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REVIEW

Peptide receptors and immune-related proteins expressed in the digestive system of a urochordate, *Ciona intestinalis*

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Abstract

The digestive system is responsible for nutrient intake and defense against pathogenic microbes. Thus, identification of regulatory factors for digestive functions and immune systems is a key step to the verification of the life cycle, homeostasis, survival strategy and evolutionary aspects of an organism. Over the past decade, there have been increasing reports on neuropeptides, their receptors, variable region-containing chitin-binding proteins (VCBPs) and Toll-like receptors (TLRs) in the ascidian, *Ciona intestinalis*. Mass spectrometry-based peptidomes and genome database-searching detected not only *Ciona* orthologs or prototypes of vertebrate peptides and their receptors, including cholecystokinin, gonadotropin-releasing hormones, tachykinin, calcitonin and vasopressin but also *Ciona*-specific neuropeptides including Ci-LFs and Ci-YFVs. The species-specific regulation of GnRHergic signaling including unique signaling control via heterodimerization among multiple GnRH receptors has also been revealed. These findings shed light on the remarkable significance of ascidians in investigations of the evolution and diversification of the peptidergic systems in chordates. In the defensive systems of *C. intestinalis*, VCBPs and TLRs have been shown to play major roles in the recognition of exogenous microbes in the innate immune system. These findings indicate both common and species-specific functions of the innate immunity-related molecules between *C. intestinalis* and vertebrates. In this review article, we present recent advances in molecular and functional features and evolutionary aspects of major neuropeptides, their receptors, VCBPs and TLRs in *C. intestinalis*.

Keywords Ascidian · Neuropeptides · Receptors · Toll-like receptor · Variable region-containing chitin-binding protein

Introduction

Ascidians or squirts are marine filter-feeding invertebrate chordates and are members of the Urochordata. Urochordates and vertebrates are sometimes called Olfactores, due to a close relationship of the two groups (Satoh 2015). Such unique feeding behavior and critical phylogenetic position as the sister group of vertebrates provide us attractive and useful targets for research into the evolution and diversification of the digestive system in chordates. The digestive systems are responsible for two major physiological actions on exogenous factors: food digestion and defensive responses. The former includes recognition and digestion of foods, intake of nutrients and excretion of waste matters. This process is believed to involve various regulatory factors including neuropeptides and gut hormones as well as digestive enzymes and nutrient transporters. Although digestive systems in ascidians have yet to be investigated, a growing body of reports has identified ascidian neuropeptides, some of which are also expressed in the digestive system and their receptors. These findings will pave the way for investigating the molecular mechanism underlying hormonal and/or neuropeptidergic digestive systems in ascidians. The latter is mainly implicated with the innate immunity against various microbes, given that no adaptive immune system has been developed in ascidians. Moreover, the immune system plays a pivotal role in the interaction with enteric bacteria. Collectively, investigation of the neuropeptidergic regulatory mechanism and innate immune systems in the digestive tract

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of ascidians will surely provide crucial clues to the verification not only of the molecular mechanism underlying physiological functions of the ascidian digestive systems but also of both common and diverged traits of the digestive systems in chordates. In this review, we focus on major neuropeptides and their receptors, variable region-containing chitin-binding proteins (VCBPs) and Toll-like receptors (TLRs) in the cosmopolitan ascidian species, *Ciona intestinalis*.

Neuropeptides and their receptors in the digestive system of *C. intestinalis*

To date, approximately 40 neuropeptides and peptide hormones (Table 1) of *C. intestinalis* have been characterized by molecular cloning, genome survey and peptidomoic approaches (Satake and Kawada 2006a; Kawada et al. 2010, 2011; Matsubara et al. 2016). *Ciona* peptides are categorized as two major groups. The first group includes homologs or prototypes of vertebrate peptides such as gonadotropin-releasing hormones (GnRHs), tachykinins (TKs), vasopressin/oxytocin (VP/OT), calcitonin (CT), insulin/relaxin and galanin. The second group includes *Ciona*-specific peptides that have not been found in any other animal species such as Ci-YFV/Ls (Table 2) and Ci-LFs (Table 3). All *Ciona* peptides are summarized in Table 1. Collectively, *Ciona* was found to possess not only more prototypes and homologs than conventional model invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* but also various *Ciona*-specific neuropeptides (Satake and Kawada 2006a; Kawada et al. 2010, 2011; Matsubara et al. 2016). These findings highlight the advantages of *C. intestinalis* in studies on the molecular and functional evolution of neuropeptides throughout chordates. Although no functions were known for *Ciona* neuropeptides in the digestive tract, the expression of the cognate receptors in this organ suggests a wide variety of biological roles of the peptides in the digestive system. In this section, we provide an overview of *Ciona* neuropeptides and their cognate receptors.

Table 1 Major peptides identified by peptidomes of the *C. intestinalis* cerebral ganglion

Gene	Peptide	Sequence
Ci-gnrh-1	t-GnRH-3	pQHWSYEFMPGa
	t-GnRH-5	pQHWSYEYMPGa
	t-GnRH-6	pQHWSKGYSPGa
Ci-GnRH-X	Ci-GnRH-X	pQHWSNWWIPGAPGYNGa
Ci-NTLP-A	Ci-NTLP-1	pQLHVPSIL
	Ci-NTLP-2	GMMGPSII
	Ci-NTLP-3	MMLGPGIL
	Ci-NTLP-4	FGMIPSII
Ci-NTLP-B	Ci-NTLP-5	NKLLYPSVI
	Ci-NTLP-6	SRHPKLYFPGIV
Ci-GALP	Ci-GALP	PFRGQGGWTLNS VGYNAGLGALRKLFE
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
Ci-YFV/L	Ci-YFV-1	ELVVRDPYFV
	Ci-YFV-2	NNQESYFV
	Ci-YFV-3	DDEPRSYFV
	Ci-YFL-1	DAARPNYYFL
Ci-VP	Ci-VP	CFFRDCSNMDWYR
Ci-TK	Ci-TK-I	HVRHFYGLMa
	Ci-TK-II	SIGDQPSIFNERASFTGLMa

pyro-Glutamate and C-terminal amide are shown by "pQ" and "a"

Cionin

Cholecystkinin (CCK)/gastrin are vertebrate brain/gut peptides and are responsible for various physiological events including the regulation of digestive functions (Johnsen 1998; Noble and Roques 1999). Moreover, these peptides share the C-terminal consensus sequence Trp-Met-Asp-Phe-NH_2 and possess a sulfated Tyr residue (Table 2), which are essential for activities of the peptides. A sulfated Tyr is located at position 7 from the C-terminus of CCK and at position 6 from the C-terminus of gastrin, respectively. Two class-A G protein-coupled receptors (GPCRs) for CCK/gastrin family peptides have been identified and shown to activate several signaling cascades (Noble and Roques 1999; Pommier et al. 2003). Sulfakinins, isolated from several insects and crustaceans, are not included in the authentic CCK/gastrin family, given that sulfakinins conserve different C-terminal consensus and do not bind to CCK/gastrin receptors (Nichols 2003; Dickinson et al. 2007; Janssen et al. 2008).

Cionin (Tables 1 and 2) is an octapeptide that was first isolated from ascidians in 1990 (Johnsen and Rehfeld 1990). Of note, cionin completely conserves the CCK/gastrin C-terminal consensus motif and harbors two sulfated Tyr residues. These structural propensities indicate that cionin is an authentic member of the CCK/gastrin family (Kawada et al. 2010; Matsubara et al. 2016). Two authentic cionin receptors, CioR1 and CioR2 were cloned and shown to activate intracellular calcium mobilization in cultured cells in response to cionin (Nilsson et al. 2003; Sekiguchi et al. 2012). Furthermore, both of the two sulfated Tyr residues were found to be essential for full activation of CioRs (Sekiguchi et al. 2012). Molecular phylogenetic tree analysis verified that

Table 2 Sequences of cholecystokinin/gastrin family peptides

Species	Peptide	Sequence
Deuterostome invertebrate		
Ciona intestinalis	**cionin**	**NYYGWMDFa**
Vertebrate		
Mammals	CCK	DYMGWMDFa
	gastrin	pQGPWLEEEEEAYGWMDFa
Protostome		
Drosophila melanogaster (fruit fly)	Dsk1	FDDYGHMRFa
Caenorhabditis elegans (nematode)	NLP12	DGYRPLQFa

Sulfated tyrosines are indicated by a double line. The ascidian peptide (cionin) is indicated in boldface. The CCK/ gastrin C-terminal consensus motif is shaded

CioRs originated from the common ancestor of vertebrate CCK gastrin receptors and that CioR1 and CioR2 were generated via gene duplication in the ascidian lineage (Sekiguchi et al. 2012). CioRs are expressed in the neural complex, digestive organs, oral/atrial siphons and ovary. The critical phylogenetic position of ascidians and the ancestral structure of cionin suggest that the fundamental CCK/gastrinergic system was first established in ancestral chordates, although cephalochordate cionin homologs remain to be elucidated.

GnRH

GnRHs are 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 et al. 2004; Millar and Newton 2013; Millar 2005) and are also involved in diverse neuroendocrine, paracrine, autocrine and neurotransmitter/neuromodulatory functions in the central and peripheral nervous systems and various peripheral tissues (Millar et al. 2004; Millar and Newton 2013; Millar 2005). GnRHs or their related peptides have thus far been identified in molluscs, echinoderms and ascidians as well as vertebrates (Table 3). Most vertebrate GnRHs, except lamprey GnRH-I and -III (Table 3), are composed of ten amino acids and conserve the consensus motif of pyro-Glu^{1}-His/Tyr^{2}-Trp^{3}-Ser^{4}, Gly^{6} and Pro^{9}-Gly^{10}-amide (Millar et al. 2004; Millar 2005; Kah et al. 2007; Kawada et al. 2009a, b; Millar and Newton 2013; Sakai et al. 2017). GnRH1 and -2 were characterized in all vertebrates and GnRH3 was found exclusively in teleost (Millar et al. 2004; Millar and Newton 2013; Millar 2005). In lamprey, one GnRH1 ortholog (l-GnRH-II) and two species-specific subtypes (l-GnRH-I and -III) were characterized (Kavanaugh et al. 2008).

Protostome and echinoderm GnRHs are composed of 11 to 12 amino acids and are partially similar to vertebrate GnRHs (Table 3). These GnRHs possess two additional amino acid residues after position 1 (Table 3). Moreover, the C-terminal Pro-Gly-amide is replaced with Pro/Ser/Ala-amide in non-cephalopod (octopus and squid) protostome GnRHs, whereas this consensus motif is found in echinoderm GnRHs (Table 3). In contrast, protostome and echinoderm GnRHs completely conserve Gly^{8} that corresponds to Gly^{6} in vertebrate GnRHs (Table 3). One GnRH sequence is encoded as a single copy in the precursor protein and this structural organization of the precursors is conserved in vertebrates and protostomes (Millar et al. 2004; Millar and Newton 2013; Millar 2005; Kah et al. 2007; Kawada et al. 2009a, b; Roch et al. 2014; Semmens et al. 2016; Sakai et al. 2017). Recently, other protostome peptides, corazonin and adipokinetic hormones, are expected to belong to the GnRH family due to the conservation of several amino acids and sequence similarity among their receptors and GnRHRs but such categorization remains controversial (Sakai et al. 2017).

