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Chapter I

Structure, Function, and Evolutionary Aspects of Invertebrate GnRHs and their Receptors

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Abstract

Gonadotropin-releasing hormones (GnRHs) play a pivotal role in the control of reproduction via the hypothalamic-pituitary-gonad (HPG) axis in vertebrates. Furthermore, GnRHs are involved in diverse neuroendocrine, paracrine, autocrine, and neurotransmitter/neuromodulatory functions in the central and peripheral nervous systems, and a wide range of peripheral tissues. While GnRH signaling is largely dependent on closed circulatory systems, GnRHs have also been identified in a variety of invertebrates including ascidians, an echinoderm, annelids, and mollusks, which do not have a closed circulation systems. Of note, these invertebrate GnRHs show higher molecular diversity than chordate orthologs. For example, two amino acids are inserted after pyro-Glu^{1}, and the C-terminal Gly^{10} is lost in several protostome GnRHs. In the ascidian *Ciona intestinalis*, multiple GnRHs and GnRH receptors (GnRHRs) have been identified, and these enhance signal transduction cascades with various ligand specificities. Moreover, a *Ciona* GnRH-related peptide (Ci-GnRH-X) exhibits moderate inhibitory activity at *Ciona* GnRH receptors (Ci-GnRHRs), and heterodimerization among Ci-GnRHR paralogs is involved in species-specific regulation of Ci-GnRHR signaling. These findings, combined with the fact that acquisition of the HPG axis was a seminal event in vertebrate evolution and there has been no pituitary gland development

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in invertebrates, suggest that GnRHs might have occurred in a common ancestor of vertebrates and invertebrates. In this model, invertebrate GnRHs would act not as a "gonadotropin-releasing hormone" but as a direct regulatory factor for various central and peripheral tissues. This chapter focuses on the molecular and functional features of the invertebrate GnRHs and their receptors, highlighting both the conservation and diversity of GnRHs between vertebrates and invertebrates.

Introduction

Ovarian functions, including the growth and maturation of oocytes and follicles, are believed to involve coordinated and multistep biological events that undergo functional regulation by a wide range of endogenous factors. Since Schally & Guillemin were awarded the Nobel Prize in Medicine or Physiology in 1977 for their discovery of gonadotropin-releasing hormone (GnRH), this peptide hormone has occupied a central position in endocrinology and reproductive biology. In vertebrates, GnRH plays a crucial role in regulating the hypothalamic-pituitary-gonadal (HPG) axis to control reproduction by releasing gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. Over the past decade, GnRH and its related peptides have been identified in the central nervous system of many invertebrates such as ascidians, an echinoderm, annelids, and mollusks. Invertebrate GnRHs have two functional "contradictions" considering their gonadotropin-releasing roles. First, most invertebrates have an open circulation system, unlike vertebrates which possess complete (closed) circulatory systems. Thus, invertebrate GnRHs cannot serve as hormones in the HPG axis but rather function as neuropeptides. Moreover, neither pituitary glands nor orthologs of gonadotropin hormones have been identified in invertebrates. In contrast, GnRH receptors are expressed in the ovary of several invertebrates, suggesting that invertebrate GnRHs are also involved in the regulation of ovarian functions in a non-endocrine manner. In this chapter, we provide current knowledge regarding primary sequences, signaling cascades, and physiological activities, and also insight into the biological roles and evolutionary processes of invertebrate GnRHs and their receptors.

Invertebrate GnRHs

Vertebrate GnRHs are composed of 10 amino acids with consensus sequences of pyro-Glu^{1}-His^{2}-Trp^{3}-Ser^{4} and Pro^{9}-Gly^{10}-amide (Millar et al., 2008). As shown in Table 1, two types of GnRH (GnRH-I and –II) have been characterized in vertebrates, whereas GnRH-III was found exclusively in teleosts (Millar et al., 2004; Millar 2005). Three GnRHs (l-GnRH-I to –III), have been identified in the sea lamprey, *Petromyzon marinu*s, which is a basal vertebrate (Kavanaugh et al., 2008). Phylogenic analyses of GnRH precursors suggested that l-GnRH-I and –III are classified as a novel GnRH type, GnRH-VI (Kavanaugh et al., 2008). Furthermore, l-GnRH-II was not categorized into any group, although the precursor sequences of GnRH-I to –III are closer to that of l-GnRH-II than of l-GnRH-I and –III (Kavanaugh et al., 2008). In addition, the amino acid sequence of l-GnRH-II differs from that of gnathostome GnRH-II by only one amino acid substitution at position 8 (Kavanaugh et al.,

2008). These results lead to the presumption that l-GnRH-II might have initially evolved from the ancestral GnRH gene, and thereafter l-GnRH-I and –III might have been generated by gene duplications.

