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Gonadotropin-Releasing Hormones and Their Receptors in the Marine Invertebrates: Aplysia californica and Branchiostoma floridae

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Gonadotropin-Releasing Hormones and Their Receptors in the Marine Invertebrates: *Aplysia californica* and *Branchiostoma floridae*

by

Lisa Hae-Won Jung

B.A., University of Colorado at Boulder, 2009
M.A., University of Colorado at Boulder, 2013

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Faculty of the Graduate School of the
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Gonadotropin-Releasing Hormones and Their Receptors  
in the Marine Invertebrates:  
_Aplysia californica and Branchiostoma floridae_  
written by Lisa Hae-Won Jung  
has been approved for the Department of Integrative Physiology

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Pei-San Tsai, PhD., Committee Chair

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David Norris, PhD.

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Daniel Medeiros, PhD.

Date__________________

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
Abstract

Gonadotropin-releasing hormone (GnRH) is a neuroendocrine activator of the reproductive axis in vertebrates. GnRH was once thought to be a hormone that exists exclusively in vertebrates. However, there is now ample evidence that support the presence of GnRH-like molecules outside of vertebrate classes. Although the vertebrate GnRH systems have been well characterized, little is known about the anatomy and function of the invertebrate GnRH systems. The aim of this thesis was to examine the anatomical distribution and perform preliminary functional studies on GnRH and its receptors in two invertebrates, a gastropod mollusk, *Aplysia californica*, and a cephalochordate, *Branchiostoma floridae*. Chapter 1 examines the mRNA and peptide distribution of *Aplysia* GnRH (ap-GnRH) in the central and peripheral tissues of *A. californica*. Results revealed that ap-GnRH is expressed in three discrete central ganglia (abdominal, cerebral, and pedal) of *A. californica*, suggesting ap-GnRH may perform a wide array of functions unrelated to reproduction. Chapter 2 examines the tissue distribution and reproductive consequence of activating endogenous GnRH receptors (GnRH-R) in *B. floridae*. RT-PCR revealed that 3 amphioxus GnRH-Rs were expressed in several body segments, indicating that these receptors may be involved in regulation of diverse functions. *In vivo* functional study revealed that ap-GnRH had a stimulatory effect on gametogenesis, suggesting a conserved functional link to
reproductive activation. Overall, work from this thesis illustrates the complex evolutionary trajectory of the GnRH system and suggests that the functional connection between GnRH and reproductive activation becomes more uncertain in organisms outside Phylum Chordata.
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Thank you for your helpful advice and guidance which enabled me to experience a wonderful research opportunity in Taiwan. Being able to work with amphioxus in Dr. Jr-Kai Yu’s laboratory was one of the highlights of my graduate career. Thank you for offering your valuable insight and taking the time to serve on my committee.

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Thank you so much for believing in me. Even though my journey through graduate school was not always easy, I felt secure knowing that you would be there for me every step of the way. I could not have survived this process without your endless encouragement and advice. Thank you for being a great mentor and a wonderful friend.

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I am so blessed to have such amazing family and friends. Your love, encouragement, and support inspire me to become a better person every day. Thank you for being my biggest cheerleaders. I am so lucky to have each and every one of you in my life.
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Introduction

Gonadotropin-releasing hormone (GnRH) is a neuropeptide indispensable for neuroendocrine activation of vertebrate reproduction. Upon release from the hypothalamus, GnRH activates its cognate receptors on gonadotropes of the adenohypophysis to stimulate the secretion of two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The gonadotropins, in turn, travel through the circulation to induce gonadal steroidogenesis and gametogenesis, thereby ensuring the fertility of the organisms.

GnRH ligand

Vertebrate GnRH

To date, 15 isoforms of GnRH have been identified in vertebrates. All vertebrate GnRHs consist of 10 amino acids and share an N-terminal pyroglutamyl residue, high conservation of the first four and last two amino acids, and a C-terminal amidated glycine residue (Figure 1) (1).

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Figure 1. Amino acid sequences of vertebrate GnRHs. GnRHs are named after the species in which they were first discovered. Universal features conserved in all vertebrate GnRHs include N-terminal pyroglutamate and C-terminal amidated residues. Excluding a few exceptions (highlighted), residues at position 1-4 and 9-10 are highly conserved in vertebrate GnRHs identified thus far.

Isoforms of vertebrate GnRH are categorized into three types based on their neuroanatomical distribution, function, and developmental origin. GnRH 1 is produced primarily within the preoptic/hypothalamic area and is the principal neurohormone that stimulates gonadotropin secretion from the pituitary (2). GnRH 2 is produced in the midbrain and is a neurotransmitter/neuromodulator implicated in a variety of functions involving reproductive behavior, energy balance (3) and suppression of feeding intake. Notwithstanding some exceptions, GnRH 2 is found in most vertebrates examined thus far that range from mammals to fishes (4). GnRH 3 is only found in the terminal nerves and anterior forebrain of some teleost fishes and is implicated in regulating the olfactory and visual system (5-7). The majority of vertebrates possesses at least two types of GnRH (GnRH 1 and 2), whereas some teleost fishes can express up to three types of GnRH in addition to lamprey (8-10).

Like most peptide hormones, the mature GnRH peptide is enzymatically cleaved from a larger preprohormone, prepro-GnRH. The prepro-GnRH consists of a signal peptide, the GnRH peptide, a dibasic processing site, and the GnRH-associated peptide (GAP) with poorly understood function (Figure 2). The GnRH gene consists of 4 exons and 3 introns in all vertebrates (11).
Figure 2. Structural organization of prepro-GnRH. GnRH peptide is preceded by signal peptide and followed by processing site and GAP.

**Invertebrate GnRH-like molecules**

Over the last decade, unprecedented advances in data mining, cloning, and phylogenetic analysis have allowed the elucidation of GnRH-like molecules in multiple protostomes and invertebrate deuterostomes (12, 13). So far, 13 forms of invertebrate GnRH-like molecules have been either molecularly or biochemically isolated. Among these, 9 have been identified in tunicates, 1 in the common octopus (*Octopus vulgaris*), 1 in the sea slug (*Aplysia californica*), 1 in the Pacific oyster (*Crassostrea gigas*) and 1 in the yesso scallop (*Pinctopecten yessoensis*) (1, 12-14). Additional GnRH-like sequences within the genomes of the owl limpet (*Lottia gigantea*), marine worms (*Capitella teleta*), a sea urchin (*Strongylocentrotus purpuratus*), and a leech (*Helobdella robusta*) were also found by data mining (1, 13).

Two major differences exist between protostomian GnRH-like molecules and chordate GnRHs. First, protostomian GnRH-like molecules have two extra residues at the N-terminus and thus deviate from the decapeptide motif universal to chordate GnRH (Figure 3). Second, most protostomian GnRH-like molecules (except for octopus GnRH) lack the universal chordate Pro⁹ and Gly¹⁰ motif at the C-terminus. However, protostomian GnRH-like molecules share many structural features common to chordate
GnRHs. For example, genes encoding protostomian GnRH-like molecule also consist of 4 exons and 3 introns that include a signal peptide, the GnRH peptide, a cleavage site and the GAP. Moreover, many protostomian GnRH-like molecules share four identical amino acid sequences at positions 1, 2, 4, and 6 (using chordate GnRH numbering) with chordate GnRH.

![Amino acid sequences of representative GnRH from vertebrates and protostomes.](image)


Although multiple protostomian GnRH-like molecules have been isolated, little is known about their biological functions. To date, functional studies using endogenous GnRHs have been done in only two protostomes, the octopus (O. vulgaris) and sea slug (A. californica). Studies from the octopus reveal that octopus GnRH (oct-GnRH) stimulates steroidogenesis from the ovary and testis (15, 16) and induces oviduct contraction. Furthermore, it has positive chronotropic and inotropic effects on the heart (15). Studies on A. californica led to surprising findings that Aplysia GnRH (ap-GnRH)
does not serve as an acute reproductive activator. Instead, it modulates a wide range of behaviors (motor and feeding) unrelated to reproduction (17). One hypothesis is that GnRH originally arose in a bilaterian ancestor as a general neural regulator. During metazoan evolution, GnRH was recruited for multiple functions that may or may not involve reproduction (18).

Despite ample evidence that protostomes produce molecules with great resemblance to chordate GnRH, orthology between protostomian and chordate GnRHs cannot be definitely established due to the difficulty in analyzing extremely short target GnRH sequences (10-12 amino acids). The substantial support for orthology concept can ultimately come from establishing the presence of GnRH system in an animal that serves as a “missing link” between chordates and non-chordates. One such animal is amphioxus (19). Unfortunately, it is presently unclear whether GnRH is produced by amphioxus. Immunohistochemical studies performed with antisera raised against chordate GnRHs detected GnRH-immunoreactivity in various neurons in the brain, Hatschek’s pit (a presumed homolog of vertebrate pituitary), and photoreceptors in amphioxus (Branchiostoma lanceolatum and Branchiostoma belcheri) (20-22), insinuating that peptides similar to chordate GnRH may be present in amphioxus.

