an antibody against phosphorylated substrates of protein kinase A (P-PKA-substrate)(Cell Signaling,#9624). As control for equal protein concentrations either an antibody against the continously expressed protein Elp (Cell Signaling, #3142) (Hubert et al., 1999) or an antibody against β-actin (Cell Signaling, #4967) was used. Bands of the protein marker are shown on the left side. Experiments were at least performed twice.

4 Discussion

Evolutionary conserved signalling mechanisms regulate developmental processes in the fox and the dog tapeworm *E. multilocularis* and *E. granulosus* (Brehm, 2010). Ligands bind to surface or intracellular receptors and activate downstream signalling pathways that influence development, metabolism and movement. Due to their early rise in metazoan evolution, cell-cell communication and important signalling pathways are well conserved between vertebrates and invertebrates (Brehm et al., 2006). Signalling molecules are structurally related and often functionally exchangeable. This allows host-parasite communication through these signalling pathways. Evidence for this hormonal cross-talk between host and *E. multilocularis* could be obtained, among others, for the insulin and epidermal growth factor pathways (Konrad et al., 2003; Spiliotis et al., 2006; Hemer, 2012). The study of evolutionary conserved signalling pathways is of great importance, not only to gain a better understanding of host-parasite interactions but also to identify possible targets for new chemotherapy.

4.1 Serotonin signalling in *Echinococcus*

Serotonin signalling is of special interest in *Echinococcus* as serotonin is an important neuroactive and morphogenetic molecule. The results of the genetic analysis indicate that *Echinococcus* is able to synthesize serotonin, transport it and perceive it with serotonin receptors. However, the canonical MAO could not be detected in the *E. multilocularis* and *E. granulosus* genomes. While in vertebrates MAO is mainly responsible for the degradation of monoamines, in invertebrates various ways of monoamine degradation have been described (Sloley, 2004). Nevertheless, MAO activity was detected in the trematode *S. mansoni* (Nimmo-Smith and Raison, 1968) and the cestode *H. diminuta* (Moreno and Barrett, 1979; Ribeiro and Webb, 1984). Therefore it is possible that *Echinococcus* has a different degradation pathway or that the MAO could not be detected in the genome due to significant divergence from vertebrate homologues. While there are 15 known subtypes of serotonin receptors in humans (Bohn and Schmid, 2010), only four serotonin receptors could be identified in the *E. multilocularis* and *E. granulosus* genomes, all of them G-Protein coupled receptors. The cation channel 5-HT_3 receptor could not be identified in the genomes. Its absence might be attributed to gene loss or considerable divergence from the mammalian homologue. In fact, no canonical 5-HT_3 receptors could be found in the genomes of *C. elegans* and *D. melanogaster* (Dent, 2006). However, the *C. elegans* genome contains a gene (*mod-1*) which encodes a new type of ionotropic 5-

HT receptor (Ranganathan et al., 2000). This receptor is not permeable for cations like the canonical 5-HT_3 receptor but rather acts as a 5-HT-gated chloride channel. As serotonin is an important neurotransmitter in *Echinococcus* (Brownlee et al., 1994; Fairweather et al., 1994; Camicia et al., 2013), it is possible that *Echinococcus* encodes some kind of serotonin gated ion channel.

Genes for serotonin biosynthesis and serotonin transport are highly conserved among flatworms but divergent from the human homologues. According to RNAseq data, expression of the tryptophan hydroxylase gene, encoding the enzyme catalyzing the rate limiting step of serotonin production, is highest in protoscoleces and adults - the stages with the most complex nervous system (Koziol et al., 2013). The high gene expression in these stages therefore could be attributed to a higher demand of serotonin in the nervous system. Low expression in the early primary cell stage and in the metacestode stage could be explained by the absence of a serotonergic nervous system in those stages (Koziol et al., 2013). Remarkably, expression of the aromatic-L-amino-acid decarboxylase gene, whose protein is also involved in serotonin biosynthesis, is highest in the metacestode. Due to a wide substrate selectivity of the enzyme (Berry et al., 1996), however, this cannot be attributed to serotonin synthesis alone. The serotonin transporter gene is highly expressed in primary cells, protoscoleces and gravid adults compared to lower expression in metacestodes and pre-gravid adults. While high expression in the protoscoleces and gravid adults could be attributed to its expression in the nervous system, high expression in the primary cell stage indicates a role outside the nervous system. Serotonin receptor genes are expressed in all stages but expression is highest in protoscoleces indicating special importance of the serotonin signalling through GPCRs in this larval stage.

Serotonin and the serotonin transporter inhibitor citalopram were shown to influence development and survival of *E. multilocularis* primary cells and metacestodes as well as *E. granulosus* protoscoleces. Serotonin induced the formation of metacestode vesicles from *E. multilocularis* primary cells in a dose dependent manner for concentrations <1 mM. Vesicle formation was completely prevented by the very high concentration of 1 mM serotonin which probably was toxic for primary cells. Inhibition of serotonin transport with citalopram reduced vesicle formation at the concentration of 10 µM citalopram and completely prevented it at 100 µM. The inhibitory effect of citalopram could not be prevented by additional application of serotonin. These results indicate that serotonin acts as a morphogen and induces the development of metacestode vesicles from primary cells. This effect could be mediated through serotonin receptors and subsequent stimulation of the cAMP signalling pathway and variation of gene expression. However, the fact that citalopram

inhibits this development suggests an essential role of serotonin transport in this process.

Serotonin induced growth of metacestode vesicles at concentrations ≥10 µM in a dose dependent manner, while concentration of 1 µM inhibited vesicle growth. On the other hand, high concentration of citalopram (100 µM) lead to disintegration of metacestode vesicles within three weeks. Lower concentrations of citalopram had no significant effect on metacestode vesicles. Evidently higher concentrations of citalpram were necessary to cause a visible effect on metacestode vesicles (100 µM) than on primary cells (10 µM). This difference could be attributed to higher sensibility of primary cells or to lower citalopram levels in the metacestode vesicle due to a selective permeability of the laminated layer (Garcia-Llamazares et al., 1998; Conchedda et al., 2004).

Application of serotonin to protoscoleces induced re-differentiation of protoscoleces in a dose dependent manner. Prolonged incubation lead to death of those protoscoleces that had not developed into pre-microcysts. Survival of protoscoleces was also reduced by high concentrations of citalopram. Interestingly, treatment of protoscoleces with a combination of serotonin and citalopram (100 µM) resulted in even earlier death of protoscoleces than treatment with citalopram alone. One possible explanation for these results is that serotonin induces re-differentiation via the nervous system. Serotonin would then influence the protoscoleces towards microcyst development. Protoscoleces unable to undergo re-differentiation would be affected by the high concentrations of serotonin and its toxic effects on the nervous system. As the pre-microcyst stage has fewer serotonergic nerve cells (Camicia et al., 2013), pre-microcysts would be less affected. Inhibition of serotonin uptake with citalopram would result in higher concentrations of serotonin in the synaptic cleft and thus be toxic for protoscoleces. This theory could also explain the higher mortality of protoscoleces treated with both serotonin and citalopram: The concentration of serotonin in the nervous system would be even higher than at treatment with citalopram alone and thus result in earlier death of protoscoleces. However, this theory alone can not explain the morphogenetic effects of serotonin and the toxic effects of citalpram on metacestode vesicles and primary cells where no serotonergic nervous system is present (Koziol et al., 2013).

Serotonin has been reported to regulate various developmental processes, such as proliferation and differentiation, apoptosis, migration and morphogenesis (Azmitia, 2001; Buznikov et al., 2001; Lauder, 1993). Serotonin acts as a morphogen in cleavage division in early sea urchin embryos (Buznikov et al., 2001; Lauder, 1993; Renaud et al., 1983), gastrulation and neurulation of chick embryos (Emanuelsson

et al., 1988), regeneration in planaians (Franquinet, 1979; Franquinet and Martelly, 1981) and miracidial transformation in *S. mansoni* (Kawamoto et al., 1989). Various mechanisms have been described to explain how serotonin acts as a morphogenetic factor although the exact mechanism is often unclear. Different receptors can produce opposite and complimentary actions on apoptosis, proliferation and maturation (Azmitia, 2001; Buznikov et al., 1996). In *S. mansoni* serotonin was found to highly activate adenylate cyclase and increase cAMP levels (Kasschau and Mansour, 1982; Estey and Mansour, 1987). Interestingly, some serotonin-mediated effects in *S. mansoni* can be revoked by inhibition of serotonin transport with SSRI, while other effects are not influenced by serotonin transport. For example, serotonin-induced motility of sporocysts is not inhibited by SSRIs (Boyle and Yoshino, 2005) whereas SSRIs abolish the stimulatory effect of serotonin on glucose uptake and lactate excretion (Harder et al., 1987). It has been proposed that serotonin could effect muscle contractility via surface receptors and a cAMP-dependent pathway without requiring serotonin transport (Day et al., 1994; Boyle and Yoshino, 2005). On the other hand, serotonin transport is required for serotonin-induced glycolysis and glucose uptake (Harder et al., 1987) and miracidial transformation (Taft et al., 2010). Serotonin transport is also essential for parasite survival (Patocka and Ribeiro, 2007; Mbah et al., 2012). These facts indicate that there is another signalling mechanism of serotonin in *Schistosoma spp.* outside surface receptors and the cAMP-dependent pathway.

Though many effects of serotonin in different species can be explained by signalling of surface receptors and G-proteins with positive or negative coupling to adenylate cyclase or phosphatidylinositol hydrolysis, these pathways do not explain why serotonin transport is necessary for some serotonin effects. Moreover, there is evidence of intracellular serotonin receptors or binding sites. Serotonin is known to induce DNA synthesis and proliferation in rat pulmonary artery smooth muscle cells (Azmitia, 2001). This effect appears to be mediated through internalization of serotonin and tyrosine phosphorylation of GTPase-activating enzyme (Lee et al., 1991, 1994, 1997; Eddahibi et al., 1999). Interestingly, serotonin is also known to stimulate DNA synthesis in planaians, presumably through a Ca^{2+}-dependent protein kinase (Franquinet and Martelly, 1981). Therefore, it is possible that the morphogenetic effects of serotonin are mediated by several different mechanisms, including surface and intracellular receptors as well as binding to cytoskeletal elements. Serotonin binds to endothelial cell stress fibers and induces actin polymerization *in vitro*. In this context it is interesting to note that, while endothelial cells take up serotonin, not all of them have serotonin surface receptors. It has been proposed that serotonin

my affect cell shape and metabolism by directly binding to actin and influencing its polymerization state (Alexander et al., 1987). In summary, various pathways have been described for the morphogenetic effects of serotonin, some of them associated with internalization of serotonin.

In this work it was shown that application of serotonin to *E. multilocularis* metacestode vesicles resulted in higher phosphorylation of protein kinase A substrates, indicating that serotonin activates the cAMP signalling pathway, possibly via serotonin surface receptors. However, it is unclear whether the observed serotonin-induced morphogenetic effects are mediated through this pathway. Serotonin transport appears to be necessary for serotonin-mediated morphogenesis as inhibition of serotonin transport prevents development of metacestode vesicles from primary cells, re-differentiation of protoscoleces and growth of metacestode vesicles. Furthermore, serotonin transport seems to be essential for parasite survival. As the concentration of 100 µM citalopram is very high, unspecific toxic effects cannot be ruled out. Nevertheless, high concentrations of citalopram could be required to cause an effect in *Echinococcus* due to highly reduced inhibitory potency. Compared to the human transporter, the *Echinococcus* serotonin transporter shows two amino acid substitutions which are crucial for affinity to citalopram. Double substitution of Y95 and I172 has been demonstrated to extremely reduce the inhibitory potency of citalopram (Barker et al., 1998*b*; Henry et al., 2006; Ravna et al., 2006). Heterologous expression of the *S. mansoni* serotonin transporter, which exhibits the same substitutions Y95F and I172T as the *Echinococcus* serotonin transporter, showed lower affinity for citalopram compared to the human transporter, requiring 120-fold higher concentration for inhibitory activity (Patocka and Ribeiro, 2007). Reduced affinity of the *Echinococcus* serotonin transporter for citalopram could account for the high concentrations required for inhibition. Serotonin concentrations used in experiments were quite high compared to basal tissue concentrations of less than 100 nM (Mossner and Lesch, 1998). However, higher concentrations have been reported during inflammation (Mossner and Lesch, 1998). Also blood concentration of 1,0 to 1,2 µM serotonin (Flachaire et al., 1990; Chauveau et al., 1991) and serotonin concentration of 2,2 µg/g tissue in dog small intestine (approximately 6 µM) (Toh, 1957) are higher than tissue concentrations. Alternatively, serotonin could be produced by the parasite itself and therefore require higher exogenous serotonin concentrations to induce development.

In conclusion, the data presented indicates that serotonin is an important developmental signal in *Echinococcus*. While it is is unclear whether the developmental effects of serotonin are mediated through the cAMP signalling pathway or a different

pathway, serotonin transport appears to be required for the developmental effects of serotonin.

4.2 cAMP signalling pathway in *Echinococcus*

The cAMP signalling pathway is known to influence a variety of biological processes, including gene transcription, differentiation and cell growth (Houslay and Milligan, 1997; Hanoune et al., 1997; Patel et al., 2001). In *E. multilocularis* six Gα, three Gβ and one Gγ subunits of G proteins as well as four catalytic and three regulatory subunits of protein kinase A subunits have already been identified (Schilling, 2010; Burkhardt, 2011). Therefore this work concentrates on the role of GPCRs and adenylate cyclase in *Echinococcus*. To gain a general overview, GPCRs of *E. multilocularis* were identified with blast searches. 76 GPCR sequences could be identified in the *E. multilocularis* genome, including members of all major families. In humans more than 800 GPCR sequences were identified (Fredriksson et al., 2003). However, less GPCR sequences were identified in flatworms; 418 in *S. mediterranea* and 117 in *S. mansoni*. This could be attributed to gene loss in platyhelminthes or gene gain in humans. To determine whether *E. multilocularis* possesses a glucagon receptor orthologue, the members of the *Secretin* family were sequenced. Nevertheless, no glucagon receptor orthologue could be identified. GPCRs are a large group of receptors in flatworms and present a variety of potential antihelmintic drug targets. With HMMsearch and blast searches four genes for adenylate cyclase type IX were identified in the *E. multilocularis* genome whereas the *E. granulosus* genome only contains three. Only one gene coding for adenylate cyclase IX could be found in *S. mansoni*. While all *Echinococcus* adenylate cyclase protein sequences showed higher homology among flatworms than with human homologues, homology of EmAC1 and EgAC1 to flatworm and human homologues was considerably higher than homology of other *Echinococcus* adenylate cyclases. Phylogenetic analysis indicated a close relationship of EmAC1 and EgAC1 with the schistosome adenylate cyclase. EmAC3 and EmAC4 only differ in one amino acid and are more closely related to each other than to the EgAC3. The varying numbers and similarities of adenylate cyclases indicate gene duplications in a common *Echinococcus* ancestor (in case of *emac1/egac1*, *emac2/egac2* and *emac3/egac3*) and in *E. multilocularis* (in case of *emac3* and *emac4*). Interestingly, only *emac1/egac1* contain introns. It is possible that *emac2/egac2*, *emac3/egac3* and *emac4* are reverse transcribed mRNA transcripts that were reintegrated into the genome. In EmAC2 and EgAC2 two amino acids required for catalytic activity are substituted (Yan et al., 1997). While EmAC2 and EgAC2 will have no catalytic function, they could act as regulators. Accord-

ing to RNAseq data, all adenylate cyclase genes in *E. multilocularis* are expressed. Compared to *emac1*, the others are expressed quite low. Hence, the main catalytic activity could be attributed to EmAC1 while the other adenylate cyclases might act as regulators and modulators.

