Sequence and expression of four coral G protein-coupled receptors distinct from all classifiable members of the rhodopsin family

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Abstract

A measure of the functional importance of G protein-coupled receptors (GPCRs) as signalling molecules is that over seven hundred have been cloned and identified in the human genome alone. Yet few have been characterized in the lower metazoan phyla, especially in the phylum Cnidaria which is well positioned phylogenetically for tracing the early evolution of GPCRs owing to their possession of the first-evolved nervous systems. We report here the cloning and characterization of four novel rhodopsin-like GPCR cDNAs from the staghorn coral Acropora millepora that share significant similarity with each other but not with the majority of other members of the rhodopsin α subfamily. The deduced proteins lack many of the conserved residues and motifs that form the signature of the different groups of α rhodopsin receptors. Maximum likelihood phylogenetic analysis likewise implies that the coral receptors do not have a simple or close relationship with any of the major groups within the α rhodopsin subfamily. In situ hybridization revealed transcripts in endodermal cells of planula larvae of all ages and in post-settlement polyps. These GPCRs appear to belong to a α rhodopsin-like group unique to corals. Comparisons with other cnidarian GPCRs suggest also that GPCRs diverged early in metazoan evolution.

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Keywords: GPCR; Cnidaria; Anthozoa; Acropora; Orphan receptor

1. Introduction

G protein-coupled receptors (GPCRs) constitute a large superfamily of proteins with seven transmembrane domains that are involved in various forms of cell signalling of great physiological significance to animals and humans (Hamm, 1998). Over 2500 GPCRs have been sequenced, with more being regularly reported from vertebrates, especially mammals. They represent different families of receptors according to ligand class and other criteria (Atwood and Findlay, 1994; Bockert and Pin, 1999; Fredriksson et al., 2003; Fredriksson and Schiöth, 2005). Members of many GPCR families are known from invertebrates, especially those with fully sequenced genomes such as the fruit fly Drosophila melanogaster and the nematode worm Caenorhabditis elegans, thus prompting the view that many GPCR families arose prior to the divergence of the Ecdysozoa from the lineage leading to chordates (Schiöth and Fredriksson, 2005). Little is known about GPCRs in invertebrates more basal than ecdysozoans. Investigating basal metazoans may lead to insights into the early diversification of GPCR structure and function.

Members of the phylum Cnidaria are the morphologically simplest animals with nervous systems and neuroendocrine activities (Mackie, 1990; Leitz, 2001; Anderson, 2004). Although a few anemogenic receptors have been reported in two cnidarians on the basis of radiobinding (Awad and Anctil, 1993a; Hajj-Ali and Anctil, 1997) and cell transduction assays (Awad and Anctil, 1993b; Chung and Spencer, 1991), the use of mammalian classification schemes to identify such receptors in a vastly distant phylum is highly problematic. It is conceivable that cnidarian
receptor proteins may diverge from their mammalian counterparts in structural features and binding configurations to an extent that casts doubt on the validity of receptor identifications based solely on pharmacological classification. Cloning and sequencing cnidian GPCRs may allow us to remove these ambiguities by deducing the structure of these proteins, thus facilitating more reliable comparisons with mammalian and other GPCRs.

Only a few cnidian GPCRs have been identified. One cloned GPCR in the sea anemone *Anthopleura elegantissima* showed structural similarity to the glycoprotein receptor subfamily (Nothacker and Grimelikhuijzen, 1993) whereas another in the sea anemone *Actinia* sp. appeared to be orphan, with no clear relationship with any known GPCR rhodopsin subfamilies (New et al., 2000). More recently two GPCRs showing strong similarity to aminergic receptors were identified in the sea pansy *Renilla koellikeri* (Bouchard et al., 2003, 2004). No ligand has been identified for any of these receptors despite extensive screening of heterologously expressed transcripts in the case of the sea pansy GPCRs (Bouchard et al., 2003, 2004).

Here we report the sequence of four GPCR cDNAs from the staghorn coral *Acropora millepora* that cannot be grouped with any of the known GPCR rhodopsin subfamilies on the basis of sequence and which may therefore represent a new subfamily of orphan receptors specific to Cnidaria. We also describe their expression in the endoderm of larvae and newly settled polyps of the staghorn coral.

