Chapter 8
Neuropeptides and Their Physiological Functions in Mollusks

Fumihiro Morishita

Abstract Neuropeptides have essential functions in the neural regulation of physiological functions of various tissues and organs, as well as of animal behaviors. Many neuropeptides have been identified in mollusks, and investigation of their functions is currently proceeding. In this review, I attempt to give an overview of the neuropeptides in mollusks. Then, regulatory actions of neuropeptides are described with a special reference to reproduction. I chose three neuropeptides: egg-laying hormone (ELH) and caudodorsal cell hormone (CDCH), gonadotropin-releasing hormone (GnRH), and APGWamide. ELH and CDCH are well-investigated peptide hormones that trigger complex egg-laying behaviors in *Aplysia* and *Lymnaea*. GnRH, which is a key peptide that induces gonadal maturation and ovulation in mammals, also regulates gonadal maturation in bivalves and cephalopods. However, evidence suggests that GnRH also mediates other activities such as feeding and locomotion in mollusks. APGWamide, which regulates the male copulatory activity in freshwater snails, seems to have pheromonal actions in bivalves and cephalopods. These facts collectively emphasize the diverse actions of neuropeptides and peptide hormones on the regulation of reproduction in mollusks.

Keywords Neuropeptide • Peptide hormone • Nervous system • Endocrine system • Reproduction

Abbreviations

ACEP	*Achatina* cardioexcitatory peptide
AGP	atrial gland peptide
AKH	adipokinetic hormone
BCP	bag cell peptide
CDC	caudodorsal cell
CDCH	caudodorsal cell hormone

F. Morishita (✉)
Department of Biological Science, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8511, Japan
e-mail: fumi425@hiroshima-u.ac.jp

T. Horiguchi (ed.), *Biological Effects by Organotins*,
DOI 10.1007/978-4-431-56451-5_8

CDCP	caudodorsal cell peptide
EC_{50}	effective concentration that induce 50 % response
EDC	endocrine-disrupting chemical
ELH	egg-laying hormone
GnRH	gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
HPLC	high-performance liquid chromatography
LC-ESI-MS/MS	liquid chromatography–electrospray ionization tandem mass spectrometry
LUQ	left upper quadrant
NMDA	*N*-methyl-*D*-aspartate
PC2	prohormone convertase 2
PCR	polymerase chain reaction
Q-PCR	quantitative PCR
PTM	post-translational modification
RT-PCR	reverse transcription PCR
TEP	*Thais* excitatory peptide

8.1 Introduction

When the internal environments of animals are challenged, stabilization of homeostasis is essential for their survival. When the animal is fully grown-up, reproduction must be started at the proper timing. In various aspects of animal life, the nervous system and endocrine system have central roles in mounting the proper reactions to environmental stimuli, which lead to the survival and breeding of species. Since various chemicals including acetylcholine, biogenic amines, amino acids, nucleotides, steroids, and peptides mediate the regulatory actions of the nervous and endocrine systems, elucidation of the function of the signal molecules is necessary to understand the cellular and molecular mechanisms of neural and hormonal control of the physiological activities and behaviors in animals.

In structure and function, the diversity of neuropeptides and peptide hormones overwhelms that of other signal molecules. In fact, many peptides were identified in mollusks in the past four decades through the combination of fractionation of peptidic extracts by high-performance liquid chromatography (HPLC) and immunological or biological screening of the obtained fractions. Identification of novel peptides is accelerated nowadays by analysis using mass spectrometry supported by genetic information such as the genome sequence and expressed sequence tag (EST) data. Through the intensive investigations on the physiological functions of the identified peptides, it is well accepted that neuropeptides and peptide hormones have central roles in regulation of homeostasis and animal behaviors, including reproduction and feeding in mollusks.

Mollusks, as the consequence of evolution, are adapted to diverse environments such as land, freshwater, and seawater. They have evolved various strategies for

reproduction including gonochorism, hermaphroditism, and sex reversal. Accordingly, the cellular and molecular mechanisms that regulate reproduction are also diverse. In bivalves and primitive gastropods, eggs and sperm are released into the seawater and fertilization occurs externally (Fretter 1984; Mackie 1984). In those animals, a mechanism that triggers the synchronized gamete release is essential. In many gonochorismic (Fretter 1984) and hermaphroditic (Geraerts and Joosse 1984; Hadfield and Switzer-Dunlap 1984; Tompa 1984) gastropods, fertilization occurs internally, after copulation with the conspecific. In those animals, neural and hormonal systems that motivate courtship behaviors concomitant with sexual maturation are important for mating. Some mollusks exhibit protandrous sex reversal once in their lifetime, and others exhibit repetitive sex reversal every mating season (Fretter 1984; Mackie 1984). In those animals, changes in a particular hormone level may induce the sex reversal.

In the 1980s, it was recognized that organotin compounds such as triphenyl tin and tributyl tin in seawater induce the secondary formation of male reproductive organs or sperm formation (imposex) in female gastropod snails (Smith 1981), which disturbs reproduction of the afflicted snails (Birchenough et al. 2002; Horiguchi 2006; Titley-O'Neal et al. 2011). It was once proposed that malfunctioning of the neuropeptide system, including the penis-morphogenic factor (Geraerts et al. 1988) and APGWamide (Oberdorster and McClellan-Green 2002), is a key step for the induction of imposex by environmental organotin. Now, accumulating evidence suggests that the primary target molecule for organotin is a nuclear receptor, retinoid X receptor (RXR) (Nishikawa et al. 2004; Castro et al. 2007). Because RXR regulates gene expression, it is possible to assume that the endocrine-disrupting chemical (EDC) induces malfunctioning of the regulatory neuropeptide system by modifying the expression of neuropeptide precursor and receptor genes.

Unfortunately, we do not have enough evidence to explain all the neural and hormonal regulation of reproduction in mollusks. Nevertheless, accumulated data have demonstrated that neuropeptides and peptide hormones, such as egg-laying hormone (ELH) and caudodorsal cell hormone (CDCH), trigger egg-laying behaviors in the gastropods *Aplysia* and *Lymnaea*. Gonadotropin-releasing hormone (GnRH)-related peptides are also found in mollusks, and their functions are currently being investigated. Therefore, I believe that it is informative for all readers who are interested in imposex of mollusks to briefly summarize how neuropeptides and peptide hormones regulate mollusk reproduction.

In this review, I provide some basic information on the molluscan neuropeptides. Then, I refer to the structure and function of neuropeptides of prosobranch gastropods so far identified. Finally, I characterize three neuropeptides, namely, APGWamide, ELH and CDCH, and GnRH, to overview the regulatory system of the reproduction in mollusks, including bivalves and cephalopods. Apparently, it is beyond my capacity to cite all the papers on reproduction in mollusks. Readers can refer to other books and review articles on the peptide biology of mollusks (Chase 2002; Morishita and Furukawa 2006; Ketata et al. 2008; Koene 2010; Morishita et al. 2010).

8.2 Basic Information on Molluscan Neuropeptides

Most of the neuropeptides found in mollusks are oligopeptides, consisting of fewer than 20 amino acids (Muneoka et al. 2000; Morishita and Furukawa 2006). These peptides are synthesized in neurons as a part of the precursor protein. On the precursors of short peptides such as APGWamide, FMRFamide, and PRQFVamide, the same peptide sequences are aligned in tandem on the respective precursor. For example, the *Lymnaea* APGWamide precursor contains 10 copies of APGWamide (Smit et al. 1992), the *Aplysia* FMRFamide precursor contains 28 copies of FMRFamide (Taussig and Scheller 1986), and the *Aplysia* PRQFVamide precursor contains as many as 34 copies of PRQFVamide (Furukawa et al. 2003). This multiplicity of peptides on a single precursor is beneficial for effective production of signal molecules from one precursor protein, as well as a backup for replacement of the amino acid by genetic mutation.