Forskolin was used to stimulate adenylate cyclase *in vitro*. Forskolin is able to activate all mammalian adenylate cyclases except adenylate cyclase IX (Hurley, 1999; Simonds, 1999). *D. melanogaster* adenylate cyclase IX, on the other hand, is sensible to forskolin (Iourgenko et al., 1997). It has been shown that a Tyr1082-to-leucine mutation converts mammalian adenylyl cyclase IX to be forskolin-sensitive (Yan et al., 1998). In *Echinococcus* all adenylate cyclases besides EmAC2 and EgAC2 show the Tyr1082 to Leucin exchange and therefore are forskolin sensible. Furthermore, in this work it was shown that forskolin increases phosphorylation of PKA substrates. For inhibition of adenylate cyclase the p-site inhibitor 2', 5' didesoxyadenosine was used. p-site inhibitors selectively inhibit adenylate cyclase in a non-competitive way with respect to activators but are competitive with respect to cAMP for the reverse reaction (Pavan et al., 2009; Tesmer et al., 1997, 2000). Inhibition is activity-dependent and inhibition is increased when adenylate cyclase is activated (Tang et al., 1995; Hurley, 1999). 2', 5' didesoxyadenosine is one of the most potent, cell permeable inhibitors (Désaubry and Johnson, 1996).

Stimulation of adenylate cyclase with forskolin induced metacestode formation from primary cells at lower concentrations and significantly inhibited vesicle formation at 100 µM forskolin. On the other hand, inhibition of adenylate cyclase with 2', 5' didesoxyadenosine had no visible effect on primary cells. It has been reported that increased cAMP levels can stimulate cell proliferation (Seuwen and Pouyssegur, 1990). High concentration of forskolin could be toxic for primary cells or inhibit vesicle formation through other effects of forskolin such as its impact on glucose transporters, voltage gated K^+ channels and glycoproteins (Hanoune et al., 1997). As 2', 5' didesoxyadenosine inhibition depends on adenylate cyclase activity (Tang et al., 1995; Hurley, 1999), low basal activity of adenylate cyclase could account for the ineffectiveness of the p-site inhibitor at this larval stage.

Forskolin slightly reduced survival of metacestode vesicles in a dose dependent manner and higher concentrations of 2', 5' didesoxyadenosine (50 µM, 300 µM) caused disintegration of metacestode vesicles. It could be speculated that integrity of metacestode vesicles is corrupted due to misregulation of cytoskeletal dynamics and cell migration by the cAMP signalling pathway and activation of PKA. PKA is known to have a paradoxical role in cell motility, both facilitating and inhibiting actin polymerisation (Howe et al., 2005). Another explanation could be the impor-

tance of adenylate cyclase in glucose uptake and glycolysis as has been shown for *S. mansoni* and *F. hepatica* (Kasschau and Mansour, 1982; Estey and Mansour, 1987). Manipulation of adenylate cyclase activity with forskolin and 2', 5' didesoxyadenosine could disturb glucose metabolism and thus be responsible for disintegration of metacestodes.

In protoscoleces both 100 µM forskolin and 100 µM 2', 5' didesoxyadenosine induced re-differentiation and subsequently death. No effects were observed at lower concentrations. Interestingly, protoscoleces treated with forskolin solved in DMSO re-differentiated and died earlier than those treated with forskolin solved in ethanol. As DMSO is toxic at higher concentrations (Pagan et al., 2006; Yuan et al., 2012), this could be attributed to an additive effect of DMSO and forskolin. cAMP levels are known to influence cell proliferation and development. In *S. mansoni* a decrease of cAMP level is required for miracidial transformation (Kasschau and Mansour, 1982; Kawamoto et al., 1989; Taft et al., 2010). It is possible that in *Echinococcus* protoscolex re-differentiation can be induced both by increased or decreased cAMP level.

In summary, it was shown that adenylate cyclase activator forskolin and adenylate cyclase inhibitor 2', 5' didesoxyadenosine influenced vesicle formation from primary cells, survival of metacestode vesicles and re-differentiation and death of protoscoleces. The influence of other mechanisms than the cAMP signalling pathway, however, cannot be ruled out. An explanation for the contradictory effects could be that developmental processes depend on a certain cAMP level and variation in either direction could cause another effect. Also spatial location and compartmentalization of cAMP signalling as well as crosstalk between signalling pathways could account for contrary effects of different forskolin concentrations in primary cells and similar effects for forskolin and 2', 5' didesoxyadenosine in protoscoleces (Houslay and Milligan, 1997). Further evidence for the importance of the cAMP pathway in *E. multilocularis* development and survival was gained by Schilling (2010) and Burkhardt (2011). Schilling (2010) could show that vesicle formation from primary cells was stimulated by forskolin and prevented by H-89, a PKA inhibitor. Metacestode integrity was affected by high concentrations of forskolin and H-89 (Schilling, 2010) but not by cholera toxin (Burkhardt, 2011). In protoscoleces cholera toxin induced re-differentiation and subsequent death while H-89 caused death without stimulating re-differentiation (Schilling, 2010; Burkhardt, 2011). Taken together these results indicate that cAMP signalling pathway plays an important role in *Echinococcus* development and survival which could be shown both for different larval stages and different enzymes of the cAMP signalling pathway.

4.3 Glucagon in *Echinococcus*

While lower concentrations (50 nM, 500 nM) of glucagon slightly stimulated vesicle formation from primary cells and higher concentration (5 µM) slightly inhibited vesicle formation, glucagon had no visible effect on metacestodes. In protoscoleces lower concentrations promoted re-differentiation and later death whereas higher concentration had no effect compared to control cultures. These effects were not statistically significant and could be attributed to unspecific reactions, especially considering that no glucagon receptor orthologue could be identified in *E. multilocularis*. Also there are no reports about effects of glucagon on platyhelminthes. In planaians glucagon proved without effect on glucose uptake (Csaba and Kadar, 1978). Thus, glucagon appears to be without consequence in platyhelmithes.

5 Summary

Alveolar and cystic echinococcosis, caused by *Echinococcus multilocularis* and *Echinococcus granulosus* respectively, are severe zoonotic diseases with limited treatment options. The sole curative treatment is the surgical removal of the complete parasite material. Due to late diagnosis, chemotherapeutic treatment often is the only treatment option. Treatment is based on benzimidazoles, which merely act parasitostatic and often display strong side effects. Therefore, new therapeutic drugs are urgently needed.

Evolutionarily conserved signalling pathways are known to be involved in host-parasite cross-communication, parasite development and survival. Moreover, they represent potential targets for chemotherapeutic drugs. In this context the roles of the serotonin- and cAMP-signalling pathways in *Echinococcus* were studied.

Genes encoding serotonin receptors, a serotonin transporter and enzymes involved in serotonin biosynthesis could be identified in the *E. multilocularis* and *E. granulosus* genomes indicating that these parasites are capable of synthesizing and perceiving serotonin signals. Also the influence of exogenous serotonin on parasite development was studied. Serotonin significantly increased metacestode vesicle formation from primary cells and re-differentiation of protoscoleces. Inhibition of serotonin transport with citalopram significantly reduced metacestode vesicle formation from primary cells and caused death of protoscoleces and metacestodes. Furthermore, it could be shown that serotonin increased phosphorylation of protein kinase A substrates. Taken together, these results show that serotonin and serotonin transport are essential for *Echinococcus* development and survival. Consequently, components of the serotonin pathway represent potential drug targets.

In this work the cAMP-signalling pathway was researched with focus on G-protein coupled receptors and adenylate cyclases. 76 G-protein coupled receptors, including members of all major families were identified in the *E. multilocularis* genome. Four genes homologous to adenylate cyclase IX were identified in the *E. multilocularis* genome and three in the *E. granulosus* genome. While glucagon caused no significant effects, the adenylate cyclase activator forskolin and the adenylate cyclase inhibitor 2', 5' didesoxyadenosine influenced metacestode vesicle formation from primary cells, re-differentiation of protoscoleces and survival of metacestodes. It was further shown that forskolin increases phosphorylation of protein kinase A substrates, indicating that forskolin activates the cAMP-pathway also in cestodes. These results indicate that the cAMP signalling pathway plays an important role in *Echinococcus* development and survival.

To complement this work, the influence of different media and additives on *E. granu-*

losus protoscoleces was investigated. Anaerobic conditions and the presence of FBS prolonged protoscolex survival while different media influenced protoscolex activation and development.
Taken together, this work provided important insights into developmental processes in *Echinococcus* and potential drug targets for echinococcosis chemotherapy.

6 Zusammenfassung

Alveoläre und zystische Echinokokkose, hervorgerufen durch *Echinococcus multilocularis* und *Echinococcus granulosus*, sind schwere zoonotische Erkrankungen mit eingeschänkten Behandlungsmöglichkeiten. Die einzig kurative Therapie besteht in der chirurgischen Entfernung des gesammten Parasitenmaterials. Aufgrund später Diagnosestellung stellt Chemotherapie oft die einzige Behandlungsmöglichkeit dar. Die derzeitige Therapie basiert auf Benzimidazolen, welche nur parasitostatisch wirken und oft schwere Nebenwirkungen hervorrufen. Neue Medikamente werden daher dringend benötigt.
Evolutionär konservierte Signalwege sind bekanntermaßen an Wirt-Parasit Kreuzkommunikation, Parasitenentwicklung und deren Überleben beteiligt. Darüber hinaus stellen sie auch mögliche Angriffspunkte für Chemotherapeutika dar. In diesem Zusammenhang wurden die Rollen des Serotonin- und des cAMP-Signalwegs in *Echinococcus* untersucht.
Gene für Serotoninrezeptoren, einen Serotonintransporter und für Enzyme, die in der Serotoninsynthese involviert sind, konnten in den *E. multilocularis* und *E. granulosus* Genomen identifiziert werden, was darauf schließen lässt, dass diese Parasiten in der Lage sind, Serotonin selbst herzustellen und zu sensieren. Des Weiteren wurde der Einfluss von exogenem Serotonin auf die Parasitenentwicklung untersucht. Serotonin förderte die Bildung von Metazestodenvesikeln aus Primärzellen und die Rückdifferenzierung von Protoskolizes signifikant. Die Hemmung des Serotonintransports mit Citalopram reduzierte die Bildung von Metazestodenvesikeln aus Primärzellen signifikant und führte zum Absterben von Protoskolizes und Metazestoden. Des Weiteren konnte gezeigt werden, dass Serotonin die Posphorylierung von Proteinkinase A Substraten erhöht. Zusammengefasst zeigen diese Ergebnisse, dass Serotonin und Serotonintransport essentiell für die Entwicklung und das Überleben von *Echinococcus* sind. Folglich stellen Komponenten des Serotoninsignalwegs potentielle Angriffspunkte für Medikamente dar.
In dieser Arbeit wurde der cAMP-Signalweg mit Schwerpunkt auf G-Protein gekoppelte Rezeptoren und Adenylatzyklasen untersucht. 76 G-Protein gekoppelte Rezep-

toren, inclusive Mitglieder aller Hauptfamilien, wurden im *E. multilocularis*-Genom identifiziert. Vier Homologe zur Adenylatzyklase IX wurden im *E. multilocularis*-Genom und drei im *E. granulosus*-Genom identifiziert. Während Glukagon keine signifikanten Effekte hervorrief, beeinflussten der Adenylatzyklase-Aktivator Forskolin und der Adenylatzyklase-Inhibitor 2', 5'-Didesoxyadenosin die Bildung von Metazestodenvesikeln aus Primärzellen, die Rückdifferenzierung von Protoskolizes und das Überleben von Metazestoden. Zudem wurde gezeigt, dass Forskolin die Phosphorylierung von Proteinkinase A-Substraten erhöht. Dies bestätigt, dass Forskolin den cAMP-Signalweg aktiviert. Diese Ergebnisse legen nahe, dass der cAMP-Signalweg eine wichtige Rolle in der Entwicklung und dem Überleben von *Echinococcus* spielt. Um diese Arbeit zu vervollständigen, wurde der Einfluss von verschiedenen Medien und Zusätzen auf *E. granulosus* Protoskolizes untersucht. Anaerobe Bedingungen und die Anwesenheit von FBS verlängerten das Überleben von Protoskolizes, während verschiedene Medien die Aktivierung und die Entwicklung von Protoskolizes beeinflussten.
Insgesamt gibt diese Arbeit wichtige Einblicke in Entwicklungsprozesse von *Echinococcus* und zeigt potentielle Angriffspunkte für Medikamente auf.

7 References

Alexander, J. S., Hechtman, H. B. and Shepro, D. (1987), 'Serotonin induced actin polymerization and association with cytoskeletal elements in cultured bovine aortic endothelium', *Biochem Biophys Res Commun* **143**(1), 152–8.

Alic, N. and Partridge, L. (2007), 'Antagonizing Methuselah to extend life span', *Genome Biol* **8**(8), 222.

Andersen, J., Taboureau, O., Hansen, K. B., Olsen, L., Egebjerg, J., Stromgaard, K. and Kristensen, A. S. (2009), 'Location of the antidepressant binding site in the serotonin transporter: importance of Ser-438 in recognition of citalopram and tricyclic antidepressants', *J Biol Chem* **284**(15), 10276–84.

Azmitia, E. C. (2001), 'Modern views on an ancient chemical: serotonin effects on cell proliferation, maturation, and apoptosis', *Brain Res Bull* **56**(5), 413–24.

Baganz, N. L. and Blakely, R. D. (2013), 'A dialogue between the immune system and brain, spoken in the language of serotonin', *ACS Chem Neurosci* **4**(1), 48–63.

