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Ascidians: New Model Organisms for Reproductive Endocrinology

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1. Introduction

Ovarian functions, including growth of oocytes and follicles, are believed to involve coordinated and multistep biological events that undergo functional regulation by a wide range of endogenous factors. Mammalian follicles consist of one oocyte surrounded by granulosa cells and theca cells (Orisaka et al., 2009). Mammalian follicular growth is basically classified into two phases: gonadotropin-independent and gonadotropin-dependent stages. The former includes early follicular growth stages, namely, primordial, primary, secondary, preantral, and antral stages, whereas follicle recruitment, selection, and ovulation occur during the latter. A great variety of intraovarian and extraovarian factors such as a pituitary hormone, gonadotropin, have been well structurally and functionally elucidated. In contrast, no signaling molecules responsible for gonadotropin-independent oocyte growth have ever been identified. ”Neuropeptides” and ”non-sexual peptide hormones” are promising candidates for regulators of early follicle growth, given that many of their receptors were found to be expressed in the ovary. Nevertheless, biological effects of neuropeptides and non-sexual hormones on the ovary still remain controversial or unknown. Such inconclusive data resulted primarily from the difficulty in elucidating *in vitro* and *in vivo* physiological functions of neuropeptides or non-sexual hormones in the mammalian ovaries due to heterogeneous quality of oocytes (the very small number of successfully growing oocytes), the low basal levels of receptor expression, and complicated sexual periods (Candenas et al., 2005; Satake & Kawada, 2006a; Debejuk, 2006; Wang & Sun 2007). Furthermore, most receptors of peptide ligands are not ubiquitously, but widely expressed, and thus, neuropeptides and peptide hormones have multiple biological roles, which frequently caused great difficulties in discriminating direct actions on the ovary from indirect actions of a peptide during functional analyses via *in vivo* administration or using a peptide- and/or a receptor-knockout mouse. In such cases, model studies using lower organisms, such as a fruitfly *Drosophila melanogaster* or a nematode *Caenorhabditis elegans*, may be useful as the primary step for studies on the biological sciences of mammals. Nevertheless, most invertebrate neuropeptides and hormones possess species-specific primary structures and biological activities. This has hindered comparative analysis and application of traditional invertebrate models to neuroscience and endocrinology research involving neuropeptides and hormones in mammals. These issues suggest a potential

requirement for new approaches and model organisms for studies on biological roles of neuropeptides and non-sexual hormone peptides in the ovary. In this chapter, we propose an ascidian, *Ciona intestinalis*, as an emerging model organism for studies of endocrinology, in particular, reproductive biology of oocytes at early stages, and review a novel oocyte growth pathway revealed by studies on biological effects of *Ciona* homologs of mammalian neuropeptides on *Ciona* oocytes, and structures and functions of ascidian neuropeptides and hormone peptides which are highly likely to participate in growth of early-stage follicles and oocytes.

2. What is an "ascidian"?

2.1 Characteristics of an ascidian, *Ciona intestinalis*, and its utility in biological sciences

Ascidians (sea squirts), invertebrate deuterostome marine animals, belong to the subphylum Tunicata or Urochodata under the Chordata phylum (Burighel & Cloney, 1997). Their critical phylogenetic position as protochordates (Fig. 1) and their simple body plan (Fig. 2) and lifecycle have provided attractive and useful targets in research in embryogenesis,

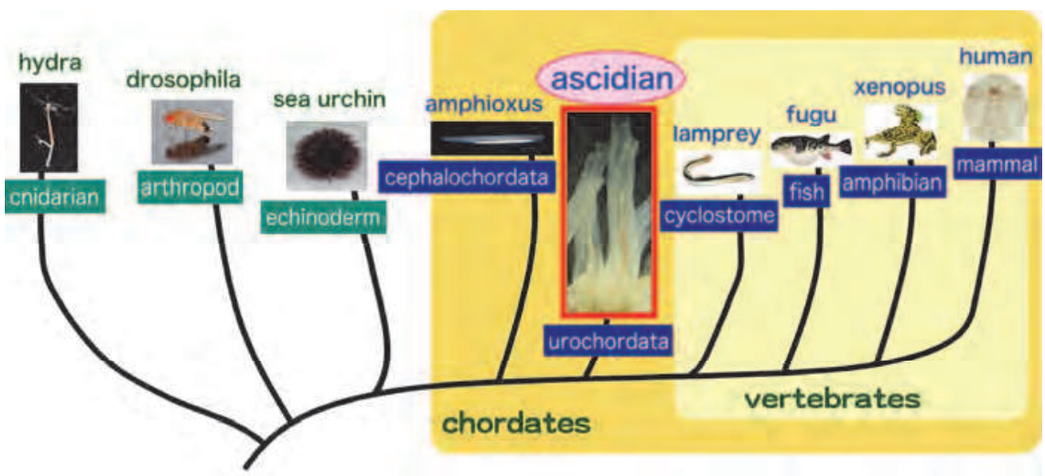

Fig. 1. Phylogenetic tree.

development, and evolution as a direct model or ancestor for vertebrates (Burighel & Cloney, 1997; Satoh, 2009). In 2002, a draft genome and expression sequence tags (ESTs) of the ascidian, *Ciona intestinalis*, (available at http://hoya.zool.kyoto-u.ac.jp/blast_kh.html http://genome.jgi-psf.org/Cioin2/Cioin2.home.html) revealed that the genome of *C. intestinalis* contains approximately 16000 protein-coding genes without the extensive gene duplications typical of vertebrates. Recently, most genes have been annotated by gene-model prediction and sequence homology analysis on the basis of 700,000 ESTs as well as genomic sequences, and the expression profiles of gene products are also available. Furthermore, the establishment of fundamental experimental methods such as morpholino DNA gene silencing and Minos transposon-based transgenic technology (Sasakura, 2007) has led to the post-genomic comprehensive studies in embryogenesis and development of whole chordates (Imai et al., 2006).

In addition to these advantages of *C. intestinalis*, characterization of *Ciona* peptide hormones and neuropeptides is expected to provide crucial clues to the elucidation of the molecular

and functional evolution of endocrine, neuroendocrine and nervous systems of chordates as well as the biological roles of the *Ciona* neuropeptides and hormones. Despite these advantages in biological research, ascidian neuropeptides and hormones have received little attention. Moreover, as stated above, complexity of tissue organization and lifecycles of mammals frequently hinders advances in research for neuropeptidergic and hormonal systems. Therefore, molecular and functional characterization of neuropeptides and peptide hormones in *C. intestinalis* is expected to lead not only to investigation of structures and biological roles of ascidian bioactive peptides, but also to establishment of ascidians as novel deuterostome invertebrate models for research in the neuroscience and endocrinology of vertebrates.

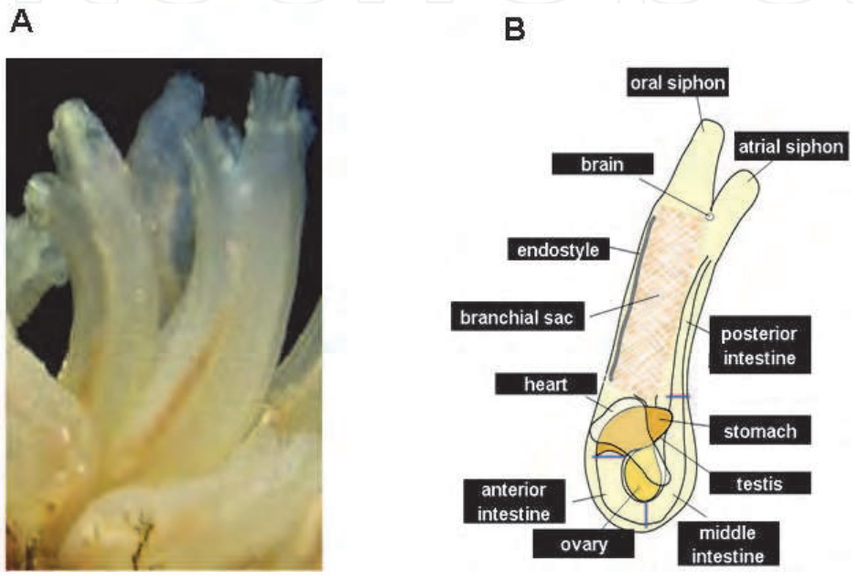

Fig. 2. *Ciona intestinalis* (A) and its tissue organization (B)