Chambery et al. (23) reported isolation of a peptide identical to mammalian GnRH in B. lancelolatum by biochemical purification followed by mass spectrometry analysis. However, extensive genome analysis has not been able to identify any GnRH-like sequences in either B. lancelolatum or its closely related species B. floridiae, suggesting these published data may be an experimental artifact. Currently, the lack of convincing evidence for the presence of amphioxus GnRH hinders the establishment of orthology
between protostomian and chordate GnRHs. However, since ligands and receptors often co-evolve (24-27), elucidating the presence of orthologous GnRH receptors in various chordate and protostomian organisms will strengthen the argument for the orthology concept.

**GnRH receptor (GnRH-R)**

*Vertebrate GnRH-R*

GnRH receptors are seven transmembrane G protein-coupled receptors (GPCR) that couple predominantly to $G_{q/11}$ and utilize primarily inositol trisphosphate ($IP_3$) as a second messenger (28). Upon activation, accumulation of $IP_3$ results in the release of intracellular $Ca^{2+}$ that promotes the release of gonadotropins from the pituitary. Multiple GnRH-R paralogs and orthologs have been identified in mammals, chicken, frogs, and fishes examined thus far (4). The receptors are phylogenetically grouped into two subtypes (GnRH-R 1 and 2). GnRH-R1 lacks the cytoplasmic tail and binds preferentially to GnRH1 whereas GnRH-R 2 possesses a cytoplasmic tail and binds preferentially to GnRH 2.

*Invertebrate GnRH-R*

The presence of GnRH-Rs is not limited to vertebrate classes. Extensive data mining has revealed invertebrate homologs to vertebrate GnRH-Rs in the genomes of octopus (*O. vulgaris*), sea slug (*A. californica*), sea urchin (*S. purpuratus*), marine worm (*C. teleta*), limpet (*L. gigantea*), oyster (*C. gigas*), tunicates (*C. intestinalis*), and amphioxus (*B. floridensis*) (1, 13, 29). However, cloning and sequencing of invertebrate GnRH-R genes have been accomplished only in tunicates (*C. intestinalis*), amphioxus (*B. floridensis*) and octopus (*O. vulgaris*) (16, 30, 31). Like vertebrate GnRH-Rs, all
invertebrate GnRH-Rs are rhodopsin-like GPCRs that are primarily coupled to the phosphatidylinositol bisphosphate pathway. They also share sequence similarity with vertebrate GnRH receptors in the transmembrane regions and at some extra- and intracellular loops (1).

Octopus GnRH-R (oct-GnRH-R) is the first protostomian GnRH-R characterized (16). Its identity as an authentic oct-GnRH-R is confirmed by its ability to become activated by oct-GnRH. Bioassays revealed that the activation of oct-GnRH-R led to increased steroidogenesis in octopus ovary and testis, suggesting a conserved functional link to reproductive activation. On the other hand, activation of oct-GnRH-R also resulted in the contraction of radula retractor muscles, which supports the notion that protostomian GnRH systems may be involved in diverse functions beyond reproduction.

Invertebrate GnRH-Rs that require special note are the four forms identified in a basal chordate, amphioxus (B. floridae) (30). These receptors are grouped into two paralogous groups. One pair is phylogenetically clustered with vertebrate GnRH-R1 and 2. The remaining pair is grouped with oct-GnRH-R/adipokinetic hormone receptor (AKH-R). In vitro pharmacological assay (30) revealed that vertebrate forms of amphioxus GnRH-Rs were only responsive to vertebrate GnRHs and are differentially activated by vertebrate GnRH types 1 and 2. In contrast, one of the protostomian forms of amphioxus GnRH-Rs was activated by only oct-GnRH and AKH (a peptide thought to be related to GnRH) with similar potencies, while the remaining receptor was unresponsive to the aforementioned ligands and may be a pseudogene (30). To date, the simultaneous presence of both chordate and non-chordate forms of GnRH-Rs has
never been reported in any other taxa other than amphioxus; this supports the status of amphioxus as a transitional model between non-chordates and chordates. Although the presence of amphioxus GnRH still remains to be elucidated, the characterization of biological functions of amphioxus GnRH-Rs will provide greater insights into the ancestral function and structure of the chordate GnRH system and provide additional support for the orthology between chordate and non-chordate GnRHs.

**Overall goals**

Within the last decade, the concept that GnRH arose in a bilaterian ancestor and persisted through protostomian and deuterostomian lineages has gained substantial support. Although vertebrate GnRH systems are characterized extensively, the anatomy and function of invertebrate GnRH systems are still poorly understood. The overall goals of this thesis are to examine the anatomical distribution and perform preliminary functional studies on GnRH and its receptors in two invertebrates, a gastropod mollusk, *A. californica*, and a cephalochordate, *B. floridae*. The first chapter examines the mRNA and peptide distribution of ap-GnRH in the central and peripheral tissues of *A. californica*. The second chapter examines the tissue distribution of GnRH-Rs and reproductive consequence of activating endogenous GnRH-Rs with various ligands (vertebrate GnRH 1 and 2, ap-GnRH, and adipokinetic hormone) in *B. floridae*. Overall, this work will provide insights into the function of invertebrate GnRH systems and contribute to further understanding of the complex evolutionary trajectory of this ancient hormone.

Abstract

Gonadotropin-releasing hormone (GnRH) plays important roles in vertebrate reproduction. Recently, molecules structurally similar to vertebrate GnRH were discovered in a gastropod mollusk, *Aplysia californica*. As a step toward better functional characterization of this hormone, the present study examined the localization of *Aplysia* GnRH (ap-GnRH) in the central and peripheral tissues of *A. californica*. Reverse transcription polymerase chain reaction (RT-PCR) revealed wide expression of ap-GnRH transcript in both central (pedal, cerebral, and abdominal ganglia) and peripheral tissues (osphradium, tail, and small hermaphroditic duct). *In situ* hybridization (ISH) performed on the central ganglia, ovotestis, and heart detected ap-GnRH transcript only in the pedal (ca. 91 neurons), cerebral (ca. 7 neurons), and abdominal (ca. 6 neurons) ganglia. Immunocytochemistry (ICC) performed on adjacent sections revealed that most neurons positive for ap-GnRH transcripts were also positive for the ap-GnRH peptide, although some discrepancies were observed in cerebral and abdominal ganglia. Overall, our data suggest that ap-GnRH is widely distributed across central ganglia and support the notion that ap-GnRH may assume multiple functions beyond reproduction.

1. Introduction

Gonadotropin-releasing hormone (GnRH) is a key neuropeptide responsible for activating the reproductive axis of vertebrates. In most vertebrates, GnRH is produced by neurons located within the preoptic area (POA)/hypothalamus and released from
their axon terminals concentrated in the median eminence. Upon release, GnRH stimulates the secretion of gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior pituitary, which in turn stimulate gametogenesis and steroidogenesis within the gonads. Until recently, the majority of GnRH research has focused on vertebrates (32-34). It was only within this decade that the presence of GnRH-like molecules in invertebrate animals has been brought into light (12-14).

To date, 28 forms of GnRH and GnRH-like molecules have been molecularly or biochemically isolated. These include fifteen forms from vertebrates, nine from tunicates, and four from mollusks (1, 14, 35, 36). Additional GnRH-like sequences within the genomes of owl limpet (Lottia gigantea), marine worm (Capitella teleta), sea urchin (Strongylocentrotus purpuratus), and leech (Helobdella robusta) were also found by data mining (1, 13). Unlike decapeptide sequences universal to chordate GnRH (Figure 1), protostomian GnRH-like molecules are either 11 or 12 amino acids in length (Figure 1) and have features that are both familiar and exotic to chordate GnRH (Figure 1). The presence of GnRH-like molecules in protostomes suggests that GnRH may have an ancient origin that predates the split between the protostomes and deuterostomes.

The function of protostomian GnRH-like molecule remains poorly understood. The earliest functional characterization of molluscan GnRH-like molecule was conducted on a cephalopod, Octopus vulgaris. Octopus GnRH-like molecule (oct-GnRH), purified from O. vulgaris, was the first protostomian GnRH-like molecule to be isolated (12). oct-GnRH transcript and peptide are present in neuronal regions associated with gonadal maturation (subpedunculate lobe and optic lobe) and
peripheral reproductive organs (oviduct and oviducal gland) in *O. vulgaris*. Further, oct-GnRH-positive neuronal cell bodies and fibers were detected in regions responsible for regulating feeding mechanism (superior buccal lobe), arm movement (pre- and postbrachial lobe), cardiac activity (palliovisceral lobe and vasomotor lobe) and the auricle of the heart. Functional studies showed that oct-GnRH stimulates steroidogenesis from the ovary and testis (16), induces oviducal contraction, and exerts positive chronotropic and inotropic effects on the heart (15). These findings suggest that oct-GnRH is a multifunctional peptide with neural, peripheral, motor, and reproductive roles (37).

