Identification of neuropeptides and evolutionarily conserved peptide receptors in *Hydra*

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Contents

Abstract

Neuropeptides are utilized as transmitters and hormones in higher metazoans. In lower metazoans, the information of neuropeptides are still limited. *Hydra*, a member of Cnidaria, is one of the most basal metazoans that have a definite body plan and nervous system. It has been implicated that developmental processes and physiology of *Hydra* are highly regulated by various signaling peptides. Furthermore, neurotransmission in Cnidaria are thought to be performed exclusively with peptides. Thus, identification and characterization of neuropeptides are of particular importance to understand both development and physiology of *Hydra*. The information obtained from *Hydra* should also shed light on evolution of peptides and their receptors.

Chapter Ⅰ Identification of evolutionarily conserved neuropeptides and prediction of their receptors in *Hydra*

Some neuropeptide families are conserved among Chordata and Arthropoda. This suggests that neuropeptides are conserved throughout animal evolution and that evolutionarily conserved neuropeptides may be identified in *Hydra*. Thus, I attempted to identify such neuropeptides in *Hydra* by in silico data mining.

First, I developed an algorithm to detect possible neuropeptides in the *Hydra* EST database. I discovered a novel precursor gene that possibly encodes neuropeptide Y (NPY)-related peptides. The deduced precursor contained a N-terminal signal peptide, 3 putative NPY-related peptides, each flanked by an amidation motif "G(K/R)" at the C-terminal side. The sequence of C-terminal 5 amino acids in one of the deduced peptides

was quite similar to neuropeptide F, a member of NPY family, of *Helix aspersa* (Brown garden snail). In situ hybridization revealed that the gene was expressed in a subpopulation of neurons distributed in tentacles and foot region of *Hydra*. Thus, the peptides were designated as *Hydra* neruropeptide Fs.

Next, I looked for homologs of *Hydra* neuropeptides, Hym-355 and GLWamides in the *Caenorhabditis elegans* EST database as a midpoint between primitive and higher organisms. Genes that possibly encode Hym355-like and GLWamide-like peptides were found. The peptides similar to these *C. elegans* peptides were searched in the insect DNA databases. *C.elegans* Hym-355-like peptides showed similarity to insect PRXamides (Ecdysis triggering hormone, CAP, Pyrokinin), and *C.elegans* GLWamide-like peptides showed similarity to insect Allatostatin type B. These findings suggest that some of the neuropeptides are evolutionarily conserved throughout animal evolution.

Neuropeptides and their receptors (GPCRs) evolve together. In fact, GPCRs form clusters in a phylogenetic tree and those in a cluster often use the same or similar ligands. In this study, sets of novel GPCR genes were identified from the *Hydra* whole genome shotgun database. Phylogenetic analysis showed that some of the *Hydra* GPCRs were highly related to neuropeptide receptors known in higher metazoans. One of them (HGR001) was related to neuropeptide Y and cholecystokinin receptors. The gene was expressed in dividing nematoblasts (sting cell precursors) suggesting its involvement in proliferation and/or early differentiation of nematocytes.

These results suggest that some of the neuropeptides and their receptors are evolutionarily conserved and based on the conservation, novel peptides and GPCRs can be identified not only in *Hydra* but also in other animals.

Chapter Ⅱ Identification of novel neuropeptide family, FRamide-1 and FRamide-2

In the course of systematic identification of peptide signaling molecules combined with EST database analysis in *Hydra*, we have identified a novel neuropeptide family that consists of two members with a C-terminal motif of FRamide; FRamide-1 (IPTGTLIFRamide) and FRamide-2 (APGSLLFRamide). The precursor sequence deduced from cDNA contained a single copy each of FRamide-1 and FRamide-2 precursor. The expression analysis by whole-mount in situ hybridization showed that the peptide encoding gene was specifically expressed in a subpopulation of neurons that were distributed throughout the body from tentacles to basal disc. In order to analyze the subpopulation of FRamide-1 and FRamide-2 expressing neuron in *Hydra*, the expression pattern was compared with other neuropeptides (Hym-176, Hym-355, GLWamides) expressing neurons. Double in situ hybridization analysis showed that the population was further subdivided into two; one consisted of neurons expressing both FRamide-1/FRamide-2 and Hym-176 encoding genes and the other consisted of neurons expressing only the FRamide-1/FRamide-2 encoding gene. The neuron population did not overlap with that expressing the Hym-355 or GLWamdie neuropeptide gene.

FRamide-1 evoked elongation of the body column of epithelial *Hydra* that is composed of epithelial cells and gland cells but lacks essentially all the cells in the interstitial stem cell lineage including neurons. In contrast, FRamide-2 evoked the body column contraction. These results suggest that both of the neuropeptides directly act on epithelial (muscle) cells as neurotransmitters and are involved in the body movement in a longitudinal direction.

General Introduction

1. Neuropeptides and their receptors

1-1. Neuropeptides

Metazoans utilizes a variety of bioactive compounds as signals for intercellular communication (e.g. biogenic amines, proteins, lipids, amino acids, nucleotides, nitric oxides, steroids, peptides etc.). Bioactive peptide is one of the major groups of intercellular signals and they are ubiquitously found in the entire animal kingdom. Bioactive peptides have been isolated from various tissues and organs (e.g. pancreas, intestine, heart, skin and neural tissues) both in vertebrates and invertebrates. Functions of these peptides widely vary depending on the tissues where they are produced. They work as hormones, neurotransmitters, cytokines, morphogenetic factors, toxins etc.

The peptides that are produced from neural tissues are classified as neuropeptides (Hokfelt, 1991). Production of neuropeptides is schematically shown in Fig. 1. A group of neuropeptides are usually included in a larger precursor protein that has a signal peptide at the N-terminus. The precursor is translocated into the lumen of the endoplasmic reticulum, and the signal peptide is removed by a signal peptidase. Then the precursor is typically processed at sites containing Lys-Arg (KR), Arg-Arg (RR), or Arg-Xaan-Arg in immature secretory granules to produce a small peptide(s)(Fricker, 2003). Processing at these basic amino acids involves cleavage by endopeptidases such as prohormone convertase 1 or 2 followed by the removal of the C-terminal basic residues by carboxypeptidase E (Manser et al., 1990). A pattern of processing of the precursor is not always same, and some examples of differential processing depending on the tissues are

known. For example, Proopiomelanocortin is differentially processed in the anterior lobe and intermediate lobe of pituitary gland in mammals, and different repertoires of peptides are expressed in each region(Tanaka, 2003). Modification at the N-terminal or C-terminal site or even at a residue within the peptide often occurs.

The most frequently observed modification is amidation at the C-terminus. It occurs in approximately one-half of the known neuropeptides and is often required for the biological activity. Enzymatic transformation of the C-terminus from a glycine to an alpha-amide is catalyzed by peptidylglycine alpha-amidating monooxygenase (Emeson et al., 1984). First, peptidylglycine is transformed into peptidyl-alpha-hydroxyglycine in the presence of copper, ascorbate, and molecular oxygen and then converted to peptide alpha-amide and glyoxylate. These enzymatic reactions occur in secretory vesicles. As shown in Fig. 2, mature vesicles containing modified neuropeptides are translocated to the nerve terminal and tethered. When the neuron is excited, the granules fuse to the cytoplasmic membrane of the nerve terminals and neuropeptide is released into the surrounding (exocytosis)(Hong and Tang, 1993; Kelly, 1991).

1-2. Three types of neuropeptide signaling

The first and major type is signaling at synapses between neurons or between a neuron and a muscle cell (neuromuscular junction). When a neuron is excited, action potential triggers exocytosis to release neuropeptides into the synaptic cleft (Fig. 2). Neuropeptides then bind to receptors in post-synaptic cells to transduce signals. The second type is neurosecretion(Scalettar,2006). This type of signaling mainly occurs in neurosecretory tissues like hypothalamus and pituitary gland. In these tissues, axon

endings are close to blood capillaries. When a neuron is excited, neuropeptides are released into blood stream to be conveyed to a target organ. The third type is called paracrine （Snyder,1985). After being released from a secretory cell, neuropeptides difuse only locally. Then, they are broken down quickly or get stuck in surrounding area. In paracrine signaling, the effect of neuropeptides is short-range and only nearby cells are affected.

1-3. Variety of neuropeptides and physiological functions

A great variety of neuropeptides have been identified form various metazonas.(Table. 1). They perform a wide repertoire of physiological functions including muscle contraction, digestive actions, memories, food intake and reproductive behaviors. Here are some examples identified in vertebrates.

In the central nervous system, neuropeptides show both long term and short term effects. A good example of the former is ghrelin. Circulating ghrelin passively crosses the blood-brain barrier from the periphery and enter the hippocampus (Banks et. atl., 2002). Ghrelin promotes the formation of dendritic spine synapses and generates long-term potentiation. Orexin is an example of the latter. Orexin binds to mesolimbic dopamine neurons in the ventral tegmental area (VTA). VTA is implicated in the regulation of reward and wakefulness/sleep, and activation of VTA neurons by orexin induces awakeness (Vittoz and Berridge 2006).

Neuropeptides have a variety of effects on various organs besides the central nervous system. Neuromedin U stimulates muscle contractions of specific regions of the gastrointestinal tract (Minamino et al., 1985). Cholecystokinin stimulates pancreas to

release digestive enzymes. It also induces the contraction of gallbladder to deliver bile (Denton et al., 1950). Recently, it was shown that some neuropeptides control human behaviors (Pedersen and Boccia, 2006). For example, oxytocin and vasopressin have striking and specific effects on social behaviors such as maternal behavior and pair bonding (Debiec , 2005).

A single neuropeptide can exhibit different biological effects according the tissue or region where it is secreted. For example, neuropeptide Y, one of the most well characterized neuropeptides, is known to have multiple functions. The peptide released in the cerebrospinal fluid activates food intake and decreases physical activity. It is also expressed in autonomic nervous system and inhibits pancreatic secretion. Recently, it becomes apparent that neuropeptide Y is also involved in memory, learning and epilepsy (Colmers et al., 2003).

Neuropeptides have been identified and analyzed also in various invertebrates belonging to the phyla Arthropoda, Mollusca, Nematoda and Cnidaria. In Arthropoda, *Bombyx mori* has been often used for comparative endocrinology. Metamorphosis is a well studied phenomenon in the animal, and several compounds involved in the metamorphosis have been identified. Some neuropeptides (e.g. allatostatin, myosupressin) have been shown to play an important role in the metamorphosis. Allatostatins are structurally diverse peptides isolated from corpora allata of *Cockroach* (Bendena et al., 1999; Kramer et al., 1991; Lorenz et al., 1995). They control metamorphosis by inhibiting the biosynthesis of juvenile hormone. Today, members of the allatostatin family were identified in numerous insect species and classified into 3 groups. FMRFamide is the first neuropeptide identified in invertebrates. It was isolated

in 1977 as a cardioexcitatory molecule from the clam *Macrocallista nimbosa* (Price and Greenberg, 1977). Since then, many FMRFamide-related peptides have been identified throughout the animal kingdom (e.g. *Drosophila,* Human, *Lymnaea, Hydra).* They have many physiological functions including muscular control, cardioregulation and pain modulation.

1-4. Receptors for neuropeptides

In elucidating functions of neuropeptides, identification of their specific receptors is indispensable. Generally, most of the neuropeptides act as ligands for G-protein-coupled receptors (GPCRs) expressed on cellular membrane and transduce the signal inside the cell (Fig. 3)(Ji and Grossman,1998)).

Members of the GPCR family share a common molecular architecture consisting of seven transmembrane alpha-helices (Palczewski et al., 2000). The seven transmembrane helices are linked to three intracellular loops and three extracellular loops. The N-terminal portion is located in extracellular space and is often glycosylated. The N-teminal portion and extracellular loops of GPCR play an important role in ligand recognition. The C-terminal portion is located in cytoplasm and contains phosphorylation sites. Intracellular regions, especially the 3rd intracellular loop and C-terminal portion, are important for signal transduction.

The signal cascade via GPCR is initiated by ligand-binding to the receptor. The ligand-bound GPCR changes its conformation so that it can interact with hetero-trimeric GTP binding proteins (G proteins) (Linder and Gilman, 1992). Association of ligand-bound receptor with the G protein complex allows its alpha-subunit to exchange GDP with

GTP. Subsequently, heterotrimeric G-proteins dissociate into subunits, and the GTP bound alpha subunit activates and modulates cellular signaling pathways synthesizing second messengers (e.g. cAMP, inositol triphosphate, diacylglycerol) or releasing calcium ion (Birnbaumer, 1990).

GPCRs constitute a large superfamily of membrane receptors that share typical seven transmembrane structures. Not only peptides, a great variety of bioactive compounds like biogenic amines, lipids, nucleotides, glycoproteins, odorants and even photon are ligands for this type of receptors. GPCRs have been classified into six families according to their structure(Bockaert and Pin, 1999). Family A, rhodopsin family; Family B, secretin receptor family; Family C, metabotropic receptor family; Family D, fungal pheromone receptor family; Family E, cyclic AMP receptor family ; Family F, Frizzled/Smoothened family. Family A includes subfamilies of receptors for peptides, biogenic amines, lipids, photons and glycoproteins. Members of a given subfamily are structurally related to each other, and they can be classified into a group in the phylogenetic tree .

In order to identify a receptor specific to a peptide ligand, various strategies have been utilized. The most standard and classic method was biochemical identification by affinity chromatography（Dean,1985). In this method, membrane fractions are applied to ligand-bound matrix. The ligand-specific receptor specifically binds to the matrix, and then the specific receptor is eluted. An alternative approach more recently developed is called "reverse pharmacology" (Reinscheid et al.,1995；Meunier et al., 1995). The feature of this approach is to identify both bioactive compounds and their receptors at the same time in exhaustive and automatic ways. First, an uncharacterized GPCR whose ligand is not identified is expressed in cultured cells. As ligand candidates for the receptor,

chemichal compounds or tissue extracts are applied to these cells. Activation of GPCR by ligand candidates is detected by generation of intracellular signaling (e.g. Ca^{2+} ,cAMP,. inward K^+ current). By using these assays, a GPCR specific ligand is selected or purified, and finally both a ligand and its receptor are identified.

Using the reverse pharmacology approach, several novel neuropeptides and their receptors have been successfully identified in mammals. Orphanin FQ is a nociceptive peptide identified in the early stage of reverse pharmacology, and it demonstrated the validity of the strategy (Reinscheid et al., 1995). Orexin is also identified by reverse pharmocology and shown to play a significant role in the regulation of food intake and sleep-wakefulness (Sakurai et al., 1998).

Today, receptors of many neuropeptides have been identified, and they facilitated understanding of neuropeptide signalling system and biological functions.

2. Neuropeptides in animal evolution

2-1. Evolutionarily conservation of neuropeptides between invertebrates and vertebrates

Until present, great efforts have been made to identify neuropeptides in invertebrates and vertebrates. As a large number of neuropeptides were uncovered, it became apparent that some neuropeptide families are conserved between vertebrates and invertebrates (Table 1). The neuropeptide Y family is a good example (Larhammar et al., 2004). Pancreatic polypeptide, one of the neuropeptide Y family, was first identified in a vertebrate, and then, neuropeptides whose C-terminal structure were similar to Pancreatic polypeptide were identified in vertebrates (Neuropeptide Y and peptide YY) (Tatemoto, 1980; Tatemoto and

Mutt, 1982). So, they were classified as neuropeptide Y family. Neruopeptide Y-related neuropeptides were also identified in *Lymnaea stagnails (*Leung et al., 1992*)*, *Drosophila melanogaster (*Brown et al., 1992*)* and other insects. Their structures were well conserved, and all are classified into the neuropeptide Y family. Not only the neuropeptide Y family, several mammalian neuropeptide families of cholecystokinin and tachykinin are also found in Arthropoda.

Not only the structures, but also physiological functions of some neuropeptide families are also conserved. In mammals, neuropeptide Y is known to be released in brain and affect the paraventricular nucleus to reduce energy expenditure and enhanced weight gain (Frankish et.al,1995). Similarly, *Lymnaea* neuropeptide Y increases the body weight by modulating specific neurons that release growth factor. Although their target organs are not necessarily the same, their effects are similar in *Lymnaea* (Jong-Brink et al., 2001).

2-2. Conservation of a neuropeptide and its receptor as a set

As described above, structures of some neuropeptides are conserved between invertebrate and vertebrates. How are the structures conserved during evolution? Neuropeptide-Receptor binding is of primary importance in signaling process. This molecular interaction between a neuropeptide and its receptor possibly has prevented their changes through the ages resulting in conservation of both the ligand and receptor (Goh et al., 2000). One of the good examples of conservation of neuropeptide/receptor pairs is the vasopressin family. Vasopressin and oxytocin are structurally related neuropeptides in vertebrates. Their amino acid sequences and the position of the disulfide bond are well

conserved. However, they exhibit different biological effects depending on receptor subtypes used. Vsopressin/oxytocin-related neuropeptide was identified also in Mollusca and named "Lys-conopressin". Lys-conopressin-specific receptor was identified, and its amino acid sequences were found to be highly related to those of vertebrate vasopressin/oxytocin receptor family (Kesteren et al., 1996). As this case clearly shows, it is generally believed that receptors for a distinct neuropeptide family have sequence homology to each other

2-3. Neuropeptides in lower invertebrates

As I have described, it is apparent that some neuropeptides and their receptors are conserved among vertebrate and higher invertebrates (Chordata, Arthropoda and Mollusca). In contrast to these higher animals, knowledge on neuropeptides and receptors in lower invertebrates is quite limited. Although a variety of neuropeptides in lower invertebrates have been identified, the number and variety are still not enough to discuss the evolutionary conservation throughout metazoan evolution.

Neuropeptides in Nematoda are relatively well studied because of the availability of DNA databases and genetic tools. It has been reported that 23 genes encode around 59 FMRFamides in *C.elagans* (Husson et al., 2005). They are expressed in approximately 10% of the neurons, including motor neurons, sensory neurons and interneurons that are involved in movement, feeding, defecation and reproduction. Some of them were functionally characterized, and receptors for some of them were identified recently. In much lower invertebrates in phyla Platyhelminthes, Gnathostomulida, Cnidaria and Porifera, the number of identified neuropeptides and receptors is still too small (Meeusen

et al., 2003).

In order to elucidate the origin and evolutionary conservation of neuropeptide families and their receptors, more data remain to be uncovered in lower invertebrates, particularly cnidarians because of its most basal position of having nervous system. Thus, *Hydra*, a member of class Hydrozoa, phylum Cnidaria should be an important model animal for such a study.

3. Hydra as a model organism in neuropeptide study

3-1. Phylogenetic position of Hydra

Cnidaria is the most basal metazoa that has a simple body plan with definite tissues and nervous system. There are 4 classes in this phylum: Anthozoa, Hydrozoa, Schyphozoa and Cubozoa. Among them, Anthozoa is thought to be the most basal. *Hydra* is a member of class Hydrozoa and has only the polyp stage in its life cycle. The *Hydra* body is composed of a single (oral-aboral) axis, a head (tentacles and mouth) at one end and a foot (pedunclue and basal disk) at the other (Fig. 4A). Tentacles contain nematocytes (sting cells) and are used for feeding and locomotion. The body column is essentially a digestive tube and a source of young buds, asexual offspring. The basal disk secretes mucous compounds and attaches to the substratum.

3-2. Body plan

The body of *Hydra* consists of a limited number of cell types (Fig. 4B,C). The body wall consists of two epithelial cell layers, the ectoderm and endoderm, separated by

extracellular matrix, mesoglea. Ectodermal and endodermal epithelial cells are muscle cells. Muscle processes of ectodermal and endodermal epithelial cells respectively run longitudinally and circumferentially. Contraction of ectodermal muscle thereby results in contraction of the body column and contraction of endodermal muscle results in elongation. Ectodermal cells of tentacle are called battery cells because they contain many namatocytes (sting cells), and ectodermal cells of basal disk are called glandular cells that secrete mucous. Since epithelial cells have a perpetual proliferative capacity and differentiate into tentacle or basal disk specific cells, they are referred to epithelial stem cells (Fig. 4C).

The most distinguished cell type in *Hydra* is multipotent interstitial stem cell (David and Gierer, 1974; Schmidt and David, 1986; Bode et al., 1987; Bosch and David, 1987). The stem cells are wedged between ectodermal epithelial cells and constantly proliferate and give rise to nerve cells and nematocytes. Once an interstitial stem cell is committed to the nematocyte differentiation pathway, it undergoes 2-4 rounds of synchronous cell division and the daughter cells are connected to each other to form a cluster or a nest. All cells subsequently start synchronous production of nematocysts in cytoplasm. Once matured a nest breaks up and nematocytes individually migrate mostly to the tentacles (David,1973;David and Challoner,1974; Fujisawa et al.,1981; Bode,1988). Nematocytes are the cells characteristic to Cnidaria. Each nematocyte contains in its cytoplasm an explosive organelle, nematocyst. Four types of nematocytes are distinguished by the nematocyst type. They are used for capturing prey and locomotion. Upon discharge, nematocysts either adhere to the prey (or substratum) or penetrate into the prey to deliver neurotoxins to paralyze (Klug et al., 1989).

Interstitial stem cells also have a capacity to give rise to gland cells (Bode et al., 1987) and germline stem cells（Littlefield et al.,1985）. Nerve cells and nematocytes are terminally differentiated cells and have no mitotic capacity. However, both gland cells and germline stem cells have perpetually proliferative capacity. Gland cells reside in the endoderm and excrete digestive enzymes. Germline stem cells produce eggs or sperms depending on their sex.

Nerve cells are also produced from interstitial stem cells (Holstein and David, 1986; Bode et al.,1990). Two types of nerve cells are morphologically distinguished; ganglionic neurons and sensory neurons. The former is located at the base of epithelial cells and extends mutipolar processes parallel to mesoglea. The latter is monopolar and is typically oriented perpendicular to mesoglea. Sensory neurons are rich in head region and are thought to sense chemical and physical stimuli from outside.