2.2 Biological aspects of *Ciona* oocytes and ovary

In the ovary of *C. intestinalis*, the large part is occupied by pre-germinal vesicle breakdown (GVBD) oocytes which are readily classified into three major growth stages on the basis of their diameter and organization of oocyte complexes (Fig. 3), even using 10-μm sections: stage I (pre-vitellogenic stage), stage II (vitellogenic stage), and stage III (post-vitellogenic stage). *Ciona* oocytes are equipped with test cells (TCs), which are believed to be functional

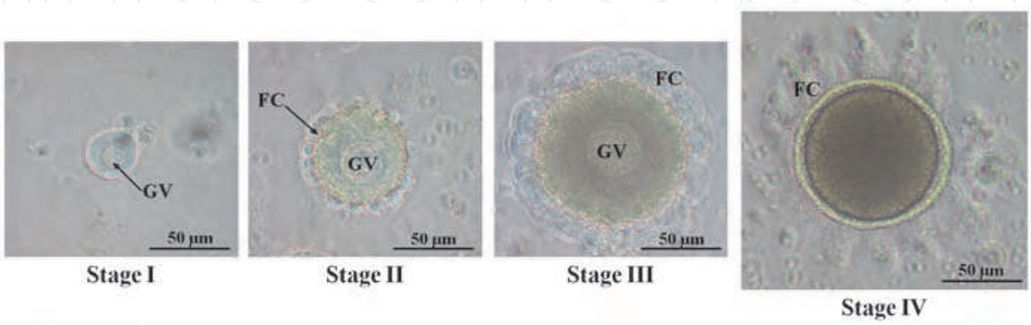

Fig. 3. *C. intestinalis* oocytes. Scale bar, 50 μm. GV, germinal vesicle; FC, follicular cells

and cytological counterparts for mammalian granulosa cells, an acellular vitelline coat, inner follicular cells, and outer follicular cells. Oocytes at stage I (less than 50 μm in diameter) contain the smallest GV and cytoplasm, and are surrounded by envelope organs consisting of undifferentiated primary follicular cells. Stage II oocytes (50-70 μm in diameter) have prominently individualized cube-shaped follicle cells surrounding the oocytes. Stage III oocytes (approximately 100 μm in diameter) have a larger cytoplasm and more outstanding inner follicle structure, and automatically cause GVBD when exposed to seawater (Burighel, & Cloney 1997; Prodon et al., 2006). In addition, stage IV (post-GVBD) oocyte (Fig.3), predominantly present in the oviduct, have the ability of fertilization (Burighel, & Cloney 1997; Prodon et al., 2006). *C. intestinalis* also displays advantages in study of reproductive biology of an ovary. Firstly, the *Ciona* ovary harbors numerous oocytes at each growth stage that are readily characterized and abundantly isolated. Secondly, numerous *Ciona* oocytes are normally grown at a high ratio or maintained for more than one week in sterile seawater. This advantage enables a variety of experiments for evaluation of biological effects of signaling molecules including neuropeptides and peptide hormones. Thirdly, ascidians are not endowed with an organ corresponding to a pituitary. This is in good agreement with the evolutionarily biological propensity of *C. intestinalis* that ascidians, unlike vertebrates, lack a complete circulation system. These findings indicate that the post-antral processes and hypothalamus-pituitary-ovary axis involving the secretion of gonadotropins in responsive to gonadotropin-releasing hormone (GnRH) has not been developed in ascidians, and reinforce the evolutionary scenario that the hypothalamus-pituitary endocrine system might have been established in concert with the acquisition of the closed circulation system in the evolutionary process of chordate invertebrates to vertebrates. Instead, as mentioned in the following sections, the functions of the *Ciona* ovary have been found to be regulated mainly by the neuroendocrine system and intraovarian paracrine system. Altogether, these findings lead to a presumption that whole *Ciona* oocyte growth process corresponds to the mammalian follicular growth process including the primordial to antral stages that is NOT subjected to the regulation by gonadotrpins, and thus, *Ciona* oocytes are excellent models for clarification of the molecular mechanisms underlying gonadotropin-independent follicular growth stages in vertebrates.

Despite these potentials of *C. intestinalis* as a novel model organism, this ascidian was not employed for study of endocrinology, neuroedocrinology, and neurology, given that only a few peptides had been identified in *C. intestinalis*. Over the past few years, however, various neuropeptides and/or hormone peptides have been characterized from *C. intestinalis*, which has paved the way for exploring the unprecedented regulatory systems for oocyte and follicle growth by neuropeptides and peptide hormones.

3. Novel oocyte growth pathway regulated by tachykinin and neurotensin

Recently, we have substantiated a novel oocyte growth pathway using *C. intestinalis*., in which growth of vitellogenic oocytes to postvitellogenic oocytes is regulated via the regulation of protease activation by tachykinin (TK) and neurotensin-like peptide (NTLP) (Aoyama et al., 2008; Kawada et al., 2011). In this section, we provide basal knowledge concerning TKs and NTs followed by the clarification of the TK- and NTLP-directed oocyte growth.

3.1 TKs in C. *intestinalis*

TKs are vertebrate multi-functional brain/gut peptides involved in smooth muscle contraction, vasodilation, nociception, inflammation, neurodegeneration, and neuroprotection in a neuropeptidergic or endocrine fashion (Satake & Kawada, 2006a). All TKs are featured by the C-terminal consensus -Phe-X-Gly-Leu-Met-NH_2 (Satake & Kawada, 2006a). The mammalian TK family consists of four major peptides: Substance P (SP), Neurokinin A (NKA), Neurokinin B (NKB), and Hemokinin-1/Endokinins (HK-1/EKs), as shown in Table 1. TKs are encoded by three genes, namely *tac1* (or *pptA*), *tac3* (or *pptB*), or *tac4* (*pptC*) gene (Page, 2004; Satake & Kawada, 2006a). *tac1* generates four splicing variants which produce SP alone or SP and NKA. Similarly, several EK isoforms are produced from the *tac4* gene. The *tac3* gene yields only NKB.

TK receptors belong to the Class A (rhodopsin-like) G protein-coupled receptor (GPCR) family. Three subtypes of TK receptors, namely NK1, NK2, and NK3, have been identified in vertebrates (Satake & Kawada, 2006a). NK1, 2, and 3 were shown to induce both elevation of intracellular calcium and production of cAMP. NK1, 2, and 3 possess moderate ligand-selectivity: SP>NKA>NKB for NK1, NKA>NKB>SP for NK2, and NKB>NKA>SP for NK3, respectively (Page, 2004; Satake & Kawada, 2006a). HK-1 and the 10-amino acid C-terminal common sequence of EKA and EKB (EKA/B) were also found to exhibit potent binding activity on all NK1-3 with the highest selectivity to NK1 (Page 2004; Satake & Kawada, 2006a). In addition, EKC and EKD are devoid of any activity on NK1-3 (Page 2004; Satake & Kawada, 2006a).

In protostomes, TK-related peptides (TKRPs) exert a TK-like contractile activity and the expression of the *tkrp* gene is observed in the central nervous system (Satake et al, 2003; Satake & Kawada, 2006a). However, they contain the analogous Phe-Xaa1-(Gly/Ala)-Xaa2-Arg-NH_2 consensus (Table 1), and TKRP precursors encode multiple TKRP sequences (Satake et al., 2003; Satake and Kawada, 2006a), which are totally distinct from those of vertebrate TKs. These findings suggest that *tkrp* genes and *tk* genes diverged in distinct evolutionary lineages. TKRP receptors were characterized from several insects, an echiuroid worm and octopus (Kawada et al., 2002; Satake et al., 2003; Satake and Kawada, 2006a, Kanda et al., 2007). These GPCRs display high similarity to vertebrate TK receptors in the sequence and exon/intron organization (Kawada et al., 2002; Satake et al., 2003; Satake & Kawada, 2006a; Kanda et al., 2007). Consequently, TK receptors and TKRP receptors share the common original GPCR gene.

Moreover, TKRP receptors were shown to stimulate the increase in intracellular calcium or generation of cAMP (Satake et al., 2003; Satake & Kawada, 2006). Nevertheless, TKRP receptors, unlike NK1-3, exhibit no significant ligand selectivity to their cognate endogenous ligand peptides (Kawada et al., 2002; Satake et al., 2003; Satake & Kawada, 2006a; Kanda et al., 2007).