Balbisi, E. A. (2004), 'Frovatriptan succinate, a 5-HT1B/1D receptor agonist for migraine', *Int J Clin Pract* **58**(7), 695–705.

Barker, E. L., Perlman, M. A., Adkins, E. M., Houlihan, W. J., Pristupa, Z. B., Niznik, H. B. and Blakely, R. D. (1998*a*), 'High affinity recognition of serotonin transporter antagonists defined by species-scanning mutagenesis. an aromatic residue in transmembrane domain I dictates species-selective recognition of citalopram and mazindol', *J Biol Chem* **273**(31), 19459–68.

Barker, E. L., Perlman, M. A., Adkins, E. M., Houlihan, W. J., Pristupa, Z. B., Niznik, H. B. and Blakely, R. D. (1998*b*), 'High affinity recognition of serotonin transporter antagonists defined by species-scanning mutagenesis. An aromatic residue in transmembrane domain I] dictates species-selective recognition of citalopram and mazindol', *J Biol Chem* **273**(31), 19459–68.

Berger, M., Gray, J. A. and Roth, B. L. (2009), 'The expanded biology of serotonin', *Annu Rev Med* **60**, 355–66.

Berry, M. D., Juorio, A. V., Li, X. M. and Boulton, A. A. (1996), 'Aromatic L-amino acid decarboxylase: a neglected and misunderstood enzyme', *Neurochem Res* **21**(9), 1075–87.

Bertaccini, G. (1960), 'Tissue 5-hydroxytryptamine and urinary 5-hydroxyindoleacetic acid after partial or total removal of the gastro-intestinal tract in the rat', *J Physiol* **153**(2), 239–49.

Berumen, L. C., Rodriguez, A., Miledi, R. and Garcia-Alcocer, G. (2012), 'Serotonin receptors in hippocampus', *ScientificWorldJournal* **2012**, 823493.

Billington, C. K. and Hall, I. P. (2012), 'Novel cAMP signalling paradigms: therapeutic implications for airway disease', *Br J Pharmacol* **166**(2), 401–10.

Billington, C. K. and Penn, R. B. (2003), 'Signaling and regulation of G protein-coupled receptors in airway smooth muscle', *Respir Res* **4**, 2.

Bockaert, J. and Pin, J. P. (1999), 'Molecular tinkering of G protein-coupled receptors: an evolutionary success', *EMBO J* **18**(7), 1723–9.

Bohn, L. M. and Schmid, C. L. (2010), 'Serotonin receptor signaling and regulation via beta-arrestins', *Crit Rev Biochem Mol Biol* **45**(6), 555–66.

Bowen, L. C., Bicknell, A. V., Tabish, M., Clegg, R. A., Rees, H. H. and Fisher, M. J. (2006), 'Expression of multiple isoforms of the cAMP-dependent protein kinase (PK-A) catalytic subunit in the nematode, *Caenorhabditis elegans*', *Cell Signal* **18**(12), 2230–7.

Boxberger, H. (2007), *Leitfaden für die Zell- und Gewebekultur: Einführung in Grundlagen und Techniken*, 1 edn, Wiley-VCH, Weinheim.

Boyle, J. P. and Yoshino, T. P. (2005), 'Serotonin-induced muscular activity in *Schistosoma mansoni* larval stages: importance of 5-HT transport and role in daughter sporocyst production', *J Parasitol* **91**(3), 542–50.

Boyle, J. P., Zaide, J. V. and Yoshino, T. P. (2000), '*Schistosoma mansoni*: effects of serotonin and serotonin receptor antagonists on motility and length of primary sporocysts in vitro', *Exp Parasitol* **94**(4), 217–26.

Brehm, K. (2010), 'The role of evolutionarily conserved signalling systems in *Echinococcus multilocularis* development and host-parasite interaction', *Med Microbiol Immunol* **199**(3), 247–59.

Brehm, K., Jensen, K. and Frosch, M. (2000), 'mRNA trans-splicing in the human parasitic cestode *Echinococcus multilocularis*', *J Biol Chem* **275**(49), 38311–8.

Brehm, K., Jensen, K., Frosch, P. and Frosch, M. (1999), 'Characterization of the genomic locus expressing the ERM-like protein of *Echinococcus multilocularis*', *Mol Biochem Parasitol* **100**(1), 147–52.

Brehm, K., Spiliotis, M., Zavala-Gongora, R., Konrad, C. and Frosch, M. (2006), 'The molecular mechanisms of larval cestode development: first steps into an unknown world', *Parasitol Int* **55 Suppl**, S15–21.

Brehm, K., Wolf, M., Beland, H., Kroner, A. and Frosch, M. (2003), 'Analysis of differential gene expression in *Echinococcus multilocularis* larval stages by means of spliced leader differential display', *Int J Parasitol* **33**(11), 1145–59.

Brownlee, D. J., Fairweather, I., Johnston, C. F. and Rogan, M. T. (1994), 'Immunocytochemical localization of serotonin (5-HT) in the nervous system of the hydatid organism, *Echinococcus granulosus* (cestoda, cyclophyllidea)', *Parasitology* **109 (Pt 2)**, 233–41.

Burkhardt, S. (2011), 'Molecular biological analysis of adenylate cyclase signalling in *Echinococcus multilocularis*: Characterization of heterotrimeric G Proteins stimulating adenylate cyclase', pp. Bachelorarbeit, Fakultät für Biomedizin, Universität Würzburg.

Buznikov, G. A., Lambert, H. W. and Lauder, J. M. (2001), 'Serotonin and serotonin-like substances as regulators of early embryogenesis and morphogenesis', *Cell Tissue Res* **305**(2), 177–86.

Buznikov, G. A., Shmukler, Y. B. and Lauder, J. M. (1996), 'From oocyte to neuron: do neurotransmitters function in the same way throughout development?', *Cell Mol Neurobiol* **16**(5), 537–59.

Camicia, F., Herz, M., Prada, L. C., Kamenetzky, L., Simonetta, S. H., Cucher, M. A., Bianchi, J. I., Fernandez, C., Brehm, K. and Rosenzvit, M. C. (2013), 'The nervous and prenervous roles of serotonin in *Echinococcus spp*', *Int J Parasitol* .

Cann, M. J., Chung, E. and Levin, L. R. (2000), 'A new family of adenylyl cyclase genes in the male germline of *Drosophila melanogaster*', *Dev Genes Evol* **210**(4), 200–6.

Chauveau, J., Fert, V., Morel, A. M. and Delaage, M. A. (1991), 'Rapid and specific enzyme immunoassay of serotonin', *Clin Chem* **37**(7), 1178–84.

Conchedda, M., Gabriele, E. and Bortoletti, G. (2004), 'Immunobiology of cystic echinococcosis', *Parassitologia* **46**(4), 375–80.

Côté, F., Thévenot, E., Fligny, C., Fromes, Y., Darmon, M., Ripoche, M. A., Bayard, E., Hanoun, N., Saurini, F., Lechat, P., Dandolo, L., Hamon, M., Mallet, J. and Vodjdani, G. (2003), 'Disruption of the nonneuronal tph1 gene demonstrates the importance of peripheral serotonin in cardiac function', *Proc Natl Acad Sci U S A* **100**(23), 13525–30.

Craig, P. (2003), '*Echinococcus multilocularis*', *Curr Opin Infect Dis* **16**(5), 437–44.

Csaba, G. and Kadar, M. (1978), 'Effects of epinephrine, glucagon and insulin on glucose metabolism of planaria', *Endokrinologie* **71**(1), 113–5.

Culpepper, L. (2012), 'The use of monoamine oxidase inhibitors in primary care', *J Clin Psychiatry* **73 Suppl 1**, 37–41.

Day, T. A., Bennett, J. L. and Pax, R. A. (1994), 'Serotonin and its requirement for maintenance of contractility in muscle fibres isolated from *Schistosoma mansoni*', *Parasitology* **108 (Pt 4)**, 425–32.

de Saram, P. S., Ressurreicao, M., Davies, A. J., Rollinson, D., Emery, A. M. and Walker, A. J. (2013), 'Functional mapping of protein kinase A reveals its importance in adult *Schistosoma mansoni* motor activity', *PLoS Negl Trop Dis* **7**(1), e1988.

Dent, J. A. (2006), 'Evidence for a diverse Cys-loop ligand-gated ion channel superfamily in early bilateria', *J Mol Evol* **62**(5), 523–35.

Désaubry, L. and Johnson, R. A. (1996), 'Adenine nucleoside 3'-tetraphosphates are novel and potent inhibitors of adenylyl cyclases', *J Biol Chem* **273**(38), 24972–7.

Dixon, J. B. (1997), 'Echinococcosis', *Comp Immunol Microbiol Infect Dis* **20**(1), 87–94.

Eckert, J. and Deplazes, P. (2004), 'Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern', *Clin Microbiol Rev* **17**(1), 107–35.

Eddahibi, S., Fabre, V., Boni, C., Martres, M. P., Raffestin, B., Hamon, M. and Adnot, S. (1999), 'Induction of serotonin transporter by hypoxia in pulmonary vascular smooth muscle cells. Relationship with the mitogenic action of serotonin', *Circ Res* **84**(3), 329–36.

Eddy, S. R. (2009), 'A new generation of homology search tools based on probabilistic inference', *Genome Inform* **23**(1), 205–11.

Eddy, S. R. (2011), 'Accelerated profile HMM searches', *PLoS Comput Biol* **7**(10), e1002195.

Edgar, R. C. (2004*a*), 'MUSCLE: a multiple sequence alignment method with reduced time and space complexity', *BMC Bioinformatics* **5**, 113.

Edgar, R. C. (2004*b*), 'MUSCLE: multiple sequence alignment with high accuracy and high throughput', *Nucleic Acids Res* **32**(5), 1792–7.

Ehlers, U. (1986), 'Comments on a phylogenetic system of the Platyhelminthes', *Hydrobiologia* **132**, 1–12.

Emanuelsson, H., Carlberg, M. and Lowkvist, B. (1988), 'Presence of serotonin in early chick embryos', *Cell Differ* **24**(3), 191–9.

Estey, S. J. and Mansour, T. E. (1987), 'Nature of serotonin-activated adenylate cyclase during development of *Schistosoma mansoni*', *Mol Biochem Parasitol* **26**(1-2), 47–59.

Estey, S. J. and Mansour, T. E. (1988), 'GTP binding regulatory proteins of adenylate cyclase in *Schistosoma mansoni* at different stages of development', *Mol Biochem Parasitol* **30**(1), 67–75.

Fairweather, I., McMullan, M. T., Johnston, C. F., Rogan, M. T. and Hanna, R. E. (1994), 'Serotoninergic and peptidergic nerve elements in the protoscolex of *Echinococcus granulosus* (Cestoda, Cyclophyllidea)', *Parasitol Res* **80**(8), 649–56.

Fernández, C., Gregory, W. F., Loke, P. and Maizels, R. M. (2002), 'Full-length-enriched cDNA libraries from *Echinococcus granulosus* contain separate populations of oligo-capped and trans-spliced transcripts and a high level of predicted signal peptide sequences', *Mol Biochem Parasitol* **122**(2), 171–80.

Fernandez, R., Tabarini, D., Azpiazu, N., Frasch, M. and Schlessinger, J. (1995), 'The *Drosophila* insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential', *EMBO J* **14**(14), 3373–84.

Flachaire, E., Beney, C., Berthier, A., Salandre, J., Quincy, C. and Renaud, B. (1990), 'Determination of reference values for serotonin concentration in platelets of healthy newborns, children, adults, and elderly subjects by HPLC with electrochemical detection', *Clin Chem* **36**(12), 2117–20.

Flavell, S. W., Pokala, N., Macosko, E. Z., Albrecht, D. R., Larsch, J. and Bargmann, C. I. (2013), 'Serotonin and the neuropeptide PDF initiate and extend opposing behavioral states in *C. elegans*', *Cell* **154**(5), 1023–35.

Förster, S. (2012), 'Nuclear Hormone Receptors and Fibroblast Growth Factor Receptor Signaling in *Echinococcus multilocularis*', pp. Naturwissenschaftliche Doktorarbeit, Graduate School of Life Sciences, Universität Würzburg.

Francis, S. H., Blount, M. A. and Corbin, J. D. (2011), 'Mammalian cyclic nucleotide phosphodiesterases: molecular mechanisms and physiological functions', *Physiol Rev* **91**(2), 651–90.

Franquinet, R. (1979), 'The role of serotonin and catecholamines in the regeneration of the planaria *Polycelis tenvis*', *J Embryol Exp Morphol* **51**, 85–95.

Franquinet, R. and Martelly, I. (1981), 'Effects of serotonin and catecholamines on RNA synthesis in planarians; *in vitro* and *in vivo* studies', *Cell Differ* **10**(4), 201–9.

Frazer, A. and Hensler, J. (1999), Serotonin Receptors, *in* G. Siegel, B. Agranoff and R. Albers, eds, 'Basic Neurochemistry: Molecular, Cellular and Medical Aspects', 6th edn, Lippincott-Raven, Philadelphia.

Fredriksson, R., Lagerström, M. C., Lundin, L. G. and Schiöth, H. B. (2003), 'The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints', *Mol Pharmacol* **63**(6), 1256–72.

Fredriksson, R. and Schiöth, H. B. (2005), 'The repertoire of G-protein-coupled receptors in fully sequenced genomes', *Mol Pharmacol* **67**(5), 1414–25.

Gao, X., Sadana, R., Dessauer, C. W. and Patel, T. B. (2007), 'Conditional stimulation of type V and VI adenylyl cyclases by G protein betagamma subunits', *J Biol Chem* **282**(1), 294–302.

Garcia-Llamazares, J. L., Alvarez-de Felipe, A. I., Redondo-Cardena, P. A. and Prieto-Fernandez, J. G. (1998), '*Echinococcus granulosus*: membrane permeability of secondary hydatid cysts to albendazole sulfoxide', *Parasitol Res* **84**(5), 417–20.

Gelmedin, V., Caballero-Gamiz, R. and Brehm, K. (2008), 'Characterization and inhibition of a p38-like mitogen-activated protein kinase (MAPK) from *Echinococcus multilocularis*: antiparasitic activities of p38 MAPK inhibitors', *Biochem Pharmacol* **76**(9), 1068–81.

Gelmedin, V., Spiliotis, M. and Brehm, K. (2010), 'Molecular characterisation of MEK1/2- and MKK3/6-like mitogen-activated protein kinase kinases (MAPKK) from the fox tapeworm *Echinococcus multilocularis*', *Int J Parasitol* **40**(5), 555–67.

Giribet, G. (2008), 'Assembling the lophotrochozoan (=spiralian) tree of life', *Philos Trans R Soc Lond B Biol Sci* **363**(1496), 1513–22.

