Distinct Expression Patterns of Glycoprotein Hormone Subunits in the Lophotrochozoan Aplysia: Implications for the Evolution of Neuroendocrine Systems in Animals

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Glycoprotein hormones (GPHs) comprise a group of signaling molecules critical for major metabolic and reproductive functions. In vertebrates they include chorionic gonadotropin, LH, FSH, and TSH. The active hormones are characterized by heterodimerization between a common α and hormone-specific β subunit, which activate leucine-rich repeat-containing G protein coupled receptors. To date, genes referred to as GPHα2 and GPHβ5 have been the only glycoprotein hormone subunits identified in invertebrates, suggesting that other GPHα and GPHβ subunits diversified during vertebrate evolution. Still the functions of GPHα2 and GPHβ5 remain largely unknown for both vertebrates and invertebrates. To further understand the evolution and putative function of these subunits, we cloned and analyzed phylogenetically two glycoprotein subunits, AcaGPHα and AcaGPHβ, from the sea hare Aplysia californica. Model based three-dimensional predictions of AcaGPHβ confirm the presence of a complete cysteine knot, two hairpin loops, and a long loop. As in the human GPHβ5 subunit the seatbelt structure is absent in AcaGPHβ. We also found that AcaGPHα and AcaGPHβ subunits are expressed in larval stages of Aplysia, and we present a detailed expression map of the subunits in the adult central nervous system using in situ hybridizations. Both subunits are expressed in subpopulations of pleural and buccal mechanosensory neurons, suggesting a neuronal modulatory function of these subunits in Aplysia. Furthermore it supports the model of a relatively diffuse neuroendocrine-like system in molluscs, where specific primary sensory neurons release peptides extrasynaptically (paracrine secretion). This is in contrast to vertebrates and insects, in which releasing and stimulating factor from centralized sensory regions of the central nervous system ultimately regulate hormone release in peripheral glands. (Endocrinology 153: 5440–5451, 2012)
date endostyle have been homologized with the vertebrate thyroid gland (reviewed in Refs. 1–4). Still, the Hatcheck’s pit and the neural gland receive chemical cues directly from the aquatic environment and therefore fulfill sensory-receptive functions in addition to their endocrine function (4). This is in clear contrast to the vertebrate HPP axis in which sensory and endocrine functions are spatially separated.

Vertebrate hormones, such as thyroid hormones and steroid hormones, have known functions in development and metabolism of invertebrate species (reviewed in Refs. 2, 5 and 6). Still, with the exception of arthropods, in particular insects (7), invertebrate endocrine systems are poorly understood in comparison with vertebrates, and the mechanistic basis for such hormonal actions in the large majority of invertebrate species remains unknown (5). Moreover, stimulating and releasing hormones from the vertebrate HPP axis generally do not have recognized homologs in classical invertebrate models such as the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, two taxa with highly derived genomes. Still, homologous genes coding for these factors have been identified in sequenced genomes of cnidarians, arthropods, molluscs, nematodes, echinoderms, and ascidians (reviewed in Refs. 5 and 6 and 8–11). Analyses of tissue specificity and putative synthesis and signaling pathways of steroid, thyroid, and glycoprotein hormones in ascidians suggest that a small subset of these hormones may have been used in ancestral bilaterians and then diversified in vertebrate lineages (6).

Glycoprotein hormones (GPHs) are heterodimers consisting of α- and β-subunits that bind to leucine-rich-repeat G protein-coupled receptors (LGRs). Specifically, GPH heterodimers bind to the large N-terminal extracellular (ecto) domain containing leucine-rich repeats (12, 13). In contrast to the common α-subunit, the β-subunit gives the hormones their specificity among vertebrates (14–16), and four such β-subunits have been identified. These genes code for chorionic gonadotropin (CG), LH, FSH, and TSH and appear to be absent from sequenced invertebrate genomes. However, two peptides related to vertebrate GPHs have recently been annotated from the fruit fly genome and were named GPHα2 and GPHβ5 after subsequent identification from humans [GPA2 and GPB5 (17)]. These two subunits represent a second α- and fifth β-subunit of the GPH family, and subsequent studies confirmed the presence of α- and β-subunits in several other invertebrate genomes including ascidians (6), echinoderms (8), and cephalochordates (18–20). Both subunits can be distinguished from GPHα1 and previously described GPHβ (i.e. CG, LH, FSH, TSH) subunits based on their phylogenetic position and specific structural characteristics such as the lack of a seatbelt structure (17, 21, 22). Together, these data suggest that all vertebrate GPHs evolved from single GPHα and GPHβ ancestral genes and that the diversification we observe in vertebrate GPH loci is likely a consequence of several gene duplication events during chordate evolution (21, 23).

