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Revisiting the evolution of Family B1 GPCRs and ligands: Insights from mollusca

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ABSTRACT

Family B1 G protein-coupled receptors (GPCRs) are one of the most well studied neuropeptide receptor families since they play a central role in many biological processes including endocrine, gastrointestinal, cardiovascular and reproduction in animals. The genes for these receptors emerged from a common ancestral gene in bilaterian genomes and evolved via gene/genome duplications and deletions in vertebrate and invertebrate genomes. Their existence and function have mostly been characterized in vertebrates and few studies exist in invertebrate species. Recently, an increased interest in molluscs, means a series of genomes have become available, and since they are less modified than insect and nematode genomes, they are ideal to explore the origin and evolution of neuropeptide gene families. This review provides an overview of Family B1 GPCRs and their peptide ligands and incorporates new data obtained from Mollusca genomes and taking a comparative approach challenges existing models on their origin and evolution.

1. Introduction

The G protein-coupled receptors (GPCRs) are a large superfamily of receptors that govern a large number of physiological processes in both vertebrate and invertebrate organisms. It is one of the largest and oldest families of receptors found in living organisms, which transmit signals from the outside of cells to the cytosol and trigger a response (Bockaert and Pin, 1999; Kroeze et al., 2003; Liu et al., 2021; Maudsley et al., 2005). These receptors exist exclusively in eukaryotes but are suggested to have evolved from the prokaryote sodium-translocating rhodopsin's, with which they share structural but low sequence similarity and they play a crucial role in a wide range of physiological processes from fungi (unicellular) up to multicellular organisms (Isom and Dohlman, 2015; King et al., 2003; Shalaeva et al., 2015). Although it should be noted their existence in plants is still controversial (Chakraborty and Raghuram, 2023; Taddese et al., 2014). Their endogenous ligands, like the receptors, are extremely diverse and include amino acids, small and large peptides, fatty acids, ions, steroids and even light and gasses. Their ancient origin and wide distribution in living organisms means they regulate a vast diversity of actions including cell growth and survival, metabolism, immunity, the stress response, cell adhesion as well as vision, olfaction, taste and cell proliferation in diverse pathologies (Ahmad and Dalziel, 2020; Lämmermann and Kastenmüller, 2019; Lappano and Maggiolini, 2012; Ridge and Palczewski, 2007; Sloop et al., 2018; Spehr and Munger, 2009).

GPCRs are structurally conserved receptors, and they are characterized by the presence of seven highly conserved transmembrane α-helix domains that anchor the receptor protein to the cell membrane. Of the two terminal regions, the extracellular N-terminus normally binds the ligand, and the intracellular C-terminus is involved in the activation of a heterotrimeric G proteins complex, which is responsible for the amplification and activation of signalling pathways that mediate the cellular response (Schiöth and Lagerström, 2008) (Fig. 1). The seven transmembrane domains of the receptor are interconnected by three short extracellular (ECL) and intracellular (ICL) loops, which maintain the conformation of the receptor and participate in signalling. The heterotrimeric G-protein complex is comprised of three protein subunits α, β and γ. The β- and γ-subunits are a membrane associated heterodimer and with α-subunit are involved in the recognition of effector molecules, which generate secondary messengers (e.g., cAMP, IP3) that trigger distinct signalling cascades (Rosenbaum et al., 2009; Strader et al., 1994) (Fig. 1).

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Abbreviations

GPCR	G-protein coupled receptors
CRH	Corticotrophin Releasing Hormone
SCT	Secretin
VIP	Vasoactive Intestinal Peptide
PACAP	Pituitary Adenylate Cyclase-Activating Polypeptide
PAC_1	PACAP receptor
VPAC	VIP receptor
GHRH	Growth Hormone Releasing Hormone
GCG	Glucagon
GLP	Glucagon-Like Peptide
GIP	Glucose Insulinotropic Peptide
PTH	Parathyroid Hormone
iPTH	invertebrate Parathyroid Hormone
iPTHR	iPTH receptor
CALC	Calcitonin
CALCR	CALC receptor
CGRP	Calcitonin Gene-Related Peptide
PDF	Pigment Dispersing Factor
PDFR	PDF receptor
DH44	Diuretic Hormone 44
DH44R	DH44 receptor
DH31	Diuretic Hormone 31
DH31R	DH31 receptor
GPS-group	GCGR, PTHR and SCTR/PAC1/VPAC group

In the human genome, approximately 800 GPCRs have been identified, while in the teleost model, the zebrafish (*Danio rerio*), over 700 have been reported (Fredriksson et al., 2003; Fredriksson and Schiöth, 2005). In the nematode worm *Caenorhabditis elegans* GPCRs are predicted to account for over 5% of the entire worm genome and in the fruit-fly *Drosophila melanogaster* there is estimated to be over 200 GPCR members (Caers et al., 2012; Fredriksson and Schiöth, 2005; Frooninckx et al., 2012). In contrast, the yeast genome contains a surprisingly small number of GPCRs – three in *Saccharomyces cerevisiae* and nine in *Schizosaccharomyces pombe* where they are involved in pheromone and glucose pathways (Hoffman, 2005; Versele et al., 2001).

Several classification systems have been used to cluster and name GPCRs. Based on structural similarity, type of activating molecules, and the ability to couple to heterotrimeric G proteins, GPCRs were grouped into six families/classes (from A to F) that include all receptors found in vertebrates and invertebrates (Bockaert and Pin, 1999; Kolakowski, 1994). In the A to F classification, Family A is the rhodopsin-receptor family, Family B is the secretin-receptor family, Family C is the metabotropic glutamate receptor family, Family D is the fungal mating pheromone receptor family, Family E is the cyclic-AMP receptor family and Family F is the Frizzled-receptor family. The secretin-receptor family (B) GPCRs are further grouped into three distinct subfamilies B1, B2 and B3 based on receptor structure and the type of activating ligands. In the case of the secretin-GPCRs they are classified in subfamily B1 (or Family B1) to distinguish them from other Family B members that possess a large N-terminal region with characteristic protein domains (subfamily B2 or Family B2) and from the methuselah-GPCRs (subfamily B3 or Family B3) that are a characteristic of insects (Harmar, 2001). An alternative GPCR classification system exists, which is based on receptor sequence similarity with human GPCRs and five main superfamilies are proposed:

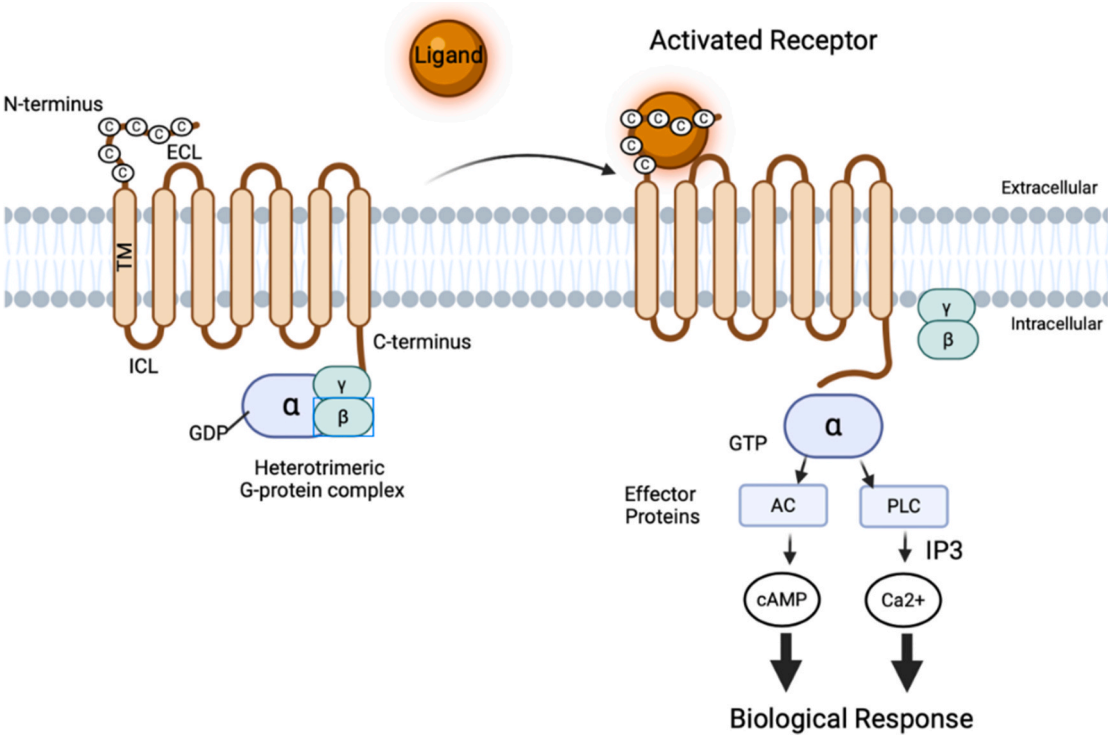


Fig. 1. General structure of a GPCR and activation. GPCRs are composed of seven transmembrane domains (TM) that anchor the receptor to the cell membrane. The N- terminus that interacts with the ligand and the C-terminus end that activates the G-protein heterotrimer complex are indicated. The G-protein heterotrimeric complex is represented by the Gα, Gβ and Gγ subunits and upon receptor activation by the ligand are involved in triggering the intracellular signalling cascade to provide the cells response. The receptor represented is a member of Family B1 GPCRs that are characterized by the presence of six conserved cysteine residues in the N-terminus that are important for the establishment of disulphide bridges critical for ligand binding (Couvineau and Laburthe, 2011; Harmar et al., 2012; Laburthe et al., 1996; Parthier et al., 2007).

Glutamate (G), Rhodopsin (R), Adhesion (A), Frizzled (F), and Secretin (S) and these receptors are collectively known as GRAFS (Fredriksson et al., 2003), and are proposed to have evolved from a common ancestral gene in early metazoans via gene/genome duplication events (Fredriksson et al., 2003) (Fig. 2). The pharmacology and the evolutionary classifications of GPCRs with the two naming systems mirror each other in most cases except for family D and E that are characteristic of Fungi and slime mold, respectively and so do not exist in humans or any other bilaterian and are not contemplated in the GRAFS classification.

The Rhodopsin or Family A GPCRs (which is the most diverse family in humans with more than 700 members) includes receptors for hormones, neurotransmitters and photons and are involved in taste, smell, and the regulation of metabolism, reproduction, and neural function and are targets for the development of drugs (Gaillard et al., 2004; Pathan and Williams, 2012; Satake et al., 2013; Yang et al., 2021). The Secretin or Family B1 GPCRs are only activated by peptide hormones, which regulate brain-gut function, calcium homeostasis and the stress response (Donnelly, 2012; Harmar, 2001; Harmar et al., 2012). The Adhesion receptors or Family B2 GPCRs are involved in cell adhesion, signalling, and immune function and with the exception of GPR56 that is activated by collagen III, most are orphans (Bjarnadóttir et al., 2007; Luo et al., 2012; Yona et al., 2008). The Glutamate or Family C GPCRs are involved in synaptic plasticity and participate in numerous functions within the central nervous system (Niswender and Conn, 2010). Frizzled or Family F GPCRs participate in the Wnt signalling pathway, cell proliferation and the control of embryogenesis (Schulte, 2010; van Amerongen and Nusse, 2009). With the exception of Adhesion GPCRs, all members of this family have been identified in fungal genomes (Krishnan et al., 2012) (Fig. 2).

GPCRs are the main receptors mediating the action of neuropeptide and peptide hormones belong to Family A subfamily β and subfamily γ and to the Family B1 GPCR superfamilies. In humans, at least 80 genes encoding for neuropeptide precursors have been described and these precursors give rise to at least 150 mature neuropeptides, of which 72% signal via GPCRs (Sahbaz and Iyison, 2018). Neuropeptide and peptide hormones are an ancient, widespread and heterogeneous group of signalling molecules involved in neuro-modulation, neuro-transduction, and hormonal functions (De Oliveira et al., 2019; Liu et al., 2008). Given the crucial physiological role of neuropeptide GPCR systems in animal physiology, identification, and functional characterization of neuropeptides and their GPCRs have contributed to increase understanding of neuropeptide-related signal transduction and are a major target for human drug development and for the generation of novel pesticides (Audsley and Down, 2015; Hauser et al., 2017). However, the role of many neuropeptides remains elusive, and many GPCRs remain orphans. Neuropeptide and peptide hormone precursors have been identified and isolated from non-bilaterians up to vertebrates including taxa with no nervous tissue, such as porifera sponges (Yañez-Guerra et al., 2022). At least 30 common neuropeptide signalling systems are shared in protostomes and deuterostomes (Elphick et al., 2018; Jékely, 2013; Mirabeau and Joly, 2013) and neuropeptide-GPCR systems are involved in a diversity of biological functions including feeding and metabolism, locomotion and muscle movement, cognition, and reproduction (Caers et al., 2012; Cardoso et al., 2012; Frooninckx et al., 2012).