Hall, T. (1999), 'Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT.', *Nucleic Acids Symposium Series* **41**, 95–98.

Hanoune, J., Pouille, Y., Tzavara, E., Shen, T., Lipskaya, L., Miyamoto, N., Suzuki, Y. and Defer, N. (1997), 'Adenylyl cyclases: structure, regulation and function in an enzyme superfamily', *Mol Cell Endocrinol* **128**(1-2), 179–94.

Harder, A., Abbink, J., Andrews, P. and Thomas, H. (1987), 'Praziquantel impairs the ability of exogenous serotonin to stimulate carbohydrate metabolism in intact *Schistosoma mansoni*', *Parasitol Res* **73**(5), 442–5.

Harder, A., Andrews, P. and Thomas, H. (1987a), 'Chlorpromazine, other amphiphilic cationic drugs and praziquantel: effects on carbohydrate metabolism of *Schistosoma mansoni*', *Parasitol Res* **73**(3), 245–9.

Hastings, K. E. (2005), 'SL trans-splicing: easy come or easy go?', *Trends Genet* **21**(4), 240–7.

Heath, D. D. and Osborn, P. J. (1976), 'Formation of *Echinococcus granulosus* laminated membrane in a defined medium', *Int J Parasitol* **6**(6), 467–71.

Hemer, S. (2012), Molecular characterization of evolutionarily conserved signaling systems of *Echinococcus multilocularis* and their utilization for the development of novel drugs against *Echinococosis*, PhD thesis, Julius-Maximilians-Universität Würzburg.

Hemer, S., Konrad, C., Spiliotis, M., Koziol, U., Schaack, D., Forster, S., Gelmedin, V., Stadelmann, B., Dandekar, T., Hemphill, A. and Brehm, K. (2014), 'Host insulin stimulates *Echinococcus multilocularis* insulin signalling pathways and larval development', *BMC Biol* **12**, 5.

Hemphill, A. and Gottstein, B. (1995), 'Immunology and morphology studies on the proliferation of *in vitro* cultivated *Echinococcus multilocularis* metacestodes', *Parasitol Res* **81**(7), 605–14.

Hemphill, A., Stadelmann, B., Scholl, S., Muller, J., Spiliotis, M., Muller, N., Gottstein, B. and Siles-Lucas, M. (2010), '*Echinococcus* metacestodes as laboratory models for the screening of drugs against cestodes and trematodes', *Parasitology* **137**(3), 569–87.

Henry, L. K., Defelice, L. J. and Blakely, R. D. (2006), 'Getting the message across: a recent transporter structure shows the way', *Neuron* **49**(6), 791–6.

Hirai, K., Tsuboi, T., Torii, M. and Nishida, H. (1987), 'Carbohydrate metabolism in intact golden hamsters infected with plerocercoids of *Spirometra erinacei* (Cestoda: Diphyllobothriidae)', *Parasitol Res* **74**(2), 183–7.

Horschitz, S., Hummerich, R. and Schloss, P. (2001), 'Structure, function and regulation of the 5-hydroxytryptamine (serotonin) transporter', *Biochem Soc Trans* **29**(Pt 6), 728–32.

Houslay, M. D. and Milligan, G. (1997), 'Tailoring cAMP-signalling responses through isoform multiplicity', *Trends Biochem Sci* **22**(6), 217–24.

Howe, A. K., Baldor, L. C. and Hogan, B. P. (2005), 'Spatial regulation of the cAMP-dependent protein kinase during chemotactic cell migration', *Proc Natl Acad Sci U S A* **102**(40), 14320–5.

Hoyer, D., Clarke, D. E., Fozard, J. R., Hartig, P. R., Martin, G. R., Mylecharane, E. J., Saxena, P. R. and Humphrey, P. P. (1994), 'International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (Serotonin)', *Pharmacol Rev* **46**(2), 157–203.

Hubert, K., Cordero, E., Frosch, M. and Solomon, F. (1999), 'Activities of the EM10 protein from *Echinococcus multilocularis* in cultured mammalian cells demonstrate functional relationships to ERM family members', *Cell Motil Cytoskeleton* **42**(3), 178–88.

Hurley, J. H. (1998), 'The adenylyl and guanylyl cyclase superfamily', *Curr Opin Struct Biol* **8**(6), 770–7.

Hurley, J. H. (1999), 'Structure, mechanism, and regulation of mammalian adenylyl cyclase', *J Biol Chem* **274**(12), 7599–602.

Iourgenko, V., Kliot, B., Cann, M. J. and Levin, L. R. (1997), 'Cloning and characterization of a *drosophila* adenylyl cyclase homologous to mammalian type IX', *FEBS Lett* **413**(1), 104–8.

Iourgenko, V. and Levin, L. R. (2000), 'A calcium-inhibited *Drosophila* adenylyl cyclase', *Biochim Biophys Acta* **1495**(2), 125–39.

Ito, A. (2002), 'Serologic and molecular diagnosis of zoonotic larval cestode infections', *Parasitol Int* **51**(3), 221–35.

Jalink, K. and Moolenaar, W. H. (2010), 'G protein-coupled receptors: the inside story', *Bioessays* **32**(1), 13–6.

Johnson, L. S., Eddy, S. R. and Portugaly, E. (2010), 'Hidden Markov model speed heuristic and iterative HMM search procedure', *BMC Bioinformatics* **11**, 431.

Jura, H., Bader, A. and Frosch, M. (1998), '*In vitro* activities of benzimidazoles against *Echinococcus multilocularis* metacestodes', *Antimicrob Agents Chemother* **42**(5), 1052–6.

Jura, H., Bader, A., Hartmann, M., Maschek, H. and Frosch, M. (1996), 'Hepatic tissue culture model for study of host-parasite interactions in alveolar echinococcosis', *Infect Immun* **64**(9), 3484–90.

Kasschau, M. R. and Mansour, T. E. (1982), 'Adenylate cyclase in adults and cercariae of *Schistosoma mansoni*', *Mol Biochem Parasitol* **5**(2), 107–16.

Kawamoto, F., Shozawa, A., Kumada, N. and Kojima, K. (1989), 'Possible roles of cAMP and Ca2+ in the regulation of miracidial transformation in *Schistosoma mansoni*', *Parasitol Res* **75**(5), 368–74.

Kern, P. (2010), 'Clinical features and treatment of alveolar echinococcosis', *Curr Opin Infect Dis* **23**(5), 505–12.

Kern, P., Wen, H., Sato, N., Vuitton, D. A., Gruener, B., Shao, Y., Delabrousse, E., Kratzer, W. and Bresson-Hadni, S. (2006), 'WHO classification of alveolar echinococcosis: principles and application', *Parasitol Int* **55 Suppl**, S283–7.

Klein, D. C. (2007), 'Arylalkylamine N-acetyltransferase: "the Timezyme"', *J Biol Chem* **282**(7), 4233–7.

Kolakowski, L. F., J. (1994), 'GCRDb: a G-protein-coupled receptor database', *Receptors Channels* **2**(1), 1–7.

Konrad, C., Kroner, A., Spiliotis, M., Zavala-Gongora, R. and Brehm, K. (2003), 'Identification and molecular characterisation of a gene encoding a member of the insulin receptor family in *Echinococcus multilocularis*', *Int J Parasitol* **33**(3), 301–12.

Korswagen, H. C., van der Linden, A. M. and Plasterk, R. H. (1998), 'G protein hyperactivation of the *Caenorhabditis elegans* adenylyl cyclase SGS-1 induces neuronal degeneration', *EMBO J* **17**(17), 5059–65.

Koziol, U., Krohne, G. and Brehm, K. (2013), 'Anatomy and development of the larval nervous system in *Echinococcus multilocularis*', *Front Zool* **10**(1), 24.

Koziol, U., Rauschendorfer, T., Zanon Rodriguez, L., Krohne, G. and Brehm, K. (2014), 'The unique stem cell system of the immortal larva of the human parasite *Echinococcus multilocularis*', *Evodevo* **5**(1), 10.

Kristensen, A. S., Andersen, J., Jorgensen, T. N., Sorensen, L., Eriksen, J., Loland, C. J., Stromgaard, K. and Gether, U. (2011), 'SLC6 neurotransmitter transporters: structure, function, and regulation', *Pharmacol Rev* **63**(3), 585–640.

Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E. L. (2001), 'Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes', *J Mol Biol* **305**(3), 567–80.

Lagerström, M. C. and Schiöth, H. B. (2008), 'Structural diversity of G protein-coupled receptors and significance for drug discovery', *Nat Rev Drug Discov* **7**(4), 339–57.

Lasda, E. L. and Blumenthal, T. (2011), 'Trans-splicing', *Wiley Interdiscip Rev RNA* **2**(3), 417–34.

Lauder, J. M. (1993), 'Neurotransmitters as growth regulatory signals: role of receptors and second messengers', *Trends Neurosci* **16**(6), 233–40.

Le, T. H., Pearson, M. S., Blair, D., Dai, N., Zhang, L. H. and McManus, D. P. (2002), 'Complete mitochondrial genomes confirm the distinctiveness of the horse-dog and sheep-dog strains of *Echinococcus granulosus*', *Parasitology* **124**(Pt 1), 97–112.

Lee, S. L., Wang, W. W. and Fanburg, B. L. (1997), 'Association of Tyr phosphorylation of GTPase-activating protein with mitogenic action of serotonin', *Am J Physiol* **272**(1 Pt 1), C223–30.

Lee, S. L., Wang, W. W., Lanzillo, J. J. and Fanburg, B. L. (1994), 'Serotonin produces both hyperplasia and hypertrophy of bovine pulmonary artery smooth muscle cells in culture', *Am J Physiol* **266**(1 Pt 1), L46–52.

Lee, S. L., Wang, W. W., Moore, B. J. and Fanburg, B. L. (1991), 'Dual effect of serotonin on growth of bovine pulmonary artery smooth muscle cells in culture', *Circ Res* **68**(5), 1362–8.

Lesurtel, M., Graf, R., Aleil, B., Walther, D. J., Tian, Y., Jochum, W., Gachet, C., Bader, M. and Clavien, P. A. (2006), 'Platelet-derived serotonin mediates liver regeneration', *Science* **312**(5770), 104–7.

Lesurtel, M., Soll, C., Graf, R. and Clavien, P. A. (2008), 'Role of serotonin in the hepato-gastrointestinal tract: an old molecule for new perspectives', *Cell Mol Life Sci* **65**(6), 940–52.

Letunic, I., Doerks, T. and Bork, P. (2012), 'SMART 7: recent updates to the protein domain annotation resource', *Nucleic Acids Res* **40**(Database issue), D302–5.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G. and Durbin, R. (2009), 'The Sequence Alignment/Map format and SAMtools', *Bioinformatics* **25**(16), 2078–9.

Li, T., Chen, X., Zhen, R., Qiu, J., Qiu, D., Xiao, N., Ito, A., Wang, H., Giraudoux, P., Sako, Y., Nakao, M. and Craig, P. S. (2010), 'Widespread co-endemicity of human cystic and alveolar echinococcosis on the eastern Tibetan Plateau, northwest Sichuan/southeast Qinghai, China', *Acta Trop* **113**(3), 248–56.

Littlewood, D. T. J., Rohde, K. and Clough, K. A. (1999), 'The interrelationships of all major groups of Platyhelminthes: phylogenetic evidence from morphology and molecules', *Biological Journal of the Linnean Society* **66**(1), 75–114.

Liu, Q., Yang, Q., Sun, W., Vogel, P., Heydorn, W., Yu, X. Q., Hu, Z., Yu, W., Jonas, B., Pineda, R., Calderon-Gay, V., Germann, M., O'Neill, E., Brommage, R., Cullinan, E., Platt, K., Wilson, A., Powell, D., Sands, A., Zambrowicz, B. and Shi, Z. C. (2008), 'Discovery and characterization of novel tryptophan hydroxylase inhibitors that selectively inhibit serotonin synthesis in the gastrointestinal tract', *J Pharmacol Exp Ther* **325**(1), 47–55.

Matsushita, H., Lelianova, V. G. and Ushkaryov, Y. A. (1999), 'The latrophilin family: multiply spliced G protein-coupled receptors with differential tissue distribution', *FEBS Lett* **443**(3), 348–52.

Mbah, A. N., Kamga, H. L., Awofolu, O. R. and Isokpehi, R. D. (2012), 'Drug Target Exploitable Structural Features of Adenylyl Cyclase Activity in *Schistosoma mansoni*', *Drug Target Insights* **6**, 41–58.

McCloskey, D. J., Postolache, T. T., Vittone, B. J., Nghiem, K. L., Monsale, J. L., Wesley, R. A. and Rick, M. E. (2008), 'Selective serotonin reuptake inhibitors: measurement of effect on platelet function', *Transl Res* **151**(3), 168–72.

Millan, M. J., Marin, P., Bockaert, J. and Mannoury la Cour, C. (2008), 'Signaling at G-protein-coupled serotonin receptors: recent advances and future research directions', *Trends Pharmacol Sci* **29**(9), 454–64.

Moreno, M. S. and Barrett, J. (1979), 'Monoamine oxidase in adult hymenolepis diminuta (cestoda)', *Parasitology* **78**(1), 1–5.

Moro, P. and Schantz, P. M. (2009), 'Echinococcosis: a review', *Int J Infect Dis* **13**(2), 125–33.

Mossner, R. and Lesch, K. P. (1998), 'Role of serotonin in the immune system and in neuroimmune interactions', *Brain Behav Immun* **12**(4), 249–71.

Nakajima-Shimada, J., Hirota, Y. and Aoki, T. (1996), 'Inhibition of *Trypanosoma cruzi* growth in mammalian cells by purine and pyrimidine analogs', *Antimicrob Agents Chemother* **40**(11), 2455–8.

Nakao, M., McManus, D. P., Schantz, P. M., Craig, P. S. and Ito, A. (2007), 'A molecular phylogeny of the genus *Echinococcu*s inferred from complete mitochondrial genomes', *Parasitology* **134**(Pt 5), 713–22.

Neubauer, H. A., Hansen, C. G. and Wiborg, O. (2006), 'Dissection of an allosteric mechanism on the serotonin transporter: a cross-species study', *Mol Pharmacol* **69**(4), 1242–50.

New, D. C. and Wong, Y. H. (2007), 'Molecular mechanisms mediating the G protein-coupled receptor regulation of cell cycle progression', *J Mol Signal* **2**, 2.

Nimmo-Smith, R. H. and Raison, C. G. (1968), 'Monoamine oxidase activity of *Schistosoma mansoni*', *Comp Biochem Physiol* **24**(2), 403–16.