Physiological functions of GPHα2 and GPHβ5 remain controversial. Nakabayashi et al. (21), for example, showed that GPHα2 and GPHβ5 heterodimerize and activate the human TSH receptor. Based on their findings, they named the GPHα2/GPHβ5 heterodimer thyrostimulin. However, functional genetic studies in mice on GPHβ5 provided very little evidence for thyroid hormone axis phenotypes (24, 25). Intriguingly, new data from Sun et al. (26) suggest a paracrine action of GPHβ5 in the mammalian ovary providing an interesting link between the reproductive and the thyroid hormone axis. Studies on the orthologous GPHα2/GPHβ5 thyrostimulin from *Drosophila* also revealed that the heterodimer is expressed throughout development and activates a leucine-rich repeat G protein-coupled receptor (DLGR1), the closest fly ortholog of TSH receptor (27, 28).

Here we provide structural and phylogenetic evidence for sequence homologs of GPHα/GPHβ subunits from the sea hare *Aplysia californica* (AcaGPHα and AcaGPHβ), a molluscan model system for neurogenomics and the molecular basis for learning and memory (29). Furthermore, our phylogenetic analysis of GPH loci from 13 animal genomes confirms a bilaterian origin of these subunits and provides new insights into the evolutionary history of the GPH gene family. Quantitative RT-PCR (qRT-PCR), transcriptome analysis using next-generation sequencing and *in situ* hybridization (ISH) reveal expression patterns of AcaGPHα and AcaGPHβ in the central nervous system (CNS) as well as during development. Based on the distribution of GPHα and GPHβ glycoprotein-related neuropeptides in subpopulations of mechanosensory neurons of the *Aplysia* CNS, we conclude that these peptides, at least in part, may be involved in the modulation of sensory input in gastropod molluscs.

### Materials and Methods

#### Molecular cloning

We amplified full-length AcaGPHα and AcaGPHβ (These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession nos ACN32202 (for GPHα) and AAX35673 (for GPHβ); note that these subunits are identified as AcaGPHα2 and AcaGPHβ5 in the databases but based on the analysis presented in this paper the nomenclature was changed to GPHα and GPHβ, respectively) from normalized *Aplysia* CNS libraries using specific primers against candidates in an anno-
tated expressed sequence tag (EST) collection of neuronal transcripts (see additional details in Ref. 29). AcaGPHα and AcaGPHβ were cloned into pCR4-TOPO vector (catalog no. K4575–02; Invitrogen, Carlsbad, CA).

Three-dimensional (3D) structure reconstruction

We used DeepView/Swiss-PDBViewer (v.3.7) for 3D reconstructions of GPHs. Sequences were aligned with ClustalX (v. 1.83) and manually adjusted. We used the crystal structure of human (h) choric gonadotropin (hCG; IHRP_B; GenBank accession no. 809146) as a template for 3D reconstruction of *Aplysia* GPHβ and human GPHβ5. We then imported the crystal structure file into DeepView/Swiss-PDBViewer and adjusted the alignment manually based on the positions of conserved cysteine residues in the sequence. Final optimization of the model was done on the SWISS-MODEL server at http://swissmodel.expasy.org/. Note that the sequence similarity between the vertebrate hCG a1 subunit and *Aplysia* a2 subunit was too low to allow a reliable 3D reconstruction. The .pdb file with the modeled 3D structure for AcaGPHβ is available electronically as Supplemental Fig. 1 published on The Endocrine Society’s Journals online web site at http://endo.endojournals.org.

Phylogenetic analysis and genomic organization of AcaGPHα and AcaGPHβ subunits

Phylogenomic analyses were conducted using an in-house bioinformatics pipeline that consisted of the following steps. First, protein sequences from AcaGPHα and AcaGPHβ, together with eight other well-characterized vertebrate and insect GPH sequences, were used as queries in BLAST (30) searches of predicted gene sets from whole genome sequences of 13 phylogenetically informative taxa (Supplemental Tables 1 and 2). Searches were conducted with an Expect value of 0.01, and the 20 highest scoring sequences were retained from each gene model (Supplemental Table 2). Next, sequences were concatenated into a single file, and redundant sequences were removed. Sequences were then aligned under the “auto” setting in MAFFT (31). Spurious sequences were removed using trimAl (32) with the settings “resoverlap” (0.75) “seqoverlap” (80), and the alignment was repeated as before. Regions of low scoring alignment were removed using the automated heuristic also in trimAl (32). Finally, the best-fit model of molecular evolution was determined using Prottest (33).

Preliminary phylogenetic analyses of the resulting dataset revealed a clade including bursicon, gremlin, and suppression of tumorigenicity (SOT) genes that is the sister group to the GPHα and GPHβ gene family. This non-GPH clade was used as the outgroup in all subsequent phylogenetic analyses. We conducted analyses using both maximum likelihood (ML) and Bayesian Monte Carlo Markov Chain (BMCMC) approaches (Supplemental Fig. 2). ML analyses were conducted in RaXML (34) under PROTGAMMAWAG (Supplemental Fig. 3). Nodal support was assessed by 1000 bootstrap replications. BMCMC analyses were conducted using the NCBI trace archive and publicly available *Aplysia* genome browser at http://genome.ucsc.edu/.