On the precursors for longer neuropeptides such as myomodulin and enterin, several copies of similar but different peptides are found. For example, the *Lymnaea* myomodulin precursor contains 5 kinds of heptapeptides sharing the C-terminal Met-Leu-Arg-Leu-NH_2 structure (Kellett et al. 1996), whereas the *Aplysia* enterin precursor includes 21 kinds of deca- and undecapeptides, most of which share the C-terminal His-Xaa-Phe-Val-NH_2 structure (Furukawa et al. 2001). These peptides are collectively referred as the family peptide, such as the enterin family peptide.

One important point is that a minor difference in amino acids greatly modifies the bioactivity of the peptides. For example, ENe (ADLGFTHSFV-NH_2) and ENh (VPGYSHSFV-NH_2) are *Aplysia* enterin family peptides found in the same precursor. However, EC_{50} of the inhibitory action of ENh on the triturating stomach is around 10^{-10} M, whereas that of ENe is a little more than 10^{-7} M. Thus, minor differences in the peptide structure affect affinity to the receptor and tolerance to degradation by the peptidase of the peptide. Thus, neurons expressing the family peptide precursor release a cocktail of the peptides with different potencies.

Recently, it was found that G protein-coupled receptors (GPCR), including neuropeptide receptors, works in forms of monomer, homodimer, or heterodimer (Milligan 2009). Satake et al. (2013) proposed a hypothesis that neuropeptide receptors modify selectivity to the ligand by changing the partner for the dimerization. If a similar receptor system is applicable to mollusks, the aforementioned ENe peptide that is less effective on the triturating stomach than ENh, for instance, may have potent action on other tissues.

Newly synthesized precursor proteins in the cell body are subjected to the post-translational modification (PTM), during it passes through the trans-Golgi network. Matured peptide is ultimately packaged in the synaptic vesicles and transported to the nerve endings (Elekes and Rozsa 1984; Reed et al. 1988). PTM is a rather complicated enzymatic and nonenzymatic reaction that includes processing, disulfide-bond formation, and C-terminal amidation. For example, the processing enzymes prohormone convertase 2 (PC2) and furin were identified in *Aplysia* (Nagle et al. 1993, 1995) and *Lymnaea* (Smit et al. 1994; Spijker et al. 1999).

PC2 cleaves the precursor at the C-terminal side of the mono- or di-basic amino acids such as Arg and Lys, which liberate neuropeptides from the precursor. Furin cleaves precursor at the tetra-basic site on the precursor. It was demonstrated that the ELH precursor in *Aplysia* is cleaved by furin, and the N-terminal and C-terminal fragments thus generated are packaged into the different synaptic vesicles. Because those vesicles are transported to the different nerve endings (Fisher et al. 1988; Sossin et al. 1990; Li et al. 1994), a single neuron can release different peptides from different nerve endings.

A unique PTM in molluscan neuropeptides is D-/L-conversion of N-terminal penultimate amino acid (Kamatani et al. 1991; Kreil 1994a, b; Morishita et al. 1997), catalyzed by the peptidyl D-/L-isomerase (Kreil 1994a, b). This modification greatly affects both the bioactivity and half-life of the peptide. However, differing from processing or amidation, neither the amino acid sequence of the precursor nor structural analysis of peptides by standard mass spectrometry shows us which peptide contains D-amino acid. One practical approach to find a D-amino acid-containing peptide is comparison of elution time between the native peptides and synthetic peptides with or without D-amino acids, on reversed-phase or ion-exchange column HPLC (Fujimoto et al. 1991; Morishita et al. 1997). Although the D-/L-conversion of neuropeptide is a rare phenomenon, it is an important issue when the bioactivities of newly identified peptides are examined.

The synthesis of a precursor normally occurs in the cell body. However, local synthesis of peptide hormone is also known in *Aplysia* (Lee and Wayne 2004) and *Lymnaea* (Van Minnen et al. 1997). In these gastropods, translation machinery such as mRNA and ribosomes is transported to the nerve endings. Local synthesis of peptide is promoted by the depolarization of the nerve endings (van Minnen and Bergman 2003).

Neuropeptides packaged in the synaptic vesicles in the nerve endings are released by exocytosis, when the synapse membrane is depolarized (Sudhof 2012). The elevation of Ca^{2+} in the presynaptic element by Ca^{2+} influx through the voltage-dependent Ca^{2+} channel triggers the exocytosis (Geiger et al. 2009). In vertebrate neurons, the fusion of the neuropeptide-containing synaptic vesicle to the presynaptic membrane requires a high concentration of Ca^{2+}. Accordingly, neuropeptide release occurs when the neuron is discharged at high frequency (Leng and Ludwig 2008). However, Vilim et al. (Vilim et al. 2000) demonstrated that, at the neuromuscular junction between the buccal B16 neuron and anterior radula closer muscle of *Aplysia*, neuropeptide release occurs at a low-frequency discharge of the neuron. Thus, co-release of ACh and myomodulin is inducible at low-frequency neuronal discharge.

The mode of action of the released neuropeptides is variable in mollusks. The central actions of a neuropeptide include postsynaptic action, which modulates the excitability of neurons, and presynaptic action that modulates neurotransmitter release (Fossier et al. 1994). Most of the peripheral actions are the control of muscle contraction in the digestive (Sweedler et al. 2002; Furukawa et al. 2003) and cardiovascular (Buckett et al. 1990; Sasaki et al. 2004) systems and reproduction-associated ducts, as well as the hormonal actions (Newcomb and Scheller 1990;

Hermann et al. 1997). In one instance, a peptide in the pedal ganglion regulates locomotion of *Aplysia* by regulating ciliary movements on the surface of the foot (Hall and Lloyd 1990). In the female reproductive gland in land snails, neuropeptides promote ovulation by squeezing the acini around the oocyte (Chase et al. 2004).

Degradation of the released peptides is another important aspect in the effectiveness of neuropeptides. The neuropeptides released into the extracellular space or the hemolymph are normally digested by peptidases (Squire et al. 1991; Owens et al. 1992; Rothman et al. 1992). Apparently, localization and activity of the peptidase are important factors that determine the half-life of the peptides. Some peptides have protections against the peptidase by various modifications, such as C-terminal amidation (De Camargo et al. 1982) and D-amino acid (Morishita et al. 2003) or proline (Mentlein 1988) at the N-terminal penultimate residue. Accordingly, the half-life of neuropeptides with those modifications is longer than that of other peptides. On the other hand, deamidase, which inactivates D-amino acid-containing neuropeptide (Morishita et al. 2003), and dipeptidyl proline aminopeptidase, that degradates peptides with N-terminal Xaa-Pro structure, such as APGWamide, are reported (Henry and Zatylny 2002).

Despite the diversity of neuropeptides so far known, only a few neuropeptide receptors are currently identified. Neuropeptide receptors cloned in mollusks include receptors for conopressin (van Kesteren et al. 1995), LyCEP (Tensen et al. 1998), and leucokinin-like peptide (Cox et al. 1997) in *Lymnaea*, tachykinin (Kanda et al. 2007) and cephalotocin (Kanda et al. 2003) in *Octopus*, and FMRFamide in *Aplysia* (Lingueglia et al. 1995: Furukawa et al. 2006). The FMRFamide receptor is a ligand-gated Na^+ channel, whereas the others are GPCRs. Information on the spatial and temporal expression of GPCRs for neuropeptides on the target tissues, as well as that on the signal transduction cascade that is downstream to the GPCR, is indispensable to elucidate the functions of neuropeptides.

8.3 Peptides Identified in Prosobranch Gastropods

Early evidence for the existence of neuropeptides in prosobranchs came from immunological studies. For example, using antibodies to vertebrate neuropeptides, the presence of neuropeptide-Y and substance P was reported in a freshwater gastropod, *Viviparus ater,* and that of calcitonin gene-related peptide and small cardioactive peptide was reported in the green ormer, *Haliotis tuberculata* (Barlow and Truman 1992; Franchini et al. 1994; Duvail et al. 1997). However, the precise structures of the immunoreactive materials are not known so far.