Table 1. Amino acid sequences of GnRHs

Vertebrate GnRHs			
Human	*Homo sapiens*	GnRH-I	pQ--HWSYGLRPGa
		GnRH-II	pQ--HWSHGWYPGa
Guinia pig	*Cavia porcellus*	GnRH-I	pQ--HWSYGVRPGa
Trout	*Oncorhynchus mykiss*	GnRH-III	pQ--HWSYGWLPGa
Lamprey	*Petromyzon marinus*	l-GnRH-I	pQ—-HYSLEWKPGa
		l-GnRH-II	pQ—-HWSHGWFPGa
		l-GnRH-III	pQ--HWSHDWKPGa
Deuterostome invertebrate GnRHs			
Tunicate	*Chelyosoma productum*	t-GnRH-1	pQ--HWSYGLRPGa
		t-GnRH-2	pQ--HWSLCHAPGa
	Ciona intestinalis	t-GnRH-3	pQ--HWSYEFMPGa
		t-GnRH-4	pQ--HWSNQLTPGa
		t-GnRH-5	pQ--HWSYEYMPGa
		t-GnRH-6	pQ--HWSKGYSPGa
		t-GnRH-7	pQ--HWSYALSPGa
		t-GnRH-8	pQ--HWSLALSPGa
		Ci-GnRH-X*	pQ--HWSNWWIPGAP GYNGa
	Ciona savignyi	t-GnRH-5	pQ--HWSYEYMPGa
		t-GnRH-6	pQ--HWSKGYSPGa
		t-GnRH-7	pQ--HWSYALSPGa
		t-GnRH-8	pQ--HWSLALSPGa
		t-GnRH-9	pQ--HWSNKLAPGa
Sea urchin	*Strongylocentrotus purpuratus*	spGnRHP	pQVHHRFSGWRPGa
Protostome GnRHs			
Octopus	*Octopus vulgaris*	Oct-GnRH	pQNYHFSNGWHPGa
Cuttlefish	*Sepia officinalis*	Oct-GnRH	pQNYHFSNGWHPGa
Swordtip squid	*Loligo edulis*	Oct-GnRH	pQNYHFSNGWHPGa
Pacific oyster	*Crassostrea gigas*	CgGnRH	pQNYHFSNGWQPa
Yesso scallop	*Patinopecten yessoensis*	py-GnRH	pQNFHYSNGWQPa
Sea hare	*Aplysia californica*	ap-GnRH	pQNYHFSNGWYAa
Owl limpet	*Lottia gigantean*	GnRH-like	pQHYHFSNGWKSa
Marine worm	*Capitella teleta*	Ca-GnRH	pQAYHFSHGWFPa
Leech	*Helobdella robusta*	le-GnRH	pQSIHFSRSWQPa
Protostome AKHs			
Fruitfly	*Drosophila melanogaster*	Dm-AKH	pQLTFSPDWa
Nematode	*Caenorhabditis elegans*	Ce-AKH	pQMTFTDQWT
Sea hare	*Aplysia californica*	ap-AKH	pQIHFSPDWGTa

Notes: The N-terminal pyroglutamic acid and C-terminal amide are shown by “pQ” and “a”, respectively. An asterisk indicates a GnRH-related peptide, Ci-GnRH-X, which has no agonistic activity for ascidian GnRH receptors.

GnRH family peptides have also been characterized from extensive invertebrate species, including mollusks, annelids, an echinoderm, and protochordates (Table 1). The first invertebrate GnRHs, named as t-GnRH-1 and -2, were isolated from the neural extract of an ascidian, *Chelyosoma productum* (Powell et al., 1996). Subsequently, further protochordate GnRHs were also identified from other ascidians, *Ciona intestinalis* and *Ciona savignyi* (Adams et al., 2003). The former ascidian produces t-GnRH-3 to -8, and the latter generates t-GnRH-5 to -9 (Adams et al., 2003). All ascidian GnRHs contain the consensus sequences of pyro-Glu-His-Trp-Ser and Pro-Gly-amide (see Table 1), indicating that GnRH consensus sequences are highly conserved in chordates. One exception is a unique GnRH-related peptide, Ci-GnRH-X, which was identified from the neural tissue of *Ciona intestinalis*, and conserves the N-terminal sequence and C-terminal Gly-amide (Kawada et al., 2009). Unlike vertebrate and other ascidian GnRHs, Ci-GnRH-X is composed of 16 amino acids and lacks the common Pro at position 2 from the C-terminus (see Table 1). It is intriguing that the incomplete GnRH-related peptide exerts not agonistic activity but antagonisitic activity for *Ciona* GnRH receptors (Kawada et al., 2009).

Recently, a novel GnRH sequence was found by a genomic database search of the sea urchin, *Strongylocentrotus purpuratus* (Rowe & Elphick, 2012). Although all chordate GnRHs are composed of 10 amino acids, the sea urchin GnRH, named spGnRHP, is a 12-residue peptide containing a Pro-Gly-amide at the C-terminus, (see Table 1). Interestingly, a Val-His sequence is inserted after pyro-Glu in spGnRHP (see Table 1), resulting in generation of the 12-residue peptide. It is noteworthy that the insertion of two amino acids occurs not only in the spGnRHP but also in all protostome GnRHs (see Table 1). Therefore, it is suggested that the two amino acids after pyro-Glu were lost from the ancestral GnRH during the chordate evolutionary process (see Figure 1), instead of being inserted in the ancestral GnRH during the invertebrate evolutionary process. In other words, ancestral GnRH might have harbored the two amino acids after position 1, unlike chordate GnRHs (see Figure 1). In addition, the Arg-Phe sequence is present in spGnRHP, rather than Trp-Ser in the N-terminal consensus sequence (see Table 1). Since the Arg-Phe sequence is absent in both vertebrate and protostome GnRHs, the substitution was likely specific to the sea urchin.