1.1. Family B1 GPCR members and their functions in vertebrates

The members of Family B1 are one of the most studied GPCR families (Cary et al., 2023). They represent one of the largest receptor families for hormones and neuropeptides, and are involved in important biological functions, in protostomes and deuterostomes, but not in cnidarian and porifera (Cardoso et al., 2006). Five receptor subfamilies: a) Corticotrophin Releasing Hormone (CRH) receptors; b) Secretin (SCT), Vasoactive Intestinal Peptide (VIP), Pituitary Adenylate Cyclase-Activating

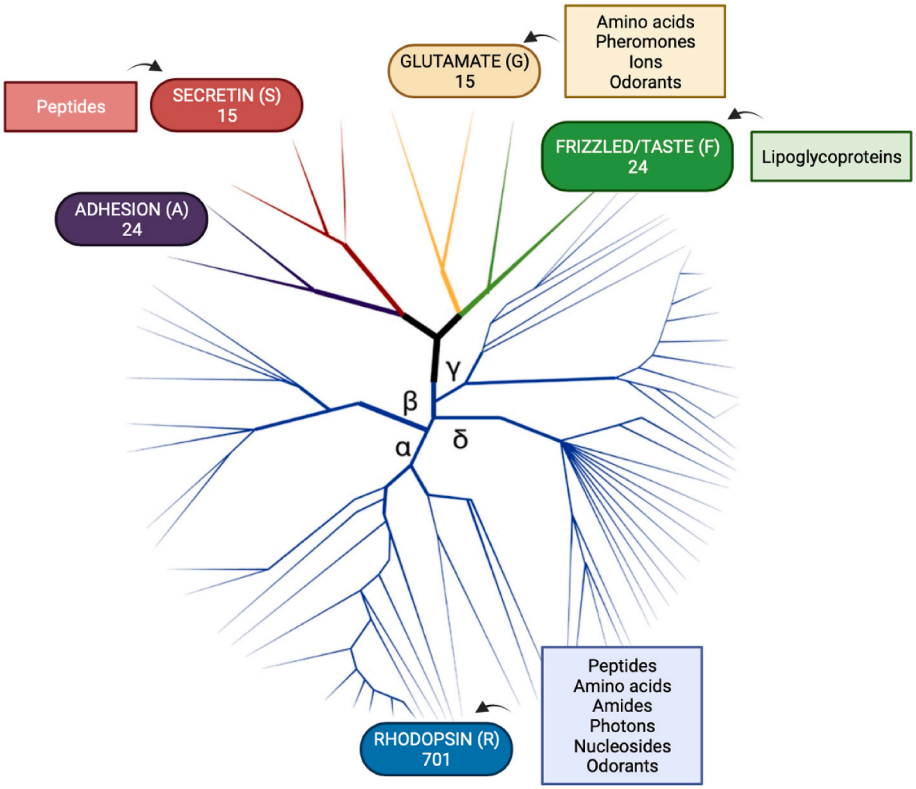


Fig. 2. The GPCR superfamilies in human. The phylogenetic tree represents the evolutionary relationship between the human GRAFS and the number of representatives is indicated within brackets (Fredriksson et al., 2003). Each GPCR superfamily is indicated in a different colour and the type of ligands that activate the members of each superfamily is indicated inside the same-coloured box. Adhesion receptors are mostly orphans. The rhodopsin superfamily contains the greatest number of receptors and is divided into four main sub-branches (α, β, γ, and δ). The figure was adapted from (Cardoso et al., 2012).

Polypeptide (PACAP) and Growth Hormone Releasing Hormone (GHRH) receptors; c) Glucagon (GCG), Glucagon-Like Peptide (GLP), Glucose Insulinotropic Peptide (GIP) receptors; d) Parathyroid Hormone (PTH) and Parathyroid hormone related peptide (PTHrP) receptors and e) Calcitonin (CALC) and Calcitonin Gene-Related Peptide (CGRP) receptors (Donnelly, 1997; Harmar, 2001; Harmar et al., 2012) exist and are activated by moderately large peptides that are members of distinct endocrine peptide families. Signature motifs which characterize Family B1 GPCRs are the presence of a large N-terminal ligand-binding ecto-domain (N-ted) with six conserved cysteines, which establish disulphide bridges and create a cleft to which ligands bind, and several N-glycosylation motifs, which play a crucial role in protein folding (Fig. 1) (Couvineau and Laburthe, 2011; Harmar et al., 2012; Laburthe et al., 1996; Parthier et al., 2007).

In humans, the CRH system is composed of two receptors and four peptide ligands (CRH and three, urocortins: UCN1, UCN2 and UCN3) that are involved in the regulation of stress, emotional behaviour, and anxiety (Cardoso et al., 2014a, 2016; Fox and Lowry, 2013; Koob and Heinrichs, 1999; Lovejoy and Balment, 1999). The members of the SCT, VIP, PACAP and GHRH receptors are the largest receptor subfamily, with 5 receptors, and 4 peptide precursors described in humans. Members of this family are mainly associated with brain-gut function, and are important neuromodulators, regulating the secretion of other peptide hormones from the pituitary, while also contributing to the regulation of the circadian rhythm, differentiation of immune cells and also the regulation of pancreatic and gastric acid secretion (Dickson and Finlayson, 2009; Lam et al., 2008; Sherwood et al., 2000; Vaudry et al., 2000). The GCG, GLP, and GIP receptors are mostly known for their role in controlling glucose and lipid metabolism and satiety. In humans four receptors and four peptides have been described (Hope et al., 2021; Kim and Egan, 2008; Sandoval and D'Alessio, 2015). The members of the PTH and PTHrP receptors and of the CALC and CGRP receptors are involved in the regulation of calcium and phosphorus homeostasis and with skeletal development and turnover, but other functions have also been assigned particularly in the case of CGRP and include cardiovascular homeostasis and nociception (Dobolyi et al., 2012; Felsenfeld and Levine, 2015; Gensure et al., 2005; Naot and Cornish, 2008).

1.2. Family B1 GPCRs in invertebrates

Sequence orthologues of the vertebrate Family B1 GPCRs have been described in invertebrate deuterostomes and protostomes (Ecdysozoans and Lophotrochozoans). This suggests Family B1 GPCRs emerged prior to the protostome-deuterostome divergence although no members have

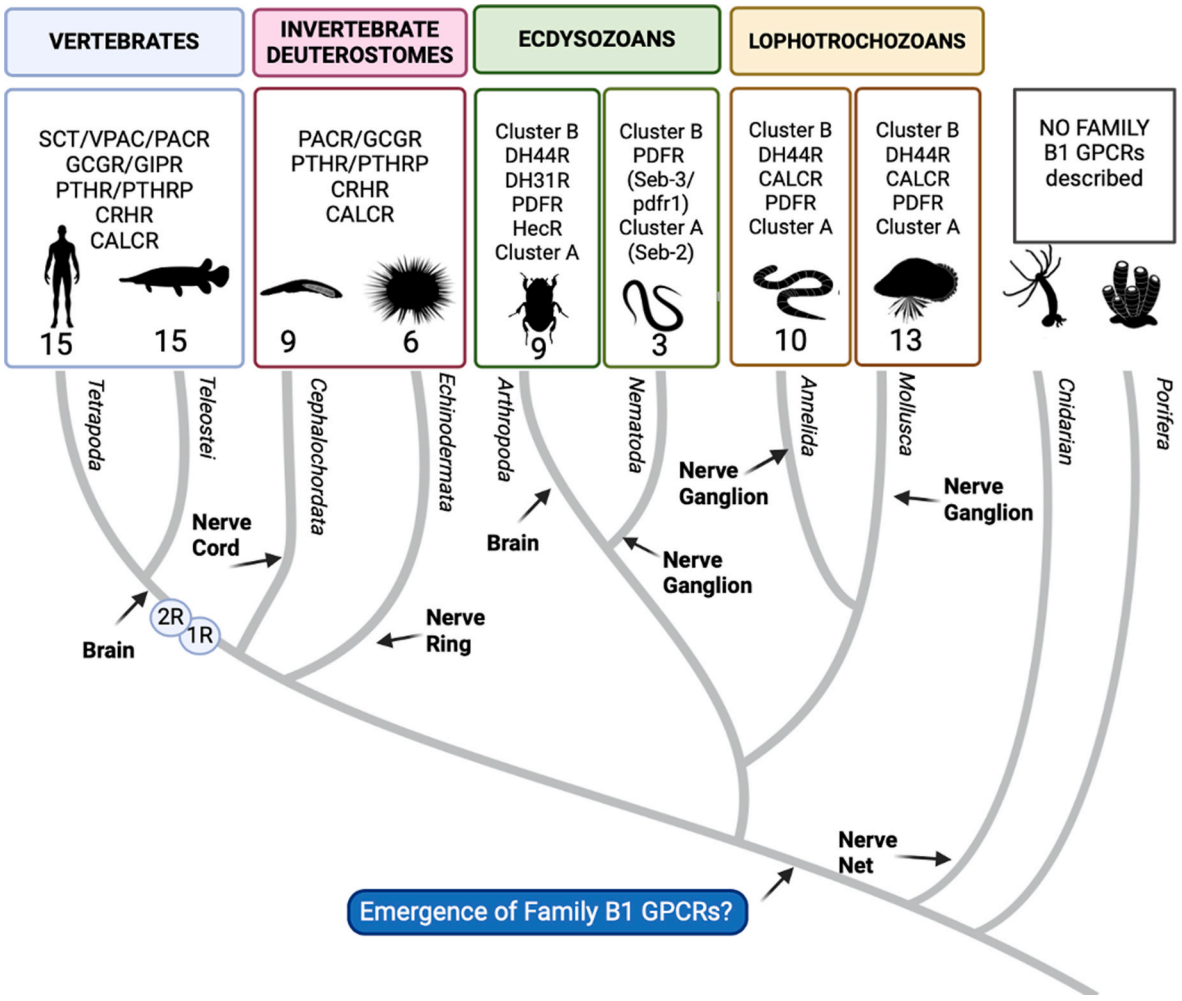


Fig. 3. Family B1 GPCRs in vertebrates and invertebrates. The main events associated with the appearance of the metazoan nervous system are also represented. Receptor data was obtained from (Cardoso et al., 2014b, 2020c; Elphick et al., 2018; Mirabeau and Joly, 2013). The genome duplication events that occurred earlier during the vertebrate radiation are indicated (1R, 2R). No Family B1 GPCRs have been identified in Cnidarians and Porifera. The evolutionary relationship between the main phylum was based on (Brunet and King, 2017). The numbers below the animal silhouettes indicate the predicted total number of Family B1 GPCRs that were previously reported in those species (Cardoso et al., 2014b, 2020b): Vertebrate: Human (*Homo sapiens*) and spotted gar (*Lepisosteus aculeatus*); Invertebrate deuterostomes: Amphioxus (*Branchiostoma floridae*) and sea urchin (*Strongylocentrotus purpuratus*); Ecdysozoans: Arthropod Flour beetle (*Tribolium castaneum*) and Nematode *C. elegans*; Lophotrochozoans: Annelid (*Capitella teleta*) and Mollusca (*Mytilus galloprovincialis*).

yet been described in non-bilaterian cnidarians and porifera (Hauser et al., 2022) (Fig. 3). In the invertebrate deuterostome, the cephalochordate, putative homologues of the vertebrate PAC1/VPAC/GCGRs, PTHR, CALCR and CRFR have been identified (Cardoso et al., 2006, 2014a, 2020c; Jékely, 2013; Mirabeau and Joly, 2013; On et al., 2015, 2019). However, in protostomes such as arthropods and molluscs, the two most diverse invertebrate phyla, only members of CALC/CGRP-like (CALCR-like and Diuretic Hormone 31 (DH31) receptors) and CRF-like receptors (Diuretic Hormone 44 receptors, DH44R) of Family B1 GPCRs have been identified (Cardoso et al., 2006, 2020b; Elphick et al., 2018). Other identified invertebrate Family B1 GPCRs are the Pigment Dispersing Factor receptors (PDFRs), which are suggested to be involved in the regulation of locomotor activity in the nematode *C. elegans* (Janssen et al., 2008; Meelkop et al., 2012) and in the fruit-fly *D. melanogaster* (Li et al., 2022).