Transcript quantification experiments

Relative expression levels for *Aplysia* GPHα and GPHβ during development were measured using qRT-PCR. We extracted RNA from seven developmental stages using an RNAqueous micro kit (Ambion, Inc., Austin, TX). Total RNA was quantified using a Nanodrop ND1000 (Thermo Scientific, Rockford, IL), and quality was assessed using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). We used the kit’s DNase treatment protocol to eliminate potential contamination with genomic DNA. cDNA synthesis was performed using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen), following the manufacturer’s recommendations. Random hexamers were used as primers. qRT-PCRs were prepared using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), following the manufacturer’s recommendations. Reactions were prepared in triplicate and run in an ABI PRISM’s 7000 Sequence Detection System (Applied Biosystems). Relative abundance levels were calculated by the ΔCt method (36), using the ribosomal protein S5 transcript (Forward primer, RPS5F: reverse primer, RPS5R) as an endogenous control gene (37) and gastrulation as a reference stage from which relative expression levels were calculated as outlined by Ref. 36. The choice for gastrulation as a reference stage was based on the fact that this stage showed the lowest expression levels (lowest Ct value). GPH primers used for this experiment were as follows: AcaGPHα forward primer (GPBF) CGGCCATACATCGCCTAGTC and reverse primer (GPBR) CACTCAGCCCCTGAGGTAATG; AcaGPHβ forward primer (GPBF) CGACTGTCGGATTCCGAT and reverse primer (GPBR) GTGGCACGCTTCCGGTAT. Primer concentrations were optimized using serial dilutions and were used at 300 μM in the final experiment, following manufacturer’s instructions (Applied Biosystems). Amplification plots were visually inspected for a single amplicons.

We also used earlier RNA-sequencing experiments (see Ref. 38 for details) and obtained complementary relative abundance levels for AcaGPHα and AcaGPHβ subunit mRNA across different neuronal populations. This approach not only allows us to assess relative expression levels but also compare them to other relevant transcripts expressed in these cells. Details about RNA isolation and RNA-seq experiments are described elsewhere (29, 38). All experiments were performed in duplicate.

Animals, tissue preparation, and ISHs

All procedures involving live animals were performed in accordance with ethical guidelines. Adult *A. californica* were obtained from the National Resource for *Aplysia* at the University of Miami. Animals were anesthetized by injection of 50% volume/body weight) isotonic MgCl₂ (337 mM) before surgical removal of the CNS. Ganglia were removed and incubated in 1% HEPES; pH 7.6) at 34 °C for 20–45 min to soften the connective tissue of the neuronal sheath. For details on CNS preparation and fixation, see Ref. 39. For ISH using CNS samples, we followed protocols previously described by Jezzini et al. (39). Some important modifications are further described below.

**In situ probes**

We used 7 μl of template (cleaned and cut plasmid prep) with 1 μl of 10 × RNA polymerase buffer, 1 μl of DIG [DIG RNA
Labeling Kit (SP6/T7) Roche; catalog no. 1175025]rsqb], 1 µl Sp6/T7/T3 RNA polymerase, and 1 µl of RNAsin and incubated for 3.5 h at 37 °C. We then added 1 µl of DNase I (NEB M0303S) and incubated for 15 min at 37 °C. After digestion we added 1.5 µl of 7.5M LiCl2 and 50 mM EDTA solution plus 38 µl of 100% EtOH, mixed gently and kept at −20 °C overnight for precipitating the RNA. The next morning we spun the reaction for 20 min at 15,000 rpm at 4 °C and removed the supernatant. We resuspended the RNA. The next morning we spun the reaction for 20 min at 15,000 rpm at 4 °C and removed the supernatant. We resuspended the RNA.

McGill and embryonic stages were reared from egg masses at the University of Guelph (Ontario, Canada). Details on rearing techniques are reported in Ref. 37. We also performed one experiment with sense probe and did not find any staining of cells above background levels.

Results

AcaGPHβ shows low overall sequence similarity to vertebrate subunits but is structurally conserved

AcaGPHα encodes a 129-amino acid protein with homology to fruit fly and human α-subunits, and AcaGPHβ encodes a 127-amino acid protein homologous to fruit fly and human β-subunits (Fig. 1). Sequence similarity between AcaGPHα and human α2 subunits is 25% and 17% between AcaGPHα and human α1. Sequence similarity between AcaGPHβ and human β5 subunit is 28% and 18% between human TSH β5 (all values expressed as exact matches). Although these values are low, structural cysteine residues are well conserved in both subunits (see also Fig. 2). Based on the manual alignment presented in Fig. 1, we predict that AcaGPHβ only forms four of the six disulfide bridges usually found in other GPHβ subunits (Figs. 1A and 1B). Note that a comparable structure is predicted for human GPHβ5 subunit. Cysteine residues contributing to four disulfide bridges appear to be largely conserved between AcaGPHα and human GPHα2 subunits (Fig. 2).