It is currently unknown whether the multifunctional nature of oct-GnRH is conserved in all mollusks. The sea hare *Aplysia californica* is a gastropod mollusk believed to have diverged from cephalopods 520 million years ago (38). The simple CNS of *A. californica* and its well-characterized female reproductive axis make this organism ideal for the functional characterization of GnRH-like molecule in gastropoda (39), the largest class in Phylum Mollusca. Comparisons between a cephalopod and gastropod offer significant evolutionary insights into the similarity and divergence in GnRH structure and function within one of the most successfully and diverse invertebrate taxa. The structure of a GnRH-like molecule (ap-GnRH) in *A. californica* was previously elucidated by molecular cloning (13) and shown to be an undecapeptide orthologous to oct-GnRH (Figure 1). As an important step towards understanding the function of ap-GnRH, this study aims to co-localize ap-GnRH transcript and peptide in central and peripheral tissues by *in situ* hybridization (ISH) and immunocytochemistry (ICC) respectively. Such an approach is enabled by the large size of *A. californica*
neurons (up to 2 mm in diameter) that permits differential detection in alternating sections. Results from this study should provide significant insights into the source and physiological role of a class of newly discovered peptides that is still in search of a function (13, 35).

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<td>His</td>
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<td>Tyr</td>
<td>His</td>
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<td>Ser</td>
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<td>Gly</td>
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<td>Lys</td>
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<tr>
<td>ys-GnRH</td>
<td>pGlu</td>
<td>Asn</td>
<td>Phe</td>
<td>His</td>
<td>Tyr</td>
<td>Ser</td>
<td>Asn</td>
<td>Gly</td>
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<td>Glu</td>
</tr>
<tr>
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<td>Tyr</td>
<td>His</td>
<td>Phe</td>
<td>Ser</td>
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<td>Gly</td>
<td>Trp</td>
<td>Phe</td>
</tr>
<tr>
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<td>Phe</td>
<td>Ser</td>
<td>Arg</td>
<td>Ser</td>
<td>Trp</td>
<td>Gln</td>
</tr>
</tbody>
</table>


2. Materials and Methods

2.1 Animals

Wild-caught A. californica were purchased from Alacrity Marine Biological Services (Redondo Beach, CA) and maintained in 400 gallons of artificial seawater (Instant Ocean™, Cincinnati, OH) recirculated through biological and chemical filters. The water temperature was kept between 15-18°C and animals were fed a rotating diet of kale, spinach, and romaine lettuce daily.

2.2 Tissue Harvest and Preparation.
*A. californica* were anesthetized by an injection of 1/2 body volume of isotonic MgCl$_2$. Central ganglia (pedal, pleural, abdominal, cerebral, and buccal) as well as peripheral tissues (ovotestis, heart, osphradium, small hermaphroditic duct, and tail) were dissected and immersion-fixed in 4% paraformaldehyde at 4°C for 6-8 hours then cryoprotected in 30% sucrose and embedded in the OCT medium (Tissue Tech, Torrance, CA). Tissues were sectioned at 12 µm thickness using a cryostat, and adjacent sections were thaw-mounted onto alternating slides. One series of the sections were processed for ISH, and the alternating sections were processed for ICC.

2.3 Reverse-transcription polymerase chain reaction (RT-PCR) analysis of ap-GnRH expression in central and peripheral tissues.

Total RNA from central ganglia (abdominal, buccal, cerebral, and pedal/pleural) and peripheral tissues (osphradium, small hermaphroditic duct, and tail) were isolated using the Trizol reagent following the manufacturer’s protocol (Invitrogen Carlsbad, CA). RNA samples were pre-treated with DNase to remove DNA contamination before the reverse transcription (RT) reaction. For RT, 1µg of total RNA was reverse transcribed using a Superscript III first-strand cDNA synthesis kit from Invitrogen (Carlsbad, CA). ap-GnRH was amplified using a 25 µl PCR reaction mixture consisting of 2µl of the first strand cDNA, 15 mM MgCl$_2$, 1x reaction buffer (Bioer, Hangzhou, China), 0.1 mM dNTP, and 1 U Taq DNA Polymerase (Bioer, Hangzhou, China), and 0.2 µM of following primer sets (ap-GnRH: ApGS4 and ApGA4) used in previous study (13). PCR of cDNA was performed in following condition: 0.5 min at 94°C, 0.5 min at 58°C, and 0.5 min at 72°C for 30 cycles, followed by a final extension for 7 min at 72°C. ap-GnRH amplicon was visualized with 1.5% agarose gel stained with ethidium bromide.
2.4 In situ hybridization (ISH) of ap-GnRH transcript.

A 604-nt digoxigenin-labeled riboprobe corresponding to the full-length ap-GnRH cDNA was generated using the digoxigenin RNA labeling mix (Roche, Indianapolis, MN). A three-day ISH procedure was carried out as described below. On Day 1, sections were deproteinized with HCl (0.2N), acetylated with acetic anhydride (0.25% acetic anhydride, 0.42% HCl, 1.4% triethylamine mixed with DEPC-treated water) and washed once with 0.1M phosphate buffered saline with 0.3% Triton X for 10 minutes at room temperature (RT), followed by 2 washes with 2x saline sodium citrate buffer (SSC) for 5 minutes each at RT. Sections were then prehybridized in a hybridization buffer (50% formamide, 10% dextran sulfate, 5x Denhardt's solution, 0.62M NaCl, 10mM EDTA, 20mM PIPES (pH 6.8), 50mM DTT, 250µg/ml yeast tRNA) for 2 hours, and then hybridized overnight at 55˚C with 200ng/ml digoxigenin-labeled probe in the hybridization buffer.

On Day 2, sections were washed twice in 2x SSC for 5 minutes each at RT, once in 0.1x SSC for 10 minutes at 55˚C, twice in 0.1x SSC for 10 minutes each at 60˚C, and twice in 2x SSC for 5 minutes each at RT. Then the tissues were blocked in blocking buffer (2% Roche powder, 10% heat inactivated normal goat serum mixed in a wash buffer (100 mM Tris HCl, pH 7.5, 150mM NaCl), and incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:800) (Roche, Indianapolis, IN) overnight at 4˚C. On Day 3, sections were washed 3 times with the wash buffer at RT, then with a detection buffer (10mM Tris HCl, pH 9.5, 0.1M NaCl, 0.05 M MgCl₂) and the color visualized at RT using 1.5% of NBT (nitro-blue-tetrazolium)/BCIP (5-bromo-4-chloro-3-indolyl-phosphate) solution (Roche, Indianapolis, IN) as substrates. Specificity
of ISH was confirmed by the absence of signals in tissues incubated with digoxigenin-labeled sense probe. All sections were counterstained with methyl green before dehydration and coverslipping.

2.4 Immunocytochemistry (ICC) of ap-GnRH peptide.

ICC was conducted on alternating sections using a specific antiserum generated against ap-GnRH (AS 203-2, 1:500) following a previous ICC protocol (39). Specificity of this antibody was confirmed in a previous study (17). ap-GnRH positive neurons were detected by diaminobenzidine as a chromagen.

3. Results

3.1. RT-PCR analysis of ap-GnRH expression in several tissues

RT-PCR analysis revealed wide distribution ap-GnRH transcript in three discrete central ganglia (abdominal, cerebral, and pedal ganglia). The presence of ap-GnRH transcript was detected in peripheral tissues (osphradium, tail, and small hermaphroditic duct) in *A. californica*.

![Figure 2. Expression of ap-GnRH in central (bag cell neurons, abdominal, buccal, cerebral, pedal/pleural ganglia) and peripheral (osphradium, tail, and small hermaphroditic duct) tissues of *A. californica*. PCR products were visualized with ethidium bromide. BCN, bag cell neurons, AbG, abdominal ganglia; Ceb, cerebral ganglia; P/P, pedal/pleural ganglia; BuG, buccal ganglia; Osp, osphradium; SHD, small hermaphroditic duct.](image)

3.2 ISH localization of ap-GnRH mRNA transcript in central and peripheral tissues of *A. californica*
ISH revealed the presence of ap-GnRH transcript in three discrete ganglia (pedal, cerebral, and abdominal). The pedal ganglia contained the highest number (ca. 91) of ap-GnRH transcript-positive neurons (Table 1, Table 2). ap-GnRH neurons were concentrated in the inferior region of the pedal ganglia (Figure 3G, Figure 5). The cerebral ganglia contained the second highest number (ca. 7) of ap-GnRH neurons (Table 1, Table 2). These neurons were located in the superior and medial region of the cerebral ganglia (Figure 3C, Figure 5). Lastly, the abdominal ganglia expressed ap-GnRH transcripts in 6 neurons (Table 1, Table 2). These neurons were localized in the medial region of the abdominal ganglia (Figure 3I, Figure 5). ap-GnRH mRNA expression was absent in the rest of the central ganglia, including the buccal (Figure 3A) and pleural (Figure 3E) ganglia, and in peripheral tissues including the ovotestis (Figure 4A), and heart (Figure 4B).