Fig. 1. Multiple sequence alignment of C014-D2, TM7-1-6, C007-H6 and A007-G11. Protein sequences were aligned with the DNASTAR Lasergene MegAlign program according to the ClustalW method. Dark shaded segments represent identity or conservative changes shared by all eight sequences, while lighter shades show high similarity shared by only some sequences. The predicted transmembrane regions TMI–TMVII are indicated by open boxes, and intracellular (I) and extracellular (E) loops are also marked. The positions of the consensus residues for Class A (rhodopsin family) GPCRs according to the position-identifier nomenclature (Shi and Javitch, 2002) are shown by asterisks. Positions for consensus residues identifying aminergic (open squares), adenosine (diamonds) and melanocortin (filled squares) receptors are also marked (see text for further details). The receptor sequences have been submitted to GenBank and assigned accession numbers DQ885472 (A007-G11), DQ885473 (C007-H6), DQ885474 (C014-D2) and DQ885475 (TM7-1-6).
2. Materials and methods

2.1. cDNA isolation

Sequences A007-G11, C007-H6 and C014-D2 were selected from an Acropora millepora EST library (see Technau et al., 2005 and Kortschak et al., 2003 for details) on the basis of their apparent similarity with aminegic GPCRs as revealed by BLAST searches. A007-G11 and C007-H6 were sequenced using vector and internal primers and contain complete open reading frames. Sequencing of C014-D2 revealed that the open reading frame was truncated at the 5′ end. A cDNA library was screened with the C014-D2 insert using standard techniques (Sambrook and Russell, 2001) in an attempt to isolate a full length version of this GPCR cDNA. Five clones were isolated, three of which matched C014-D2, but failed to provide any additional sequence, and two of which were derived from a related GPCR transcript named AmTM7-1-6. Details of library construction and clone isolation have been published previously (Brower et al., 1997).

2.2. RACE

In order to obtain the full open reading frame of the transcript corresponding to C014-D2, a 5′ RACE using the SMART kit (BD Biosciences) was performed with the primer 5′-TGCCAGACTAAGAACAGAAGCGGTGG-3′ designed from the original cloned sequence C014-D2 using the following conditions: 5 cycles of 94 °C for 5 s, 72 °C for 3 min; 5 cycles of 94 °C for 5 s, 70 °C for 10 s, 72 °C for 3 min; 25 cycles of 94 °C for 5 s, 68 °C for 10 s, 72 °C for 3 min. The PCR products were purified from agarose gels and ligated into pGEM-T Easy (Promega).

2.3. DNA sequencing and analysis

Sequencing was performed using Big Dye Terminator v. 3.1 (Applied BioSystems). Reactions were run on an ABI 3730 sequencer at the Biomolecular Resource Facility (JCSMR, ANU). DNA sequence analysis was performed using MacVector 7.2.2 (Applied BioSystems). Reactions were run on an ABI 3730 sequencer at the Biomolecular Resource Facility (JCSMR, ANU). DNA sequencing and analysis were performed using the maximum likelihood method. The amino and carboxy ends of the sequences could not be unambiguously aligned, and were removed to leave 349 alignment positions spanning the carboxy and amino acids of the sequences could not be unambiguously aligned, and were removed to leave 349 alignment positions spanning the C014-D2 and C007-H6 sequences. Amino acid alignments were then performed using ClustalW and GeneDoc software.

2.4. In situ hybridization

Embryos, planula larvae and postmetamorphic specimens of Acropora millepora were fixed for 10–20 min in 3.7% formaldehyde in Millipore-filtered (0.22 μm) sea water buffer to pH 8 with HEPES buffer. After repeated rinses in buffered sea water, they were dehydrated through a graded series of methanol and stored at −20 °C in absolute methanol until used.

Prior to the hybridization procedure the specimens were dehydrated to PBS containing 0.1% Triton-X-100 (PBS-T). Lipids were removed by treating specimens in the RIPA detergent cocktail (Rosen and Beddington, 1993) overnight at 4 °C, followed by rinses in PBS-T, dehydration in a graded ethanol series to absolute ethanol and clearing in xylene for 2 h. After dehydration in PBS-T, whole-mount hybridization proceeded as described by Kucharsky et al. (2000), including probe production. The DIG-labelled antisense probes corresponded to the entire length of the cDNA inserts. Hybridization was carried out at 55 °C for 72 h. DIG labelling was detected with an alkaline phosphatase-labelled anti-DIG antibody (Roche) diluted 1:2000 in PBS-T. Either BCIP/NBT (Vector) or BM Purple (Roche) was used as substrate.