TKs were not identified in deuterostome invertebrates including ascidians till 2004. Two authentic TK peptides, Ci-TK-I and -II, were detected in the neural complex of *C. intestinalis* using a mass spectrometric analysis (Satake et al., 2004). The TK consensus motif is completely conserved in both the ascidian peptides (Table 1). Ci-TKs were found to elicit a TK-typical contraction of the guinea pig ileum, as seen in administration of SP. This data confirmed that Ci-TKs are conferred with the essential pharmacological characteristics of vertebrate TKs, given that protostome TKRPs are devoid of any effects on mammalian tissues (Satake et al., 2003; Satake & Kawada, 2006a). The Ci-TK precursor encodes both Ci-TK-I and Ci-TK-II, and shows structural organization similar to γ-*tac*1 (Satake et al., 2004;

Species	Peptide	Sequence
Chordate tachykinins		
Mammals	Substance P	RPKPQQFFGLM-amide
	Neurokinin A	HKTDSFVGLM-amide
	Neurokinin B	DMHDFVGLM-amide
Rat and mouse	Hemokinin-1	SRTRQFYGLM-amide
Human	Endokinin A/B	GKASQFFGLM-amide
	Endokinin C	KKAYQLEHTFQGLL-amide
	Endokinin D	VGAYQLEHTFQGLL-amide
Ascidian (*Ciona intestinalis*)	**Ci-TK-I**	**HVRHFYGLM-amide**
	Ci-TK-II	**ASFTGLM-amide**
Protostome tachykinin-related peptides		
Fruitfly	DTK-1	APTSSFIGMR-amide
Echiuroid worm	Uru-TK-I	LRQSQFVGAR-amide

Table 1. Tachykinins (TKs) and Tachykinin-related peptides (TKRPs). The TK and TKRP consensus moieties are underlined and dotted, respectively. *C. intestinalis* tachykinins (Ci-TK-I and –II) are indicated in boldface.

Satake & Kawada, 2006a). These findings provide evidence that the TK family is conserved as neuropeptides in chordates as well as in vertebrates, and indicate that the *ci-tk* gene is a direct prototype of vertebrate TKs. However, Ci-TK-I and -II sequences are located in the same exon, indicating that no alternative splicing of the *ci-tk* transcript occurs. Therefore, it is presumed that alternative production of TK peptides was established during the evolution of vertebrates (Satake et al., 2004). In combination, these findings indicate that the "prototype" *tk* gene, organized similarly to the *ci-tk*gene, originally encoded two TKs in the same exon, and then was divided by intron insertsion followed by acquirement of the alternative splicing system during the divergence of the ancestral gene into *tac*1, *tac*3, and *tac*4 during evolution of vertebrates.

The endogenous Ci-TK receptor, Ci-TK-R, was also identified in *C. intestinalis*. A sequence comparison verified that the transmembrane domain of Ci-TK-R displayed high sequence similarity (30-43%) to those of mammalian TK receptors (Satake et al., 2004). The phylogenetic analysis of TK receptors and TKRP receptors revealed that Ci-TK-R belongs to the vertebrate TK receptor clade (Satake & Kawada, 2006a). Moreover, *Ciona* database-searching for TK receptors detected only Ci-TK-R as an ortholog of TK receptors, indicating that ascidians possess one TK receptors (Satake et al., 2004). In combination, these findings suggest that Ci-TK-R is an ancestor of vertebrate TK receptors and that three vertebrate TK receptor genes were generated from a single common ancestor via gene duplication in the evolutionary process of vertebrates. Application of Ci-TK-I to Ci-TK-R evoked a typical intracellular calcium elevation (Satake et al., 2004). Additionally, SP and NKA also exhibited comparable responses to Ci-TK-I (Satake et al., 2004). These pharmacological analyses

indicated that Ci-TK-R lacked the ligand selectivity typical of NK1-3 (Satake & Kawada, 2006a). Consequently, it is suggested that the ancestral TK receptor is highly likely to possess no significant ligand-selectivity and that the ligand-selectivity of TK receptors, along with the alternative production of TK ligands, were established during generation of NK1-3 in vertebrates.

3.2 NTLPs in *C. intestinalis*

Neurotensin (NT) is a vertebrate brain/gut peptide involved in dopamine transmission, pituitary hormone secretion, hypothermia, and analgesia as a neuromodulator (Evers, 2006). NT is yielded from a single precursor with a structurally related peptide, neuromedin N. These family peptides are featured by a Pro-Tyr-Ile-Ile C-terminal consensus sequence (Table 2). The NT family has so far been characterized only in mammals and birds (Evers, 2006). NT receptors, NTR1, 2, and 3 have been identified in mammals. NTR1 and -2 are Class A GPCRs, which trigger an elevation of intracellular calcium ion (Evers, 2006). NTR3 (or sortilin) is a non-GPCR membrane protein, and bound to NT. However, the resultant signal transduction has yet to be detected.

Peptide	Sequence
Neurotensin (rat)	pQLYENKPRRPYIL
Neurotensin (chicken)	pQLHVNKARRPYIL
Neuromedin N (rat)	KIPYIL
LANT 6(chicken)	KNPYIL
Ci-NTLP-1	**pQLHVPSIL**
Ci-NTLP-2	**GMMGPSII**
Ci-NTLP-3	**MMLGPGIL**
Ci-NTLP-4	**FGMIPSII**
Ci-NTLP-5	**NKLLYPSVI**
Ci-NTLP-6	**SRHPKLYFPGIV**

Table 2. Neurotesin (NT) and its related peptides. The NT consensus moiety is underlined. *C. intestinalis* NT-like peptides (Ci-NTLP-1 to -6) are indicated in boldface.

Quite recently, we have identified six *Ciona* NT-like peptides, Ci-NTLP-1 to -6, by a peptidomic approach (Kawada et al., 2011). As shown in Table 2, Ci-NTLPs share the Pro-Ser/Gly-Ile/Val-Ile-Leu, which is reminiscent of the NT C-terminal consensus (Table 2). Ci-NTLPs are encoded by two genes; Ci-NTLPs were encoded in two precursors; *ci-ntlp-A* encodes Ci-NTLP-1 to -4, while *ci-ntlp-B* encodes Ci-NTLP-5 and -6 (Kawada et al., 2011). Of particular interest is that *ci-ntlp-A* is expressed exclusively in neurons of the brain ganglion, whereas the abundant expression of *ci-ntlp-B* is detected in the ovary. Consequently, it is presumed that Ci-NTLP-1 to 4 serve as neuropeptides, while Ci-NTLP-5 and -6 are ovarian hormones. This is the first characterization of NT-related peptides in invertebrates.

3.3 The TK- and NTLP-regulated oocyte growth in *C. intestinalis*

The biological roles of TKs in the ovary remained to be elucidated. The ascidian ovary harbors numerous oocytes at each growth stage (Prodon et al., 2006; Aoyama et. al., 2008). Furthermore, *Ciona* oocytes are readily classified into four major growth stages on the basis of their dimeter and organization: previtellogenic (stage I), vitellogenic (stage II),

postvitellogenic (stage III), and mature (stage IV) stages (Prodon et al., 2006). These advantages indicate the possibility that the *Ciona* ovary and oocytes are suitable models for functional analyses of ovarian TKs. The immunoreactivity of Ci-TK-R was detected exclusively in test cells residing in late stage-II oocytes (Aoyama et al., 2008), which are believed to be functional counterparts for mammalian granulosa cells and to be involved in growth of oocyte bodies and follicle cells (Burighel & Cloney, 1997).

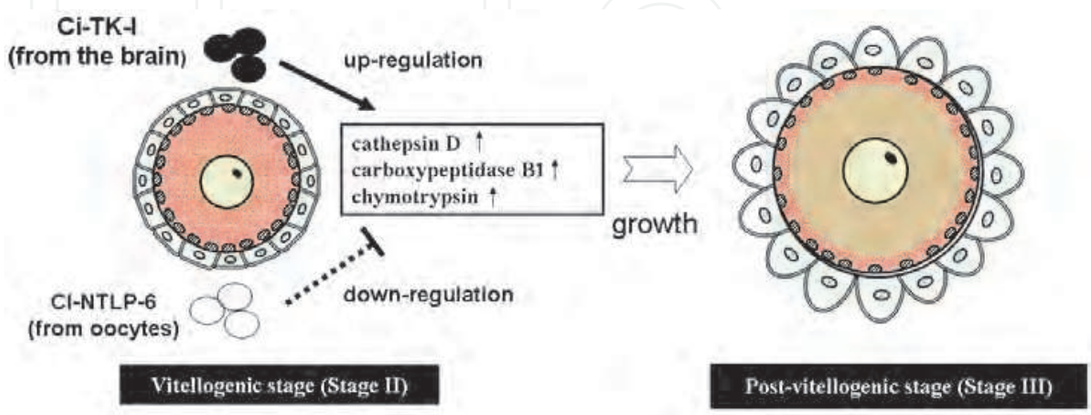

Fig. 4. Scheme of the oocyte growth regulated by Ci-TK-I and Ci-NTLP-6 in *C. intestinalis*.