3.2. Distribution of ap-GnRH-immunoreactivity (ir) in central and peripheral tissues of A. californica

ICC was performed on sections adjacent to those used for ap-GnRH ISH to confirm successful translation of ap-GnRH transcript into a peptide. In the pedal ganglia, there was a close match on the location and number of neurons positive for ap-GnRH transcript and peptide (Figures 3G, H, Figure 5, Table 1). However, such a match was not observed in cerebral and abdominal ganglia. In the cerebral ganglia, some neurons expressed both the transcript and peptide (Figure 3C, D, solid arrows), while others expressed either the transcript (Figure 3C, D, dotted arrows) or peptide (Figure 3C, D, dashed arrow). Overall, there were a greater number of neurons that expressed ap-GnRH peptide than those that expressed ap-GnRH transcript in the cerebral ganglia.
(Figure 5, Table 1). In the abdominal ganglia, almost every neuron (including the neurons in the BCN) stained above background for ap-GnRH-immunoreactivity (ap-GnRH-IR) (Figure 3J). However, based on the previous observation that paraffin-embedded tissues, which are resistant to non-specific staining, contained no ap-GnRH-positive signals in the abdominal ganglia (17), we believe that this ubiquitous signal is non-specific. The ubiquitous signal in the abdominal ganglia most likely resulted from paraformaldehyde fixation and could not be abolished by preincubation of the antiserum with excess ap-GnRH (unpublished observation). Further blocking may resolve this issue in the future. Next, ap-GnRH-ir was not detected in the buccal ganglia. Lastly, none of the cell bodies tested positive for ap-GnRH in the pleural ganglia (Figure 3F). However, abundant ap-GnRH-ir was observed in nerve fibers projecting to the neurons of pleural ganglia (Figure 3F, dotted arrow). ap-GnRH-ir was not detected in peripheral tissues including ovotestis and heart (data not shown).
Figure 3. ISH of buccal (A), cerebral (C), pleural (E), pedal (G) and abdominal (I) ganglia. ICC of buccal (B), cerebral (D), pleural (F), pedal (H) and abdominal (J) ganglia. Diagram of *A. californica* CNS (K) showing the general location of each ganglia. Arrows of the same shape indicate identical neurons in the adjacent sections. Purple = ap-GnRH transcript; Brown=ap-GnRH peptide; Green = methyl green nuclear stain. Scale bar = 200 µm in Figures 3E and 3F and 100 µm in the remaining panels. Figure 3C and 3D: solid arrows point to the identical neuron that expresses both the transcript and peptide. The dotted arrows point to the identical neuron that expresses only the transcript, while the dashed arrows point to the identical neuron that expresses only the peptide. Figure F: solid arrow points specifically to the identical neuron that appears to have GnRH fibers impinging on it, while dotted arrow points to GnRH fibers.
Figure 4. ISH of ovotestis (A) and heart (B). A. Solid and dashed arrows point to oocytes and spermatogenic tissues, respectively. Green=methyl green nuclear stain. All sections are devoid of positive signals. ap-GnRH-ir was not detected with ICC (data not shown). Scale bar=100µm

Distribution of ap-GnRH Neurons in *Aplysia* CNS

Figure 5. Distribution of neurons positive for ap-GnRH transcript (ISH) or peptide (ICC) in *A. californica* CNS. Purple (ap-GnRH transcript) and brown (ap-GnRH peptide) circles indicate the relative abundance and location of positive neurons. The distribution of ap-GnRH peptide in the abdominal ganglia could not be confirmed due to non-specific staining observed in these ganglia.
Table 1. Comparison of total number of neurons positive for ap-GnRH peptide or transcript in central ganglia. The total number of neurons positive for ap-GnRH peptide in abdominal ganglia could not be confirmed due to non-specific staining.

<table>
<thead>
<tr>
<th>Method</th>
<th>ISH</th>
<th>ICC</th>
</tr>
</thead>
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<tr>
<td>Pedal</td>
<td>91</td>
<td>79</td>
</tr>
<tr>
<td>Cerebral</td>
<td>6.5</td>
<td>12</td>
</tr>
<tr>
<td>Abdominal</td>
<td>5.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. A summary of the relative abundance of ap-GnRH-positive neurons detected by ISH or ICC. The mismatch occurs in the abdominal and cerebral ganglia. The number of positive signs indicates the relative abundance of transcripts or peptide found in each ganglion. Negative sign indicates absence of transcript or peptide.

*Relative abundance of ap-GnRH peptide in abdominal ganglia could not be confirmed due to high background staining.

**Discussion**

Although GnRH-like molecules have been discovered in multiple non-chordate organisms, the function of these molecules is still poorly understood. A growing body of evidence supports molluscan GnRH-like molecules’ involvement in diverse functions (15, 17), adding to the notion that GnRH is not solely an activator of reproduction (40, 41). In 2008, our laboratory isolated the full length cDNA of a GnRH-like molecule (ap-GnRH) from a gastropod mollusk, *A. californica* (42). Previous radioimmunoassay (RIA) and ICC studies have revealed the wide distribution of ap-GnRH in the central ganglia of *A. californica* (17). However, discrepancies in the location of ap-GnRH peptide led to some confusions regarding the site of ap-GnRH production. For example, ap-GnRH was detected in all central ganglia, using RIA. However, ap-GnRH was only detected in
pedal and cerebral ganglia, using ICC. As a step toward understanding where ap-GnRH is synthesized and localized, we examined the expression as well as the co-localization of ap-GnRH transcript and peptide in the central and peripheral tissues of A. californica. The co-localization study is enabled by the large neuronal size in A. californica (up to 2 mm in diameter) which permits the differential detection of ap-GnRH mRNA and peptide in alternating sections.

Initially, RT-PCR analysis revealed that ap-GnRH transcript is widely distributed in the central (abdominal, cerebral, and pedal ganglia) and peripheral tissues (osphradium, small hermaphroditic duct, and tail). ISH showed that ap-GnRH transcript is most abundantly expressed in the cell bodies of pedal ganglia followed by the cerebral and then abdominal ganglia. ICC on alternating sections further confirmed that most neurons positive for ap-GnRH transcripts were also positive for the ap-GnRH peptide. Abundant ap-GnRH-ir was also observed in the nerve fibers projecting to the neurons of pleural ganglia. In the pedal ganglia, there was a close match in the location and number of neurons positive for ap-GnRH transcript and peptide; however, such a match was not observed in abdominal and cerebral ganglia. The discrepancy between ISH and ICC observed in these ganglia could be due to the (1) uptake of ap-GnRH peptide by neurons that do not produce ap-GnRH, (2) the extended half-life of ap-GnRH peptide in neurons that once produced the transcript, or (3) the crossreactivity of the antiserum to a structurally related peptide. Lastly, we examined the ovotestis and heart to characterize the peripheral functions of ap-GnRH. Neither ISH nor ICC could detect ap-GnRH in these tissues. Collectively, these data suggest that ap-GnRH is produced
exclusively by the CNS, predominantly by the pedal ganglia, and secondarily by the cerebral and the abdominal ganglia.

Diverse functions of ap-GnRH can be inferred from its distribution within the various tissues of *A. californica*. Pedal ganglia, the ganglia with the highest expression of ap-GnRH transcript and peptide, contain motor neurons that directly innervate the foot and the parapodia via pedal nerves P1, P8, and P9 (43) and are well known for its role in generating movement and maintaining muscle tone of the foot (44). Our previous studies have provided strong evidence that ap-GnRH is involved in motor regulation by establishing a neuroanatomical overlap between pedal motor neurons and ap-GnRH neurons (41) and projection of ap-GnRH neurons to efferent pedal motor nerves (P1, P8, and P9). This notion was further validated by our previous functional study in which we observed prominent parapodial opening and increased foot attachment to the substrate upon ap-GnRH injections (17). The robust expression of ap-GnRH transcript and peptide in the pedal ganglia seen in our present study demonstrates that pedal ganglia is the major site of ap-GnRH production and adds to the growing body of evidence that ap-GnRH is utilized as a central motor regulatory neurotransmitter in *A. californica*.

Expression of ap-GnRH outside of pedal ganglia suggests that function of ap-GnRH is not limited to motor behavior. The cerebral ganglia, ganglia with the second highest expression of ap-GnRH, are known to control feeding posture and appetitive arousal associated with feeding behavior (45, 46). It is currently believed that upon exposure to food stimuli, inter-ganglionic interactions between cerebral, pedal, and buccal ganglia induce appetitive and consummatory responses (47). Interestingly, ap-
GnRH was demonstrated to inhibit feeding in our previous functional study (17). Although the exact mechanism in which ap-GnRH inhibits feeding remains unclear, based on the wide presence of ap-GnRH neurons and ap-GnRH-ir nerve fibers in the pedal and cerebral ganglia, there is enough preliminary evidence to speculate: (1) ap-GnRH neurons can modulate polysynaptic inputs involved in feeding circuitry to induce anorexic effects, (2) ap-GnRH modulates other interreleated feeding behavior, such as head movement controlled by the pedal ganglia, that may inhibit appetite (17, 48). However, further studies are needed to validate these conclusions.