3-3. Nervous system and neuropeptides in *Hydra*

The nervous system most likely originated in the ancestor of cnidarians. The present day cnidarians were considered to preserve a simple net-like nervous system. However, recent studies using various molecular markers have revealed that the nervous system of *Hydra* is not so simple as previously conceived (Hansen et al., 2000; Koizuimi et al., 1988; Hayakawa et al., 2004). *Hydra* nervous system is composed of several subpopulations of nerve cells that are intricately connected to each other.

It has been believed that all neurotransmission in Cnidaria is mediated only by neuropeptides. Cnidarian FMRFamides are relatively well studied neuropeptides in *Hydra* (Grimmelikhuijzen et al., 1982). As mentioned above, the FMRFamide family was

originally identified in Mollusca, and antibody against the C-terminus of FMRFamide is widely used to isolate holomogs from other animals. *Hydra* FMRFamide-related neuropeptides were identified in 1982. Subsequently, the precursor genes encoding these FMRFamide-related peptides were identified. Now, it becomes apparent that *Hydra* contains at least 9 FMRFamide-related peptides (Darmer et al., 1998) (Table2). Their physiological function is not completely elucidated, but some of them are involved in contraction of body column and peduncle.

Until now, a dozen of neuropeptides have been identified in *Hydra* (Table 2), but FMRFamide-related peptides (Darmer et al., 1987) are the only example of evolutionarily conserved neuropeptides in *Hydra*. Since numerous neuropeptides are still to be identified in *Hydra*, it is expected that more evolutionarily conserved neuropeptides are obtained. As will be shown later, I have obtained results by phylogenetic comparison of structures and functions that suggest evolutionary conservation of neuropeptides throughout invertebrates and vertebrates. Thus, *Hydra* will prove to be important for comparative study of neuropeptides.

Identification of novel neuropeptides may also serve as tools to understand primitive nervous system.

Chapter Ⅰ

Identification of evolutionarily conserved neuropeptides and predicted receptors

1. Introduction

A great variety of neuropeptides have been identified in higher animals. Some neuropeptides families exist both in Arthropoda and Chordata. This suggests that neuropeptides have been conserved through animal evolution (Conlon, 2005; Park et al., 2003). However, the variety of neuropeptides identified in primitive animals is quite limited to indicate evolutionary conservation. In *Hydra*, FMRFamide-like neuropeptides are the only example (Darmer et al., 1998). In order to reveal evolutionary conservation of neuropeptides, it is necessary to obtain data in lower animals to link them to those of higher animals.

In this study, two alternative approaches were taken. In the first approach, a collective database of precursor proteins for presumptive neuropeptides was constructed from the *Hydra* EST database. Then known neuropeptides from higher animals were searched in the database. This fished out *Hydra* neuropeptide F-like neuropeptides. In the second approach, *Hydra* neuropeptides were first searched in the databases of nematodes and then that of *Drosophila*. By this stepwise search, two neuropeptide familes were linked together among, *Hydra*, nematodes and *Drosophila*.

Neuropeptide specific receptors are also important to demonstrate the conservation of neuropeptides signaling in *Hydra*. In this study, a set of novel GPCR genes were systematically identified from the *Hydra* whole genome shotgun database. Phylogenetic analysis using *Hydra* GPCRs and neuropeptide GPCRs of higher animals revealed their clustering suggesting their evolutionary conservation.

Together with systematic identification of neuropeptides and prediction of their receptors, it was suggested that the neuropeptide families and receptors were conserved throughout the animal evolution.

2. Matterial and Methods

2-1. Prediction and identification of conserved neuropeptides from DNA databases

2-1-1.Database construction

A set of cDNA sequences of each animal (*Anopheles gambiae, Apis mellifera, Drosophila melanogaster, Caenorhabditis elegans, Ciona intestinalis, Schistosoma japonicum, Schistosoma mansoni, Hydra magnipapillata, Strongylocentrotus purpuratus, Branchiostoma floridae*) were collected from the NCBI UniGene archive. The cDNA sequences were classified according to their animal phyla and processed by Perl scripts as follows. All cDNA sequences were translated into amino acid sequences in six frames. One that gave the longest open reading frame was selected and registered in a translated database. By automatic PROSITE motif scan program (ps_scan) all the translated sequences were scanned (Gattiker et al., ,2002).

Prosite motif information on each translated sequences was used as follows. In case that any of the neuropeptide-related PROSITE motifs (PS00265; PANCREATIC_HORMONE_1; 1, PS50276; PANCREATIC_HORMONE_2; 1, PS00259; GASTRIN; 1, PS00259; GASTRIN; 2, PS00267; TACHYKININ; 2. PS00257; BOMBESIN; 1, PS00473; GNRH; 1, PS00264; NEUROHYPOPHYS_HORM; 1, PS00967; NMU; 1.) were found in a sequence, the entry was retained in the translated database. In case that other PROSITE motifs than the neuropeptide-related ones were found, the entry was removed. In case that no PROSITE motif was found in the sequence, the entry was retained in the translated database.

2-1-2. Search and prediction of evolutionarily conserved neuropeptide

The sequences of known neuropeptides were collected from the Swiss-Prot protein database (http://www.expasy.org/sprot/). Then, amidated neuropeptides were selected and classified into families according to the classification in the Swissprot database. In case that there is no description about the classification, neuropeptides were classified manually according to the description in the original articles. Furthermore, if amidated neuropeptide families were identified in more than one sub-phyla, they were selected and used as queries in the following search process. *Hydra* neuropeptides, that were already identified biochemically and characterized, (Table 2) were also used as queries.

The C-terminal 5 amino acid sequence of each neuropeptide collected by the method described above was set as query and searched against a translated database from each animal species described above. In most of the amidated neuropeptides in the families, 5 amino acid motifs at the C-termini are highly conserved and therefore they were used as queries. Search was done by using String::Approx perl module (Hietaniemi, 2001) to see if these 5 amino acid sequences are flanked by a possible amidation motif G(K/R)(K/R)) in a given translated sequence. The String::Approx module calculates the difference between query and 5 amino acid sequence in the N-terminal side of a possible amidation motif. When 3 or more of the 5 amino acids are conserved between query and the target sequences, the targets were recorded as candidates of the neuropeptide homolog genes.

After the systematic search described above, the selected candidate genes were further analyzed. Hydrophobicity of the N-terminal sequence of a translated protein was analyzed by SignalP program (Nielsen et al., 1997). The homology of translated gene to other proteins were checked by BLAST homology search system. The genes that survived these screening methods were considered as possible neuropeptide genes.

2-2. Animals and culture

The 105 strain of *Hydra magnipapillata* was cultured in *Hydra* culture solution (1 mM NaCl, 1 mM $CaCl_2$, 0.1 mM KCl, 0.1 mM $MgSO_4$, 1mM Tris pH7.6) at 18 ℃. Freshly hatched brine shrimp (*Artemia* nauplii) were given daily to *Hydra*, and the culture solution was changed a few hours after feeding.

2-3. Cloning of neuropeptide and neuropeptide receptor genes from *Hydra*.

Total RNA was extracted from strain 105 polyps by using the AGPC method (Chomcynski and Sacchi, 1987) and used as the template for cDNA synthesis. Reverse transcription was carried out by using SuperScript II (Clontech) and a oligo-dT primer according to the protocol recommended by the manufacturer.

Synthesized cDNA was used for polymerase chain reaction (PCR) to amplify the genes encoding *Hydra* neuropeptide F precursor and GPCR HGR001. All PCR reactions were carried out by using the High Fidelity PCR (Roche) mixture according to the protocol recommended by the manufacturer. For cloning a gene encoding neuropeptide F precursor, PCR reaction by using sense primer (5'ATGATCTTTAAGATGCTTGCAT, corresponding to the N-terminus) and antisense primer(5' CTTCCAAATCTAGACAATGG, corresponding to the C-terminal region) was carried out. The primers were designed from the *Hydra* EST sequence for neuropeptide F (Acc# CN625699 in NCBI). The PCR mixture was incubated in a thermal cycler (Takara) with 30 cycles for 1min at 94 ℃, 1min at 50 ℃ and 2 min at72 ℃.

The original short cDNA fragment of HGR001 was obtained accidentally in another work. Now, a full length of HGR001 DNA sequence can be found in NCBI trace archive

(Trace identifier (Ti) numbers 1236957410). For cloning GPCR HGR001, PCR reaction by using sense primer (5'GCGGATGGTAATTTCACCACA, corresponding to the N-terminal sequence) and antisense primer (5'TAAAAATTAGGTCCGTTATTATCAAC, corresponding to the C-terminal sequence) were used. The PCR was carried out in the same condition as described above. Amplified PCR products were separated on an agarose gel and cloned into a vector pCRII-topo (invitrogen). The plasmid was used for sequencing and amplifying the inserts.

2-4. Wholemount in situ hybridization

Digoxygenin (DIG)-labeled antisense and sense riboprobes were prepared in the following way. Neuropeptide F precursor cDNA and HGR001 cDNA were amplified using the plasmid DNA as a template and primers corresponding to T7 and T3 promoter regions by PCR and the amplified products were used as templates for RNA probe synthesis. Digoxygenin labeled antisense and sense RNA probes were prepared using the DIG labeling Mix (Roche)

Whole mount in situ hybridization was carried out according to Grens et al. (1996) with a slight modification. *Hydra* starved for 48 hours were relaxed in culture solution containing 2% urethane for a few minutes and fixed in 4 % parafromaldehyde overnight at 4℃. The fixative was removed and 100% methanol was poured to dehydrate. The samples can be stored at -20℃ for a long time. The dehydrated samples were then rehydrated in a 75%, 50%, 25% methanol/PBT series and washed twice in PBT. They were treated with 10 mg/ml proteinase K in PBT for 10 minutes at room temperature, and the reaction was stopped by washing in 4 mg/ml Glycine solution. After washing in PBT twice, the samples were incubated in 0.1 M triethanolamine for 5minutes twice. Then, they were incubated in 2.5 mg/ml acetic anhydrite/0.1 M triethanoleamine solution for 10 minutes twice on a shaker.

After washing in PBT twice, samples were fixed with 4% paraformaldehyde in PBT at room temprature for 20 minutes. Then, the samples were washed in PBT five times for 10 minutes followed by heating at 80 ℃ for 30 minutes to inactivate endogenous alkaline phosphatases

The samples were incubated in mixture of 50% PBT/ 50% hybridization solution for 10 minutes. The make up of hybridization solution was as follow: 50% formamide, 5xSSC, 0.1% tween20 (Wako), 0.1% CHAPS (sigma), 1xDenhardt's (0.02% BSA(PAESEL+LOREI), 0.02% PVP, 0.02% Ficoll-400)), 0.01% heparin, 0.2 mg/ml yeast tRNA(Roche). They were then incubated in 100% hybridization solution at 55℃ for two hours. Synthesized DIG-labeled RNA probes were denatured at 80℃ for 5minutes and added to hybridization solution at a concentration of 1μg/ml. Hybridization were performed at 55℃ for approximately 60 hours. After that, the samples were washed for 10 minutes in a series of 25%, 50%, 75% 5xSSC in hybridization solution at 55℃. They were then incubated twice in 2xSSC, 0.1% Chaps solution for 30 minutes at 55℃. After washing samples in MAB(100 mM maleic acid (pH7.5), 150 mM NaCl) at room temperature, they were incubated in 1% BSA in MAB for one hour at room temperature. They were further incubated in blocking solution (20% sheep serum and 1% BSA in MAB) at 4℃ for 2 hours. In the meantime, alkaline phsphatase-conjugated Anti-Dig antibody was preabsorbed with fixed *Hydra,* and diluted 1:2000 in blocking buffer. Then, samples were incubated in antibody containing blocking solution at 4℃ overnight.

Post-antibody wash was carried out 8 times in MAB solution for one hour at room temperature. Samples were washed 3 times in NTMT (100 mM NaCl, 100 mM Tris-HCl (pH9.5), 50 mM $MgCl_2$, 0.01% Tween20, 1 mM Levamisole (Sigma)) for 5minutes, and then incubated in color reaction solution (18.75 mg/ml NBT (Roche), 9.4 mg/ml BCIP(Roche) in NTMT). The reaction was stopped by removing reaction solution and washing in NTMT.

Stained samples were fixed in 3.7 % formaldehyde and dehydrated with ethanol. Samples were mounted with Euparol (ASCO laboratories) on cover slips.

2-5. Identification of Hydra GPCR genes

Hydra whole genome shotgun (WGS) sequences were obtained from NCBI trace archives. Amino acid sequences of all neuropeptide GPCR in human, *Drosophila* and *C. elegans* were acquired from GPCRDB (Horn et al., 1998) (http://www.gpcrdb.org). These amino acid sequences of GPCRs were used in tBLASTn (Thresshold e-value was set at 0.1) against the *Hydra* WGS database. WGS fragments selected by tBLASTn search were translated into amino acid sequences in six frames. One that gave the longest open reading frame was selected as deduced amino acid sequence. For each translated sequence, the hydrophobicity was calculated by DNASIS(Hitachi software), and the seven transmembrane(TM) regions were identified. Furthermore, well defined GPCR conserved amino acid motifs Gly-Asn(GN) motif in TM1, ERY motif just after TM3, CWLP motif in TM6, NP motif in TM7 were checked.

2-6. GPCR sequence alignment and phylogenetic analysis

Multiple alignment was constructed by using GPCR sequences from animals listed in Table 3. All the GPCR sequences were acquired from GPCRDB. The multiple alignment includes all subfamilies of GPCR family 1 (receptors for neuropeptides, biogenic amines, nucleotide, lipids, glyco-protein, photon) (Table 3). For the alignment, the N-terminal extracellular portion, the C-terminal extracellular portion, intracellular loops and extracellular loops were excluded as shown in Fig.5 because they are poorly conserved and not necessary for subclassfication

of GPCR families (Strader et al. 1994).

Each *Hydra* GPCR sequence was mutiple-aligned with the GPCR family1 sequences already produced as described above. First, a phylogenetic tree for whole GPCR family 1 including Hydra GPCR was constructed by the neighbor-joining method (Saitou and Nei, 1987). *Hydra* GPCR which was clustered with a distinct neuropeptide GPCR sub-group was selected as a candidate for neuropeptide GPCR. Then, another multiple alignment with known neuropeptide GPCRs and selected candidates for *Hydra* neuropeptide GPCRs was constructed and processed for phylogenetic tree construction by the neighbor-joining method, followed by bootstrap analysis. All alignments and phylogenetic tree in this study was constructed by using CLUSTALX (Thompson et al., 1997).

Results

3-1. Identification of neuropeptide F in *Hydra*

By the method described in Materials and Methods, neuropeptide subfamilies were selected and listed in Table 1. Conserved C-terminal five amino acid sequences were used as queries and homologous sequences were searched against the Hydra translated UniGene database. When the C-terminus of molluscan neuropeptide F (a member of neuropeptide Y family) was used as a query, a novel gene that encodes neuropeptide Y-related peptides(Acc#CN625699) was found. Fig.6 shows its cDNA sequence and deduced amino acid sequence of the precursor protein. The precursor contained a typical signal sequence at the N-terminus and three neuropeptide Y-related sequences flanked by G(K/R/D), a possible amidation motif in the C-terminal side. The acidic amino acid D after G in amidation motif was found only in *Hydra* (Yum et al., 1998). These possible neuropeptides were named as neuropeptide F 1,2,3 for the following reasons. As shown in Table 4, all members of the neuropeptide Y family in invertebrates contain phenylalanine (F) at their C-termini and they are called neuropeptide F(de Jong-Brink et al., 2001). Hydra neuropeptides predicted in Fig. 6 also have the C-terminal F. Furthermore, almost all members in the family share a C-teminal RXRFamide motif. *Hydra* neuropeptide F1 also has same motif. Finally, the C-terminal five amino acid motif of Mollusca neuropeptide F (...GRTRFa) exactly matches to the C-terminus of *Hydra* neuropeptide F 1 sequence (Leung wt al., 1992). Because there was no conventional processing site in the N-terminal side of each peptide, the exact N-terminus of each peptide is unclear. However, many of the cnidarian neuropeptides contain pyro-glutamic acid at the N-teminus or a proline residue at the second position from the N-terminus. Since presumptive *Hydra* neuropeptide F1 and 3 have similar amino acid motif "QN" at 16th position from C-

terminus, it was postulated that N-termini of *Hydra* neuropeptide F1 and 3 start with pyro-Glu. If so, *Hydra* neuropeptide F2 also might start with pyro-Glu.

Expression pattern of the gene encoding Hydra neuropeptide F was examined by WISH using an antisense RNA probe. As shown in Figure 7, the gene was expressed in neurons in the ectoderm of tentacles (Fig. 7A) and peduncle region (Fig. 7C). This suggests that the deduced Hydra neuropeptide F members are neuropeptides. The neurons expressing the neuropeptide F encoding gene were located at the base of ectodermal epithelial cells. This is a typical morphological feature of ganglionic neuron in *Hydra*(Fig 7B). Neurons expressing the gene were not observed in the basal disk (Fig. 7D). Also, no staining was observed in the gastric region.

3-2. A neuropeptide, Hym-355 was a member of evolutionarily conserved PRXamide family

Evolutionary conservation of Hydra neuropeptide in higher animals was examined. The first example was *Hydra* Hym-355 (Takahashi et al., 2000). By using Hym-355 sequence as a query, systematic peptide search was done against all the translated UniGene databases I had constructed. A possible neuropeptide precursor gene highly related to Hym-355 encoding gene was found only in *C.elegans* (Acc#Y23B4A.2). A homologous gene was also obtained in *C.briggsae* (Acc#CBG14780) by searching in the wormbase. As shown in (Fig. 8A,B), both nematode genes encode proteins of 87 amino acids. The proteins in nematodes, contain a typical signal sequence at the N-terminus and three Hym-355-like sequences flanked by G(KR). This suggests that mature forms of all three peptides are amidated at their C-termini. Table 5 show the structure of Hym-355 and nematode presumptive Hym-355-like peptides. Most of the nematode peptides contain a PRXamide motif at the C-terminus as *Hydra* Hym-

355 does. The N-terminal regions showed poor similarity (Table 5) as in other PRXamides. The precursor structure of nematode Hym355-like shows strong similarity to that of Hydra Hym-355 in the C-terminal region. In the C-terminal region of the Hym-355 precursor, Hym-355 sequence is followed by GKR, then another peptide plus GK at the C-terminal end. Exactly the same arrangement was found in both *C. elegans* and *C. briggsae* in the C-terminal regions PRX amide (Fig 8C). In the nematode genes an extra copy of Hym-355-like was found in the middle of the precursor.

Next, nematode Hym-355-like neuropeptides were used as query and searched against translated UniGene database of *Anopheles gambiae* and *Drosophila melanogaster.* A sequence similar to *C. elegans* Hym-355-like1 (APHPSSALLVPYPRVa)was found in the Cardio acceleratory peptide (CAP) precursor gene in *Anopheles gambiae* translated Unigene database. CAP precursors have been already identified in *D. melanogaster* and *A. gambiae*, both of which contains 3 copies of CAP neuropeptides (Table 5)*(*Iversen et al., 2002*)*. *C. elegans* Hym-355-like 1 sequence (APHPSSALLVPYPRVa) matched well to mosquito CAP-related 2 (QGLVPFPRVa) in the C-terminal half. 6 out of 7 of C-terminal amino acids are conserved between them. CAP neuropeptides belong to PRXamide neuropeptide family (e.g. Ecdysis triggering hormone, hug_gamma, Neuromedin U) (Zitnan et al., 1996; Tatemoto et al., 1986; Choi et al., 2001). As shown in Table 5, other Nematoda Hym-355-like peptide also showed sequence similarity to other PRXamide neuropeptides. Alignment of *Hydra* Hym-355, nematoda Hym-355-like peptides and insect PRXamide family members indicates conservation in PR sequence at the C-terminus and phenylalanine or tyrosine residue at the fifth position from C-terminus.

3-3. Assignment of GLWamides as members of an evolutionarily conserved allatostatin type B family

In the search of Hym-355 related peptides in other vertebrates, nematode databases proved useful, probably because of their more basal position in phylogeny. GLWamides have been identified in other cnidarians, all of which possess a GLWamide motif at the C-terminus. Amino acid sequences of *Hydra* GLWamides (Hym-51, 53, 54, 248, 249, 331, 370) were searched against the *C. elegans* translated UniGene database. The search detected a hypothetical protein that contains presumptive GLWamide-like peptides (Q8T3G1). As shown in fig 9, the precursor protein contains 3 copies of GLWamie-like sequences, each flanked by G(KR) on the C-terminal side. This suggests that mature forms of all three peptides are amidated at the C-terminus. There are mono- or dibasic residues, possible processing sites, 12th, 13th or 12th position from each G(K/R) at the C-teminus.

As summarized in table 6, the C-termini of the nematode peptides were assumed to have GLWamide or LWamide at the C-termini like cnidarian GLWamides. No significant sequence similarity was observed in the N-terminal part between *Hydra* GLWamides and the nematode peptides.

In the next step, *C. elegans* GLWamide peptide sequences were searched against the translated UniGene databases from arthropods, as in PRXamides. Four out of 5 C-terminal amino acids of nematode GLWamide-like 2(LNSLWa) matched to the C-terminal region of prothoracicostatic peptide (AWQDLNSAWG(K/R)), whose sequence was found in the translated protein database from Bombyx (Hua et al., 1999). The search also detected other allatostatin type B neuropeptides (allastosatin type B from *Gryllus bimarculatus* and Drostatins from *D. melanogaster*) as homologs of nematode GLWamides. These sequences were listed in Table 6. "WN" sequence in the N-terminal side was conserved between

nematode GLWamides and insect allatostatin type B members.

3-4. Prediction of *Hydra* neuropeptide specific receptors

I searched the *Hydra* whole genome shotgun sequence database with the goal of identifying the evolutionarily conserved GPCRs for neuropeptides. In this study, almost 400 genes structurally similar to GPCR family 1 were identified. Each *Hydra* GPCR and all the GPCR family members (Table 3) were used to construct a neighbor-joining phylogenetic tree. According to the position of each *Hydra* receptor in a tree, it was classified into subfamilies (receptors for i)peptides, ii)biogenic amines, iii)opsins, iv)nucleotides and lipid receptors and v)chemokines)(Birnbaumer, 1990).