We used the published crystal structure of hCG β subunit (Swissprot no. P01233; PDB no. 1HCC) as a template to reconstruct the putative 3D structure of the AcaGPHβ subunit. Based on this structure (Fig. 2A) we modeled the 3D structure of both, AcaGPHβ (Fig. 2B) and human (Fig. 2C) GPHβ5. In hCG (Figs 1A and 2B) the heterodimer between α- and β-subunit is stabilized by a seatbelt structure (reviewed in Ref 16). This structure is buckled by a disulfide bridge formed between cysteine residues 3 (positions 26 and 110) marked in Fig. 1 and shown in Fig. 2A. Note that these cysteine residues are missing in AcaGPHβ (B) and human GPHβ5 (C) and it is therefore assumed that a seatbelt and buckle structure is not formed in these subunits. Still, as confirmed from the alignment presented in FIG. 1. A, Amino acid sequence of the newly identified A. californica GPHβ (AcaGPHβ) subunit and B) AcaGPHα subunit with other GPH subunits. For AcaGPHβ (A) only four of the six disulfide bonds found in human hCG (HomohCG) β-subunit are conserved in Aplysia, Drosophila, and human GPHβ5. Numbers in the header row indicate cysteines involved in disulfide bonds for human hCG. Stars indicate conservation of these cysteines across all species aligned. Note that there are six predicted disulfide bridges in human GPHβ8 subunits (here represented by hCG) and five in human GPHα1 subunit. Dashed lines in the footrow indicate hairpin loops (HP1 and HP2) for GPHβ5. Solid line with arrows marks the position of the long loop (LL) for GPHβ5. Shading marks degree of conservation of residues. Note that conservation between GPHα2 and GPHα1 was too low to predict specific structural features from the alignment (B). HomohCG, Homo sapiens hCG: Uniprot, P01233; HomoGPH5, H. sapiens glycoprotein β 5 subunit: Uniprot, Q86YW7; Drosophila GPHβ5, D. melanogaster GPH β 5 subunit: Uniprot, Q86LTE; HomoGPHα1, H. sapiens GPH α subunit: Uniprot, P01215; Drosophila GPHα2, D. melanogaster glycoprotein α 2 subunit: Uniprot, Q56L88; HomoGPHα2, H. sapiens GPH α 2 subunit: Uniprot, Q96T91; AcaGPHα, A. californica GPH β subunit; AcaGPHα, A. californica GPH α subunit.
Fig. 2. The 3D reconstruction of AcaGPHβ subunit confirms conserved GPH structure and reveals putative new structural elements. A, We used the crystal structure of hCG β subunit (A: hCG: Uniprot P01233; PDB 1HCN) and aligned the AcaGPHβ (B) sequence manually to it in PYMOLTM v. 0.99rc6. The same method was used to model human Thyrostimulin β subunit (C) (HsGPHβ5: Uniprot Q86YW7), an ortholog of the AcaGPHβ subunit. Both hairpin loops (HP1 and HP2) as well as the long loop (LL) appear to be structurally largely conserved in Aplysia with the exception that the model used predicts an α-helix in hairpin loop 1 potentially involved in receptor and/or α-subunit interaction. Arrows point to disulfide bonds (indicated by yellow cylinders) that form the cysteine knot. Both proteins are predicted to form four disulfide bonds. In hCG (A) the heterodimer between α- and β-subunit is stabilized by a seatbelt sequence. This structure is buckled by a disulfide bridge formed between cysteine residues 3 (positions 26 and 110) marked in Fig. 1 and shown in panel A. Note that these cysteine residues are missing in AcaGPHβ subunits (B) and human GPHβ5 (C), and it is therefore assumed that a seatbelt and buckle structure is not formed.

Fig. 1, AcaGPHβ forms a total of four disulfide bridges. Other structural elements of AcaGPHβ appear to be well conserved including two hairpin loops and one long loop (Fig. 2). Based on information from the Aplysia EST database (29) and the emerging Aplysia genome sequencing data (http://genome.ucsc.edu/) AcaGPHα and AcaGPHβ are the only members of the GPH family in the Aplysia genome. The AcaGPHα has a gene length of 5481 bp with three exons whereas AcaGPHβ has a gene length of 13,353 bp and two exons as summarized in Supplemental Fig. 4.

Phylogenetic analysis

Our phylogenetic analysis provides a detailed picture of the evolutionary history of the GPH gene family (Fig. 3). Despite our inclusion of a broader phylogenetic diversity of taxa than prior explorations of GPH phylogeny, our findings are largely consistent with previous studies (17, 18, 23, 40–42). First we recover strongly supported GPHα and GPHβ clades comprised of genes from each of the bilaterian taxa included in the study. The resulting tree topology supports the hypothesis that GPHα and GPHβ gene families originated from a single gene duplication that occurred before the evolution of bila-
teria. Our phylogenomic pipeline contained whole-genome data from four nonbilaterian taxa including the sponge, Amphimedon queenslandica, the placozoan Trichoplax adhaerens, and two cnidarians, Hydra magnipapillata and Nematostella vectensis. No GPH gene was recovered from any of these taxa, indicating that GPH genes arose along the lineage leading to Bilateria (Fig. 4). However, two genes were recovered from nonbilaterian taxa that group with outgroup gene sequences: a burscin-like gene from N. vectensis and a SOT-like gene from T. adhaerens.