Studies on protostome neuropeptides have shown the presence of GnRHs in several mollusks and annelids (see Table 1); an octopus, *Octopus vulgaris* (Iwakoshi et al., 2002), a cuttlefish, *Sepia officinalis* (Di Cristo et al., 2009), a swordtip squid, *Loligo edulis* (Onitsuka et al., 2009), a pacific oyster, *Crassostrea gigas* (Bigot et al., 2012), a yesso scallop, *Patinopecten yessoensis* (Treen et al., 2012), a sea hare, *Aplysia californica* (Zhang et al., 2008), an owl limpet, *Lottia gigantean* (Veenstra, 2010), a marine worm, *Capitella teleta* (Veenstra, 2011), and a leech, *Helobdella robusta* (Roch et al., 2011; Sun et al., 2012). However, protostome GnRHs fail to completely conserve in the vertebrate GnRH consensus sequences. The most outstanding characteristic in protostomes GnRHs is the two amino acid insertion after position 1 (see Table 1). Furthermore, Trp^3 in the N-terminal consensus motif is substituted with Phe in protostome GnRHs (see Table 1). Of particular interest is that the C-terminal Pro-Gly-amide is replaced with Pro-amide in most protostome GnRHs (Table 1).

These observed differences in GnRH C-termini have led to two evolutionary hypotheses. First, the Gly was lost from the ancestral GnRH during the protostome evolutionary process (see Figure 1A). Second, Gly was inserted in the ancestral GnRH during the deuterostome evolutionary process (see Figure 1B). Further identification of invertebrate GnRHs is required to address this question. Interestingly, an octopus GnRH, named oct-GnRH, possesses the

Pro-Gly-amide at the C-terminus (Iwakoshi et al., 2002), although the octopus belongs to the protostome family (*Cephalopoda*).

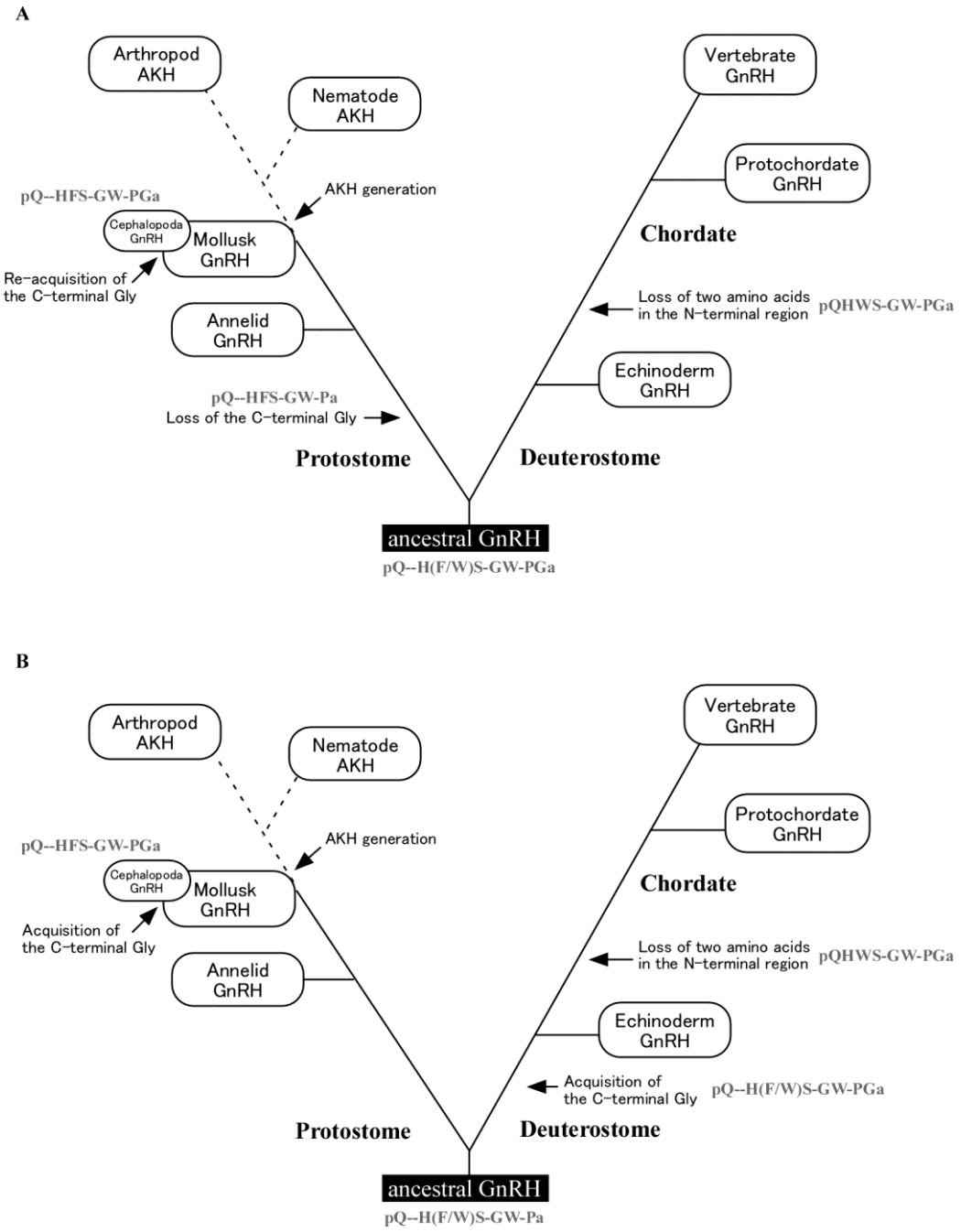

Figure 1. Scenarios of molecular evolution of GnRHs. In the case of (A), ancestral GnRH might have possessed a Pro-Gly-amide at the C-terminus, and a C-terminal Gly residue has been lost during the protostome evolutionary process. Meanwhile, in (B) the Gly residue has been inserted in the C-terminus of ancestral GnRH during deuterostome evolutionary process.