The stained preparations were dehydrated through a graded series of glycerol/PBS mixtures and mounted in 90% glycerol. DAPI-stained preparations were processed using Adobe Photoshop. The stained preparations were dehydrated through a graded series of glycerol/PBS mixtures and mounted in 90% glycerol. DAPI-stained preparations were processed using Adobe Photoshop. The stained preparations were dehydrated through a graded series of glycerol/PBS mixtures and mounted in 90% glycerol. DAPI-stained preparations were processed using Adobe Photoshop.

Table 1

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The percentages correspond to the transmembrane regions.
3. Results

3.1. Characterization of cloned receptors

We obtained the complete ORF of four DNA sequences encoding GPCRs. Sequence analysis revealed a 1014-bp ORF encoding 338 amino-acid (aa) residues for A007-G11, 924 bp and 308 aa for C007-H6, 981 bp and 327 aa for C014-D2, and 1077 bp and 377 aa for TM7-1-6 (Fig. 1). All four deduced translations include the seven membrane-spanning segments typically present in GPCRs as revealed by hydrophobicity analysis (Tusnady and Simon, 2001). The N-terminal segment as well as the first intracellular loop and the first extracellular loop is especially short in all four receptors (Fig. 1).

The 4 sequences possess for the most part the attributes of GPCRs belonging to the rhodopsin α subfamily in the GRAFS classification scheme (Fredriksson et al., 2003) such as the conserved motif NPxxY in TMVII (Fig. 1, bracket) which appears to be involved in ligand-induced receptor internalization (Kurten, 2003). They share consensus TM residues that are also highly conserved in these receptors: N1.50, D2.50, R3.50, W4.50, W6.50, and P7.50, with the exception of P5.50 (Fig. 1, asterisks), according to the indexation method for positioning key residues (Ballesteros and Weinstein, 1995; Shi and Javitch, 2002). On the other hand, they depart from the highly conserved DRY motif at the cytoplasmic end of TMIII, which is considered important for G protein coupling (Dixon et al., 1987; Fraser, 1989), by substituting a phenylalanine (C014-D2, C007-H6 and TM7-1-6) or a leucine (A007-G11) for the tyrosine (DRF or DRL). In addition, the cysteine residues of the first and second extracellular loops, which are typically involved in stabilizing receptor conformation through disulfide bridges (Dixon et al., 1987; Fraser, 1989), are lacking in the A. millepora receptors. Another motif of interest is the consensus N-glycosylation site in the short N-terminal (Fig. 1, boxes) of all receptors except for C007-H6 where none is found.

Table 1 shows identity and similarity scores for the entire sequences of the A. millepora cloned receptors and some of the sequences of the other receptors belonging to various groups of the rhodopsin α subfamily. Alignment was performed according to the ClustalW algorithm and the tree was constructed using the Molephy implementation of the maximum likelihood method. Numbers on nodes indicate the percentage of 1000 bootstrap replicates supporting the topology shown. Ren1 was used as outgroup representative. The coral clade is shaded. Aj = Artibeus jamaicensis (Mammalia); Am = Acropora millepora (Cnidaria); Asm = Astetina miniata (Echinodermata); Bf = Branchiostoma floridae (Cephalochordata); Bl = Branchiostoma lanceolatum (Cephalochordata); Cc = Cyprinus carpio (Pisces); Cf = Canis familiaris (Mammalia); Cp = Cavia porcellus (Mammalia); Dr = Danio rerio (Pisces); Gg = Gallus gallus (Aves); Hs = Homo sapiens (Mammalia); Ma = Myzopoda aurita (Mammalia); Mc = Mus masculus (Mammalia); Om = Oncorhynchus mykiss (Pisces); Rk = Rentilla koellikeri (Cnidaria); Sp = Stronglylocentrotus purpuratus (Echinodermata); Tg = Taricha granulosa (Amphibia); Tn = Tetraodon nigroviridis (Pisces).
their closest relatives (see below). In the more conserved TM segments there is 71% similarity (50% identity) between C007-H6 and TM7-1-6, 55% (31%) between C007-H6 and A007-G11, and 55% (27%) between TM7-1-6 and A007-G11. There is 55% similarity (27% identity) between C014-D2 and A007-G11, 75% (53%) between C014-D2 and C007-D5, and 93% (84%) between C014-D2 and TM7-1-6. As shown also in Table 1, C014-D2 and TM7-1-6 appear very closely related to each other, as expected from the cloning of TM7-1-6 during a screen with the C014-D2 cDNA, and to C007-H6. Only A007-G11 stands apart from the 3 others.