Olson, P. D., Zarowiecki, M., Kiss, F. and Brehm, K. (2011), 'Cestode genomics - progress and prospects for advancing basic and applied aspects of flatworm biology', *Parasite Immunol* .

Ostad Haji, E., Tadić, A., Wagner, S., Dragicevic, A., Müller, M. J., Boland, K., Rao, M. L., Fric, M., Laux, G. and Hiemke, C. (2011), 'Association between citalopram serum levels and clinical improvement of patients with major depression', *J Clin Psychopharmacol* **31**(3), 281–6.

Pagan, O. R., Rowlands, A. L. and Urban, K. R. (2006), 'Toxicity and behavioral effects of dimethylsulfoxide in planaria', *Neurosci Lett* **407**(3), 274–8.

Parkinson, J., Wasmuth, J. D., Salinas, G., Bizarro, C. V., Sanford, C., Berriman, M., Ferreira, H. B., Zaha, A., Blaxter, M. L., Maizels, R. M. and Fern'andez, C. (2012), 'A transcriptomic analysis of *Echinococcus granulosus* larval stages: implications for parasite biology and host adaptation', *PLoS Negl Trop Dis* **6**(11), e1897.

Pascual, J. (2004), 'A review of rizatriptan, a quick and consistent 5-HT1B/1D agonist for the acute treatment of migraine', *Expert Opin Pharmacother* **5**(3), 669–77.

Patel, T. B., Du, Z., Pierre, S., Cartin, L. and Scholich, K. (2001), 'Molecular biological approaches to unravel adenylyl cyclase signaling and function', *Gene* **269**(1-2), 13–25.

Patocka, N. and Ribeiro, P. (2007), 'Characterization of a serotonin transporter in the parasitic flatworm, *Schistosoma mansoni*: cloning, expression and functional analysis', *Mol Biochem Parasitol* **154**(2), 125–33.

Pavan, B., Biondi, C. and Dalpiaz, A. (2009), 'Adenylyl cyclases as innovative therapeutic goals', *Drug Discov Today* **14**(19-20), 982–91.

Pierce, K. L., Premont, R. T. and Lefkowitz, R. J. (2002), 'Seven-transmembrane receptors', *Nat Rev Mol Cell Biol* **3**(9), 639–50.

Ponce Gordo, F. and Cuesta Bandera, C. (1997), '*Echinococcus granulosus*: characterization of the Spanish strains using *in vitro* vesicular development', *J Helminthol* **71**(1), 61–7.

Premont, R. T., Matsuoka, I., Mattei, M. G., Pouille, Y., Defer, N. and Hanoune, J. (1996), 'Identification and characterization of a widely expressed form of adenylyl cyclase', *J Biol Chem* **271**(23), 13900–7.

Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J., Heger, A., Holm, L., Sonnhammer, E. L., Eddy, S. R., Bateman, A. and Finn, R. D. (2012), 'The [Pfam protein families database', *Nucleic Acids Res* **40**(Database issue), D290–301.

Ranganathan, R., Cannon, S. C. and Horvitz, H. R. (2000), 'MOD-1 is a serotonin-gated chloride channel that modulates locomotory behaviour in *C. elegans*', *Nature* **408**(6811), 470–5.

Ravna, A. W., Sylte, I., Kristiansen, K. and Dahl, S. G. (2006), 'Putative drug binding conformations of monoamine transporters', *Bioorg Med Chem* **14**(3), 666–75.

Renaud, F., Parisi, E., Capasso, A. and De Prisco, P. (1983), 'On the role of serotonin and 5-methoxy-tryptamine in the regulation of cell division in sea urchin eggs', *Dev Biol* **98**(1), 37–46.

Reuter, S., Buck, A., Manfras, B., Kratzer, W., Seitz, H. M., Darge, K., Reske, S. N. and Kern, P. (2004), 'Structured treatment interruption in patients with alveolar echinococcosis', *Hepatology* **39**(2), 509–17.

Ribeiro, P. and Webb, R. A. (1984), 'The occurrence, synthesis and metabolism of 5-hydroxytryptamine and 5-hydroxytryptophan in the cestode *Hymenolepis diminuta*: a high performance liquid chromatographic study', *Comp Biochem Physiol C* **79**(1), 159–64.

RKI (2006), 'Epidemiologie der Fuchsbandwurmerkrankungen in Deutschland Daten des Echinokokkose Registers', *Epidemiologisches Bulletin* **15**, S283–7.

RKI (Date of inquiry: 15.11.12), *http://www3.rki.de/SurvStat/* .

Robinson, J. T., Thórvaldsdottir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G. and Mesirov, J. P. (2011), 'Integrative genomics viewer', *Nat Biotechnol* **29**(1), 24–6.

Rodriguez-Caabeiro, F. and Casado, N. (1988), 'Evidence of *in vitro* germinal layer development in *Echinococcus granulosus* cysts', *Parasitol Res* **74**(6), 558–62.

Rogan, M. T., Hai, W. Y., Richardson, R., Zeyhle, E. and Craig, P. S. (2006), 'Hydatid cysts: does every picture tell a story?', *Trends Parasitol* **22**(9), 431–8.

Römpler, H., Stäubert, C., Thor, D., Schulz, A., Hofreiter, M. and Schöneberg, T. (2007), 'G protein-coupled time travel: evolutionary aspects of GPCR research', *Mol Interv* **7**(1), 17–25.

Saitou, N. and Nei, M. (1987), 'The neighbor-joining method: a new method for reconstructing phylogenetic trees', *Mol Biol Evol* **4**(4), 406–25.

Saudou, F., Boschert, U., Amlaiky, N., Plassat, J. L. and Hen, R. (1992), 'A family of *Drosophila* serotonin receptors with distinct intracellular signalling properties and expression patterns', *EMBO J* **11**(1), 7–17.

Schilling, M. (2010), 'Charakterisierung von Proteinkinase A-Untereinheiten von *Echinococcus mulitlocularis*, Bachelorarbeit, Fakultät für Biomedizin, Universität Würzburg'.

Schiöth, H. B. and Fredriksson, R. (2005), 'The GRAFS classification system of G-protein coupled receptors in comparative perspective', *Gen Comp Endocrinol* **142**(1-2), 94–101.

Schiöth, H. B., Nordström, K. J. and Fredriksson, R. (2007), 'Mining the gene repertoire and ESTs for G protein-coupled receptors with evolutionary perspective', *Acta Physiol (Oxf)* **190**(1), 21–31.

Schultz, J., Milpetz, F., Bork, P. and Ponting, C. P. (1998), 'SMART, a simple modular architecture research tool: identification of signaling domains', *Proc Natl Acad Sci U S A* **95**(11), 5857–64.

Seuwen, K. and Pouyssegur, J. (1990), 'Serotonin as a growth factor', *Biochem Pharmacol* **39**(6), 985–90.

Simonds, W. F. (1999), 'G protein regulation of adenylate cyclase', *Trends Pharmacol Sci* **20**(2), 66–73.

Singh, S. K., Piscitelli, C. L., Yamashita, A. and Gouaux, E. (2008), 'A competitive inhibitor traps LeuT in an open-to-out conformation', *Science* **322**(5908), 1655–61.

Slater, G. S. and Birney, E. (2005), 'Automated generation of heuristics for biological sequence comparison', *BMC Bioinformatics* **6**, 31.

Sloley, B. D. (2004), 'Metabolism of monoamines in invertebrates: the relative importance of monoamine oxidase in different phyla', *Neurotoxicology* **25**(1-2), 175–83.

Smyth, J. D. (1968), '*In vitro* studies and host-specificity in *Echinococcus*', *Bull World Health Organ* **39**(1), 5–12.

Smyth, J. D., Howkins, A. B. and Barton, M. (1966), 'Factors controlling the differentiation of the hydatid organism, *Echinococcus granulosus*, into cystic or strobilar stages *in vitro*', *Nature* **211**(5056), 1374–7.

Smyth, J. D., Miller, H. J. and Howkins, A. B. (1967), 'Further analysis of the factors controlling strobilization, differentiation, and maturation of *Echinococcus granulosus in vitro*', *Exp Parasitol* **21**(1), 31–41.

Sossin, W. S. and Abrams, T. W. (2009), 'Evolutionary conservation of the signaling proteins upstream of cyclic AMP-dependent kinase and protein kinase C in gastropod mollusks', *Brain Behav Evol* **74**(3), 191–205.

Spiliotis, M. and Brehm, K. (2009), 'Axenic *In Vitro* Cultivation of *Echinococcus multilocularis* Metacestode Vesicles and the Generationof Primary Cell Cultures', pp. 245–62.

Spiliotis, M., Konrad, C., Gelmedin, V., Tappe, D., Bruckner, S., Mosch, H. U. and Brehm, K. (2006), 'Characterisation of EmMPK1, an ERK-like MAP kinase from *Echinococcus multilocularis* which is activated in response to human epidermal growth factor', *Int J Parasitol* **36**(10-11), 1097–112.

Spiliotis, M., Kroner, A. and Brehm, K. (2003), 'Identification, molecular characterization and expression of the gene encoding the epidermal growth factor receptor orthologue from the fox-tapeworm *Echinococcus multilocularis*', *Gene* **323**, 57–65.

Spiliotis, M., Lechner, S., Tappe, D., Scheller, C., Krohne, G. and Brehm, K. (2008), 'Transient transfection of *Echinococcus multilocularis* primary cells and complete *in vitro* regeneration of metacestode vesicles', *Int J Parasitol* **38**(8-9), 1025–39.

Spiliotis, M., Tappe, D., Sesterhenn, L. and Brehm, K. (2004), 'Long-term *in vitro* cultivation of *Echinococcus multilocularis* metacestodes under axenic conditions', *Parasitol Res* **92**(5), 430–2.

Sutherland, E. W. and Rall, T. W. (1958), 'Fractionation and characterization of a cyclic adenine ribonucleotide formed by tissue particles', *J Biol Chem* **232**(2), 1077–91.

Taft, A. S., Norante, F. A. and Yoshino, T. P. (2010), 'The identification of inhibitors of *Schistosoma mansoni* miracidial transformation by incorporating a medium-throughput small-molecule screen', *Exp Parasitol* **125**(2), 84–94.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007), 'MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0', *Mol Biol Evol* **24**(8), 1596–9.

Tang, W. J., Krupinski, J. and Gilman, A. G. (1991), 'Expression and characterization of calmodulin-activated (type i) adenylylcyclase', *J Biol Chem* **266**(13), 8595–603.

Tang, W. J., Stanzel, M. and Gilman, A. G. (1995), 'Truncation and alanine-scanning mutants of type I adenylyl cyclase', *Biochemistry* **34**(44), 14563–72.

Terenina, N. B., Gustafsson, M. K. and Reuter, M. (1995), 'Serotonin, reserpine, and motility in *Mesocestoides tetrathyridia*. An experimental spectrofluorometry and immunocytochemistry study', *Parasitol Res* **81**(8), 677–83.

Terry, A. V., J., Buccafusco, J. J. and Wilson, C. (2008), 'Cognitive dysfunction in neuropsychiatric disorders: selected serotonin receptor subtypes as therapeutic targets', *Behav Brain Res* **195**(1), 30–8.

Tesmer, J. J., Dessauer, C. W., Sunahara, R. K., Murray, L. D., Johnson, R. A., Gilman, A. G. and Sprang, S. R. (2000), 'Molecular basis for P-site inhibition of adenylyl cyclase', *Biochemistry* **39**(47), 14464–71.

Tesmer, J. J. and Sprang, S. R. (1998), 'The structure, catalytic mechanism and regulation of adenylyl cyclase', *Curr Opin Struct Biol* **8**(6), 713–9.

Tesmer, J. J., Sunahara, R. K., Gilman, A. G. and Sprang, S. R. (1997), 'Crystal structure of the catalytic domains of adenylyl cyclase in a complex with Gsalpha.GTPgammaS', *Science* **278**(5345), 1907–16.

Toh, C. C. (1957), 'The presence of a 5-hydroxytryptamine (serotonin) liberator in the gastro-intestinal tract', *J Physiol* **138**(3), 488–94.

Trapnell, C., Pachter, L. and Salzberg, S. L. (2009), 'TopHat: discovering splice junctions with RNA-Seq', *Bioinformatics* **25**(9), 1105–11.

Tsai, I. J., Zarowiecki, M., Holroyd, N., Garciarrubio, A., Sanchez-Flores, A., Brooks, K. L., Tracey, A., Bobes, R. J., Fragoso, G., Sciutto, E., Aslett, M., Beasley, H., Bennett, H. M., Cai, J., Camicia, F., Clark, R., Cucher, M., De Silva, N., Day, T. A., Deplazes, P., Estrada, K., Fernandez, C., Holland, P. W., Hou, J., Hu, S., Huckvale, T., Hung, S. S., Kamenetzky, L., Keane, J. A., Kiss, F., Koziol, U., Lambert, O., Liu, K., Luo, X., Luo, Y., Macchiaroli, N., Nichol, S., Paps, J., Parkinson, J., Pouchkina-Stantcheva, N., Riddiford, N., Rosenzvit, M., Salinas, G., Wasmuth, J. D., Zamanian, M., Zheng, Y., Cai, X., Soberon, X., Olson, P. D., Laclette, J. P., Brehm, K. and Berriman, M. (2013), 'The genomes of four tapeworm species reveal adaptations to parasitism', *Nature* **496**(7443), 57–63.

Turlejski, K. (1996), 'Evolutionary ancient roles of serotonin: long-lasting regulation of activity and development', *Acta Neurobiol Exp (Wars)* **56**(2), 619–36.

Tusády, G. E. and Simon, I. (2001), 'The HMMTOP transmembrane topology prediction server', *Bioinformatics* **17**(9), 849–50.

Tusnády, G. E. and Simon, I. (1998), 'Principles governing amino acid composition of integral membrane proteins: application to topology prediction', *J Mol Biol* **283**(2), 489–506.

Vuitton, D. A. (2004), 'Echinococcosis and allergy', *Clin Rev Allergy Immunol* **26**(2), 93–104.

Walker, M., Rossignol, J. F., Torgerson, P. and Hemphill, A. (2004), '*In vitro* effects of nitazoxanide on *Echinococcus granulosus* protoscoleces and metacestodes', *J Antimicrob Chemother* **54**(3), 609–16.

Wettschureck, N. and Offermanns, S. (2005), 'Mammalian G proteins and their cell type specific functions', *Physiol Rev* **85**(4), 1159–204.

Yamashita, A., Singh, S. K., Kawate, T., Jin, Y. and Gouaux, E. (2005), 'Crystal structure of a bacterial homologue of Na+/Cl–dependent neurotransmitter transporters', *Nature* **437**(7056), 215–23.