In the 1990s, Muneoka and colleagues purified several bioactive peptides from a peptidic extract of the ganglia of the spindle snail, *Fusinus ferrugineus*, through fractionation by reversed-phase or cation-exchange HPLC, which was followed by bioassay with the radula retractor muscle of the animal. Identified peptides are

Table 8.1 Structures of neuropeptides isolated from prosobranch gastropods

Fusinus ferrugineus		*Thais clavigera*	
Name	Structure	Name	Structure
FRFamide	GSLFRFa	FRFamide	GSLFRFa
	SSLFRFa		SSLFRFa
FMRFamide	FMRFa	Tachykinin	FHPSAFFGSRa
FLRFamide	FLRFa	WWamide	WKSMSKVWa
	ALTNDHFLRFa	TEP-1	KCSGKWAIHACWGGNa
myomodulin	PMSMLRLa	TEP-2	KCYGKWAMHACWGGNa
	PMNMLRLa		
APGWamide	APGWa		
FEP	GFRMNSSNRVAHGFa		

Two cysteine residues in TEP-1 and TEP-2 are linked by intramolecular disulfide bond

a C-terminal amide

LSSFVRIamide, ALTNDHELRFamide, GSLFRFamide, SSLFRFamide, allatotropin-like tetradecapeptide (*Fusinus* excitatory peptide 4, FEP-4), myomodulin, and APGWamide (Kanda et al. 1990; Kuroki et al. 1990; Harada et al. 1993; Kuroki et al. 1993) (Table 8.1).

Bioactivities of those peptides are mainly examined on the isolated preparation of some muscular tissues of *Fusinus*. For example, FEP-4 and APGWamide potentiate electrically induced twitch contraction of the radula muscle of *F. ferrugineus* and the rapa whelk, *Rapana thomasiana* (Minakata et al. 1991; Harada et al. 1993), whereas FRFamide showed inhibitory action on the contraction of the radula retractor muscle (Kuroki et al. 1993). Detailed characterization of those *Fusinus* peptides, such as localization in the nervous tissues and molecular cloning of the precursor, has not been reported. Accordingly, the actions of those peptides on the gonad or reproduction-associated organs are largely unknown.

Another instance of the peptide identification in prosobranchs was our study on the rock shell, *Thais clavigera*. In this study, we identified tachykinin-related peptide, WWamide, FRFamide (Morishita et al. 2006a), and *Thais* excitatory peptide (TEP)-1 and -2 (Morishita et al. 2006b). Of those peptides, WWamide and FRFamide reduced the contractile activities of the esophagus and penial complex whereas others showed excitatory actions on those tissues. Both TEP-1 and TEP-2 induce contraction of the penial complex, as well as the esophagus, of *T. clavigera*. Molecular cloning of the TEP precursor revealed that TEP-1 and TEP-2 are encoded on a distinct precursor protein (Morishita et al. 2015), and the

two precursor genes are expressed in different subsets of neurons in the central nervous system. The biological significance of this distinct expression between TEP-1 and TEP-2 is currently unknown.

Recent progress in techniques for the analysis of nucleotide sequences enabled us to predict the amino acid sequences of neuropeptide precursors from the genome DNA. With this technique, Veenstra (2010) predicted the amino acid sequences of neuropeptide precursors in an owl limpet, *Lottia gigantea*. Predicted precursors included some molluscan neuropeptides such as ELH, myomodulin, APGWamide, and enterin, as well as some unique peptides such as bursicon that mediate cuticle tanning in insects (Luo et al. 2005). This approach greatly improved our knowledge on the structures of peptides in prosobranchs. However, their physiological functions in the limpet are largely unknown.

Recently, York et al. (2012) cloned several neuropeptide precursor cDNAs in the Indo-Pacific tropical abalone, *Haliotis asinina*. In this study, cDNA prepared from the cerebral and pleuropedal ganglia that regulate reproduction was subtracted by cDNA prepared from other ganglia. Thus, it is likely that the cloned precursors encode neuropeptides that mediate reproduction. The cloned precursors included APGWamide, myomodulin, and whitnin. The APGWamide precursor encoded 8 copies of authentic APGWamide, whereas the myomodulin precursor encoded 14 copies of distinct peptides sharing the C-terminal Leu-Arg-Leu-NH_2 structure. The whitnin-related precursors have been found in *Lottia* (Veenstra 2010), *Lymnaea* (Koert et al. 2001) and *Aplysia* (Moroz 2006), as well. On those precursors, the proctolin-related hexa-peptide (PKYMDT) is conserved in the middle region, while a 22-mer peptide with one intramolecular disulfide bond and a C-terminal amide is conserved in the C-terminal region. In *Lymnaea*, a serotonergic cerebral giant cell in the cerebral ganglion contains the peptide (Koert et al. 2001). However, the physiological functions of those peptides are currently unknown.

Because the timing of the spawning of *Haliotis asinina* is well synchronized with the lunar and tidal cycle, the authors determined the changes in the expression levels of the precursor mRNA by quantitative polymerase chain reaction (Q-PCR) at the different times of spawning (York et al. 2012). The results demonstrated that expression of neuropeptide precursors in the cerebral and pleuropedal ganglia was higher on the day of spawning than the days before or after spawning. This result suggests that external cues such as tidal cycle, and internal cues such as circadian rhythm, augmented the de novo biosynthesis of neuropeptides for spawning. The next question to be answered is how external or internal stimuli regulate the gene expression in *Haliotis*.

In the following section, I describe the regulation of reproductive activity by selected peptides in mollusks.

8.4 APGWamide

APGWamide was originally identified in a prosobranch, *Fusinus ferrugineus* (Kuroki et al. 1990). However, regulatory action of APGWamide, especially on male reproductive behavior, is well investigated in the freshwater snail *Lymnaea stagnalis*. In this snail, the penial complex is located just behind the right tentacle and is retracted into the body during the resting phase. Although *Lymnaea* is hermaphroditic, one snail behaves as a male that transfers sperm, and the other behaves as a female that receives sperm. When the male snail is motivated to mate, he climbs up on the shell of the partner female snail and approaches the female genital pore from behind (Van Duivenboden and Ter Maat 1988). Then, the preputium of the penial complex is everted and inserted into the female genital pore before intromission is completed.

APGWamide-containing nerve endings were found in the body wall around the male genital pore, as well as male sex-associated organs such as penial complex, vas deferens, and prostate gland (Croll and Van Minnen 1992; de Lange and van Minnen 1998). APGWamide inhibited the contraction of vas deferens that has been induced by a neuropeptide, conopressin, and an injection of APGWamide into the snail induced eversion of the preputium (De Boer et al. 1997). Moreover, the right hemisphere of the cerebral ganglion of *Lymnaea* contains a cluster of more than 100 neurons, which is immunoreactive to specific anti-APGWamide antibody (Croll and Van Minnen 1992). It was also suggested that axon terminals of these APGWamide-containing neurons are located in the aforementioned male reproductive organs. Moreover, the firing rate of the APGWamide-containing neurons in the right cerebral hemisphere is increased when the snail everts the preputium (De Boer et al. 1997). These results strongly suggested that APGWamide regulates male reproductive activity in *Lymnaea*. However, regulation of the reproductive activities of the snail may not be so simple, because nerve processes in the male reproductive organ contains various kinds of neuropeptides such as FMRFamide and myomodulin (De Lange et al. 1998).

As is in *Lymnaea*, localization of APGWamide-containing neurons in the right cerebral hemisphere was also found in *Aplysia californica*, and in the land snails *Helix pomatia* and *Achatina fulica* (Koene et al. 2000), but not in the common periwinkle *Littorina littorea* (Croll and Van Minnen 1992) or in the rock shell *Thais clavigera* (Fig. 8.1) (Morishita, unpublished data). Thus, it is likely that localization of the APGWamide neuron in the right cerebral hemisphere is characteristic of hermaphroditic gastropods.