Oct-GnRH has been found in other cephalopods, *Sepia officinalis* (cuttlefish) (Di Cristo et al., 2009) and *Loligo edulis* (Swordtip squid) (Onitsuka et al., 2009) (see Table 1), suggesting that oct-GnRH might have acquired the C-terminal Gly in the *Cephalopoda* evolutionary process (see Figure 1). Furthermore, the Gly^6-Trp^7 motif in vertebrate GnRH-II and -III is also conserved in protostome GnRHs and spGnRHP (see Table 1), suggesting that ancestral GnRH also contained the Gly-Trp sequence (see Figure 1). In contrast, no Gly-Trp sequence is conserved in other chordate GnRHs such as GnRH-I, l-GnRH-I, -III, and ascidian GnRHs (see Table 1), resulting from diversification during the chordate evolutionary process. Altogether, it appears that the ancestral GnRH sequence was likely to be composed of pQ--H(F/W)S-GW-PGa or pQ--H(F/W)S-GW-Pa (see Figure 1), and thereafter, original GnRHs might have diverged via various substitution and deletion in the evolutionary process of each species; for instance, deletion of two amino acids after position 1, acquisition or deletion of the C-terminal Gly, and substitution of the Trp^3 (or Phe) and Gly^6-Trp^7 with other amino acids.

Vertebrate GnRHs are encoded as a single copy in the precursor, and its organization is conserved in protostome GnRHs and in the sea urchin GnRH (Kawada et al., 2010). In contrast, two *Ciona intestinalis* GnRH genes, *Ci-gnrh-1* and *-2*, encode three different GnRH peptide sequences, t-GnRH-3, -5, -6 and t-GnRH-4, -7, -8, respectively (Adams et al., 2003). Moreover, these triplet GnRH sequence organizations are observed in the *Ciona savignyi* GnRH genes, *Cs-gnrh-1* and *-2*. *Cs-gnrh-1* encodes two t-GnRH-5 sequences and one t-GnRH-6, and *Cs-gnrh-2* encodes one sequence each of t-GnRH-7, -8, and -9 (Adams et al., 2003). These findings demonstrate that, unlike vertebrate and protostome GnRH precursors, three copies of GnRH are present in one precursor in ascidians, and that the structural organization of *Ciona* GnRH genes occurred from the ancestral GnRH gene during an evolutionary process unique to *Ciona* species.

Invertebrate GnRH Receptors

GnRH receptors (GnRHRs) belong to the Class A (rhodopsin-like) G protein-coupled receptor (GPCR) family. In most vertebrates, two or three molecular forms of GnRHRs are present (Millar, 2005). Based on phylogenetic analyses, vertebrate GnRHRs have mainly been classified into three groups, type-I, -II and -III (see Figure 2). The type-I GnRHRs were characterized from a wide range of vertebrate species such as teleosts, amphibians, reptiles, birds, and mammals (Sower et al., 2012). Mammalian type-I GnRHRs completely lack the C-terminal tail region which is found in its non-mammalian counterparts (Millar et al., 2004; Millar 2005). The type-II *gnrhr* gene is present in the genome of amphibians, reptiles, avian species, and mammals (Sower et al., 2012). Most mammalian type-II *gnrhr* is likely silenced due to deletion of functional domains or interruption of full-length translation by the presence of a stop codon, whereas functional type-II GnRHRs were identified in several monkeys, pigs, and other non-mammalian vertebrates (Millar 2005; Kah et al., 2007). Type-I GnRHRs show high affinity for both GnRH-I and -II, whereas type-II GnRHRs are specific to GnRH-II (Lu et al., 2005). Furthermore, it is presumed that GnRH-I and -II induce different active conformations of mammalian type-I GnRHRs, given that the binding affinity for GnRH-II (but not for GnRH-I) is affected by several point mutations in regions distant from the ligand-

binding site in the receptor (Lu et al., 2005). Type-III GnRHRs were found in not mammals but teleosts, amphibians, reptiles, and birds (Sower et al., 2012). In the chicken, type-III GnRHR exhibits a 35-fold higher affinity for GnRH-II than for GnRH-I (Joseph et al., 2009).