Next, a phylogenetic tree of GPCRs specific to neuropeptides was constructed. Hydra GPCRs that make monophyletic groups with distinct neuropeptide receptor families (e.g. neuropeptide Y receptor family, PRXamide receptor family, Allatostatin type B receptor family) were selected as candidates for *Hydra* neuropeptide GPCRs.

3-5. Neuropeptide Y and cholecystokinin receptor subfamily

In the phylogenetic tree of GPCRs specific to neuropeptides, neuropeptide Y receptors and cholecystokinin receptors (Saito et al., 1980) made a monophyletic group (Fig. 10). The group comprises of receptors for neuropeptide Y, neuropeptide F, cholecystokinin, *Drosophila* cholecystokinin homologs (Kubiak et al., 2002).

Five novel GPCRs from *Hydra*, HGR001, HGR045, HGR122, HGR138 and HGR139, appeared to be members of this cluster (Fig. 10). HGR001 and HGR139 branch out from the base of neuropeptide Y and cholecystokinin receptor groups. HGR045 is more related to

QRFP receptor of human in the tree. QRFP is a neuropeptide that shows sequence similarity to neuropeptide Y family (Table 4). HGR138 and HGR122 were not highly related to neuropeptide Y receptors in the tree.

Expression pattern of a gene encoding a *Hydra* novel GPCR, HGR001, was examined by whole mount in situ hybridization using an antisense RNA probe. As shown in Fig. 11, HGR001 was expressed in nematoblasts distributed throughout the body column. Nematoblasts comprising 4 to 16 cell clusters expressed HGR001, but no single or paired interstitial cells expressed the gene. The stained nematoblasts appeared not to contain visible nematocysts. Therefore, it was concluded that the gene was expressed in proliferating and differentiating nematoblasts.

3-6. PRXamide receptor subfamily

The neuropeptide PRXamide family consists of CAP, ETH and neuromedin U. All of them contain a PRXamide motif at the C-terminus. Receptors for these neuropeptides make a monophyletic group in the phylogenetic tree (Fig. 12). As exceptions, mammalian receptors for GHS and neurotensin, which contain no PRXamide motif, were clustered in this group. Two novel GPCRs from Hydra, HGR125 and HGR131 belonged to this cluster. HGR125 is most related to uncharacterized GPCR(F02E8.2b) from *C. elegans* and these two branched out from the base of arthropod and mammalian PRXamide receptors (ETHR,CAPR, NMUR). The receptor for thyrotropin releasing factor (TRF) is known to be located on the midpoint between the FMRFamide receptor cluster and the PRXamide receptor cluster (Duthie et al., 1993; Park et al., 2002). The TRF receptor branches out from the base of HGR131 and other known PRX amide receptor cluster. Therefore, HGR131 appears to be related to the PRXamide receptor cluster.

4. Discussion

4-1. Prediction of evolutionarily conserved neuropeptides

As systematic DNA sequencing data (e.g. EST projects, genome projects) accumulate, it has become possible to find evolutionarily conserved proteins or genes in the databases of primitive animals by homology search programs (e.g. BLAST, FASTA). These programs based on sequence alignments are generally excellent tools to find the conserved long sequences such as genes and proteins in database. However, it is still difficult to identify evolutionarily conserved short neuropeptides or their precursors by conventional homology search algorithm. In most C-terminally amidated neuropeptides, only a short sequence at the C-terminus that is necessary for receptor activation is conserved during evolution. The precursor sequence except for neuropeptide regions is even less conserved. Furthermore, the number of encoded neuropeptides in the precursor changes by exon duplication or exon loss (Hoyle, 1999). Thus, the precursor sequence as a whole is less conserved during evolution. Consequently, the number of reports describing identification of conserved neuropeptides by conventional homology search programs is quite limited.

Previously, in silico analysis to predict bioactive peptide precursor gene in *C. elegans* was carried out and 92 precursor genes were found (Brownlee et al., 1999; Nelson et al., 1998). Also in *Drosophila*, 28 genes possibly encoding bioactive peptides were predicted by using similar data mining technique (Vanden Broeck J, 2001). In these studies, neuropeptide precursor genes were mainly predicted by the existence of a signal peptide and amidation motifs. But, other non-neuropeptide secretory proteins also contain signal peptide, and amidation motif G(K/R) is too short to predict precisely neuropeptide precursors. By this strategy, proteins that has no relationship to neuropeptide might be incidentally predicted as

neuropeptide precursors.

In this study, two approaches were taken to identify evolutionarily conserved neuropeptides. In the first approach, conserved C-terminal five amino acid sequences among subfamilies were used to find their relatives in the translated *Hydra* UniGene database. Close relationship between neuropeptides and their receptors was also considered to predict evolutionarily conserved peptides. In the second approach, *Hydra* neuropeptides were searched in nematode translated databases. When their relatives were found, new relatives were searched in insect translated databases. By this stepwise search, evolutionarily conserved peptides have been successfully identified. Although, the results should be still carefully evaluated, I believe the methods described here are sufficiently powerful for further identification of evolutionarily conserved peptides.

4-2. Evolutionary conservation of neuropeptide Y family

Pancreatic polypeptide was originally identified in the the pig pancreas. Subsequently, other pancreatic polypeptide-related peptides (e.g. neuropeptide Y, peptide YY) were identified in mammals and they were classified as neuropeptide Y family. Members of a neuropeptide Y family were also found in higher invertebrates. The first identification of neuropeptide Y homolog in invertebrate was from tapeworm *Monieza expansa* (Maule, 1992). The peptide was designated as neuropeptide F because its C-terminal end was an amidated pheynylalanine. Various Neuropeptide Fs were identified later in *Lymnaea* (Leung, 1992) and *Drosophila* (Brown et al., 1999)(Table 4).

Hydra neuropeptide F members predicted in this study were relatively shorter than the other family members, but the following findings support that *Hydra* neuropeptide Fs belong

to neuropeptide Y family. It is reported that about 12 amino acids at the C-terminus of neuropeptide Y are important to activate receptors (SickingerI et.al., 1990). The importance of C-terminal motif is also reported in neuropeptide F in *Platyhelminthes* (Humphries et.al., 2004). Therefore, the length of presumptive *Hydra* neuropeptide Fs predicted from precursor gene are sufficient for the activity. Furthermore, short form members of neuropeptide Fs are also identified in *D. melanogaster* and are designated as short neuropeptide Fs (Mertens, 2002). These structural characteristics in neuropeptide Y family indicate that *Hydra* neuropeptide Fs are members of neuropeptide Y family. This is the first report suggesting the existence of neuropeptide Y family in *Cnidaria*. Thus, *Hydra* neuropeptide Fs seem the most primitive form of evolutionarily conserved neuropeptide Y.

Expression analysis showed that *Hydra* neuropeptide F encoding gene is expressed in neurons in tentacles and peduncle. This suggests that presumptive neuropeptide F-like peptides are neuropeptides. In *Hydra*, other neuropeptide encoding genes (Hym355 precursor, Hym176 precursor, GLWamide precursor, RFamide precursors) are known to be expressed in neurons that are distributed in tentacles or peduncle. In order to elucidate the relationship of neurons expressing these genes and those expressing neuropeptide F gene, additional expression anaysis using double in situ hybridization is necessary.

Although precursor gene of *Hydra* neuropeptide Fs is identified in this study, mature peptides of *Hydra* neuropeptide F1, 2, 3 have not been isolated from *Hydra* tissue. For the final proof for neuropeptides, it is necessary to demonstrate the existence of mature neuropetide Fs in *Hydra* biochemically.

4-3. Phylogenetic analysis of neuropeptide Y receptor family

Neuropeptide Y and cholescystokinin are called "brain-gut peptides" because of their

characteristic expression in brain and digestive organs (Holicky et al., 2001). Amino acid sequences of neuropeptide Y and cholecystokinin are not so highly related, but C-terminal amino acid sequence of *Drosophila* cholecystokinin homolog DSK1(..GHMRFa) is similar to molluscan neuropeptide Y(..GRPRFa)(Table 4). Physiological functions of neuropeptide Y and cholecystokinin are similar. They are involved in food intake, secretion of digestive enzymes and contraction of the digestive tract. Together with close phylogenetic relationship of their receptors, it is suggested that cholecystokinin and neuropeptide Y are evolutionarily related. In accordance with this, their receptors form close clusters in a phylogenetic tree (Fig. 10). Five *Hydra* GPCRs were classified in the vicinity of these families (Fig. 10); two of them HGR001 and HGR139 branched out from the base of neuropeptide Y and cholecystokinin receptors. Another GPCR, HGR045 related to mammalian QRFP receptor branched out from the base of neuropeptide Y and cholecystokinin receptor clusters, HGR001 and HGR139. Since the bootstrap values are small, the exact order of branching is still debatable.

QRFP is known to be released in central nervous system and control feeding behavior. Although the detail of signaling cascade is unclear, it is suggested that its effect is detected after increased general locomotor activity and metabolic rate (Takayasu et al., 2006). Furthermore, I found that QRFP contained a C-terminal RFamide motif that appears in almost all neuropeptide Y related neuropeptides and shared several amino acids in the N-terminal side with neuropeptide Y (Table 4). These findings and the phylogenetic position of its receptor suggest that neuropeptide Y, cholecystokinin and QRFP are evolutionarily related and a ligand to *Hydra* HGR045, may belong to the QRFP family.

Although the phylogenetic analysis suggested that newly identified *Hydra* GPCRs were related to neuropeptide Y receptor, cholecystokinin receptor and QRFP receptor families,

it does not necessarily indicate that ligands of these *Hydra* GPCRs belong to the neuropeptide Y family. To show this, it is necessary to identify these *Hydra* GPCRs and their specific ligands by signaling assays such as those used in reverse pharmacology.

As described above, neuropeptide Y and cholecystokinin are expressed in brain and guts. However, one of *Hydra* GPCRs, HGR001 was expressed only in dividing nematoblasts. It suggests that HGR001 and its ligand are involved in nematocyte differentiation pathway but not in digestion. In any case, this is the first report suggesting peptide and GPCR signaling is involved in nematocyte differentiation.

4-4. Evolutionary relationship between Hym-355 and PRXamides

Neuropeptide Hym-355 was originally isolated from *H. magnipapillata* and was shown to enhance neuron differentiation in *Hydra*. In this study, the presence of Hym-355-like peptides in nematodes was suggested for the first time (Fig. 8). The arrangement of peptides in the C-terminal regions of *Hydra* and *C. elegans* precursors was quite similar. Using the Hym-355-like sequences of *C. elegans*, their relatives, CAPs were found in fly and mosquito. All of them share a C-terminal PRXamide motif (Table 5). The approach that resulted in the identification of PRXamides from *Hydra*, *C.elegans* to *Drosophila* seems to be a reasonable method to identify evolutionarily conserved peptides. Interestingly, Table 5 contains human neuromedin U which also has PRXamide. In the search for relatives of *C.elegans* Hym355-like peptide, no hit was found in the *Ciona* translated UniGene database. Thus, no attempt was made to search for chordate counterparts. However, since neuromedin U came up, It might be worthwhile to look into other mammals more carefully. Previous study suggested the co-evolution of PRXamides and their receptors (Park et.al., 2002). In the phylogenetic

tree of PRXamide receptors, *C. elegans* GPCR(F02E8.2b) and *Hydra* HGR125 formed a cluster indicating sequence similarity. It is possible that Hym-355 and Hym-355-like peptides are their respective ligands. To prove this, it is again required to perform signaling assays. However, it has not been performed yet.

4-5. Evolutionary relationship between GLWamide and insect allatostatins

Cnidarian neuropeptides, GLWamides were originally isolated from the sea anemone *Anthopleura elegantissima* as a metamorphosis inducer of planula larvae of *Hydractinia echinata*(Leitz et al., 1994). GLWamides, however, are multifunctional peptides. They not only promote metamorphosis of planula but also induce muscle contraction in *Hydra*. Previously Ishihara et al (unpublished), identified a gene from *C. elegans*, which appears to encode GLW(or LW)amides. The gene is expressed in a small number of neurons in the nerve ring near pharynx, but its function has not been elucidated. The analysis presented in this study was done independently and ended up with the same gene in *C.elegans*. Furthermore, *C.elegans* GLWamide 2 and 3 were recently isolated biochemically by using two dimensional liquid chromatography and mass spectrometry (Husson et al., 2005). These results demonstrate the validity of the strategy used in this study.

By sequence comparison between cnidarian GLWamides and insect allatostatin type B (Williamson M et al., 2002), it is not easy to see evolutionary relationship. The N-terminal sequences are not conserved, and some amino acids at the C-terminus changed even within insects (see Table 6). However, if *C.elegans* GLWamides were placed inbetween, the evolutionary relationship of these peptides in three phyla began to appear. Furthermore, there may be a common functional feature among GLWamides and allatostatin type B. That

is metamorphosis. Cnidarian GLWamides enhance metamorphosis of planula larvae, although their targets and signaling cascade are not known (Leitz et al., 1994). Allatostain type B is also involved in metamorphosis in insects. It blocks the synthesis of juvenile hormone (a sesquiterpenoid) in the corpora allata. Juvenile hormone allows larval molting in response to ecdysteroids, but prevents the switching of gene expression necessary for metamorphosis (Riddiford, 1996). In C. *elegans*, the cells expressing a GLWamide gene are expressed in a pair of interneurons (Ishihara et al, unpublished). Involvement of neurons in subpharyngeal region including the pair in dauer formation has been suggested (Ishihara, personal communication). It may be possible to consider dauer formation in nematodes as a kind of metamorphosis. The common function in different animals may be a coincidence, and a farfetched interpretation should be avoided. Nevertheless, it is tempting to assume that GLWamides and allatostain type B have a common evolutionary origin. If this holds true, one of the ways to identify the evolutionary relationship among less conserved molecules like peptides is to compare molecules between two close phyla stepwise. As discussed above, receptors for evolutionary common ligands cluster in the phylogenetic tree. Therefore, final answers to the evolutionary relationship of these peptides may be waited until the signaling cascades of these peptides including their receptors are revealed and compared.

4-6. Future plans

In this study, amidated neuropeptides were selected to find evolutionary conservation of neuropeptides. This was because the C-terminal amidation neuropeptides was commonly observed and an amidation motif of G(K/R) increased a chance to find conserved short peptides in a database. As to non-amidated neuropeptides, the C-terminal region is not

necessarily conserved. Therefore, it is difficult to search their homologs by the method described in this study. Improvement of search argorithm is absolutely necessary to predict evolutionary conserved non-amidated neuropeptides in-silico.

In this study, neuropeptides were searched against translated Unigene database of *Hydra* and other invertebrates. It was partially successful. However, both quantity and quality of Unigene databases of lower metazoans are still incomplete and insufficient. When these databases are upgraded, the present approaches should identify more evolutionarily conserved neuropeptides.

Evolutionarily conserved receptors for neuropeptides in *Hydra* were predicted in this study. But, their specific ligands, target cells and physiological functions are still unknown. In higher metazoans, molecular signaling analysis by using GPCR expressing cultured cells has been successfully used to identify ligands and their receptors. Although the results are not included in this thesis, I have attempted to establish a so called reverse pharmacological technique and screened for the ligands by Ca^{++} imaging. The cultured mammalian cells expressing Hydra GPCRs were established but screening for specific ligands has been unsuccessful.

In order to carry out the ligand screening successfully, other techniques must be applied. They may be following. i) *Hydra* GPCR expression in other cell culture systems (e.g. insects) or in Xenopus oocytes. It is known that proteins derived from primitive animals cannot interact with host proteins appropriately. Therefore, changing expression system might be effective to express *Hydra* GPCR functionally. Sometimes culturing temperature affects proper functioning of GPCR or their downstream pathways (Kubiak et al., 2003). ii)Binding assay by using RI-labelled neuropeptide ligands. By applying labeled neuropeptide to GPCR expressing cell, binding of ligand to GPCR will be detected. This

does not involve functional assays and might be easier. iii) Monitoring GPCR internalization by ligand binding. It is known that GPCR is internalized into cysoplasmic space after ligand binding (Conway et al., 1999). By conjugating GFP to GPCR, ligand binding will be detedced as GFP signal pattern changes on cell surface.

By identification of specific GPCRs for *Hydra* neuropeptides, it would provide a good chance to reveal target cells, signalling cascade and even evolutionary relationship to other neuropeptides.

Chapter Ⅱ　Identification of novel neuropeptide family, FRamide-1 and FRamide-2

1. Introduction

In order to reveal a whole functional repertoire of peptides in an animal, systematic identification of peptide signaling molecules in *Hydra* has been carried out in the Fujisawa lab. As a result, many novel peptides involved not only in neurotransmission but also in cell differentiation and morphogenesis were identified (Takahashi et al., 1997, 2000, 2003, 2005; Yum et al., 1998a; Grens et al., 1999; Harafuji et al., 2001). Hym-355 promotes the differentiation of multipotent stem cell into neurons. Hym-323 enhances foot formation by increasing foot activating potential in epithelial cells. GLWamides induce contraction of ectodermal circular muscle at the base of buds thereby causing detachment of buds from the parental polyp. Hym-176 is expressed in peduncle region and evokes contraction of the body column.

In the present study we have identified a novel family of neuropeptides, FRamide-1 and FRamide-2 by searching amino acid sequences obtained from the *Hydra* Peptide Project in the *Hydra* EST database. The precursor sequence deduced from cDNA contained a single copy each of FRamide-1 and FRamide-2. The identification of cell types that express the genes and the functions of these peptides will be reported here.

2. Materials and Methods

2-1. Animals and culture

The 105 strain of *Hydra magnipapillata* was cultured as described in Chapter Ⅰ. Epithelial *Hydra*, which lacks all the cells in the interstitial cell lineage except for gland cells, was produced from strain 105 by colchicine treatment (Campbell, 1976) and have been maintained for several years in the laboratory by hand feeding according to Nishimiya-Fujisawa and Sugiyama (1993).

2-2. Purification of FRamide-1 and FRamide-2

Extraction and purification of peptides from *Hydra* was carried out as described previously (Takahashi et al. 1997a). In brief, about 500g of frozen hydra tissue was homogenized in 2 liters of cold acetone. The homogenate was centrifuged at 16,000 x g for 30 min at 4˚C. The precipitate was resuspended in 5 % acetic acid, homogenized and centrifuged again. The first and second supernatants were pooled and concentrated by rotary evapolation. To this solution, 1/10 volume of 1N HCl was added, mixed and centrifuged as described above. The supernatant was applied to C-18 cartridges (Mega Bond-Elut, Varian). After washing the cartridges with 10 % methanol in 0.1% trifluoroacetic acid (TFA) (pH 2.2), the retained material mainly containing peptides was eluted with 60% methanol in 0.1% TFA. The 60%-methanol eluate (RM60) was then fractionated in a C-18 reversed-phase HPLC column (Capcell pack C18; 10 mm x 250 mm, Shiseido, Japan) with a linear gradient of 0-60% acetonitrile (ACN) in 0.1% TFA at a flow rate of 1 ml/min for 120 min (Fig. 13A). The chromatography was monitored at 220 nm. The fractions eluted between

5% and 37% ACN were divided into 15 groups. Groups 6 and 8 eluted around 19-23% ACN were selected and peptides present in these groups were systematically purified without bioassay.

FRamide-1 was purified in the following steps of HPLC. Group 8 in Fig. 13A was applied to a cation-exchange column (SP-5PW, Tosoh; 7.5 mm x 75 mm) and the column was eluted with a 70-min linear gradient of 0-0.7 M NaCl in 10 mM phosphate buffer (pH 7.2) at a flow rate of 0.5 ml/min (Fig. 13B). The fraction indicated by a bar was subsequently subjected to C-18 reversed-phase HPLC (ODS-80TM, Tosoh; 4.6 mm x 150 mm) with a 75-min linear gradient of 18-33% ACN in 0.1% TFA at a flow rate of 0.5 ml/min (Fig. 13C). The peak indicated by an arrow was subjected to the final HPLC (ODS-80TM) with an isocratic elution of 24% ACN in 0.1% TFA at a flow rate of 0.3 ml/min, and purified as a single peak (FRamide-1) (Fig. 13D). FRamide-2 was purified in a similar fashion. Group 6 from Fig. 13A was applied to a SP-5PW column and eluted as in Fig. 13B (Fig. 13E). The fraction indicated by a bar was subjected to C-18 reversed-phase HPLC (ODS-80Ts, Tosoh; 4.6 mm x 250 mm) with a 150-min linear gradient of 18-48% ACN in 0.1% TFA at a flow rate of 0.5 ml/min (Fig. 13F). The peak indicated by an arrow was subjected to the next HPLC (ODS-80Ts) with an isocratic elution of 23% ACN in 0.1% TFA at a flow rate of 0.5 ml/min (Fig. 13G). Finally, the peak indicated by an arrow was purified as a single peak (FRamide-2) using the same column with an isocratic elution of 23% ACN in 0.1% TFA at a flow rate of 0.5 ml/min (Fig. 13H).

2-3. Structure analysis and peptide synthesis

Amino acid sequences of FRamide-1 and FRamide-2 were determined by peptide

sequencers (PPSQ-10, Shimadzu and Procise 491, ABI) and also by tandem mass spectrometry (MS) (Q-TOF, Micromass). FRamide-1 and FRamide-2 were synthesized using a standard solid-phase method (433A, ABI), followed by TFA-anisol cleavage and HPLC purification. The structure of each synthetic peptide was confirmed by tandem MS.

2-4. Identification of a precursor for FRamide-1 and FRamide-2

All *Hydra magnipapillata* ESTs were collected from GenBank and these sequence data were processed by Perl Scripts. First, *Hydra* ESTs were translated into amino acid sequences in six frames. One that gave the longest open reading frame was selected and registered in a translated database. Amino acid sequences of the purified peptides were searched against the translated database to identify their possible precursors.