Of the two GPH genes recovered from Aplysia is one closely related to an annelid Capitella GPHα (the other lophotrochozoan in the analyses and a member of a sister group to Mollusca) and is a member of a clade comprised of protostome GPHα genes. Consistent with previous results (17, 18, 23), only vertebrates possess GPHα1 and GPHα2 paralogs in our topology; however we note paraphyletically in Fig. 3 instances in which the α2 distinction has been used for other invertebrate GPHα genes previously. The other GPH gene that was recovered from Aplysia falls out in a protostome clade of GPHβ genes as expected. Again, instances in which the GPHβ nomenclature has been previously used are indicated in Fig. 3. Other GPHβ paralogs such as LH, TSH, and FSH, are found only in vertebrate genomes.

Because several previously undescribed subunits were identified by our phylogenetic analyses, we used the following rules to name them: 1) Previously used terms for genes are used when possible and are indicated in the phylogeny in parenthesis after the generic name; 2) When there is no previous nomenclature, we use only the terms α and β; 3) In either case, subscripts are used to denote specific paralogs.

AcaGPHα and AcaGPHβ are expressed in Aplysia embryonic and larval development

We used qRT-PCR to quantify the mRNA expression levels of GPHα and GPHβ in Aplysia embryonic and larval development (Fig. 5). Although both transcripts are expressed at low levels, they show increased expression levels during larval and metamorphic stages. Specifically, we identified increased levels of expression in hatching, before metamorphosis (stage 6) and after metamorphosis (stage 7) (for staging of Aplysia larvae see also Ref. 37). Note that all expression levels are relative to gastrulation (we chose this stage as the reference because the expression levels were lowest). We noticed a strong positive correlation between the expression levels of these two subunits and developmental progression from embryonic to larval stages. Figure 5B illustrates the developmental stages used...
Spatial transcription patterns of glycoprotein α- and β-subunits in the Aplysia CNS

We performed a detailed analysis of GPHα and GPHβ mRNA expression patterns in all ganglia of the Aplysia CNS. In summary we found that both subunits are expressed in all ganglia of the CNS including a subgroup of cells within a larger cluster of the pleural mechanosensory neurons as well as many unidentified cells. We also found AcaGPHα to be expressed in more cells than AcaGPHβ throughout all ganglia. However, AcaGPHβ is expressed at higher levels than AcaGPHα in pleural mechanosensory neurons as was confirmed by RNA-seq experiments (see Supplemental Fig. 5). The patterns of their distribution have been observed to be variable in some instances between the eight different ISH experiments that were performed. Nevertheless, consistent expression was observed in the majority of cells reported here. Thus, the patterns described below summarize only the consensus from these experiments. Note that approximate counts of neurons expressing AcaGPHα and AcaGPHβ are provided in Table 1.

Buccal ganglia

Both subunits are largely expressed outside, but in close proximity, of two well-defined clusters of mechanosensory cells. For example, AcaGPHβ is expressed in few cells of both the caudal and rostral area of the buccal ganglia (Fig. 6, B–B′). AcaGPHα is abundantly expressed on the rostral side of the buccal ganglia and highly symmetrical between the left and right side of the buccal ganglia.

Cerebral ganglia

GPHβ5 is most abundantly expressed on the dorsal side of the cerebral ganglia (Fig. 6, C–C″). Compared with AcaGPHα, AcaGPHβ is more abundantly expressed. Both subunits are symmetrically expressed with respect to right and left ganglia and show partial overlap in expression on the dorsal side. Neither AcaGPHα nor AcaGPHβ are expressed in the F cluster or A, B, and C clusters of the cerebral ganglia (Fig. 6C), regions of the Aplysia CNS previously implicated in neuroendocrine functions.

Pedal and pleural ganglia

AcaGPHβ is expressed within a defined subset of mechanosensory neurons of the pleural ganglia (Fig. 6, D–D″). These sensorin-containing cells are among the best characterized neurons in Aplysia with many critical roles in learning and memory of this model organism (43, 44). AcaGPHα is also expressed in a smaller subset of mechanosensory neurons of the pleural ganglia. However, overall relative expressions of both AcaGPHα and AcaGPHβ subunits in pleural mechanosensory neurons are significantly lower than for the neuropeptide sensorin (see also Supplemental Fig. 5).

In addition, we identified several distinct clusters in the proximity of LPL1 that express AcaGPHα but not AcaGPHβ. In the pedal ganglia AcaGPHβ is expressed in several dispersed cells in a symmetrical pattern. AcaGPHα
expression patterns in the pedal ganglia are also distinct from AcaGPHβ and dispersed.

**Abdominal ganglia**

AcaGPHβ and AcaGPHα are both expressed in small populations of currently unidentified neurons on the ventral side of the abdominal ganglia (Fig. 6, E—E′). On the dorsal side, we also detected expression of two large yet unidentified cells that stained only for AcaGPHα.