ISH and ICC failed to detect ap-GnRH in the heart of A. californica. This observation was surprising since cardio-excitatory effect of GnRH is well established in both vertebrates (40) and the octopus (16), suggesting conservation of cardiac function of GnRH in some protostomian and deuterostomian lineages. However, in A. californica, another peptide may have assumed cardio-excitatory functions through the course of evolution, while ap-GnRH was recruited to assume roles in other tissues.

Although there is strong evidence that ap-GnRH is involved in diverse functions, it is currently unclear whether it assumes a reproductive role. A. californica is a hermaphrodite with a well characterized female reproductive axis. Upon receiving stimulatory signal from the cerebral ganglia, two clusters of bag cell neurons (BCN) located in the abdominal ganglia undergo a highly characteristic pattern of electrical firing called after discharge (AD) (49). AD results in the secretion of a peptide functionally analogous to vertebrate luteinizing hormone (LH) in charge of ovulation, egg-laying hormone (ELH). ELH, in turn, stimulates ovulation and egg laying behavior in A. californica (50). Unexpectedly, our ISH and ICC failed to detect ap-GnRH in the
BCN, suggesting that BCN is not the source of ap-GnRH. These results differed from RT-PCR data, which detected low levels of ap-GnRH transcript in BCN. However, the RT-PCR results are inconsistent with the negative ISH and ICC data and suggest that ap-GnRH is produced at a biologically insignificant level in this region. Overall, our data suggest that ap-GnRH is not produced locally in the BCN at sufficient levels for significant transcription and translation.

In addition to BCN, *A. californica* hermaphroditic reproductive tract (small hermaphroditic duct) and gonad (ovotestis) were examined to further investigate the peripheral role of ap-GnRH in reproduction. RT-PCR revealed the presence of ap-GnRH transcript in small hermaphroditic duct, raising the possibility that ap-GnRH may serve as a paracrine factor that regulates the contraction of the reproductive tract. Interestingly, ISH and ICC revealed that ap-GnRH was not present in the ovotestis. This observation is somewhat puzzling since protostomian GnRH-like molecules have been shown to induce gonadal steroidogenesis (testosterone, progesterone, and 17β-estradiol) and gametogenesis in other mollusks (14, 16). However, these observations must be interpreted with caution for the following reasons. First, it is currently unclear whether endogenous biosynthesis of vertebrate steroids occurs in mollusks. To date, the genes for the key enzymes that transform cholesterol into vertebrate-type steroids have not been identified in molluscan genome, raising doubts regarding whether steroids detected in molluscan tissues are endogenously produced or have been absorbed from the environment (51). Without concrete proof of endogenous steroid production, it is difficult to infer whether steroidogenesis induced by protostomian GnRH-like molecules is a valid reproductive effect. Second, even if mollusks are
capable of producing vertebrate type sex steroids, it is unclear whether these steroids assume biological roles. Molluscan estrogen receptor orthologue is a ligand-independent transcription factor that is constitutively activated (52, 53). Moreover, there is no concrete evidence supporting the existence of a functional molluscan androgen receptor (18, 54). Although we certainly cannot preclude the possible role of molluscan sex steroid in reproduction, current data suggests the direct activation of *A. californica* gonads by sex steroids is highly unlikely. Therefore, it is presently unclear whether steroidogenesis induced by molluscan GnRH-like molecules ultimately leads to reproductive activation.

The ambiguous relationship between molluscan GnRH-like molecules and reproduction raises the question of whether ap-GnRH plays a role in reproductive physiology of this organism. While the present data certainly do not rule out the reproductive role of ap-GnRH, previous functional studies indicate that ap-GnRH is not an acute reproductive activator. Injection of ap-GnRH in both sexually immature and mature *A. californica* had no effect on ovotestis mass, reproductive tract mass, egg-laying and penile eversion. Moreover, ap-GnRH failed to alter oocyte growth, ELH accumulation or secretion (17). Taken together, these studies suggest that ap-GnRH does not serve as a potent reproductive activator but functions as a general multifunctional neural regulator in *A. californica*. This notion should not come as a surprise since there is ample evidence that both vertebrates and octopus express their respective GnRH and GnRH-Rs in diverse regions of CNS and peripheral tissues. Both vertebrate GnRH and oct-GnRH assume diverse neural regulatory roles as well as peripheral functions (16, 40). The diverse functions assumed by vertebrate GnRH and
protostomian GnRH-like molecules characterized thus far provide compelling evidence for the hypothesis that GnRH may have originally arose as a general neural regulator in a bilaterian ancestor. Throughout metazoan evolution, different selection pressures may have rendered GnRH to specialize in different functions in different evolutionary lineages, including reproduction in vertebrates (18).

Although unprecedented development in data mining and phylogenetic analysis has led to significant breakthroughs in our understanding of GnRH biology, many questions still remain regarding the origin and functional changes that GnRH underwent over the course of evolution. Further physiological and functional characterization of non-chordate GnRH-like molecules in multiple species will further our understanding of the complex evolutionary trajectory of GnRH.
Chapter 2: Differential regulation of GnRH receptor gene expression and reproductive effects of *in vivo* GnRH application in a basal cephalochordate, *Branchiostoma floridae*

Abstract

In vertebrates, activation of pituitary gonadotropin-releasing hormone receptor (GnRH-R) is critical to the secretion of gonadotropins, hence gonadal development. Recently, four receptors orthologous to vertebrate and protostomian GnRH-R families were identified in a basal chordate, the amphioxus (*Branchiostoma floridae*). However, the biological functions of these receptors are not fully understood. To infer whether reproductive activation initiated by GnRH-R signaling seen in vertebrates is conserved in amphioxus, tissue distribution of GnRH-Rs in *B. floridae* at various gonadal maturation stages was examined by reverse-transcription polymerase chain reaction (RT-PCR). Further, the effects of *in vivo* activation of GnRH-R on gonadal development were examined by immersion treatment of *B. floridae* with vertebrate GnRH types 1 and 2 and protostomian hormones (*Aplysia* GnRH and *Heliothis zea* adipokinetic hormone [AKH]). RT-PCR revealed that amphioxus GnRH-R 1, 2, and 3 were expressed in various body segments, whereas amphioxus GnRH-R4 was not detectable in any of the tissues examined. *In vivo* treatment revealed that only ap-GnRH was capable of increasing the mean gonadal density (MGD), suggesting that activation of protostomian form of GnRH-R results in gametogenesis. Taken together, our data suggest that amphioxus GnRH-Rs are widely distributed in various body segments of *B. floridae* and assume diverse roles including, but not limited to, reproduction.
1. Introduction

Gonadotropin-releasing hormone (GnRH) is universally critical to the activation of the vertebrate reproductive axis. The activation of reproduction by GnRH has been observed in vertebrates ranging from lampreys to mammals (55, 56). The recent advances in data mining, cloning, and phylogenetic analysis has allowed the discovery of GnRH-like molecules and GnRH receptor (GnRH-R) orthologs in various organisms outside the vertebrate classes (1, 12, 13). The invertebrate GnRH-like molecules and their cognate receptors exhibit features highly characteristic of their vertebrate counterparts (1). Both the ligands and their receptors have been implicated in the regulation of various reproductive and non-reproductive functions in invertebrate animals (14, 16, 17). The current belief is that protostomian and deuterostomian forms of GnRH and GnRH-R arose in a common ancestor (1, 13, 16, 57). However, the ultimate validation of this notion can only come from an animal that serves as a “missing link” between invertebrates and vertebrates.

Amphioxus (genus Branchiostoma, Asymmetron, and Epigonichthys) occupies a key phylogenetic position at the base of Phylum Chordata and is believed to be the “missing link” between chordates and non-chordates (19). Because of their transitional status, they can provide valuable insights and bridge the poorly defined evolutionary gaps between chordate and protostomian endocrine systems. At present, the presence of GnRH in amphioxus is still under active debate. Chambery et al., (23) reported the identification of the mammalian form of GnRH from a species of amphioxus, Branchiostoma lanceolatum, by biochemical purification followed by mass spectrometry analysis. However, extensive genome analysis has not been able to identify any
GnRH-like sequences in either *B. lancelolatum* or its closely related species *B. floridæ*, suggesting these published data may be an experimental artifact.

Although the presence of amphioxus GnRH-like molecules is at present unclear, four GnRH-Rs were cloned and characterized from *B. floridæ* (30). Two of these receptors were phylogenetically grouped with vertebrate GnRH-R1 and 2 and were termed amphioxus GnRH-R1 and GnRH-R2. The remaining two receptors were clustered with octopus GnRH receptor (oct-GnRH-R)/adipokinetic hormone receptor (AKH-R) and were termed amphioxus GnRH-R3 and GnRH-R4. Functional receptor assays revealed that amphioxus GnRH-R1 and 2 were activated by only vertebrate GnRHs. amphioxus GnRH-R1 had a preference for GnRH 2, whereas amphioxus GnRH-R2 had a preference for GnRH1. Among the two remaining protostomian forms of amphioxus GnRH-Rs, amphioxus GnRH-R3 was activated by only oct-GnRH and *Bombyx mori* (silkworm) AKH with similar potencies. Interestingly, amphioxus GnRH-R4 was unresponsive to all of the ligands tested, raising questions regarding the functionality of this receptor.