Eleven ascidian GnRHs have so far been identified: t-GnRH1 and -2 in *Chelyosoma productum*, (Powell et al. 1996); t-GnRH3 to -8 in *Ciona intestinalis* (Adams et al. 2003); t-GnRH-5 to -9 in *Ciona savignyi* (Adams et al. 2003); and t-GnRH10, and -11 in *Halocynthia roretzi* (Hasunuma and Terakado 2013). All ascidian GnRHs completely conserve the consensus sequences of pyro-Glu-His-Trp-Ser and Pro-Gly-amide (Table 3). Unlike GnRH genes of any other species, the *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 and t-GnRH-4, -7 and -8 are encoded in *Ci-gnrh*-2. Likewise, such a structural organization of *C. intestinalis* GnRH precursors were conserved in *Cs-gnrh*-1 and 2 of *C. savignyi* (Adams et al. 2003). In *Halocynthia roretzi*, the *H. roretzi* GnRH gene encodes t-GnRH-10 and -11 (Hasunuma and Terakado 2013). These findings highlighted a unique molecular diversity of ascidian GnRHs. Another *Ciona*-specific GnRH-like peptide, Ci-GnRH-X (Kawada et al. 2009a, b), was also characterized from the neural complexes. This peptide bears several GnRH consensus sequences, including the

Table 3 Sequences of GnRHs

Species	Peptide	Sequence
Deuterostome invertebrate		
Chelyosoma productum	**t-GnRH-1**	**pQHWSDYFKPGa**
	t-GnRH-2	**pQHWSLCHAPGa**
Ciona intestinalis	**t-GnRH-3**	**pQHWSYEFMPGa**
	t-GnRH-4	**pQHWSNQLTPGa**
	t-GnRH-5	**pQHWSYEYMPGa**
	t-GnRH-6	**pQHWSKGYSPGa**
	t-GnRH-7	**pQHWSYALSPGa**
	t-GnRH-8	**pQHWSLALSPGa**
Ciona savignyi	**t-GnRH-9**	**pQHWSNKLAPGa**
Ciona intestinalis	**Ci-GnRH-X**	**pQHWSNWWIPGAPGYNGa**
Halocynthia roretzi	**t-GnRH-10**	**pQHWSYGFSPGa**
	t-GnRH-11	**pQHWSYGFLPGa**
Branchiostoma floridae (amphioxus)	Amph.GnRHv	pQEHWQYGHWYa
	Amph.GnRH	pQILCARAFTYTHTWa
Strongylocentrotus purpuratus (sea urchin)	Sp-GnRHP	pQVHHRFSGWRPGa
Asterias rubens (starfish)	Ar-GnRH	pQIHYKNPGWGPGa
Vertebrate		
Homo sapiens (human)	GnRH1	pQHWSYGLRPGa
	GnRH2	pQHWSHGWYPGa
Cavia porcellus (guinea pig)	GnRH1	pQHWSYGVRPGa
Oncorhynchus mykiss (rainbow trout)	GnRH3	pQHWSYGWLPGa
Petromyzon marinus (sea lamprey)	l-GnRH-I	pQHYSLEWKPGa
	l-GnRH-II	pQHWSHGWFPGa
	l-GnRH-III	pQHWSHDWKPGa
Protostome		
Octopus vulgaris (octopus), *Sepia officinalis* (cuttlefish) and *Loligo edulis* (swordtip squid)	Oct-GnRH	pQNYHFSNGWHPGa
Crassostrea gigas (oyster)	Cg-GnRH	pQNYHFSNGWQPa
Patinopecten yessoensis (yesso scallop)	Py-GnRH	pQNFHYSNGWQPa
Aplysia californica (sea hare)	Ap-GnRH	pQNYHFSNGWYAa
Capitella teleta (marine worm)	Ca-GnRH	pQAYHFSHGWFPa
Helobdella robusta (leech)	Hr-GnRH	pQSIHFSRSWQPa

The N-terminal pyroglutamic acid and C-terminal amide are shown by "pQ" and "a," respectively. The ascidian peptides are indicated in boldface. Chordate GnRH consensus motifs are shaded

N-terminal pGlu-His-Trp-Ser and a C-terminal amidated Gly. In contrast, Ci-GnRH-X is composed of 16 amino acids and lacks the common Pro at position 2 from the C-terminus of other chordate GnRHs (Table 3). Additionally, the *t-gnrh-X* gene encodes a single sequence of Ci-GnRH-X (Kawada et al. 2009a, b), which is typical of non-ascidian GnRH genes.

GnRH receptors (GnRHRs) induce elevation of intracellular calcium ion mobilization (Millar et al. 2004; Millar and Newton 2013; Millar 2005; Kah et al. 2007; Kawada et al.

2009a, b, 2013; Sakai et al. 2017). GnRH1 and -2 interact with type-I GnRHRs, whereas type-II GnRHRs are specifically responsive to GnRH2. The octopus GnRHR was the only receptor that was shown to be responsive to the cognate ligand and to trigger mobilization of intracellular calcium ions in molluscs (Kanda et al. 2006; Kawada et al. 2013; Sakai et al. 2017). Ar-GnRH specifically triggers intracellular $Ca2^{+}$ mobilization of Ar-GnRHR in the starfish, *Asterias rubens* (Tian et al. 2016).

In *C. intestinalis*, four GnRH receptors, Ci-GnRHR-1, -2, -3 and -4, have been identified (Kusakabe et al. 2003; Tello et al. 2005). Ci-GnRHR-1 displays 70%, 38% and 36% sequence identity to Ci-GnRHR-2, -3 and -4, respectively and all Ci-GnRHRs are ca. 30% similar to vertebrate Type I GnRHR (Kusakabe et al. 2003; Tello et al. 2005). The molecular phylogenetic tree demonstrated that Ci-GnRHRs were generated via *Ciona*-specific gene duplication (Kusakabe et al. 2003; Tello et al. 2005). These receptors are found to possess unique ligand-selectivity and second messenger generation. The elevation of intracellular calcium, which is typical of other GnRHRs (Millar et al. 2004; Millar 2005; Tello et al. 2005; Kanda et al. 2006; Tello and Sherwood 2009; Kawada et al. 2013; Tian et al. 2016; Sakai et al. 2017), is only activated by t-GnRH-6 and Ci-GnRHR-1 pair (Tello et al. 2005). t-GnRH6, -7 and -8 enhance the cAMP production via Ci-GnRHR-2, whereas t-GnRH3 and -5 activate cAMP production via Ci-GnRHR-3 with high specificity. Ci-GnRHR-4 exhibited neither elevation of intracellular calcium nor cAMP production (Tello et al. 2005) but participates in unique fine-tuning of t-GnRH signaling via heterodimerization of other subtypes; a very unique functional role in *C. intestinalis* GnRH signaling (Fig. 1). Heterodimerization of Ci-GnRHR4 with Ci-GnRHR-1 enhances the t-GnRH6-specific elevation of intracellular calcium, both calcium-dependent and -independent protein kinase C and ERK phosphorylation (Sakai et al. 2010). Ci-GnRHR-4 also forms a heterodimer with Ci-GnRHR-2 (Sakai et al. 2012), which results in the downregulation of t-GnRH7 and -8-responsive cAMP production by 50% in a non-ligand selective manner via shifting of activation from Gs protein to Gi protein, compared with the Ci-GnRHR-2 monomer/homodimer (Sakai et al. 2012). These findings provide evidence that Ci-GnRHR-4 acts as a protomer of GPCR heterodimers rather than a ligand-binding receptor (Satake et al. 2013), indicating ascidian-specific molecular and functional evolution of GnRHergic systems.

Ci-GnRH-X also exhibits unique activity at Ci-GnRHRs. This *Ciona*-specific GnRH-related peptide moderately (10–50%) suppressed the elevation of the intracellular calcium and cAMP production by t-GnRH-6 at Ci-GnRHR-1 and cAMP generation by t-GnRH-3 and -5 at Ci-GnRHR-3 (Kawada et al. 2009a). In contrast, no effect of Ci-GnRH-X at Ci-GnRHR-2 was observed (Kawada et al. 2009a).

t-GnRH-3 and -5 increased the gemate spawning activity and water flow (Adams et al. 2003). Moreover, tGnRH-3 and -5 arrest the growth of adult organs by downregulation of cell cycle progression and tail absorption in the *Ciona* larva (Kamiya et al. 2014). Combined with the fact that *C. intestinalis* possesses no pituitary and gonadotropins, these findings suggest that t-GnRHs are responsible for the regulation of various biological events that are distinct from gonadotropin release.

Tachykinin

Tachykinins (TKs) are 9-14-amino acid vertebrate brain/gut peptides regulating a wide variety of biological functions such as smooth muscle contraction, vasodilation, nociception,

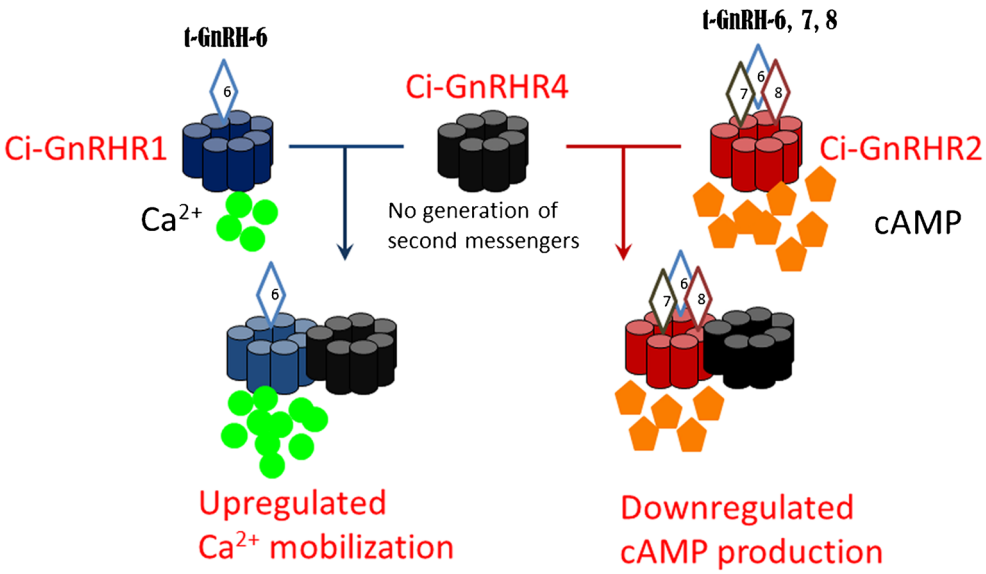

Fig. 1 Heterodimerization-directed regulation of *Ciona* GnRH receptor signaling. Ci-GnRHR1-R4 heterodimer elicits 10-fold more potent Ca^{2+} mobilization than Ci-GnRHR1 alone in response to t-GnRH6. R2-R4 heterodimer produces less than 50% cAMP than Ci-GnRHR2 in response to t-GnRH6, 7 and 8

inflammation, neurodegeneration and neuroprotection in a neuropeptidergic or endocrine manner (Millar and Newton 2013; Steinhoff et al. 2014). The mammalian TK family consists of four major peptides, substance P (SP), neurokinin A (NKA), neurokinin B (NKB) and homeokinin-1/endokinins (HK-1/EKs) (Table 4). TKs are encoded by three genes: *TAC1* (or *PPTA*), *TAC3* (or *PPTB*), or *TAC4* (*PPTC*) gene (Satake and Kawada 2006b; Page 2006; Steinhoff et al. 2014). *TAC1* generates four splicing variants that yield SP alone or SP and NKA (Satake and Kawada 2006b; Page 2006; Steinhoff et al. 2014). Tetrapod *TAC3* gene encodes only NKB (Satake and Kawada 2006b; Page 2006; Steinhoff et al. 2014), whereas the teleost counterpart encodes another TK peptide, NKF (Biran et al. 2012). Major vertebrate TKs share the C-terminal consensus -Phe-X-Gly-Leu-Met-NH_2 (Table 4)