Recently, three novel members of the invertebrate Family B1 GPCRs were identified, some of which are absent from the genomes of the most studied protostome models, the nematode *C. elegans* and the insect fruit fly *D. melanogaster*. The novel GPCRs identified in protostomes were named Cluster A, Cluster B and PDFR-related clusters, and their existence reveals that the Family B1 GPCR subfamilies evolved differently from the vertebrate receptors (Fig. 3) (Cardoso et al., 2014b). The protostome Cluster B members shared a common origin with the deuterostome GCGR, PTHR and Secretin receptors (GPS-group), while Cluster A are related in sequence with the metazoan CALCRs (Fig. 4). Analysis of available genomes and transcriptomes of invertebrate taxa suggests that some receptor clades of Family B1 GPCRs greatly expanded, and the predicted number of receptors is more numerous and variable than in chordates. Moreover, genome, transcriptome and proteome studies in protostome have only yielded a handful of potential peptide precursors that are likely to encode the cognate receptor activating peptides. This raises fundamental questions about the functional significance of the receptors, their origin, evolution, and the mechanisms behind the establishment of the ligand-receptor pairs during the metazoan radiation.

Mollusca are the second largest and most diverse group of invertebrate protostomes. They are Lophotrochozoans, and possess less modified genomes than nematodes and insects as their genome organization and gene structure and function is more like deuterostomes and non-bilaterian metazoans (cnidarians and sponges) (Miller and Ball, 2009; Raible et al., 2005; Simakov et al., 2013; S. Wang et al., 2017). Thus, genes that have been deleted or highly modified during the Ecdysozoan radiation are likely to have been maintained in Lophotrochozoan and thus studies of their genomes have the potential to expand our knowledge about the inventory of genes in the last common bilaterian ancestor. For this reason, studies of the GPCRs in the Lophotrochozoans may contribute to a better understanding of metazoan genomes and gene family evolution, by creating a link between Ecdysozoans and deuterostomes. For example, a recent comparative study of GPCR evolution identified a novel Mollusca Galanin receptor (GALR)-like clade, which is the sequence orthologue of the deuterostome GALRs, but which only persisted in cephalopods, thus challenging the previously accepted paradigm that Allatostatin A receptors/Buccalin receptors (AST-AR/Buccalin-R), another sequence related family, were the protostome GALR representatives (Li et al., 2021).

The aim of the present review is to delve into the evolution of the GPCRs and their cognate neuropeptide ligands in the Mollusca. By taking advantage of the plethora of Mollusca genomes in publicly available databases and integrating previously published data an updated model was generated to explain the origin and evolution of Family B1 GPCRs in Mollusca and deuterostomes. In relation to function we have previously highlighted the need for caution when analysing data about GPCR neuropeptide ligands generated before the era of molecular studies when non-homologous peptides and antisera were used (Cardoso et al., 2010, 2020c).

1.3. The Molluscs

The Molluscs are a large, speciose, and monophyletic superphylum of protostomes that comprises nearly one-third of documented marine species (Kocot et al., 2011; Smith et al., 2011; Vinther, 2015; Wanninger and Wollesen, 2019). They are among the most ancient animal taxa, since through fossilized shell records they can be dated back to the Early Cambrian period. The evolutionary success of the Mollusca has been

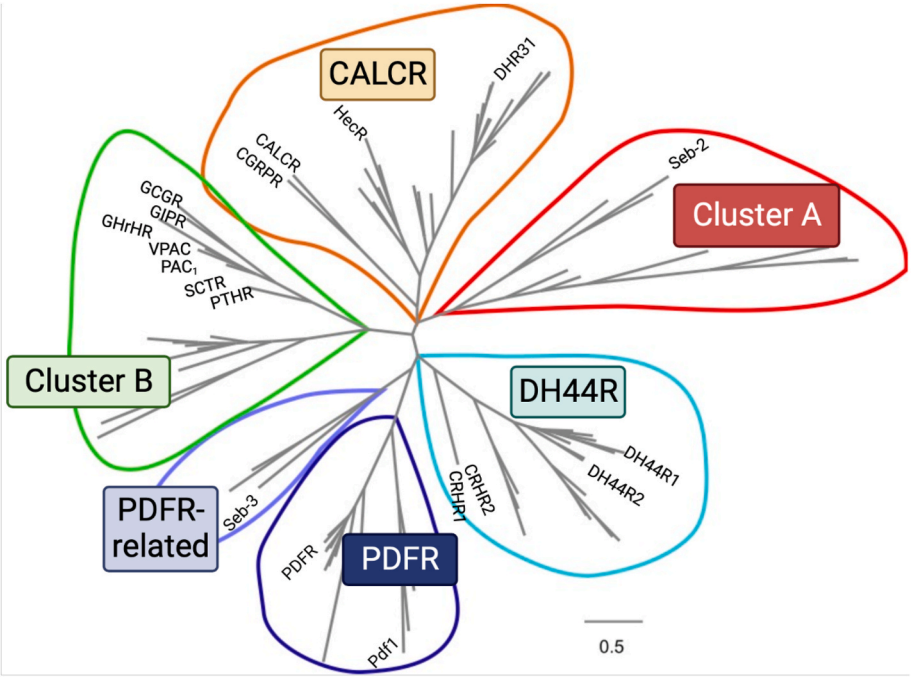


Fig. 4. Evolutionary relationships of the metazoan Family B1 GPCRs. The metazoan receptor groups identified are annotated in colour. Analysis was based on the amino acid sequence alignment of the TM regions of Family B1 GPCRs receptors, and the figure was adapted from (Cardoso et al., 2014b).

linked to their capacity to adapt to a wide variety of habitats, and they have been found in almost all ecosystems on Earth. They diverged from the Ecdysozoans (arthropods, nematodes and other related phyla), another superphylum, that diverged early during the metazoan radiation. The Mollusca phylum is divided into two main early lineages, the Aculifera (shell-less aplacophorans and multi-shelled polyplacophorans) and Conchifera (all other, primarily uni-shelled groups), which possess a hard-biomineralized shell that provides protection and serves as an exoskeleton (Wanninger and Wollesen, 2019) (Fig. 5).

The Conchifera contains seven classes, and the most well studied are the Gastropoda (snails and slugs), Bivalvia (clams, oysters, and mussels) and Cephalopoda (squid, octopus, and nautilus) (Vinther, 2015; Wanninger and Wollesen, 2019) (Fig. 5). The members of each class are characterized by the possession of unique anatomical and physiological features, including variations in shell morphology, nervous system complexity, and feeding mechanisms. Despite their differing morphological characteristics, they all possess an unsegmented body that is often protected by a hard outer shell. The classes, Gastropod and Bivalve, are richest in terms of species and include several of the most economically important species for fisheries and aquaculture.

The growing richness of publicly available genomes, transcriptomes and proteomes are providing new insights into their biology, morphological diversity, and adaptive response to different environments and their response to anthropogenic driven changes in the marine environment (Davison and Neiman, 2021). Recent studies have uncovered the neuropeptide and GPCR repertoire in some Mollusca, and includes members of Family B1 GPCRs and the cognate putative neuropeptide precursors (Bose et al., 2017; Cardoso et al., 2020b, 2020c; De Oliveira et al., 2019; Jékely, 2013; Mirabeau and Joly, 2013; Stewart et al., 2014; Thiel et al., 2021; Veenstra, 2010). However, there are far fewer studies about the functional importance of GPCRs and their cognate ligands in mollusc physiology. Furthermore, the sheer diversity of mollusc species and their low commercial importance means there is a lack of public genome and transcriptome resources for many taxa. In contrast, numerous resources are available for protostome models such as the arthropods (*D. melanogaster*), nematodes (*C. elegans*), and annelids (*Platynereis*), where GPCRs have been identified in several species, neuropeptide precursors have been described and some receptors deorphanized and their function *in vivo* studied (Bauknecht and Jékely, 2015; Caers et al., 2012; Frooninckx et al., 2012).

There is growing interest in molluscs due to their importance for ecosystem functioning, nutrient cycling, carbon sequestration, and more recently as an important source of food, materials and fertilizers (Ramón and Galimany, 2022) and this means that the number of publicly available genomes and transcriptomes have increased significantly. Genomes for 143 members of Mollusca phyla are now available in NCBI (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/), the large majority of which (84%) were deposited in the last 5 years. Genomes for species representative of the six Mollusca classes are available and include 57 Gastropods, 57 Bivalves, 21 Cephalopods, 3 Polyplacophora, 2 Solenogastres and 1 Agaricomycetes. Moreover, improved genome assemblies have been released for the Pacific oyster (*Crassostrea gigas*) (Peñaloza et al., 2021), scallop (*Pecten maximus*) (Zeng et al., 2021) and Mediterranean mussel (*Mytillus galloprovincialis*) (Simon, 2022) and for the gastropod, the freshwater snail (*Biomphalaria glabrata*)(GCF_947242115.1).

Despite the large amounts of genome data publicly available, genome data assembled in chromosomes only exists for 53 species, while annotated genomes with predicted protein coding genes are even fewer. For example, annotated genomes with predicted proteins are available for 13 bivalves, 16 gastropods and 4 cephalopod species (as of October 2023) and for the present analysis of the GPCRs and their putative neuropeptide ligands 5, 5 and 2, representative species from each class, respectively were selected. The presence in each of the selected genomes of Family B1 GPCRs and their putative neuropeptide ligands was analysed to a) determine the Family B1 GPCR repertoire and novel receptors, b) to identify putative orthologues of the vertebrate and invertebrate GPCR neuropeptide ligands and c) to reconstruct existing evolutionary models for the receptors and potential ligands.

1.4. Family B1 receptors in Mollusca

In molluscs, orthologues of the vertebrate and invertebrate specific Family B1 GPCRs have been described (Fig. 3). The first isolated receptor in members of this phylum was described in the bivalves, where a putative CALCR-like was identified in the Pacific oyster and was found to be involved in osmoregulation and expressed in a variety of tissues but mostly in the gills and mantle (Dubos et al., 2003). Subsequently, in mantle transcriptomes of the Antarctic clam (*Laternulla eliptica*), CALCR-like and PTHR-like receptors were identified (Clark et al., 2010). However, only when sequenced genomes of molluscs became available was a thorough examination of the GPCR gene repertoire performed (Jékely, 2013; Mirabeau and Joly, 2013). In bivalves and gastropods orthologues of the six invertebrate GPCR gene families have been identified, with Cluster B, that shares a common origin with the deuterostome GCGR, PTHR and Secretin receptors, being most diversified, followed by Cluster A and more specifically CALCR in some lineages (Cardoso et al., 2020b). In addition, the number or members found within each receptor group was species-specific and the members of the PDFR and its related family (PDFR-related genes) were not found in some gastropods and PDFR-related genes were absent in mollusc species (Cardoso et al., 2020b) (Fig. 3).

Our searches, that integrated a larger number of sequences and representative species of Mollusca than previously, confirmed that species from all classes had GPCRs belonging to each of the metazoan receptor subfamilies. Comparison of the identified Family B1 GPCRs from the different Mollusca classes, revealed that bivalves had the largest number of receptors, varying from 30 in *Mya arenaria* to 17 in *Mizuhopecten yessoensis* genomes. The number of predicted receptors within each subfamily was species-specific, with the most diverse subfamily being Cluster B (Table 1,Supplementary Fig. 1). The main characteristics of the Mollusca GPCRs will now be briefly considered in relation to previous studies about their evolution, abundance, and function in the metazoans.

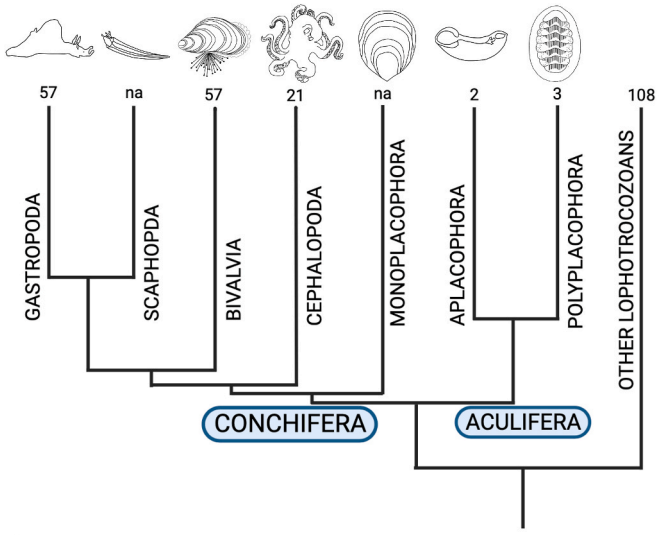


Fig. 5. The Mollusca phylum. The number of publicly available genome data for the representatives of each class available from the NCBI database (October 2023) is indicated. The dendrogram was based on the scheme of (Wanninger and Wollesen, 2019).