Amino acid sequences of APGWamide precursor were available in the GenGank for a mussel, *Mytilus edulis* (Q25461), an oyster, *Crassostrea gigas* (EKC38991.1), an abalone, *Haliotis asinina* (AFN20271.1), a freshwater snail, *Lymnaea stagnalis* (1811269A), a sea hare, *Aplysia californica* (NP_001191561.1), and a sea slug, *Tritonia diomedea* (ABU82758.1). In the octopus *Octopus vulgaris*, cDNA encoding a part of the APGWamide precursor is available in the GenBank

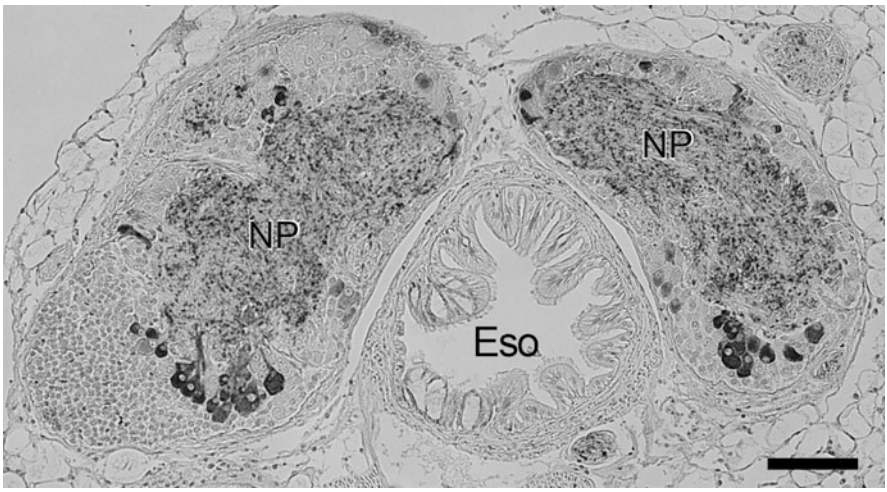

Fig. 8.1 APGWamide-containing neurons in the cerebral ganglion of *Thais clavigera*. A cross section of the cerebral ganglion was immunostained with anti-APGWamide antibody. Immunopositive neurons were found in the ventral side of the *right* and *left* hemispheres. Diffused positive signals were also found in the neuropile (*NP*). *Eso* esophagus. *Scale* 100 μm

(JR446524). The APGWamide precursor of *Lottia gigantea* was predicted by data mining on the genome sequences of this species (Veenstra 2010).

In the bivalves, APGWamide-containing neurons were demonstrated in the cerebral, pedal, and parietovisceral ganglia of a scallop, *Placopecten magellanicus*, an oyster, *Crassostrea virginica*, and *Mytilus edulis*, by immunohistochemistry with anti-APGWamide antibody. Immunopositive nerve processes were found in various tissues including the gonad of the scallop. However, the cloned precursor encoded RPGW-NH_2, TPGW-NH_2 and KPGW-NH_2, but not authentic APGWamide, in *M. edulis* (Favrel and Mathieu 1996). Presence of the three peptides in the *Mytilus* tissues was confirmed by precise mass spectrometry in the extract of the cerebral ganglion and pedal retractor muscle (Henry et al. 2000). With a similar technique, existence of APGWamide was also confirmed in the ganglionic extract of *Crassostrea gigas* (Bernay et al. 2006).

In *M. edulis*, synthetic peptides induced the contraction of pedal retractor muscle and anterior byssus retractor muscle (ABRM) (Henry et al. 2000). A simple interpretation of this result is that neurons containing RPGWamide, TPGWamide, and KPGWamide regulate the locomotion of the mussel. Functional relevance of APGWamide-related peptides to the regulation of reproduction is unclear in this animal. In *C. gigas*, APGWamide was also detected in the seminal fluid in the seminal duct, suggesting the pheromonal action of the peptide (Bernay et al. 2006). In this context, it is noteworthy that *C. gigas* initiates the open/closure response of the shell when the oyster is immersed in seawater containing APGWamide (Bernay et al. 2006), which is an important response for the effective release of gametes into the external seawater,

In cephalopods, APGWamide and TPGWamide were identified in the optic lobe and supra- and subesophageal masses of the brain and oviducal gland of the squid *Sepia officinalis* by liquid chromatography–electrospray ionization mass/mass spectrometry (LC-ESI-MS/MS) (Henry and Zatylny 2002). Because the two peptides inhibit the motility of the oviduct, involvement in female reproductive activity by modifying the transportation of oocytes through the oviduct is suggested. APGWamide was also found in seminal fluid obtained from the spermatophore, suggesting the exocrine or pheromonal actions of APGWamide in male *Sepia officinalis*. Involvement of APGWamide in the regulation of male reproductive activity is also suggested in a pygmy squid, *Idiosepius pygmaeus*. In this squid, APGWamide localizes in nerve processes in the male reproductive organs, as well as the brain regions such as the supraesophageal mass, palliovisceral lobe of the subesophageal mass, and olfactory lobe.

Henry et al. (1997) identified the dipeptide GW-NH_2 from the peptidic extract of the optic lobe of *Sepia officinalis* by the combination of HPLC fractionation and bioassay on the oviduct. The dipeptide showed potent inhibitory action on the oviduct. Minakata et al. (1991) examined the effects of APGWamide analogues on the ABRM of *M. edulis* and the radula retractor muscle of *Rapana thomasiana* and reported that the potency order was GWa> > APGWa > FAPGWa > PGWa. The peptides containing the N-terminal penultimate proline residue are digested by dipeptidyl proline aminopeptidase, which removes the N-terminal Xaa-Pro residue (Mentlein 1988). Thus, it is possible to assume that dipeptidyl proline aminopeptidase liberates GW-NH_2 from APGWamide. In fact, the optic gland of *S. officinalis* contains dipeptidyl proline aminopeptidase activity, because incubation of APGWamide with the extract of the gland generated GW-NH_2 (Henry et al. 1997). It is an interesting issue to be examined if generation of GW-NH_2 is occurring in the extracellular space or in the intracellular space as a step of post-translational modification.

In *Octopus vulgaris*, distribution of APGWamide was demonstrated by immunohistochemistry (Di Cristo et al. 2005). In this study, APGWamide-containing neurons were found in the inferior frontal lobe in the brain and posterior olfactory lobe on the optic tract, as well as the glandular cell of the oviducal gland. It is likely that the APGWamide neuron in the olfactory lobe regulates the activity of the optic gland, whereas APGWamide-containing cells in the oviducal gland secrete the peptide into the extracellular space to modify oviduct motility (Di Cristo and Di Cosmo 2007). Verification of this hypothesis is the next work to be conducted.

As already mentioned, the nucleotide sequence of cDNA encoding APGWamide precursor is found on the database among the transcriptome analysis data of *Octopus* (Zhang et al. 2012). The translated protein includes several copies of the APGW sequence flanked by the dibasic cleavage site and amidation signal in the C-terminal region of the polypeptide. However, this precursor seems to be a fragment, because it does not include the C-terminal signal peptide that is characteristic of the neuropeptide precursor. Therefore, more APGWamide, or a structurally related peptide such as TGPWamide as identified in *Sepia*, could be encoded in the missing N-terminal region.

8.5 Egg-Laying Hormone (ELH) and Caudodorsal Cell Hormone (CDCH)

ELH in *Aplysia californica*, and CDCH in *Lymnaea stagnalis*, are good examples that show us how peptide hormone regulates reproduction-associated behaviors. Egg laying in *Aplysia* is a series of behaviors including ovulation, packaging fertilized eggs into the egg string, and transportation of the string to the head through the genital duct and genital groove. Then, *Aplysia* attaches the egg string to a solid surface by waving the head to left and right. The bag cells, a cluster of neurosecretory cells in the abdominal ganglia, are crucial in initiation of this egg-laying behavior (Kupfermann and Kandel 1970). When an external or internal stimulus activates the bag cells, the cells initiate a series of synchronized discharges (Blankenship and Haskins 1979; Haskins and Blankenship 1979), which continues for some time even after the stimulation is terminated (afterdischarge) (Kupfermann and Kandel 1970).

Because the injection of the bag cell extract into matured *Aplysia* triggered egg laying (Kupfermann 1970), the bag cell contains signal molecules that trigger the egg laying. Those peptides include a 36-mer peptide, ELH (Chiu et al. 1979), and four short peptides, α-, β-, γ-, and δ-bag cell peptide (BCP) (Rothman et al. 1983a; Scheller et al. 1983; Nagle et al. 1990) (Figs. 8.2 and 8.3). ELH and BCPs are encoded on the same precursor (Fig. 8.2). The massive release of ELH and BCPs occurs from nerve endings of the bag cells during the afterdischarge of the cell (Nagle et al. 1988), which in turn induces egg-laying behaviors in *Aplysia* (Kupfermann and Kandel 1970; Pinsker and Dudek 1977; Dudek et al. 1979).