2-5. Cloning of a gene encoding FRamide-1 and FRamide-2

Total RNA was extracted from strain 105 polyps and was used for cDNA synthesis according to the protocol recommended by the producer (Clontech) as discrived in chapter Ⅰ. By using the DNA sequence of FRamide-1 and FRamide-2 encoding gene, primers were designed (sense primer, GTTTATATAAAGTAAGTGGATTGG; antisense primer, CTAGACAATTTCTTCGCCTGAG) to amplify the full coding region gene by PCR. Amplified DNA was separated on an agarose gel and cloned into pCRII-topo (invitrogen). The plasmid was used for sequencing and synthesizing RNA probes.

2-6. Wholemount in situ hybridization

Whole-mount in situ hybridization (WISH) using digoxygenin-labeled antisense and sense probes was carried out as described by in chapter Ⅰ. Double WISH was carried out using digoxygenin-labeled and FITC-labeled antisense probes as described by Mochizuki et al. (2000).

2-7. Biological Assay

Normal and epithelial *Hydra* starved for 24 hours were used to test the activity of FRamide-1 and FRamide-2. Before the assay the polyps were transferred to a 24 well-plate and kept still for 1hour before the peptide application. Synthetic FRamide-1 and FRamide-2 were respectively dissolved in water (10^{-3} M) as stock solutions and stored at –20 ˚C . For biological assays, the peptide solutions were diluted in *Hydra* culture solution (Sugiyama and Fujisawa 1977) before use and an aliquot was added gently to the well containing polyps. Movements of animals were video-recorded using a binocular attached with a CCD camera. Myoactivity of FRamide-1 and FRamide-2 was quantitated by measuring the body length (between hypostome and basal disk) on a monitor screen after recording.

3. Results

3-1. Purification of FRamide-1 and FRamide-2 from *Hydra* Tissue

Peptides were extracted from 500g of *Hydra magnipapillata* polyps, and untargeted purification was performed as described previously and also in Experimental Procedures with a series of HPLC until each peptide gave a single peak in a final chromatography (Takahashi et al., 1997). The purified peptides were systematically subjected to amino acid sequencing. Fig. 13 shows the HPLC elution profiles during purification of FRamide-1 (Fig. 13A, B, C, D,) and FRamide-2 (Fig. 13A, E, F, G, H). The amino acid sequences of FRamide-1 and FRamide-2 were tentatively determined by peptide sequencers as IPTGTLIFR and APGSLLFR, respectively.

3-2. Identification of the gene encoding FRamide-1 and FRamide-2 and structural determination of the peptides

A gene that encodes FRamide-1 and FRamide-2 was identified by searching the unmodified peptide sequences in the translated *Hydra* EST database (see Materials and Methods). Two possible full-length cDNAs (Acc# DR435693 and DT605584) that encode both FRamide-1 and FRamide-2 unmodified sequences were detected. A full-length cDNA was obtained by PCR as described in Materials and Methods. Fig.14 shows the cDNA sequence and the deduced amino acid sequence of the precursor protein. The precursor contained a typical signal sequence at the N-terminus and a single copy each of unmodified FRamide-1 and FRamide-2 sequences that were flanked by GKR, a possible amidation motif, on the C-terminal side. In the N-terminal side, serine and glycine

respectively preceded FRamide-1 and FRamide-2. These residues are thought to be unconventional processing sites in cnidarians (Grimmelikhuijzen et al., 1996).

In order to examine if FRamide-1 and FRamide-2 are amidated in the C-terminus, mass analyses of purified FRamide-1 and FRamide-2 were carried out. The expected masses (M+H)+ for amidated peptide and non-amidated peptide were 1016.63 and 1017.61, respectively for FRamide-1 and 859.51 and 860.50 for FRamide-2. The measured mass of native FRamide-1 was 1016.57 and that of FRamide-2 was 859.47. The results indicate the C-terminal residues of these peptides are amidated. Mass analysis of chemically synthesized amidated and non-amidated versions of FRamide-1 and FRamide-2 showed that the masses of native peptides coincided with those of amidated versions (data not shown). Thus, the final structure of FRamide-1 is IPTGTLIFRamide, and FRamide-2 APGSLLFRamide. Cross Species MegaBLAST search (NCBI) identified genome sequences (e.g. Trace identifier (Ti) numbers 688964329 and 1246173617) that contained the gene encoding FRamide-1 and FRamide-2. No intron was found within the gene (data not shown).

3-3. Expression analysis of the gene encoding FRamide-1 and FRamide-2

Expression pattern of the gene encoding FRamide-1 and FRamide-2 was examined by WISH using antisense and sense RNA probes. As shown in Fig. 15, this gene is expressed in neurons in the ectoderm throughout the body. No staining was obtained with a sense probe (data not shown). This indicates that FRamide-1 and FRamide-2 are neuropeptides. In *Hydra* two types of neurons are morphologically distinguished; ganglionic neuron and sensory neuron. Neurons expressing the gene encoding FRamide-

1/FRamide-2 were located at the base of ectodermal epithelial cells extending processes along the mesoglea (Fig. 15B). This is a typical morphological feature of ganglionic neurons in *Hydra*.

However, their relatively low number indicated that they only represented a subpopulation of neurons. In order to examine this, double in situ hybridization was carried out using RNA probes for the gene encoding FRamide-1 and FRamide-2 combined with that encoding neuropeptides Hym-176, Hym-355 or LWamides. Neurons strongly expressing the Hym-176 encoding gene are present in the peduncle region, while those in the gastric region weakly express the gene (Yum et al., 1998). No such neurons were found in the tentacles, hypostome and basal disk. In contrast, neurons expressing the Hy-355 or LWamides encoding gene are distributed throughout the body from the tentacles to the basal disk (Takahashi et al., 2000; Mitgutsch et al., 1999). Figure 16 shows the results. Neurons in the peduncle expressed the gene encoding Hym-176 (red) (Fig. 16A). Fig. 16B shows neurons in the same region as 16A but also expressed the gene encoding FRamide-1/FRamide-2 (blue). Double positive neurons show the purple color. The result indicated that neurons expressing the Hym-176 encoding gene completely overlapped with those expressing the FRamide-1/FRamide-2 encoding gene in the peduncle. However, there were also neurons stained in dark blue. They expressed only the FRamide-1/FRamide-2 encoding gene. Therefore, the population of neurons expressing the FRamide-1/FRamide-2 encoding gene could be classified into two subsets: One expressing only the FRamide-1/FRamide-2 encoding gene and the other expressing both FRamide-1/FRamide-2 and Hym-176. The single positive neurons appeared to express the gene more strongly than those expressing the two genes. Figure 16C,D respectively show

double staining using RNA probes for genes encoding FRamide-1/FRamide-2 (blue) and Hym-355 or LWamide (red) in tentacle region. Neurons expressing the former gene did not overlap with those expressing the latter, indicating that they belong to completely different subpopulations. The same was observed in other regions (data not shown).

3-4. Bioactivity of FRamide-1 and FRamide-2

Application of FRamide-1 and/or FRamide-2 to normal *Hydra* did not reveal any obvious effects. Normal *Hydra* exhibits constant movements, which could mask the effects exerted by peptides. In contrast, the effects of FRamide-1 and FRamide-2 were clearly shown when epithelial *Hydra* was used for a biological assay. Since epithelial *Hydra* consists essentially of muscle cells, and completely lacks interstitial stem cells and their derivatives (neurons, nematocytes and germ cells), spontaneous movement hardly occurs (Marcum and Campbell 1978; Sugiyama and Fujisawa 1978). Thus, epithelial *Hydra* is by far the best in vivo system to examine the direct effect of neuropeptides on muscle cells.

Epithelial polyps were treated with varying concentrations of FRamide-1 and FRamide-2 (10^{-7} - 10^{-4} M) and the effects were analyzed after video-recording. The results are shown in Fig. 17. FRamide-1 at 10^{-4}M induced drastic elongation of the body length over 30 min after the peptide application (Fig. 17A, crosses). The polyps slowly shortened after 1 hour. This effect was concentration-dependent. At 10^{-6}M the effect was marginal (Fig. 17A, squares) but at 10^{-7} M no obvious effect was detected (data not shown). The action of FRamide-1 was reversible, because the original body length was recovered after removing the peptide (data not shown).

FRamide-2, on the contrary, exhibited an opposite effect to FRamide-1. The treatment (10^{-7} - 10^{-4} M) caused body contraction of epithelial polyps in a concentration-dependent manner (Fig. 17B). The effect was also reversible (data not shown). Concomitant treatment with the two peptides at the same concentration always showed the dominant effect by FRamide-1.

4. Discussion

4-1. Identification of FRamide-1 and FRamide-2

Physiological functions, developmental processes and behaviors of *Hydra* are highly regulated by varieties of bioactive peptides (Schaller and Bodenmueller, 1981; Hoffmeister, 1996; Takahashi, et al., 1997; Yum et al., 1998; Grens et al., 1999; Takahashi et al., 2000; Lohmann and Bosch, 2000; Harafuji et al., 2001; Takahashi et al., 2003; Takahashi et al., 2005). Systematic identification of peptide signaling molecules in *Hydra* (the *Hydra* Peptide Project) has been carried out and FRamide-1 was purified and sequenced earlier in the project. In the original identification method, we screened for peptides that had activity to alter gene expression patterns in *Hydra* by differential display PCR (DD-PCR) (Takahashi et al., 1997). FRamide-1 had given a negative result in that screen and was set aside. Now that *Hydra* EST and genome databases are available, more rapid identification of signaling peptides and their genes has become possible by searching amino acid sequences of purified peptides in the translated *Hydra* database and examining the presumptive precursors carefully. Novel peptides FRamide-1 and FRamide-2 were identified just by this approach. The precursor of FRamide-1/FRamide-2 has a typical structural feature of neuropeptides: the N-terminal signal sequence, plural copies of peptides flanked by an amidation motif at the C-termini. Mass analysis easily identified the C-terminal modification and yielded a final sequence. The expression of the gene encoding FRamide-1/FRamide-2 in ganglionic neurons (Fig. 15) showed that FRamide-1 and FRamide-2 were neuropeptides. So far, no other peptide with a FRaminde motif at the C-terminus is known. Thus, FRamide-1 and FRamide-2 form a distinct peptide family.

4-2. A distinct subpopulation of neurons expressing the gene encoding FRamide-1 and FRamide-2

Nervous system of *Hydra* is composed of several different subpopulations of neurons (Dunne et al., 1985; Mitgutsch et al., 1999; Yum et al., 1998b; Takahashi et al., 2000; Hansen et al., 2000; Hayakawa et al., 2004). The present study added another group of neurons to these subpopulations. Double in situ hybridization analysis showed that neurons expressing the FRamide-1/FRamide-2 encoding gene were different from those expressing the Hym-355 or LWamides encoding genes (Fig. 16C,D). Also, they consisted of at least two subgroups; one expressing only the FRamide-1/FRamide-2 encoding gene and the other expressing the FRamide-1/FRamide-2 and Hym-176 genes in the peduncle. Since neurons expressing the Hym-176 gene also express the RFamide preprohormone-A (Hansen et al., 2000), they express at least 3 different neuropeptide genes at a time. Furthermore, Hym-176 is also involved in body contraction (Yum et al, 1998a). Therefore, the neurons in the peduncle release different but functionally related neuropeptides. These findings draw a rather complicated picture of the *Hydra* nervous system. Namely, the system consists of many subgroups of ganglionic and sensory neurons and a single neuron releases several types of neuropeptides. How the expression of these genes are regulated, whether the peptides are localized in the same vesicles or not, how the release of the peptides are controlled remain to be seen.

4-3 Possible functions of FRamide-1 and FRamide-2

FRamide-1 induced body elongation and FRamide-2 body contraction of epithelial *Hydra*

which consists of only epithelial cells (ectodermal muscle cells and endodermal muscle cells) and gland cells. Muscle processes of ectodermal epithelial cells run longitudinally and those of endodermal epithelial cells run circumferentially. Thus, FRamide-1 and FRamide-2 appear to act directly at neuro-muscular junctions of ectodermal and endodermal muscles, respectively as possible neurotransmitters. As can be seen in Fig. 15, neurons expressing the FRamide-1/FRamide-2 encoding gene were localized only in the ectoderm. Therefore, some of the neurons must extend processes to have junctions with endodermal muscle cells through mesoglea.

There are other *Hydra* neuropeptides that affect body elongation and contraction; e.g. Hym-176 (Yum et al., 1998), one of the GLWamides Hym-248 (Takahashi et al., 2003) and RFamide III/IV (Shimizu et al., 2003). It is not obvious why a simple metazoan like *Hydra* contains so many types of neuropeptides involved in similar functions. One possibility is that the current bath application may evoke less specific response. Another possibility is that coordination or combination of several peptides is required for regulation of precise movements of *Hydra*.

FRamide-1 and FRamide-2 exhibited opposite effects on epithelial *Hydra*, despite the fact they were encoded in the same gene. There are at least two possible explanations for this seemingly contradictory observation. One is that the release of the pepdies are regulated. For example, in bending the body column, FRamide-2 is released in the one side of the body, while FRamide-1 is released in the other side. Another possibility is that processing of the peptides occurs in the different neurons. In this case, a population of neurons contains one type of peptide and innervate to the ectodermal muscles, while another contains the other type innervating to the endodermal muscles.

Proopiomelanocortin is differentially processed in the anterior lobe and intermediate lobe of pituitary gland in mammals and the different repertoires of peptides are expressed in each region (Eipper and Mains, 1980; Rosa et al., 1980). Similarly, the precursor of FMRFamides in *Drosophila* is processed differentially resulting in a unique non-overlapping cellular localization (McCormick et al., 1999). To examine this possibility the production of peptide-specific antibodies is underway.

In higher animals, most of neuropeptides act as ligands for G-protein-coupled receptors expressed on cellular membrane of a target cell and transduce the signal inside the cell. Identification of FRamides-specific receptors is essential to reveal target cells, and intracellular signaling and eventually functions of peptides.

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Tables and figures

Table 1

Table 1. List of amidated neuropeptide familes.

Neuropeptide family	Animal phyla	spiecies	Peptide name	Peptide Sequence
Neuropeptide Y	Chordata	Homo sapiens	Neuropeptide Y	YPSKPDNPGEDAPAEDMAR**Y**YSALRH**Y**INLIT**R**Q**R**Ya
	Chordata	Homo sapiens	Pancreatic hormone	APLEPVYP**GD**NAT**P**EQMAQ**Y**AADLRR**Y**INMLT**R**P**RY**a
	Mollusca	Lymnaea stagnail	Neuropeptide Y	TEAMLTPPQRPEEFKN**P**NELRK**Y**LKALNE**Y**YAIVG**R**P**R**Fa
	Cnidaria		not identified	
Cholecystokinin	Chordata	Homo sapiens	Cholecystokinin 8	**DY**MG**W**M**D**F**a**
	Chordata	Ciona intestinalis	Cionin	**NYYGWMDF**a
	Arthropoda	*Drosophila melanogaster*	DSK1	FD**DYG**H**M**R**F**a
	Cnidaria		not identified	
Tachykinin	Chordata	Homo sapiens	substance P	RPKPQQ**F**F**G**LMa
	Arthropoda	*Drosophila melanogaster*	Tachykinin1	APTSS**F**I**G**MRa
	Cnidaria		not identified	
FMRF related(Short RF)	Chordata	Homo sapiens	NPVF	VPNLPQ**RF**a
	Arthropoda	*Drosophila melanogaster*	DPKQDFMRFamide	DPKQDFM**RF**a
	Cnidaria	*Hydra magnipapillata*	RFamide I	pEWLGG**RF**a
Neuromedin B/Bombesin	Chordata	Homo sapiens	Neuromedin B	GNLWATGHFMa
	Arthropoda		not identified	
	Cnidaria		not identified	
PRXamide	Chordata	Homo sapiens	Neuromedin U	EEFQSPFASQSRGYFLFR**PR**Na
	Arthropoda	*Drosophila melanogaster*	Ecdysis triggering hormone 1	DDSSPGFFLKITKNV**PR**La
	Cnidaria		not identified	
Vasopressin	Chordata	Homo sapiens	Arg-vasopressin	CYFQ**NCP**R**G**a
	Mollusca	*Lymnaea stagnails*	Lys-conopressin G	CFIR**NCP**K**G**a
	Cnidaria		not identified	
Gonadotropin-releasing hormone	Chordata	Homo sapiens	Gonadotropin-releasing hormone	pEHWSYGLRPGa
	Arthropoda,Mollusca	Octopus Vulgris	GnRH-related peptide	pENYHFSNGWHPGa
	Cnidaria		not identified	
Allatostatin	Chordata		not ideitified	
	Arthropoda	*Drosophila melanogaster*	Drostatin B1	AWQSLQSSWa
	Cnidaria		not identified	

Amino acid residues that are consereved in each families are shown in bold. C-terminal alpha-amidation is shown as “a”. Pyro-glutamic acid is shown as “pE”.

Table 2. List of *Hydra* neuropeptides

Designation	Peptide sequence	Family
RFamide I	pEWLGGRFa	RMRFamides
RFamide II	pEWFNGRFa	
RFamide III/IV	KPHLRGRFa	
RFamide III	AKPHLIGRFa	
RFamide IIIB	KPHYRGRFa	
RFamide A	pELMTGRFa	
RFamide B	pELMSGRFa	
RFamide C	pELMRGRFa	
RFamide D	pELLRGRFa	
Hym-51	NPYPGLWa	GLWamides
Hym-53	NPYPGLWa	
Hym-54	GPMTGLWa	
Hym-248	EPLPIGLWa	
Hym-249	KPIPGLWa	
Hym-331	GPPPGLWa	
Hym-338	GPPGLWa	
Hym-370	KPNAYKGKLPIGLWa	
Hym-355	FPQSFLPRGa	other
Hym-176	APFIFPGPKVa	
Hym-357	KPAFLFKGYKa	

C-terminal alpha-amidation is shown as “a”. Pyro-glutamic acid is shown as “pE”.

Table 3. List of GPCRs used for neighbor-joining phylogenetic tree construction.

ID	AccessionNo.	Subfamily
OPSB_HUMAN	(P03999)	
OPSG_HUMAN	(P04001)	
OPSR_XENLA	(O12948)	
OPS1_DROPS	(P28678)	Opsin
OPS2_DROME	(P08099)	
Q9NJC9_SCHMA	(Q9NJC9)	
O57422_XENLA	(O57422)	
5HT1A_HUMAN	(P08908)	
5HT1R_DROME	(P20905)	
5HTR_LYMST	(Q25414)	
5HTB1_APLCA	(Q16950)	
O15969_DUGJA	(O15969)	
O17470	(O17470)	
AA1R_HUMAN	(P30542)	
AA2DA_BRARE	(Q8JG70)	
ACM1_HUMAN	(P11229)	
ACM2_HUMAN	(P08172)	
ACM5_HUMAN	(P08912)	
ACM1_DROME	(P16395)	BiogenicAmine
ACM1_CAEEL	(Q18007)	
ACM2_CAEEL	(Q09388)	
ACM3_CAEEL	(Q9U7D5)	
ADA2C_BRARE	(Q90WY6)	
ADRB1_HUMAN	(P08588)	
DOPR1_DROME	(P41596)	
DRD1_HUMAN	(P21728)	
DRD5_XENLA	(P42290)	
O44198_APIME	(O44198)	
GPR9_BALAM	(Q93126)	
Q9VEG1_DROME	(Q9VEG1)	
HRH1_HUMAN	(P35367)	
MTR1A_HUMAN	(P48039)	
P87499_XENLA	(P87499)	
AA1R_HUMAN	(P30542)	
P2RY4_HUMAN	(P51582)	Nucleotide like
LT4R1_HUMAN	(Q15722)	
CNR2_HUMAN	(P34972)	Cannabinoid
CNR2_RAT	(Q9QZN9)	
PE2R1_HUMAN	(P34995)	
O00325_HUMAN	(O00325)	
EDG1_HUMAN	(P21453)	Prostanoid etc
EDG2_HUMAN	(Q92633)	
EDG5_HUMAN	(O95136)	
CC1L1_MOUSE	(P51676)	
CCBP2_HUMAN	(O00590)	
CCR2_HUMAN	(P41597)	
CCR9_HUMAN	(P51686)	Chemokine
CCRL1_MOUSE	(Q924I3)	
CX3C1_HUMAN	(P49238)	
FSHR_HUMAN	(P23945)	
LSHR_HUMAN	(P22888)	Glyco hormone
Q7ZZC0_BRARE	(Q7ZZC0)	
PAR1_HUMAN	(P25116)	
Q5U791_XENLA	(Q5U791)	
FPR1_HUMAN	(P21462)	
GP173_HUMAN	(Q9NS66)	
CXCR1_HUMAN	(P25024)	other
C3AR_HUMAN	(Q16581)	
DUFFY_HUMAN	(Q16570)	
PKR1_HUMAN	(Q8TCW9)	

ID	AccessionNo.	Subfamily
ACTHR_HUMAN	(Q01718)	
PRLHR_HUMAN	(P49683)	
ACTHR_HUMAN	(Q01718)	
AGTR1_HUMAN	(P30556)	
AGTRL_XENLA	(P32303)	
BKRB1_HUMAN	(P46663)	
APJ_HUMAN	(P35414)	
EDNRA_HUMAN	(P25101)	
ETBR2_HUMAN	(O60883)	
MC3R_HUMAN	(P41968)	
O73769_CARAU	(O73769)	
UR2R_HUMAN	(Q9UKP6)	
MCHR1_HUMAN	(Q99705)	
CAPAR_DROME	(Q8ITC7)	
BRS3_HUMAN	(P32247)	
CCKAR_HUMAN	(P32238)	
CCKAR_XENLA	(P70031)	
GALR1_HUMAN	(P47211)	
GASR_HUMAN	(P32239)	
GHSR_HUMAN	(Q92847)	
GNRHR_HUMAN	(P30968)	
GNRR2_HUMAN	(Q96P88)	
GPR54_ORENI	(Q6BD04)	
MTR_BUFMA	(Q90252)	
NK1R_HUMAN	(P25103)	
NMBR_HUMAN	(P28336)	
NMUR1_HUMAN	(Q9HB89)	
NPFF1_HUMAN	(Q9GZQ6)	peptides
NPY1R_HUMAN	(P25929)	
NPYR_DROME	(P25931)	
NTR1_HUMAN	(P30989)	
O43192_HUMAN	(O43192)	
O76873_DROME	(O76873)	
O77152_DROME	(O77152)	
Q7M3J6_DROME	(Q7M3J6)	
OPRD_HUMAN	(P41143)	
Q98UH1_BRARE	(Q98UH1)	
Q4VBP0_HUMAN	(Q4VBP0)	
Q8UWL5_FUGRU	(Q8UWL5)	
ANR_EISFO	(Q75W84)	
O43192_HUMAN	(O43192)	
Q25396_LYMST	(Q25396)	
OX1R_HUMAN	(O43613)	
QRFPR_HUMAN	(Q96P65)	
CAPAR_DROME	(Q8ITC7)	
Q330M5_ANOGA	(Q330M5)	
Q717T5_DROME	(Q717T5)	
Q8MX81_DROME	(Q8MX81)	
TRFR_HUMAN	(P34981)	
Q86RK9_DROME	(Q86RK9)	

ID and Accession No. respectively indicate the entry name and the accession number of each GPCR in Swiss-Prot protein database.