Table 1
Number of Family B1 GPCRs members in representatives of the Mollusca phylum searched in this study. The number of receptors identified in the main receptor families are indicated. Within the PDFR group the forward slash (/) separates the different receptor clusters (PDFR/Mollusca PDFR/PDFR-related). The phylogenetic tree where data was retrieved is available from Supplementary Fig. 1.

	CALCR	Cluster A	PDFR	CRHR/DH44R	Cluster B	TOTAL
BIVALVE						
Crassostrea gigas	7	5	1/1/1	1	12	28
Mytilus galloprovincialis	6	5	2/2/1	1	10	27
Mizuhopecten yessoensis	3	4	1/3/1	1	4	17
Mercenaria mercenaria	4	8	1/1/1	1	6	22
Mya arenaria	6	15	2/0/1	1	5	30
GASTROPOD						
Pomacea canaliculata	2	5	1/1/2	1	6	18
Biomphalaria glabrata	2	0	1/1/0	3	6	13
Aplysia californica	3	3	1/1/2	1	3	14
Lottia gigantea	3	3	1/1/0	1	7	16
Gigantopelta aegis	3	3	1/3/1	1	6	18
Haliotis rufescens	3	3	1/2/2	1	5	17
CEPHALOPOD						
Sepia pharaonis	2	2	0/1/0	0	4	9
Octopus bimaculoides	4	5	2/1/1	2/1	6	22

1.5. Neuropeptide precursors in Mollusca

Many neuropeptides have been identified in molluscs, and most are oligopeptides of less than 20 amino acids that are generated from cleavage of larger precursor proteins. One of the first neuropeptides isolated from a mollusc was the FMRF-amide peptide, which is a 4 amino acid peptide that was extracted from the neuronal ganglia of the bivalve clam *Macrocallista nimbosa* and had cardio-excitatory actions in functional tests (Price and Greenberg, 1977). Since then, gastropods have become the focus for neuropeptide discovery in Mollusca and several neuropeptides have been extracted and isolated, using peptide chemistry, from the nervous tissue of Californian sea hare *Aplysia californica* and from the great pond snail *Lymnaea stagnalis*. The first description of the neuropeptide gene repertoire in Mollusca was only available when the genome of the gastropod *Lottia gigantea* (the first mollusc to have its genome sequenced) was sequenced, this revealed genes coding for 67 neuropeptides/neurohormones (Veenstra, 2010). Today, the availability of transcriptomes and proteomes of several bivalves, gastropods and cephalopods suggests that Mollusca ganglia and neurons express a large spectrum of neuropeptides and that most neuropeptides show sequence or structure conservation with neuropeptides in Arthropoda and Vertebrata, suggesting that they evolved from common ancestral genes (Adamson et al., 2015; Bose et al., 2017; De Oliveira et al., 2019; Réalis-Doyelle et al., 2021; Stewart et al., 2014; T. Wang et al., 2017;

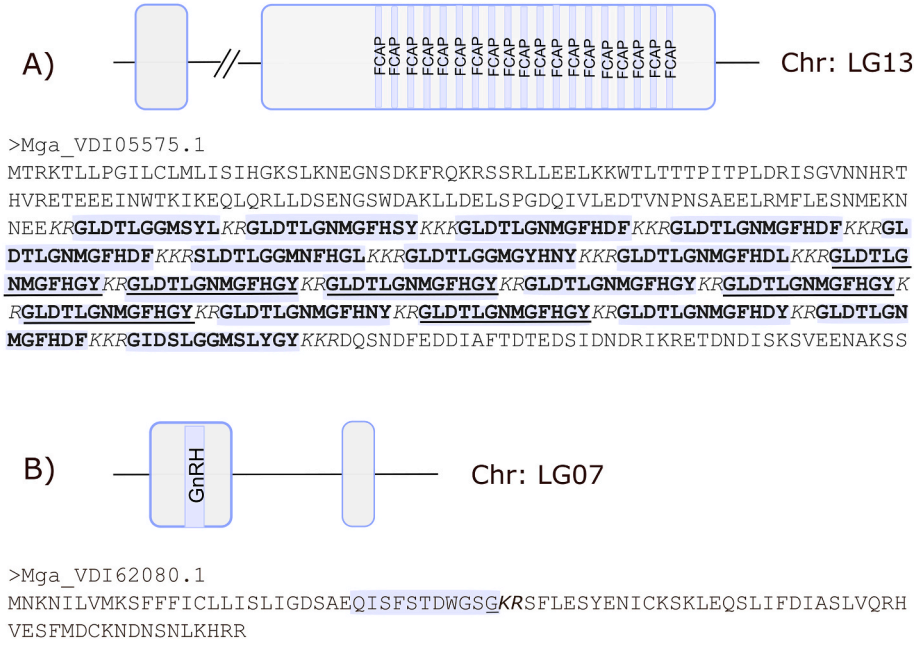


Fig. 6. Organization of two neuropeptide precursors in the genome of the bivalve Mediterranean mussel (*Mytilus galloprovincialis*). The **A)** Feeding Circuit-Activating Peptide (FCAP) and **B)** Gonadotropin-Releasing Hormone (GnRH) genes and deduced mature peptide precursors are represented. The predicted mature peptides are annotated in bold; the proteolytic cleavage sites are in italics and underlined and the signal peptide is underlined. In **A)** the genes are localized in LG13 and the multiple copies of FCAP peptides derive from a large protein precursor and are released by proteolytic cleavage. In grey is highlighted an example of the same peptide sequence within the FCAP precursors. In **B)** the GnRH precursor gene is localized in chromosome LG07 and encodes a single peptide. The Mollusca GnRH mature peptide is C-terminal amidated and the glycine residue likely to be converted into a C-terminal amide is in bold and italics. The accession numbers of the mussel protein coding genes are indicated. The gene structures were deduced by searching with the deduced mature protein precursor against the latest version of the mussel genome (MgalMED). Exons are represented by boxes and the lines indicate the introns and the localization of the mature peptides inside the exons is indicated. The C-terminal G amide is underlined.

Zatylny-Gaudin et al., 2016; Zhang et al., 2018). Neuropeptide/peptide families that are specific to Lophotrochozoans or that are only found in Mollusca have also been described and generally represents 34% of the minimum proneuropeptide/peptide prohormone complement described (De Oliveira et al., 2019). Nonetheless, their biological functions currently remain poorly understood when compared to arthropod peptides and those that have been described are related to reproduction, water and ion balance, muscle contraction and feeding (Di Cosmo and Polese, 2013; Kiss, 2011; Morishita, 2017; Morishita et al., 2010).

In common with the arthropods, several Mollusca neuropeptides derive from large protein precursors that encode multiple short mature peptides which are released by proteolytic cleavage, but there are others that encode single peptides (Fig. 6). In some cases, there is a multiplicity of the same peptide sequence within a single precursor and this has been suggested to be beneficial for amplifying the peptide biological signal, as well as serving as a backup if there is modification of amino acids caused by genetic mutations (Morishita, 2017). The Mollusca-specific Feeding Circuit-Activating Peptide (FCAP) precursors and the Pedal neuropeptide precursor are amongst the richest peptide containing precursors and in *Lottia*, respectively encode for 26 mature peptides and 20, 42 and 23 for the three (A, B and C) Pedal precursor genes identified (Veenstra, 2010). In the marine bivalve, *M. galloprovincialis* two FCAP predicted protein coding precursors (VDI05575.1 and VDI05576.1) have been deduced and both encode 19 peptides. However, searches in this species genome revealed that they come from a single gene on chromosome LG13 (Fig. 6). The *M. galloprovincialis* Pedal neuropeptide precursor is also a large precursor, encoding numerous 23-amino acid mature peptides and is also localized on chromosome LG13.

Neuropeptides that are likely activators of Family B1 GPCRs have also been identified in Mollusc genomes, but their biological role remains poorly understood. The orthologues in Mollusca of the vertebrate CRH family members are the DH44/ELH peptides (Favrel et al., 2024; Li et al., 2001). Orthologues of the vertebrate CALC have also been found (Cardoso et al., 2020b; Schwartz et al., 2019) and the peptide cerebrin, which was first isolated from the cerebral ganglia of *A. californica*, is the sequence orthologue of the arthropod PDF peptide (Veenstra, 2010). The existence of other Family B1 GPCR peptides orthologous in sequence to the vertebrate PACAP, VIP, GCG and CGRP peptides are reported to be expressed in Mollusca tissues, but heterologous antibodies raised against the mammalian mature peptides were used raising questions about their authenticity (Kiss and Pirger, 2013; Lafont et al., 2007a; Ottaviani et al., 1992; Ottaviani and Cossarizza, 1990; Pirger et al., 2016). Surprisingly, although molecular datasets from different Mollusca are available the peptide genes or precursor transcripts for them remain to be isolated (Cardoso et al., 2010, 2020c). Below, an update of the molecular and functional data available for the different Family B1 GPCR receptors and their potential peptide ligands in members of the Mollusca phyla are described.

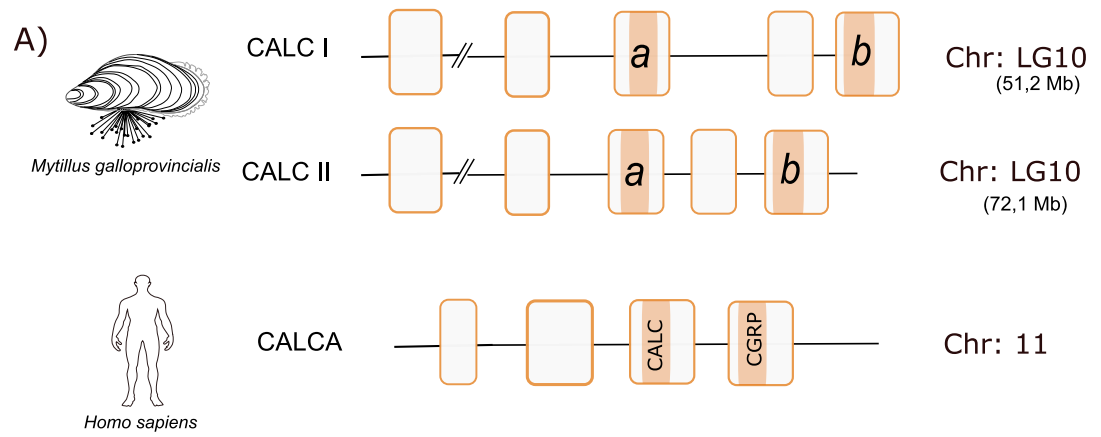
1.6. The Mollusca Calcitonin and Cluster A receptor systems

The metazoan Calcitonin-like and the protostome Cluster A (also named as I-Calc) receptors are very similar in sequence and those from *M. galloprovincialis* share ~30 % amino acid sequence identity. Both receptor subfamilies are likely to have a shared common ancestry, but while CALCRs exists in protostome and deuterostome genomes, Cluster A have only been reported in protostomes (Cardoso et al., 2014b, 2020b; Elphick et al., 2018). In vertebrates, the calcitonin system consists of a CALC peptide of 32 amino acids, and the presence of two conserved cysteines cause the peptide to form a cyclic structure when a disulphide bond forms, and this is essential for bioactivity. Additionally the calcitonin system contains four other structurally related peptides, which evolved from the same common gene precursor (Ogoshi et al., 2006) and which activate two structurally similar receptors, CALCR and CGRPR (or CALCLR), coupled to receptor activity-modifying proteins (RAMPs) (Hay and Pioszak, 2016). The RAMPs are a group of auxiliary transmembrane proteins (3 identified in humans, RAMP1, RAMP2 and RAMP3) that are involved in receptor activation since they can regulate signalling, receptor trafficking and interaction with intracellular Gαs proteins (Dickerson, 2013). The vertebrate CALCR system is best known for its role in bone homeostasis but it has other functions such as cardiovascular homeostasis, glucose metabolism, and feeding behaviour (Felsenfeld and Levine, 2015; Findlay and Sexton, 2004; Sekiguchi, 2018). This diversity of actions may be explained by the shift in ligand preference of CALCR for CGRP or adrenomedullin (ADM) and adrenomedullin 2 (ADM2) peptides when RAMPS bind (Kotliar et al., 2022). In Ecdysozoans, DH31R and its cognate peptide (DH31) were proposed to be the function and sequence orthologues of the vertebrate CALC system, and were described to be involved in water excretion, diuresis and digestive functions (Coast et al., 2001; Veenstra et al., 2008; Zandawala, 2012).