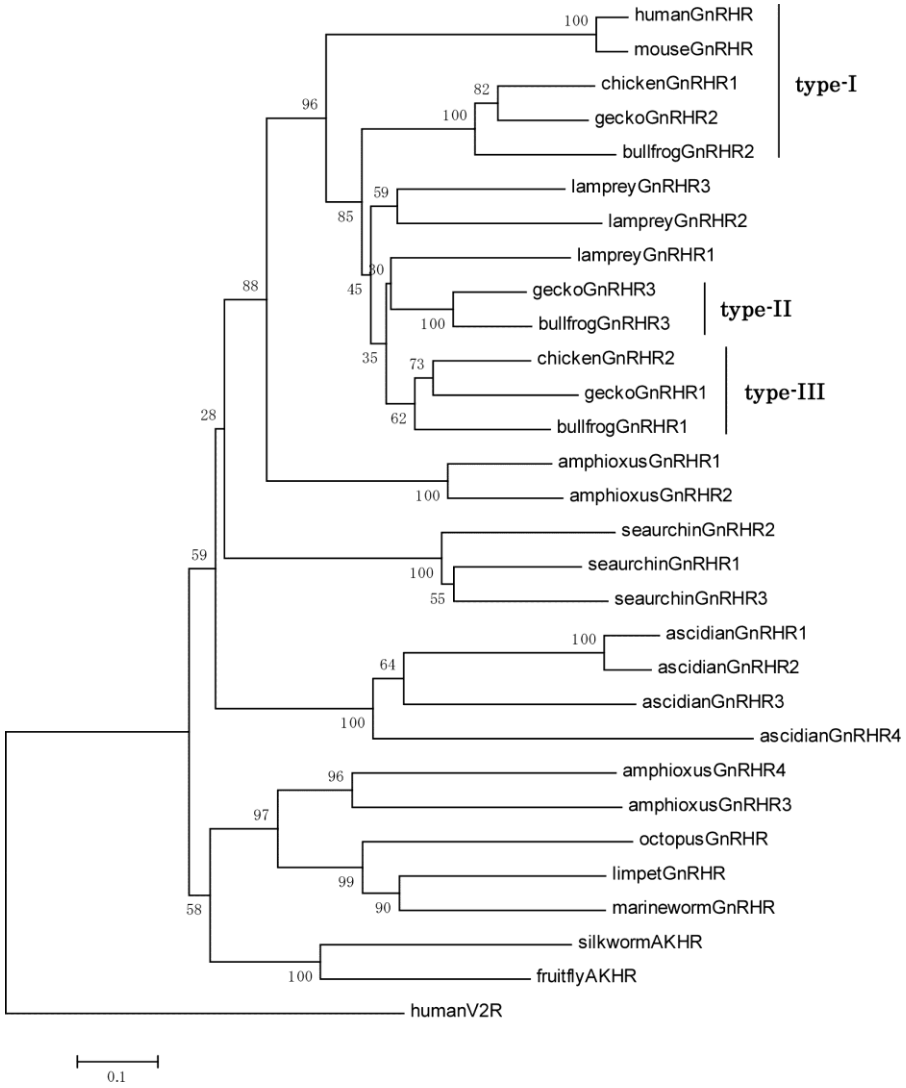

Figure 2. Phylogenetic analysis of GnRH receptors. A phylogenic tree of GnRH receptors was constructed by the neighbor-joining method. The scale bar indicates the evolutionary distance of 0.1 amino acid substitutions per protein. The sequences used were as follows: human GnRHR (L03380); mouse GnRHR (L01119); chicken GnRHR1 (AJ304414) and GnRHR2 (AY895154); gecko GnRHR1 (DQ269481), GnRHR2 (AB109032), and GnRHR3 (DQ269482); bullfrog GnRHR1 (AF144063), GnRHR2 (AF153913), and GnRHR3 (AF224277); lamprey GnRHR1 (AF439802), lamprey GnRHR2 (HM641828), and lamprey GnRHR3 (HM641829); ascidian GnRHR1 (AY742888), ascidian GnRHR2 (AY742889), ascidian GnRHR3 (AY742890), and ascidian GnRHR4 (AY742891); amphioxus GnRHR1 (EU433377), amphioxus GnRHR2 (EU433378), amphioxus GnRHR3 (EU433380), and amphioxus GnRHR4 (FJ426561); sea urchin GnRHR1 (NM_001123518), sea urchin GnRHR2 (NM_001123520), and sea urchin GnRHR3 (NM_001123519); octopus GnRHR (AB185200); limpet GnRHR (jgi|Lotgi1|103088); marine worm GnRHR (jgi|Capca1|126901); silkworm AKHR (AF403542); fruit fly AKHR (AF522194); human vasopressin 2 receptor (AF101727).

The sea lamprey, *Petromyzon marinus*, possesses three GnRHRs although it has been suggested that lamprey GnRHRs should be classified into two type-III GnRHRs and one non-categorized GnRHR (Joseph et al., 2012), our phylogenetic analysis demonstrates that all lamprey GnRHRs are likely to be non-categorized GnRHRs (see Figure 2). This discrepancy is most likely due to the fact that prior work analyzed phylogenetic relationships using full-length amino acid sequences of GnRHRs, whereas our investigations employed the sequences between GnRHR transmembrane domain-I to -VII, leading to the result that lamprey were assigned to a different position in the phylogenetic tree. These findings, combined with the phylogenetic position of the sea lamprey as a basal vertebrate, suggest that a lamprey type-III-like GnRHR may be a prototype of an authentic type-III GnRHR found in advanced vertebrates.

Invertebrate GnRHRs were cloned and characterized from ascidians, amphioxus and octopus. Furthermore, *in silico* gene predictions suggest the presence of putative GnRHRs in other invertebrates, namely, three sea urchin GnRHRs, a hemichordate GnRHR, a marine worm GnRHR, a limpet GnRHR, and a sea hare GnRHR (Roch et al., 2011; Sun et al., 2012). To date, four GnRH receptors, Ci-GnRHR-1, -2, -3, and -4, have been identified in the ascidian, C*iona inetestinalis* (Kusakabe et al., 2003; Tello et al., 2005). One Ci-GnRHR homolog, Pm-GnRHR, has been cloned from another ascidian *Polyandrocarpa miskiensis* (Kobayashi et al., 2005). Ci-GnRHR-1, -2, and -3 sequences were found to harbor a long C-terminal tail, whereas a short tail is present in the C-terminus of Ci-GnRHR-4 (Tello et al., 2005). Moreover, Tello et al. identified four GnRHRs from an amphioxus, *Branchiostoma foetida* (Tello & Sherwood, 2009). Amphioxus GnRHRs are categorized into two paralogous phylogenetic groups: amphioxus GnRHR-1 and -2, which are closer in sequence to vertebrate GnRHRs than other non-chordate GnRHRs, and amphioxus GnRHR-3 and -4, which are highly homologous to the protostome GnRHRs (see Figure 2). However, amphioxus GnRHR-1 and -2 are not categorized into vertebrate type-I to -III GnRHR groups, as well as amphioxus GnRHR-3 and -4, protostome GnRHRs, and ascidian GnRHRs (see Figure 2). Since invertebrate GnRHRs can be separated from vertebrate GnRHRs by phylogenic analysis, type-I to -III GnRHR subtypes have likely been generated by gene duplications during the vertebrate evolutionary process. Of particular interest is that the octopus GnRHR and amphioxus GnRHR-3 and -4 are homologous to adipokinetic hormone (AKH) receptors (see Figure 2). AKH is a lipid-mobilizing hormone in insects, and AKH family peptides have been identified from other protostomes including crustaceans, nematodes, and mollusks (Lindemans et al., 2009; Roch et al., 2011). Although AKH, like GnRH, is a short peptide hormone harboring pyro-Glu at the N-terminus, no other consensus sequences of GnRH are conserved in AKH (see Table 1). In addition, both AKH and GnRH have been found in two mollusks, a sea hare and a limpet (Roch et al., 2011). Although several invertebrate GnRHRs are highly homologous with AKH receptors, the evolutionary relationship between GnRH and AKH remains unclear.