The central nervous system of *Aplysia* is covered by a ganglionic sheath made by the connective tissue. There is a space between the surface of the ganglion and the covering sheath, and the bag cells release ELH and BCPs into this space (Chiu and Strumwasser 1981). Because this space is connected to the circulatory system (Furgal and Brownell 1987), released peptides are delivered to the broad area of the central nervous system. For example, ELH induces ovulation in the ovotestis (Dudek and Tobe 1978; Dudek et al. 1980; Rothman et al. 1983b) and augments the neuronal activity of the R15 neuron in the abdominal ganglion (Mayeri et al. 1985; Levitan et al. 1987). The R15 neuron modulates the motility of the hermaphroditic duct that transports the egg codon to the genital pore (Alevizos et al. 1991). Recently, it was reported that ELH switches jaw movement in *Aplysia kurodai*, so that food ingestion is reduced (Narusuye et al. 2013). Feeding activity and reproductive activity are negatively correlated in mollusks, and this result suggests that ELH switches the two activities.

On the other hand, α- and β-BCPs reduce neural activity of the left upper quadrant (LUQ) neurons in the abdominal ganglion (Sigvardt et al. 1986) that regulate circulation and kidney function (Koester and Alevizos 1989). The peptides have an autocrine action that augments the excitability of the bag cell (Kauer et al. 1987; Brown and Mayeri 1989). This action of the BCPs is important for the prolonged discharge of the cells. During the afterdischarge, Ca^{2+} is mobilized

A) Alignment of ELH and related peptides in mollusks.

```
Aplysia       ELH       ---ISINQDLKAITDMLLTEQIRERQ----RYLADLRQRLLEK---------
              Califin   ---ISINQDLKAITDMLLTEQIQARR----RCLDALRQRLLDL---------
Lymnaea       CDCH-I    ---LSITNDLRAIADSYLYDQNKLRE----RQEENLRRRFLEL---------
              CDCH-II   ----SITNDLRAIADSYLYDQHKLRE----QQEENLRRRFYELSLRPYPDNL
Lottia        ELH-1     AGRLSINGALSSLADLLVSENQRRDR----LESMELRQRLQYL---------
              ELH-2     -SRLSINQELKSLANLLVLRENKRRE----AQKTKLRSKLLSI---------
Haliotis      ELH       ---LSITNDLRAIADSYLYDQNMLRE----RQEENLRRRFLRL---------
Pinctata      ELH-1     -TYISLNGDMRSLAKMLMRHYGNRSVKRPVENYTSLRKKLYAL---------
              ELH-2     -QRLSVNSALASLADMVSADGHRRMK----EEMSSNHQRLLGL---------
Crassostrea   ELH-1     -GRLSLTADLRSLARMLEAHR-KRFIASRFP-YDSIRKKLFRY---------
              ELH-2     -QRLSVNGALSSLADMLAANGRQRMM----SEMAMNRQRLFGL---------
                           *:.  : :::                      : ::
```

B) Alignment of the bag cell peptides and caudo-dorsal cell peptides.

```
Aplysia       Peptide A IFVPNRAVKLSSDGNYPFDLSKEDGAQPYFMTPRLRFYPI
              α-BCP     ------------------------------APRLRFYSL
              β-BCP     --------------------------------RLRFH--
              γ-BDP     --------------------------------RLRFSD-
Lymnaea       α-CDCP    -------------------------------EPRLRFHDV
              β1-CDCP   --------------------------------RLRFH--
              β1-CDCP   --------------------------------RLRAS--
              β1-CDCP   --------------------------------RLRFN--
                                                        ***
```

Fig. 8.2 Alignments of egg-laying hormone (ELH) precursor-associated peptides. (**A**) Alignment of ELH and ELH-related peptides in mollusks. (**B**) Alignment of the bag cell peptides and caudodorsal cell peptides. Alignments were calculated by the ClustalW-multialign (Mobyle portal). *Asterisks*, *colons*, and *periods* beneath the sequences represent amino acid residues conserved among all data, those with high similarity, and those with moderate similarity, respectively

from the extracellular space through a Ca^{2+} channel, or from the intracellular Ca^{2+} store (Wayne and Frumovitz 1995; Magoski 2004). Increase in the Ca^{2+} level in the bag cells promotes the de novo synthesis of ELH (Wayne et al. 2004). Thus, coordination of ELH and BCPs is the key for egg-laying behaviors in *Aplysia*.

The atrial gland, an exocrine organ located on the large hermaphroditic tract of *Aplysia* (Painter et al. 1985), contains several egg laying-associated peptides, such as califin and atrial gland peptide (AGP)-A and -B (Table 8.2). AGP-A and AGP-B trigger the afterdischarge of the bag cell through the mediation of certain neurons in the pleural or cerebral ganglia (Heller et al. 1980; Painter et al. 1988), whereas califin regulates the excitability of neurons located in the left lower quadrant of the abdominal ganglion (Rothman et al. 1986). AGP-A and califin are encoded on the same precursor. The APG-B precursor is a C-terminal-truncated version of AGP-A precursor. Accordingly, the AGP-B precursor does not include the califin (Scheller et al. 1983; Nagle et al. 1986; Kurosky et al. 1997).

The overall structures of the AGP-A and AGP-B precursors are quite similar to that of the ELH precursor. However, 81 amino acids of the ELH precursor, which include β-, γ-, and δ-BCPs, are missing in the AGP-A and AGP-B precursors. The C-terminal regions of AGP-A and AGP-B are quite similar to that of α-BCP, and

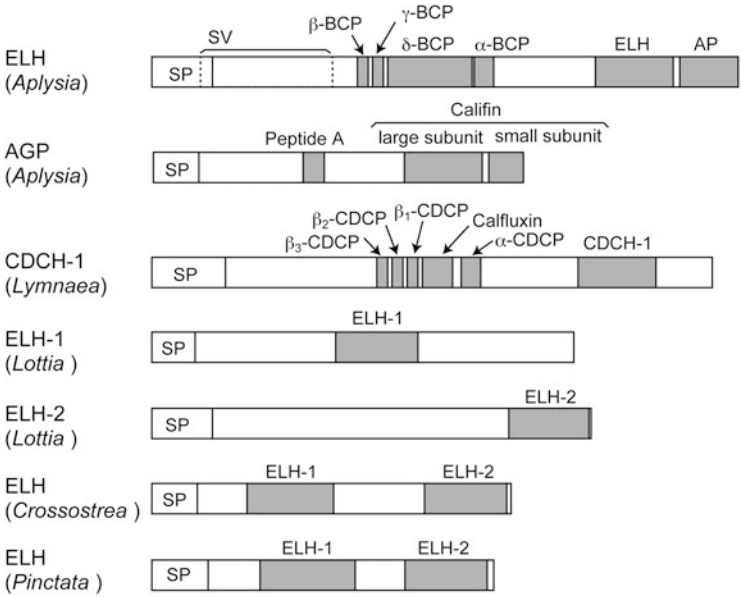

Fig. 8.3 Scale drawings represent localizations of egg-laying hormones and other associated peptides on the respective precursors. Drawings are based on the amino acid sequences of precursors for ELH in *Aplysia californica* (accession: P01362.2), *CDCH* in *Lymnaea stagnalis* (accession: P06308), *AGP* in *A. californica* (accession: P01360.2), ELH in *Lottia gigantea* (accession: XP_009066138.1), and ELH in *Crassostrea gigas* (Veernstra 2010). Note that in the *Aplysia* ELH precursor there is a splicing variant that lacks the N-terminal region (indicated by SV). *Shaded bars* represent the localizations of bioactive peptides. *AGP* atrial gland peptide, *AP* acidic peptide, *BCP* bag cell peptide, *CDCH* caudodorsal cell hormone, *CDCP* caudodorsal cell peptide, *ELH* egg-laying hormone, *SP* signal peptide

the N-terminal region of califin is identical to that of ELH (Figs. 8.2 and 8.3). As the ELH precursor gene, but not the AGP-A and AGP-B precursor, are found in other opisthobranch gastropods, it is likely that the peptide A/califin-precursor gene emerged from the duplication and deletion of the ancestral ELH precursor gene (Nambu and Scheller 1986).