In Mollusca, CALCRs and Cluster A receptors were found, and our searches confirmed that the largest number of members were identified in bivalves, with 15 Cluster A receptor genes in *M. arenaria* and 7 CALCRs genes in *C. gigas*, thus suggesting a large expansion occurred in the members of both receptor groups, and that this occurred in a species-specific manner (Table 1). Our phylogeny revealed that Cluster A sequences grouped with the invertebrate CALCRs, unlike previous analysis that placed them in a separate independent cluster (Supplementary Fig. 1A). More extensive sampling of a larger number of representative species and more functional studies will be required to establish if Cluster A represents an independent invertebrate receptor group or has the same evolutionary origin as the vertebrate CALCRs.

Putative sequence and structure orthologues of the vertebrate calcitonin peptides were also found in Mollusca, where in the genomes analysed two peptide precursors exist (CALC precursor I and II), each of which is likely to produce two mature (Calca and Calcb), peptides with a similar structure (Fig. 7, Supplementary Fig. 2). All Mollusca mature peptides share low sequence similarity (<30 % aa sequence similarity) with human CALC (Cardoso et al., 2020b)). In *M. galloprovincialis*, the precursors share a similar gene structure and are localized next to each other on chromosome LG10 and likely resulted from a tandem gene duplication event (Fig. 7). At the N- terminus, the Mollusca mature peptides, like the human CALC, possess two conserved cysteines that are likely responsible for the formation of a disulphide bridge and a C-terminal proline that is amidated and both structures in humans are essential for the bioactivity of the peptide (Wimalawansa, 1996). In humans, the *CALCA* gene is localized on Chr 11 and is composed of 4 exons and like the mussel precursor, codes for two peptides: CALC (32 aa) in exon 3 and CGRP (37 aa) in exon 4 (Fig. 7). CGRP is a member of the calcitonin peptide family and shares structural similarity with CALC (but lower sequence identity) however, each of the peptides have different physiological roles. In humans, (in contrast to the mussel) the two peptides are expressed in different precursors that are generated by alternative splicing of the primary mRNA transcript, leading to the translation of the two peptides in a tissue-specific manner, with CALC being more specific to the thyroid gland and playing a role in bone homeostasis, while CGRP is more related to the nervous system and is implicated in vasodilation (Sekiguchi, 2018; Wimalawansa, 1996).

An immunologically-related peptide to vertebrate CGRP was detected in the haemolymph and several tissues in cephalopods using a human CGRP-specific antisera and receptor binding displacement assays in *Sepia officinalis* and *Nautilus macromphalus* brain, optical lobes, heart and kidney (Lafont et al., 2007a, 2007b). Our searches identified a putative Calc mature peptide gene sequence on Chr5 (NC_068985.1) of the octopus (*O. bimaculoides*) genome but the full-length peptide precursor was not identified and a gene with a similar gene organization to the bivalve and gastropod precursors was not found (Supplementary Fig. 2). Based on these observations we propose that the cephalopod CGRP previously reported likely corresponds to Mollusca Calc which, in the few species where it has been described, has a widespread tissue distribution including in the nervous system (Cardoso et al., 2020b;



B)

>Mga_CalcI

MHQCLWRCIYLIIVLQLIEHLRADQREEDARRLMESLNQAQNDISSMMSTVDGLDNYLTTQ*KKR***ACNLGLNSHHC**
ALADLDNQLQSREWLSNGHSPG*KR*TTLKEEHKEKLSEVEIKRLIEDIVRKRLDLKNIRTLLEDANGSIIENS*KR***T**
CTVELGGACRTEWASSIADQYYYLMSPHSPG*RKRR*SFRKRMSPLRLFHKRLQQIKKRHSNN

a
b

>Mga_VD151118.1

MVETYMWLLITAILFLTTSAAVEDDVTNRGKQLIQQLDDVRHNLKSVTESLEKLSDVIEE**ECTWGGGMSDEMCST**
VDIDEIQRSFQVIHDRNSPG*KR*SVPSMDPSLRQFLQDLLRKKEMIQRLKHILTYADNAVHEE*RKK***SCQLNLGFHC**
QTQEYSAIADMFNFLGSGRSPG*KRR*RRSIQQILKTKETL

a
b

C)

	Hsa_AAA58403.1	--------**C**GN-----LST**C**MLGTYTQDFNKFHTFPQTAIGVGA**P**-*amide*
Precursor I	**Mga_CalcIa**	-------A**C**NL--GLNSHH**C**ALADLDNQLQSRE-WLSNGHS---**P**-*amide*
	Mga_CalcIb	-------T**C**TV--ELG-GA**C**RTEWASSIADQYY-YLMSPHS---**P**-*amide*
	Cgi_XP_011441374.2a	--------ETQ--ERQNNL**C**RGMGPNNHPCGLTSFDVRNV-----
	Cgi_XP_011441374.2b	-------T**C**AV--ELG-GA**C**RTEWASAIADQYY-YLMGPHG---**P**-*amide*
	Lgi_XP_009043607.1a	-------T**C**AF--GINSHQ**C**TLTSLNNKMMSQA-WLSDGMS---**P**-*amide*
	Lgi_XP_009043607.1b	-------S**C**SL--RLG-GM**C**LTENLNAAANQYE-YLSSGLS---**P**-*amide*
	Pca_XP_025087211.1a	-------S**C**EL--GINSHH**C**ALAHLDDVMMARD-FLRSGYS---**P**-*amide*
	Pca_XP_025087211.1b	-------T**C**QF--RLG-NH**C**LTEALDRAASQYY-YLKSALS---**P**-*amide*
Precursor II	**Mga_VD151118.1a**	-------E**C**TWGGGMSDEM**C**STVDIDEIQRSFQ-VIHDRNS---**P**-*amide*
	Mga_VD151118.1b	-------S**C**QL--NLG-FH**C**QTQEYSAIADMFN-FLGSGRS---**P**-*amide*
	Cgi_XP_011456505.1a	------EA**C**AL--SLNVDI**C**TEKYIEETADHQSKLQNLIEGN--**P**-*amide*
	Cgi_XP_011456505.1b	-------S**C**TL--NLA-YH**C**QTSEYAGLTDLYN-YLNSNAS---**P**-*amide*
	Lgi_XP_009062318.1	-------S**C**NL--NLG-FH**C**QTDEYSSIADMYD-FLQSALS---**P**-*amide*
	Pca_XP_025088744.1a	ADATDSSS**C**T---GFRDSI**C**QAMELLKYLDSQNGYLGNPAS---**P**-*amide*
	Pca_XP_025088744.1b	-----WFS**C**HL--NLG-FT**C**QTQEYADIADYYD-FLNSAES---**P**-*amide*

*

Fig. 7. The bivalve calcitonin precursors. A) Gene structures of the two calcitonin precursors (CALCI and CALCII) in the genomes of the bivalve Mediterranean mussel (*M. galloprovincialis*) and of the human (*H. sapiens*) calcitonin genes. The two mussel genes share a similar gene structure and are localized in the same chromosome (LG10). Exons are represented by boxes and the lines indicate the introns and the localization of the mature peptides inside the exons is indicated. In the mussel gene the two peptides are encoded by different exons Calca in exon 3 (Ia 32 aa, IIa 33a) and Calcb in exon 5 (Ib 31 aa, IIb 31 aa). The human *CALCA* gene is localized in Chr 11 and is composed of 4 exons and encodes the CALC (32 aa) in exon 3 and the Calcitonin Gene Related Peptide (CGRP, 37 aa) in exon 4. The human gene produces and expresses the two peptides in different precursors, generated by alternative splicing of the primary mRNA transcript that leads to the translation of the two peptides in a tissue-specific manner (Wimalawansa, 1996). The gene structures were deduced by searching the species deduced mature protein precursor against the latest version of the mussel (MgalMED) and human (GRCh38.p14) reference genomes available at NCBI. **B)** Deduced sequence of the peptide precursors of mussel CALC I and CALCII. The deduced mature peptides (a and b) inside the precursors are highlighted in colour and the proteolytic cleavage sites are in italics. No consensus proteolytic cleavage site was identified at the N-terminus of CalcIIa. **C)** Multiple sequence alignment of the Mollusca mature Calc deduced peptides with the human orthologue with all conserved motifs of the "typical" CALC peptide highlighted in bold: the two conserved cysteines and the C-terminus amidated proline. Both human and Mollusca peptides are C-terminal amidated except for the oyster Cgi-CalcIa. The accession numbers of the sequences are indicated, and the full precursors are available as Supplementary Fig. 2. For the Lottia precursor II, only one deduced Calc peptide was predicted. Hsa- *Homo sapiens*, Mga- *Mytilus galloprovincialis*, Lgi- *Lottia gigantea*, Pca- *Pomacea canaliculata*, Cgi- *Crassostrea gigas.*

Schwartz et al., 2019). In the gastropod *L. gigantea* a precursor (precursor II) encoding a single Calc-like peptide was found and, in *C. gigas* the first peptide encoded by CalcI (CalcIa) has a highly divergent sequence from other Mollusca and human orthologues and it is not amidated at the N-terminus (Fig. 7, Supplementary Fig. 2).

For Cluster A receptors, no bioactive peptide precursor has yet been described in Mollusca. The reasons why CALCR and Cluster A members have undergone a large gene family expansion in bivalve genomes raises interesting questions and may be linked to the importance and multiplicity of their biological role. The studies that exist for the CALC system suggests some functions have been conserved during evolution. For example, in common with the vertebrate CALC peptides those in bivalves are involved in calcium regulation, although linked to shell formation, rather than skeletal homeostasis (Cardoso et al., 2020b; Clark et al., 2010; Dubos et al., 2003; Schwartz et al., 2019). Bivalves have two highly mineralized valves (a.k.a., shells) that are produced by the mantle and are primarily composed of calcium carbonate. The shell is essential for bivalve survival since it protects them from predators and desiccation and is a reservoir of minerals (Marin et al., 2012; Suzuki and Nagasawa, 2013). Studies on the CALC system in *C. gigas* and *M. galloprovincialis* revealed that the mantle and gills have an abundant expression of both the peptide precursor and the receptors, and that changes in the salinity of seawater modifies their expression, indicating that they also probably have a role in ion balance. Moreover, *ex-vivo* studies using mantle explants, revealed that incubation with CALC peptides promoted calcium mobilization to isolated mantle cells strongly supporting their importance in calcium transport during Mollusca biomineralization (Cardoso et al., 2020b). Furthermore, in a recent study we revealed that *M. galloprovincialis* CalcIa and CalcIIa rescued shell growth and mineral microstructure in an *in vivo* shell repair model, and this corroborates previous observations about their importance in bivalve shell building. Moreover, we demonstrated that the CALC peptides likely act via CALCR in *M. galloprovincialis* and *C. gigas* since they activated both adenylyl cyclase and phosphoinositide transduction pathways in mammalian cells transfected with the receptors (Cardoso et al., 2020b; Schwartz et al., 2019).

Revisiting the phylogeny of the CALCR gene repertoire in *C. gigas*, including sequences retrieved from a larger number of Mollusca and other invertebrate taxa, revealed that the two functionally characterized oyster CALCRs (Schwartz et al., 2019) grouped within the Cluster A clade (Supplementary Fig. 1A) (Cardoso et al., 2020b). In *C. gigas*, these receptors have been deorphanized using the deduced mature peptides encoded in CalcI (Ia and Ib) and CalcII (IIa and IIb) precursors and in *C. gigas* only the peptides CalcIb and CalcIIb (a.k.a, CT1b and CT2 in (Schwartz et al., 2019)) activated the two *C. gigas* CALCRs. Interestingly, in *M. galloprovincialis* the peptide CalcIIa activated a CALCR (Cardoso et al., 2020b) (Fig. 7). Furthermore, it reveals that the Mollusca Calc peptide precursor is likely to produce bioactive peptides that activate both CALCRs and Cluster A receptors. Further work will be required to deorphanize the CALCR repertoire across the Mollusca to consolidate models about the evolution of their sequence and function as well as the scope of their biological functions. In this context although there is overall low amino acid sequence conservation of the deduced Mollusca CALCs, key amino acids responsible for vertebrate CALC bioactivity are conserved, suggesting the system and its activity has been well conserved as they evolved (Cardoso et al., 2020b).