The TK receptor family is constituted by NK1, NK2 and NK3 in vertebrates (Satake and Kawada 2006b; Page 2006; Steinhoff et al. 2014). All TK receptors induce both elevation of intracellular calcium and production of cAMP with moderate ligand-selectivity: SP > NKA > NKB for NK1, NKA > NKB > SP for NK2 and NKB > NKA > SP for NK3, respectively (Satake and Kawada 2006b; Page 2006; Steinhoff et al. 2014). HK-1 and the 10-amino acid C-terminal common sequence of EKA and EKB (EKA/B) exhibited potent binding activity with the highest selectivity to NK1 (Satake and Kawada 2006b; Page 2006; Steinhoff et al. 2014) and the teleost-specific peptide, NKF, specifically activates NK3 (Biran et al. 2012). TK receptors are expressed abundantly in the brain, gut, smooth muscle and genital organs (Satake and Kawada 2006b; Page 2006; Biran et al. 2012; Millar and Newton 2013; Steinhoff et al. 2014)

In protostomes, TK-related peptides (TKRPs) have been identified in the central nervous system of various protostomes (Satake et al. 2003; Satake and Kawada 2006b; Jiang et al. 2013; Satake et al. 2013). These peptides conserve the Phe-Xaa1-(Gly/Ala/Pro)-Xaa2-Arg-NH_2 consensus (Table 4) that is analogous to that of vertebrate TKs. Unlike TK precursors, TKRP precursors encode multiple (five to ten) TKRP sequences (Satake et al. 2003, 2013; Satake and Kawada 2006b; Jiang et al. 2013). These findings suggest that TKRP and TK genes might have diverged in distinct evolutionary lineages. TKRP receptors were characterized from several protostomes (Satake et al. 2003, 2013; Satake and Kawada 2006b; Jiang et al. 2013). These receptors display high sequence similarity to vertebrate TK receptors and the molecular phylogenetic tree demonstrated that TK receptors and TKRP receptors share the common ancestral gene (Satake et al. 2003, 2013; Satake and Kawada 2006b; Jiang et al. 2013). TKRP

Table 4 Tachykinin family peptides

Species	Peptide	Sequence
Deuterostome invertebrate tachykinins		
Ciona intestinalis	**Ci-TK-I**	**HVRHFYGLMa**
	Ci-TK-II	**SIGDQPSIFNERASFTGLMa**
Vertebrate tachykinins		
Mammals	Substance P	RPKPQQFFGLMa
	Neurokinin A	HKTDSFVGLMa
	Neurokinin B	DMHDFVGLMa
Rattus norvegicus (rat) and *Mus musculus* (mouse)	Hemokinin-1	SRTRQFYGLMa
Homo sapiens (human)	Endokinin A/B	GKASQFFGLMa
	Endokinin C	KKAYQLEHTFQGLLa
	Endokinin D	VGAYQLEHTFQGLLa
Danio rerio (zebrafish)	Neurokinin F	YNDIDYDSFVGLMa
Protostome tachykinin-related peptides (TKRPs)		
Locusta migratoria (locust)	Lom-TK-I	GPSGFYGVRa
Drosophila melanogaster (fluit fly)	DTK-1	APTSSFIGMRa
	Natalisin-1	EKLFDGYQFGEDMSKENDPFIGPRa
Urechis unicinctus (echiuroid worm)	Uru-TK-I	LRQSQFVGARa

C-terminal amide is shown by "a." The ascidian peptides are indicated in boldface. The chordate TK consensus motif and its related sequences are underlined by plain and dotted lines, respectively. The protostome TKRP consensus motif and its related sequence are indicated by wavy lines. Tachykinins and TKRPs consensus motifs are shaded

receptors were shown to stimulate the increase in intracellular calcium or generation of cAMP in response to cognate ligands (Satake et al. 2003, 2013; Satake and Kawada 2006b; Jiang et al. 2013). In contrast, TKRP receptors exhibit almost redundant ligand selectivity to TKRPs (Satake et al. 2003, 2013; Satake and Kawada 2006b; Jiang et al. 2013). In *C. intestinalis*, two TK peptides, Ci-TK-I and Ci-TK-II, have been identified in the neural complex (Satake et al. 2004). Ci-TKs conserve the complete vertebrate TK consensus (Table 4) and Ci-TKs elicit a TK-typical contraction of the mammalian smooth muscle (Satake et al. 2004). Furthermore, the Ci-TK precursor encodes both Ci-TK-I and Ci-TK-II, of which structural organization is reminiscent of that of TAC1 (Satake et al. 2003; Satake and Kawada 2006b). On the other hand, unlike the Tac1 gene, the Ci-TK gene encodes Ci-TK-I and -II in the same exon, indicating that no splicing variants are yielded (Satake et al. 2004. Consequently, alternative splicing of Tac1 and Tac4 genes might have been acquired during the evolution of vertebrates.

The cognate Ci-TK receptor, Ci-TK-R, was identified in *C. intestinalis*. Ci-TK-R displayed high sequence homology (30–43%) to mammalian TK receptors (Satake et al. 2003, 2004, 2013; Satake and Kawada 2006b; Jiang et al. 2013; Steinhoff et al. 2014). The molecular phylogenetic tree indicated that Ci-TK-R is included in the vertebrate TK receptor clade, not in the protostome TKRPR clade (Satake et al. 2003, 2004, 2013; Satake and Kawada 2006b; Jiang et al. 2013; Steinhoff et al. 2014). Ci-TK-I elicited a canonical intracellular calcium mobilization at Ci-TK-R (Satake et al. 2004). Moreover, SP and NKA also exhibited intracellular calcium mobilization at Ci-TK-R to a similar degree to Ci-TK-I (Satake et al. 2004), verifying that Ci-TK-R is not endowed with the ligand selectivity typical of NK1-3 (Satake et al. 2004, 2013; Matsubara et al. 2016).

The Ci-TK gene is expressed in the neural complex and several peripheral tissues including unidentified small cells in the intestine and the Ci-TK-R gene was expressed abundantly in the brain, intestine and ovary (Satake et al. 2004). Although the function of Ci-TK in the digestive system has yet to be investigated, Ci-TK-I specifically enhances follicular development via upregulation of enzymatic activities of cathepsin D, chymotrypsin and carboxypeptidase B1 (Aoyama et al. 2008, 2012; Matsubara et al. 2016). The abundant expression of Ci-TK-R in the alimentary tracts suggests multiple roles for Ci-TK in the digestive systems.

Oxytocin/vasopressin

Oxytocin (OT) and vasopressin (VP) are nonapeptides and share Asn^5, Pro^7, Gly^9, C-terminal amidation. An intramolecular disulfide bridge between conserved Cys^1 and Cys^6 is essential for their activities (Table 5). The VP family peptides contain a basic amino acid (Lys or Arg) at position 8, whereas the OT family peptides share a neutral aliphatic one (Leu, Ile, Val or Thr) in vertebrates. They have so far been identified from a great variety of animal species from protostomes to mammals (Hoyle 1998; Satake et al. 1999; Kawada et al. 2008; Stafflinger et al. 2008; Aikins et al. 2008; Elphick and Rowe 2009; Kawada et al. 2009b; Beets et al. 2012). OT participates in reproductive behaviors including uterine contraction, milk ejection and male reproductive tract stimulation (Gimpl and Fahrenholz 2001; Kawada et al. 2004, 2009b) and VP is responsible for osmoregulation, control of blood pressure and anti-diuretic effect (Gimpl and Fahrenholz 2001; Frank and Landgraf 2008). OT and VP play multiple roles in learning, social behavior, anxiety and autism (Gimpl and Fahrenholz 2001; Frank and Landgraf 2008). One VP family peptide and one OT family peptide are conserved in most jawed vertebrates, although only a single OT/VP superfamily peptide has ever been identified in cyclostomes and most invertebrates (Hoyle 1998; Kawada et al. 2009b). In keeping with this, molecular phylogenetic analysis suggested that the OT and VP family might have occurred via gene duplication of the common ancestral gene during the evolution from agnathans to gnathostomes.

OT/VP superfamily peptides have also been identified in diverse invertebrates (Hoyle 1998; Satake et al. 1999; Kawada et al. 2008, 2009b; Aikins et al. 2008; Stafflinger et al. 2008; Elphick and Rowe 2009; Beets et al. 2012). Various protostomes and non-ascidian deuterostome invertebrates conserve OT/VP superfamily nonapeptides with the OT/VP consensus amino acids (Table 5). Both OT and VP precursors are organized with major three domains: a signal peptide, an OT or VP sequence flanked by a glycine C-terminal amidation signal followed by dibasic endoproteolytic site and a neurophysin containing 14 conserved cysteines (Hoyle 1998; Satake et al. 1999; Kawada et al. 2008, 2009b; Aikins et al. 2008; Stafflinger et al. 2008; Elphick and Rowe 2009; Beets et al. 2012). Seven disulfide bridges between each of the 14 cysteines are prerequisites for adoption of a functional tertiary structure to interact with OT/VP (Hoyle 1998; Satake et al. 1999; Kawada et al. 2008, 2009b; Aikins et al. 2008; Stafflinger et al. 2008; Elphick and Rowe 2009; Beets et al. 2012). This structural organization as well as peptide consensus residues are conserved in invertebrates except for *C. intestinalis* counterparts.