Although the presence of both protostomian and vertebrate forms of GnRH-Rs has been established in amphioxus, endogenous ligands that activate these receptors have not been identified. These GnRH-Rs are essentially orphan receptors in search of functions. As a first step toward understanding the biological roles of these receptors, we investigated the tissue distribution of GnRH-Rs in *B. floridæ* at various reproductive stages. In vertebrates, GnRH-R plays a dominant role in reproductive activation, and thus its expression is differentially regulated according to animal's reproductive status (58-60). Next, we examined if the *in vivo* activation of GnRH-R in amphioxus by
heterologous ligands can induce gonadal growth and reproductive activation, both of which are hallmark effects of GnRH-R activation in vertebrates. We chose to use four ligands (vertebrate GnRH type 1 and 2, oct-GnRH and AKH) previously demonstrated to activate amphioxus GnRH-Rs in vitro. However, due to the unavailability of oct-GnRH, this ligand was substituted with another form of protostomian GnRH-like molecule, Aplysia GnRH (ap-GnRH). ap-GnRH shares 88.3% sequence similarity with oct-GnRH (13). Although ap-GnRH's ability to activate amphioxus GnRH-R has never been tested, examining the in vivo effect of ap-GnRH may shed light on whether the conformation of amphioxus GnRH-R3 allows the binding of multiple protostomian GnRH-like molecules, and if the activation of amphioxus GnRH-R3 results in gonadal growth. Overall, our study has the potential to infer if reproductive activation by GnRH-R signaling is an ancestral chordate condition or a feature unique to vertebrate evolution. The results from this study will aid in further characterization of the functional evolution of this receptor family.

2. Materials and methods

2.1 Animals

_B. floridae_ were collected from Dr. Jr-Kai Yu’s amphioxus culture facility (Institute of Organismic Biology, Academia Sinica, Taipei, Taiwan) during early July through mid-August, 2012. The animals were hatched and reared in the facility. Animals were kept in a 12-L tank with natural seawater prefILTERED through 1µm Chanson SF-815C water filter. Sand and water was replaced every two weeks. Animals were maintained between 22-23°C and were subjected to a 16L: 8D photoperiod. Animals were fed twice daily with a mix of microalgae grown at the culture facility.
2.2 Reverse transcription polymerase chain reaction (RT-PCR)

Gonadal stage (0-5) was determined by microscopic examination (Table 1).

Following the initial staging, the animals were classified into three maturational classes:

Undeveloped (Stage 0), unripe (Stage 1-2), and ripe gonads (Stage 4-5).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>No gonadal sac can be observed.</td>
<td></td>
</tr>
<tr>
<td>Stage 1</td>
<td>Clear outline of gonadal sacs appear under the myocoel. Germinal epithelium which may develop into oocytes or spermatocytes appears as dots or a ring shaped sac. Animals are sexually indistinguishable.</td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td>Cloud shaped testes begin to appear under the myocoel.</td>
<td>Oocytes begin to appear under the myocoel.</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Ventral region of gonads start to fill up with sperm.</td>
<td>Ventral region of gonads start to fill up with oocytes.</td>
</tr>
<tr>
<td>Stage 4</td>
<td>Dorsal region of gonad starts to fill up with sperm.</td>
<td>Dorsal region of gonad starts to fill up with oocytes. Oocytes are loosely packed within the gonadal sac.</td>
</tr>
<tr>
<td>Stage 5</td>
<td>Gonadal sac is densely packed with sperm.</td>
<td>Oocytes are densely packed within gonadal sac. No clear outline of eggs are observed.</td>
</tr>
</tbody>
</table>

Table 1: Morphological features of each gonadal stage in *B. floridae*. These features were based on unpublished observations by J.K. Yu.

Each animal was dissected into three parts: the head, gonad, and tail. Tissues from 3 animals were pooled into a tube and the total RNA from each body part was isolated by Trizol (Invitrogen, Carlsbad, CA). RNA samples were pre-treated with DNase to remove DNA contamination before the reverse transcription (RT) reaction. For RT, 1μg of total RNA was reverse transcribed using a Superscript III first-strand cDNA synthesis kit from Invitrogen. PCR amplification of four amphioxus GnRH-Rs was carried out in 25 μl reaction mixtures consisting of 2 μl first strand cDNA, 15 mM MgCl₂,
1x reaction buffer, 0.1 mM dNTP, 1 U Taq DNA polymerase (Bioer, Hangzhou, China), and 10 pM of each of the following primer sets (Table 2). Negative controls to rule out genomic DNA contamination include the use of RNA samples that have not been reverse transcribed as PCR templates for amphioxus GnRH-R1. Positive controls include PCR with primers for amphioxus actin. PCR for amphioxus GnRH-R 1, 2, and 3 was performed at the following condition: 5 min at 95°C, 30 sec at 95°C, 30 sec at 56°C and 20 sec at 72°C (35 cycles), followed by a final extension for 10 min at 72°C. PCR for amphioxus GnRH-R 4 was performed at the following condition: 5 min at 95°C, 30 sec at 95°C, 30 sec at 58°C and 20 sec at 72°C (35 cycles), followed by a final extension for 10 min at 72°C. PCR products were visualized on a 2% agarose gel by ethidium bromide staining. Each PCR reaction is performed at least twice to monitor consistency.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin-F</td>
<td>5’ AGG TAT CGT GCT GGA CTC TG 3’</td>
</tr>
<tr>
<td>Actin-R</td>
<td>5’ TCC AGA GCC ACA TAG CAC AG 3’</td>
</tr>
<tr>
<td>amp-GnRH-R1-F</td>
<td>5’ TCG GCT TTC GTC ATC AAC TG 3’</td>
</tr>
<tr>
<td>amp-GnRH-R1-R</td>
<td>5’ GGT GTA CGG GCT CTC ATT GG 3’</td>
</tr>
<tr>
<td>amp-GnRH-R2-F</td>
<td>5’ GAC GGA CGC CTA CAA GAG AT 3’</td>
</tr>
<tr>
<td>amp-GnRH-R2-R</td>
<td>5’ GGC GTC ACG AGA TGG AAA AT 3’</td>
</tr>
<tr>
<td>amp-GnRH-R3-F</td>
<td>5’ TGG GTT CAG AGG GTA GCA TT 3’</td>
</tr>
<tr>
<td>amp-GnRH-R3-R</td>
<td>5’ TGT CGT CGC TGT AAT GCT TG 3’</td>
</tr>
<tr>
<td>amp-GnRH-R4-F</td>
<td>5’ CTG TAC CCG TTT GGC GTG TC 3’</td>
</tr>
<tr>
<td>amp-GnRH-R4-R</td>
<td>5’ TCC CGC TGC TCG CTT CTC CT 3’</td>
</tr>
</tbody>
</table>

Table 2. Primers used for PCR analysis.

2.3 In vivo characterization of GnRH/AKH treatments on gonadal maturation

A total of 180 Stage 1 animals were collected over the course of two months.

Three days prior to the hormone treatment, animals (n=3 per beaker) were maintained
in 200-ml beakers with 50g of sterilized sand and 100-ml of prefiltered natural seawater at 22-24°C with a 16L:8D photoperiod. Each beaker was aerated to ensure proper oxygen supply for the animals.

2.3.1 Hormones

In a previous study (30), vertebrate GnRHs (1 and 2) as well as protostomian hormones (oct-GnRH and AKH) were demonstrated to activate respective GnRH-R subtypes in *B. floridae*. Hormones were synthesized based on published sequences of GnRH1 (pQHWSYGLRPG-amide); the prevalent mammalian form of GnRH (61), GnRH2 (pQHWSHGWPG-amide); the universal sequence of GnRH2 (34), *Heliothis zea* AKH (pQLTFTSWG-amide) (62), and ap-GnRH (pQNYHFSNGWYA-amide) (13) by GenScript (Piscataway, NJ Piscataway, NJ). Parent stocks of vertebrate GnRH1, GnRH2, and AKH were diluted in deionized water to a concentration of 1μg/μl. Due to low solubility of ap-GnRH in water, ap-GnRH was diluted to a parent stock of 500μg/μl in DMSO (dimethylsulfoxide), then diluted to 1μg/μl concentration with deionized water.

2.3.2 Hormone administration

*B. floridae* were immersion-treated with three concentrations (10μg/100ml, 50μg/100ml, 100μg/100ml) of GnRH1, GnRH2, ap-GnRH, or AKH to determine whether different doses of these hormones could accelerate gonadal maturation. During the trial, animals were housed in 200-ml glass beakers containing 50g of sterilized sand and 100-ml of prefiltered natural seawater. The vehicle groups consist of only natural seawater with no additive. On Day 1, photographs of gonads were captured under a stereomicroscope (Leica Z16APO with DFC300 FX camera) at 0.57x magnification. The animals were then immediately returned to the beakers. The hormones were dispensed
into designated beakers, and the animals were immersed with each hormone for the next 48 hours. The water was then changed daily for the next five days without hormone treatments. On Day 7, gonads were photographed and animals sacrificed (Figure 1). Each experiment was repeated four times.