Such specific expression of Ci-TK-R revealed that test cells of the late stage II oocytes are the sole targets of Ci-TK-I in the ovary. Comprehensive gene expression profiles between the untreated ovary and ovary treated with Ci-TK-I verified that the cathepsin D, chymotrypsin, and carboxypeptidase B1 genes are upregulated by Ci-TK-I (Aoyama et al., 2008). The enzymatic activities of these proteases were also shown to be enhanced by Ci-TK-I (Aoyama et al., 2008). Furthermore, treatment of *Ciona* late stage II oocytes with Ci-TK-I resulted in the growth to stage III, which was completely blocked by a Ci-TK-R antagonist or inhibitors of cathepsin D, chymotrypsin, and carboxypeptidase B1 (Aoyama et al., 2008). These findings revealed that Ci-TK-I is responsible for oocyte growth via the activation of the proteases. The involvement of these proteases in the *Ciona* oocyte growth is compatible with their functions in other species. For instance, leupeptin, which has the ability of inhibiting activity of chymotrypsin, was shown to suppress progression of oocyte growth at pre-GVBD stages in an ascidian *Halocynthia roretzi* (Sakairi and Shirai, 1991) and a starfish *Asterina pectinifera* (Takagi et al., 1989; Tanaka et al., 2000). In *Drosophila*, fluorescent-conjugated chymotrypsin inhibitors were localized to the growing oocyte-somatic follicle cells (Jakobsen et al., 2005). Carboxypeptidase B1 was also shown to play a crucial role in proteolytic processing of several component proteins for zona pellucida in mammalian oocytes at an early growth stage (Litscher et al., 1999). Cathepsin D was found to participate in production of yolk proteins and follicular component in various vertebrate oocytes at the stage prior to GVBD (Carnevali et al., 2006). Collectively, these results led to the conclusion that Ci-TK-I enhances growth of the vitellogenic oocytes via upregulation of gene expression and enzymatic activities of the proteases for vitellogenesis and oogenesis (Fig. 4), and suggested that the TK-regulated oocyte growth is an evolutionary origin of the TKergic functions in the ovary. More recently, a *Ciona* NT-like peptide, Ci-NTLP-6, has been shown to arrest the protease-associated oocyte growth at stage II via down-regulation of the gene expression of the Ci-TK-induced proteases (Kawada et al., 2011). It should be noteworthy

that Ci-NTLP-6 serves as an oocytic hormone, given that the *ci-ntlp-B* gene expression was detected in the ovary (Kawada et al., 2011). This is the first clarification of the biological roles of TKs and NTs in the ovary and the underlying essential molecular mechanism, leading to a substantiation of a novel regulatory pathway for oocyte growth (Fig. 4). In addition, our preliminary work suggests that the protease-associated oocyte growth pathway is essentially conserved in mammals (unpublished data). Altogether, these data reinforce the view that ascidians are one of the most useful model organisms for functional analyses of neuropeptides and hormones conserved in vertebrates and ascidians.

4. Neuropeptides and peptide hormones in *C. intestinalis*

In addition to Ci-TKs and Ci-NTLPs, a wide variety of neuropeptides and peptide hormones have been identified in *C. intestinalis*. In this section, we present the *Ciona* homologs of mammalian peptides which are highly likely to be involved in ovarian functions due to the expression of several of their receptors or receptor candidates in the ovary or their potential reproductive functions.

4.1 Recent identification of *Ciona* neuropeptides and hormone peptides

Recently, two approaches for characterization of peptides and/or their genes are available. The first one is homology search on a genome/EST database of an organism of interest. This procedure is useful for detection of peptides containing long sequences and/or conserving consensus motifs. For instance, genes of *Ciona* GnRHs, calcitonin (CT), insulin-like peptides (INS-Ls), and corticotrophin-releasing factor (CRF) were identified by homology search on the *Ciona* genome/EST database (Adams et al., 2003., Olinski et al., 2006; Sekiguchi et al., 2009; Lovejoy & Barsyte-Lovejoy, 2010). In contrast, homology-based search is frequently useless for detection of small peptides or their genes, given that major neuropeptides and peptide hormones contain short sequences, and that their precursors have slight homology, even though homologous peptide hormones and neuropeptides harbor complete consensus motifs. Additionally, novel peptides or peptide homologs with partial consensus motifs cannot be detected by any form of homology search. The second one is mass spectrometry (MS)-based peptidomic analysis. This procedure enables direct characterization of peptide sequences present in the tissues of interest. The most important step in peptidomic analysis is to discriminate biologically significant peptides with protein fragments. In general, neuropeptide or peptide hormone precursors harbor a hydrophobic signal peptide sequence at the N-terminus, and the mature peptide sequences there are flanked by endoproteolytic mono- or dibasic sites (Lys-Lys / Lys-Arg / Arg-Arg / Arg). According to these criteria, authentic neuropeptides and peptide hormones can be discriminated from degraded protein fragments, when their precursors of candidates for neuropeptides and peptide hormones sequenced by peptidomic analysis are available. A genome/EST database allows us to detect precursor sequences of the peptide candidates by referencing the obtained sequences, but not by the cloning of their cDNAs. In conclusion, a combination of MS-based peptidomic analysis and database-referencing of the resultant sequence (Fig. 5) is the most reliable and efficient methods for characterization of neuropeptides and peptide hormones. Based on such a screening strategy, we eventually identified 33 *Ciona* peptides (Table 3).

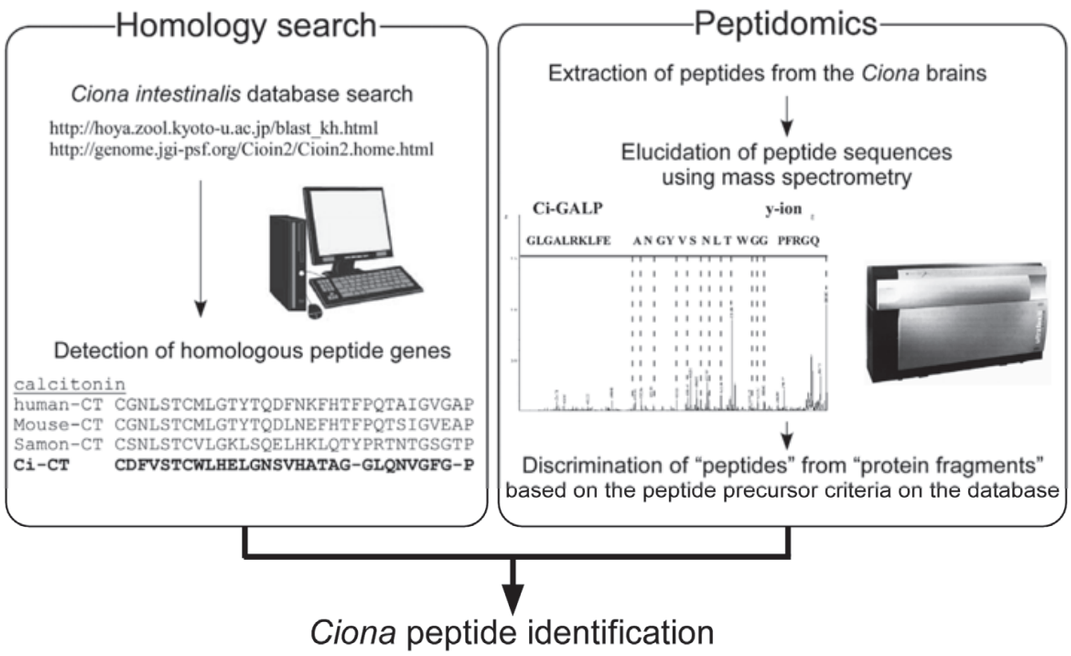

Fig. 5. Scheme of *Ciona* peptide identification

These *Ciona* peptides are largely classified into three categories: (i) prototypes and homologs of vertebrate peptides such as cholecystokinin (CCK), GnRHs, TK, CT, INS-Ls, CRF, galanin/galanin-like peptide (GALP), (ii) peptides partially homologous with vertebrate peptides including vasopressin (VP), GnRH-related peptide, NTLPs, (iii) novel peptides such as LF-family peptides and YFL/V peptides. Of particular significance is that *C. intestinalis* has been shown to possess the prototypes and homologs of vertebrate peptides, including CCK, TK, CT, CRF, galanin/GALP, and NTs, which have been never found in any other invertebrates including *Drosophila melanogaster*, *Caenorhabditis elegans* and *Stronglyocentrotus purpuratus* (Satake & Kawada, 2006b, Kawada et al., 2010). These findings provide indisputable evidence that *C. intestinalis* possesses a greater number of homologs to vertebrate peptides compared with other invertebrates, and suggest that many peptide prototypes originated from common ancestor chordates of ascidians and vertebrates, not from ancient vertebrates.