The vertebrate OT/VP receptors belong to the Class A GPCR family (Hoyle 1998; Gimpl and Fahrenholz 2001; Frank and Landgraf 2008). The OT/VP superfamily peptide receptors display high sequence similarity with one another, indicating that they evolved from a common ancestor (Hoyle 1998; Gimpl and Fahrenholz 2001; Frank and Landgraf 2008). 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, whereas V2R induces the

Table 5 Oxytocin/vasopressin family peptides

Species	Peptide	Sequence
Deuterostome invertebrate		
Ciona intestinalis	**Ci-VP**	**CFFRDCSNMDWYR**
Styela plicata	**SOP**	**CYISDCPNSRFWSTa**
Branchiostoma floridae (amphioxus)	[Ile^4]-vasotocin	CYIINCPRGa
Strongylocentrotus purpuratus (sea urchin)	echinotocin	CFISNCPKGa
Vertebrate		
Mammals	oxytocin	CYIQNCPLGa
	Arg-vasopressin	CYFQNCPRGa
	Lys-vasopressin	CYFQNCPKGa
Neoceratodus forsteri (lungfish), Non-mammalian tetrapods,etc.	mesotocin	CYIQNCPIGa
Osteichthyian (bony fish)	isotocin	CYISNCPIGa
Squalus acanthias (dogfish)	valitocin	CYISNCPVGa
	aspargtocin	CYINNCPLGa
Other vertebrates	vasotocin	CYIQNCPRGa
Protostome		
Erpobdella octoculata (leech), *Lymnaea stagnail* (sea snail), *Aplysia kurodai* (sea hare)	Lys-conopressin	CFIRNCPKGa
Lymnaea stagnail (sea snail)	Arg-conopressin	CIIRNCPRGa
Einsenia foetida (earthworm)	annetocin	CFVRNCPTGa
Octopus vulgaris (octopus)	cephalotocin	CYFRNCPIGa
	octopressin	CFWTSCPIGa
Locusta migratoria (locust), *Tribolium castaneum* (red flour beetle)	inotocin	CLITNCPRGa
Caenorhabditis elegans (nematode)	nematocin	CFLNSCPYRRYa

"a" denotes C-terminal amide. The ascidian peptides (Ci-VP and SOP) are indicated in boldface. Consensus cysteine residues are shaded

production of cAMP (Hoyle 1998; Gimpl and Fahrenholz 2001; Frank and Landgraf 2008).

Two ascidian OT/VP superfamily peptides were identified in different species. Ci-VP is the OT/VP family peptide from *C. intestinalis* (Kawada et al. 2008) and *Styela* oxytocin-related peptide (SOP) was characterized from another ascidian, *Styela plicata* (Ukena et al. 2008). The most outstanding characteristic of these ascidian OT/VP superfamily peptides is an elongated C-terminal sequence, compared with other OT/VP superfamily peptides (Table 5). Ci-VP and SOP consist of 13 and 14 amino acids, respectively, which are in contrast to typical OT/VP superfamily peptides comprising nine amino acids (Table 5). Moreover, Ci-VP exceptionally bears the non-amidated C-terminus (Table 5). In contrast, the N-terminal circular region of these ascidian peptides displays high sequence homology to other OT/VP superfamily peptides (Table 5). The Ci-VP precursor also encoded a neurophysin-like domain but the *Ciona* neurophysin possesses only ten cysteines (Kawada et al. 2008), whereas the SOP precursor encodes the 14-cysteine neurophysin domain (Ukena et al. 2008). In addition, the 13-residue and C-terminally non-amidated Ci-VP peptide sequence and the 10-cysteine neurophysin region were found in the genome of the closely related ascidian species, *Ciona savignyi*. These findings demonstrate the species-specific molecular diversification of the OT/VP superfamily in ascidians.

C. intestinalis possesses a single Ci-VP-receptor, Ci-VP-R, which also displayed a high amino acid sequence similarity (35–56%) to those of vertebrate and protostome OT/VP superfamily peptide receptors and the molecular phylogenetic tree indicated that Ci-VP-R is a member of 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). Ci-VP-R is expressed in the

neural complex, digestive tract, endostyle and gonad (Kawada et al. 2008), suggesting diverse biological roles of Ci-VP. The expression of SOP mRNA in hypotonic sea water was twofold greater than those in isotonic and hypertonic sea water (Ukena et al. 2008). Furthermore, SOP stimulated contractions with increased tonus in the siphon of *Styela* (Ukena et al. 2008). These results suggested the biological role of SOP in osmoregulation in the ascidian.

Calcitonin

Calcitonin (CT) is a 32-amino acid peptide and is released from various tissues including the C cells of the thyroid gland in mammals and the ultimobranchial gland in non-mammalian vertebrates except cyclostomes (Conner et al. 2004). CTs are responsible for calcium metabolism via suppression of osteoclasts activity in bones of tetrapods and teleost scales. CTs harbor a C-terminally amidated proline and N-terminal circular structure formed by a disulfide bridge between Cys^1 and Cys^7. In vertebrates, the CT family (Table 6) includes CT, CT gene-related peptide (CGRP), amylin (AMY), adrenomedullin (AM) and CT receptor-stimulating peptide (CRSP) (Katafuchi et al. 2003, 2009; Conner et al. 2004). CGRPs are generated from the CT gene via alternative splicing (α-CGRP) and another CGRP gene (β-CGRP) in the central and peripheral neurons and acts not only as a vasodilator but also as a neuromodulation (Conner et al. 2004). Other family peptides are also encoded by different genes and play various and specific roles (Conner et al. 2004).

The CT superfamily peptides interact with two Class B GPCRs, CT receptor (CTR) and CTR-like receptor (CRLR) (Conner et al. 2004). Furthermore, three receptor activity-modifying proteins 1 to -3 (RAMP1–3) form a heterodimer with CTR or CRLR and regulate the ligand-receptor specificity (Conner et al. 2004).

Regardless of the lack of skeletal tissues, the CT-related peptides have been found in several invertebrate deuterostomes (Sekiguchi et al. 2009; Rowe and Elphick 2012; Rowe et al. 2014; Sekiguchi et al. 2016; Semmens et al. 2016; Suwansa-Ard et al. 2018; Cai et al. 2018). In *C. intestinalis*, *Ciona* CT (Ci-CT) was characterized from the adult neural complex (Sekiguchi et al. 2009). Ci-CT harbors the N-terminal circular region formed by a disulfide bond between Cys^1 and Cys^7 and the C-terminal amidated Pro (Table 6), which is reminiscent of the sequence characteristics of vertebrate CTs (Sekiguchi et al. 2009). However, CT and CGRP peptides are produced from the CT gene via alternative splicing (Conner et al. 2004), whereas Ci-CT gene encodes only a Ci-CT peptide sequence and no splicing variant was detected (Sekiguchi et al. 2009). In addition, putative genes for AM, AMY, CRSP and β-CGRP were not detected in the *Ciona* genome (Sekiguchi et al. 2009), indicating that Ci-CT is the sole *Ciona* peptide of the CT/CGRP family peptides and that alternative splicing of the CT gene was acquired during the evolution of vertebrates.

Ci-CT mRNA is localized to the endostyle and the neural gland (a non-neuronal ovoid body of spongy texture lying immediately ventral to the cerebral ganglion), suggesting that Ci-CT serves not as a neuropeptide but as an endocrine/paracrine factor in the neural gland and endostyle of ascidians (Sekiguchi et al. 2009). Unfortunately, direct evidence for the interaction of Ci-CT with the putative endogenous receptor candidate, Ci-CT-R, has yet to be obtained (Sekiguchi et al. 2009). On the other hand, three CGRP-like peptides, Bf-CTFP1 to -3 (Table 6) were identified and localized to nervous tissues in another invertebrate chordate, an amphioxus

Table 6 The CT/CGRP superfamily peptides

Species	Peptide	Sequence
Deuterostome invertebrate		
Ciona intestinalis	**Ci-CT**	**----------CDGVSTCWLHELGNSVHATAGGKQNVGFGPa**
Branchiostoma floridae (amphioxus)	Bf-CTFP1	----------DCSTLTCFNQKLAHELAMDNQRTDTANPYSPa
	Bf-CTFP2	----------GKIACKTAWCMNNRLSHNLSSLDNPTDTGVGAPa
	Bf-CTFP3	----------KCESGTCVQMHLADRLRLGLGHNMFTNTGPESPa
Vertebrate		
Homo sapiens (human)	CT	----------CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAPa
	CGRP	----------ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAFa
	Adrenomeullin	YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGYa
	Amylin	----------KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTYa
Sus scrofa (pig)	CRSP	----------SCNTATCMTHRLVGLLSRSGSMVRSNLLPTKMGFKVFGa

"a" denotes C-terminal amide. The ascidian peptide (Ci-CT) is indicated in boldface. Consensus motif is underlined and consensus cysteine residues are shaded

Branchiostoma floridae (Sekiguchi et al. 2016, 2017). These results provide evidence that Bf-CTFP serve as neuropeptides, which is in contrast to Ci-CT that functions as a non-neural factor. Furthermore, unlike *C. intestinalis*, one Bf-CTFP receptor (Bf-CTFP-R) and three RAMP-like proteins (Bf-RAMP-LPs 1–3) were identified in *B. floridae* (Sekiguchi et al. 2016). Notably, heterodimerization of Bf-CTFP-R with Bf-RAMP-LPs is requisite for translocation of Bf-CTFP-R to the plasma membrane and signaling via binding to Bf-CTFPs (Sekiguchi et al. 2016). On the other hand, unlike mammalian RAMPs, Bf-RAMP-LPs fail to alter ligand selectivity of Bf-CTFP-R but modulate the potency of Bf-CTFPs (Sekiguchi et al. 2016). These findings indicate that the CT family evolved in distinct lineages between ascidian and amphioxus. Interestingly, both Ci-CT and Bf-CTFPs elicited vertebrate CT-specific suppression of osteoclastic activity (Sekiguchi et al. 2017, 2009), suggesting that these peptides adopt similar active conformation despite low sequence similarity (Table 6).

Such molecular and functional differences between relatively close invertebrate chordates suggest puzzling evolutionary processes of the CT family in chordates. Three possible roles for an original CT/CGRP family peptide in chordate ancestors can be presumed. Firstly, an original CT/CGRP family peptide, like Bf-CTFPs, might have served as brain/gut peptides and thereafter, vertebrates and ascidians might have acquired hormonal functions. Additionally, the role as a neuropeptide might have been abolished in ascidian-specific lineages. Secondly, an ancestral CT/CGRP family peptide might have acted as a non-neural hormone, as Ci-CT does. If this is true, vertebrate CGRPs and BF-CTFPs might have acquired the functions as neuropeptides and CT and the other related peptides might have conserved and diverged their hormonal roles. Thirdly, a CT/CGRP family peptide might have played various roles both as a non-neural hormone and as a neuropeptide in ancestral chordates. If so, specialization of neuropeptidic (for all Bf-CTFPs) and gut hormonal (for Bf-CTFP2 and Bf-CTFP3) functions might have occurred in amphioxus. Further investigation of the physiological roles of the CT-family peptides of other deuterostome invertebrates may provide crucial clues to understand the molecular and functional evolution and diversification of CT/CGRP family peptides.