**Discussion**

The present study reveals new insights into the structure, evolutionary history, and expression of two putative GPH subunits in the life history and CNS of the lophotrochozoan *A. californica*. The CNS of the sea hare has large identifiable neurons that have been extensively studied both on a physiological and molecular level. These properties contributed to the development of *Aplysia* as a model organism for advancing understanding of the cellular and molecular basis of learning and memory (29, 45, 46). In contrast to the CNS, the neuroendocrine system (NES) of *Aplysia* is less understood, and fundamental information on the function of most hormones and peptides remains to be elucidated (but see Refs 47 and 48).

GPHs are an important group of pituitary hormones with essential functions in vertebrate metabolism and reproduction. Recent studies also reveal potential actions of these hormones in the brain (i.e. Ref. 49) suggesting a novel function of these hormones in modulating reproductive physiology and behavior. The analysis of several invertebrate genomes has revealed close relatives of GPH α and β subunits (9, 16–19, 22, 23, 40), and several studies have explored the distribution and function of these subunits in basal chordates, *i.e.* cephalochordates and urochordates (6, 18, 19, 50). Additional work has established the presence and distribution of these genes in ecdysozoan species such as the fruit fly *D. melanogaster* (17) and the nematode *C. elegans* (17, 23, 51). Still, little to no information exists on GPHs in lophotrochozoan species. Therefore, by analyzing the sequence and structure of GPH subunits in *Aplysia*, we gain novel insights into the evolution of the GPH family in animals. By comparing the distribution of GPH subunits in the *Aplysia* CNS to the distribution patterns of other *Aplysia* peptides and hormones, we also gain novel insights into the extent and potential function of the molluscan NES.

**Structure and evolution of AcaGPH subunits**

Cysteine-knot proteins form a large assembly of molecules that function as growth factors, morphogens, and hormones (42). Cysteine residues are essential for disulfide bonds, which make a critical contribution to protein folding and subunit interaction (16, 42). For example, GPHs form a complete cysteine knot and additional disulfide bonds, the so called “seatbelt” and “buckle” structure, which can be involved in heterodimer formation between the common α- and hormone-specific β-subunits. Together these subunits give the identity to the four biologically active hormones in humans and other vertebrates: CG, LH, FSH, TSH (52). Signal transduction is established by heterodimers activating LGRs (53), a subgroup of G...
protein-coupled receptors with members in both vertebrates and invertebrates (54). Based on well-annotated EST collections of neuronal transcripts (29) and publicly available genome information, AcaGPHα and AcaGPHβ appear to be the only GPH-like genes in Aplysia and show numerous structural similarities to vertebrate α2 and β5 subunit types, respectively. Our phylogenetic analysis also identifies AcaGPHα and AcaGPHβ as strongly supported homologs of α- and β-subunit types (Fig. 3). This is the first time that data from a lophotrochozoan species have been included in studies of animal GPH phylogeny. Our findings support the view that both α- and β-subunits are present in each of the major clades of bilaterians, including deuterostomes and protostomes, and that the two subunit types likely originated from a gene duplication event that occurred before the deuterostome/protostome split (6, 9, 17, 40). Interestingly, we found no evidence for either GPH subunit types outside of bilaterian animals. Our phylogenomic analyses also provide information on likely outgroups to GPHs. These include the tanning hormone bursicon in insects (55), the bone morphogenetic protein agonist gremlin (56), and the tumor suppressor SOT. The latter two genes are present in the nonbilaterian genomes included in this study. Finally our phylogeny revealed that the diversification of vertebrate α-subunits, which gave rise to α1 and α2 paralogy groups, and the diversification of β-subunits, giving rise to β5, GC, LH, TSH, and FSH, occurred along the lineage leading to vertebrates. Therefore, the invertebrate α- and β-subunits described here, including AcaGPHα and AcaGPHβ, are, in fact, equally related to their vertebrate homologs. Because of this, the current nomenclature, which identifies all invertebrate subunits as α2 and β5, is in need of revision. We have made an attempt here to correct this for the Aplysia subunits described. We suggest that in future studies, invertebrate subunits be identified based on phylogenetic criteria as α- or β-subunits (not α2 and β5).

In vertebrates, GPHs function as heterodimers comprised of α- and β-subunits. However, in the majority of neurons in the Aplysia CNS, the expression patterns of GPH subunits do not overlap (Fig. 5). Further 3D modeling of the β-subunit demonstrates the absence of a seatbelt and buckle structure (Fig. 2), critical for establishing a connection between the α- and β-subunit during heterodimerization (42). Together these data indicate that Aplysia GPH subunits may function individually or as homodimers, as has been hypothesized for vertebrate and insect GPHα2 and GPHβ5 subunits (16–18, 23). Still, our 3D model of the AcaGPHβ tertiary structure revealed additional structural elements that have not been observed in other subunits so far and are therefore not understood in detail (Fig. 2). For example, the short α-helix on hairpin loop 1 has not been confirmed from any other subunit and may be a unique feature of AcaGPHβ potentially involved in protein-protein interactions. Further studies including

![Image](https://example.com/image.png)

**FIG. 5.** Temporal expression patterns of AcaGPHβ and AcaGPHα mRNA during development (A) show increased abundance of mRNA levels in larval stages during larval stages and a strong correlation between α- and β-subunits. All values were calculated as relative expression levels to the gastrula stage using ribosomal protein S5 from Aplysia as a control gene. Therefore, the expression levels for the relative expression levels for the gastrula stage are set at 0 in this diagram and not shown. Schematic representation of juvenile nervous system shows maturation of the ganglionic CNS with respect to developmental stages. B, representative developmental stages of Aplysia show stages tested for qRT-PCR in panel A. CI, Cleavage stages; Tr, Trochophore stage; Tr-Vel, Trochophore-veliger transition; PreH, prehatching; preM, pre-metamorphic/metamorphically competent; PostM, post-metamorphosis.