This view is compatible with the fact that ascidians occupy a phylogenetic position which is closer to vertebrates in evolutionary lineages of animals than any other invertebrates. It should be noteworthy that the *Ciona* homologs of vertebrate neuropeptides and/or peptide hormones from various glands including the hypothalamus have been identified, but no homologs of vertebrate pituitary hormones, such as ACTH, TSH, FSH, LH, GH, and PRL, have ever been reported (Kawada et al., 2010; Kawada et al., 2011). This is consistent with the fact that ascidians are not endowed with an organ corresponding to a pituitary (Burighel & Cloney, 1997). Collectively, these findings lead to a presumption that *C. intestinalis* shows applicability to the investigation of biological functions and their evolutionary aspects of neuropeptides and non-pituitary peptide hormones of vertebrates. In the following sections, we summarize *Ciona* homologs of vertebrate peptides, of which receptors or their candidates were shown to be expressed in the ovary.

Accession Number	Gene	Peptide	Peptide Sequence
BR000879	*ci-ntlp-B*	Ci-NTLP-5	NKLLYPSVI
		Ci-NTLP-6	SRHPKLYFPGIV
BR000880			AVLHLAINEFQRL
BR000876	*ci-ntlp-A*	Ci-NTLP-1	pQLHVPSIL
		Ci-NTLP-2	MMLGPGIL
		Ci-NTLP-3	GMMGPSII
		Ci-NTLP-4	FGMIPSII
BR000877	*ci-galp*	Ci-GALP	PFRGQGGWTLNSVGYNAGLGALRKLFE
BR000881	*ci-lf*	Ci-LF-1	*FQSLF*
		Ci-LF-2	YPGFQGLF
		Ci-LF-3	HNPHLPDLF
		Ci-LF-4	YNSMGLF
		Ci-LF-5	SPGMLGLF
		Ci-LF-6	SDARLQGLF
		Ci-LF-7	YPNFQGLF
		Ci-LF-8	GNLHSLF
BR000882			GFQNNAEGPV
			SADLFGAPMYII
AB219239	*ci-gnrh-x*	Ci-GnRH-X	pQHWSNWWIPGAPGYNG-amide
AY204706	*ci-gnrh-1*	t-GnRH-3	pQHWSKGYSPG-amide
		t-GnRH-5	pQHWSYEFMPG-amide
		t-GnRH-6	pQHWSYEYMPG-amide
BR000878			GEKESRPLSSYPGSV
BR000883			*DPLTNIM*
BR000884			WLRYDA
BR000885	*ci-yfv/l*	Ci-YFV-1	ELVVRDPYFV
		Ci-YFV-2	*NNQESYFV*
		Ci-YFV-3	DDEPRSYFV
		Ci-YFL-1	DAARPNYYFL
AB432887	*ci-vp*	Ci-VP	CFFRDCSNMDWYR
AB175738	*ci-tk*	Ci-TK-I	HVRHFYGLM-amide
		Ci-TK-II	SIGDQPSIFNERASFTGLM-amide
BR000886			NLLSLLQHAIETANNAYRSPR

Table 3. *C. intestinalis* peptides identified by a peptidomic approach.

4.2 GnRHs in *C. intestinalis*

GnRH (previously designated luteinizing-hormone-releasing hormone = LH-RH), has a critical role in reproductive development and function, which is released via the hypothalamic-hypophysial portal system to regulate the synthesis and release of pituitary gonadotropins that in turn trigger the steroidogenesis and stimulate gonadal maturation in vertebrates (Millar, 2005). Furthermore, GnRHs are involved in diverse neuroendocrine,

Peptide	Sequence
human GnRH-I	pQHWSYGLRPG-amide
human GnRH-II	pQHWSHGWYPG-amide
GnRH-III (fish)	pQHWSYGWLPG-amide
t-GnRH-3	**pQHWSYEFMPG-amide**
t-GnRH-4	**pQHWSNQLTPG-amide**
t-GnRH-5	**pQHWSYEYMPG-amide**
t-GnRH-6	**pQHWSKGYSPG-amide**
t-GnRH-7	**pQHWSYALSPG-amide**
t-GnRH-8	**pQHWSLALSPG-amide**
Ci-GnRH-X	**pQHWSNWWIPGAPGYNG-amide**
octopus-GnRH	pQNYHFSNGWHPG-amide

Table 4. GnRHs. The GnRH consensus moiety is underlined. *C. intestinalis* GnRH family peptides (t-GnRH-3 to -8 plus Ci-GnRH-X) are indicated in boldface.

paracrine, autocrine, and neurotransmitter/neuromodulatory functions in the central and peripheral nervous systems, and a wide range of peripheral tissues (Millar 2005). GnRH was isolated from protostomes, urochordates and vertebrates (Table 4). Vertebrate GnRHs are composed of 10 amino acids with the consensus sequences of pyro-Glu^{1}-His/Tyr^{2}-Trp^{3}-Ser^{4} and Pro^{9}-Gly^{10}-amide (Millar, 2005).

As shown in Table 4, two types of GnRH, GnRH-I and II were characterized in vertebrates, whereas GnRH-III was also found exclusively in teleosts (Millar, 2005). In lamprey GnRH, one GnRH-I orthlog (l-GnRH-II) and two paralogs (l-GnRH-I and –III) were identified (Kavanaugh et al., 2008). Protostome GnRHs were characterized from molluscs (Iwakoshi et al., 2002; Zhang et al, 2008). These molluscan GnRHs, composed of 12 amino acids, essentially conserve the GnRH consensus sequence (Table 4). All GnRHs are encoded as a single copy in the precursor, whose organization is conserved in vertebrates and protostomes (Iwakoshi-Ukena et al., 2004; Millar, 2005; Kavanaugh et al., 2008).

GnRH receptors (GnRHRs) belong to the class A (rhodopsin-like) GPCR family, and regulate elevation of intracellular calcium ion, generation and inhibition of cAMP production by coupling to different G-proteins (Millar et al., 2005). GnRHRs display some species-specific distribution, although at least one GnRHR was identified in all vertebrates (Kah et al., 2007). Mammalian type-I GnRHR completely lacks the C-terminal tail region which is found in non-mammalian counterparts (Millar et al., 2004; Millar 2005). Species-distribution of type-II GnRHR is relatively confounding; human, chimp, cow, and sheep type-II *gnrhr* gene is likely silenced due to deletion of functional domains or interruption of full-length translation by the presence of a stop codon, whereas a functional type-II GnRHR was identified in several monkeys, pigs, reptiles, and amphibians (Millar 2005; Kah et al., 2007). Moreover, type-II *gnrhr* genes have not been found in mouse and fish. Instead, fish possesses another GnRH receptor, type-III GnRHRs which are included in the subgroup separate from type-I and II GnRHRs (Millar 2005; Kah et al., 2007). Type-I GnRHRs show high affinity to both GnRH-I and II, whereas the type-II receptor is specific to GnRH-II. In

protostomes, an octopus GnRHR was found to possess a C-terminal tail and to trigger mobilization of intracellular cellular calcium ions (Kanda et al., 2006).

In *C. intestinalis*, six authentic GnRH peptides (t-GnRH-3 to -8) and one structurally related peptide were identified (Adams et al., 2003; Kawada et al., 2009a). Of particular interest is that two *Ciona* GnRH genes, *ci-gnrh-1* and *-2*, encode three different GnRH peptide sequences (Adams et al., 2003): t-GnRH-3, -5, and -6 are encoded in *ci-gnrh-1*, whereas t-GnRH-4, -7, and -8 are encoded in *ci-gnrh-2*. All GnRH sequences are flanked by mono- or dibasic endoproteolytic sites at their N- and C-termini (Adams et al., 2003). These triplet GnRH sequence organizations were also observed in *cs-gnrh-1* and *2* of *Ciona savignyi*, an ascidan species closely related to *C. intestinalis* (Adams et al., 2003). These findings have established the basis that three copies of GnRH are present in one precursor in ascidians, unlike vertebrate and protostome GnRH precursors that encode only a single GnRH sequence, and that the structural organization of *Ciona gnrh* genes occurred from the ancestral GnRH gene during an ascidian-unique evolutionary process.