The innate immune systems in *C. intestinalis*

Innate immunity is a primary defensive system against invading pathogens in invertebrates, in which adaptive immune systems have not been fully developed. Ascidian innate immune systems consist of diverse pathogen recognition systems including hemolymph coagulation, lectin-mediated complement activation, antimicrobial peptides, Toll-like receptors (TLRs) and variable region-containing chitin-binding proteins (VCBPs) (Nonaka 2001; Fujita 2002; Khalturin et al. 2004; Iwanaga and Lee 2005; Rast and Messier-Solek 2008; Fedders et al. 2008; Bonura et al. 2009; Satake and Sasaki 2010; Nonaka and Satake 2010; Dishaw et al. 2011; Liberti et al. 2015). Particularly, TLRs and VCBPs are responsible for innate immunity in the digestive systems. In this section, we provide an essential overview of the *Ciona* VCBPs and TLRs.

VCBPs in *C. intestinalis*

VCBPs have thus far been identified in an amphioxus *B. floridae* and an ascidian *C. intestinalis* (Liberti et al. 2015). In *C. intestinalis*, four subtypes, VCBP-A to -D have been characterized (Dishaw et al. 2011; Liberti et al. 2015). As depicted in Fig. 2, VCBP-A to -C are organized with an N-terminal leader peptide, two V-type immunoglobulin domains and chitin-binding domain (CBD), while VCBP-D lacks the CBD. Various single nucleotide polymorphisms (SNPs) were found in coding regions as well as introns in the gene of VCBP-A to -C (Dishaw et al. 2011). The VCBP-A and -C genes are expressed in the developing and matured digestive tract, suggesting the developmental and immunological role of these VCBPs (Dishaw et al. 2011; Liberti et al. 2015). Moreover, VCBP-B is localized to the digestive tract and blood cells in the adults (Dishaw et al. 2011; Liberti et al. 2015). The expression of VCBP-D remains to be elucidated.

VCBP-C is secreted into the lumen and acts as an opsonin via binding to various bacteria (Dishaw et al. 2011). Furthermore, VCBP-C plays a vital role in the formation of gut biofilms via interaction with the *Ciona* endogenous chitin and bacteria, leading to co-localization of bacteria with chitin-rich mucus matrix (Dishaw et al. 2016). In addition, a growing body of reports has identified microbes, including bacteriophage and viruses, in the *C. intestinalis* gut (Leigh et al. 2017; Leigh et al. 2018) and has verified the roles of the chitin-rich mucus in protection against colitis (Nakashima et al. 2018; Liberti et al. 2018). Accumulation of the findings as to such pathological and microbiological studies is expected to contribute a great deal to the verification of the net molecular mechanism underlying the maintenance of the host-microbe interaction and innate immunity systems in *C. intestinalis*.

TLRs of *C. intestinalis*

TLR was originally identified as a mammalian ortholog of a *Drosophila melanogaster* transmembrane protein, Toll, which plays a central role in antifungal defense and dorsal/ventral pattern determination (Lemaitre et al. 1996; Hoffmann 2003). TLRs are type I transmembrane proteins and consist of an intracellular Toll/Interleukin-1 receptor (TIR) domain and leucine rich repeat (LRR) motifs in the extracellular domain (Takeda and Akira 2005; Dunne and O'Neill 2005). TLRs have key roles in the innate immune system in vertebrates. LRRs exhibit specific pathogenic ligand recognition

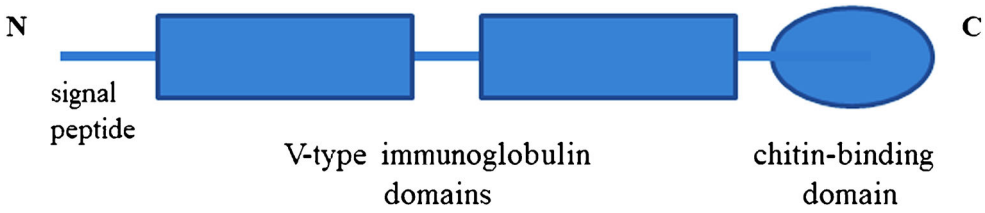

Fig. 2 Structural organization of VCBPs. "N" and "C" indicate the N and C terminus, respectively.

and TIR participates in activation of the following signaling pathways. To date, ten TLRs have been identified in human (Takeda and Akira 2005; Dunne and O'Neill 2005). As summarized in Table 7, each human TLR has a unique LRR structural organization and binds to its specific pathogen-associated molecular patterns (PAMPs). The diversity in the numbers and organization of LRR domains is believed to enable the specific and sensitive recognition of PAMPs by each TLR (Takeda and Akira 2005; Dunne and O'Neill 2005). TLR1, TLR2, TLR4, TLR5 and TLR6 are present on plasma membranes and recognize extracellular microbial pathogenic components, whereas TLR3, TLR7, TLR8 and TLR9 are localized to endosomes and are responsive to viral DNA or RNA incorporated into the cytoplasm (Takeda and Akira 2005; Dunne and O'Neill 2005). These findings substantiate that TLRs are categorized into the plasma membrane TLR group (TLR1, -2, -4, -5 and -6) and endosome TLR group (TLR3, -7, -8 and -9) in light of their intracellular localization. The tertiary structures of several TLR-ligand complexes have also been reported (Liu et al. 2008; Park et al. 2009). Binding of TLRs to their specific PAMPs stimulates signal transduction pathways for the upregulation of a variety of inducible transcriptional factors such as NF-κB, AP-1 and IRF via adaptor proteins (MyD88, TIRAP, TRIF and TRAM), eventually initiating production of inflammatory cytokines such as tumor-necrosis factor (TNF)-α, chemokines and/or type I interferon (Takeda and Akira 2005; Dunne and O'Neill 2005). TLRs are expressed not only in immune cells such as lymphocytes, macrophages and dendritic cells but also the lung, kidney, small intestine, stomach and testis (Takeda and Akira 2005; Dunne and O'Neill 2005). These findings indicate that TLRs exhibit innate immunity in various tissues including the digestive system. In non-human vertebrates, 10–22 TLRs have been identified (Tassia et al. 2017), although their PAMPs or immunological roles have been poorly investigated. TLR-related structures have also been detected in non-vertebrate deuterostomes, such as *C. intestinalis*, *Branchiostoma floridae* (amphioxus) and *Strongylocentrotus purpuratus* (sea urchin). Genomic analysis of the three organisms verified that 2, 72 and 253 putative TLR genes are encoded in the genome of *C. intestinalis*, *B. floridae* and *S. purpuratus* (Hibino et al. 2006; Rast et al. 2006; Huang et al. 2008; Sasaki et al. 2009; Tassia et al. 2017), highlighting high diversity in the number of putative TLR genes among deuterostome invertebrates. It was recently presumed that TLRs and the canonical signaling pathways might have been acquired in common deuterostome ancestors and have diverged into the respective phyletic lineages (Tassia et al. 2017; Ji et al. 2018), although the biochemical and immunological functions of TLRs or putative TLRs of non-mammalian deuterostomes, except for a few fish and *C. intestinalis*, have yet to be investigated.

The presence of only two *Ciona* TLR (Ci-TLRs) suggested that *Ciona* is a relevant model for the evolution of the biological and immunological functions of deuterostome TLRs as well as the biological roles of TLRs in *C. intestinalis*. Two *Ciona* TLRs, Ci-TLR1 and -2 have thus far been identified (Sasaki et al. 2009). Ci-TLR1 and Ci-TLR2 possess a TIR, transmembrane and LRR domain, which is typical of TLRs, whereas 7 and 13 LRRs are encoded in Ci-TLR1 and Ci-TLR2, respectively (Fig. 3). The *ci-tlr1* and *ci-tlr2* genes were expressed intensively in the stomach, intestine and numerous hemocytes and, to a lesser degree, the central nervous system (Sasaki et al. 2009). These findings indicate that Ci-TLRs function mainly in the digestive system and hemocytes. Of note, both Ci-TLRs were present on both the plasma membrane and a number of late endosomes, which is contrast with localization of mammalian TLRs to plasma membranes or endosomes (Sasaki et al. 2009). Furthermore, Ci-TLR1 and Ci-TLR2 transfected into HEK293MSR cells and activated a typical NF-κB signaling in response to multiple TLR ligands (Table 7), which are recognized by different mammalian TLRs: zymosan (*Saccharomyces cerevisiae* cell wall) for TLR2, heat-killed *Legionella pneumophila* (HKLP, a Gram-negative bacterium) for TLR2, double-stranded RNA, poly(I:C) for TLR3 and *Salmonella typhimurium* Flagellin (the major component of the bacterial flagellar filament) for TLR5, showed an induction of transcriptional induction by NF-κB in the *ci-tlr1*- or *ci-tlr2*-expressing cells (Sasaki et al. 2009). Poly(I:C) and flagellin also triggered Ci-TNFα expression in the stomach and anterior and/or middle intestine in which *ci-tlr1* or *ci-tlr2* were abundantly expressed but not in the posterior intestine where no expression of *ci-tlr*s was observed (Sasaki et al. 2009). These studies shed light on the common and unique propensities of Ci-TLRs, compared with other deuterostome TLRs. Firstly, Ci-TLRs, like vertebrate TLRs, directly recognize PAMPs and trigger the induction of NF-κB, which is atypical of *Drosophila* and other protostome Tolls (Hoffmann 2003). In other words, *C. intestinalis* shares the TLR-directed PAMP recognition and signaling

Table 7 Structural organization, PAMPs and intracellular localization of hTLRs and Ci-TLRs

TLR	Number of LRR	PAMPs	Intracellular localization
Ci-TLR1	*7*	*Zymosan (yeast cell wall)*	*Plasma membrane and endosome*
Ci-TLR2	*13*	*Heat-killed Legionella pneumophila (HKLP, Gram-negative)* *Double-stranded-RNA (poly(I:C))* *Flagellin (bacterial flagellar filament)*	
hTLR1 (with TLR2)	5	Triacylated lipoprotein	Plasma membrane
hTLR2	9	Zymosan (yeast cell wall) 1, 3-β-glucan Lipoarabinomannan	Plasma membrane
		Heat-killed *Legionella pneumophila* (HKLP, Gram-negative) Heat-killed *Staphylococcu aereus* (HKSA, Gram-positive)	Plasma membrane
		Glycosylphosphatidylinositol (GPI)-anchored glycoprotein	
hTLR3	17	Double-stranded-RNA (poly(I:C))	Endosome
hTLR4 (with MD2)	11	lipopolysaccharide from Gram-negative bacteria (LPS) Lipid A (lipid component of LPS)	Plasma membrane
hTLR5	10	Flagellin (bacterial flagellar filament)	Plasma membrane
hTLR6	6	MALP-2 (micoplasma-derived macrophage-activating lipopeptide) Micoplasma-derived lipoprotein (FSL1)	Plasma membrane
hTLR7	14	Imidazoquimod (imidazoquinolone amino acid analog)	Endosome
hTLR8	16	Single-stranded RNA	Endosome
hTLR9	19	Unmethylated CpG DNA	Endosome

Ciona TLRs and their LRR numbers, PAMPs, and intracellular localization are described in italic

mechanisms with vertebrates. Secondly, Ci-TLRs respond to multiple PAMPs of respective hTLRs. The PAMP recognition by Ci-TLRs is in good agreement with intracellular localization to both the plasma membrane and endosome, given that poly(I:C) is recognized by hTLR3 present on the endosome. Altogether, these multiple PAMP recognitions and intracellular localization provide evidence that Ci-TLRs are functionally hybrid TLRs of vertebrate cell-surface TLRs (hTLR1, 2, 4, 5, 6) and endosome TLRs (hTLR3, 7, 8, 9). Thirdly, such functions of Ci-TLRs are not predicted on the basis of sequence homology to hTLRs and X-ray tertiary structures of hTLRs, leading to the unique location in the molecular phylogenetic trees of chordate TLRs and their candidates. It is noteworthy that both Ci-TLRs exhibited equipotent NF-κB induction in response to the same ligands, whereas Ci-TLRs possess a different structural organization of LRRs (Fig. 3, Table 7). These findings suggest that recognition of PAMPs and the resultant signaling of deuterostome invertebrate TLRs including Ci-TLRs cannot be elucidated or even predicted by comparison of primary sequences or tertiary structures.