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<th>Ganglia</th>
<th>AcaGPHα2</th>
<th>AcaGPHβ5</th>
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<td>45 ± 9</td>
<td>46 ± 4</td>
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<tr>
<td>Cerebral</td>
<td>71 ± 3</td>
<td>119 ± 13</td>
</tr>
<tr>
<td>Pleural</td>
<td>70 ± 9</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>Pedal</td>
<td>52 ± 9</td>
<td>17 ± 8</td>
</tr>
<tr>
<td>Abdominal</td>
<td>86 ± 10</td>
<td>49 ± 10</td>
</tr>
<tr>
<td>Total average</td>
<td>324</td>
<td>291</td>
</tr>
</tbody>
</table>

Numbers of cells were averaged from four complete Aplysia CNS. Note that these numbers are approximations only because the exact number of cells is difficult to count in some cases. Variance expressed as ± 1 SD from the mean.
GPH expression and putative function in Aplysia

AcaGPHα- and AcaGPHβ-expressing cells in the Aplysia CNS are relatively small in number and show a highly dispersed pattern of expression (Fig. 5), and both subunits are expressed at very low levels (see Supplemental Data, Appendix 5) in comparison with well-established neuropeptides identified in this organism. Our data also show that AcaGPHβ is expressed at relatively higher levels than AcaGPHα (Appendix 5). Although most of the neurons revealed in the ISH experiments are not yet identified, AcaGPHs are consistently expressed in two subclusters of pleural mechanosensory neurons (44). These cells have been previously mapped using RNA probes for sensorin A in the Aplysia CNS (44, 57) and represent approximately 5–10% of all neurons in the Aplysia CNS (44). These cells project to the entire body surface, and sensorin A release can be stimulated in response to mechanical stimulation, which has been viewed in light of a potential nociceptive and defense function of these neurons (44). GPH expression in these sensory cells indicates that AcaGPH hormone subunits may be involved in the modulation of sensory input to the brain. These results also suggest that this cell population is much more heterogeneous than was previously proposed (reviewed by Refs. 43 and 44). Although the hypothesized functions remain to be tested in future studies, the expression of GPH subunits in these regions of the CNS has important implications for the structure and function of the Aplysia NES that will be discussed further below. Note also that observed variation in expression between nervous systems indicates that the expression levels may be, to some extent, dependent on the physiological state of the individual. This could be a consequence of how the individuals were handled before the dissection but could also include physiological and life history parameters such as reproductive state. Although we did not specifically test for such factors, we are confident that the cells described here represent a relatively

the crystallography of AcaGPHβ will be necessary to gain better insights into this question. Future studies with synthetic AcaGPHs will also be able to investigate binding affinities between the Aplysia glycoprotein subunits and their putative receptors (LGRs) and their role in nervous system function, behavior, and development. In the shorter term, it will be critical to examine the mRNA distribution of these LGRs in the Aplysia CNS and test whether they colocalize with GPHs in putative target cells.