BLAST searches produced relatively low-score matches with a varying mixture of adenosine, melanocortin/EDG and aminergic receptor sequences (Table 1). However, none of the 4 coral receptors possesses any of the key residues for aminergic ligand binding (Shi and Javitch, 2002), such as D6.51, S5.42, S5.43, S5.46, (F/Y)6.51, p6.52, N6.55, N7.39 and W7.40 (Fig. 1, open squares). Similarly, the key residues for adenosine binding (Fredholm et al., 2001) are lacking, e.g. E1.39, H6.52, (T/S)7.42 and H7.43, with the single exception of T7.46 (Fig. 1, diamonds). Some residues that appear to be important for ligand binding by melanocortin receptors (Schiöth and Fredriksson, 2005) are also lacking, e.g. F1.39, C2.44 and E3.29, while others such as (I/V)1.57, I3.34, S3.35 and F5.42, are present (Fig. 1, filled squares). While these receptors are not readily classifiable on the basis of structure and putative ligand class, they appear to be closer to the melanocortin/EDG/cannabinoid/adenosine (MECA) cluster than to other clusters within the rhodopsin α subfamily (Fredriksson et al., 2003; Schiöth and Fredriksson, 2005) as suggested by their recurrent

![Fig. 3. Expression of A007-G11 at various developmental stages of *A. millepora* as revealed by whole-mount in situ hybridization.](image)

(A) Expression is seen in the endoderm (en) of an early planula larva, while the ectoderm (ec) is devoid of staining. Short arrows mark the mesogleal boundary between ectoderm and endoderm here and in B. (B) Expression is associated with a granular appearance, due to the staining of scattered small cells, in the endoderm (en) of a later stage planula. (C) Flattened disc stage of an early post-settlement juvenile with expression in the endoderm (en), but not in the ectoderm (ec) of the body wall or the calciblastic ectoderm joining the mesenteries. (D) Later post-settlement stage (oral view of a primary polyp rising from the basal disc) showing stained cells located in the endoderm (en) of the flattened disc and lining the body wall of the polyp.

![Fig. 4. Expression of C014-D2 in *A. millepora*.](image)

(A) Planula showing expression in the endoderm. Arrows mark the mesogleal boundary between ectoderm (ec) and endoderm (en). (B) Post-settlement flattened disc displaying stained cells in the endoderm (en) which lines each of the cavities of which the body is composed. There are no staining cells in the ectoderm (ec) which forms the outer wall of the polyp or in the calciblastic ectoderm which abuts the mesenteries. (C) Expression in endodermal cells as in B, at higher magnification. Staining cells do not appear to be randomly distributed, but tend to occur in clusters and lines (arrows). Scale bar=50 μm. (D) A tentacle bud from a juvenile polyp to show the granular pear-shaped or elongated cells (dark blue) present in the endoderm (en) but not in the adjoining ectoderm (ec). Scale bar=40 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
homologies with these receptors and their possession of some key residues for melanocortin ligand binding.

In ML phylogenetic analyses (Fig. 2), the four A. millepora GPCRs form a distinct clade that is well supported and resolved from both the amergic receptor and MECA GPCR clades. The adenosine receptor clade is the sister group to that consisting of the coral GPCRs, but this relationship has only relatively weak bootstrap support. On this basis, the novel coral GPCRs appear to constitute a distinct group within the rhodopsin α subfamily of GPCRs.

3.2. Distribution and cellular localization of transcripts

In situ hybridization revealed that all four GPCR transcripts are expressed similarly in the endoderm of larval and post-settlement polyps. The following description is based on the A007-G11 and C014-D2 transcripts because of their stronger signals. For a description of embryonic development of A. millepora, see Ball et al. (2002).

A007-G11 expression is first observed throughout the endoderm as the larva elongates (Fig. 3A). At the swimming planula stage expression in the endoderm appears stronger (Fig. 3B). As the planula transforms into a flattened disc during settlement, the gene continues to be expressed in cells scattered throughout the endoderm (Fig. 3C). As the primary polyp rises from the basal disc, A007-G11 expression can be seen in the endoderm that lines the polyp wall, but not in ectoderm that forms the outer wall of the polyp or the calicoblastic ectoderm which abuts the mesenteries (Fig. 3D).