1.7. The Mollusca PDF/cerebrin receptor system

The PDFR and PDFR-related receptors are two sequence related receptor subfamilies that have been described in invertebrates. While the PDFR cluster groups the Mollusca orthologues with the *D. melanogaster* and *C. elegans* PDFRs, no PDFR-related receptors have yet been identified in insects (Cardoso et al., 2014b). In deuterostomes, elements of both receptor clusters have been predicted in invertebrate deuterostomes, the echinoderm *Strongylocentrotus purpuratus* (PDFR)) and in the cephalochordate *Branchiostoma floridae* (PDF-related receptors) but not in other chordates (Cardoso et al., 2020b). Our searches on Mollusca genomes confirmed that members of PDFR and PDFR-related families exist but a novel group clustering only Mollusca receptors (Mollusca-PDFR) was found, and it seems probable that members of this family emerged from the same ancestral gene as other invertebrate PDFRs (Table 1, Fig. 8). This highlights the increased diversity of Mollusca receptors compared to other invertebrates, although the reason for this is unknown but may be linked to acquisition of novel functions associated with their evolutionary success and the associated speciation and range-expansion (Krug et al., 2008). To date no functional studies on members of the PDFR clade have been described in Mollusca.

PDF is a small peptide hormone encoded by a single precursor mainly studied in insects, crustaceans and nematodes (Helfrich-Förster, 2009; Meelkop et al., 2011). This hormone was first discovered in insects due to its pigment dispersing action in crustaceans, when applied exogenously. The biological action of the insect peptide on crustacea is due to high sequence similarity with crustacean pigment dispersing hormones (PDHs), which regulates light-dependent pigment dispersion and migration in chromophores and photoreceptors (Rao and Riehm, 1989; Shafer and Yao, 2014). In *D. melanogaster* PDF regulates the circadian rhythm (Mezan et al., 2016), feeding, sleeping, reproduction, activity, and arousal (Chen et al., 2016; Talsma et al., 2012; Veenstra et al., 2008), and in *C. elegans* it also regulates circadian rhythm, locomotion, reproduction, and gastrointestinal function (Frooninckx et al., 2012; Janssen et al., 2008; Meelkop et al., 2012). In crustaceans, PDF (a.k.a., PDH- Pigment Dispersing hormone) is a pigment dispersing hormone, and a neurotransmitter/neuromodulator (Harzsch et al., 2009; Mangerich et al., 1987; Nussbaum and Dircksen, 1995).

Sequence orthologues of the protostome PDF peptides have been predicted in invertebrate deuterostomes (Elphick et al., 2018; Mirabeau and Joly, 2013) and are absent from vertebrates, but it has been suggested that they are the functional analogues of mammalian VIP, due to their similar role in circadian systems (Janssen et al., 2008). In Mollusca the peptide, cerebrin, is the orthologue of arthropod PDF and was first isolated from the cerebral ganglia of *A. californica* (Veenstra, 2010) and is involved in feeding behaviour (Li et al., 2001). Orthologues of *A. californica* cerebrin/PDF have been identified in several molluscs, but their function remains uncharacterized (Fig. 9). In the bivalve *M. galloprovincialis* genome, the cerebrin precursor is located on chromosome LG02 and is composed of 3 exons, with the mature peptide being encoded by exon 3. In contrast, the precursor for PDF in the genome of *D. melanogaster* is encoded in a single exon on chromosome 3R. The mature Mollusca cerebrin peptide shares less than 30% amino acid sequence identity with *D. melanogaster* PDF, but both peptides are likely to be amidated at the C-terminus and share three conserved amino acid residues (Asparagine, Lysine and Proline) (Fig. 9).

1.8. The Mollusca CRH/DH44 receptor system

In vertebrates, the CRH systems is comprised of 5 structurally related neuropeptides that shared common ancestry and evolved through gene duplication. The peptides signal via two CRH receptors and the system regulates the stress response, emotional behaviour, and anxiety (Cardoso et al., 2020a; Fox and Lowry, 2013; Lovejoy and Balment, 1999; Vale et al., 1981). In invertebrates, the DH44Rs are the orthologues of the vertebrate CRHRs (Cardoso et al., 2014a). Putative DH44Rs have been described in Mollusca and in common with the arthropods vary from 1 in bivalves to 3 in the gastropod, *B. glabrata*, and cephalopod, *O. bimaculoides*, (Table 1). Based on the clustering of the receptors in the phylogenetic analysis, the *B. glabrata* receptors share high sequence similarity and are likely to have arisen from a recent species-specific duplication event (Supplementary Fig. 1B). However, in *O. bimaculoides*, one of the identified receptors clustered with the arthropod orthologues and the two other receptors clustered with the Mollusca sequences. Based on the outcome of the phylogenetic analysis

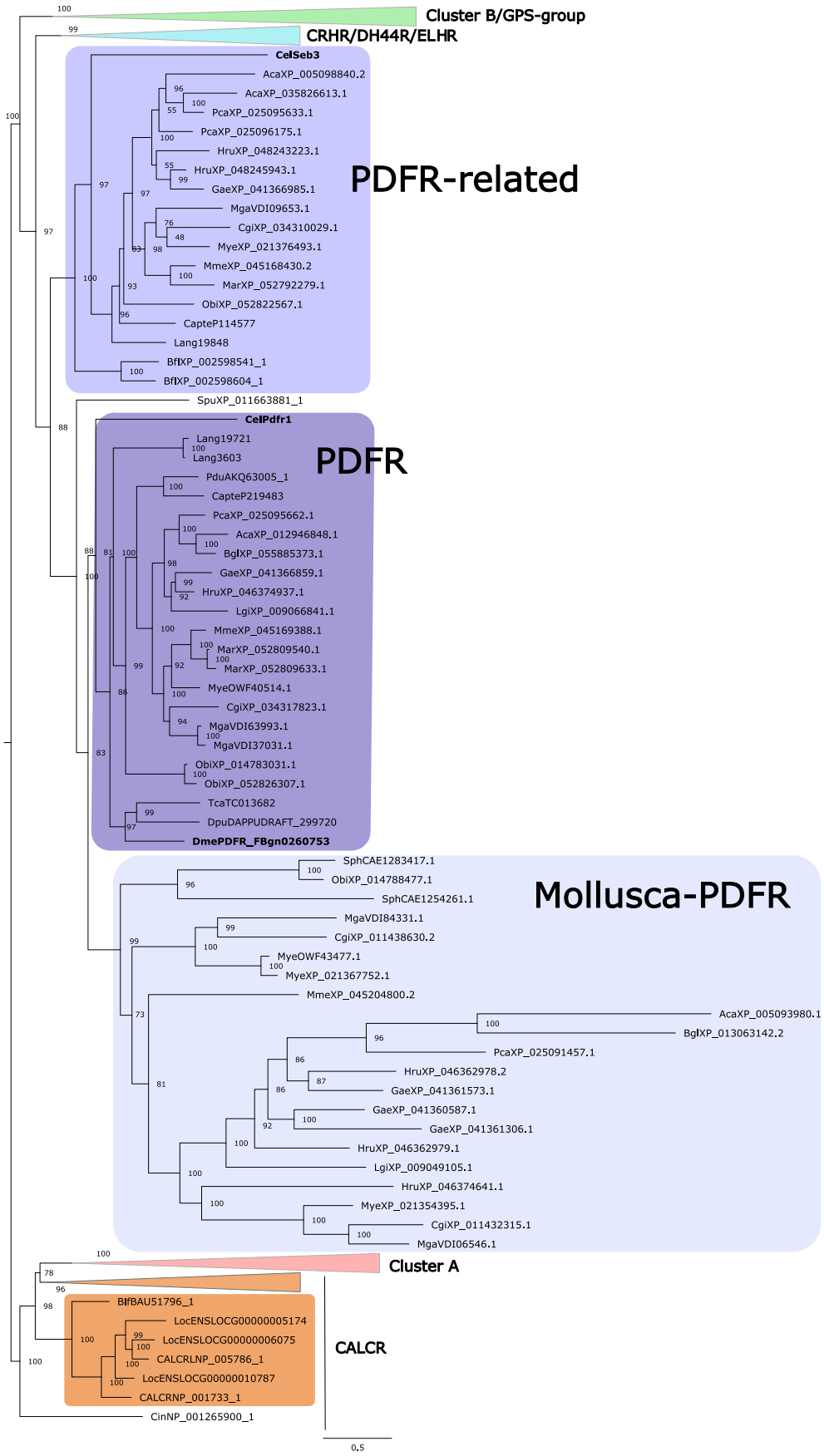


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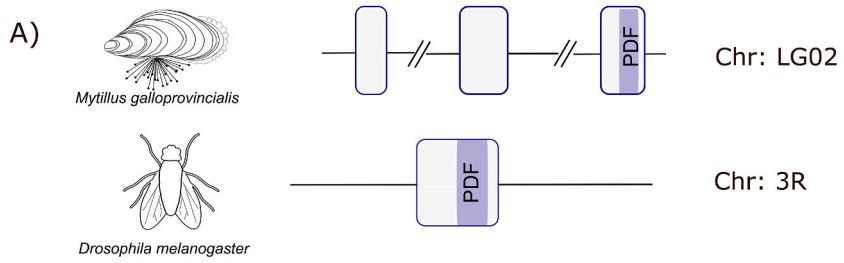
Fig. 8. Phylogenetic tree showing the novel Mollusca receptor group within the PDFR cluster. The tree was constructed using the ML method. The metazoan receptor groups identified are annotated in colour. Analysis was implemented with a multiple sequence alignment of the edited receptor sequences performed with MUSCLE in the Aliview platform (Larsson, 2014). The tree was built using the Maximum Likelihood test (ML) using IQ-tree multicore version 1.6.12 (Nguyen et al., 2015) and using a VT model selected based on ModelFinder using an Ultrafast bootstrap approximation with 1000 replications. The complete tree can be found in Supplementary Fig. 1.



B)

>Mga_VDI56898.
1MKYGRVLNVNYNLFGDIVKTDNISLSDPPTTSVILDMFWSKCLLCFIIVICICLDVIMSSPLRSYQDV
LDQEDRKMVALAHIMRLAMALPDNGMFLD*KR***NAGTVDTLYNLPDLFTAG***R*

C)

Dme_NP_524517.1	**N**SELINS**L**LSL**P**KNMNDA-*amide*
Mga_VDI56898.1	**N**AGTVDT**L**YNL**P**DLFTA--*amide*
Lgi_XP_009054938.1	**N**AGTVDH**L**LNF**P**DLSV---*amide*
Pca_PVD29851.1	**N**GGTLDT**L**YNL**P**NLNNI--*amide*
Obi_XP_014770792.1	**N**HGMIDT**L**INI**P**DLDKV--*amide*
	* :: * .:*.