In intact animals, external or internal stimuli, such as temperature and growth and maturation of the animal, initiate egg laying in *Aplysia*. How do these stimuli activate the bag cells and atrial gland? It was demonstrated that neurons located in the cerebral and pleural ganglion have a neural connection to the bag cells (Brown et al. 1989) and that these neurons relay sensory input to the bag cells to induce their discharge. Although injection of AGP-A or califin triggers egg laying of *Aplysia*, it is unclear how the atrial gland is involved in the regulation of egg laying. Because the atrial gland is located near the genital pore, it is an attractive hypothesis that copulation stimulates the secretion of peptide hormone from the gland. However, as the peptides are packaged in the secretory vesicles in the glandular cells, the peptides are normally released into the lumen of the hermaphroditic duct but not

Table 8.2 Structures of gonadotropin-releasing hormone (GnRH), adipokinetic hormone, and corazonin

Phyla	Order	Species	Common name	Structure
GnRH				
Chordata	Mammalia	*Mus musculus*	mice	pQ---HWSYGLRPGa
	Aves	*Gallus gallus*	chicken	pQ---HWSYGLQPGa
	Cyclostmata	*Petromyzon marinus*	lamprey	pQ---HWSHQWFPGa
	Ascidiacea	*Ciona intestinalis*	tunicate	pQ---HWSDYFFPGa
Mollusca	Cephalopoda	*Octopus vulgaris*	octopus	pQ-NYHFSNGWHPGa
	Gastropoda	*Aplysia californica*	sea hare	pQ-NYHFSNGWYA-a
		Lottia gigantea	limpet	pQ-HYHFSAGWLS-a
	Bivalvia	*Crossosteria gigas*	oyster	pQ-NYHFSNGWQP-a
		Patinopectin yessoensis	scallop	pQ-NFHYSNGWEP-a
Adipokinetic hormone				
Arthropoda	Insecta	*Locusta migratria*	locust	pQLNF--TFNW-GTa
Corazonin				
Arthropoda	Insecta	*Aedes gambiae*	mosquito	pQ-TFQYSRGW-TMa

Note that several gaps (indicated by hyphens) were placed in the peptide sequence for alignment

pQ pyroglutamine, *a* C-terminal amide

into the hemolymph. One possibility is that peptides in the atrial gland have pheromonal action (Susswein and Benny 1985).

Lymnaea stagnalis lays eggs in an egg mass that contains about 50–100 eggs in a gelatinous sheath. The egg laying of *Lymnaea* consists of four sequential phases: resting, shell turning, oviposition, and inspection of the egg mass (Ter Maat et al. 1989). As in the bag cells in *Aplysia*, the neurosecretory caudodorsal cell (CDC) triggers the sequential behaviors (Geraerts and Bohlken 1976). CDC located in the cerebral ganglion sends to the axon terminals on the cerebral commissure and releases several peptide hormones (Geraerts et al. 1983; Vreugdenhil et al. 1985) when the CDC is activated to initiate the afterdischarge. For more information on egg laying of *Lymnaea*, refer to the recent review by Koene (2010).

The structures of the peptide hormones released from CDC are quite similar to those released from the bag cells in *Aplysia*. For instance, caudodorsal cell hormone (CDCH) in *Lymnaea* consists of 36 amino acids, and the 16 residues are identical to those of ELH (Ebberink et al. 1985) (Fig. 8.2). Moreover, the organization of the CDCH precursor is quite similar to that of ELH (Vreugdenhil et al. 1988) (Fig. 8.3). As is the ELH precursor, CDCH is located in the C-terminal region, whereas α-, β_2-, and β_3-caudodorsal cell peptides (CDCPs), which correspond to α-, β-, and γ-BCP, respectively, are located in the middle region. Although the overall similarity of the amino acid sequences between ELH precursor and CDCH precursor is 25 %, similarities in amino acids in the peptide-coding regions are 50–70 %. CDCH and CDCP coordinate actions to induce egg laying, including auto-excitation and modulation of the excitability of the right pedal N motor neuron (RPeN) (Hermann et al. 1997).

In prosobranch gastropods, the ganglionic factor that induces egg laying was reported in the flat-top shell, *Gibbula umbilicalis* (Clare 1986). In this study, extract of the cerebral ganglion, but not of the visceral ganglion, induced egg laying in the closely related gastropod *Gibbula cineraria* when the snail was in reproductive season. Because the same batch of the brain factor failed to induce egg laying in the nonreproductive female, it appears that the brain factor does not promote the sexual maturation of females. The brain factor also does not induce sperm release in the male. Although the entity of the factor is unknown, it seems to be a certain peptide, because egg-laying activity was diminished by protease digestion but not by heating or acid treatment.

The extract of the parietal ganglion of the whelk *Busycon* also induces egg laying in this animal (Ram 1977). It is suggested that the factor is a ELH-related peptide because behavior of the factor on gel filtration chromatography is quite similar to that of *Aplysia* ELH, and injection of *Aplysia* ELH also induces egg laying (Ram et al. 1982). In *Haliotis asinina*, immunohistochemistry with anti-*Haliotis* ELH antibody demonstrated the localization of ELH-related peptide-containing neurosecretory cells in the cerebral, pleuropedal, and visceral ganglia (Saitongdee et al. 2005). The numbers of the neurosecretory cells are high in cerebral and pleuropedal ganglia, and much fewer in the visceral ganglion. The localization of the peptide, together with the fact that the extract of the cerebral and pleuropedal ganglia of *G. umbilicalis* contained the egg laying-inducing activity, suggest that, as in *Aplysia* and *Lymnaea*, ELH-related peptides derived from neurosecretory cells in the brain regulate egg laying in prosobranch gastropods. Immunostaining also demonstrated that the follicular and glandular cells in the ovary contained ELH-related peptide (Saitongdee et al. 2005). ELH in the ovary may have local actions, such as oocyte maturation and ovulation.

Recent progress in EST analysis and data mining on the genome sequences showed the structure of precursor proteins for the ELH-related peptides in several mollusks, such as the prosobranch *Lottia gigantea* (Veenstra 2010), the pearl oyster *Pinctada fucata*, and *Crassostrea gigas* (Stewart et al. 2014). In these animals, amino sequences of the predicted ELH-related peptide are similar to those of ELH and CDCH (Figs. 8.2 and 8.3). However, organizations of precursor proteins are somewhat different from those of ELH and CDCH. For instance, BCP or CDCP peptides are not found on the precursor proteins of *Pinctada*, *Crassostrea*, and *Lottia*. In *Pinctada* and *Crassostrea*, ELH-related peptides are duplicated on the respective precursors. Considering that egg-laying behaviors in *Aplysia* and *Lymnaea* are inducible through the coordination of different peptides derived from the same precursor, it is an interesting question how ELH-related peptides are involved in the reproduction of the animals.

8.6 Gonadotropin-Releasing Hormone (GnRH)

In mammals, GnRH is a key peptide hormone to assure the proper development and function of the gonad (Guillemin 1978; Schally 1978; Iversen et al. 2000). The mammalian GnRH is a 10-mer peptide with N-terminal pyroglutamine and C-terminal Pro-Gly-NH_2 structures (Baba et al. 1971; Matsuo et al. 1971). More than 20 kinds of GnRH have been found in chordates so far (Roch et al. 2011). All share the aforementioned structural features, although some differences in amino acids were found in the middle region of the peptides (Table 8.2).