A number of GnRH studies have demonstrated that GnRHRs activate the phospholipase C / inositol phosphate (IP) / Ca^{2+} pathway via Gq/11 (Millar et al., 2004 & 2008). Moreover, several GnRHRs, coupled to Gs or Gi, trigger an increase or decrease of intracellular cAMP (Millar et al., 2004 & 2008). The signaling pathways of only three GnRHRs were investigated in invertebrates, namely, an octopus, an amphioxus, and an ascidian (Tello et al., 2005; Kanda et al., 2006; Lindemans et al., 2009; Sakai et al., 2010 & 2012). Administration of oct-GnRH to *Xenopus* oocytes expressing the cognate GnRHR, oct-GnRHR, induced membrane Cl^-

currents coupled to the Ca^{2+} pathway, whereas oct-GnRHR failed to exhibit significant responses to even 10^{-5} M mouse GnRH-I, chicken GnRH-I, or –II (Kanda et al., 2006). Intriguingly, an oct-GnRH synthetic analog with a Asn^2-Tyr^3 deletion abolished the ability to activate the Ca^{2+} pathway via oct-GnRHR, whereas a chicken GnRH-II synthetic analog with an Asn-Tyr insertion after position 1 exhibited weak activation (Kanda et al., 2006). These findings verify that Asn^2-Tyr^3 is required for the activation of oct-GnRHR, suggesting that the two amino acids after position 1 in non-chordate GnRHs play a crucial role in activating cognate GnRHRs.

A peptide with an identical sequence to that of mammalian GnRH-1 has been identified from the European amphioxus, *Branchiostoma lanceolatum* (Chambery et al., 2009). However, no gene encoding GnRH-I was detected in the *Branchiostoma* genome database (Chambery et al., 2009). Thus, whether the peptide is an authentic *Branchiostoma* GnRH is highly questionable. As stated above, four amphioxus receptors have been cloned from *Branchiostoma foetida*. Intracellular inositol phosphate accumulation assays have been performed using amphioxus GnRHR-expressing COS7 cells and GnRHs of other species; mammalian GnRH-I, chicken GnRH-II, oct-GnRH, and AKH (Tello & Sherwood, 2009). Amphioxus GnRHR-1, and -2 were responsive to both GnRH-I and –II at physiological concentrations, and amphioxus GnRHR-2 demonstrated high selectivity for GnRH-I (Tello & Sherwood, 2009). In addition, amphioxus GnRHR-3 responded not only to GnRH-II but also to oct-GnRH and AKH (Tello & Sherwood, 2009), indicating that amphioxus GnRHR-3 exhibits extensive ligand-selectivity for GnRH family peptides. In contrast, none of these ligands induced intracellular IP accumulation via amphioxus GnRHR-4 (Tello & Sherwood, 2009). The characteristics of amphioxus GnRHRs suggest that amphioxus also possesses multiple GnRHs and demonstrates complicated signaling pathways via four GnRHRs. Identification of authentic an amphioxus GnRH will clarify the precise ligand-receptor pairs and the relevant signaling pathways in this GnRHergic system.

In the ascidian, *Ciona intestinalis*, six GnRHs and four GnRHRs were characterized as stated above, and regulate exceptionally complicated signaling pathways involving ligand-receptor selectivities, coupling with multiple G-protein subtypes, and receptor heterodimerization.

It is noteworthy that the elevation of intracellular calcium, which is a typical response of GnRHR activation, was observed only in the t-GnRH-6 and Ci-GnRHR-1 pair (see Table 2 and Figure 3). t-GnRH-6 also induces cAMP production via Ci-GnRHR-1 (see Table 2 and Figure 3). Moreover, Ci-GnRHR-2 stimulates cAMP production in response to t-GnRH-7, 8, 6 in this order of potency, whereas t-GnRH-3 and -5 evoke cAMP production via Ci-GnRHR-3 to a similar extent (see Table 2 and Figure 3). Ci-GnRHR-4 exhibited neither elevation of intracellular calcium nor cAMP production (see Table 2 and Figure 3). Intriguingly, Ci-GnRHR-4 heterodimerizes with Ci-GnRHR-1 and then potentiates the elevation of intracellular calcium (see Figure 3) via both calcium-dependent and –independent protein kinase C subtypes, and ERK phosphorylation in a ligand-selective fashion (Sakai et al., 2010). These data verify that Ci-GnRHR-4 serves as a protomer of GPCR heterodimers rather than a ligand-binding GPCR. Quite recently, Ci-GnRHR-4 was also found to heterodimerize with Ci-GnRHR-2 (Sakai et al., 2012). The Ci-GnRHR-2 / -4 heterodimer decreases cAMP production by 50% in a non-ligand selective manner by shifting of activation from Gs protein to Gi protein by Ci-GnRHR-2, compared with the Ci-GnRHR-2 monomer/homodimer (see Figure 3).