FIG. 6. Spatial expression patterns of AcaGPHα and AcaGPHβ mRNA in ganglia of the CNS of A. californica detected with antisense probe using ISHs. For detailed quantitative expression data see Table 1. A, Schematic representation of ganglionic nervous system of Aplysia CNS indicating major ganglia: BG, buccal ganglia; CG, cerebral ganglia; PG, pedal ganglia; PlG, pleural ganglia; AG, abdominal ganglia. B, Rostral surface of buccal ganglion (BGr) showing AcaGPHβ localization in cells in proximity of buccal sensory cells (circle). B′, Caudal surface of buccal ganglion (BGC) showing highly specific localization of AcaGPHβ staining in three cells that have not yet been described. B″, Rostral surface of buccal ganglion showing abundant localization of AcaGPHα. C, Dorsal surface of cerebral ganglion (CGd) showing AcaGPHβ staining in various yet unidentified cells. MCC and CPC (cerebropedal connectivity) are given as landmarks. C′, Ventral surface of cerebral ganglion (CGv) showing AcaGPHβ staining. MCC cells are indicated as landmarks. Very few cells were identified on the ventral side of the cerebral ganglion (arrows). C″, Partial view of dorsal side of cerebral ganglion (CGd) showing GPHα2 staining. None of these cells could directly be associated with any known cell type in the cerebral ganglia. Position of MCC cells is given as a landmark. D, Dorsal view of left pleural ganglion (PlGd) stained for AcaGPHβ subunit mRNA. In addition to a few unknown cells in the central part of the ganglia, the main staining is localized in the pleural sensory cell cluster (circle). Note that this cluster is also staining for AcaGPHα probe (E′ and E″). D′, Ventral view of left pleural-pedal ganglion (PlGv) stained with AcaGPHα antisense probe. Staining occurs in pleural sensory cluster (circle) and some associated cells that have not been previously identified. Note that this staining is highly similar to the staining of the GPHβ5 subunit seen in panel E. D″, Dorsal view of pedal-plural ganglion (PG+PlGd) stained with AcaGPHα antisense probe shows positive staining in the pleural sensory cluster. Note that this staining is highly similar to the staining of the AcaGPHβ subunit seen in panel E. EIPC (pedalpleural connectivity) and nerves PS and P9 are given as landmarks. E, Ventral view of abdominal ganglion (AGv) showing expression of AcaGPHβ in unknown cells (arrows). Note that these cells also express AcaGPHα as seen in E′. Nerve A2 is given as a landmark. E′, Ventral view of abdominal ganglion showing expression of AcaGPHα (arrows). Note that these cells also express AcaGPHβ as seen in E. Nerve A2 is given as a landmark. E″, Dorsal view of abdominal ganglion shows asymmetric expression of AcaGPHα (arrows).
consistent pattern because these patterns were repeatedly found in eight individuals. It is also interesting to note that a neuronal modulatory function of GPHs has been proposed for vertebrate GPH subunits in vertebrates (49). Furthermore, data on the nematode *C. elegans* show a function of these subunits on the level of the brain (51), and GPH distribution has been documented in the cephalochordate nerve cord. It is therefore worthwhile to consider that neuronal paracrine action of these hormones may be a widespread feature among animals.

In addition to the mature CNS, we also tested the expression levels of GPH subunits in *Aplysia* development (Fig. 4). *Aplysia* development is characterized by a drastic metamorphosis during which the larval body is transformed into a juvenile/adult (37). Immediately preceding metamorphosis, the larvae enter a stage of metamorphic competence during which they are responsive to settlement cues from the environment, which are likely detected by larval sensory organs such as the apical sensory organ. Thus, endocrine activity for the apical sensory organ is a possibility (58). Although we were unable to test this hypothesis using ISHs for GPH subunits in embryonic and larval stages (due to the overall low abundance of transcripts), we did find evidence for transcriptional up-regulation of both subunits later in larval development using qRT-PCR. The expression pattern matches profiles of several other hormones and peptides (37) and suggests a developmental function before metamorphosis or even during the metamorphic transition. Furthermore, previously published data on the fruit fly, *D. melanogaster*, have also found expression of these subunits in development (21), suggesting that a regulatory role of these hormone subunits should be considered and investigated in future studies.

**Implication for the evolution of neuroendocrine systems in bilaterian animals**

The HPP axis in vertebrates is a highly specialized and complex regulatory system critical for a broad array of biological functions such as reproduction, development, and metabolism. Tissues and receptor/ligand systems in the HPP axis are highly conserved among vertebrates. In contrast, clearly homologous morphological structures are absent in the majority of invertebrate phyla. Still, several studies, including the present one, document the presence of signaling molecules, receptors, and synthesis enzymes from this axis.

Several endocrine and neuroendocrine signaling molecules have been identified in the *Aplysia* CNS. These include *Aplysia* insulin-like peptide (48), GnRH (11, 59), and egg-laying hormone (47). Insulin-like peptide-secreting cells are primarily found in the F and C cluster of the cerebral ganglia but have also been identified in other regions of the CNS (48). Egg-laying hormone is secreted from a conglomerate of cells in the abdominal ganglia (47), and GnRH is secreted from specific cells found in the majority of ganglia (11, 59). Based on these distributions and our new data from GHPs, we can conclude that the majority of putative hormones and neurohormones that have been examined in *Aplysia* are not released from centralized clusters of cells but instead are released in a more diffuse pattern.

Over the course of evolution, animal NES became specialized in different ways, adding to the complexity and diversity of such systems in contemporary taxa (60). For example, in annelids and molluscs, clusters of neuroendocrine cells project neurosecretory peripheral axons directly to contact blood vessels or peripheral endocrine glands (61, 62). Therefore we hypothesize that in *Aplysia*, no central control mechanism regulates the release of GPHs. Instead, the release is directly dependent on sensory stimulation. This is in contrast to the NES of arthropods and vertebrates, which tightly regulate the release of reproductive and metabolic hormones from peripheral glands such as the corpora allata and corpora cardiaca in insects or the pituitary gland in vertebrates (7, 60, 63, 64). The distribution of GPH subunits in *Aplysia* within a cluster of mechanosensory neurons (44) provides preliminary evidence for this hypothesis. It will be critical to investigate the specific hormone and peptide receptors in *Aplysia* to gain better insights into the target sites of these signaling molecules. Furthermore it will be interesting to analyze the expression of these peptides in development. As pointed out above, our data suggest an increase of GPH subunit expression that correlates with the development of the juvenile nervous system, and modulatory functions in development ought to be considered as well.

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