Table 3

Table 4

Table 4. List of neuropeptide Y family and *Hydra* neuropeptide Fs.

Designation	Animal phyla	Species	Peptide sequence
Neuropeptide Y	Chordata	*Homo sapiens*	YPSK**P**DNP**GE**-DAPAEDMAR**Y**YSALRH**Y**INLIT**R**Q**RYa**
NeuropeptideY	Chordata	*Brachydanio rerio*	YPTK**P**DNP**GE**-DAPAEELAK**Y**YSALRH**Y**INLIT**R**Q**RYa**
Pancreatic hormone	Chordata	*Homo sapiens*	APLE**P**VYP**GD**-NAT**P**EQMAQ**Y**AADLRR**Y**INMLT**R**P**RYa**
Peptide YY	Chordata	*Bos taurus*	YPAK**P**QAP**GE**-HAS**P**DELNR**Y**YTSLRH**Y**LNLVT**R**Q**RFa**
QRFP	Chordata	*Homo sapiens*	...FL**P**-AA**GE**KTSG**P**LGNLAEELNG**Y**SRKKGGFSF**RFa**
Neuropeptide F	Arthropoda	*Drosophila melanogaster*	SNSR**P**PRKNDVNTMADAYK**F**LQDLDT**Y**YGDRA**R**V**RFa**
Short neuropeptide F 1	Arthropoda	*Drosophila melanogaster*	AQRSPSL**R**L**RFa**
Short neuropeptide F 2	Arthropoda	*Drosophila melanogaster*	W**F**GDVNQKPIRSPSL**R**L**RFa**
Neuropeptide Y	Mollusca	*Helix aspersa(Brown garden snail)*	TQMLS**P**PERPREFRHPNERQ**Y**LKELNE**Y**YAIM**GR**T**RFa**
Neuropeptide Y	Mollusca	*Lymnaea stagnalis*	TEAMLTP**P**QRPEEFKN**P**NELRK**Y**LKALNE**Y**YAIV**GR**P**RFa**
Neuropeptide F	Platyhelminthes	*Moniezia expansa*	PDKDFIVNPSDLVLDNKAALRDYLRQINEYFAII**GR**P**RFa**
Neuropeptide F	Platyhelminthes	*Schistosoma mansoni*	AQALAKLMSLFYTSDAFNK**Y**MENLDA**Y**YMLR**GR**P**RFa**
Neuropeptide F1	**Cnidaria**	*Hydra magnipapillata*	pENQYIETISSL**GR**T**RFa**
Neuropeptide F2	**Cnidaria**	*Hydra magnipapillata*	pEYNPSF**R**E**RFNa**
Neuropeptide F3	**Cnidaria**	*Hydra magnipapillata*	pENYENRGANVVPLS**RFa**

Amino acid residues that are conserved in neuropeptide Y family are shown in bold. C-terminal alpha-amidation is shown as “a”.
Pyro-glutamic acid is shown as “pE”.

Table 5

Table 5. List of PRXamide family members including Hydra Hym-355 and nematode Hym-355-like peptides.

Designation	Animal phyla	Species	peptide sequence
NeuromedinU	Chordata	*Homo sapiens*	EEFQSPFASQSRG**Y**FL**F**R**PR**Na
CAP1	Arthropoda	*Drosophila melanogaster*	GANMGLYAF**PR**Va
CAP2	Arthropoda	*Drosophila melanogaster*	ASGLVAF**PR**Va
CAP3	Arthropoda	*Drosophila melanogaster*	TGPSASSGLW**F**G**PR**La
hug_gamma	Arthropoda	*Drosophila melanogaster*	pELQSNGEPA**Y**RVRT**PR**La
PK-2	Arthropoda	*Drosophila melanogaster*	SVP**F**K**PR**La
ETH1	Arthropoda	*Drosophila melanogaster*	DDSSPGFFLKITKNV**PR**La
ETH2	Arthropoda	*Drosophila melanogaster*	GENFAIKNLKTI**PR**Ia
CAP related 1	Arthropoda	*Anopheles gambiae*	GPTVGLFAF**PR**Va
CAP related 2	Arthropoda	*Anopheles gambiae*	QGLVPF**PR**Va
CAP related 3	Arthropoda	*Anopheles gambiae*	GGTGANSAMW**F**G**PR**La
Nematoda Hym355-like1	Nematoda	*C.elegans*	APHPSSALLVPY**PR**Va
Nematoda Hym355-like2	Nematoda	*C.elegans*	SNILNNNSESQNSVQKRL**Y**MA**R**Va
Nematoda Hym355-like3	Nematoda	*C.elegans*	AFF**Y**T**PR**Ia
Nematoda Hym355-like1	Nematoda	*C.briggsae*	APHPSSALLVPY**PR**Va
Nematoda Hym355-like2	Nematoda	*C.briggsae*	SVNGPRHLESRNTAQKRL**Y**MA**R**Va
Nematoda Hym355-like3	Nematoda	*C.briggsae*	AFF**Y**A**PR**Va
Hym-355	Cnidaria	*Hydra magnipapillata*	FPQS**F**L**PR**Ga
Hym-355 related peptide	Cnidaria	*Hydra magnipapillata*	DAR**PR**Aa

Amino acid residues that are conserved in this family are shown in bold. C-terminal alpha-amidation is shown as “a”.

Table 6

Table 6. List of Allatostatin type B family, Hydra GLWamides and Nematoda GLWamides.

Designation	Animal phyla	Species	Peptide sequence
drostatinB1	Arthropoda	*Drosophila melanogaster*	A**W**QSLQSS**W**a
drostatinB2	Arthropoda	*Drosophila melanogaster*	A**W**KSMNVA**W**a
drostatinB3	Arthropoda	*Drosophila melanogaster*	RQAQG**WN**KFR**G**A**W**a
drostatinB4	Arthropoda	*Drosophila melanogaster*	EPT**WN**NLK**G**M**W**a
drostatinB5	Arthropoda	*Drosophila melanogaster*	DQ**W**QKLH**G**G**W**a
Prothoracicostatic peptide	Arthropoda	*Bombyx mori*	A**W**QDLNSA**W**a
AllatostatinB1	Arthropoda	*Gryllus bimaculatus*	G**W**QDLN**G**G**W**a
AllatostatinB2	Arthropoda	*Gryllus bimaculatus*	G**W**RDLN**G**G**W**a
AllatostatinB6	Arthropoda	*Gryllus bimaculatus*	A**WN**NLGSA**W**a
GLWamide-like1	Nematoda	*Caenorhabditis elegans*	VLG**WN**KAH**GLW**a
GLWamide-like2	Nematoda	*Caenorhabditis elegans*	TPQN**WN**KLNS**LW**a
GLWamide-like3	Nematoda	*Caenorhabditis elegans*	SPAQ**W**QRAN**GLW**a
Hym-51/53	Cnidaria	*Hydra magnipapillata*	NPYP**GLW**a
Hym-54	Cnidaria	*Hydra magnipapillata*	GPMT**GLW**a
Hym-248	Cnidaria	*Hydra magnipapillata*	EPLPI**GLW**a
Hym-249	Cnidaria	*Hydra magnipapillata*	KPIP**GLW**a
Hym-331	Cnidaria	*Hydra magnipapillata*	GPPP**GLW**a
Hym-370	Cnidaria	*Hydra magnipapillata*	KPNAYKGKLPI**GLW**a

Amino acid residues that are conserved in this family are shown in bold. C-terminal alpha-amidation is shown as “a”.

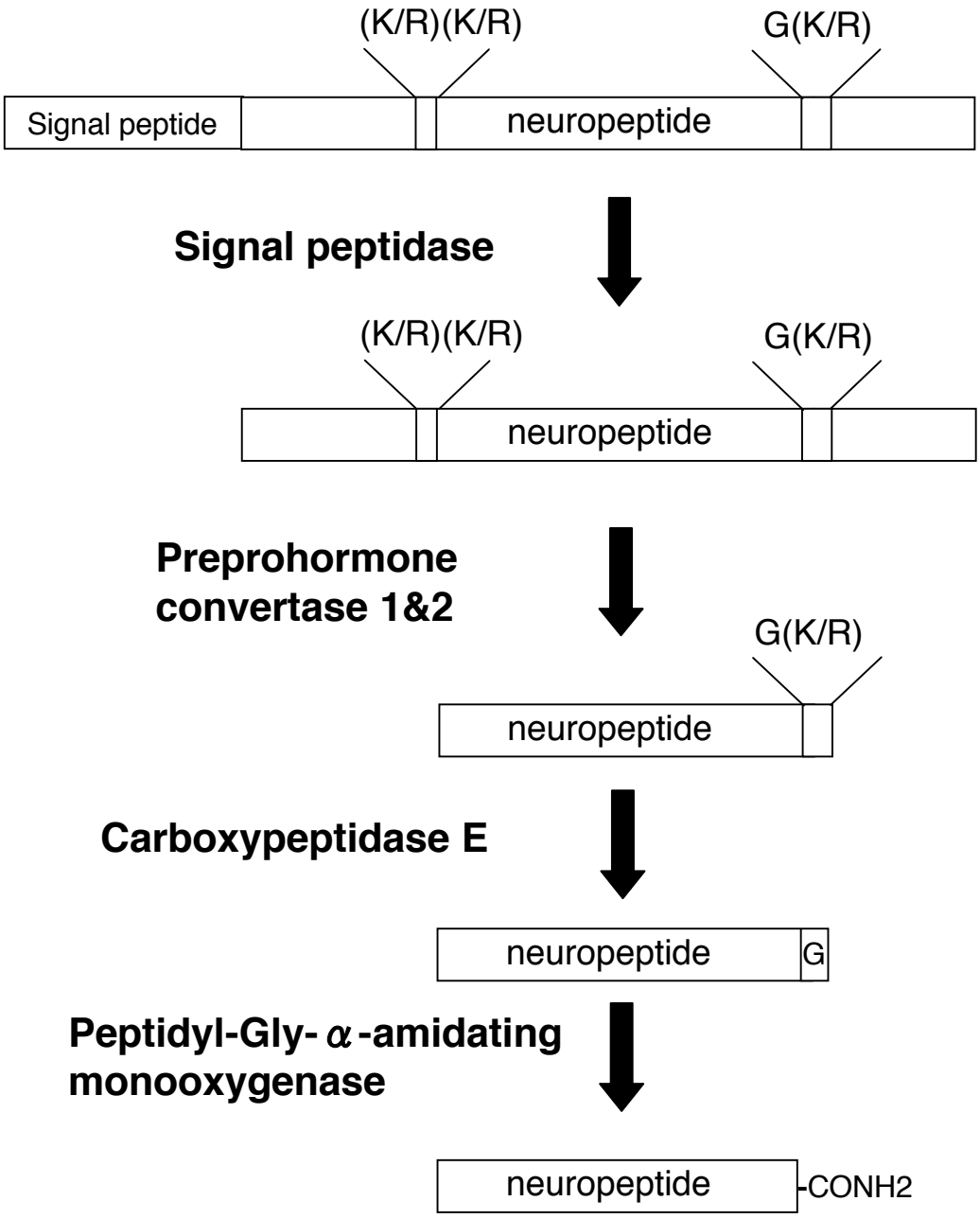

(K/R)(K/R)
G(K/R)
Signal peptide
neuropeptide
Signal peptidase
(K/R)(K/R)
G(K/R)
neuropeptide
Preprohormone convertase 1&2
G(K/R)
neuropeptide
Carboxypeptidase E
neuropeptide
G
Peptidyl-Gly-α-amidating monooxygenase
neuropeptide
-CONH2

Fig. 1

Fig.1. Scheme of neuropeptide production from the precursor.

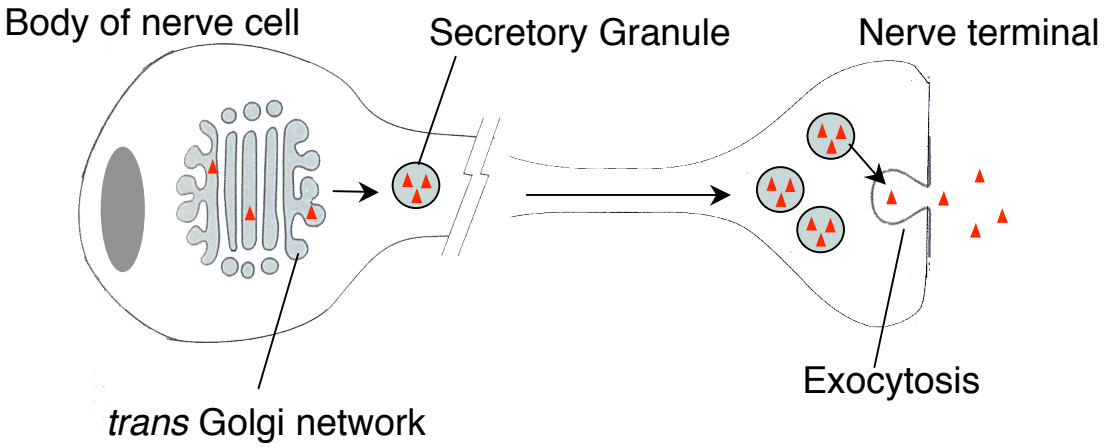

Body of nerve cell
Secretory Granule
Nerve terminal
Exocytosis
trans Golgi network

Fig. 2

Fig.2. Translocation and secretion of neuropeptide in the neuron. In trans golgi network, neuropeptides are packed into secretory granules, and transferred to the nerve terminal. Upon appropriate stimulation, granules fuse to the membrane of nerve terminal and neuropeptides are released by exocytosis.

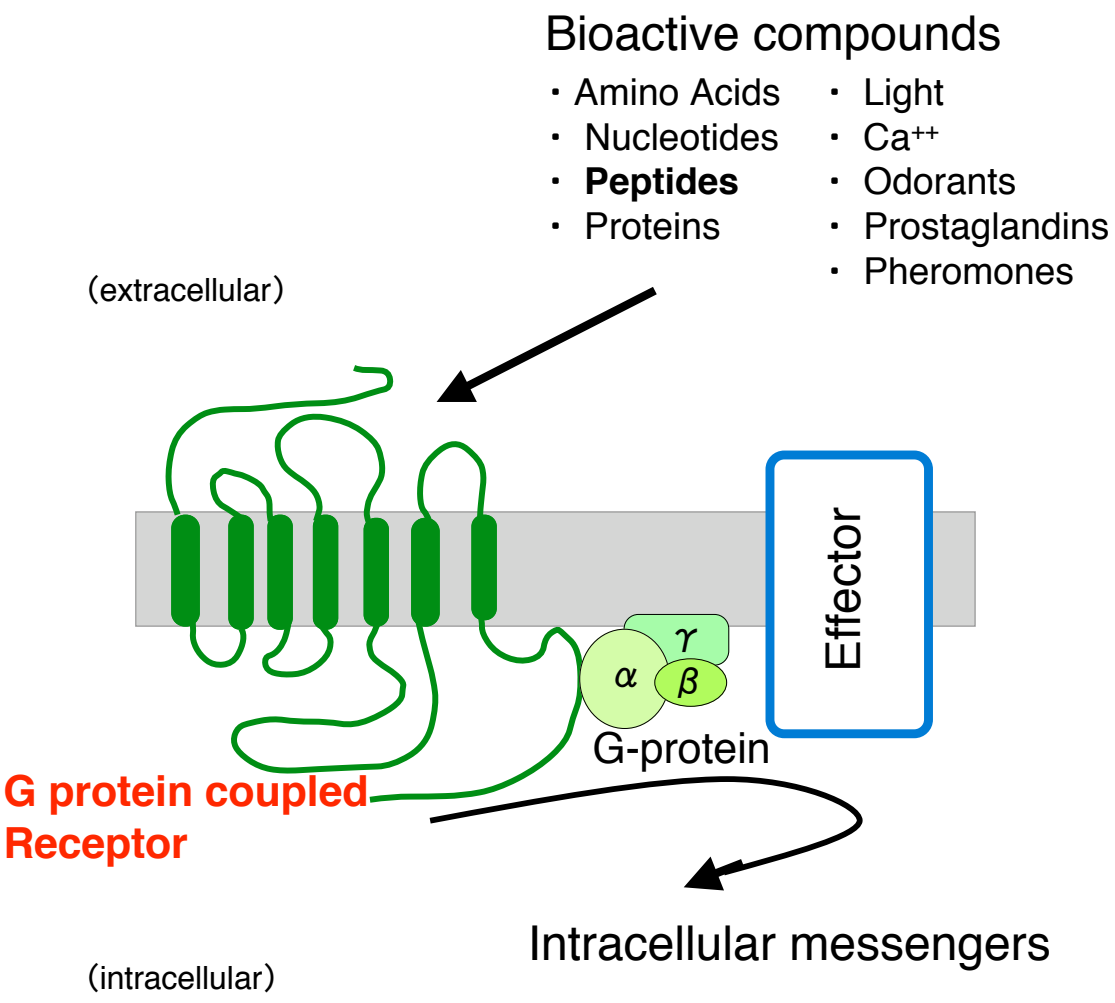

Bioactive compounds
・Amino Acids
・Nucleotides
・**Peptides**
・Proteins
・Light
・Ca++
・Odorants
・Prostaglandins
・Pheromones
(extracellular)
Effector
γ
α
β
G-protein
G protein coupled
Receptor
Intracellular messengers
(intracellular)

Fig. 3

Fig.3. Signaling system of GPCR superfamily. The illustration depicts the GPCR and related molecules around the cellular membrane.