Evolutionary processes of TLRs in deuterostomes

Amphioxus and sea urchin possess a great number of TLRs or their candidate genes: 36–72 amphioxus genes (Huang et al. 2008) and 253 sea urchin genes (Hibino et al. 2006; Rast et al. 2006; Tassia et al. 2017; Ji et al. 2018). Furthermore, the molecular phylogenetic analyses demonstrated that most of these genes were generated via species-specific gene duplication, suggesting that these deuterostome invertebrates expand TLRs or their related genes in unique lineages of innate immunity, if most of the genes are functional (Hibino et al. 2006; Rast et al. 2006; Tassia et al. 2017; Ji et al. 2018). Recently, deuterostome invertebrate TLRs were presumed to have originated from

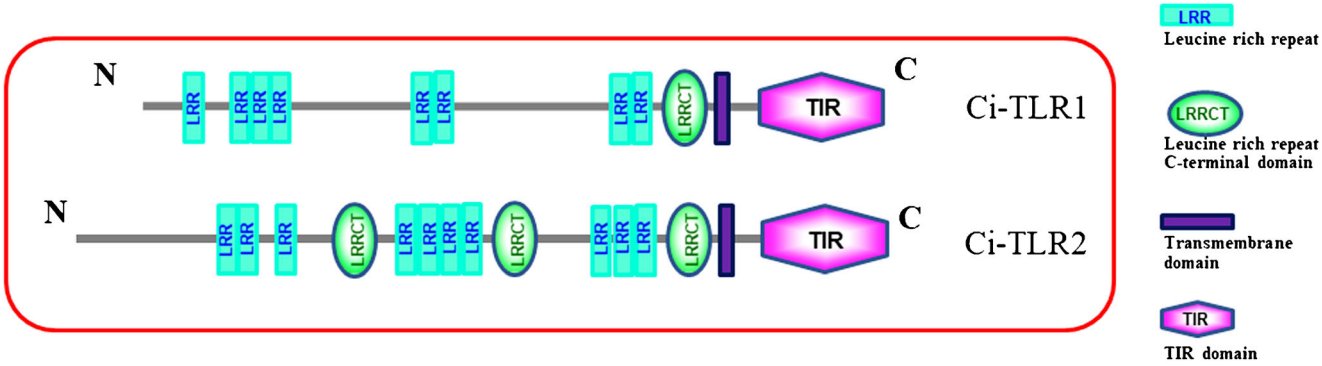

Fig. 3 Structural organization of Ci-TLRs. "N" and "C" indicate the N and C terminus, respectively.

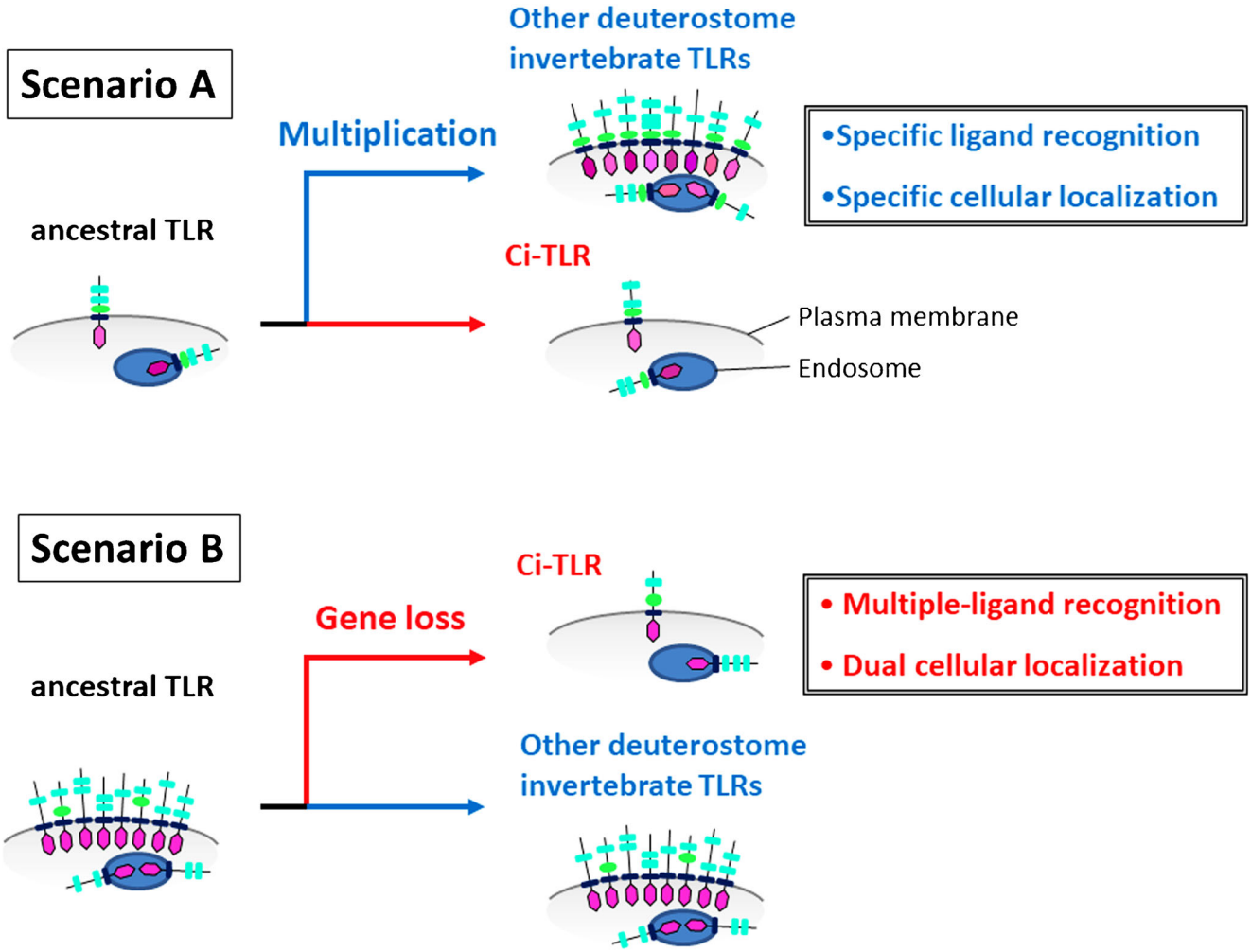

Fig. 4 Possible evolutionary scenarios of deuterostome invertebrate TLRs. **a** A few TLR ancestral genes might have existed on the cell surface and on endosomes in a common deuterostome antecedent and, like Ci-TLR, might have responded to multiple PAMPs that are differentially recognized by respective currently existing vertebrate TLRs. In this case, *C. intestinalis* conserves the ancestral characteristics, whereas sea urchins, amphioxus and vertebrates expanded the variation of their TLRs. Non-ascidian deuterostome TLRs might have acquired their specific PAMP recognition and intracellular localization during their evolution. **b** Numerous TLR family genes might have been developed in a common deuterostome antecedent. *C. intestinalis* should have abolished a large part of ancestral TLR genes during the evolutionary process and have developed multiple PAMP recognition and intracellular localization of Ci-TLRs in the species-specific lineage

mammalian TLR3-type ancestors (Tassia et al. 2017). On the other hand, another deuterostome invertebrate most phyletically close to vertebrates, *C. intestinalis*, possesses only two authentic TLRs (Sasaki et al. 2009). These findings lead to the presumption of two evolutionary processes of the TLR family (Fig. 4) (Satake and Sekiguchi 2012). First, a few TLR genes might have been originated in a common deuterostome antecedent and might have recognized multiple PAMPs both on the cell surface and on endosomes. If this is true, sea urchins and amphioxus expanded their TLR or their related genes, while *C. intestinalis* conserved the ancestral characteristics. This presumption is also compatible with the aforementioned molecular phylogenetic analyses of sea urchin and amphioxus TLR or their related genes. Consistently, vertebrate TLRs might have acquired their specific PAMP recognition and intracellular localization of their evolutionary lineages (Satake and Sekiguchi 2012). Alternatively, a deuterostome antecedent might have possessed multiple TLR family genes. In this case, *C. intestinalis* should have lost a large part of ancestral TLR family genes and have innovatively acquired Ci-TLRs that recognize multiple PAMPs (Satake and Sekiguchi 2012). Collectively, functional characterization of PAMPs and intracellular localization of sea urchin and amphioxus TLRs will provide crucial clues to understanding their immunological roles and evolutionary pathways of functions of deuterostome TLRs family.