Expression of C014-D2 is also observed in the endoderm of the planula stage (Fig. 4A). The staining in the planula is mainly associated with round or ovoid cells of 4–6 μm diameter, the staining intensity of which varies widely. In newly settled animals, which have the shape of flattened discs, the endoderm lining the gastrovascular cavity contains abundant stained cells (Fig. 4B). In shape these cells (Fig. 4C) resemble those seen in planula larvae (Fig. 4A), but they are larger (8–10 μm). At the juvenile polyp stage numerous cells in the endoderm of the budding tentacles express C014-D2 (Fig. 4D), whereas the ectoderm of both the oral disc and tentacles is devoid of transcript. The stained endodermal cells are largely pear- or club-shaped, are located in an epithelium adjoining the ectoderm and their content appears granular (Fig. 4D).

4. Discussion

4.1. The coral GPCRs form a distinctive group within the rhodopsin family

The sequence alignment and in situ hybridization results in the present study show that C014-D2, TM7-1-6, C007-H6 and A007-G11 represent a separate group of related 7-transmembrane receptors belonging to a rhodopsin subfamily of GPCRs and expressed in endodermal cells. They add to the four GPCRs previously cloned from other cnidarians, two from sea anemones (Nothacker and Grimmelikhuijzen, 1993; New et al., 2000), and two others from the sea pansy (Bouchard et al., 2003, 2004).

The multiple alignment analysis (Fig. 1, Table 1) clearly shows that the four receptors are structurally related, although A007-G11 displays the lowest identity with the others. While they possess nearly all the defining residues of the rhodopsin family of GPCRs, the DRY motif at the TMIII/I2 transition and the cysteine residues bridging TMIII and E2 are not conserved in the A. millepora receptors. These discrepancies are shared with Ren2 of the sea pansy (Bouchard et al., 2004). The DRF and DRL motifs of the coral, as well as the DRC motif of Ren2, have in common that tyrosine is substituted by hydrophobic residues. A recently described crustacean serotonin-like receptor with a DRF motif does display agonist independent activity in HEK cells (Clark et al., 2004). Whether C014-D2, C007-H6 and TM7-1-6 possess constitutive activity remains to be determined by functional expression studies. However, the observation that Ren2 expressed in HEK293 and LTK cells shows no constitutive activity (Bouchard et al., 2004) in contrast to Ren1, which has conserved the DRY motif (Bouchard et al., 2003), makes a direct link between DRY substitutions and constitutive activity highly unlikely. It is known that other residues interact with DRY to determine constitutive activity (Parnot et al., 2002). Shapiro et al. (2002) showed that agonist-induced activity was dependent on disruption of a strong ionic interaction between the arginine residue of DRY and a glutamate residue at the cytoplasmic end of TM6, and that substitutions of these residues led to constitutive activity. As all four coral GPCRs have conserved both residues (Fig. 1), it is highly unlikely that agonist independent activity occurs at these receptors on this basis.

The extremely short E2 and the concomitant lack in the coral receptors of the highly conserved cysteines in E2/TMIII and E2 are also shared with Ren2 (Bouchard et al., 2004). Because the disulfide bond was demonstrated to be critically important for the stabilization of the active receptor and to be physically close to the binding site of most GPCRs (Shi and Javitch, 2002; Karnik et al., 2003), its absence in all four coral receptors as well as in Ren2 and in the Actinia sp. GPCR (New et al., 2000), is highly significant. It was noted that the majority of GPCRs classified as putative/orphan lack this disulfide bond (Rader et al., 2004). Whether this means that these coral receptors are incapable of accommodating ligand interactions (and therefore are true orphans) or that alternative receptor interactions substitute for the disulfide bond (Karnik et al., 2003), thereby allowing ligand binding, remains to be determined. The disulfide bond is missing in six out of the eight known cnidarian GPCRs indicating that it is not a conserved character in this phylum.