Yan, S. Z., Huang, Z. H., Andrews, R. K. and Tang, W. J. (1998), 'Conversion of forskolin-insensitive to forskolin-sensitive (mouse-type IX) adenylyl cyclase', *Mol Pharmacol* **53**(2), 182–7.

Yan, S. Z., Huang, Z. H., Shaw, R. S. and Tang, W. J. (1997), 'The conserved asparagine and arginine are essential for catalysis of mammalian adenylyl cyclase', *J Biol Chem* **272**(19), 12342–9.

Yuan, Y., Tsoi, K. and Hunt, R. H. (2006), 'Selective serotonin reuptake inhibitors and risk of upper GI bleeding: confusion or confounding?', *Am J Med* **119**(9), 719–27.

Yuan, Z., Zhao, B. and Zhang, Y. (2012), 'Effects of dimethylsulfoxide on behavior and antioxidant enzymes response of planarian *Dugesia japonica*', *Toxicol Ind Health* **28**(5), 449–57.

Zamanian, M., Kimber, M. J., McVeigh, P., Carlson, S. A., Maule, A. G. and Day, T. A. (2011), 'The repertoire of G protein-coupled receptors in the human parasite *Schistosoma mansoni* and the model organism *Schmidtea mediterranea*', *BMC Genomics* **12**(1), 596.

Zavala-Gongora, R., Kroner, A., Bernthaler, P., Knaus, P. and Brehm, K. (2006), 'A member of the transforming growth factor-beta receptor family from *Echinococcus multilocularis* is activated by human bone morphogenetic protein 2', *Mol Biochem Parasitol* **146**(2), 265–71.

8 List of abbreviations

5-HT	5-Hydroxytryptophan (serotonin)
AC	adenylate cyclase
BC	brood capsules
bp	base pair
C. elegans	Caenorhabditis elegans
cAMP	cyclic adenosine monophosphate
cit	citalopram
CREB	cAMP responsive element-binding protein
d	dead protoscolex
D. melanogaster	Drosophila melanogaster
DAT	dopamine transporter
E. coli	Echerichia coli
EST	expressed sequence tag
ev	evaginated protoscolex
F. hepatica	Faciola hepatica
FBS	fetal bovine serum
GEF	guanine nucleotide exchange factors
GPCR	G-Protein coupled receptor
GRK	GPCR kinase
iv	invaginated protoscolex
MAO	monoamine oxidase
PBS	phosphate buffered saline
PC	primary cell
PCR	polymerase chain reaction
PKA	Protein kinase A
PS	protoscoleces
RACE	Rapid amplication of cDNA-ends
S. mansoni	Schistosoma mansoni
Ser	serotonin
SERT	serotonin transporter
SL	spliced leader
SLC	solute carrier
TM	transmembrane
ves	vesicularized protoscolex

9 Supplement

9.1 Sources of genome sequences, ESTs, transcriptome data and protein predictions

ftp://ftp.sanger.ac.uk/pub/pathogens/Echinococcus/multilocularis/
ftp://ftp.sanger.ac.uk/pub/pathogens/Echinococcus/granulosus/
ftp://ftp.sanger.ac.uk/pub/pathogens/Schistosoma/mansoni/
http://www.genedb.org/Homepage

9.2 Queries for identification of *E. multilocularis* and *E. granulosus* homologues

9.2.1 Protein sequences

protein	**accession number**	**organism**
adenylate cyclase	XP_393016.3|	*Apis mellifera*
	gi|325296841|	*Aplysia californica*
	XP_002129241.1|	*Ciona intestinalis*
	gi|321455067|	*Daphnia pulex*
	sp|O60503|	*Homo sapiens*
	XP_002424824|	*Pediculus humanus corporis*
aromatic-L-amino-acid decarboxylase	dbj|GAA57180.1|	*Clonorchis sinensis*
	dbj|BAF73419.1|	*Dugesia japonica*
	ref|XP_002573855.1|	*Schistosoma mansoni*
	emb|CCD58963.1|	*Schistosoma mansoni*
serotonin receptor	gb|AAC78396.1|	*Ascaris suum*
	ref|XP_001898000.1|	*Brugia malayi*
	gb|AAC15778.1|	*Caenorhabditis elegans*
	dbj|GAA36232.2|	*Clonorchis sinensis*
	dbj|BAA22404.1|	*Dugesia japonica*
	gb|AAO45883.1|	*Haemonchus contortus*
serotonin transporter	gb|ABI33999.1|	*Cepaea nemoralis*
	dbj|GAA48758.1|	*Clonorchis sinensis*

	gb|ABI34000.1|	*Elliptio dilatata*
	gb|ABI34003.1|	*Lumbricus terrestris*
	gb|ABA39884.1|	*Schistosoma mansoni*
	gb|ABK51389.1|	*Schistosoma mansoni*
	gb|ABA60792.1|	*Schistosoma mansoni*
tryptophan hydroxylase	gb|AAD30115.1|AF135186_1	*Caenorhabditis elegans*
	dbj|BAF79887.1|	*Dugesia japonica*
	ref|XP_003137735.1|	*Loa loa*
	b|AAD01923.1| t	*Schistosoma mansoni*
	gb|AAW25092.1|	*Schistosoma japonicum*
	gb|AEE89450.1|	*Schmidtea mediterranea*

9.2.2 ESTs

EST for	**accession number**	**organism**
EmAC1	SmlC46e10.q1k, SmlC46e10.q1k, SmlC53f09.q1k, SmlC7f05.q1k, gb|CD068436.1|CD068436, gb|CD069474.1|CD069474, gb|CD114417.1|CD114417, gb|CD122712.1|CD122712, gb|CD123010.1|CD123010, gb|CD168576.1|CD168576, gb|CD187557.1|CD187557, gb|CD202220.1|CD202220, gb|CD202828.1|CD202828, gb|CD080971.2|CD080971	*Schistosoma mansoni*
EmAC3	gb|CD069474.1|CD069474, gb|CD202220.1|CD202220	*Schistosoma mansoni*
EmAC4	gb|CD202220.1|CD202220, gb|CD069474.1|CD069474, gb|CD068436.1|CD068436	*Schistosoma mansoni*
EmGPCR3	ECH2-109I16	*Echinococcus multilocularis*

9.3 Transcriptom data sets used for expression calculation

9.3.1 Transcriptom data sets used for expression calculation of genes putatively involved in serotonin pathway and metabolism

name	stage
PC_2d_G8065-774_8_5	primary cells after 2 days culture
PC_11d_G8065-7745_8_6	primary cells after 11 days culture
MetaVesicles_noBC_G8065-7745_8_7	early metacestode vesicles
MetaVesicles_LateBC-5477_3	metacestode vesicles with brood capsules
PS_noact_MS1010-7745_8_4	non-activated protoscoleces
PS_act_MS1010-7745_8_3	activated protoscoleces
EmPreAWDog-5817_2	pre-gravid adults
EmAdultGravide-5817_1	gravid adults

9.3.2 Transcriptom data sets used for expression calculation of adenylate cyclases in *E. multilocularis*

name	stage
ag1w	primary cells
mm_aRNA_1.3	early metacestode vesicles
MetaVesicles_LateBC	metacestode vesicles with brood capsules
psno_1	non-activated protoscoleces
ps_act_1	activated protoscoleces
EmPreAWDog	premature adults
EmAdultGravide	gravide adults

9.4 Protein sequences for phylogenetic analysis of SERT

protein	accession number	organism
A. californica SERT	ref|NP_001191502.1|	*Aplysia californica*
A. mellifera DAT	dbj|BAH22564.1|	*Apis mellifera*
C. elegans DAT	ref|NP_499043.1|	*Caenorhabditis elegans*
C. sinensis SERT part	dbj|GAA48758.1|	*Clonorchis sinensis*
D. melanogaster DAT	gb|AAF57986.2|	*Drosophila melanogaster*
D. melanogaster SERT	gb|AAF47200.1|	*Drosophila melanogaster*
D. rerio DAT	ref|NP_571830.1|	*Danio rerio*
D. rerio SERT	ref|NP_001035061.1|	*Danio rerio*
E. multilocularis SERT	EmW_000391300,	*Echinococcus multilocularis*

	equates EmuJ_000391300	
E. granulosus SERT	deduced from genome and *E. multilocularis* sequence, equates EgrG_00039130	*Echinococcus granulosus*
H. sapiens DAT	ref|NP_001035.1|	*Homo sapiens*
H. sapiens SERT	ref|NP_001036.1|	*Homo sapiens*
M. musculus DAT	emb|CAB51926.1|	*Mus musculus*
M. musculus SERT	gb|AAI08980.1|	*Mus musculus*
S. mansoni DAT	gb|ADM94221.1|	*Schistosoma mansoni*
S. mansoni SERT	gb|ABK51389.1|	*Schistosoma mansoni*
X. tropicalis DAT	ref|XP_002933174.1|	*Xenopus tropicalis*
X. tropicalis SERT	ref|XP_002943421.1|	*Xenopus tropicalis*

9.5 Sequences of adenylate cyclase and GPCRs of the Secretin family

Predicted sequences

protein	**name in prediction**	**prediction**
EgAC1	deduced from genome and E. multilocularis sequence,	
	equates EgrG_000840050	GeneDB
EmAC2	pathogen_EMU_contig_3320|846192-849642	Augustus_AA_old
	|g74.t1|score:0.29|cov:66.7,	by Ference Kiss
	equates EmuJ_000483100	GeneDB
EgAC2	deduced from genome and E. multilocularis sequence,	
	equates EgrG_000483100	GeneDB
EmAC3	pathogen_EMU_scaffold_007780.g9645.t1 AA96-1054,	GeneDB
	equates EmuJ_001004700	GeneDB
EgAC3	deduced from genome and E. multilocularis sequence,	
	equates EgrG_001004700	GeneDB
EmAC4	pathogen_EMU_scaffold_007780.g9623.t1	GeneDB
	equates EmuJ_001002400	GeneDB

***emac1* coding sequence with 5' and 3' UTR**

(start and stop codon marked in gray)

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  1 GGTAGTGCTT GCGTTGGGGC TCTGACGTTC CCTGTTCTAT TTTGTTGGAC TAGAGCGAAT
 61 CATATTTCGA CAAGCTGTCC ACAAACTGCA AGATAATGGG AACGAAATAT CGCGGAATCC
121 TATCATTGGC AAGAAATTTC GATTTCACCT CTTTGAACGG TGCTCTGGTG GAGTATTGAA
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 181 TCTGCGGTTT GATTCTACGG TTCTGGAGAG TTATTATACT CTCTGCTCGT TCCCTCATTC
 241 TTTGTCCAGA TTTCAAATAG CGGTATCATA TTTTGCTCTT GTTTGTGCAA CTTGGATGAT
 301 ATTTTTTGTC ACCTCAGGAA CGGGACAATG GATGATATAC GTTGCTGGAG CTGGCACGGG
 361 TCTTATAGTG GCGATTGTTC TGTTTGCACT TACCTGTTCA AAAGCCGTGT TTGAGAAGAA
 421 CTTCCTTGTT TTGTACATAA TACTAGCATT ATTCTTCAGT TTATTGTTTC TACTTGCATT
 481 TGTTCCTCCA ACCGTCGGAG TCTCTTCGGC GTTTAACGCT AGTCTGATTG TTCAATTGCT
 541 TCTGATAATC TACATCAACG TCCCGCTTCG ACTTTGGCAA GTAATACTTA TTTGTGGACC
 601 TGTCAGCATT ATACACGTGG TGCTGTCGTG TACTGTTTGT GGAAAGATAA ATTCCCGGTT
 661 AACTTGTATA TATGTTCTCT TGCACTGCTG CATTCATATG ATTGGATTCG TACAACATAT
 721 TTTATCACAA GTTCGACGGC GCTCAACATT TATGCGCCTC GGTCACAGTG CTCTGATGCG
 781 CAAAGCCTTG GAGAAGGAGC AGCAGATCCA AAATAAGATG ATCCAGTCCC TAATGCCTCA
 841 GAAGGTCGCG CACGAAGTAA TGCAGGGCTC CTACAACAGC GCAGACGAGG ATGAGGCAAA
 901 CGAAGACGAA GGCGGGAATG AATTGCGAAT GGTTAGTTTT CGCAAGGGAA AGAAGGATGG
 961 AAAAGAGACT AATCATGAGA AGATAAACGC CAAGGGAAGG GGAAGTTCCA GCTTCTCTGA
1021 CGATACGGAT GACTATAGCG CCGATGAAAG TGAGCCCTTG AGAGGAATGG AGGGTAGTAC
1081 ATCCTACAGA GAGCATAGGG GACACAATCC TGATGTGAAA ATTGCGTCTT CGTCCAGCTT
1141 GCCGCGACCC TCAGCCGGAC ATGCTGTGAA GTTTCGTAAA TTCCACGTCA GTCAGATGGA
1201 AAATGTCAGC ATCCTCTTTG CTGATATTGT GGGGTTCACG AATATGAGCT CCAACAAATC
1261 TGCTTCTCAA CTCTTGCTTC TTCTTAATGA TCTCTTTGGA CGGTTCGACA GCCTTTGTGA
1321 GCTGAACCAG TGTGAGAAGA TAGCGACGTT GGGGGACTGC TACTACTGCG TGTCGGGCTG
1381 TCCCAACGCC GTGCCTGACC ATGCAGAGAG AATTGTCGAA ATGGGTCGCT CTATGTGCGT
1441 GGCTATCCAG CAGTTTGACG ATGATCACGC CGAACAGGTG AATATGCGTG TCGGAGTGCA
1501 TACTGGAAAG GTGATTTGTG GATTGGTCGG AACGCGCCGC TTCAAATTCG ACGTCTGGTC
1561 AAACGATGTC ACCATAGCTA ATCAGATGGA GTCTTCAGGT AAGGCAGGTA AAGTGCACAT
1621 CTCCGAAACC ACACTGGAGT TTGTCAAAGA TATCTACGAA GTTTCAGAGG GCGAACCAGT
1681 TCCTGACATT CGGAAAGTAA AAGTATTAAT TGAATATTAC AACAAGGAGG ATCAACGTTA
1741 CGCCATTAAA CATACTCGAG ATCAAGCTCT TATTAAAACC TACTTCATTG AGAGACGTTT
1801 CGACAACAAA CCGATTATCA TGCTAGATAA GGATCAGCCA AAGACGGCGG TAGACCAGAT
1861 CATCGCATCA CCAACGGGAT CAAACGAATT GACCCTCACA ACATCTCCCG GATCTGATGA
1921 ACCGGAGGTG AGGGCTCGTG GTTCCGATGT GGAGATGTTG GACGCTCTGT GGAATCTCTC
1981 CAAGCCGGAA GAGGTCTTCA AATTCCCACC GATTTCACGC TTTTCTCTTT GCTTTCTCTC
2041 ACAATCTCTT GAACAGACCT ACCGGCGGCA GGTGCTTCGT GTTCCCCGTC AGTCTGTCCT
2101 CATCACACTT GCAACACCCC GCCTAACCCC TGTCACCAAT GGGCTGGCTC ACTTCCTCTT
2161 CTTCCTCCTC GTCTCCTTGG CTTGTTTCAT TAACTTTCCC AATTTAACAA ACAGTCGACT
2221 GGCGCTCGTG ACACCCTTTG TCGTGTTTGC ATTGGCTCTC TGTTTTAACT GCCTATTCAT
2281 AGCTATTGTT TTTTCGGATT TGCTAGCATG GGGAGGATTC TGTCTCCCCG AAAAGCGCCT
2341 TCAGCACATC TACCGGTTTC TATTCAACTG GTACACCAGG AACATCATCG GGGTGCTGAT
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2401 GCTCTGTAGC CCTGCGGCCT TTGTTCTCTC CAATTTCCAA ATTTGTCTCT TTTGGTTCAG
2461 TCGATCGGCG GATGTCGCAG CAGTGGCCGA TTTCTCAACT GGAGAATACC GGATGGTTCA
2521 TGGGATCTTG TTCACTTTCA TGCTGTTTAA TTTGACACTG TTTCCAAACT ACTCCTCATG
2581 GACGAAATCT CTCTCGGCAA TGACCTTATG CATCATTGCT TGTTTGTTGA TACATTGGCC
2641 CTTCGTGGGT TTTTCCTATG CGTACTCCGA CCAAGTGGCT GGCTTTGCTG CCGTGACCAC
2701 AGCTAAAGAC TTAAGCACCT CTCCTATCTG GTTGGCTGCT GCTGAAAGTA GCCTATTTCC
2761 ATGGGAAATG ACCGTTATAC TCATCCTGAA TCTCATCCTC ATATTCTTTC TCAACCGCGA
2821 CATCGACATC AGCTTTCGTG TCTCTTTCAA CCGCGACTTT GAGGCGAGCC GTGCCAAGAA
2881 GGCCATTACT CGAGAGAAGA TGCAGGGTGA ATGGTTGCTG GAAAATATCA TTCCACGCTT
2941 TGTGCTTACC GACCTGAGGA AGACCAACAA GTACTCTCAA CACGTGACCG ATGCTGCTGT
3001 TATGTTTGCG TCAATAGCCA ACTTTTCAGA GTTCTACGAT GAGCAGTATC AAGGTGGACA
3061 GGAGATGTTG CGGGTGTTAA ACGAAATTTT TGCAGATTTT GAGCACCTCC TCTCCTCTGT
3121 GAAATTTAAG GATGTGGAGA AGATCAAGAC AATTGCCGAG TGTTTCATGG CCGCTTCCGG
3181 CCTGAATTTG GTTCAGCGGG CGCAGAACAC GTCACTGGAT GATCATTTGT GTGCTCTAAT
3241 GGACTTTGCC ATTGAGCTTC TTAAGACTCT GGATGACTTC AACCGACAAA TGTTCAACTT
3301 TCAGTTTGAG TTGAAGATCG GCTACAATAT AGGAGAGGTG ACAGCGGGTG TCATAGGCAC
3361 GACAAAGTTG CTCTACGATA TTTGGGGCGA CACTGTGAAC GTGGCCAGCC GGATGTATTC
3421 GACAGGTCAG AAAGGTCGGG TGCAGGTGAC TGAGGAGGTG GCGCGACGCC TCACCAAGCA
3481 CTACGAATTT GAGTATCGTG GTAATGTTTT CGTCAAGGGC AAAGGCGAGA TGCGCACCCA
3541 CCTCCTGGTT GGCCAAAAGG CGGGAACCTC GTAAGAAAAC AGGACTTCGT GTTAGGTTAG
3601 TATCATATCC CCCCCTCTCC ATGCCCATTT ATTTATATCG AGGAGCCTCC GGATCGTGCA
3661 TTCATTACGA TCTCCCCCAT TGTGGCCTCT TTATCTTCAT GTCTACGCTC ACGTCTACCT
3721 GTTCTCGTTA ATTTCCTTAC AACTGAATTT GACTCCTCAC CTTATCTACC TTTGTGCTTC
3781 TACTATTATT ATAAGATCAA TTAATTT
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EmAC1