Conclusion remarks

As stated above, major players in neuropeptidergic regulation and innate immunity in the digestive systems of ascidians

have been identified over the past decade. These findings will contribute a great deal to the research into the physiology and immunology of urochordates and the evolutionary processes of these systems in chordates. In vertebrates, various neuropeptides stimulate contraction of smooth muscles of the intestine, enhance the secretion of digestive enzymes and regulate the enteric nervous system. However, ascidians lack smooth muscles of the intestine and enteric neurons, suggesting that neuropeptides mainly participate in food digestion, nutrient uptake and cell metabolism. Ascidian innate immune systems, unlike vertebrate ones, have not been investigated in vivo. The localization of neuropeptide receptors and immunity-related proteins will lead to the elucidation of the cells that play major roles in enteric functions and innate immunity in the ascidian digestive systems. Furthermore, development of tempo-spatial or tissue-specific gene knockdown methods would dramatically facilitate the investigation of the functions of neuropeptides, VCBPs and TLRs in the digestive system and the molecular and functional evolutionary processes of the digestive systems throughout chordates.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Adams BA, Tello JA, Erchegyi J, Warby C, Hong DJ, Akinsanya KO, Mackie GO, Vale W, Rivier JE, Sherwood NM (2003) Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, *Ciona intestinalis*. Endocrinology 144:1907–1919

Aikins MJ, Schooley DA, Begum K, Detheux M, Beeman RW, Park Y (2008) Vasopressin-like peptide and its receptor function in an indirect diuretic signaling pathway in the red flour beetle. Insect Biochem Mol Biol 38:740–748

Aoyama M, Kawada T, Fujie M, Hotta K, Sakai T, Sekiguchi T, Oka K, Satoh N, Satake H (2008) A novel biological role of tachykinins as an up-regulator of oocyte growth: identification of an evolutionary origin of tachykininergic functions in the ovary of the ascidian, *Ciona intestinalis*. Endocrinology 149:4346–4356

Aoyama M, Kawada T, Satake H (2012) Localization and enzymatic activity profiles of the proteases responsible for tachykinin-directed oocyte growth in the protochordate, *Ciona intestinalis*. Peptides 34:186–192

Beets I, Janssen T, Meelkop E, Temmerman L, Suetens N, Rademakers S, Jansen G, Schoofs L (2012) Vasopressin/oxytocin-related signaling regulates gustatory associative learning in *C. elegans*. Science 338: 543–545

Biran J, Palevitch O, Ben-Dor S, Levavi-Sivan B (2012) Neurokinin Bs and neurokinin B receptors inzebrafish-potential role in controlling fish reproduction. Proc Natl Acad Sci U S A 109:10269–10274

Bonura A, Vizzini A, Salerno G, Parrinello N, Longo V, Colombo P (2009) Isolation and expression of a novel MBL-like collectin cDNA enhanced by LPS injection in the body wall of the ascidian *Ciona intestinalis*. Mol Immunol 46:2389–2394

Cai W, Kim CH, Go HJ, Egertová M, Zampronio CG, Jones AM, Park NG, Elphick MR (2018) Biochemical, anatomical, and pharmacological characterization of calcitonin-type neuropeptides in starfish: discovery of an ancient role as muscle relaxants. Front Neurosci 12:382

Conner AC, Simms J, Hay DL, Mahmoud K, Howitt SG, Wheatley M, Poyner DR (2004) Heterodimers and family-B GPCRs: RAMPs, CGRP and adrenomedullin. Biochem Soc Trans 32:843–846

Dickinson PS, Stevens JS, Rus S, Brennan HR, Goiney CC, Smith CM, Li L, TowleDW, Christie AE (2007) Identification and cardiotropic actions of sulfakinin peptidesin the American lobster Homarus americanus. J Exp Biol 210:2278–2289

Dishaw LJ, Giacomelli S, Melillo D, Zucchetti I, Haire RN, Natale L, Russo NA, De Santis R, Litman GW, Pinto MR (2011) A role for variable region-containing chitin-binding proteins (VCBPs) in host gut-bacteria interactions. Proc Natl Acad Sci U S A 108:16747–16752

Dishaw LJ, Leigh B, Cannon JP, Liberti A, Mueller MG, Skapura DP, Karrer CR, Pinto MR, De Santis R, Litman GW (2016) Gut immunity in a protochordate involves a secreted immunoglobulin-type mediator binding host chitin and bacteria. Nat Commun 7:10617

Dunne A, O'Neill LA (2005) Adaptor usage and Toll-like receptor signaling specificity. FEBS Lett 579:3330–3335

Elphick MR, Rowe ML (2009) NGFFFamide and echinotocin: structurally unrelated myoactive neuropeptides derived from neurophysin-containing precursors in sea urchins. J Exp Biol 212:1067–1077

Fedders H, Michalek M, Grötzinger J, Leippe M (2008) An exceptional salt-tolerant antimicrobial peptide derived from a novel gene family of haemocytes of the marine invertebrate *Ciona intestinalis*. Biochem J 416:65–75

Frank E, Landgraf R (2008) The vasopressin system–from antidiuresis to psychopathology. Eur J Pharmacol 583:226–242

Fujita T (2002) Evolution of the lectin-complement pathway and its role in innate immunity. Nat Rev Immunol 2:346–353

Gimpl G, Fahrenholz F (2001) The oxytocin receptor system: structure, function, and regulation. Physiol Rev 81:629–683

Hasunuma I, Terakado K (2013) Two novel gonadotropin-releasing hormones (GnRHs) from the urochordate ascidian, *Halocynthia roretzi*: implications for the origin of vertebrate GnRH isoforms. Zool Sci 30:311–318

Hibino T, Loza-Coll M, Messier C, Majeske AJ, Cohen AH, Terwilliger DP, Buckley KM, Brockton V, Nair SV, Berney K, Fugmann SD, Anderson MK, Pancer Z, Cameron RA, Smith LC, Rast JP (2006) The immune gene repertoire encoded in the purple sea urchin genome. Dev Biol 300:349–365

Hoffmann JA (2003) The immune response of *Drosophila*. Nature 426: 33–38

Hoyle CHV (1998) Neuropeptide families: evolutionary perspectives. Regul Pept 73:1–33

Huang S, Yuan S, Guo L, Yu Y, Li J, Wu T, Liu T, Yang M, Wu K, Liu H, Ge J, Yu Y, Huang H, Dong M, Yu C, Chen S, Xu A (2008) Genomic analysis of the immune gene repertoire of amphioxus reveals extraordinary innate complexity and diversity. Genome Res 18:1112–1126

Iwanaga S, Lee BL (2005) Recent advances in the innate immunity of invertebrate animals. J Biochem Mol Biol 38:128–150

Janssen T, Meelkop E, Lindemans M, Verstraelen K, Husson SJ, Temmerman L, Nachman RJ, Schoofs L (2008) Discovery of a

cholecystokinin-gastrin-like signaling system in nematodes. Endocrinology 149:2826–2839

Ji J, Ramos-Vicente D, Navas-Pérez E, Herrera-Úbeda C, Lizcano JM, Garcia-Fernàndez J, Escrivà H, Bayés À, Roher N (2018) Characterization of the TLR family in *Branchiostoma lanceolatum* and discovery of a novel TLR22-like involved in dsRNA recognition in amphioxus. Front Immunol 9:2525

Jiang H, Lkhagva A, Daubnerová I, Chae HS, Šimo L, Jung SH, Yoon YK, Lee NR, Seong JY, Žitňan D, Park Y, Kim YJ (2013) Natalisin, a tachykinin-like signaling system, regulates sexual activity and fecundity in insects. Proc Natl Acad Sci U S A 110:E3526–E3534

Johnsen AH (1998) Phylogeny of the cholecystokinin/gastrin family. Front Neuroendocrinol 19:73–99

Johnsen AH, Rehfeld JF (1990) Cionin: a disulfotyrosyl hybrid of cholecystokinin and gastrin from the neural ganglion of the protochordate *Ciona intestinalis*. J Biol Chem 265:3054–3058

Kah O, Lethimonier C, Somoza G, Guilgur LG, Vaillant C, Lareyre JJ (2007) GnRH and GnRH receptors in metazoa: a historical, comparative, and evolutive perspective. Gen Comp Endocrinol 153: 346–364

Kamiya C, Ohta N, Ogura Y, Yoshida K, Horie T, Kusakabe TG, Satake H, Sasakura Y (2014) Nonreproductive role of gonadotropin-releasing hormone in the control of ascidian metamorphosis. Dev Dyn 243:1524–1535

Kanda A, Takahashi T, Satake H, Minakata H (2006) Molecular and functional characterization of a novel gonadotropin-releasing-hormone receptor isolated from the common octopus (*Octopus vulgaris*). Biochem J 395:125–135

Katafuchi T, Kikumoto K, Hamano K, Kangawa K, Matsuo H, Minamino N (2003) Calcitonin receptor-stimulating peptide, a new member of the calcitonin gene-related peptide family. Its isolation from porcine brain, structure, tissue distribution, and biological activity. J Biol Chem 278:12046–12054

Katafuchi T, Yasue H, Osaki T, Minamino N (2009) Calcitonin receptor-stimulating peptide: its evolutionary and functional relationship with calcitonin/calcitonin gene-related peptide based on gene structure. Peptides 30:1753–1762

Kavanaugh SI, Nozaki M, Sower SA (2008) Origins of gonadotropin-releasing hormone (GnRH) in vertebrates: identification of a novel GnRH in a basal vertebrate, the sea lamprey. Endocrinology 149: 3860–3869

Kawada T, Kanda A, Minakata H, Matsushima O, Satake H (2004) Identification of a novel receptor for an invertebrate oxytocin/vasopressin superfamily peptide: molecular and functional evolution of the oxytocin/vasopressin superfamily. Biochem J 382:231–237

Kawada T, Sekiguchi T, Itoh Y, Ogasawara M, Satake H (2008) Characterization of a novel vasopressin/oxytocin superfamily peptide and its receptor from an ascidian, *Ciona intestinalis*. Peptides 29:1672–1678

Kawada T, Aoyama M, Okada I, Sakai T, Sekiguchi T, Ogasawara M, Satake H (2009a) A novel inhibitory gonadotropin-releasing hormone-related neuropeptide in the ascidian, *Ciona intestinalis*. Peptides 30:2200–2205

Kawada T, Sekiguchi T, Sugase K, Satake H (2009b) Evolutionary aspects of molecular forms and biological functions of oxytocin family peptides. In: Jastrow H, Feuerbach D (eds) Handbook of oxytocin research: synthesis, storage and release, actions and drug forms. Nova Science Publishers, Hauppauge, pp 59–85

Kawada T, Sekiguchi T, Sakai T, Aoyama M, Satake H (2010) Neuropeptides, hormone peptides, and their receptors in *Ciona intestinalis*: an update. Zool Sci 27:134–153

Kawada T, Ogasawara M, Sekiguchi T, Aoyama M, Hotta K, Oka K, Satake H (2011) Peptidomic analysis of the central nervous system of the protochordate, *Ciona intestinalis*: homologs and prototypes of vertebrate peptides and novel peptides. Endocrinology 152:2416–2427

Kawada T, Aoyama M, Sakai T, Satake H (2013) Structure, function, and evolutionary aspects of invertebrate GnRHs and their receptors. In: Scott-Sills E (ed) Gonadotropin-releasing hormone (GnRH): production, structure and functions. Nova Science Publisher Inc., New York, pp 1–16

Khalturin K, Panzer Z, Cooper MD, Bosch TCG (2004) Recognition strategies in the innate immune system of ancestral chordates. Mol Immunol 41:1077–1187

Kusakabe T, Mishima S, Shimada I, Kitajima Y, Tsuda M (2003) Structure, expression, and cluster organization of genes encoding gonadotropin-releasing hormone receptors found in the neural complex of the ascidian *Ciona intestinalis*. Gene 322:77–84