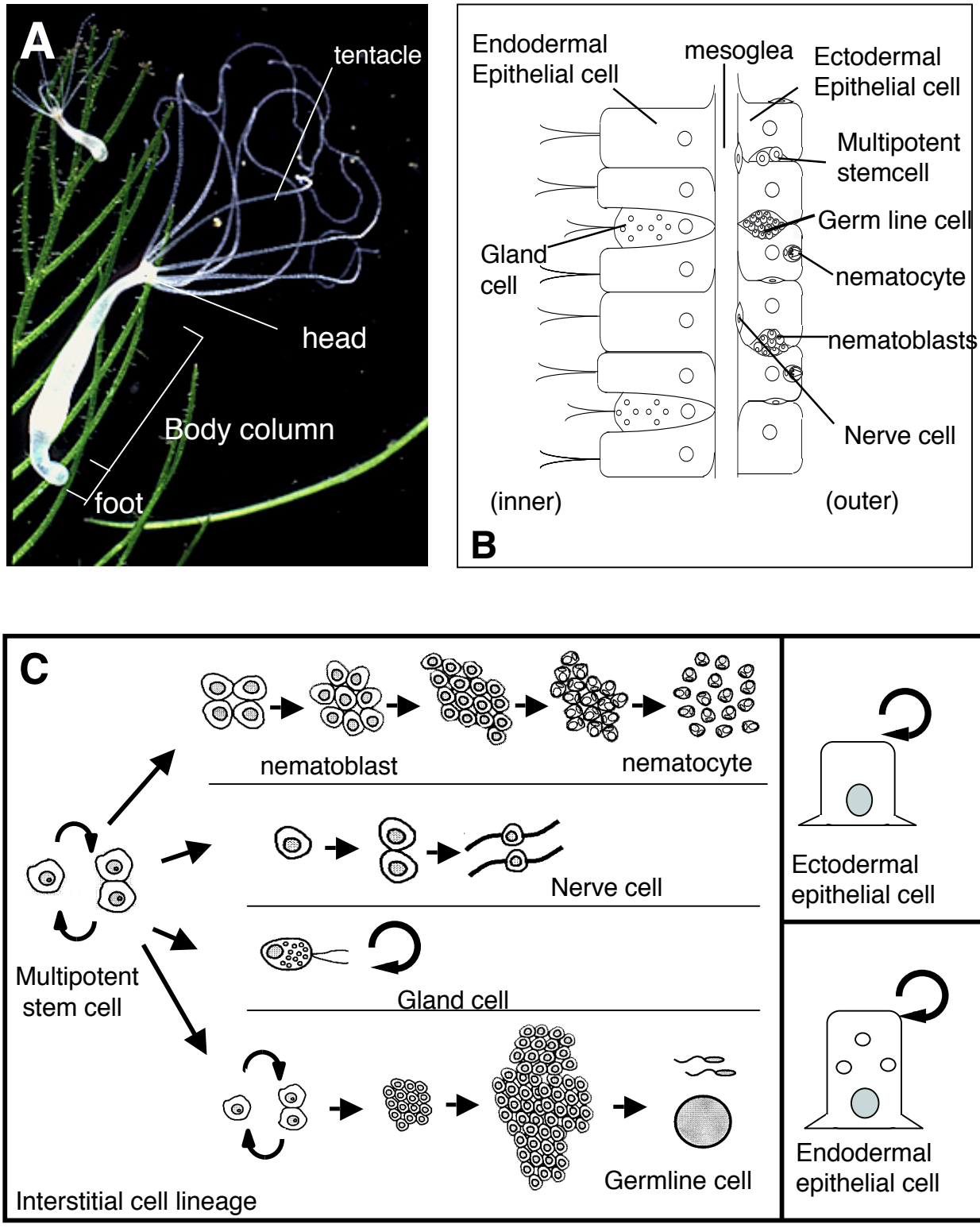
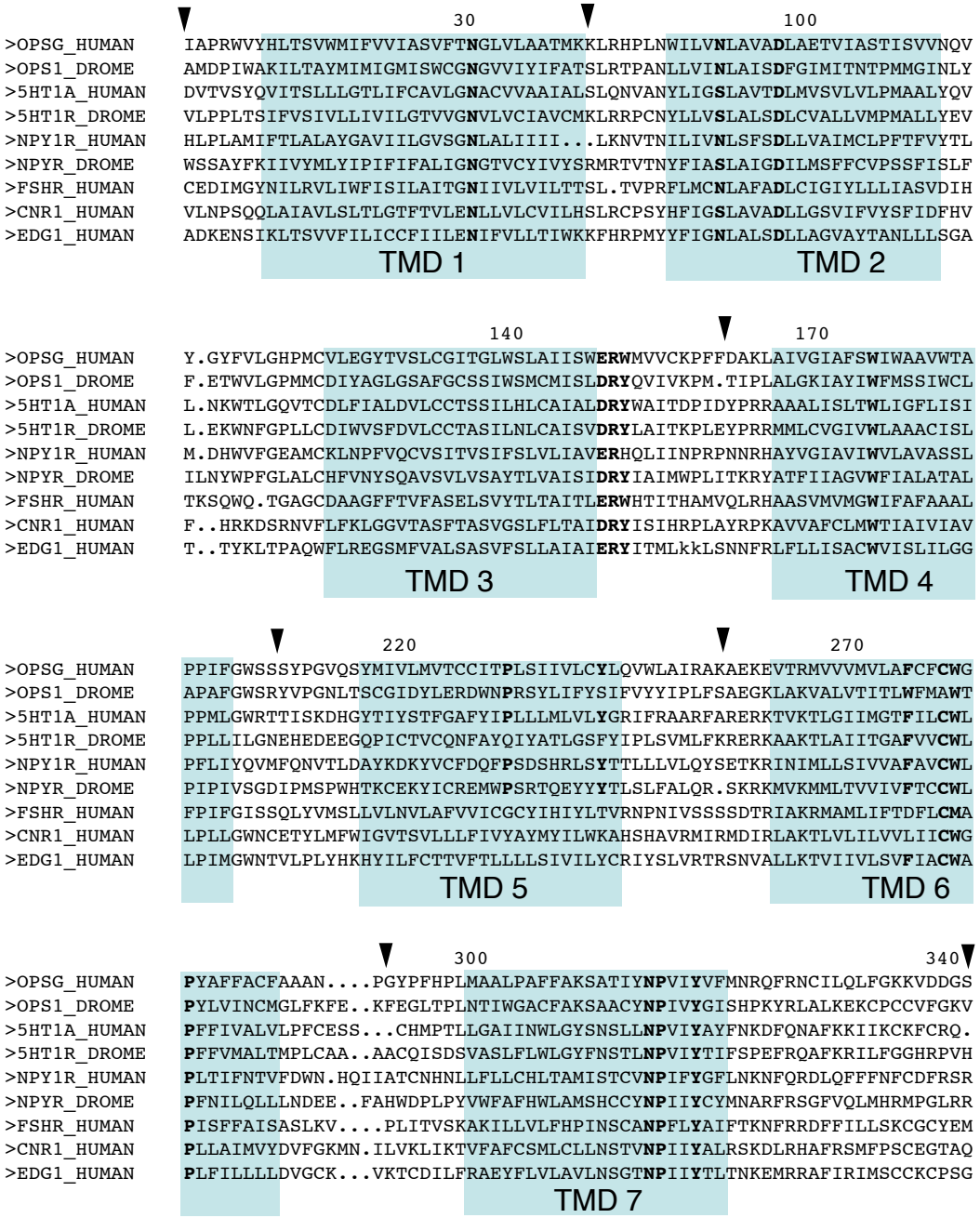

A
tentacle
head
Body column
foot
Endodermal
Epithelial cell
mesoglea
Ectodermal
Epithelial cell
Multipotent
stemcell
Germ line cell
Gland
cell
nematocyte
nematoblasts
Nerve cell
(inner)
(outer)
B
C
nematoblast
nematocyte
Nerve cell
Multipotent
stem cell
Gland cell
Germline cell
Interstitial cell lineage
Ectodermal
epithelial cell
Endodermal
epithelial cell

Fig. 4

Fig.4. Body plan and cell lineages of *Hydra*. (A) *Hydra* polyp (B) Illustration of two cellular layers of the body column. (C)Schematic drawing of three independent cell lineages in *Hydra*.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100
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>5HT1A_HUMAN DVTVSYQVITSLLLGTLIFCAVLGNACVVAAIALSLQNVANYLIGSLAVTDLMVSVLVLPMAALYQV
>5HT1R_DROME VLPPLTSIFVSIVLLIVILGTVVGNVLVCIAVCMKLRRPCNYLLVSLALSDLCVALLVMPMALLYEV
>NPY1R_HUMAN HLPLAMIFTLALAYGAVIILGVSGNLALIIII..LKNVTNILIVNLSFSDLLVAIMCLPFTFVYTL
>NPYR_DROME WSSAYFKIIVYMLYIPIFIFALIGNGTVCYIVYSRMRTVTNYFIASLAIGDILMSFFCVPSSFISLF
>FSHR_HUMAN CEDIMGYNILRVLIWFISILAITGNIIVLVILTTSL.TVPRFLMCNLAFADLCIGIYLLLIASVDIH
>CNR1_HUMAN VLNPSQQLAIAVLSLTLGTFTVLENLLVLCVILHSLRCPSYHFIGSLAVADLLGSVIFVYSFIDFHV
>EDG1_HUMAN ADKENSIKLTSVVFILICCFIILENIFVLLTIWKKFHRPMYYFIGNLALSDLLAGVAYTANLLLSGA
TMD 1
TMD 2
140
170
>OPSG_HUMAN Y.GYFVLGHPMCVLEGYTVSLCGITGLWSLAIISWERWMVVCKPFFDAKLAIVGIAFSWIWAAVWTA
>OPS1_DROME F.ETWVLGPMMCDIYAGLGSAFGCSSIWSMCMISLDRYQVIVKPM.TIPLALGKIAYIWFMSSIWCL
>5HT1A_HUMAN L.NKWTLGQVTCDLFIALDVLCCTSSILHLCAIALDRYWAITDPIDYPRRAAALISLTWLIGFLISI
>5HT1R_DROME L.EKWNFGPLLCDIWVSFDVLCCTASILNLCAISVDRYLAITKPLEYPRRMMLCVGIVWLAAACISL
>NPY1R_HUMAN M.DHWVFGEAMCKLNPFVQCVSITVSIFSLVLIAVERHQLIINPRPNNRHAYVGIAVIWVLAVASSL
>NPYR_DROME ILNYWPFGLALCHFVNYSQAVSVLVSAYTLVAISIDRYIAIMWPLITKRYATFIIAGVWFIALATAL
>FSHR_HUMAN TKSQWQ.TGAGCDAAGFFTVFASELSVYTLTAITLERWHTITHAMVQLRHAASVMVMGWIFAFAAAL
>CNR1_HUMAN F..HRKDSRNVFLFKLGGVTASFTASVGSLFLTAIDRYISIHRPLAYRPKAVVAFCLMWTIAIVIAV
>EDG1_HUMAN T..TYKLTPAQWFLREGSMFVALSASVFSLLAIAIERYITMLkkLSNNFRLFLLISACWVISLILGG
TMD 3
TMD 4
220
270
>OPSG_HUMAN PPIFGWSSSYPGVQSYMIVLMVTCCITPLSIIVLCYLQVWLAIRAKAEKEVTRMVVVMVLAFCFCWG
>OPS1_DROME APAFGWSRYVPGNLTSCGIDYLERDWNPRSYLIFYSIFVYYIPLFSAEGKLAKVALVTITLWFMAWT
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>5HT1R_DROME PPLLILGNEHEDEEGQPICTVCQNFAYQIYATLGSFYIPLSVMLFKRERKAAKTLAIITGAFVVCWL
>NPY1R_HUMAN PFLIYQVMFQNVTLDAYKDKYVCFDQFPSDSHRLSYTTLLLVLQYSETKRINIMLLSIVVAFAVCWL
>NPYR_DROME PIPIVSGDIPMSPWHTKCEKYICREMWPSRTQEYYYTLSLFALQR.SKRKMVKMMLTVVIVFTCCWL
>FSHR_HUMAN FPIFGISSQLYVMSLLVLNVLAFVVICGCYIHIYLTVRNPNIVSSSSDTRIAKRMAMLIFTDFLCMA
>CNR1_HUMAN LPLLGWNCETYLMFWIGVTSVLLLFIVYAYMYILWKAHSHAVRMIRMDIRLAKTLVLILVVLIICWG
>EDG1_HUMAN LPIMGWNTVLPLYHKHYILFCTTVFTLLLLSIVILYCRIYSLVRTRSNVALLKTVIIVLSVFIACWA
TMD 5
TMD 6
300
340
>OPSG_HUMAN PYAFFACFAAAN....PGYPFHPLMAALPAFFAKSATIYNPVIYVFMNRQFRNCILQLFGKKVDDGS
>OPS1_DROME PYLVINCMGLFKFE..KFEGLTPLNTIWGACFAKSAACYNPIVYGISHPKYRLALKEKCPCCVFGKV
>5HT1A_HUMAN PFFIVALVLPFCESS...CHMPTLLGAIINWLGYSNSLLNPVIYAYFNKDFQNAFKKIIKCKFCRQ.
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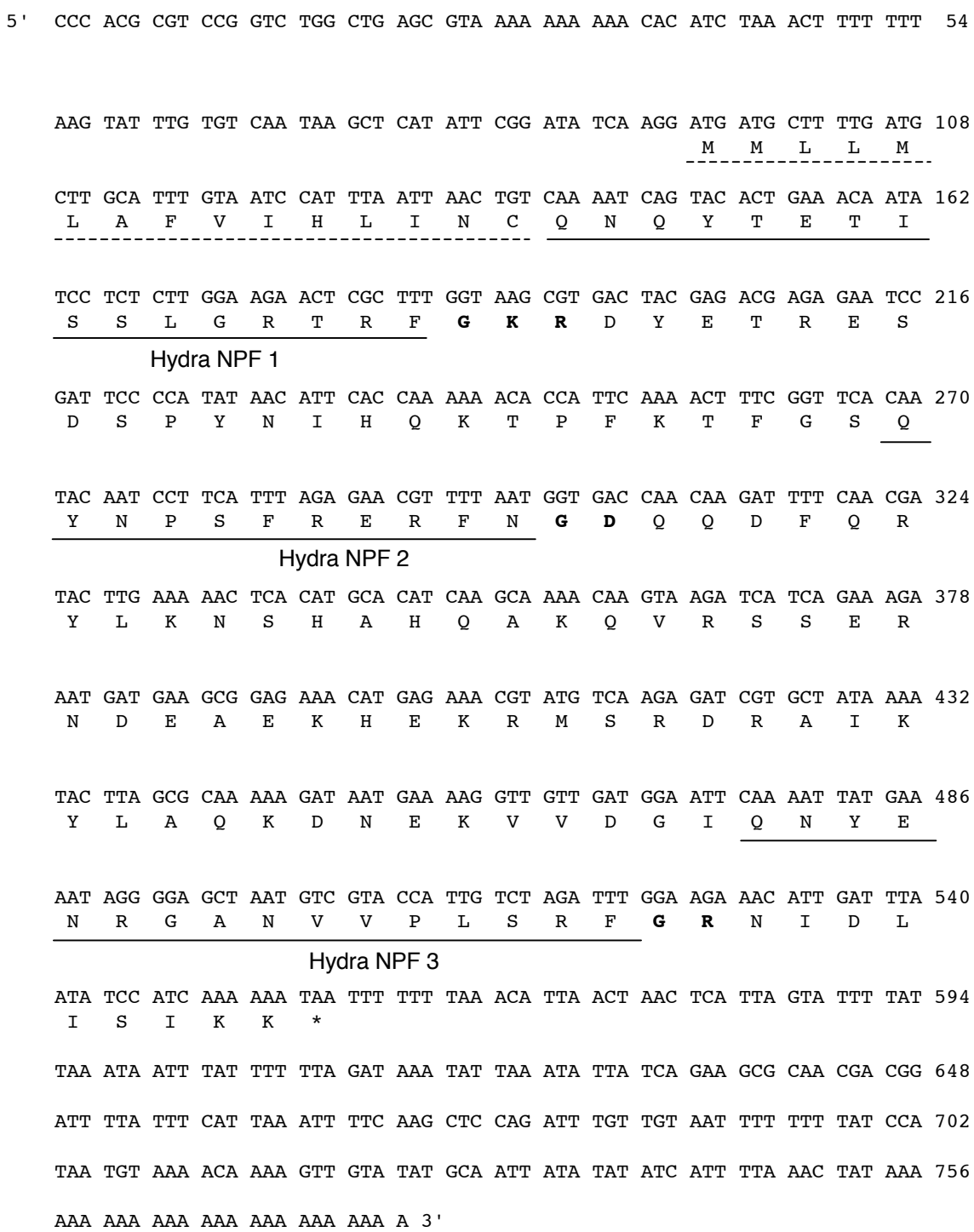
>NPYR_DROME PFNILQLLLNDEE..FAHWDPLPYVWFAFHWLAMSHCCYNPIIYCYMNARFRSGFVQLMHRMPGLRR
>FSHR_HUMAN PISFFAISASLKV....PLITVSKAKILLVLFHPINSCANPFLYAIFTKNFRRDFFILLSKCGCYEM
>CNR1_HUMAN PLLAIMVYDVFGKMN.ILVKLIKTVFAFCSMLCLLNSTVNPIIYALRSKDLRHAFRSMFPSCEGTAQ
>EDG1_HUMAN PLFILLLLDVGCK...VKTCDILFRAEYFLVLAVLNSGTNPIIYTLTNKEMRRAFIRIMSCCKCPSG
TMD 7

Fig. 5

Fig. 5. Multiple alignment of GPCR family1 members. The alignment is comprised of sequences of OPSG_Human, OPS1_Human, 5HT1A_Human, 5HT1R_*Drosophila*, NPY1R_Human, NPYR_*Drosophila*, FSHR_Human,CNR1_Human,EDG1_Human. Numbers at the top of alignment shows the amino acid number of OPSG_Human. Highly conserved amino acid residues are shown in *bold*. Transmembrane domains (TMDs) are boxed. N-terminal region, extracellular loop regions, intrarellular loop regions and C-terminal region were omitted. A*rrowhead* indicates the positions at which these sequences were removed. (OPSG)Green opsin; (OPS1)Opsin 1; (5HT1A)5HT receptor 1; (NPY1R)Neuropeptide Y receptor 1; (NPYR)Neuropeptide Y receptor; (FSHR) Follicle-stimulating hormone receptor; (CNR) Cannabinoid receptor;(EDG1)Sphingosine 1-phosphate receptor.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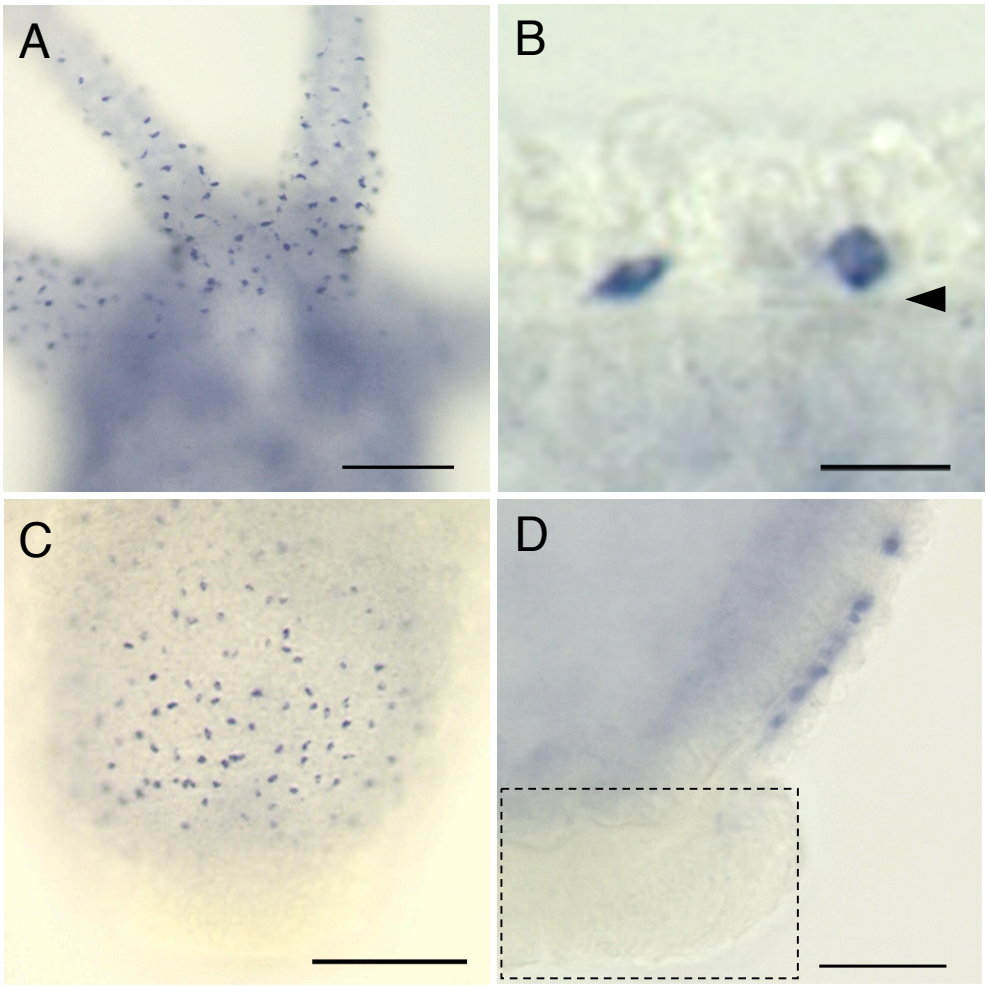
AAG TAT TTG TGT CAA TAA GCT CAT ATT CGG ATA TCA AGG ATG ATG CTT TTG ATG 108
M M L L M
CTT GCA TTT GTA ATC CAT TTA ATT AAC TGT CAA AAT CAG TAC ACT GAA ACA ATA 162
L A F V I H L I N C Q N Q Y T E T I
TCC TCT CTT GGA AGA ACT CGC TTT GGT AAG CGT GAC TAC GAG ACG AGA GAA TCC 216
S S L G R T R F G K R D Y E T R E S
Hydra NPF 1
GAT TCC CCA TAT AAC ATT CAC CAA AAA ACA CCA TTC AAA ACT TTC GGT TCA CAA 270
D S P Y N I H Q K T P F K T F G S Q
TAC AAT CCT TCA TTT AGA GAA CGT TTT AAT GGT GAC CAA CAA GAT TTT CAA CGA 324
Y N P S F R E R F N G D Q Q D F Q R
Hydra NPF 2
TAC TTG AAA AAC TCA CAT GCA CAT CAA GCA AAA CAA GTA AGA TCA TCA GAA AGA 378
Y L K N S H A H Q A K Q V R S S E R
AAT GAT GAA GCG GAG AAA CAT GAG AAA CGT ATG TCA AGA GAT CGT GCT ATA AAA 432
N D E A E K H E K R M S R D R A I K
TAC TTA GCG CAA AAA GAT AAT GAA AAG GTT GTT GAT GGA ATT CAA AAT TAT GAA 486
Y L A Q K D N E K V V D G I Q N Y E
AAT AGG GGA GCT AAT GTC GTA CCA TTG TCT AGA TTT GGA AGA AAC ATT GAT TTA 540
N R G A N V V P L S R F G R N I D L
Hydra NPF 3
ATA TCC ATC AAA AAA TAA TTT TTT TAA ACA TTA ACT AAC TCA TTA GTA TTT TAT 594
I S I K K *
TAA ATA ATT TAT TTT TTA GAT AAA TAT TAA ATA TTA TCA GAA GCG CAA CGA CGG 648
ATT TTA TTT CAT TAA ATT TTC AAG CTC CAG ATT TGT TGT AAT TTT TTT TAT CCA 702
TAA TGT AAA ACA AAA GTT GTA TAT GCA ATT ATA TAT ATC ATT TTA AAC TAT AAA 756
AAA AAA AAA AAA AAA AAA AAA A 3'

Fig. 6

Fig. 6. Nucleotide and deduced amino acid sequences of the *Hydra* neuropeptide F precursor gene. The predicted neuropeptide F1, 2 and 3 sequences are underlined. Amidation motifs are shown in bold. The predicted signal sequence is underlined with a dotted line.The asterisk indicates a stop codon.



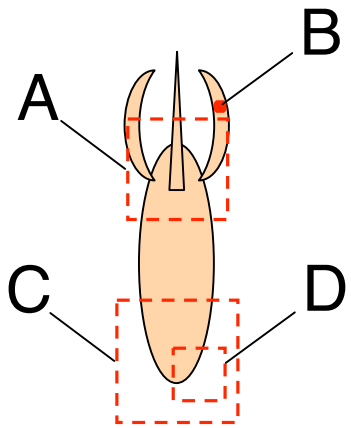


Fig. 7

Fig. 7. Expression of the *Hydra* neuropeptide F precursor gene analyzed by whole mount in istu hybridization of *Hydra*. (A)Head region. (B) High magnification of tentacles to show the basal localization above mesoglea. *Arrow head* indicates mesoglea. (C)Foot region.(D)Side view of foot region. Basal disc is boxed. Bars, 100 μ m(A,C) 10 μ m(B)50 μ m(D). The illustration shows the areas where the photos (A-D) were taken.