2.3.3 Morphometric analysis

Morphometric analysis of gonadal height and density was conducted to quantify gonadal maturation via an image analysis software (ImageJ; http://rsb.info.nih.gov/ij). All photographs were coded to conceal their identity and analyzed by an investigator who had no knowledge of the code.

Figure 2. Representative photomicrographs of reproductively immature (Stage 1) B. floridæ. Stage 1 is characterized by gonadal sacs (dotted circles) that have not yet developed oocytes or sperm. Animals were photographed with ventral side on top and anterior side to the left. Starting at the posterior end, three alternating gonadal sacs (#2, #4, #6) were chosen for gonadal height and gonadal density measurements. Maximum diameter of gonadal sac (d) was measured for each gonadal sac. Body height (D) was measured from the tallest body region. Dotted circle indicates area measured for gonadal density. The background intensity (square outlined in white) was measured from superior and middle region of each photograph, and this value was subtracted from gonadal density of each gonadal sac to normalize for variations in lighting.

Gonadal height to body height ratio (GBR)
For every animal, three gonadal sacs were selected as illustrated in Figure 2. Gonadal sac height and body height were measured using the ImageJ software. Measurements of the gonadal sac height were averaged and then divided by the body height to obtain the gonadal height to body height ratio (GBR). The following equation was used:

\[
\text{GBR} = \frac{\text{Average of height measurements from 3 gonadal sacs}}{\text{body height}}
\]

Mean Gonadal density (MGD)

Three gonadal sacs were chosen in the same fashion described above. The perimeter of each gonadal sac was manually traced using a selection tool from ImageJ and the optical density of the selected area measured. The background optical density was obtained from the superior and middle region of each photograph (Figure 2), and this value was subtracted from optical density measurement of each gonadal sac to normalize for variations in lighting. The resulting values, called gonadal density (GD), were averaged to calculate the mean gonadal density (MGD) of an animal. The following equations were used:

1. \( GD \text{ of gonadal sac} (GD_g) = \text{Optical density of gonadal sac} - \text{background intensity} \)
2. \( \text{MGD of animal} = \frac{GD_{g1} + GD_{g2} + GD_{g3}}{3} \)

The MGD is intended as a surrogate measurement for gamete density. To validate this relationship, we performed a linear regression analysis to correlate MGD with the number of oocytes in 7 gonadal sacs from 7 separate animals. The validation is presented in Figure 5.

2.3.4 Statistical analysis
Paired $t$-test was used to compare differences in GBR and MGD before and after hormone treatments. Although each experiment consisted of 3 animals per treatment group, it was still considered as $n = 1$ since they were housed and treated within the same beaker. As each experiment was repeated four times, our sample size was $n = 4$ for all GBR and MGD measurements. Correlation between oocyte number and MGD was performed using the linear regression analysis. Statistical analysis was performed using InStat (GraphPad, San Diego, CA).

3. Results

3.1 Distribution of amphioxus GnRH-R in B. floridae at different gonadal maturation stages

Representative data (Figure 3) showed that GnRH-R1 is most consistently expressed in the head region compared to other tissues. A repetition of the PCR using less cDNA (1uL) resulted in similar observation on the prevalence of its expression in the head region, but also suggest the possibility of its expression in additional tissues (data not shown). GnRH-R 2 and 3 are ubiquitously expressed in the head, the gonad, and the tail in both sexes of gonadally undeveloped, unripe, and ripe animals (Figure 3). amphioxus GnRH-R 4 is not expressed in any of the aforementioned tissues in any of the animals examined (Figure 3).
3.2 Effects of vertebrate GnRH (1 and 2), ap-GnRH, and AKH on GBR

Exposure to various forms of GnRH or AKH for 48 hours did not alter GBR in *B. floridæ* (Figure 4). There was a trend towards increased GBR in animals treated with 10µg of AKH, although the increase was not significant (*p*=0.0522).

3.3 Effect of vertebrate GnRH (1 and 2), ap-GnRH, and AKH on MGD

In addition to GBR, MGD was measured to examine the effect of various GnRHs and AKH on gonadal density. MGD was used as a surrogate of gamete density; as gametes became more numerous, they became more densely packed, thereby giving rise to higher optical density. The measurement of MGD was less time-consuming and error-prone than counting gametes. Using the linear regression analysis, we validated the use of MGD by showing that it correlated linearly with oocyte number and could be...
used reliably as an indirect measurement of gamete number, in particular oocyte number (Figure 5).

After validating the use of MGD, we examined if GnRH or AKH treatments altered this parameter. Paired t-test comparisons of MGD before and after 48 hours of hormone treatments revealed ap-GnRH (10 µg) significantly increased MGD. No significant differences were observed in other treatment groups (Figure 6). Representative photographs of gonadal sacs before and after treatments are shown in Figure 7.
Figure 4. GBR is not significantly altered by given treatments. Effects of 10µg, 50µg, and 100µg of A) AKH, B) ap-GnRH, C) GnRH 1, and D) GnRH 2 on GBR. Each bar represents mean ± SEM, n=4. No significant difference was observed between before and after treatment with any hormones at any doses.
Figure 5. Validation of MGD as a reliable surrogate of oocyte number by linear regression analysis. Measurements were taken from 7 different animals. $r^2 = 0.9710$. P< 0.0001.
Figure 6. **ap-GnRH significantly increased MGD.** Effects of A) AKH, B) ap-GnRH, C) GnRH 1, and D) GnRH 2 on GD. Each bar represents mean ± SEM, n=4. Asterisk (*) above the bars indicate P < 0.05 compared to before treatment. ap-GnRH (10µg) significantly increased MGD (P=0.0015).
Discussion

Amphioxus occupies a phylogenetic position at the base of Phylum Chordata and serves as an important model for inferring the ancestral function and structure of the chordate GnRH system. Recently, GnRH-Rs capable of being activated by vertebrate GnRHs or oct-GnRH and AKH were cloned and characterized in amphioxus (30). This report validated the presence of multiple GnRH-R paralogs in amphioxus, but little was known about their biological function. As a first step towards understanding the physiological roles of these receptors, the current study first investigated the tissue distribution of GnRH-Rs with the assumption that the expression of these GnRH-Rs is likely to be concentrated in reproductively relevant regions and vary according to
animal’s reproductive status. Furthermore, we examined if the in vivo activation of specific GnRH-Rs could lead to enhanced gonadal growth, a hallmark effect of vertebrate GnRH-R activation.

Using RT-PCR, we found that amphioxus GnRH-R 1 was most consistently and robustly expressed in the head. Its expression in the gonad and tail was not reliably detected. Its dominant expression in the head region suggests a possible involvement in the central nervous system function. Based on the spatiotemporal expression alone, it is difficult to determine whether this receptor is linked to reproduction. If it plays a reproductive role, this role may not directly involve the gonad; rather, it may be central. The ubiquitous expression of amphioxus GnRH-Rs 2 and 3 was consistent with the wide distribution of GnRH-R documented in vertebrates (40), tunicates (31), and octopus (16). In some protostomes and deuterostomes, the activation of GnRH-R resulted in reproductive stimulation (55, 63) as well as changes in various physiological and behavioral parameters (16, 17, 64, 65). The wide distribution of amphioxus GnRH-R 2 and 3 suggests that these receptors assume diverse regulatory roles similar to the actions of deuterostomian and protostomian GnRH-Rs characterized thus far.

The absence of amphioxus GnRH-R4 expression was puzzling since this receptor was successfully cloned in the previous study (30), thus amphioxus GnRH-R4 transcript should be expressed in B. floridae. The precise cause underlying our inability to detect amphioxus GnRH-R4 is unclear, but may be related to methodological differences, such as the nature and amount of RNA and cDNA used, as well as differences in the animals used, such as wild-caught (30) vs. lab-reared (current study). Interestingly, amphioxus GnRH-R4 was the only receptor that could not be activated by
any of the ligands tested in an *in vitro* functional assay (30). This observation, combined with an expression level that is undetectable in the current study, suggests that amphioxus GnRH-R 4 may be non-functional and could be in the process of becoming a pseudogene (30). Overall, our expression studies provide evidence that only three of the four amphioxus GnRH-Rs are expressed. These three receptors may assume diverse central and peripheral functions that remain to be characterized.

To further explore the relationship between GnRH-R and reproduction in a basal chordate, we examined the effects of *in vivo* application of various forms of GnRHs and AKH on gonadal development. Only ap-GnRH (and only the lowest dose applied, 10µg) induced a significant increase in MGD (Figure 6); none of the ligands at any concentration had a significant effect on GBR (Figure 4). Since *in vitro* functional assay on amphioxus GnRH-Rs has never been performed with ap-GnRH, the identity of the GnRH-R activated by ap-GnRH remains unclear. However, indirect evidence favors amphioxus GnRH-R3 as the receptor activated by ap-GnRH to induce gonadal growth. In the previous pharmacological study (30), only amphioxus GnRH-R3 was activated by oct-GnRH and AKH. This suggests that unlike amphioxus GnRH-R1 and 2, the unique conformation of amphioxus GnRH-R3 allows the binding of ligands that deviate from decapeptide motif universal to all vertebrate GnRHs. Since ap-GnRH is an undecapeptide that shares 88.3% sequence identity with oct-GnRH (13), it seems highly likely that ap-GnRH exerted its effects through its interaction with amphioxus GnRH-R3.