Fig. 9. The bivalve cerebrin precursor. A) Gene structure of the Mediterranean mussel (*M. galloprovincialis*) cerebrin precursor (LG02) and of the orthologue in the genome of the fruit-fly (*Drosophila melanogaster*, 3R). The gene structures were deduced by searching with the species deduced mature protein precursor against the latest version of the mussel (MgalMED) and fruit fly (Release 6 plus ISO1 MT) genome assembly. Exons are represented by boxes and the lines indicate the introns and the localization of the mature peptides inside the exons is indicated. **B)** Deduced sequence of the cerebrin peptide precursors of the mussel. The deduced mature peptide is highlighted in colour and the proteolytic cleavage sites are in italics. **C)** Multiple sequence alignment of the Mollusca mature cerebrin deduced peptides with the fruit fly orthologue. Conserved aa are in bold and the insect and mollusca mature peptides are C-terminal amidated. The complete and annotated precursor sequences for the Mollusca neuropeptides are available in Supplementary Fig. 2. Dme- *Drosophila melanogaster,* Mga- *Mytilus galloprovincialis*, Lgi- *Lottia gigantea*, Pca- *Pomacea canaliculata*, Obi- *Octopus bimaculoides.*

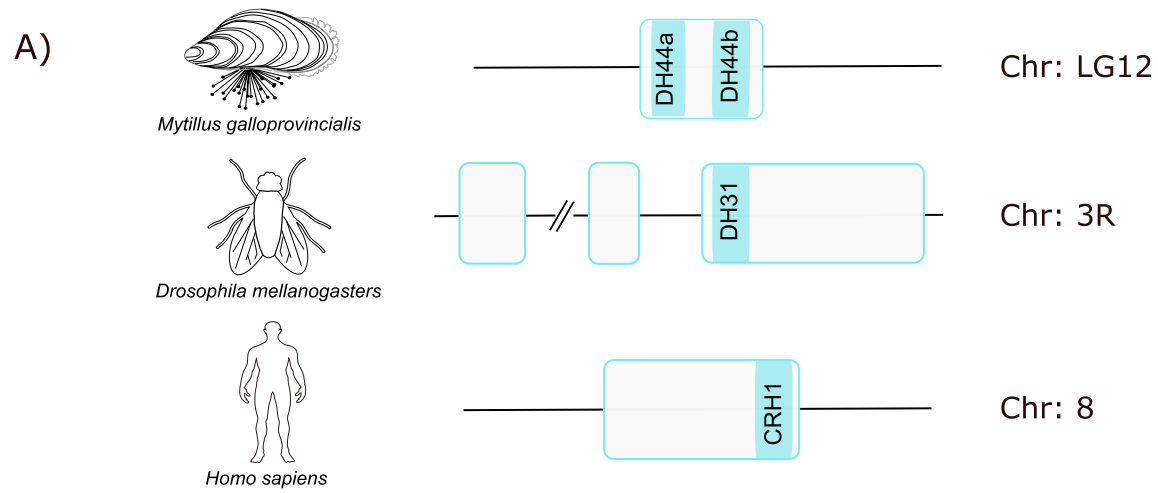
and the CRHR clustering we reveal for the first time the existence of two different types of DH44Rs in the Mollusca and more specifically the cephalopod. One of the cephalopod receptors resembles the arthropod DH44R that is absent from the genomes of bivalves and gastropods, and the other two cephalopod receptors clustered with the Mollusca DH44R isoforms. This may suggest that the arthropod-like DH44R resembles more closely the ancestral form of the receptor and during the radiation of the Mollusca was retained in cephalopods but lost in gastropods and bivalves.

The DH44 peptide is the orthologue of the vertebrate CRH peptide and in *D. melanogaster*, DH44 is involved in water regulation and nutrient metabolism (Cabrero et al., 2002; Nussbaum and Dircksen, 1995). In Mollusca, gene orthologues have been described but have a different organization from that of insect peptides, containing a greater number of mature peptides (Fig. 10, Supplementary Fig. 2). In *M. galloprovincialis*, the DH44 gene is localized in LG12 and is composed of only one exon. In contrast, the DH44 precursor in *D. melanogaster* is composed of 3 exons and only exon 3 encodes the peptide. The *M. galloprovincialis* DH44 precursor encodes two mature peptides (DH44a and DH44b), which are likely released by proteolytic cleavage. In contrast, in the gastropods the DH44 precursor is very different and contains a single peptide with a highly divergent sequence from the DH44 peptide in other Mollusca (De Oliveira et al., 2019) and is known as Egg Laying Hormone (ELH) since it stimulates ovulation and egg laying in *A. californica* and *L. stagnalis* (Ebberink et al., 1985; Morishita et al., 2010; Vreugdenhil et al., 1988). The function of the Mollusca DH44/ELH peptides in other molluscs is less studied but in the Sydney Rock oyster (*Saccostrea glomerata*) it stimulates spawning (In et al., 2016) and recently the receptor-ligand pair was explored in *C. gigas* and shown to be involved in bivalve reproduction (Favrel et al., 2024). No orthologues of the DH44/ELH precursors or mature peptides have been identified in cephalopods (Zatylny-Gaudin et al., 2016)

1.9. The Mollusca Cluster B receptor system

Cluster B receptors were recently identified in protostomes as a group of receptors that share a common origin with the deuterostome GPS-group (Cardoso et al., 2014b, 2020b) (Supplementary Fig. 1C). Characterization of Family B1 GPCRs in tunicates and cephalochordates identified putative PTHR and PACAP/GCG-receptors in an invertebrate, suggesting they emerged after the protostome-deuterostome divergence (Cardoso et al., 2006; On et al., 2015). However, many authors continue to designate members of Cluster B as the protostome PTHRs (p-PTHRs). In Mollusca, this family is highly diverse and numerous species-specific duplicates were identified in our searches. However, to date the receptors remain orphans, as their cognate peptide ligands remain to be identified.

The PACAP peptide is the ligand for the vertebrate PAC_1/VPAC receptors and is one of the best studied neuropeptides and its existence has been described in several Mollusca species (Cardoso et al., 2010, 2020c). PACAP-like cDNA and peptide fragments have been described in the brain of the gastropods the garden snail (*Helix pomatia*) and the pond



B)

>Mga_VDI02572.1

MNLNGIHIVLVFVSAFVVCKAMYLPNEDDDLQSDNTETGSEYRGGDISLQ*KRR***GLSVRGDLRALARML**
RFQQNKRRQGQTSVLPSHTQLIGLG*KR*NDLTVADDEGNRIKRLQDAFNSVI*KR***QRLSINGALHSLSDM**
LASSGRTRLSEEVSSNKNRLMHLG*RK*

a

b

C)

```
Hsa_NP_000747        SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII-----amide
Dme_AAF5442          -NKPSLSIVNPLDVLRQRLLLEIARRQMKENSRQVELNRAILKNV-amide

Mga_VDI02572.1a      ---RGLSVRGDLRALARMLRFQQNKRRQGQTSVLPSHTQLIGL---amide
Mga_VDI02572.1b      ---QRLSINGALHSLSDMLASSGRTRLSEEVSSNKNRLMHL-----amide
Cgi_FQ667900_a       ---GRLSLTADLRSLARMLEAHRKRFIASRFPYDSIRKKLFRY---amide
Cgi_FQ667900_b       ---QRLSVNGALSSLADMLAANGRQRMMSEMAMNRQRLFGL-----amide
Lgi_XP_009066138.1   ---SRLSINQELKSLANLLVLRENKRREAQKTKLRSKLLSI-----amide
                          :*:   :  *   *             .     :
```

Fig. 10. The bivalve DH44 precursor. A) Gene structure of the Mediterranean mussel (*M. galloprovincialis*) DH44 precursor, the orthologue in the genome of the fruit-fly (*D. melanogaster*) and the human CRH precursor. The gene structures were deduced by searching the deduced mature protein precursor of each species against the latest version of the mussel (MgalMED), fruit fly (Release 6 plus ISO1 MT) and human (GRCh38.p14) genome assembly. Exons are represented by boxes and the lines indicate the introns and the localization of the mature peptides inside the exons is indicated. **B)** Deduced sequence of the DH44 peptide precursors in the mussel. The localization of the deduced mature peptides (a and b) is highlighted in colour and the proteolytic cleavage sites are in italics. **C)** Multiple sequence alignment of the Mollusca mature DH44 deduced peptides, with the human and fruit-fly orthologues. Conserved aa are in bold and the Mollusca, insect and human mature peptides are C-terminal amidated. The complete and annotated precursor sequences for the Mollusca neuropeptides are available in Supplementary Fig. 2. Hsa- *Homo sapiens*, Dme- *Drosophila melanogaster,* Mga- *Mytilus galloprovincialis*, Lgi- *Lottia gigantea,* Cgi- *Crassostrea gigas.*

snail (*L. stagnalis*) (Kiss and Pirger, 2013; Pirger et al., 2010, 2016). However, there are many studies that have used antisera prepared against the vertebrate peptides PACAP, VIP, GCG and PTH to identify orthologues in molluscs and caution is required in the interpretation of results based on the use of heterologous antisera. For example, in the bivalve *M. galloprovincialis* VIP-like immunoreactivity was detected in the mantle. In the sea hare (*Aplysia kurodai*), snail (*H. pomatia*) and *L. stagnalis* VIP-like immunopositive cells were detected in the nervous system and in innate immune cells of two freshwater snails (*Planorbarius corneus* and *Viviparus ater*) (Kuramoto et al., 1985; Licata et al., 2003; Ottaviani et al., 1992) (Schot et al., 1981). GCG/GLP-like and SCT-like immunoreactivity was detected in immune cells of two freshwater snails (Ottaviani et al., 1992; Ottaviani and Cossarizza, 1990) and PTH-like proteins were detected in snail neuronal tissues using antibodies raised against the vertebrate peptides (Hull et al., 2006; Wendelaar Bonga et al., 1989). To date no molecular evidence exists demonstrating the existence of the genes for these peptides in molluscs raising questions about the previous results obtained with heterologous antisera (Cardoso et al., 2020c).

Recently, the insect Cluster B (named iPTHR (Li et al., 2013),) was deorphanized in the flour beetle, by a peptide that has no sequence orthologue in vertebrates (Xie et al., 2020). Gene knock-down studies revealed that receptor ablation resulted in defects in wing exoskeleton maturation and the fecundity of the flour beetle *Tribolium castaneum* (Xie et al., 2020). The peptide activating iPTHR was previously named PXXX-amide (where X represents any amino acid) and was first found in the cephalopod *Sepia officinalis* supra esophageal mass and optic lobe tissues by mass spectrometry, and in the flour beetle the orthologue was designated insect parathyroid hormone (iPTH). Orthologues of *T. castaneum* iPTH are absent from Diptera and Lepidoptera genomes but are present in other arthropod taxa and the peptide is the cognate ligand of the two *T. castaneum* Cluster B receptors. To date, no functional studies of the Cluster B system has been described in Mollusca. However, sequence similarity searches in the present study uncovered several

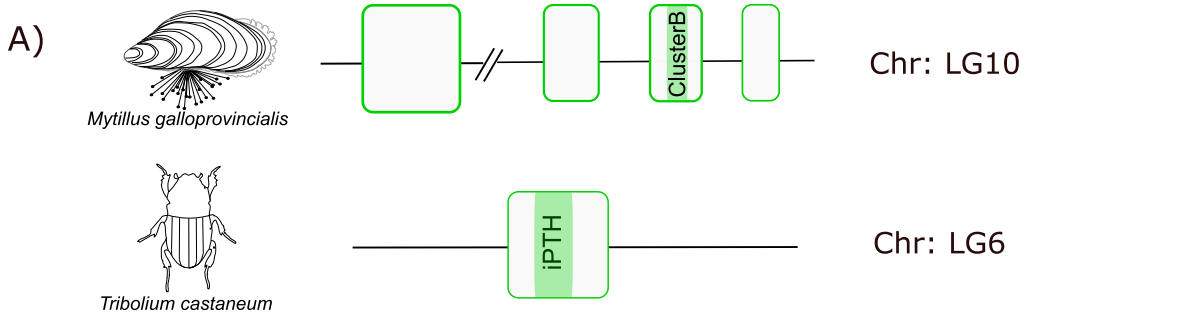
orthologues of the recently identified insect peptide precursor. The identified Mollusca PXXX-amide gene encoded a putative mature peptide precursor, and this raises the intriguing possibility that it may be the cognate ligand of members of the Mollusca Cluster B receptors (Fig. 11, Supplementary Fig. 2). The Mollusca sequence orthologues shared 37–50% sequence identity with the *T. castaneum* iPTH peptide and had five conserved amino acid positions and an amidated C-terminus. In the bivalve *M. galloprovincialis* genome the peptide precursor mapped to chromosome LG10 and the gene is composed of 4 exons, with the mature peptide being encoded by exon 3. This differs from the structure of the beetle *T. castaneum* iPTH gene, where only one exon was found (Fig. 11). In common, with the *T. castaneum* iPTH and insect precursor in general, the Mollusca precursor encoded a single peptide. However, a point to note is that many Cluster B receptor members are present in Mollusca and although one-to-many peptide-receptor interactions can exist it seems likely that there are other putative bioactive peptides that remain to be identified and their function tested.

1.10. Call for a common invertebrate GPCR nomenclature

The official nomenclature for the vertebrate Family B1 GPCRs was established by the International Union of Pharmacology (IUPHAR) (Alexander et al., 2017). However, no official nomenclature recommendations have been proposed for the invertebrate orthologues and it is urgent to remedy this situation. The recently described arthropod PTHR (iPTHR) and the activating peptide PTH (iPTH), that are proposed to be the counterparts of the vertebrate PTH system (Li et al., 2013; Xie et al., 2020), are incorrectly annotated. The preceding example coupled to the increase in data availability and the potential of non-vertebrate GPCRs as interesting targets for control highlights the need for a coherent and ratified nomenclature.