(A)

```
5'  ATG CTT CTC TGG ATC GTC GCC ACT TTG CTC ATT TTT AGC CTT CCA GTG TCA ACG  54
     M   L   L   W   I   V   A   T   L   L   I   F   S   L   P   V   S   T

    GCT TTA GAT TAT AAT GAT TTC TCC TTG CAA CGA ATT GCT CGG GCT CCA CAT CCA 108
     A   L   D   Y   N   D   F   S   L   Q   R   I   A   R   A   P   H   P

    TCA TCT GCT CTC TTA GTG CCA TAC CCA AGA GTC GGA AAA CGA AGT AAC ATT TTG 162
     S   S   A   L   L   V   P   Y   P   R   V   G   K   R   S   N   I   L
                         Hym-355-like 1
    AAT AAC AAT TCC GAA TCA CAA AAC TCT GTC CAA AAG AGA CTC TAC ATG GCG CGT 216
     N   N   N   S   E   S   Q   N   S   V   Q   K   R   L   Y   M   A   R
                                                               Hym-355-like 2
    GTT GGA AAA CGA GCA TTT TTC TAC ACT CCA CGT ATT GGG AAA TAA 3'
     V   G   K   R   A   F   F   Y   T   P   R   I   G   K   *
                                Hym-355-like 3
```

(B)

```
5'  ATG CTC CTC TGG TTG GTC GCC ACT TGG CTC ATC CTC ACC GCC GTT CCT GCT ACA 54
     M   L   L   W   L   V   A   T   W   L   I   L   T   A   V   P   A   T

    TGC TTA GAC TAT AAT GAT TAC TCT CTT CAA CGG ATC GCT CGG GCT CCA CAT CCG 108
     C   L   D   Y   N   D   Y   S   L   Q   R   I   A   R   A   P   H   P

    TCG TCT GCC CTT TTG GTA CCG TAC CCA CGA GTT GGA AAA CGG AGC GTG AAC GGA 162
     S   S   A   L   L   V   P   Y   P   R   V   G   K   R   S   V   N   G
                 Hym-355-like 1
    CCA CGG CAT CTG GAA TCA AGG AAC ACC GCT CAA AAA CGG CTT TAC ATG GCT CGC 216
     P   R   H   L   E   S   R   N   T   A   Q   K   R   L   Y   M   A   R
                                                           Hym-355-like 2
    GTT GGG AAA CGA GCA TTC TTC TAT GCT CCG CGT GTT GGA AAA TGA 3'
     V   G   K   R   A   F   F   Y   A   P   R   V   G   K   *
                              Hym-355-like 3
```

(C)

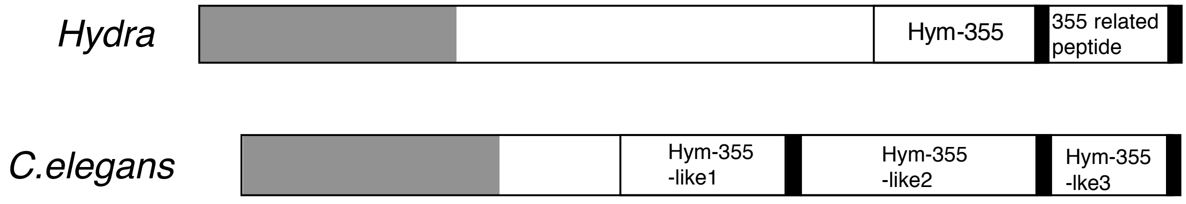
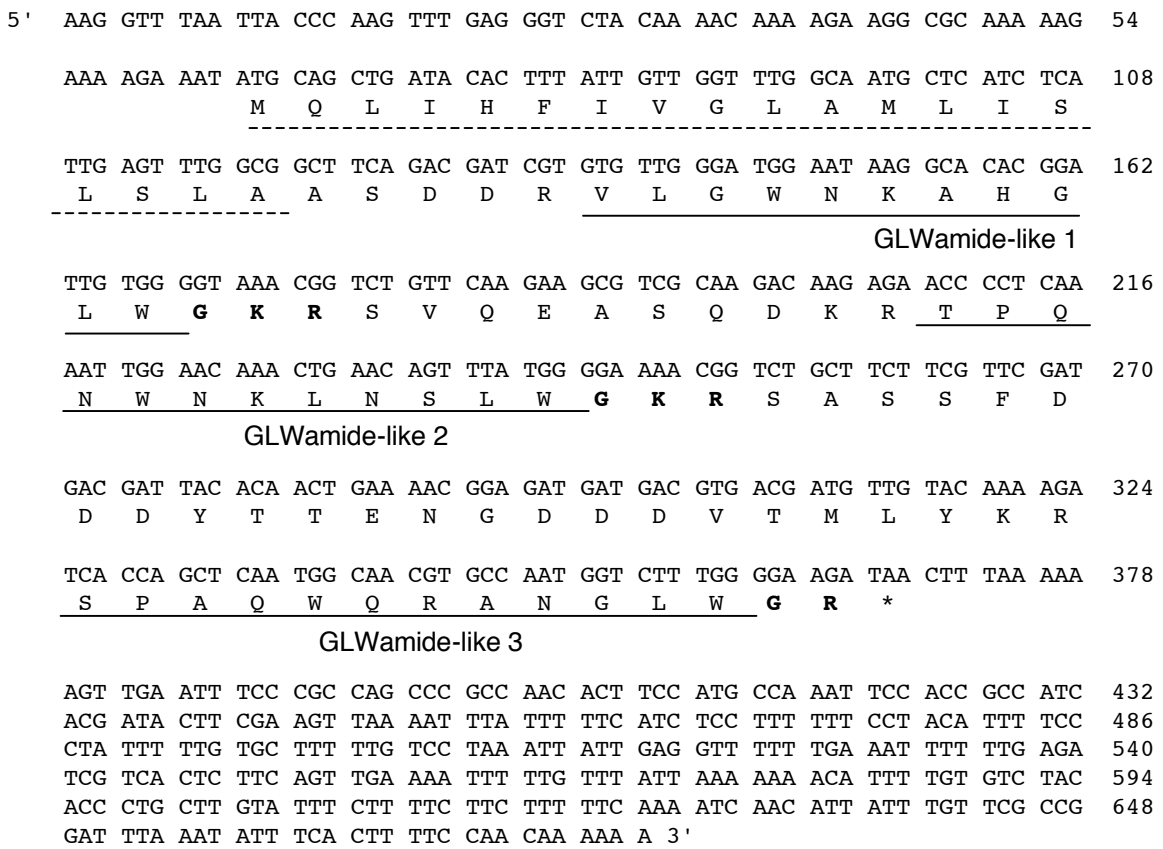


Fig. 8

Fig. 8. Nucleotide and deduced amino acid sequences of Hym-355-like peptide encoding genes from nematodes. (A) *C.elegans* Hym-355-like peptide encoding gene. (B) *C. briggsae* Hym-355-like peptide encoding gene. The predicted Hym-355-like sequences are underlined. Amidation motifs are shown in bold. The predicted signal sequence is underlined with a dotted line. The asterisk indicated a stop codon. (C)Illustration showing precursor structures with *Hydra* Hym-355 and *C. elegans* Hym355-like peptides. Signal peptide is shown by gray box and amidation motif are shown by black box.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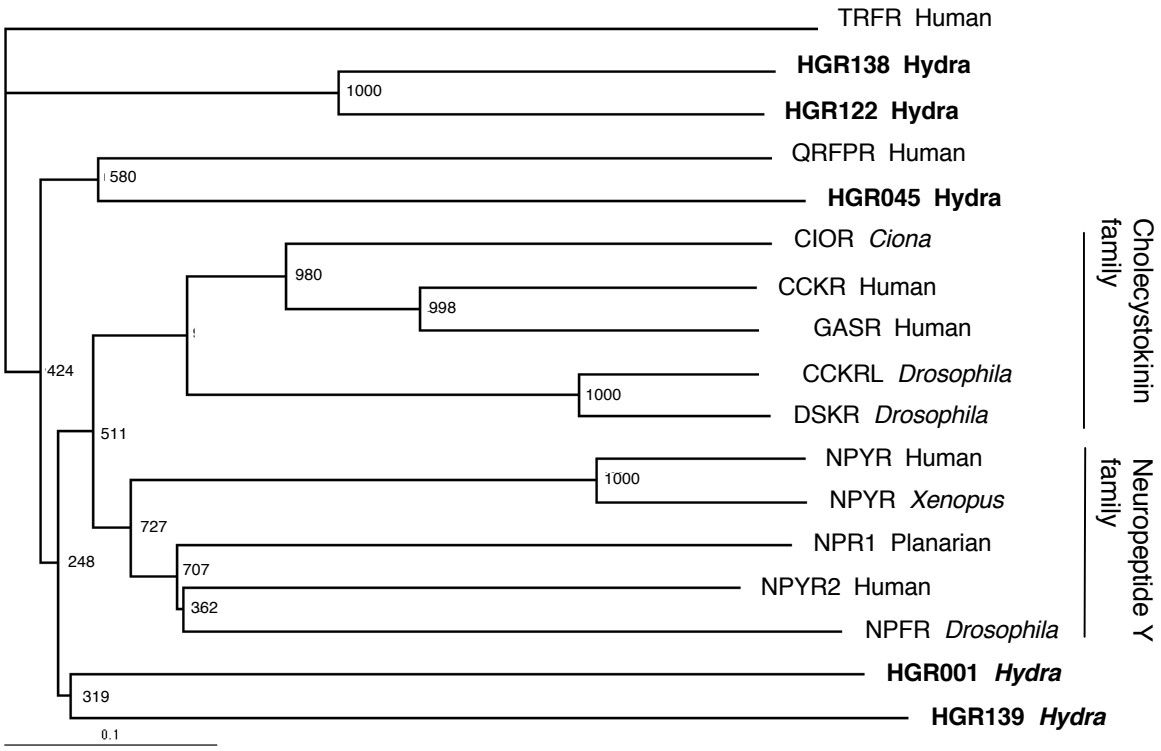
AAA AGA AAT ATG CAG CTG ATA CAC TTT ATT GTT GGT TTG GCA ATG CTC ATC TCA 108
M Q L I H F I V G L A M L I S
TTG AGT TTG GCG GCT TCA GAC GAT CGT GTG TTG GGA TGG AAT AAG GCA CAC GGA 162
L S L A A S D D R V L G W N K A H G
GLWamide-like 1
TTG TGG GGT AAA CGG TCT GTT CAA GAA GCG TCG CAA GAC AAG AGA ACC CCT CAA 216
L W G K R S V Q E A S Q D K R T P Q
AAT TGG AAC AAA CTG AAC AGT TTA TGG GGA AAA CGG TCT GCT TCT TCG TTC GAT 270
N W N K L N S L W G K R S A S S F D
GLWamide-like 2
GAC GAT TAC ACA ACT GAA AAC GGA GAT GAT GAC GTG ACG ATG TTG TAC AAA AGA 324
D D Y T T E N G D D D V T M L Y K R
TCA CCA GCT CAA TGG CAA CGT GCC AAT GGT CTT TGG GGA AGA TAA CTT TAA AAA 378
S P A Q W Q R A N G L W G R *
GLWamide-like 3
AGT TGA ATT TCC CGC CAG CCC GCC AAC ACT TCC ATG CCA AAT TCC ACC GCC ATC 432
ACG ATA CTT CGA AGT TAA AAT TTA TTT TTC ATC TCC TTT TTT CCT ACA TTT TCC 486
CTA TTT TTG TGC TTT TTG TCC TAA ATT ATT GAG GTT TTT TGA AAT TTT TTG AGA 540
TCG TCA CTC TTC AGT TGA AAA TTT TTG TTT ATT AAA AAA ACA TTT TGT GTC TAC 594
ACC CTG CTT GTA TTT CTT TTC TTC TTT TTC AAA ATC AAC ATT ATT TGT TCG CCG 648
GAT TTA AAT ATT TCA CTT TTC CAA CAA AAA A 3'

Fig. 9

Fig. 9. Nucleotide and deduced amino acid sequences of *C. elegans* GLWamide encoding gene. The predicted GLW amide sequences are underlined. Amidation motifs are shown in bold. The predicted signal sequence is underlined with a dotted line. The asterisk indicates a stop codon.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HGR138 Hydra
1000
HGR122 Hydra
QRFPR Human
580
HGR045 Hydra
CIOR Ciona
980
CCKR Human
998
GASR Human
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CCKRL Drosophila
1000
DSKR Drosophila
511
NPYR Human
1000
NPYR Xenopus
727
NPR1 Planarian
248
707
NPYR2 Human
362
NPFR Drosophila
HGR001 Hydra
319
HGR139 Hydra
0.1
Cholecystokinin family
Neuropeptide Y family

Fig. 10

Fig.10. Neighbor-joining phylogenetic tree of the neuropeptide Y and cholecystokinin receptor families. *Hydra* GPCRs were shown in bold. (TRF) Thyrotropin releasing factor receptor; (QREPR) QRFP receptor; (CIOR) Cionin receptor; (CCKR)Cholecystokinin receptor; (GASR) Gastrin receptor; (CCKRL) Cholecystokinin receptor-like GPCR; (DSKR) Drosulfakinin receptor; (NPYR) Neuropeptide Y receptor; (NPYR2) Neuropeptide Y receptor 2; (NPFR) Neuropeptide F receptor. Thyrotropin releasing factor receptor was utilized as an out group for the tree. Numbers at the nodes are results of a bootstrap analysis, out of 1000 replicates. The scale bar corresponds to 0.1 estimated amino acid substitutions per site

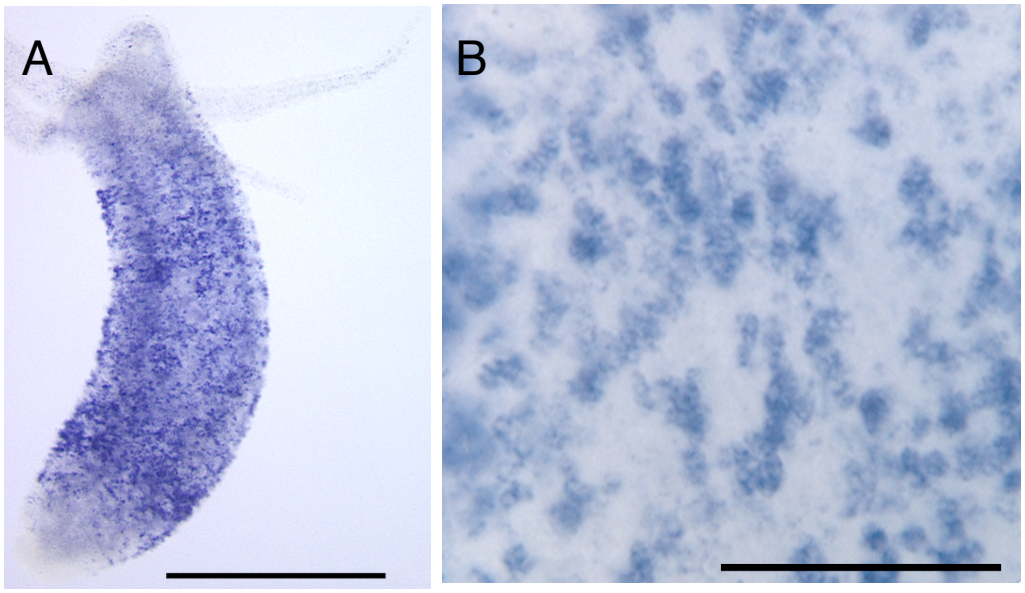
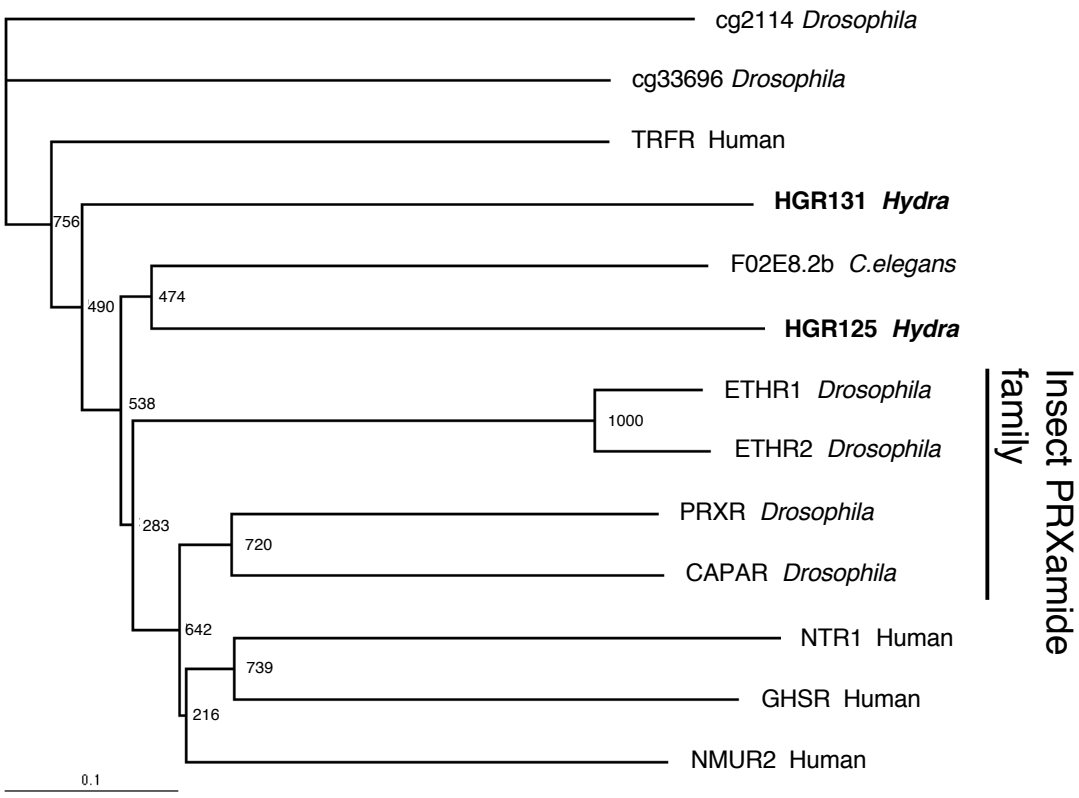


Fig. 11

Fig. 11. Expression of a *Hydra* GPCR, HGR001 analyzed by whole mount in situ hybridization in *Hydra*. A) Whole animal. (B) High magnification of body column. Bars, 0.5 μ m (A) 0.1 μ m(B)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cg33696 Drosophila
TRFR Human
HGR131 Hydra
F02E8.2b C.elegans
HGR125 Hydra
ETHR1 Drosophila
ETHR2 Drosophila
PRXR Drosophila
CAPAR Drosophila
NTR1 Human
GHSR Human
NMUR2 Human
756
490
474
538
1000
283
720
642
739
216
0.1
Insect PRXamide family

Fig. 12

Fig.12. Neighbor-joining phylogenetic tree for the PRXamide receptor family. (TRF) Thyrotropin releasing factor receptor; (ETHR1) Ecdysis triggering hormone receptor 1; (ETHR2) Ecdysis triggering hormone receptor 2; (PRXR)PRX-pyrokinin receptor; (CAPAR) CAPa receptor; (NTR1) Neurotensin receptor; (GHSR) Growth hormone secretagogue receptor; (NMUR2) Neuromedin U receptor 2. cg2114 (FMRFamide receptor) was utilized as an out group for the tree. Numbers at the nodes are results of a bootstrap analysis, out pf 1000 replicates The scale bar corresponds to 0.1 estimated amino acid substitutions per site