In mollusks, the existence of GnRH-like peptides was initially demonstrated by immunohistochemistry with an antibody that recognizes vertebrate GnRH or by testing the actions of vertebrate GnRH in the chiton (Amano et al. 2010b), oyster and mussel (Nakamura et al. 2007; Pazos and Mathieu 1999), abalone (Amano et al. 2010a; Nuurai et al. 2014), freshwater snail (Goldberg et al. 1993; Young et al. 1999), sea hare (Zhang et al. 2000), and octopus (Di Cosmo and Di Cristo 1998). Now, structures of GnRH-related peptides are known in those animals (Table 8.2). Interestingly, invertebrate GnRHs have two amino acid insertions between the N-terminal pyroglutamine and histidine, and, except for octopus GnRH, the C-terminal glycine residue is missing. Thus, octopus GnRH is a 12-mer peptide whereas other invertebrate GnRHs are 11-mer peptides.

Immunohistochemistry demonstrated that GnRH-containing neurons were found in the cerebral ganglion of the Pacific abalone, *Haliotis discus hannai* (Amano et al. 2010a), and in the cerebral and pleuropedal ganglia of *H. asinina* (Nuurai et al. 2014). In both studies, the authors suggested the existence of several distinct GnRH-related peptides, because the anti-GnRH antibody recognized multiple peptides in the extract of the nervous system. Because immunopositive nerve fibers were not found on the gonad, they hypothesized that *Haliotis* GnRH has hormonal action on the tissue. It is noteworthy that, in the ovary of *H. asinina*, oocytes in an early stage of gonadal maturation are immunopositive to anti-lamprey GnRH antibody (Nuurai et al. 2010). It is plausible that GnRH is involved in oocyte maturation in this animal. Although it was reported that repetitive injections of salmon GnRH analogue induced maturation of the gonad in the Hawaiian limpet *Cellana* (Hua and Ako 2013), the functional relevance of GnRH to gonadal maturation in prosobranchs has not been fully understood.

In the nervous tissue of a freshwater pulmonate, *Helisoma trivolvis*, neurons immunopositive to the anti-mammalian GnRH antibody were diffusely distributed in all the circumesophageal ganglia (Young et al. 1999). In the peripheral nervous system, immunopositive nerve processes were found in the reproduction-associated organs such as penial complex, vas deferens, oviduct, and ovotestis. GnRH-containing neurons in the left cerebral ganglia appeared to be involved in the regulation of the penial complex, because retrograde filling of the fluorescent dye, Lucifer Yellow, from the cut end of the penis nerve stained those neurons (Young et al. 1999).

In *Aplysia californica*, a GnRH precursor predicted by the transcriptome data of *Aplysia* (Zhang et al. 2008) consisted of 147 amino acids, including N-terminal

signal peptide (27-mer) and a single copy of GnRH. Immunohistochemistry with a specific antibody to *Aplysia* GnRH demonstrated that GnRH-containing neurons were mainly located in the pedal and cerebral ganglia of the animal (Zhang et al. 2000; Jung et al. 2014). Immunopositive nerve processes were found in the neuropil region in the head ganglia, but not in the peripheral nerve on the reproductive organs. Hormonal action of GnRH on the reproductive organs is not likely in this animal, because repetitive injection of *Aplysia* GnRH to the sexually immature *Aplysia* failed to induce gonadal maturation but induced acute changes in behaviors such as feeding and locomotion (Tsai et al. 2010). Moreover, bath application of GnRH to isolated preparation of pedal ganglia modified the firing rate of several neurons in the ganglia (Seaman et al. 1980). Thus, the primary function of *Aplysia* GnRH seems to be a regulation of behavior rather than that of gonadal maturation (Sun and Tsai 2011).

In bivalves, the structure of GnRH was determined in *Crassostrea gigas* as pGNYHFSNGWQP-NH_2 for *C. gigas* (Bigot et al. 2012) and pGNFHYSNGWQP-NH_2 for *Patinopecten yessoensis* (Treen et al. 2012), respectively, Recently, the structure of GnRH of *Pinctada fucata* was reported by predicting the precursor cDNA through analysis of the genome DNA (Stewart et al. 2014).

When the expression of the GnRH precursor in *C. gigas* was quantified by Q-PCR, expression was high in the visceral ganglia, whereas it was negligible in other tissues tested, including gonadal tissue (Bigot et al. 2012). In fact, GnRH-containing neurons visualized by immunohistochemistry were found in the central nervous system but not in other tissues. The expression level of GnRH precursor mRNA in the visceral ganglion is not constant during the development of the gonad. There is a tendency that, in the male oyster, expression of GnRH precursor is higher in gonadal maturation, whereas it is higher in the gonadal proliferation and sexual maturation phases in the female oyster.

Identification of the GnRH receptor is successful in *C. gigas*. The GnRH receptor cloned in *C. gigas* (cg-GnRH-R) is a GPCR, sharing 20–30 % homology with vertebrate GnRH receptors (Rodet et al. 2005). The consensus sequences of mammalian GnRH receptors for ligand binding are conserved in the cg-GnRH-R. The cg-GnRH-R gene consists of six exons, and it was predicted that alternative splicing generates four cg-GnRH-R subtypes with different lengths of N-terminal and C-terminal intracellular regions and different numbers of transmembrane regions (Rodet et al. 2008).

Reverse transcription PCR (RT-PCR) confirmed the expression of three of the four subtypes in peripheral tissues including the gonad. Variety in the receptor structure, together with the wide distribution of cg-GnRH-R in nonreproductive tissues, implies that GnRH is a multifunctional peptide in this animal. Apparently, the next important issue to be confirmed is the endogenous ligands to those receptors. Using an expression system such as the *Xenopus* oocyte, determination of the affinity orders of those receptors to GnRH, corazonin, and adipokinetic hormone (AKH) is attractive (see below).

Several lines of functional assay suggest that GnRH mediates the gonadal maturation in bivalves. For instance, vertebrate GnRH increased ^{3}H-thymidine incorporation into dispersed gonadal cells of *Mytilus edulis* and *Crassostrea gigas*, suggesting that proliferation of gonadal cells is promoted (Pazos and Mathieu 1999). A GnRH antagonist effectively inhibited the mitogenic action of mammalian GnRH. GnRH likely has a hormonal action on the gonad of *M. edulis* because immunohistochemistry with anti-human GnRH antibody demonstrated GnRH-positive neurons in the cerebral and pedal ganglia, but not in the nerve endings on the gonad.

In the scallop, it was reported that peptidic extract of the cerebrapedal ganglia of the scallop promoted BrdU incorporation into cultured gonadal tissue, which was blocked by pre-incubation with anti-mammalian GnRH antibody (Nakamura et al. 2007). Immunostaining with the same antibody demonstrated immunopositive neurons in the ganglia. However, as in the oyster, no immunopositive fibers were found around the gonad. These results suggest that GnRH-related peptide promotes proliferation of gonadal cells via a hormonal action. Unfortunately, those experiments were conducted with exogenous GnRH. Now, structures of GnRHs are known in those animals. Confirmation of the mitogenic action of endogenous GnRH on the gonad will be necessary.

The sex differentiation of bivalves is rather a complicated phenomenon (Mackie 1984). For instance, *Mytilus* is a rather rigid gonochorist, and sex reversal is hardly inducible. By contrast, *Crassostrea* is a protandrous hermaphrodite, and sex reversal is naturally inducible. In *Crassostrea*, the undifferentiated gonad in nonmating season rapidly differentiates to the matured ovary or testis in a few months (Enriquez-Diaz et al. 2009). Involvement of GnRH in this dynamic remodeling of the gonad is an interesting issue to be examined.

GnRH in cephalopods was initially demonstrated by immunohistochemistry with anti-chicken GnRH antibody (Di Cosmo and Di Cristo 1998); then, it was chemically isolated from the brain of the common octopus, *Octopus vulgaris* (Iwakoshi et al. 2002). Differing from other molluscan GnRHs, octopus GnRH shares the same C-terminal structure (–Pro-Gly-NH_2) with mammals.