In vertebrates, PTH is a polypeptide synthesized and cleaved in the parathyroid gland into an active 34 amino acid form and it is best known for its involvement in calcium metabolism, acting on the bone and kidneys and indirectly on the intestine (Potts, 2005; Rejnmark and Ejlsmark-Svensson, 2020). The vertebrate PTH peptide is part of the PTH-peptide family which includes other sequence and structure related peptides (Dettori et al., 2023; Geara et al., 2010; Schlüter, 1999). These peptides activate two types of receptors PTH1R and PTH2R in mammals and other vertebrates and a further receptor, PTH3R, in non-mammalian vertebrates (Gensure et al., 2005; Pinheiro et al., 2012; Rubin and Jüppner, 1999; Usdin et al., 1995). In invertebrate deuterostomes, sequence orthologues of the vertebrate receptors and peptides have been identified and their function characterized (Mirabeau and Joly, 2013; On et al., 2015; Pinheiro et al., 2012; Suarez-Bregua et al., 2017).

The arthropod "PTH peptide" needs to be reassigned, it is unrelated in sequence and structure to the vertebrate and cephalochordate PTH. Global evolutionary studies based on sequence comparisons and phylogeny of Family B1 GPCRs including the deuterostome PTHR and other protostome and deuterostome receptor subfamilies indicate the deuterostome PTHR clade shared a common origin with the PAC/VPAC clade and GCGR/GIPR clades. Moreover, the three deuterostome



B)

>Mga_VDI22276.1
MSDSFKQLSHICCHVNGRSSSFRTVCSHFFLQTSDTVFDWSLEVYIASIRGIANVPIASRINRKKSINIE
KSKLIFHLLLNLDLRKTNSFPEKMNMKQNTTSIIVAVTILLACVVQARYTDDDLDRFE*KR*TSSDQRIAEL
QALIALSRGSGIVGHGRIDPYGIG*RR*KRDTGILRTLINKNRLLQNSDDSHL

C)

```
Tca_TC015123          -VSDAHLADLQSRIALNNKLKGVSVTMPVGGGRIDPGRI-amide
Ame_XP_026300078.1    -ISDQMLAEADTRSGLSNVKG-MVKTVPVGFGVFNLEQI-amide
Mga_VDI22276.1        TSSDQRIAELQALIALSRGSG------IVGHGRIDPYGI-amide
Lgi_XP_009051665.1    SGADQRIAELQALIALTSHGG------MVAHGQFDPLRI-amide
Pca_XP_025088440.1    SSSDQRIAELQALLALTGNGG------RVAHGQFDPLRI-amide
Cgi_XP_019920821.1    TSSDQRIAELQALIALVHGRG------NVAHGQLDPALI-amide
Obi_XP014776777.1     TSSDQRIAELKALLAMSR---------VVGHGQIDVDAI-amide
                        :*  :*: .:  .:           *. * ::   *
```

Fig. 11. The putative bivalve Cluster B receptor activating peptide. A) Gene structure of the Mediterranean mussel putative cluster B receptor activating peptide (LG10) and the orthologue in the genome of the flour beetle *Tribolium castaneum* (LG6). The gene structures were deduced by searching the species deduced mature protein precursor against the latest version of the mussel (MgalMED) and flour beetle (Tcas5.2). Exons are represented by boxes and the lines indicate the introns and the localization of the mature peptides inside the exons is indicated. **B)** Deduced sequence of the mussel peptide precursors. The deduced mature peptide is highlighted with colour and the proteolytic cleavage sites are in italics. **C)** Multiple sequence alignment of the Mollusca mature peptides with the *T. castaneum* orthologue. Conserved aa are in bold and the insect and mollusc peptides are C-terminal amidated. The complete and annotated precursor sequences for the Mollusca neuropeptides are available from Supplementary Fig. 2. Tca- *Tribolium castaneum,* Mga- *Mytilus galloprovincialis*, Cgi- *Crassostrea gigas*, Lgi- *Lottia gigantea*, Pca- *Pomacea canaliculata*, Obi- *Octopus bimaculoides.*

receptor groups (GPS-group) are sister clades of the protostome Cluster B group, and they have all emerged from a single ancestral receptor gene prior to the protostome and deuterostome radiation (Cardoso et al., 2014b, 2020b, 2020c) (Fig. 12). We propose that the protostome receptors previously designated iPTHR but assigned in our phylogenetic analysis to the Cluster B group, should be designated Cluster B GPCRs, and the activating peptides as cluster B peptides and this highlights the need for robust evolutionary phylogenetic analysis before naming invertebrate receptors.

Establishing a naming convention for invertebrate GPCRs is an imperative for both scientific and socioeconomic reasons. From a scientific perspective the Worlds biodiversity is mainly non-vertebrate and larger parts of it are being sampled. Furthermore, the falling cost of sequencing has resulted in a massive increase in the number of non-vertebrate organisms with their genomes and transcriptomes sequenced. This with the "global open access data policy" means that sequence data is becoming increasingly open and available to the public and is increasingly scrutinized and analysed. In the case of invertebrates and their role as causal factors of crop loss, disease, and a suite of other undesirable but also desirable characteristics means there is increased interest in identifying non-pesticide/non-transgenic-based approaches for their control (Bass et al., 2014; Lu et al., 2010; Scharf et al., 2022; Simon and Peccoud, 2018). The importance of GPCRs in regulating myriad biological processes essential for survival and their presence in all invertebrates makes them of interest as "druggable targets" (Sriram and Insel, 2018) and reinforces the need for rigorous characterization, including naming, of GPCRs (Audsley and Down, 2015; Birgül Iyison et al., 2021).

Additional arguments for a consolidated nomenclature for GPCRs in Mollusca but also other invertebrates come from the growing need to monitor the impact of environmental change on ecosystems during the Anthropocene. Human activities and the release of industrial and domestic effluents worldwide is having a significant impact on the environment and the organisms that live in them (Persson et al., 2022). The effect of endocrine disruptors (EDs), compounds that interfere with hormonal systems, is well recognised (Crane et al., 2022; La Merrill et al., 2020). A high-profile case for the Mollusca was tributyltin (TBT) an organotin that was used as an antifouling agent in paints for boats and marine structures. This chemical was found to cause imposex in many marine gastropods, more specifically female sea snails developed male sex organs with devastating consequences (Castro et al., 2007). The development of imposex and related conditions was linked to abnormal modulation of the retinoid X receptor (RXR, a nuclear receptor), when it interacted with TBT (Castro et al., 2007). There is a vast literature about ED chemicals and their action in vertebrates and although less common in invertebrates it is primarily focussed on the effects triggered by disruption of nuclear receptors (i.e. steroid hormone disruptors) (Le Ferrec and Øvrevik, 2018). However, taking into consideration the importance of GPCRs as targets for human therapeutics, and as targets for pesticides it seems likely that such chemicals are already present in the environment and are likely to bind to GPCRs making them toxicological targets. To detect and understand the effects on Mollusca GPCRs of environmental EDs, much more work is required to characterize their function. The Mollusca may be particularly at risk from GPCR EDs since they are often found in contaminated coastal areas and bioaccumulate toxins, which is why they are often used as bioindicator organisms (Oehlmann and Schulte-Oehlmann, 2003). The diversity of Mollusca and their habitats makes comparative studies of them essential if robust risk assessment procedures are to be established and a well-established nomenclature system and correct assignment of GPCRs and their cognate ligands is crucial.

2. Conclusion

Members of the five metazoan Family B1 GPCR subfamilies were identified in Mollusca and found to display far higher diversity than that described for other metazoans. The origin and evolution of the numerous GPCRs in the Mollusca appear to be the result of lineage and species-specific evolutionary events which may have been driven in part by their speciation and associated adaptation as their range expanded. This includes a new receptor PDFR clade described here for the first time and only found in the genome of the Mollusca representatives that were the targets of this study.

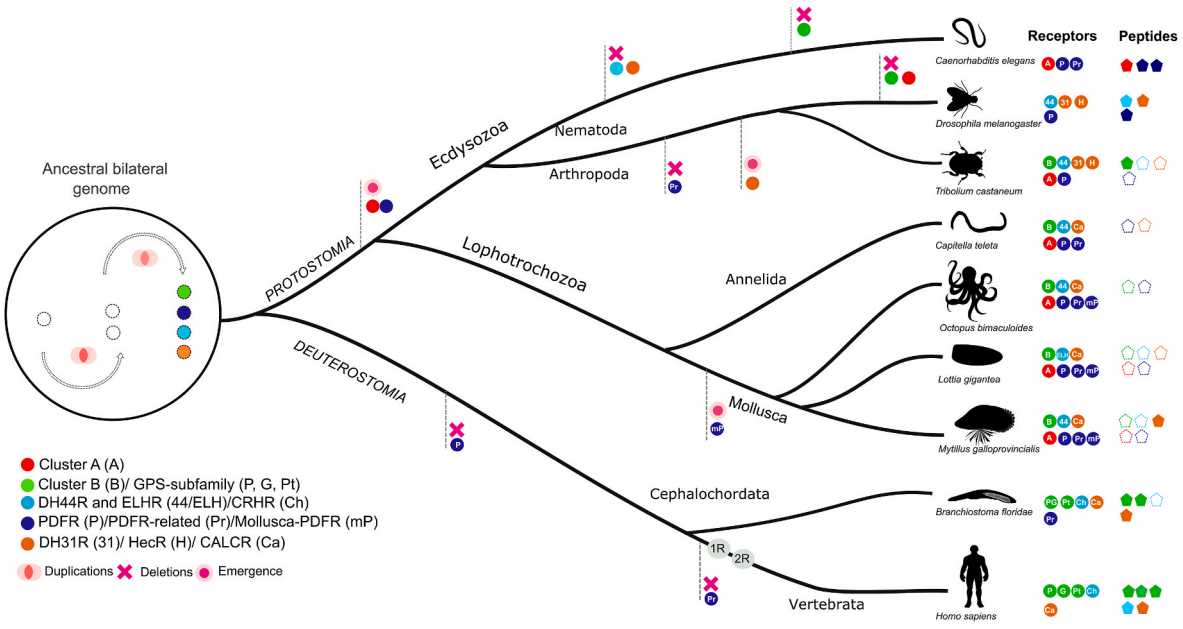


Fig. 12. Proposed model for the metazoan Family B1 GPCRs system evolution. All receptors are likely to have descended from a unique bilaterian ancestral gene, by a process of gene duplication prior to the protostome-deuterostome split. Whether the receptor peptide ligands emerged from a common ancestral gene, remains unclear. Circles represent receptors and pentagons represent the neuropeptide precursors. The full pentagons indicate the mature peptide that were used to test the function of the receptors. Dashed pentagons indicate that the peptide precursor sequence has been isolated but the mature peptide has not yet been tested for function with the cognate receptors. The potential receptors and ligands pairs are indicated in the same colour. The Mollusca neuropeptides were obtained during this study (Supplementary Fig. 2), the others were obtained from (Mirabeau and Joly, 2013). The figure was modified from (Cardoso et al., 2020c).

Putative activating peptides, orthologues of those described in other protostomes, as well as in deuterostomes, have been described for some of the GPCRs identified in Mollusca but the number of peptide precursors is fewer than the potential interacting GPCR partners. In molluscs the peptide precursors PDF, CALC and DH44/ELH, which are orthologues of peptides identified in the Ecdysozoan and vertebrates exist, and in this study, we uncovered the genes of the potential peptide precursors and mature peptides for Cluster A and Cluster B receptors. The deduced putative peptide precursors encode for one or two peptides suggesting promiscuity in ligand-receptor interactions may exist and that peptides are likely to activate more than one receptor, as was recently described in *C. elegans* (Beets et al., 2022).

In Molluscs, as well as in other Lophotrochozoan, many Family B1 GPCRs potentially involved in the regulation of key physiological processes exist but their function remains mostly unknown, and for the large majority no putative ligands have been assigned. Our study confirmed that the Family B1 system is likely to have emerged prior to the protostome-deuterostome divergence and so ligand-receptor pairs were probably established very early during metazoan evolution (Fig. 12). In bilaterian genomes the receptors descended from a unique ancestral gene by gene duplication however their peptide ligands are likely to have had a serendipitous origin.

CRediT authorship contribution statement

João C.R. Cardoso: Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization. **Jennifer C. Mc Shane:** Writing – review & editing, Visualization, Methodology, Data curation. **Zhi Li:** Writing – review & editing, Visualization. **Maoxiao Peng:** Writing – review & editing, Visualization. **Deborah M. Power:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data used in this manuscript is available in the supplementary materials or are available in public databases.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2024.112192.

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