Leigh BA, Karrer C, Cannon JP, Breitbart M, Dishaw LJ (2017) Isolation and characterization of a Shewanella phage-host system from the gut of the tunicate, *Ciona intestinalis*. Viruses 9

Leigh BA, Djurhuus A, Breitbart M, Dishaw LJ (2018) The gut virome of the protochordate model organism, Ciona intestinalis subtype A. Virus Res 244:137–146

Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA (1996) The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell 86: 973–983

Liberti A, Leigh B, De Santis R, Pinto MR, Cannon JP, Dishaw LJ, Litman GW (2015) An immune effector system in the Protochordate gut sheds light on fundamental aspects of vertebrate immunity. In: Hsu E, Du Pasquier L (eds) Pathogen-host interactions: antigenic variation v. somatic adaptations. Results and problems in cell differentiation, vol 57. Springer, Cham, pp 159–173

Liberti A, Zucchetti I, Melillo D, Skapura D, Shibata Y, De Santis R, Pinto MR, Litman GW, Dishaw LJ (2018) Chitin protects the gut epithelial barrier in a protochordate model of DSS-induced colitis. Biol Open 7

Liu L, Botos I, Wang Y, Leonard JN, Shiloach J, Segal DM, Davies DR (2008) Structural basis of toll-like receptor 3 signaling with double-stranded RNA. Science 320:379–381

Matsubara S, Kawada T, Sakai T, Aoyama M, Osugi T, Shiraishi A, Satake H (2016) The significance of Ciona intestinalis as a stem organism in integrative studies of functional evolution of the chordate endocrine, neuroendocrine, and nervous systems. Gen Comp Endocrinol 227:101–108

Millar RP (2005) GnRHs and GnRH receptors. Anim Reprod Sci 88:5–28

Millar RP, Newton CL (2013) Current and future applications of GnRH, kisspeptin and neurokinin B analogues. Nat Rev Endocrinol 9:451–466

Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR (2004) Gonadotropin-releasing hormone receptors. Endocr Rev 25: 235–275

Nakashima K, Kimura S, Ogawa Y, Watanabe S, Soma S, Kaneko T, Yamada L, Sawada H, Tung CH, Lu TM, Yu JK, Villar-Briones A, Kikuchi S, Satoh N (2018) Chitin-based barrier immunity and its loss predated mucus-colonization by indigenous gut microbiota. Nat Commun 9:3402

Nichols R (2003) Signaling pathways and physiological functions of Drosophila melanogaster FMRFamide-related peptides. Annu Rev Entomol 48:485–503

Nilsson IB, Svensson SP, Monstein HJ (2003) Molecular cloning of a putative *Ciona intestinalis* cionin receptor, a new member of the CCK/gastrin receptor family. Gene 323:79–88

Noble F, Roques BP (1999) CCK-B receptor: chemistry, molecular biology, biochemistry andpharmacology. Prog Neurobiol 58:349–379

Nonaka M (2001) Evolution of the complement system. Curr Opin Immunol 13:69–73

Nonaka M, Satake H (2010) Urochordate immunity. Adv Exp Med Biol 708:302–310

Page NM (2006) Characterization of the gene structures, precursor processing and pharmacology of the endokinin peptides. Vasc Pharmacol 45:200–208

Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO (2009) The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature 458:1191–1195

Pommier B, Marie-Claire C, Da Nascimento S, Wang HL, Roques BP, Noble F (2003) Further evidence thatthe CCK2 receptor is coupled to two transduction pathways using site-directed mutagenesis. J Neurochem 85:454–461

Powell JF, Reska-Skinner SM, Prakash MO, Fischer WH, Park M, Rivier JE, Craig AG, Mackie GO, Sherwood NM (1996) Two new forms of gonadotropin-releasing hormone in a protochordate and the evolutionary implications. Proc Natl Acad Sci U S A 93:10461–10464

Rast JP, Messier-Solek C (2008) Marine invertebrate genome sequences and our evolving understanding of animal immunity. Biol Bull 214: 274–283

Rast JP, Smith LC, Loza-Coll M, Hibino T, Litman GW (2006) Genomic insights into the immune system of the sea urchin. Science 314:952–956

Roch GJ, Tello JA, Sherwood NM (2014) At the transition from invertebrates to vertebrates, a novel GnRH-like peptide emerges in amphioxus. Mol Biol Evol 31:765–778

Rowe ML, Elphick MR (2012) The neuropeptide transcriptome of a model echinoderm, the sea urchin *Strongylocentrotus purpuratus*. Gen Comp Endocrinol 179:331–344

Rowe ML, Achhala S, Elphick MR (2014) Neuropeptides and polypeptide hormones in echinoderms: new insights from analysis of the transcriptome of the sea cucumber *Apostichopus japonicus*. Gen Comp Endocrinol 197:43–55

Sakai T, Aoyama M, Kusakabe T, Tsuda M, Satake H (2010) Functional diversity of signaling pathways through G protein-coupled receptor heterodimerization with a species-specific orphan receptor subtype. Mol Biol Evol 27:1097–1106

Sakai T, Aoyama M, Kawada T, Kusakabe T, Tsuda M, Satake H (2012) Evidence for differential regulation of GnRH signaling via heterodimerization among GnRH receptor paralogs in the protochordate, *Ciona intestinalis*. Endocrinology 153:1841–1849

Sakai T, Shiraishi A, Kawada T, Matsubara S, Aoyama M, Satake H (2017) Invertebrate gonadotropin-releasing hormone-related peptides and their receptors: an update. Front Endocrinol (Lausanne) 8:217

Sasaki N, Ogasawara M, Sekiguchi T, Kusumoto S, Satake H (2009) Toll-like receptors of the ascidian, *Ciona intestinalis*: prototypes with hybrid functionalities of vertebrate Toll-like receptors. J Biol Chem 284:27336–27343

Satake H, Kawada T (2006a) Neuropeptides, hormones, and their receptors in ascidians: emerging model animals. In: Satake H (ed) Invertebrate neuropeptides and hormones: basic knowledge and recent advances. Transworld Research Network, Kerala, pp 253–276

Satake H, Kawada T (2006b) Overview of the primary structure, tissue-distribution, and functions of tachykinins and their receptors. Curr Drug Targets 7:963–974

Satake H, Sasaki N (2010) Comparative view on Toll-like receptors of lower animals. Zool Sci 27:154–161

Satake H, Sekiguchi T (2012) Toll-like receptors of deuterostome invertebrates. Front Immunol 3:34

Satake H, Takuwa K, Minakata H, Matsushima O (1999) Evidence for conservation of the vasopressin/oxytocin superfamily in Annelida. J Biol Chem 274:5605–5611

Satake H, Kawada T, Nomoto K, Minakata H (2003) Insight into tachykinin-related peptides, their receptors, and invertebrate tachykinins: a review. Zool Sci 20:533–549

Satake H, Ogasawara M, Kawada T, Masuda K, Aoyama M, Minakata H, Chiba T, Metoki H, Satou Y, Satoh N (2004) Tachykinin and tachykinin receptor of an ascidian, *Ciona intestinalis*: evolutionary origin of the vertebrate tachykinin family. J Biol Chem 279:53798–53805

Satake H, Matsubara S, Aoyama M, Kawada T, Sakai T (2013) GPCR Heterodimerization in the reproductive system: functional regulation and implication for biodiversity. Front Endocrinol (Lausanne) 4:100

Satoh N (2015) Two decades of ascidian developmental biology: a personal research story. Curr Top Dev Biol 117:289–300

Sekiguchi T, Suzuki N, Fujiwara N, Aoyama M, Kawada T, Sugase K, Murata Y, Sasayama Y, Ogasawara M, Satake H (2009) Calcitonin in a protochordate, *Ciona intestinalis*-the prototype of the vertebrate calcitonin/calcitonin gene-related peptide superfamily. FEBS J 276: 4437–4447

Sekiguchi T, Ogasawara M, Satake H (2012) Molecular and functional characterization of cionin receptors in the ascidian, *Ciona intestinalis*: the evolutionary origin of the vertebrate cholecystokinin/gastrin family. J Endocrinol 213:99–106

Sekiguchi T, Kuwasako K, Ogasawara M, Takahashi H, Matsubara S, Osugi T, Muramatsu I, Sasayama Y, Suzuki N, Satake H (2016) Evidence for conservation of the calcitonin superfamily and activity-regulating mechanisms in the basal chordate Branchiostoma floridae: insights into the molecular and functional evolution in chordates. J Biol Chem 291:2345–2356

Sekiguchi T, Shiraishi A, Satake H, Kuwasako K, Takahashi H, Sato M, Urata M, Wada S, Endo M, Ikari T, Hattori A, Srivastav AK, Suzuki N (2017) Calcitonin-typical suppression of osteoclastic activity by amphioxus calcitonin superfamily peptides and insights into the evolutionary conservation and diversity of their structures. Gen Comp Endocrinol 246:294–300

Semmens DC, Mirabeau O, Moghul I, Pancholi MR, Wurm Y, Elphick MR (2016) Transcriptomic identification of starfish neuropeptide precursors yields new insights into neuropeptide evolution. Open Biol 6:150224

Stafflinger E, Hansen KK, Hauser F, Schneider M, Cazzamali G, Williamson M, Grimmelikhuijzen CJ (2008) Cloning and identification of an oxytocin/vasopressin-like receptor and its ligand from insects. Proc Natl Acad Sci U S A 105:3262–3267

Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW (2014) Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. Physiol Rev 94:265–301

Suwansa-Ard S, Chaiyamoon A, Talarovicova A, Tinikul R, Tinikul Y, Poomtong T, Elphick MR, Cummins SF, Sobhon P (2018) Transcriptomic discovery and comparative analysis of neuropeptide precursors in sea cucumbers (Holothuroidea). Peptides 99:231–240

Takeda K, Akira S (2005) Toll-like receptors in innate immunity. Int Immunol 17:1–14

Tassia MG, Whelan NV, Halanych KM (2017) Toll-like receptor pathway evolution in deuterostomes. Proc Natl Acad Sci U S A 114:7055–7060

Tello JA, Sherwood NM (2009) Amphioxus: beginning of vertebrate and end of invertebrate type GnRH receptor lineage. Endocrinology 150:2847–2856

Tello JA, Rivier JE, Sherwood NM (2005) Tunicate gonadotropin-releasing hormone (GnRH) peptides selectively activate *Ciona intestinalis* GnRH receptors and the green monkey type II GnRH receptor. Endocrinology 146:4061–4073

Tian S, Zandawala M, Beets I, Baytemur E, Slade SE, Scrivens JH, Elphick MR (2016) Urbilaterian origin of paralogous GnRH and corazonin neuropeptide signaling pathways. Sci Rep 6:28788

Ukena K, Iwakoshi-Ukena E, Hikosaka A (2008) Unique form and osmoregulatory function of a neurohypophysial hormone in a urochordate. Endocrinology 149:5254–5261