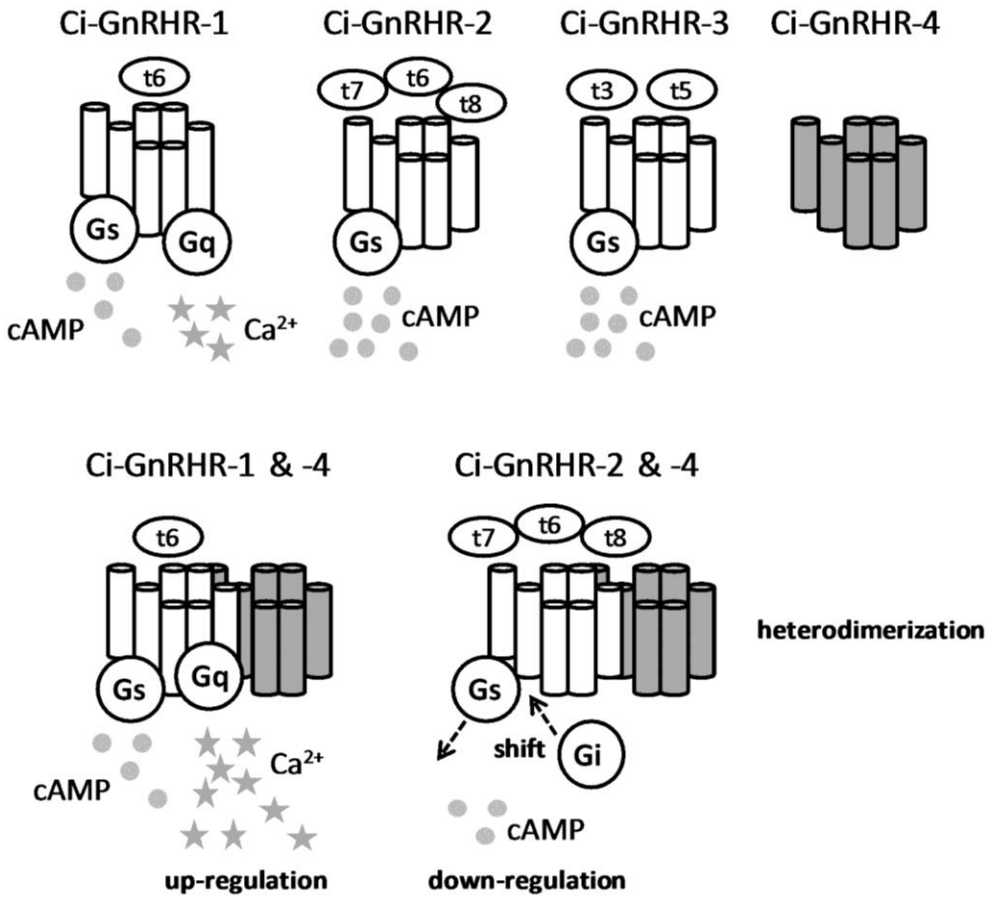

Figure 3. Scheme of signaling pathways activated by interactions between t-GnRHs and Ci-GnRHRs.

Table 2. Characteristics of ascidian GnRH receptors

receptor	preferable ligand	G protein	signaling pathway	effect by Ci-GnRH-X
Ci-GnRHR-1	t-GnRH-6	Gq, Gs	Ca^{2+}, cAMP	moderate inhibition
Ci-GnRHR-2	t-GnRH-7,-8,-6	Gs	cAMP	no effect
Ci-GnRHR-3	t-GnRH-3,-5	Gs	cAMP	moderate inhibition
Ci-GnRHR-4	no ligand	none	none	none

Collectively, these results indicate that Ci-GnRHR-4 regulates differential GnRH signaling cascades via heterodimerization with Ci-GnRHR-1 and -2 as an endogenous allosteric modulator. Furthermore, we reported a unique activity of Ci-GnRH-X at Ci-GnRHRs (Kawada et al. 2009). Intriguingly, this GnRH-related peptide exhibited moderate (10-50%) inhibitory activity against t-GnRHs at their cognate receptors (see Table 2). Ci-GnRH-X moderately inhibited the elevation of intracellular calcium and cAMP production by t-GnRH-6 at Ci-GnRHR-1, and cAMP production by t-GnRH-3, and t-GnRH-5 via Ci-GnRHR-3 was also inhibited by Ci-GnRH-X (see Table 2). In contrast, no inhibitory effect of

Ci-GnRH-X at Ci-GnRHR-2 was observed (see Table 2). These findings provide evidence that t-GnRHs and Ci-GnRHRs have not redundant but specific biological roles (Kawada et al., 2009).

In summary, multiple GnRHs and GnRHRs have emerged in an ascidian, *Ciona intestinalis*, leading to complicated signaling responses. Moreover, an amphioxus may also establish elaborate GnRH signaling cascades owing to the presence of four GnRHRs, as seen in *C. intestinalis*. In contrast, *in silico* analyses of protostome genomes showed that only one GnRH and GnRHR pair is present in a sea hare and a marine worm, and more than two GnRHs or GnRHRs have not been identified from one protostome species. These results suggest that a protostome species possesses a simple GnRH signaling pathway. Since most GnRHRs activate the Ca^{2+} pathway, protostome GnRH may induce elevation of intracellular Ca^{2+} via GnRHR, as seen in the oct-GnRH and oct-GnRHR pair.