A novel GnRH-like peptide, Ci-GnRH-X (Kawada et al., 2009a), was also characterized from the neural complexes of adult *C. intestinalis*. The *Ciona* peptide was shown to contain the GnRH consensus sequences, such as the N-terminal pQHWS sequence and a C-terminal amidated Gly. However, Ci-GnRH-X, unlike the 10-residue chordate or 12-residue protostome GnRHs, is composed of 16 amino acid residues, and lacks the common Pro at position 2 from the C-termini of the GnRH family peptides (Table 4). It is noteworthy that the *t-gnrh-X* gene encodes only a single Ci-GnRH-X. This organization is closer to those of vertebrate *gnrh* genes than *ci-gnrh-1* and *-2*.

To date, four GnRH receptors, namely, Ci-GnRHR-1, -2, -3, and -4, have been identified in *C. inetestinalis* (Kusakabe et al., 2003; Tello et al., 2005). Ci-GnRHR-1 share 70%, 38%, and 36% sequence homology to Ci-GnRHR-2, -3, and -4, respectively, and all Ci-GnRHRs display approx. 30% sequence identity to human type-I GnRHR (Kusakabe et al., 2003; Tello et al., 2005). The phylogenetic analyses demonstrated that Ci-GnRHR-2, -3, and -4 are not homologs of vertebrate GnRHR subgroups but *Ciona*-specific paralogs of Ci-GnRHR-1 (Kusakabe et al., 2003; Tello et al., 2005). Of particular significance is the distinct ligand-selective production of second messengers mediated by Ci-GnRHRs. The elevation of intracellular calcium, which is typical of vertebrate and octopus GnRH and receptors (Millar 2005; Kanda et al., 2006), was only observed in administration of t-GnRH-6 to Ci-GnRHR-1, while other Ci-GnRHRs failed to stimulate the calcium elevation with any t-GnRHs (Tello et al., 2005). Instead, Ci-GnRHR-2 is responsive to t-GnRH-7, 8, 6 in this order of potency, while t-GnRH-3 and -5 specifically activate Ci-GnRHR-3 to a similar extent. Ci-GnRHR-4 exhibited neither elevation of intracellular calcium nor cAMP production (Tello et al., 2005). Instead, Ci-GnRHR-4 was shown to heterodimerize with Ci-GnRHR-1 and then potentiate the elevation of intracellular calcium, both calcium-dependent and –independent protein kinase C subtypes, and ERK phosphorylation in a ligand-selective fashion, verifying that Ci-GnRHR-4 serves as a protomer of GPCR heterodimers rather than a ligand-binding GPCR (Sakai et al., 2010). Intriguingly, Ci-GnRH-X was found to moderately (10-50%) inhibited the elevation of the intracellular calcium and cAMP production by t-GnRH-6 at Ci-GnRHR-1, and cAMP production by t-GnRH-3 and -5 via Ci-GnRHR-3 (Kawada et al., 2009a). In contrast, no inhibitory effect of Ci-GnRH-X at Ci-GnRHR-2 was observed (Kawada et al., 2009a). These findings provide evidence that t-GnRHs and Ci-GnRHRs have not redundant

but specific biological roles. Adams et al. (2003) demonstrated that t-GnRH-3 and -5 elicited the efficient spawning activity. These findings provide evidence that a major function of t-GnRHs is the regulation of gamete release, suggesting that t-GnRHs plays a major role in gamete release in protochordates. Finally, t-GnRHs and Ci-GnRH-X were found to be expressed exclusively in neurons in the brain ganglion, whereas Ci-GnRHRs, including the Ci-GnRHR-1&R-4 heterodimer, were detected in test cells of vitellogenic oocytes as well as the brain ganglion (Adams et al., 2003, Kusakabe et al., 2003; Tello et al., 2005; Kawada et al., 2009a; Sakai et al., 2010). Altogether, it is concluded that *C. intestinalis* GnRHs participate in various reproduction-relevant functions as multifunctional neuropeptides. In other words, *C. intestinalis* has evolved neuropeptidergic GnRH-directed regulation of the ovary, which is highly likely to be a functional ancestor of vertebrate GnRHergic endocrine and neuroendocrine systems

4.3 Vasopressin/oxytocin in *C. intestinalis*

Oxytocin (OT) and vasopressin (VP) have so far been characterized from a great variety of animal species from protostomes to human (Kawada et al., 2009b). In mammals, it is well known that OT is responsible for reproductive behavior: uterine contraction, milk ejection, and male reproductive tract stimulation (Gimpl & Fahrenholz, 2001). On the other hand, VP plays a major part in osmoregulation including up-regulation of blood pressure and anti-diuretic effect (Frank & Landgraf, 2008). Moreover, a number of studies suggested that OT and VP served as multifunctional peptides in the central and peripheral tissues (Gimpl & Fahrenholz, 2001; Frank & Landgraf, 2008). OT and VP are likely to be also involved in highly advanced central functions and disorders including learning, social behavior, anxiety, and autism (Donaldson & Young, 2008; Frank & Landgraf, 2008).

OT and VP are composed of nine amino acids, and bear a circular structure formed by an intramolecular disulfide bridge between conserved Cys^1 and Cys^6 (Table 5). Moreover, Asn^5, Pro^7, Gly^9, and C-terminal amidation are conserved in most OT/VP superfamily peptides (Table 5). OT and VP are discriminated by an amino acid present at position 8; VP family peptides contain a basic amino acid (arginine or lysine), whereas a neutral amino acid (leucine, isoleucine, valine, or threonine) is located at this position of OT family peptides (Table 5). The sub-mammalian vertebrate VP homolog, vasotocin, also contains Arg at position 8, while the sub-mammalian vertebrate OT homologs, mesotocin and isotocin, harbor Ile at this position. Notably, one VP family peptide and one OT family peptide have been identified in most jawed vertebrates (Hoyle, 1998; Gimpl and Fahrenholz, 2001), although only one type of the OT/VP superfamily peptide has ever been found in cyclostomes and most invertebrates (Hoyle, 1998; Kawada et al., 2009b). Combined with the phylogenic position of cyclostomes as the stem species of vertebrates, the presence of vasotocin as the single member of the OT/VP superfamily in cyclostomes suggests that the OT family and VP family occurred via a gene duplication of the common ancestral gene during evolution from jawless fish to jawed fish. OT/VP superfamily peptides have also been identified in invertebrates: molluscs, annelids, insects, sea urchins, amphioxus, and ascidians (Hoyle, 1998; Kawada et al., 2009b). Protostome and non-ascidian deuterostome invertebrate OT/VP superfamily peptides are all composed of nine amino acids, and share the OT/VP consensus amino acids (Table 5).

Peptide	Sequence	Species
Protochordate		
Ci-VP	**CFFRDCSNMDWYR**	***Ciona intestinalis***
SOP	CYISDCPNSRFWST-amide	*Styela plicata*
Vertebrate		
oxytocin	CYIQNCPLG-amide	mammal
mesotocin	CYIQNCPIG-amide	non-mammalian tetrapod
isotocin	CYISNCPIG-amide	fish
vasopressin	CYFQNCPRG-amide	mammal
vasotocin	CYIQNCPRG-amide	non-mammalian vertebrates
Protostome		
Lys-conopressin	CFIRNCPKG-amide	*Lymnaea stagnail*
annetocin	CFVRNCPTG-amide	*Eisenia foetida*
octopressin	CFWTSCPIG-amide	*Octopus vulgaris*

Table 5. The oxytocin/vasopressin superfamily peptides. The *Ciona* peptide (Ci-VP) is indicated in boldface.

Both OT and VP precursors are composed of major three regions: a signal peptide, an OT or VP sequence flanked by a putative glycine C-terminal amidation signal and a dibasic endoproteolytic site, and a neurophysin featured by 14 highly conserved cysteines (Hoyle, 1998; Kawada et al., 2009b). Seven disulfide bridge pairs between each of the 14 cysteines are responsible for formation of correct tertiary structure to interact with OT/VP (Hoyle, 1998; Kawada et al., 2009b). This structural organization is conserved in all OT/VP superfamily peptide precursors with an exception of a *Ciona* counterpart as described later. Taken together, molecular characterstics of OT/VP superfamily peptides and their precursors are highly conserved in wide species from protostomes to human.

The OT/VP superfamily peptides manifest their activities through their receptors, which belong to the Class A GPCR family (Gimpl & Fahrenholz, 2001; Frank & Landgraf, 2008). The OT/VP superfamily peptide receptors display high sequence similarity with one another, indicating that they are included in a cognate GPCR superfamily. To date, three VP receptors (V1aR, V1bR, and V2R) and one OT receptor (OTR) have been identified in mammals. V1aR, V1bR and OTR have been shown to trigger an increase in the intracellular calcium ions (Gimpl & Fahrenholz, 2001; Frank & Landgraf, 2008), whereas V2R induces the production of cAMP (Frank & Landgraf, 2008).