The coral receptor sequences show greater identity between themselves than with their closest matches in GenBank (Table 1). These GenBank matches belong to several groups of the rhodopsin α subfamily, but the consensus residues of these groups that are important for specific ligand binding are absent in the coral GPCR sequences except for a few consensus sites shared with melanocortin/EDG and adenosine members of the MECA subfamily. These findings suggest that coral GPCRs constitute a distinct group within the rhodopsin α subfamily and that they cannot be readily clustered with any other group on the basis of...
ligand class (Fig. 2). The apparent relative closeness of the coral sequences to MECA sequences rather than to aminergic receptor sequences may be related to the observation that, like the coral receptors, all of the cannabinoid and EDG members of the MECA receptors within the rhodopsin α subfamily of GPCRs lack the disulfide bond (Rader et al., 2004). This shared feature further differentiates these receptors from the rest of the large rhodopsin family.

That these coral GPCRs constitute a distinct group within the rhodopsin family is also supported by examination of the Nematostella EST database (http://www.stellabase.org; see Sullivan et al., 2006) in which many contig sequences display significant identity with the coral GPCRs. BLAST searches against these contigs yielded a mixture of hits (aminergic/adenosine/melanocortin) reminiscent of those obtained with the coral GPCRs (not shown). This is consistent with the existence of a distinct group of α rhodopsin GPCRs to which these anthozoan GPCRs belong. Because coral GPCR structures provide few clues about the identity of their natural ligands, and because cnidarian GPCRs remain orphans even when receptor structure provides seemingly unequivocal clues (Bouchard et al., 2003, 2004), new classes of GPCR ligands, possibly derived from currently unknown ligand classes, are likely to emerge in corals in the future. This situation is reminiscent of the Acropora nuclear receptors (Grasso et al., 2001), which also all lack known ligands, seemingly indicating that there is still a great deal to learn about molecular interactions in the Cnidaria.

4.2. The coral GPCR transcripts are present in specific endodermal cells

Transcripts from the four GPCR genes were found abundantly and exclusively in the endoderm at larval stages and in post-settlement juveniles (Figs. 3 and 4), suggesting that these coral receptors are involved in endoderm-specific physiological processes throughout coral development. A large number of the endodermal cells appear to be yolk cells, but other, poorly characterized cell types are scattered among them (Ball et al., 2002). The cells expressing the GPCRs in planula larvae and in early post-settlement juveniles appear to represent one morphological type of these cells interspersed among large, unstained yolk cells. In contrast, the expressing cells in the endodermal epithelium of tentacle buds of primary polyps are morphologically distinct from those of earlier developmental stages, perhaps correlated with a change in the functional role of these GPCRs. While this role cannot precisely be known at this time, the granular texture of the stained endodermal cells of tentacle buds resembles that observed in gland cells of anthozoans (Fautin and Mariscal, 1991).

4.3. Evolutionary implications

The A. millepora GPCRs stand out by their unorthodox sequence features which they share with a very small minority of the several thousand known GPCRs (Karnik et al., 2003; Rader et al., 2004). It is becoming increasingly clear that the genomes of anthozoans are more similar to the genomes of higher metazoans than are those of members of other cnidarian classes and may therefore more closely reflect the genome of Uribilateria (Bridge et al., 1992; Collins, 2002; Kortschak et al., 2003; Ball et al., 2004). Thus the observation that six out of the eight characterized cnidarian GPCRs lack key conserved features implies that the latter were unimportant for the early evolved receptors and that the dominance of the so-called conserved features of GPCRs emerged later during metazoan evolution.

The apparent association of the coral GPCR transcripts with endodermal cells of larvae and post-settling juveniles is the first evidence that GPCRs of the large rhodopsin family may play a role in cnidarian developmental processes. A Frizzled gene, which codes for a protein belonging to a class of heptahelicoidal receptors and has the signalling factor Wnt as ligand, was recently identified in a sponge (Adell et al., 2003) as well as in the cnidarians Hydra vulgaris (Minobe et al., 2000) and Hydractinia echinata (Teo et al., 2006). In both cnidarians the transcripts were localized in the endoderm as are the GPCRs in A. millepora, and in the planula and postmetamorphic stages of H. echinata the signal was traced to interstitial stem cells (Teo et al., 2006). Together with these findings our results point to the early emergence of the role of heptahelicoidal receptors in developmental signalling during metazoan evolution.

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References


Clark, M.C., et al., 2004. Arthropod 5-HT2 receptors: a neurohormonal receptor in decapod crustaceans that displays agonist independent activity resulting from an evolutionary alteration to the DRY motif. J. Neurosci. 24, 3421–3435.