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  1 MAVHKLQDNG NEISRNPIIG KKFRFHLFER CSGGVLNLRF DSTVLESYYT LCSFPHSLSR
 61 FQIAVSYFAL VCATWMIFFV TSGTGQWMIY VAGAGTGLIV AIVLFALTCS KAVFEKNFLV
121 LYIILALFFS LLFLLAFVPP TVGVSSAFNA SLIVQLLLII YINVPLRLWQ VILICGPVSI
181 IHVVLSCTVC GKINSRLTCI YVLLHCCIHM IGFVQHILSQ VRRRSTFMRL GHSALMRKAL
241 EKEQQIQNKM IQSLMPQKVA HEVMQGSYNS ADEDEANEDE GGNELRMVSF RKGKKDGKET
301 NHEKINAKGR GSSSFSDDTD DYSADESEPL RGMEGSTSYR EHRGHNPDVK IASSSSLPRP
361 SAGHAVKFRK FHVSQMENVS ILFADIVGFT NMSSNKSASQ LLLLLNDLFG RFDSLCELNQ
421 CEKIATLGDC YYCVSGCPNA VPDHAERIVE MGRSMCVAIQ QFDDDHAEQV NMRVGVHTGK
481 VICGLVGTRR FKFDVWSNDV TIANQMESSG KAGKVHISET TLEFVKDIYE VSEGEPVPDI
541 RKVKVLIEYY NKEDQRYAIK HTRDQALIKT YFIERRFDNK PIIMLDKDQP KTAVDQIIAS
601 PTGSNELTLT TSPGSDEPEV RARGSDVEML DALWNLSKPE EVFKFPPISR FSLCFLSQSL
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 661 EQTYRRQVLR VPRQSVLITL ATPRLTPVTN GLAHFLFFLL VSLACFINFP NLTNSRLALV
 721 TPFVVFALAL CFNCLFIAIV FSDLLAWGGF CLPEKRLQHI YRFLFNWYTR NIIGVLMLCS
 781 PAAFVLSNFQ ICLFWFSRSA DVAAVADFST GEYRMVHGIL FTFMLFNLTL FPNYSSWTKS
 841 LSAMTLCIIA CLLIHWPFVG FSYAYSDQVA GFAAVTTAKD LSTSPIWLAA AESSLFPWEM
 901 TVILILNLIL IFFLNRDIDI SFRVSFNRDF EASRAKKAIT REKMQGEWLL ENIIPRFVLT
 961 DLRKTNKYSQ HVTDAAVMFA SIANFSEFYD EQYQGGQEML RVLNEIFADF EHLLSSVKFK
1021 DVEKIKTIAE CFMAASGLNL VQRAQNTSLD DHLCALMDFA IELLKTLDDF NRQMFNFQFE
1081 LKIGYNIGEV TAGVIGTTKL LYDIWGDTVN VASRMYSTGQ KGRVQVTEEV ARRLTKHYEF
1141 EYRGNVFVKG KGEMRTHLLV GQKAGTS*
```

***emac2* partial coding sequence with 3' UTR**

(stop codon marked in gray)

```
   1 CTACACACTG TCCTCGCTGA TGTCTATAAT CACTGCATGG ATTCTGGTAG TGTTTGTAGC
  61 TCGGTCCAAC GAGGCCACCT GCCGCCTGCG CTTCTATATG GTTATGGGGG TAGAAAGAGC
 121 TGCTGATACT ACAGCACGAG CGATGCGGGA ATGTGACACC TTCATTAACA ATGTAATTCC
 181 TATGCACGTA GTTAGAAGCC TGCTAGCCGA AGGTGTTCAG ACATTGAATA TCAACTCTGT
 241 CAACCATGCT GCACTCGTTC CACAGGTGGG AATCGCTTTT ATCCGCTTGA CCAACTTCTT
 301 CAACAACTAC TACCGTGAAG ACTATCACAG TGGAAAACAT GCAATTGGAC TGTTGAATCA
 361 AATAATCTGC ATGTTTGATC GACGGCTGCG CAGACCTGAA TACAAAGATG TGGAAAAGCT
 421 GAAGACCCAC AACGACTCAT ACATGGTAGC TGCAGGTTTG GATTTACGTC AGAGGGAGCA
 481 AAACTCGGAT CAGGCAATGC ACTTGTTGAA ATTGCTACGA TTTTGCTATA GTCTTTTCAA
 541 ATTGATCAAA AAGTTCAATG GGAAATTTAT TTTGGGTCAG GATAATGCAT TTGAGCTGGG
 601 TATCGGTGTG GACGTGGGTC CAGTGTGTGC CGGTCTCATT GGTTCTGCGC AGCCCTACTA
 661 TCACGTGATT GGTAGACCAG CAGACATTGC CTATCTTCTG CATCTCACCT CCCCGCCAGG
 721 AAAAATTGCT GTCACTGACA ACGTAAGAGT GGCATTGATT TCTCATTTTC ATTTTGAGGA
 781 GGCTGTATTA CCAAACCCTC CCAGAGTGGA TCAGTCGTAC TACTATTGTG TCTAACTATG
 841 TAATGCCATT TGTTAAATGA ACATCAATCC TATCCCAAAT ATTACCATCC ATTCACTAAA
 901 TCTAGCATGA ATTCATC
```

EmAC2 partial

```
   1 YTLSSLMSII TAWILVVFVA RSNEATCRLR FYMVMGVERA ADTTARAMRE CDTFINNVIP
  61 MHVVRSLLAE GVQTLNINSV NHAALVPQVG IAFIRLTNFF NNYYREDYHS GKHAIGLLNQ
 121 IICMFDRRLR RPEYKDVEKL KTHNDSYMVA AGLDLRQREQ NSDQAMHLLK LLRFCYSLFK
 181 LIKKFNGKFI LGQDNAFELG IGVDVGPVCA GLIGSAQPYY HVIGRPADIA YLLHLTSPPG
 241 KIAVTDNVRV ALISHFHFEE AVLPNPPRVD QSYYYCV*
```

***emgpcr1* coding sequence with 5' and 3' UTR**

(start and stop codon marked in gray)

```
   1 GCGGGGCACA AGGCGATGCC GACAGACAGG TAAACATGGA GTCACCTTCA AACTCGACTG
  61 GAGGCCATTC TGTAGGCCTG GATTCAGTCT GTGGCCAAAT CTTCAAGGAC GTCCTCGCTG
 121 CCATAAAGGA GCAAGGAGGC ACAGAATACT GTCCCGCGGT GTACGACGGC GTGATGTGCT
 181 GGCCTCCTGC ATTATCGGGG ACAACAGTCC GCCTCTCTTG TCCAGCCTTT TTTGAAGGAG
 241 CAACTTACAA TGCATTGTCC AACGCTTCGA GAACTTGTGA AGCCAATGGC ACCTGGGCTA
 301 ACCTGACCGA CTACTCCGCG TGCATATTGA AGGAGGGCCT GACGGACAGA GGTGGAACGC
 361 TCTACCTTCA GGCAATATTC TGCAGTGGTT ATTGTTTCTC CCTCCTCAGC CTCCTCATTG
 421 CGCTTCTAAT TTTTCAGCAT TTCAGATCCC TGCACTGTCT GAGGAATTAC ATCCACGCTC
 481 ATCTAATGGT CACCTTGGTG ATTCGTGTAG TCGCCTGGCT GACTCTCTAT GGCACCACAA
 541 GGCTAGACAC GTTGATTTTG ACCTACCTCT ACAACGCGAC AACATCGGTT CAAGCATGGG
 601 CCAGTCTGGC AATGCTCTGT TGGATGTTTG TAGAAGGTGC CTACTTATTG AATATTGTCT
 661 ACTGGACCTT TCGTCTTCAT CAAGTCCGAA TTTGGCACTA CGCTCTATTT GGATGGGGTA
 721 TTCCGGCGAT TTTCGTCTCC ATTTGGACCA CCATTCACGC GATCTACCAT CCCAACACAC
 781 GGTGGATTGA ACAGAGTCAG GACTTTTACC TTATCTCTCT GCCCTCCATC ATCATTTTGT
 841 CGAGCAACGC CCTCGTCCTC ATCGCCATCA TCTACGCCCT CATCTTCCGA CTGAAAGCAC
 901 CAAAGAACTC TTCACCCCCC TTCCCATCCA ACGACCTGGA AGTGACCAAC ACACGAGGTG
 961 ACAAATCGAG TTGGTTTGAT GCGCGACGAC GACACAGCTC AAATCGTTCA CGCTTTCGAC
1021 TTTCCGCACG GTTCAATAGG TCCGAGTCGA TGAAATCGTT GAAGGCCTGT CTTACGCTGA
1081 TTCCACTATT GGGGATTCCA CAGGTCATTT TTATTGTTCC ATATCATCCT TCAGTTGTAC
1141 AAATCTTCAC CTACGTCAAT GCTGTTGTCA CATCTACTCA GGGTTTCTGG GTTGCTCTCA
1201 TCTACTGTTT TCTGAATGAG GAGGTTCGAT TGCTCCTACG CGGCACTCTG CAGAAGGTAC
1261 TTCTACGAAA ACACCTAAGA AGGCATCGGC AGAGTTGTCA AATATCGGGA CGTCCGCGCC
1321 AACGATCTAC ACTTGATTTA ACCAACAAAT TTGTCATTGT GTCGGGGAGA GGGGCTTCAA
1381 GTACTGGCTC CGATTTATAG ATGGGAGACC TTGAACATGA ACAAGAAATA TATCGAATTT
1441 TCTGATTTGT TGATA
```

EmGPCR1

```
   1 MESPSNSTGG HSVGLDSVCG QIFKDVLAAI KEQGGTEYCP AVYDGVMCWP PALSGTTVRL
  61 SCPAFFEGAT YNALSNASRT CEANGTWANL TDYSACILKE GLTDRGGTLY LQAIFCSGYC
 121 FSLLSLLIAL LIFQHFRSLH CLRNYIHAHL MVTLVIRVVA WLTLYGTTRL DTLILTYLYN
 181 ATTSVQAWAS LAMLCWMFVE GAYLLNIVYW TFRLHQVRIW HYALFGWGIP AIFVSIWTTI
 241 HAIYHPNTRW IEQSQDFYLI SLPSIIILSS NALVLIAIIY ALIFRLKAPK NSSPPFPSND
 301 LEVTNTRGDK SSWFDARRRH SSNRSRFRLS ARFNRSESMK SLKACLTLIP LLGIPQVIFI
 361 VPYHPSVVQI FTYVNAVVTS TQGFWVALIY CFLNEEVRLL LRGTLQKVLL RKHLRRHRQS
 421 CQISGRPRQR STLDLTNKFV IVSGRGASST GSDL*
```

***emgpcr2* coding sequence with 3' UTR**

(start and stop codon marked in gray)