Sexual maturation of the female octopus is hormonally controlled by a pair of optic glands that lies on the optic tract in the vicinity of the optic lobe. The gland regulates various reproductive events such as proliferation of gonadal cells and yolk-protein synthesis (O'Dor and Wells 1973), probably by secreting the steroid hormone. Immunohistochemistry with antibodies against chicken GnRH or octopus GnRH demonstrated that octopus GnRH-containing neurons were diffusely distributed in various lobes in the octopus brain, including the subpedunculate and the posterior olfactory lobes (Iwakoshi et al. 2002; Iwakoshi-Ukena et al. 2004). Because GnRH-containing nerve processes are found in the optic tract and optic gland, it is suggested that GnRH neurons stimulate optic gland activity, which results in gonadal maturation. Di Cosmo et al. (2003) reported that the activity of the optic gland is inhibited by FMRFamide-containing neurons in the subpedunculate lobe. Accordingly, antagonistic regulation of the optic gland is suggested. These regulatory pathways via the subpedunculate lobe–optic gland axis

are quite similar to the hypothalamo–hypophysial axis in vertebrates. In addition, octopus GnRH has direct action on the gonad, which promotes steroid synthesis (Kanda et al. 2006).

The olfactory lobe, located in the vicinity of the optic gland, is a region that receives sensory information through the olfactory nerve (Budelmann 1995). In this lobe, sensory inputs such as chemoreception are integrated with the visual information to initiate reproduction with the proper timing. GnRH neurons in the olfactory lobe appeared to regulate the activity of the optic gland, because GnRH-containing nerve processes emanating from the lobe make contact with the glandular cells in the optic gland. It was reported that a glutamine receptor agonist, *N*-methyl-*D*-aspartate (NMDA), elevated the expression level of GnRH precursor mRNA in GnRH neurons in the olfactory lobe (Di Cristo et al. 2009). It is plausible that glutaminergic neural input to the GnRH neurons in the olfactory lobe promotes gonadal maturation through augmentation of the GnRH signaling system.

Recently, a novel GnRH receptor was identified in *Octopus vulgaris* (Kanda et al. 2006). The homology of the amino acid sequence between the octopus GnRH receptor and vertebrate GnRH-receptors is around 30 %, and octopus GnRH activates the GnRH receptor expressed in *Xenopus* oocytes. RT-PCR and in situ hybridization demonstrated that the octopus GnRH receptor is expressed in various brain regions including the pedunculate, olfactory, and optic lobes, as well as reproductive organs including ovary and oviduct. Thus, regulatory action of GnRH on female reproduction is suggested. Moreover, the GnRH receptor was also expressed in the digestive system and cardiovascular system. The GnRH system may regulate various physiological and behavioral responses in *Octopus*, such as memory formation, feeding, and circulation (Iwakoshi-Ukena et al. 2004).

By analyzing the similarity in amino acid sequences of neuropeptide receptors, it was postulated that corazonin receptor, AKH receptor, and AKH/corazonin-related peptide receptor in insects are evolved from the ancestral GnRH receptor, during the evolution of Protostomia that led to the divergence of Mollusca, Annelida, and Arthropoda (Hauser and Grimmelikhuijzen 2014). AKH is a peptide hormone that regulates energy metabolism (Stone et al. 1976) and corazonin regulates heartbeat (Veenstra 1989) in insects. In this theory, ligand peptides were also evolved from the ancestral GnRH precursor.

In fact, GnRH shares structural similarity with AKH, corazonin, and corazonin-related peptides (Table 8.2). Moreover, corazonin precursor genes were found in the genome sequences of several mollusks, including *Aplysia*, *Lottia*, and *Crassostrea* (Hauser and Grimmelikhuijzen 2014). In this context, it is noteworthy that anti-GnRH antibody recognizes multiple peptides in the nervous tissues of several mollusks. Apparently, elucidation of the entity of immunoreactivity to anti-GnRH antibody is the next important issue.

Recently, De Lisa et al. (2013) proposed that changes in the expression level of GnRH-precursor mRNA could be a biomarker for monitoring the effect of EDC on the rayed Mediterranean limpet, *Patella caerulea*, which is an interesting approach to elucidate how EDC affects the regulatory neuropeptide system of mollusks. However, as I have described, GnRH has a broad range of physiological functions

including promotion of gonadal development, regulation of feeding, and locomotion. Even a pheromonal action that triggers the release of gametes was reported in the chiton *Mopalia* sp. (Gorbman et al. 2003). Moreover, multiple GnRH-related peptides including AKH-related peptide could be functional in the mollusks so far examined. Accordingly, careful characterization of GnRH is essential before discussing the relevance between the GnRH system and EDC. For further information on molluscan GnRH, readers can refer to recent reviews (Minakata et al. 2009; Sun et al. 2012; Di Cristo 2013; Osada and Treen 2013).

8.7 Perspectives

In the past four decades, many peptides have been identified in mollusks. Now, in the post-genome era, identification of neuropeptides is accelerated through the combination of the prediction of neuropeptide precursors by the annotation of genome sequences and the structural analysis of tissue extracts with mass spectrometry. Accordingly, the importance of the functional analysis of identified peptides is expanding more and more. However, except for a few gastropods such as *Aplysia* and *Lymnaea*, functional analysis of neuropeptides is hampered by the fact that the nervous system consists of small-sized neurons and the soft body is covered with a hard shell. Moreover, considering the length of the reproductive cycle and long larval stages, including several steps of metamorphosis, it may not be easy to obtain gene-manipulated mollusks.

One of the practical approaches is to select the appropriate target animal, clarify its genetic background to predict a precursor gene for neuropeptides and peptide hormones, and then conduct the analysis of peptide structure by mass spectrometry on nervous tissue or reproduction-related organs. Novel approaches with techniques such as microanalysis of trace amounts of peptides in hemolymph and the expression of the hybrid gene of the 5′-upstream region of the neuropeptide precursor gene and appropriate reporter genes such as luciferase on the cultured cell, which are compatible with standard biochemical, molecular biological, and physiological techniques, are encouraged to clarify the physiological functions of identified peptides. Noninvasive observation of the gonad by magnetic resonance imaging is an interesting approach to investigate the changes in the structure of the gonad during reversible sex reversal (Davenel et al. 2006).

The receptor systems for peptide ligands are another important issue to be examined. Besides the neuropeptide receptors discussed in this review, several neuropeptide receptors including for FMRFamide (Linguelia et al. 1995), vasopressin/oxytocin-related peptides (van Kesteren et al. 1995), and *Achatina* cardioexcitatory peptide (ACEP)-1-like peptide (Tensen et al. 1998) have been cloned in mollusks. However, considering the diversity of neuropeptides and peptide hormones, many receptors still remain to be identified. For the identification of the neuropeptide receptors, molecular cloning of the orphan GPCR is one of the well-accepted approaches. In addition, recent progress in annotation of the

genome sequences, together with the massive sequence data obtained by transcriptome analyses, may be helpful in predicting the nucleotide sequence of mRNA for neuropeptide receptors. Characterization of cloned receptors is possible on an appropriate expression system such as *Xenopus* oocytes.

Now, nuclear receptor RXR is recognized to be the target molecule of organotin (Nishikawa et al. 2004; Castro et al. 2007). Because RXR regulates gene expression, directly or indirectly, it is possible to assume that EDC modifies expression of precursor genes of neuropeptides and their receptors in mollusks. In this context, analysis of the 5′-upsteam region of those genes on the genome sequences is also important to understand when and how the expression of the precursor gene is regulated.

Because EDC generally disturbs reproduction, it reduces the population of mollusks in the field and has profound effects on ecological balance. EDC also causes damage to the fishery cultivation industry by augmenting the costs of cultivation, because, under the influence of EDC, released juveniles of commercially valuable mollusks, such as abalone and clam, do not reproduce, even after they are fully grown-up. If EDCs have more acute and drastic effects, such that the compounds kill animals or induce apparent deformity in the animals, people will be more careful about using and disposing such chemicals. In reality, the actions of EDC are silent and creeping; hence, we underestimate their threat.

Unfortunately, at present, we cannot give a satisfactory explanation for the influence of EDC on regulatory neuropeptide and peptide hormone systems. If we could see, however, the large picture of the neuropeptide–peptide hormone systems of mollusks in the near future, we would be able to predict how a particular chemical affects the regulatory peptide systems, which will consequently minimize the impact of EDC on both ecological balance and the fishery industry. Because mollusks hold an important place in both the environment and the fishery industry, research on molluscan neuropeptides will satisfy not only our scientific interest in the peptide biology, but also our appetite.

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