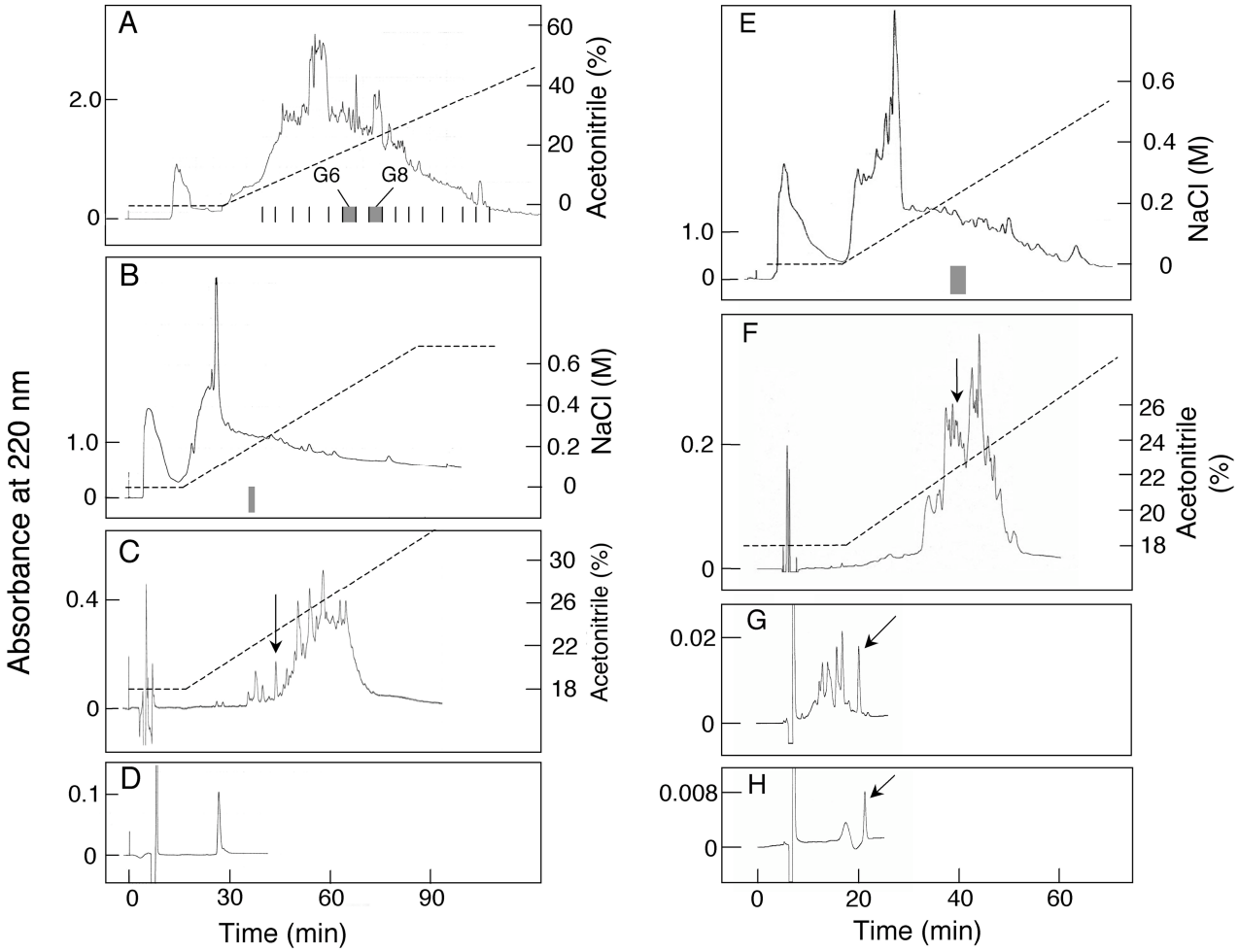
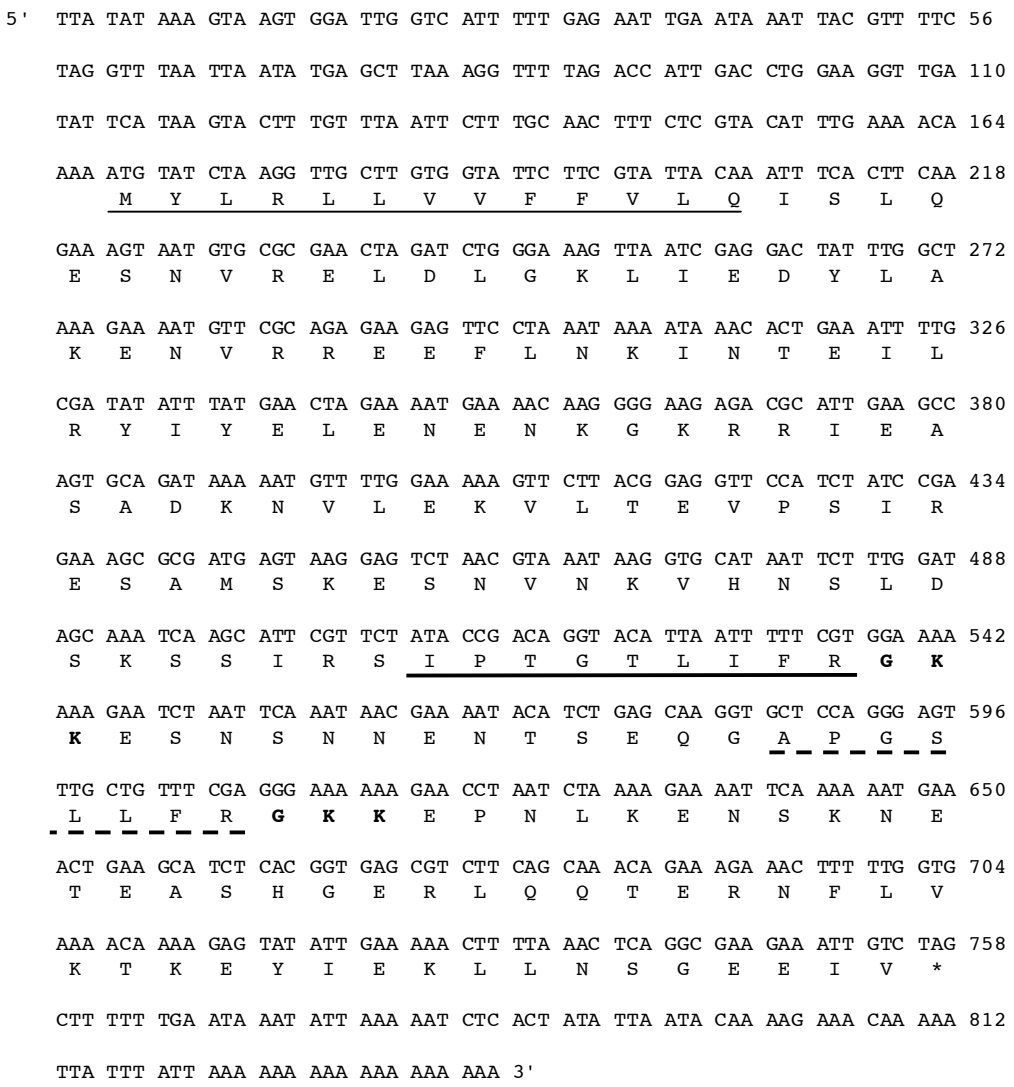

A
B
C
D
E
F
G
H
G6
G8
Absorbance at 220 nm
Acetonitrile (%)
NaCl (M)
Time (min)

Fig. 13

Fig. 13. Purification steps of FRamide-1 (A,B,C,D) and FRamide-2 (A, E, F, G, H). FRamide-1 was isolated as following steps. (A) Step1: C-18 reverse-phase HPLC. The fractions were divided into 15 groups, as indicated on the elution profile. This step is also used for FRamide-2 isolation. (B) Step2: cation-exchange HPLC of group 9 (G9) from A. The fraction indicated by the bar was subjected to further purification. (C) Step3: C-18 reverse phase HPLC. The peak indicated with the arrow was used in the next step. (D) Step4: HPLC with an isocratic elution of 20 % acetonitrile(ACN). On the other hand, FRamide-2 was isolatd as following steps. (E) Step2: cation-exchange HPLC of group 6(G6) from A. The fraction indicated by the bar was subjected to further purification. (F) Step3: C-18 reverse phase HPLC. The peak indicated with the arrow was used in the next step. (G) Step4: HPLC with an isocratic elution of 23 % ACN. (H) Step5: HPLC with an isocratic elution of 23% ACN.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TAT TCA TAA GTA CTT TGT TTA ATT CTT TGC AAC TTT CTC GTA CAT TTG AAA ACA 164
AAA ATG TAT CTA AGG TTG CTT GTG GTA TTC TTC GTA TTA CAA ATT TCA CTT CAA 218
M Y L R L L V V F F V L Q I S L Q
GAA AGT AAT GTG CGC GAA CTA GAT CTG GGA AAG TTA ATC GAG GAC TAT TTG GCT 272
E S N V R E L D L G K L I E D Y L A
AAA GAA AAT GTT CGC AGA GAA GAG TTC CTA AAT AAA ATA AAC ACT GAA ATT TTG 326
K E N V R R E E F L N K I N T E I L
CGA TAT ATT TAT GAA CTA GAA AAT GAA AAC AAG GGG AAG AGA CGC ATT GAA GCC 380
R Y I Y E L E N E N K G K R R I E A
AGT GCA GAT AAA AAT GTT TTG GAA AAA GTT CTT ACG GAG GTT CCA TCT ATC CGA 434
S A D K N V L E K V L T E V P S I R
GAA AGC GCG ATG AGT AAG GAG TCT AAC GTA AAT AAG GTG CAT AAT TCT TTG GAT 488
E S A M S K E S N V N K V H N S L D
AGC AAA TCA AGC ATT CGT TCT ATA CCG ACA GGT ACA TTA ATT TTT CGT GGA AAA 542
S K S S I R S I P T G T L I F R G K
AAA GAA TCT AAT TCA AAT AAC GAA AAT ACA TCT GAG CAA GGT GCT CCA GGG AGT 596
K E S N S N N E N T S E Q G A P G S
TTG CTG TTT CGA GGG AAA AAA GAA CCT AAT CTA AAA GAA AAT TCA AAA AAT GAA 650
L L F R G K K E P N L K E N S K N E
ACT GAA GCA TCT CAC GGT GAG CGT CTT CAG CAA ACA GAA AGA AAC TTT TTG GTG 704
T E A S H G E R L Q Q T E R N F L V
AAA ACA AAA GAG TAT ATT GAA AAA CTT TTA AAC TCA GGC GAA GAA ATT GTC TAG 758
K T K E Y I E K L L N S G E E I V *
CTT TTT TGA ATA AAT ATT AAA AAT CTC ACT ATA TTA ATA CAA AAG AAA CAA AAA 812
TTA TTT ATT AAA AAA AAA AAA AAA AAA 3'

Fig. 14

Fig. 14. Nucleotide and deduced amino acid sequence of the FRamide-1/FRamide-2 encoding gene. The predicted signal sequence is thinly underlined. The FRamide-1 sequence is thickly underlined and the FRamide-2 sequence is underlined with a dotted line. The *asterisk* indicates a stop codon.

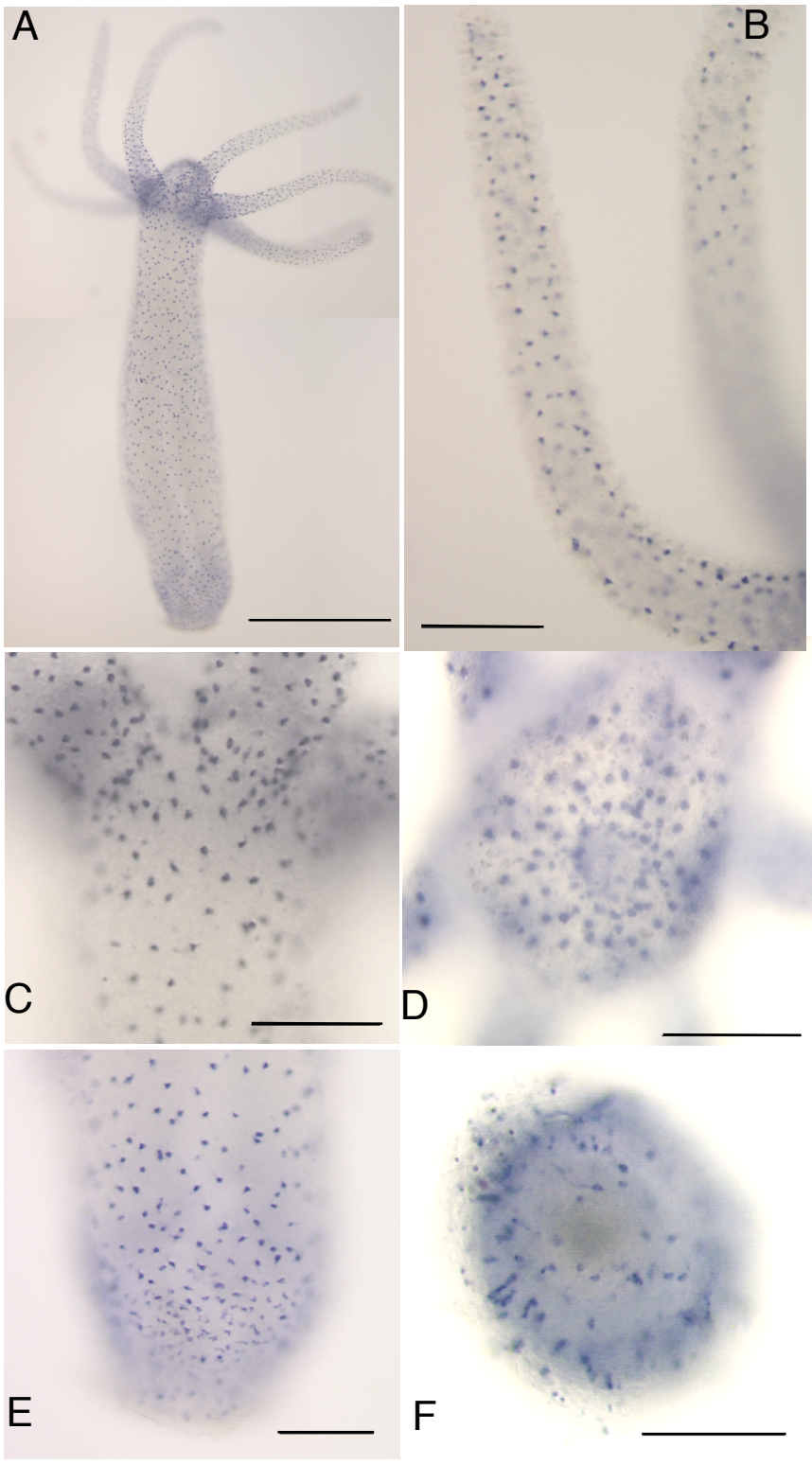


Fig. 15

Fig. 15. Expression of the FRamide-1/FRamide-2 encoding gene analyzed by whole mount in situ hybridization. (A) Whole animal. (B)Tentacles. (C)Head region. (D) Hypostome as observed from the top. (E) Foot region. (F) Basal disk as observed from the bottom. Bars, 0.5 mm (A) and 0.1 mm (B, C, D, E, F).

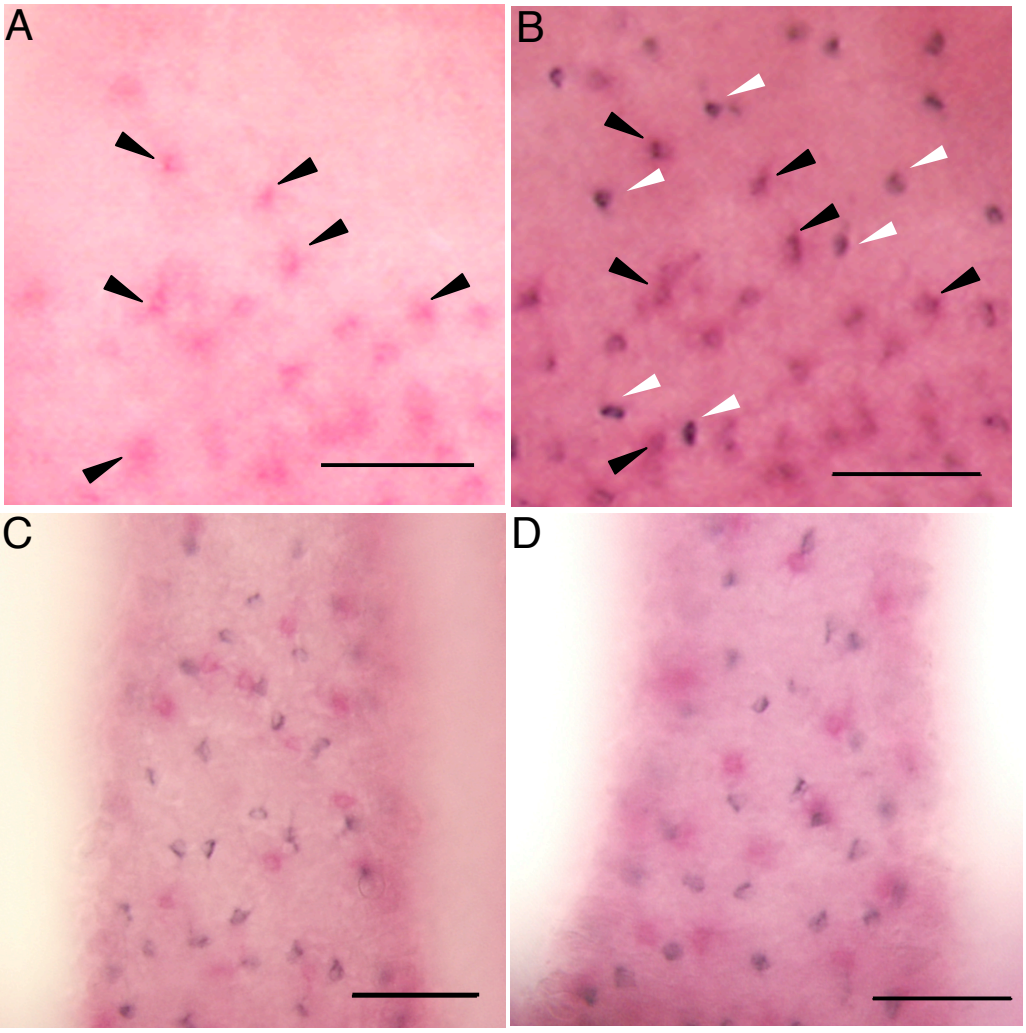
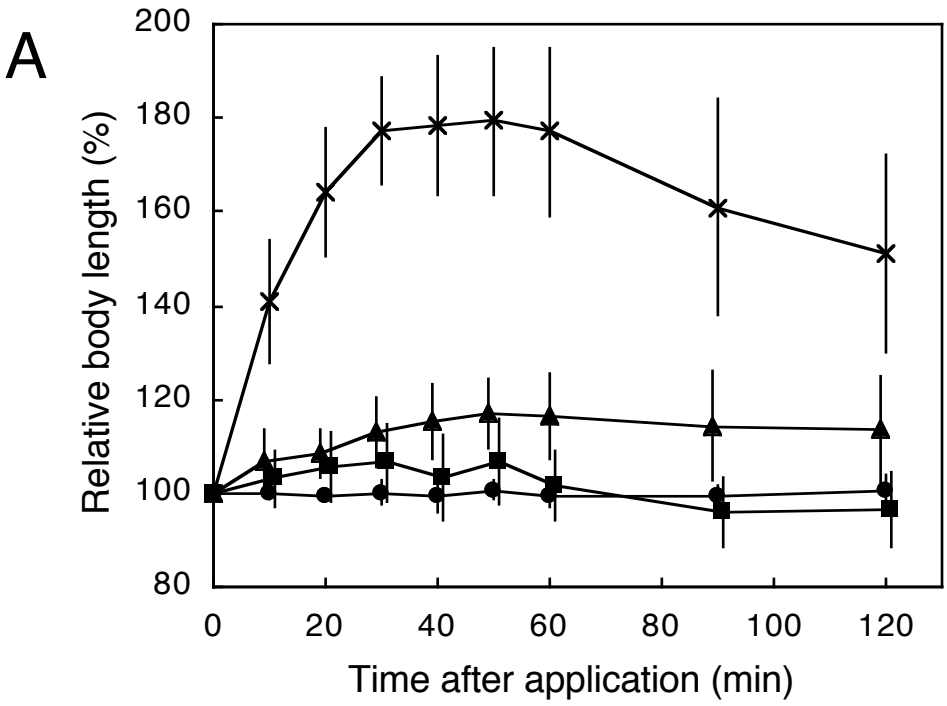


Fig. 16

Fig. 16. Double in situ hybridization of the FRamide-1/FRamide-2 encoding gene and Hym-176, Hym355 or LWamide encoding gene. (A) Neurons expressing the Hym-176 precursor gene (*red*) detected after first staining in foot region. (B) The same region as in A after second staining. *Black* arrowheads show the same neurons expressing the Hym-176 encoding gene (A) as well as FRamide-1/FRamide-2 (*blue*) encoding gene. *White* arrowheads show some neurons expressing only FRamide-1/FRamide-2 . (C) Neurons independently expressing the FRamide-1/FRamide-2 (*blue*) and Hym-355(*red*) encoding genes in the tentacle region. (D) Neurons independently expressing the FRamide-1/FRamide-2 (*blue*) and LWamide(*red*) encoding genes in the tentacle region. *Scale bars* = 50 μm


A
Relative body length (%)
200
180
160
140
120
100
80
0
20
40
60
80
100
120
Time after application (min)

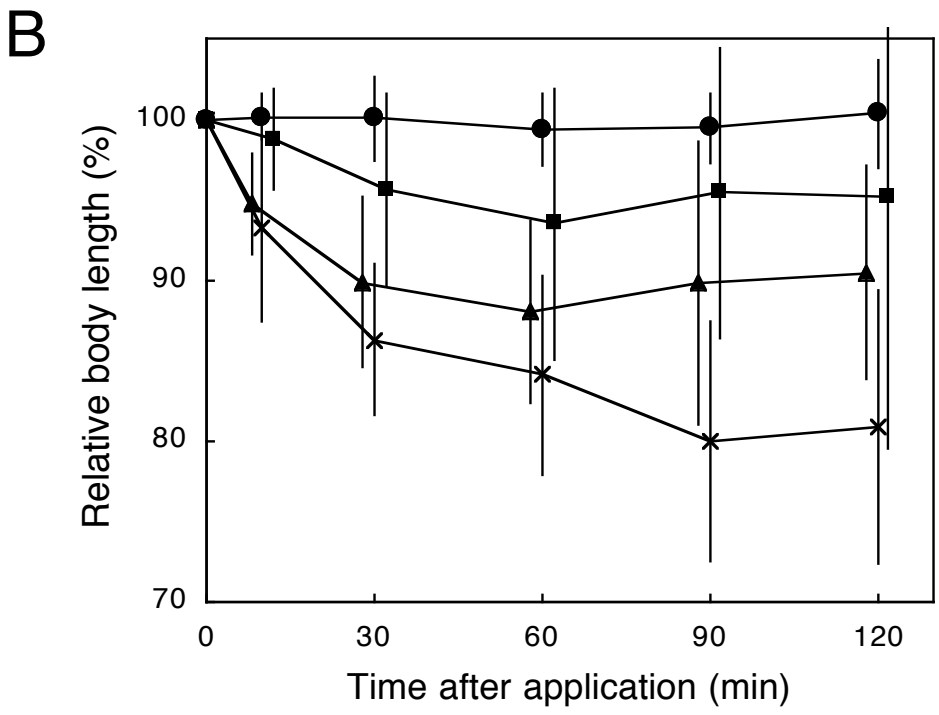

B
Relative body length (%)
100
90
80
70
0
30
60
90
120
Time after application (min)

Fig. 17

Fig. 17. Effects of FRamide-1 (A) and FRamide-2 (B) on body elongation and contraction of epithelial *Hydra*. *Filled circles* control, *filled squares* 10^{-6} M , *filled triangles* 10^{-5} M, *crosses* 10^{-4} M of a peptide. Each point represents mean value obtained from 10-12 animals. Vertical bars indicate standard deviation of the mean.