```
   1 ATGCTGGGTT TTCCACTGTT ACTTCTTTTT ACCTTCTCAG CGTGGGGAGA GGACTTCAAG
  61 AGCACCCTCC TAGGCGAGTG CATGAGTGGC GTAGGGTGCT TCTGCGACTA CAGATGCCAA
 121 GAGTTAAACG ACTGTTGTGC CGCTGTAAAC AGCACCCTGT CGCCACTTTC ATCCGAAGAA
 181 GATGCTGTCC CATCAGTCTG CCTGAGTATC GAAAACGTAT TTCTAGAGGA TATGATATTG
 241 CCAAGCGGCG ATTATGTCAG TGCAATCACC CACTGTCCAT CCGAAAAGCC AATTAGTGAA
 301 AAAGTGAAAT TAGGCTGCGA AGAGGCTGAT CTGATGCTGG TTGCTAGCCG GGAGGAGAAC
 361 CTTTTTCAAT GGCTACGAAC ACACGCGCGT TCAGTAGTTC CTGTAGTAAG TTTCCGCAAC
 421 AGGCGACTTT ATGGCAACGT GTTTTGTGCC ATTTGCAATG GACAATCTCA AGATCAAGTT
 481 TACTTCTCAC CAATTCACTT GGGTTGCAGA AAAGAAGGCA ATACAACGGA ATGCCTTGTT
 541 AGCGTAAAAC TTCCAGCGAG TTTGAGTCGG CCTTGCATTC CTCAGCGGTC TAGACTCCTT
 601 AGCCCAAAAG TCAGTTTTCT CAGTATGGAC GATCTCTTCA TCATACCCGA TGAATATCTC
 661 AACAGCCAAT ACATCACTCT TCCTCTCAAT TTTGGCAAAG ATGACAAAAA CAGCAACAGT
 721 ACCAACAGCA ACAAAACACA GGTTTCCAGT GCGTCATCAA ATCCAGTGTA CGATACAGAC
 781 AATAGAAATG CAGTTTACAC TTGGGTTCAA TTAGCACTGC TCATCTTCTC CGTCGTTGGG
 841 CTAACTCTTA TGCTCGTAGT CTACGGTCTG ACTGCCAAAT TGCGGCGATC GTTAGCCGGC
 901 ACCTTGACCA TGGGACTGGG AATAGCACTG CTAATGATGG AGGCGACCTT TTTGATGGTC
 961 GCATTTGCCG TCTCTCATGT GGAAAATCGC GGATTTTGTG TCTTCATGGT GGCGTTGCTC
1021 CTTTACTCGC TCCTCTGTAG TTTCACATGG ATGACGCTCT TTGCTACGCA GCTTCTCCTC
1081 ACTTTCGGAA ACTGTAGAAA CTGCTTTACT ATATGCTGGA AGTTCATCAC TTGTCAATGG
1141 AACAAAAAAA CGTCGACAGC AATATGCTCC CGTGGATACA AGCACAGCAG AGCAAGAAAA
1201 CCATTTCGCA AATACCTATT AGTCGCAACC ATCCTCCCCC TCTGCCTTGT CCTACCGGCC
1261 ACTACCATTA ACGAACATGC CTTCAATCTG CTCTCATCGG CATATTTCAA CCTCTCATCA
1321 GTCTCTCAGC CTTCGACCTT TGACGAAGCC AAAACCGAAG ACGAATTCGC CAGTTTCGAC
1381 ATCACTACTG CGGAAGTCCT TGGAGAGTCA TTGGCCGCCG TCTATCCAGG TTTCTGTCCG
1441 GATGGTGCGC GTTCCTGGTT CACCAACCTC GGAGGGCTGA TCGTTTGGTT CCTTGTGCCT
1501 GCGGGCACCA TGATCACCTT TAACTCTCTC GCTCTCCTTG TGGTCTGTGT CCAAATCTGT
1561 CGCCTTTCTA AGGAAGCACA GTTGCACTCC TCCCCTCAGA ATGAACAGGA GCGGGAACGG
1621 CGCAAAAAGT CAAAAAACCT TGCTGGAATC TGCGCTAGAC TGGCCATTAT CCTCGGCGCC
1681 AGTTGGTTTG CGCAACTTTT TGCTGGATGG TGGTCCCAAC TCTTCGTAAT GCGAAGAATA
1741 CTGGGCCTGG TAAACAGCGC TCAGGGAGGC GTGATAGCAG TATCTATGCT TGCCAGTGTC
1801 AAGGCCAAAC GCGCTCTGGC CAATGTCCTG CCTGAATGCT GCCGAGTCTT AGTTGGTCAT
1861 AATTCCAGCA CACAACAGTC CACGTCGAGA GAAAAAGAGA AACTGACCTC GACCACTTCC
1921 AAGACATGGA CGTCGGTGCT GCTACCGAGA CGAAATCGAC AGCAAATCCA AAGTATCGGA
1981 GAATCAAGCG GTTAGCGAAC TTACATCAGT CTATTTTCCC ATGCACTCAC CATTCTCGCC
2041 ATTAGTAATG GCTCATGATT TATTTATGTT GTGCTAACTG ACTATATTCT CACTATCTGT
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2101 TTCTCGTCCG CATTTCAAAG AGACTTTGAA ATCAAATAAT TGCAAAAATT GTCTCCTTCA
2161 CA
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EmGPCR2

```
  1 MLGFPLLLLF TFSAWGEDFK STLLGECMSG VGCFCDYRCQ ELNDCCAAVN STLSPLSSEE
 61 DAVPSVCLSI ENVFLEDMIL PSGDYVSAIT HCPSEKPISE KVKLGCEEAD LMLVASREEN
121 LFQWLRTHAR SVVPVVSFRN RRLYGNVFCA ICNGQSQDQV YFSPIHLGCR KEGNTTECLV
181 SVKLPASLSR PCIPQRSRLL SPKVSFLSMD DLFIIPDEYL NSQYITLPLN FGKDDKNSNS
241 TNSNKTQVSS ASSNPVYDTD NRNAVYTWVQ LALLIFSVVG LTLMLVVYGL TAKLRRSLAG
301 TLTMGLGIAL LMMEATFLMV AFAVSHVENR GFCVFMVALL LYSLLCSFTW MTLFATQLLL
361 TFGNCRNCFT ICWKFITCQW NKKTSTAICS RGYKHSRARK PFRKYLLVAT ILPLCLVLPA
421 TTINEHAFNL LSSAYFNLSS VSQPSTFDEA KTEDEFASFD ITTAEVLGES LAAVYPGFCP
481 DGARSWFTNL GGLIVWFLVP AGTMITFNSL ALLVVCVQIC RLSKEAQLHS SPQNEQERER
541 RKKSKNLAGI CARLAIILGA SWFAQLFAGW WSQLFVMRRI LGLVNSAQGG VIAVSMLASV
601 KAKRALANVL PECCRVLVGH NSSTQQSTSR EKEKLTSTTS KTWTSVLLPR RNRQQIQSIG
661 ESSG*
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***emgpcr3* coding sequence with 5' and 3' UTR**

(start and stop codon marked in gray)

```
  1 GAAAAATTAG ACACGGTGAC ATTCAAGTAT GTTAATTGCA GTAATTCATA TTGCACTGCT
 61 TCTCGTGCCA CAAGTATGCA GTAAGACAAC GACTTCTGGC GGATCGGCTG GAAGCGGCGA
121 ATTCGGTCTC TTTCGCAGCC GTGACAATAC AAGCACAGGT GAAGCAGAAA AGTATGCAAT
181 GGCCAATCCG AGGTTACAGT GCATTGGAGT CATGCGCGGC CTCCTCCTCA CAGCTGGGAT
241 CCATTTGGAG CCGCGTGTCA TGGCCATTGG TCAGTGTCCG TTAGAAGCAC CGCCCACTTT
301 AGCAACCCTC TGCTCCACCG ACATCCCTGT ATTTCCTCCA GAGGTGGAAT CCGTCAGTAA
361 ACTGCAAAAT CAACTCCTTT CAAAACTTAA GACCGGAAAT AAACGAAACT TAGGCATGCA
421 TTTAAACCTA GCTATTCGAA AAGTTTCTCC GGTTGTCGAC TCCAAATCGA ATGATATATA
481 CGCAAATATA TACTGTGCAC AGTGTCATAA TGTTTCTTCC AGCAAACGGA TAAAACGCCT
541 TCCAAAACGA CTACTTTGTG ACAATCAGGA GGATGCTATT AAATGTTTCG TCCAGGCTGA
601 AATGCCGAAA AATGTAAGCT TCTGTGACTC ATACGCATTA GTCCTACGAT CCTTCTTCAC
661 CTGGGACTCC ATATTCACGC TTCCAGAAGA AGATGAAGCG GATACAGAGA GGTCCTCTAC
721 AAAATCTGTT TTTGAGTTGA TTCAATGGAT CTGCTGCTCC CTCTCAATGA TCGCACTGGT
781 TATCGCCATC TACTTATTTG CCAGCTCGGC GCGATTGAGG AGACCGCTCC CAGGCAAAAT
841 GATGATCGCG CTCTGCTGCA CCCTGCTCAG TGCGCTGGTA GCTTTCCTCC TCGCCAGTGG
901 CGTCATGGAC TTCCTCAGTG CGCCTCTCCG ACCAATGTGC GTTATCTTCG CCTGCCTCCT
961 CCAGCTCCTC CTTCTTGCCG CTTTCGTTTG GATGGCAATT TTTGCCACGG AATTATTACG
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1021 TACCTTCGGT CTTACTCGAG TGTTCCAGCA TGCTTTTCTG TGTTACTGCT GCAGGAAAGT
1081 GAGAGACAAA AACACCAAAG CAATGGGTAT GATGCTTCGC GCGCGTGGGT CAGATCCTAA
1141 CTCCAACAGC CGTTTCAAGC ATCAAATAAT TGTAGCAATT ATACTTCCTC TCTGTTGCAC
1201 CATCCCTACT TTGATCATCA ACGAATACGC CTACCACCGT CTGAAACCGT TGTACGATAC
1261 CAGTGCTAAT AGCAATTGGT CCCTCAGTTC TCAAGAGGCG GAACTTGGGC CTGCTCTATA
1321 CCGCCTCAAT CCTGGCTTCT GCCCCGTCAA TAATCCGAAC TACGCCTGGT TTAAGGGGCA
1381 TCACGTAGGC CTTTTGGTCT GGTTTCTTAT ACCCTCAGGA GTCACGATCT TCTTTAATCT
1441 CTTTGCCCTC ATTATTGTGT GCATCCAAAT CTGCCGACTA AAACGGGAGA CAGGACTGTC
1501 GCCGAGCGCT GGAAGCCCAC AGATCGATGC CCACACGAAG CCCTCCAAGT CTATCGTCGC
1561 AGTATGTACG AAACTGGCGG TCATTCTGGG CGCCAGTTGG TCTATCCAAC TTCTCGCGGG
1621 TCTGAGTCCC CAACTGGAGC TGCTGAGGCA GTTGGCGGGT CTAGTGAACA GCGCTCAGGG
1681 CGGCATAATT GCGGTCTCGA TGCTAGCGAG CTCGAAGGCT CGGCGCGTGA TGGCGCAGAG
1741 GCTCCCCGCA AAGTGCCGCA AGGCCCTTGG CGTCAGCGAG TCTGCTTCAC TCAGCATCCA
1801 GGAAATGACC ACCTCCGCCA AATCCAGGTC ACGACTCACG GGCCAAATCA ACTCGCAGAC
1861 TACCAGCCTT CTCCAAAGTG ATGGCTCCTG AAAACCTTAT ACTCACGGAT TGTGTGCATT
1921 GAATTAATGG AGAATCAGTC TTAAATCTTT ATCCGCGGGA TTGTTTTTAT GTACAATAAC
1981 TATCATTGAA ATAGTGCTGT TA
```

EmGPCR3

```
  1 MLIAVIHIAL LLVPQVCSKT TTSGGSAGSG EFGLFRSRDN TSTGEAEKYA MANPRLQCIG
 61 VMRGLLLTAG IHLEPRVMAI GQCPLEAPPT LATLCSTDIP VFPPEVESVS KLQNQLLSKL
121 KTGNKRNLGM HLNLAIRKVS PVVDSKSNDI YANIYCAQCH NVSSSKRIKR LPKRLLCDNQ
181 EDAIKCFVQA EMPKNVSFCD SYALVLRSFF TWDSIFTLPE EDEADTERSS TKSVFELIQW
241 ICCSLSMIAL VIAIYLFASS ARLRRPLPGK MMIALCCTLL SALVAFLLAS GVMDFLSAPL
301 RPMCVIFACL LQLLLLAAFV WMAIFATELL RTFGLTRVFQ HAFLCYCCRK VRDKNTKAMG
361 MMLRARGSDP NSNSRFKHQI IVAIILPLCC TIPTLIINEY AYHRLKPLYD TSANSNWSLS
421 SQEAELGPAL YRLNPGFCPV NNPNYAWFKG HHVGLLVWFL IPSGVTIFFN LFALIIVCIQ
481 ICRLKRETGL SPSAGSPQID AHTKPSKSIV AVCTKLAVIL GASWSIQLLA GLSPQLELLR
541 QLAGLVNSAQ GGIIAVSMLA SSKARRVMAQ RLPAKCRKAL GVSESASLSI QEMTTSAKSR
601 SRLTGQINSQ TTSLLQSDGS *
```

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Curriculum vitae

Name	Michaela Herz
Oct 2009 - Nov 2014	Clinical Studies of Human Medicine University of Würzburg, Germany MD thesis: Molecular characterization of the serotonin and cAMP-signalling pathways in *Echinococcus* Supervision: Prof. Klaus Brehm (Würzburg, Germany) Prof. Cecilia Fernández (Montevideo, Uruguay)
Dec 2013 - Mar 2014	Internship in Internal Medicine University of Coimbra, Portugal
Jan 2010 - Mar 2013	Studies of Experimental Medicine University of Würzburg, Germany
Oct 2007 - Aug 2009	Preclinical Studies of Human Medicine University of Würzburg, Germany

Würzburg, March 18, 2015