In many invertebrates, gametogenesis or gamete release is induced by the direct stimulation of GnRH-Rs in the gonads (14, 16, 66). If amphioxus GnRH-R3 was in fact the receptor activated by ap-GnRH to stimulate MGD development, its ubiquitous
expression would not permit us to infer if the effect was induced directly by stimulation of this receptor on the gonad or indirectly by activating receptor located in other regions that control gonadal function, such as the central nervous system. Further localization of amphioxus GnRH-R3 by \textit{in situ} hybridization or immunohistochemistry could provide the spatial resolution needed to infer the pathway of gonadal growth.

One may wonder why ap-GnRH stimulated MGD but not GBR given that both measurements should be correlated with gametogenesis. This observation is not surprising given that gonadal maturation is marked by an initial increase in gonadal density as oocytes or sperm begin to develop within the gonadal sac. As gametes accumulate, a noticeable change is then observed in gonadal height. The short duration of our experiment may not have permitted sufficient gamete accumulation to result in a noticeable change in gonadal height.

While ap-GnRH was capable of increasing MGD, another protostomian hormone, AKH, failed to induce such a change. This observation was interesting since AKH has also been shown to activate amphioxus GnRH-R3 \textit{in vitro} (30). If ap-GnRH stimulated MGD by interacting with amphioxus GnRH-R3, AKH should also have a similar effect. The precise cause underlying this discrepancy is unclear, but may be related to the difference in the solubility between ap-GnRH and AKH and perhaps also a difference in their susceptibility to degradation. ap-GnRH is more hydrophobic than AKH (P.-S. Tsai, unpublished observation) and may be better able to diffuse into the animal. This could contribute to the greater biological effect of ap-GnRH compared to AKH. In addition, AKH may be more susceptible to degradation in the sea water, which may
have shortened its biological half-life compared to ap-GnRH. Regardless, further studies are needed to validate these hypotheses.

Vertebrate GnRHs (1 and 2) also failed to promote gametogenesis. In the previous pharmacological study (30), these two ligands selectively activated amphioxus GnRH-R1 and 2 in vitro. As such, our data suggest that the activation of amphioxus GnRH-R1 and 2 may not result in gonadal maturation; rather, it may result in changes in various behavioral or physiological parameters unrelated to reproduction as seen in other chordates and non-chordate organisms (16, 17, 40).

In conclusion, these data demonstrated that transcripts of three amphioxus GnRH-Rs (1, 2, and 3) are expressed in various tissues of B. floridae. Although the precise expression level of these receptors at various reproductive stages still needs to be quantified, RT-PCR data suggest that the expression of GnRH-Rs is not directly correlated with the animal’s sexual maturity. Next, our in vivo study revealed that the activation of amphioxus GnRH-R was correlated with an increase in MGD, an indicator of gametogenesis. This is possibly via the activation of amphioxus GnRHR-3. Lastly, the wide distribution of amphioxus GnRH-R2 and 3 suggests that they may assume diverse central and peripheral functions. Overall, these data provide initial proof that reproductive activation by GnRH-R signaling is a phenomenon conserved in a basal chordate. Detailed characterization of individual amphioxus GnRH-Rs represents the next important step towards understanding the functional evolution of GnRH-R.
Conclusions

GnRH is a key neuropeptide for regulating reproduction in vertebrates. Although GnRH was once thought to be a relatively “new” hormone that arose during vertebrate evolution, the presence of GnRH-like molecules in various invertebrates suggests that GnRH may have arisen in an ancestral bilaterian that gave rise to both protostomes and deuterostomes. To validate this notion, homology must be established between non-chordate and chordate GnRHs. This task requires the: (1) comparative analysis of functional conservation and diversification of non-chordate GnRH-like molecules, (2) characterization of non-chordate GnRH receptor (GnRH-R), which often co-evolve with their ligand (24-27), and (3) establishment of the presence of GnRH and GnRH-R in an animal that serves as a missing link between chordate and non-chordate organisms.

The current thesis aims to enhance our understanding on the evolution of metazoan GnRH system through the initiation of some of the aforementioned studies. Chapter 1 investigated the anatomical distribution of Aplysia GnRH (ap-GnRH) in a gastropod mollusk, A. californica. In situ hybridization and immunocytochemistry revealed that ap-GnRH is widely distributed in the central nervous system of A. californica, implicating ap-GnRH as a general neuroregulator charged with diverse roles in feeding, movement, and autonomic functions. This hypothesis was in agreement with the findings from previous functional studies (17), which uncovered novel roles of ap-GnRH in motor behaviors (stimulation of substrate attachment and parapodial opening) and in inhibition of feeding. This study revealed a functional dissociation between ap-GnRH and acute reproductive activation, supporting the hypothesis that ap-GnRH does not undertake a traditional role as a reproductive activator. This hypothesis gained
Further support from the current study that consistently failed to detect ap-GnRH in tissues (bag cell neurons and ovotestis) implicated in reproductive stimulation. Taken together, these observations provide strong evidence that ap-GnRH has taken a different functional trajectory during 650 million years of evolution and has assumed functions that deviate from reproductive activation assumed by vertebrate GnRH.

Although there is ample evidence that supports the presence of GnRH-like molecules in non-chordate invertebrates, it is presently unclear whether homology exists between chordate and non-chordate GnRHs. The homology concept can be validated by the presence of a GnRH system in a taxon that serves as a “missing link” between chordates and non-chordates. One such example is amphioxus (19).

Although the definitive presence of amphioxus GnRH still remains to be elucidated, four GnRH-Rs have been identified and cloned from one amphioxus species, *B. floridae* (30). At present, these GnRH-Rs are essentially orphan receptors in search of functions (30). To gain greater insights into the functions of these novel receptors, Chapter 2 examined the tissue distribution of amphioxus GnRH-Rs at various stages of gonadal maturation. In addition, the reproductive consequence of activating endogenous GnRH-R with various ligands (vertebrate GnRH 1 and 2, ap-GnRH, and adipokinetic hormone) was investigated to infer whether reproductive activation by GnRH-R signaling seen in vertebrates is conserved in a basal chordate.

Study on the expression pattern of amphioxus GnRH-Rs revealed that amphioxus GnRH-R4 was not detectable, whereas amphioxus GnRH-R 1, 2, and 3 were expressed in several body segments, with the expression of amphioxus GnRH-R1 being more restricted to the head segment and the remaining two receptors more
ubiquitous. These observations suggest that amphioxus GnRH-Rs may be involved in the regulation of diverse central and peripheral functions that remain to be characterized. *In vivo* activation of amphioxus GnRH-R resulted in increased gonadal density in these animals, suggesting the functional link between GnRH-R and reproductive activation persists even in an animal lacking a pituitary. Interestingly, only a protostomian form of GnRH (ap-GnRH) was able to stimulate gonadal density, providing indirect proof that a protostomian form of GnRH-R (amphioxus GnRH-R3) mediates the effect of gonadal stimulation. Overall, these studies verify the expression of three amphioxus GnRH-Rs and provide a glimpse into the reproductive and non-reproductive functions assumed by these receptors in a basal chordate with transitional features.

The findings from both chapters add to a growing body of knowledge on the presence and function of GnRH-like molecules and GnRH-Rs in chordate and non-chordate invertebrates (1, 14, 15, 17, 40, 63). The wide central expression of ap-GnRH as well as the whole-body expression of at least two forms of amphioxus GnRH-Rs suggest that invertebrate GnRH systems, whether chordate or non-chordate, regulate diverse functions. Results from the present studies echo the previously proposed idea that the activation of GnRH system induces changes that are not always associated with reproductive stimulation (16, 17, 40).

Results from this thesis provide support for a previously proposed theory on the ancestral function of GnRH (18), which stipulates that GnRH originally arose as a general neuroregulator capable of serving diverse autonomic functions. Throughout the course of evolution, gene or whole genome duplications gave rise to multiple GnRH and
GnRH-R paralogs that were subjected to three fates. Some underwent neofunctionalization and later emerged as key reproductive activators in some taxa, whereas a few were subjected to subfunctionalization and became suboptimal paralogs that could serve its original function only in conjunction with other paralogs. The remaining paralogs were subject to non-functionalization which rendered them inactive and subsequently lost from the genome (18).

The work accomplished by this thesis has just scratched the surface on a topic that probes the complex evolutionary history of GnRH. Although there is now ample evidence that supports the presence of invertebrate GnRH-like molecules and their cognate receptors, limited data on phylogenetic distribution, functional characterization, and homology of non-chordate GnRH systems still leave us with many questions regarding the origin and function of ancestral GnRH. These questions need to be addressed via the additional characterization of GnRH-like molecules and their cognate receptors in diverse invertebrate taxa. Such efforts will ultimately aid in a more complete understanding of the structural and functional evolution of this ancient hormone.
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