Biological Activity of GnRHs in Invertebrates

GnRH controls reproductive function in the HPG axis of vertebrates. GnRH synthesized in the hypothalamus is transported to the pituitary, where it induces gonadotropin-release from the pituitary to the blood stream. Subsequently, gonadotropins are transferred to the gonad, resulting in gonadal steroidogenesis and gametogenesis. It is thought that the HPG axis was established during the vertebrate evolutionary process, because the hypothalamus and pituitary are absent in the invertebrate brain. Therefore, the function of GnRH as a "gonadotropin-releasing hormone" is likely to be restricted in vertebrates.

Several physiological studies have shown that invertebrate GnRHs are involved in reproductive functions. In vertebrates, GnRH acts not only as a hypothalamic hormone but also as a peripheral bioactive peptide. For instance, GnRH induces the synthesis and release of sex steroids in vertebrate reproductive tissues (Leung & Steele, 1992; Chang & Leung, 2005). In octopus, administration of oct-GnRH to the follicle and spermatozoa led to release of sex steroids, including testosterone, progesterone, and 17β-Oestradiol (Kanda et al., 2006). Moreover, oct-GnRH induced contraction of the octopus oviduct (Iwakoshi-Ukena et al., 2004). These findings suggest that oct-GnRH directly promotes reproductive behaviors on the gonadal organs as a bioactive peptide. In another mollusk, the yesso scallop (*Patinopecten yessoesis*), py-GnRH enhanced BrdU uptake into cultured scallop testicular cells, indicating that py-GnRH induces testicular cell proliferation (Treen et al., 2012). Furthermore, an ascidian GnRH, t-GnRH-1, was found to increase water flow and then induce the release of eggs and sperm by injection into the gonaducts, ovary, stomach, and posterior body cavity of *C. intestinalis* (Terakado, 2001). t-GnRH-2 exhibited less potent, but similar activity on gamete release (Terakado, 2001). A similar effect was observed after administration of t-GnRH-3 to -9 to *C. intestinalis* (Adams et al., 2003). These findings provide evidence that a major function of t-GnRHs is the regulation of gamete release.

Tsai et al. (2010) investigated the reproductive function of the sea hare GnRH, ap-GnRH. However, injection of ap-GnRH into sexually mature and immature sea hares exhibited no effects on ovotestis mass, reproductive tract mass, egg-laying, or penile eversion. Moreover, ap-GnRH also failed to alter oocyte growth and egg-laying hormone accumulation and secretion (Tsai et al., 2010).

Instead of reproductive activities, ap-GnRH exerted other biological actions such as stimulation of the parapodial opening, inhibition of feeding, and promotion of substrate attachment. A putative ap-GnRH receptor gene is expressed in several tissues including the central nervous system, posterior foot, ovotestis, and heart (Sun et al., 2012). Likewise, GnRHR mRNAs are exclusively distributed in various tissues of an octopus and an ascidian. *Oct-gnrhr* is expressed in the central nervous system, digestive tissues, aorta, heart, salivary gland, branchia, radula retractor muscle, egg, and genital organs (Kanda et al., 2006), while Ci-GnRHR mRNAs are distributed in the neural complex, heart, intestine, endostyle, and branchia sac, and ovary (Tello et al., 2005; Sakai et al., 2010 & 2012). These findings suggest that octopus and ascidian GnRHs induce not only reproductive responses but also other various biological behaviors. Indeed, oct-GnRH induced contraction of the radula retractor muscle expressing *oct-gnrhr* (Kanda et al., 2006). Of particular interest is the fact that the *Ci-gnrhr* genes are expressed in the larva of *C. intestinalis* (Kusakabe et al., 2012). In the ascidian larva, all Ci-GnRHR genes are expressed in the brain vesicle, and Ci-GnRHR-1 and -2 mRNAs are distributed in the motor ganglion (Kusakabe et al., 2012). Furthermore, in the tail of larva, *Ci-gnrhr-1* and *-2* genes are expressed in muscle cells, while *Ci-gnrhr-3* gene is expressed in notochord cells (Kusakabe et al., 2012). These results imply that t-GnRHs play a crucial role in the process of development and/or metamorphosis. As mentioned above, the biological activities of GnRHs in invertebrates are restricted. To elucidate the biological roles and functional evolution of invertebrate GnRHs, further physiological studies of invertebrate GnRHs are required.

Conclusion

There has been a growing body of information concerning the molecular characterization of invertebrate GnRHs, which have gradually shed new light on the evolutionary origins of GnRHs. The evolutionary aspects and diversity in biological function of GnRHs cannot be clarified without studies on invertebrate GnRHs. In contrast, the biological roles of invertebrate GnRHs in reproduction, and the underlying molecular mechanisms largely remain to be elucidated.

This is mainly due to limited experimental data regarding sequences, signaling cascades, and rigorous localization of GnRH receptors in invertebrates (an exception of the ascidian, *C. intestinalis*). To be sure, molecular and functional characterization of invertebrate GnRH receptors will markedly facilitate verification of the biological roles of invertebrate GnRHs, which will eventually lead to an improved understanding of the biological significance and evolutionary processes of GnRHs.

Author’s Note

During the editorial process of this manuscript, two novel GnRH peptides, tGnRH-10 and tGnRH-11, were identified in another ascidian, *Halocynthia roretzi*. These peptides were encoded in the single precursor (Hasunuma and Terakado, *Zoolog. Sci.* 2013;30:311-318).

Disclosure Summary

None of the authors declare any conflict of interest.

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