The essential biological roles for the supefamily peptides are conserved in protostomes. An earthworm OT/VP superfamily peptide, annetocin, showed an OT-like physiological action. Injection of annetocin into the earthworm resulted in induction of the stereotyped egg-laying behavior (Oumi et al., 1996; Satake et al., 1999). Furthermore, the annetocin receptor gene was expressed specifically in the nephridia located in the clitellum region, suggesting that annetocin induced egg-laying behavior through the osmoregulatory action on the nephridia (Kawada et al., 2004). On the other hand, the osmoregulatory function was exhibited by inotocin that is an OT/VP superfamily peptide identified from a red flour beetle, *Tribolium castaneum*. Inotocin indirectly stimulated the Malpighian tubules through the central nervous system including the endocrine organs corpora cardiaca and corpora allata, leading to induction of the diuretic activity (Aikins et al., 2008).

OT/VP superfamily peptides were identified in different ascidians. Ci-VP is the first deuterostome invertebrate OT/VP family peptide from *C. intestinalis* (Kawada et al., 2008),

and SOP was characterized from another ascidian, *Styela plicata* (Ukena et al., 2008). The most outstanding feature of the ascidian OT/VP superfamily peptide sequences is an elongation of the C-termini as compared with other OT/VP superfamily peptides (Table 5). Ci-VP and SOP are composed of 13 and 14 amino acids, respectively, whereas typical OT/VP superfamily peptides are comprised of 9 amino acids (Table 5). In particular, Ci-VP is the only OT/VP superfamily peptide that bears the non-amidated C-terminus (Table 5). Meanwhile, the N-termini of these ascidian peptides display high sequence homology to other OT/VP superfamily peptides (Table 5). Such unique forms of Ci-VP and SOP were presumed to have evolved in ascidian-specific evolutionary lineages.

OT/VP superfamily precursors harbor a neurophysin domain featured by 14 highly conserved cysteines including two doublet cysteines (Kawada et al., 2009b). The Ci-VP precursor also encoded a neurophysin-like domain, but the *Ciona* neurophysin was found to possess only 10 cysteines. However, 10 cysteines in the *C. intestinalis* neurophysin, including two cysteine doublets, are positioned almost identically to those of other neurophysin domains (Kawada et al., 2008). Additionally, the 13-residue and C-terminally non-amidated Ci-VP peptide and the 10-cysteine neurophysin domain were detected in the genome database of the closely related species, *Ciona savignyi*. In contrast, the 14-cysteine neurophysin domain is completely conserved in the SOP gene (Ukena et al., 2008). These findings indicate the intraphyletic molecular diversity of neurophysin domains as well as the hormone sequences within ascidian species.

C. intestinalis possesses the sole Ci-VP-receptor, Ci-VP-R, which displayed high amino acid sequence similarity (35–56%) to those of vertebrate and protostome OT/VP superfamily peptide receptors (Kawada et al., 2008). The molecular phylogenetic analysis also confirmed that Ci-VP-R belongs to the OT/VP superfamily peptide receptor family. Furthermore, Ci-VP-R specifically evoked an intracellular calcium elevation in response to Ci-VP (Kawada et al., 2008). These results lead to the conclusion that Ci-VP-R is an endogenous Ci-VP receptor in *C. intestinalis.*

The *ci-vp* gene was expressed predominantly in several neurons of the brain ganglion (Kawada et al., 2008). Ci-VP-R mRNA was detected in various tissues: the neural complex, alimentary tract, endostyle, heart, and ovary (Kawada et al., 2008). The distribution of Ci-VP and Ci-VP-R mRNAs suggested that Ci-VP served as a multifunctional neuropeptide and was transferred from the brain ganglion to target peripheral tissues followed by exertion of physiological functions. SOP mRNA was also distributed in the neural ganglion, and immunohistochemistry of SOP in the neural complex demonstrated that SOP was localized to the neuropil of the brain (Ukena et al., 2008). Intriguingly, the expression of SOP mRNA in hypotonic sea water was 2-fold greater than those in isotonic and hypertonic sea water (Ukena et al., 2008). Furthermore, SOP evoked contractions with increased tonus in the siphon of the ascidian (Ukena et al., 2008). These results proved the functional correlation of SOP with osmoregulation. Further studies are required for the elucidation of the physiological mechanism of ascidian OT/VP superfamily peptides.

4.4 Calcitonin in *C. intestinalis*

Calcitonin (CT) is a 32-amino acid peptide, and is synthesized mainly in the C cells of the thyroid gland in mammals and the ultimobranchial gland in non-mammalian vertebrates expect cyclostomes (Hull et al., 1998). CTs play a pivotal role in calcium metabolism via suppression of osteoclasts activity in bones and teleost scales. CTs conserve a C-terminal

Peptide	Sequence
Vertebrate CT/CGRP family peptide	
Human CT	**C**GNLST**C**MLGTYTQDFNKFHTFPQTAIGVGAP-amide
Human CGRP	A**C**DTAT**C**VTHRLAGLLSRSGGVVKNNFVPTNVGSKAF-amide
Human Adrenomeullin	YRQSMNNFQGLRSFG**C**RFGT**C**TVQKLAHQIYQFTDKDKDNVAPRSKISPQGY-amide
Human Amylin	K**C**NTAT**C**ATQRLANFLVHSSNNFGAILSSTNVGSNTY-amide
Pig CRSP	S**C**NTAT**C**MTHRLVGLLSRSGSMVRSNLLPTKMGFKVFG-amide
Ascidian CT/CGRP family peptide	
Ci-CT	**CDGVSTCWLHELGNSVHATAGGKQNVGFGP-amide**

Table 6. The CT/CGRP superfamily peptides. The consensus motif is underlined. The *Ciona* peptide (Ci-CT) is indicated in boldface

amidated proline and N-terminal circular structure formed by a disulfide bridge between Cys^1 and Cys^7. In vertebrates, CT, CT gene-related peptide (CGRP), Amylin (AMY), Adrenomedullin (AM), and CT receptor-stimulating peptide (CRSP) belong to the CT/CGRP family peptide (Hull et al., 1998; Katafuchi et al., 2003). Although they display low sequence similarity, they share the essential secondary structure with CTs (Table 6). CGRPs are yielded from the *ct* gene via alternative splicing (α-CGRP) and another CGRP gene (β-CGRP) in the central and peripheral neuron, and acts not only as a vasodilator but also as a neuromodulator (Hull et al., 1998). AMY is secreted from pancreatic β-cells, and inhibits insulin-induced glucose uptake and glycogen synthesis in the skeletal muscle (Cooper et al., 1988). AM is initially isolated from phaechromyctoma, and elicits a vasodilatory effect and reduces the blood pressure (Kitamura et al., 2002). CRSP was involved in suppression of food intake (Sawada et al., 2006). Although the CT/CGRP family peptides except CRSP were detected in most vertebrates, CRSP was identified in pigs and dogs of the Laurasiatheria (Katafuchi et al., 2003).

Ligand	Receptor	RAMP
Calcitonin	CTR	none
CRSP	CTR	none
CGRP and Amylin	CTR	RAMP1
Amylin	CTR	RAMP3
CGRP	CRLR	RAMP1
Adrenomedullin and CGRP	CRLR	RAMP2

Table 7. Ligand selectivity of CTR/RAMP and CRLR/RAMP complexes

Two Class B GPCRs, CT receptor (CTR) and CTR-like receptor (CRLR), have so far been shown to be the receptors for CT/CGRP family peptides (Conner et al., 2004). Furthermore, as summarized in Table 7, three receptor activity-modifying proteins (RAMPs), single-

transmembrane spanning proteins, have been shown to form a heterodimer with CTR or CRLR, and then to modulate the ligand-receptor specificity (Conner et al., 2004).
Although no CT/CGRP family peptide was identified in invertebrates till 2008, a *Ciona* CT (Ci-CT) gene was cloned from the adult *Ciona* neural complex (Sekiguchi et al., 2009). As summarized in Table 6, the deduced amino acid sequence of Ci-CT displays that the N-terminal circular region formed by a disulfide bond between Cys^1 and Cys^7 and the C-terminal amidated Pro are almost completely conserved, indicating that Ci-CT possesses the essential sequence characteristics of vertebrate CTs (Sekiguchi et al., 2009). In contrast, the *ci-ct* gene showed the four-exon/three-intron structure, whereas the most vertebrate *ct* genes are composed of six exons and five introns (Sekiguchi et al., 2009). CT or CGRP peptide is generated from the CT gene via alternative splicing (Hull et al., 1988), whereas the *ci-ct* gene encodes a Ci-CT peptide sequence alone, and no splicing variant was detected (Sekiguchi et al., 2009). No candidate for AM, AMY, CRSP, and beta CGRP genes was detected in the *Ciona* genome (Sekiguchi et al., 2009), suggesting that Ci-CT is the sole *Ciona* peptide of the CT/CGRP family peptides.
In *Ciona* juveniles, the transcript of Ci-CT was detected in the neural complex, stigumata cell of gill, gastrointestinal tract, blood cells, and endostyle (Sekiguchi et al., 2009). These multiple tissue-distribution implied that an original CT/CGRP family peptide might have played various physiological roles of current vertebrate CT/CGRP family peptides in common ancestral chordates, and current tissue-specific gene expressions and physiological roles of CT/CGRP family peptides diverged from those of a Ci-CT-like ancestor in concert with multiplication of the family gene members via gene duplications and advances of tissue organizations during evolution of protochordates to vertebrates. Ci-CT mRNA is localized to the neural gland, which is a non-neuronal ovoid body spongy texture lying immediately ventral to the brain ganglion, suggesting that Ci-CT serves as an endocrine/paracrine factor, not as a neuropeptide, in the neural gland of ascidians. Unfortunately, direct evidence for the interaction of Ci-CT with the endogenous receptor candidate, Ci-CT-R, has yet to be obtained (Sekiguchi et al., 2009). Further investigation of Ci-CT physiological activity will provide new insights into the functional evolution of chordate CT/CGRP family peptide.

4.5 Insulin and related peptides in *C. intestinalis*

The *Ciona* insulin-like peptides were also detected. Conserved synteny between the regions hosting the human insulin/relaxin (RLN) paralogs and the region hosting the three *Ciona* insulin-like proteins (INS-L1, -L2, and -L3) suggested that *Ciona* INS-Ls are putative orthologs of the vertebrate insulin–RLN family (Olinski et al., 2006a). Olinski et al. (2006b) also revealed that *Ciona* INS-L1 is orthologous to the vertebrate insulin-like/RLN genes, INS-L2 to insulin genes and INS-L3 to IGF genes by analysis of the gene structure, on the basis of the presence of the conserved protein motifs, the predicted maturation mode of the peptide precursors, putative receptor binding sites and the relative expression level of the *Ciona* INS-Ls. The ligand-receptor pairs and their biological actions await further study.

4.6 Galanin-like peptides in *C. intestinalis*

Galanin and galanin-like peptide (GALP) are vertebrate brain/gut peptides involved in reproduction and feeding (Lang et al., 2007). In particular, GALP is believed to stimulate LH secretion via up-regulation of GnRH secretion (Lang et al., 2007). The N-terminal region of

Peptide	Sequence
Galanin (quail)	GWTLNSAGYLLGPHAVDNHRSFNDKHGFTa
Galanin (goldfish)	GWTLNSAGYLLGPHAIDSHRSLGDKRGVAa
Galanin (human)	GWTLNSAGYLLGPHAVGNHRSFSDKNGLTSa
GALP (human)	PAHRGRGGWTLNSAGYLLGPVLHLPQMGDQDGKRETALEILDLWKAIDGLPYSHPPQPS
Ci-GALP	**PFRGQGGWTLNSVGYNAGLGALRKLFE**
	** ******* ** *

Table 8. The galanin/galanin-like peptide (GALP) family peptides. The *Ciona* peptide (Ci-GALP) is indicated in boldface. The galanin/GALP consensus motif is underlined. Asterisks denote amino acids highly conserved in vertebrate and *Ciona* galanin/GALP family peptides.

galanin is critical for its receptor binding and biological function, supported by the fact that the 13-amino acid N-terminal Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro sequence is completely conserved in galanin of mammals, quail, and goldfish. On the other hand, GALP has thus far been characterized only in mammals. GALP also contains the identical consensus sequence in the N-terminal region. However, there are three different features between galanin and GALP sequences (Table 8). First, GALP has a longer sequence than galanin. Second, GALP is N-terminally elongated by a Pro-Ala-His-Arg-Gly-Arg-Gly sequence upstream of the consensus sequence, whereas galanin contains no amino acids at this region . Third, the C-terminus of galanin is amidated, while that of GALP is not. A single copy of galanin and GALP is encoded by a separate gene (Lang et al., 2007). Galanin and GALP share two Class A GPCRs, GLR1 and 2, while galanin- or GALP-specifc receptors have not ever been identified (Lang et al., 2007).

The peptidomic analysis also detected a *Ciona* galanin/GALP-related peptide, Ci-GALP (Kawada et al., 2011). The detected peptide sequence conserves the galanin/GALP consensus-like sequence, Gly-Trp-Thr-Leu-Asn-Ser-Val-Gly-Tyr-Asn-Ala-Gly-Leu, whereas it has a C-terminally truncated sequence compared with galanin and GALP (Table 8). Furthermore, the *Ciona* peptide possesses a Pro-Phe-Arg-Gly-Gln-Gly sequence at the N-terminus which is homologous with the Pro-Ala-His-Arg-Gly-Arg-Gly sequence in GALP (Table 8). Consequently, we designated the peptide as Ci-GALP. Intriguingly, the C-terminus of Ci-GALP, unlike GALP, is not amidated as it is in galanin (Table 8). In addition, no other homologous galanin/GALP-like peptide was found in *C. intestinalis*. These features support the notion that Ci-GALP is a prototype of galanin and GALP, and vertebrate galanin and GALP diverged from a Ci-GALP-like ancestor during the evolution. This is also the first characterization of galanin/GALP peptides in invertebrates.

5. Conclusion

As reviewed in this chapter, *C. intestinalis* has been found to conserve major homologs and prototypes of mammalian neuropeptides and peptide hormones, and *Ciona* oocytes and ovary have been shown to possess prominent advantages in studies of gonadotropin-independent growth stages regulated by neuropeptides and non-sexual hormones. Since peptide receptors are targets of various novel drugs, elucidation of biological functions of peptides in the ovaries is expected to lead to advances in peptidergic drug development in two regards: exploration of new drug targets in the ovary and reduction of side effects on

ovarian functions. Consequently, functional analyses of *Ciona* peptides in oocyte growth as primary model studies, leading to the verification of biological actions of peptides on the ovary and oocytes in mammals, is expected to eventually construct diverse fundamentals on the development of novel clinical pharmaceuticals or health diets for reproductive diseases or deficiencies. Such research strategies are now being attempted in our laboratory.

6. Acknowledgment

We thank Ms. Kazuko Hirayama and all members of the Maizuru Fisheries Research Station for cultivation of the ascidians. All ascidians (*C. intestinalis*) were provided by Kyoto University through the National Bio-Resource Project of the MEXT, Japan. This study is in part financially supported by JSPS (to H. S., K.T., and T. Sa.) and JST (to H. S.).

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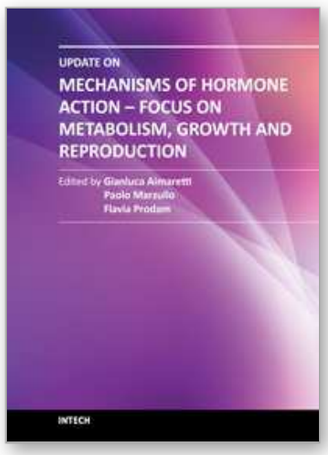

Update on Mechanisms of Hormone Action - Focus on Metabolism, Growth and Reproduction

Edited by Prof. Gianluca Aimaretti

ISBN 978-953-307-341-5
Hard cover, 470 pages
Publisher InTech
Published online 26, October, 2011
Published in print edition October, 2011

The purpose of the present volume is to focus on more recent aspects of the complex regulation of hormonal action, in particular in 3 different hot fields: metabolism, growth and reproduction. Modern approaches to the physiology and pathology of endocrine glands are based on cellular and molecular investigation of genes, peptide, hormones, protein cascade at different levels. In all of the chapters in the book all, or at least some, of these aspects are described in order to increase the endocrine knowledge.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Honoo Satake, Tsuyoshi Kawada, Masato Aoyama, Toshio Sekiguchi and Tsubasa Sakai (2011). Ascidians: New Model Organisms for Reproductive Endocrinology, Update on Mechanisms of Hormone Action - Focus on Metabolism, Growth and Reproduction, Prof. Gianluca Aimaretti (Ed.), ISBN: 978-953-307-341-5, InTech, Available from: http://www.intechopen.com/books/update-on-mechanisms-of-hormone-action-focus-on-metabolism-growth-and-reproduction/ascidians-new-model-organisms-for-reproductive-endocrinology

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